Targeting HMGB1 in Cellular Milieu and Elucidating its Effect in Mice Model of Endotoxemia

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CERTIFICATE

The work contained in this thesis entitled **"*Targeting HMGB1 in Cellular Milieu and Elucidating its Effect in Mice Model of Endotoxemia*",** is the bonafide research work of **Asma Bashir** and is worthy of consideration for the award of Doctor of Philosophy in Biotechnology.

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DECLARATION

The research work entitled, ***"Targeting HMGB1 in cellular Milieu and elucidating its effect in mice model of endotoxemia",*** presented in the thesis embodied results of the original work done by me for the **Ph.D** Degree in the Department of Biotechnology at University of Kashmir, Srinagar. This work has not been submitted in part or in full for any other degree or diploma.

Asma Bashir

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Bacterial endotoxin stimulates macrophages / monocytes to release various cytokines early (e.g., TNF-α, IL-1β, and IFN-γ) and late (HMGB-1) which then mediate sepsis (or endotoxemia). HMGB1 recently discovered as late mediator of sepsis, is now seen as one of main mediator of sepsis lethality and prompting investigations for development of new drugs. Present study was undertaken to screen some novel target for ameliorating HMGB1 release and investigate their effect in mice model of endotoxemia.

Here we demonstrate that psychosine increases the HMGB1 in primary peritoneal macrophage cells. The psychosine induced HMGB1 may have some interesting role in pathobiology of Krabbe disease.

Aloe-emodin was seen to abrogate HMGB1 release dose dependently in both RAW 264.7 cells and primary peritoneal macrophage cells. The aloe-emodin was observed to attenuate the release of pro-inflammatory cytokines (TNF-α, IL1β) and LPS – induced oxidative stress markers iNOS, HO-1. The aloe-emodin showed protective effect in endotoxemia rescuing mice from endotoxemia lethality. Aloe-emodin also decreased the systemic accumulation of proinflammatory mediators (TNF-α, IL1-β) within hours in endotoxemic mice. Endotoxemia induced multi-organ dysfunction was also ameliorated by aloe-emodin treatment depicted by serum biochemistry (ALT, ALP, BUN and creatinine) and histopathology of lung, liver and kidney. The neutrophil infiltration was also reduced in lung tissues of aloe-emodin treated mice.

The inhibition of HMGB1 release by aloe-emodin and rescue of endotoxemic mice makes aloe-emodin a potential candidate for sepsis therapy.

Sepsis is a clinical condition caused by the body's systemic response to an acute infection, which can develop into severe sepsis, which is accompanied by single/ multiple organ failure or dysfunction, leading to patient's death. It is a major cause of mortality, killing large number of people (1,400 people approx) worldwide every day (Bone *et al.*, 1992). Despite recent advances in intensive care and antibiotic therapy, sepsis still happens to be the most common cause of death in the intensive care units. The pathogenesis of sepsis is attributable to dysregulated systemic inflammatory responses characterized by excessive accumulation of various proinflammatory mediators such as tumour necrosis factor- α (TNF-α), interleukin (IL)-1 (Dinarello and Thompson, 1991), interferon (IFN)-γ (Yin *et al.*, 2005), and nitric oxide (Fink and Payen, 1996; Vincent *et al.*, 2000). However inhibition of any of these mediators showed no significant relief in amelioration of sepis. Recently it was seen that high mobility group box 1 (HMGB1), a ubiquitous protein, is released by activated macrophages/ monocytes (Rendon-Mitchell *et al.*, 2003; Wang *et al.*, 1999a), and functions as a late mediator of lethal endotoxemia and sepsis (Li *et al.*, 2007; Yang *et al.*, 2004). Circulating HMGB1 levels are elevated in a delayed fashion (after 16–32 h) in endotoxemic and septic mice (Yang *et al.*, 2004), and in patients with sepsis (Angus *et al.*, 2007) which gives HMGB1 more wider window for therapy than other cytokines. Recent research has shown that anti-HMGB1 antibodies or inhibitors significantly protect mice against LPS-induced acute tissue injury (Abraham *et al.*, 2000; Ueno *et al.*, 2004), and lethal endotoxemia (Chen *et al.*, 2005; Ulloa *et al.*, 2002). It is therefore important to search for other agents capable of inhibiting HMGB1 and investigate their effect in sepsis.

1.1 HMGB1

High mobility group box 1 (HMGB1) also referred as amphoterin was described over three decades ago as a 30 kDa protein which was first co-purified from nuclei with histones. It was termed as 'high mobility group 1' (HMG-1) protein because of rapid migration in electrophoretic gels, later renamed to HMGB1 by nomenclature (Bustin, 2001). HMGB1 is an abundant non histone protein and is produced by nearly all cell types, however cellular levels vary with development and age (Prasad and Thakur, 1988). Cellular localization studies have revealed that HMGB1 can migrate between the cytoplasm and nucleus in a cell cycle-dependent fashion. HMGB1 is a nuclear

protein but the location varies in different cells, lymphoid cells contain HMGB1 in both cytoplasm and nucleus (Landsman and Bustin, 1993), whereas cells in liver and brain tissues contain HMGB1 predominantly in the cytoplasm (Mosevitsky *et al.*, 1989). Over the years HMGB1 has been studied and besides its originally described nuclear functions, additional properties have been revealed. Extracellular HMGB1 (released in various conditions) induces migration, recruits stem cells, possesses antibacterial functions and complexed HMGB1 induces cytokine production (Andersson and Tracey, 2011).

1.1.1 Structure

HMGB1 is a member of the high mobility group box (HMGB) family of chromosomal proteins consists of three proteins, HMGB1, HMGB2, and HMGB3 sharing a common structure. HMGB1 is highly conserved between species with a sequence homology of 99% between the human and rodent forms, and is present in all mammalian cells/tissues. HMGB1 comprises a single polypeptide chain of 214 amino acids (the gene encodes for 215 amino acids residues but the initial methionine is not expressed). HMGB1 is a member of the high-mobility group (HMG) protein superfamily which includes HMGB1, HMGB2, HMGB3 and SP100HMG. The amino acid sequences within the HMGB family are highly conserved and all members consist of three distinct domains. The two DNA-binding elements of HMGB1, marked as the A and B boxes respectively, are made up of approximately 80 amino acid residues arranged in three alpha helices (**Fig 1.1**) and are strongly positively charged. The A and B boxes (DNA-binding) are followed by a highly acidic 30 amino acid tail containing only aspartic and glutamic acids which can interact with and fold over the HMG boxes and may thereby interfere with their intermolecular activity (Bustin, 1999). The positively charged amino acids may be the reason for its migration as a 30kDa molecule in SDS-PAGE gels even though HMGB1 has a molecular weight of approximately 25kDa. The large number of charged residues (43 lysines and 9 arginines in the N-terminal DNA binding domains and 36 glutamic acids and 20 aspartic acids in the C-terminal acidic tail) gives the protein strong bipolar properties. These bipolar features may promote binding to endogenous and exogenous components. HMGB1 has an uneven number of cysteines (a feature seen in IL-1β and IL-18, which is presumed to form complex with other proteins.

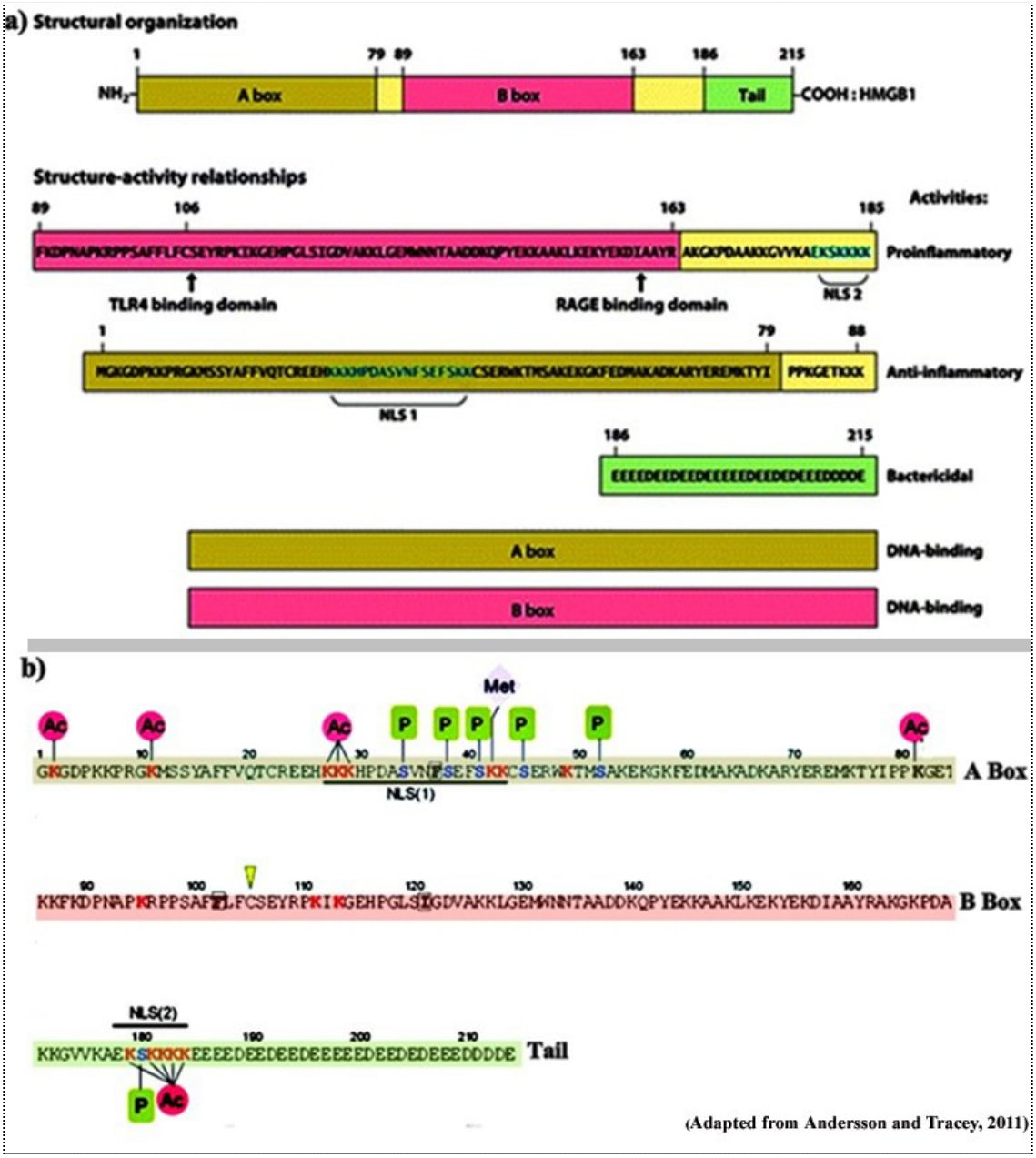


Figure1.1: Domain structure of HMGB1 and post-translational modifications. Structure of human HMGB1, a 25-kDa protein of 215 amino acids. HMGB1 has three domains A box, B box (which are positively charged DNA-binding structures) and a negatively charged acidic tail (composed of 30 glutamic and aspartic acid)(Andersson and Tracey, 2011).

1.1.2 Post-translational modifications

Besides its native form HMGB1 can exist in several different forms or conformations generated through post-translational modifications. HMGB1 actively secreted from monocytes can be acetylated in all positions, while protein derived from necrotic cells can be acetylated on lysines at positions 2 and 11, segments 27-43 and 178-184 are affected particulary, yielding 10 isoforms (at least) of the protein (Bonaldi *et al.*, 2003). These two positively charged segments act as nuclear localization signals (NLSs) and neutralization of these NLSs results in relocation of HMGB1 from the nucleus into the cytoplasm. Moreover, phosphorylated HMGB1 is also translocated into the cytoplasm and prevented from reentering the nucleus. However, phosphorylated HMGB1 has not been demonstrated extracellularly (Youn and Shin, 2006). Lastly, mono-methylation of HMGB1 on lysine 42 can occur in neutrophils. This methylated HMGB1 can be both relocated into the cytoplasm and secreted (Ito *et al.*, 2007). All these modifications diminish HMGB1-chromatin interactions but it is not clear whether they are required for lysosomal and plasma membrane passage. HMGB1 from metabolically stressed cells can also be translocated to the cytoplasm without undergoing acetylation or any other known modification (Hamada *et al.*, 2008).

1.1.3 HMGB1 as Nuclear protein

In the nucleus, HMGB1 plays an important role in transcription regulation, modifying the structure of DNA and stabilizing nucleosomes (Bianchi and Beltrame, 2000; Bustin, 1999). HMGB1 binds the minor groove of DNA without sequence specificity and induces bends, but has a preference for binding sharply bent structures (Bianchi et al., 1989). This binding of HMGB1 to DNA facilitates physical interactions between DNA and transcription factors, including p53, homeobox-containing proteins, steroid hormone receptors and recombination activating gene 1/2 (RAG1/2) proteins which are needed for VDJ recombination in T and B lymphocytes (Brickman et al., 1999; Mouri et al., 2008). There is one intriguing report about HMGB1 transactivating the human IL1-β gene promoter through association with an Ets transcription factor (Mouri et al., 2008). There is also one report demonstrating that HMGB1 may directly bind to the RRS sequence in the TNF promoter in osteoclasts to activate TNF

transcription (Yamoah *et al.*, 2008). Binding of HMGB1 to undamaged DNA is a rapid and transient process, while HMGB1 binds tightly to sites of distorted DNA (Agresti *et al.*, 2003). HMGB1 constantly shuttles within the nucleus and between the nuclear and cytoplasmic compartments (Bustin and Neihart, 1979). HMGB1 has two nuclear localisation signals that direct the protein to the nucleus and can in addition bind to calmodulin which also can target HMGB1 to the nucleus (Bonaldi *et al.*, 2003; Hanover *et al.*, 2007; Youn and Shin, 2006). Export of HMGB1 from the nucleus to the cytoplasm is independent of protein synthesis and is mediated by the chromosome region maintenance 1 protein (CRM1)(Tang *et al.*, 2007; Youn and Shin, 2006). The nuclear function of HMGB1 has been demonstrated to be essential to life since HMGB1 knockout mice die 24-48 hours after birth due to hypoglycemia (Calogero *et al.*, 1999). Phenotypic features include ruffled, small size and disorganised fur, absence of fat and long hind paws. Cells lines deficient in HMGB1 have an abnormal gene expression of different genes such as the glucocorticoid receptors, but display a normal growth (Calogero *et al.*, 1999).

1.1.4 Cytoplasmic role of HMGB1

HMGB1 plays an important role in migration, interaction with RAGE mediating cellular neurite outgrowth and tumor formation (Huttunen *et al.*, 2002a; Rauvala and Pihlaskari, 1987). HMGB1 added to normal and dystrophic mouse muscles attracts mesoangioblasts, further supporting a role for HMGB1 as a chemoattractant (Palumbo *et al.*, 2004). In addition, HMGB1 promotes angiogenesis, the process leading to formation of new blood vessels during development, growth, tissue repair and tumor growth (Chavakis *et al.*, 2007; Mitola *et al.*, 2006; Schlueter *et al.*, 2005). HMGB1 have been placed in the antibacterial barrier defence system, as HMGB1 in purified form (isolated from human adenoid glands) eliminates bacteria within a few minutes in cultures (Zetterstrom *et al.*, 2002).

1.1.5 Release of HMGB1

HMGB1 is constitutively expressed in quiescent cells and commonly stored in the nucleus, because it contains two lysine-rich nuclear localization sequences that direct the protein to the nucleus (Bonaldi *et al.*, 2003). HMGB1 release into extracellular milieu is a prerequisite to exert its inflammatory effects. Levels of HMGB1

significantly increase in serum of patients with sepsis (Wang *et al.*, 1999a). In healthy animals and normal human subjects, HMGB1 is present at an undetectable plasma level of 5 ng/ml, but HMGB1 increase to an average of 25.2 and 83.7 ng/ml in survivors and non-survivors in septic patients, respectively (Wang *et al.*, 1999a). Several studies also show after treatment with endotoxin or various proinflammatory cytokines such as TNF-α, IL-1β, or IFN-γ that HMGB1 is released from activated monocytes/macrophages more than 8 h and reaches a plateau in expression levels around 18–24 h (Andersson *et al.*, 2000; Wang *et al.*, 1999a; Wang *et al.*, 1999b; Youn *et al.*, 2008). Despite its clinical importance, the exact mechanism of HMGB1 release has largely remained unknown, but some progress has been made. HMGB1 can be released from cells in either active or passive way.

1.1.5.1 Active secretion of HMGB1

Intriguingly, HMGB1 lacks a classic leader peptide and does not travel through the endoplasmic reticulum and the Golgi apparatus, but large amounts of HMGB1 are released into the extracellular space by activated monocyte/macrophages (Degryse *et al.*, 2001). Recent evidences suggest that the secretion of HMGB1 involve at least three steps : (a) exit from the nucleus into the cytoplasm, (b) translocation from the cytosol into cytoplasmic organelles, and (c) exocytosis (Gardella *et al.*, 2002). Macrophages/monocytes activated by endotoxin or various proinflammatory cytokines acetylate HMGB1 at lysine-rich nuclear localization sequences, leading to translocation of nuclear HMGB1 into cytoplasmic vesicles and subsequent release into the extracellular milieu (Bonaldi *et al.*, 2003; Gardella *et al.*, 2002; Nickel, 2003; Rendon-Mitchell *et al.*, 2003). LPS and TNF-α stimulate macrophages/monocytes to release HMGB1 through different pathways. LPS stimulates macrophages to release HMGB1 by hyper-acetylation partly through CD14- and TNF-dependent pathway (Chen *et al.*, 2004; Youn and Shin, 2006) and IFN-β-mediated JAK/STAT pathway (Kim *et al.*, 2009). Other studies revealed HMGB1 needs to be phosphorylated for secretion, and HMGB1 is phosphorylated by the classical protein kinase C(cPKC) and is secreted by a calcium-dependent mechanism in LPS-stimulated monocytes/macrophages (Oh *et al.*, 2009). However, TNF-α stimulates macrophages to secrete HMGB1 through phosphorylation (Wang *et al.*, 1999a; Youn and Shin, 2006). HMGB1 levels in serum begin to increase in a delayed manner compared with

the early mediators of endotoxin such as TNF-α and IL-1β. Studies show HMGB1 is secreted from not only activated macrophages and monocytes, but also from NK cells, DCs, and endothelial cells, neurons, smooth-muscle cells, osteoclasts, and intestinal epithelial cells (Lotze and Tracey, 2005). HMGB1 secretion can be induced in the pituicyte in response to IL-1 or TNF-α stimulation (Wang *et al.*, 1999b). Enterocytes secret HMGB1 following stimulation with proinflammatory cytokines (Liu *et al.*, 2006). Hepatocytes also can secrete HMGB1 in hypoxic conditions and involve Toll-like receptor 4 dependent reactive oxygen species production and calcium-mediated signaling (Tsung *et al.*, 2007).

1.1.5.2. Passive release of HMGB1

Not only can cells actively secrete HMGB1 in response to proinflammatory stimuli, but cells undergoing unprogrammed cell death can passively release HMGB1 also, which aggravate and prolong inflammation (Scaffidi *et al.*, 2002). HMGB1 is bound loosely to the chromatin in whole cell cycle (both interphase and mitosis), and is leaked into the medium when membrane integrity is lost in necrotic or permeabilized cells (Falciola *et al.*, 1997; Kokkola *et al.*, 2002; Muller *et al.*, 2001). Necrotic cells from $Hmgb1^{-/-}$ mice have a greatly reduced ability to promote inflammation, proving necrotic cells release HMGB1 that mediate inflammation (Kokkola *et al.*, 2002). In contrast, apoptotic cells do not release HMGB1 even after undergoing secondary necrosis and partial autolysis, and thus fail to trigger inflammation even if not cleared promptly by phagocytic cells (Scaffidi *et al.*, 2002).

In apoptotic cells, HMGB1 is bound tightly to chromatin because HMGB1 is not acetylated, and not released into extracellular milieu (Bustin, 2002; Scaffidi *et al.*, 2002). However, recent studies show that apoptotic cell can passively release HMGB1 at least in some cell types and likely occur during late apoptosis (Bell *et al.*, 2006; Jiang *et al.*, 2007), indicating that the original dichotomy between necrosis and apoptosis may be incorrect. Wang and co-workers also found apoptotic cells can stimulate macrophages to release HMGB1 in mice with severe sepsis and splenectomy protects against sepsis lethality by reducing serum HMGB1 levels (Huston *et al.*, 2008; Qin *et al.*, 2006).

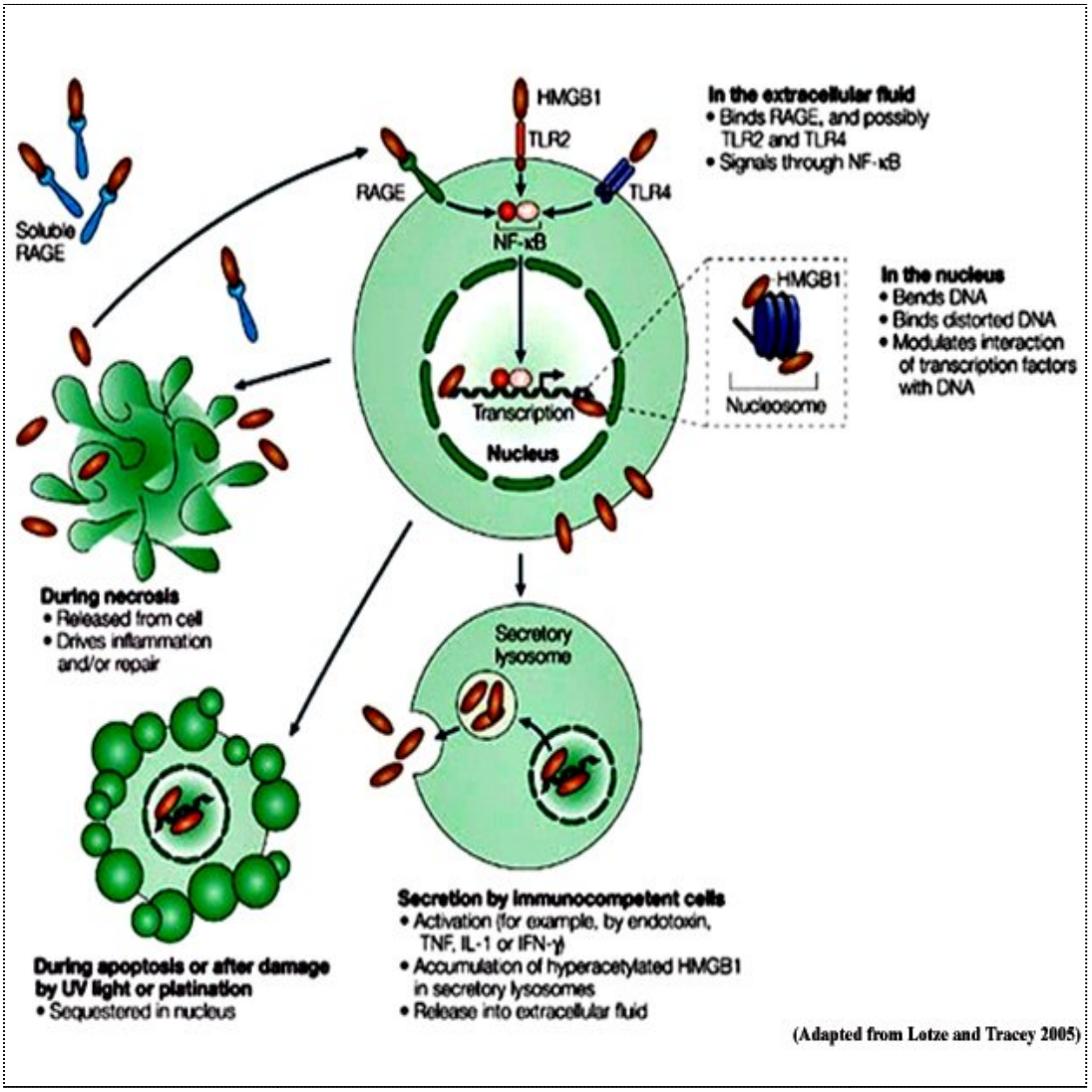


Figure 1.2: HMGB1 release. HMGB1 is associated loosely and transiently with nucleosomes. Extracellular HMGB1 signals through TLR4, TLR2 and RAGE activating various pathways involving nuclear factor-κB (NF-κB) and the mitogen-activated protein kinase (p38). HMGB1 is secreted by immune cells and released by bacterial products, such as endotoxin and/or pro-inflammatory cytokines, such as interleukin-1 (IL-1β), tumour-necrosis factor (TNF-α) and interferon-γ (IFN-γ).

1.1.6 Receptors of HMGB1

The first described receptor for HMGB1 was the receptor for advanced glycation end products (RAGE) (Parkkinen *et al.*, 1993). However, since anti-RAGE antibodies only partially suppressed the activity of HMGB1 and RAGE-deficient cells were shown to still be able to respond to HMGB1 stimulation, RAGE is not believed to be the only receptor for HMGB1 (Li *et al.*, 2003). Recently, toll-like receptors (TLR) 2 and 4 were suggested to interact with HMGB1 (Apetoh *et al.*, 2007; Park *et al.*, 2006; Park *et al.*, 2004). RAGE belongs to the immunoglobulin (Ig) superfamily and comprises of three extracellular immunoglobulin domains, a single trans-membrane segment and a short cytoplasmic tail. RAGE- deficient mice are viable and less susceptible to sepsis (Liliensiek *et al.*, 2004). As a pattern recognition receptor it interacts with several ligands such as amyloid-β, multiple members of the S100 protein family and advanced glycation end products (AGEs) (Bierhaus *et al.*, 2005). RAGE is only highly constitutively expressed in lung and skin tissues, but can be up-regulated in almost every tissue (Shirasawa *et al.*, 2004).

Binding of HMGB1 to RAGE has two main consequences: activation of intracellular signal transduction through mechanisms involving Cdc42 and Rac, guanosine triphosphatases (GTPase) that regulate cell motility and neurite outgrowth, and the other pathway activates mitogen-activated protein kinases (MAPKs) and nuclear factor kappa B (NF-κB) (Merenmies *et al.*, 1991; Taguchi *et al.*, 2000). In macrophages, Caco-2 epithelial cells and neutrophils the MAPKs activated by HMGB1-RAGE interaction are ERK1/2, p38 and p42/44 kinases and stress-activated protein kinase/c-Jun N-terminal kinase (Huttunen *et al.*, 2002a; Park *et al.*, 2003; Sappington *et al.*, 2002). Smooth muscle cell migration mediated by HMGB1 involves MAPK pathways and a G-protein-coupled receptor (Degryse *et al.*, 2001). The only known adaptor molecules binding to the cytoplasmic tail of RAGE are ERK 1/2 and diaphanous-1 (Ishihara *et al.*, 2003). A soluble isotype splice form called sRAGE exists. The function of this isoform is incompletely understood but does provide protective effects in many diseases. An HMGB1-dependent activation and recruitment of neutrophils has been demonstrated to require interplay between RAGE and Mac-1 (Orlova *et al.*, 2007), indicating the ability of HMGB1 to interact with other immunostimulatory molecules in order to amplify their activity. HMGB1 has

been described, as mentioned above, to be a ligand of TLRs. The binding of HMGB1 to TLR leads to activation of downstream signalling cascades ultimately leading to NFκB nuclear translocation leading to e.g. transcription of inflammatory genes and DC maturation (Takeda *et al.*, 2003).

1.1.7 HMGB1 as proinflammatory cytokine

Several proinflammatory activities of HMGB1 have been revealed from studies of isolated cell cultures. A picture has emerged of HMGB1, when released into the extracellular milieu show significantly proinflammatory functionality. The HMGB1 response participates in the mediation of down-stream pathophysiological responses in infectious and non-infectious inflammatory diseases characterized by a significantly delayed kinetic response relative to TNF and IL-1.

The first description of a specific proinflammatory cytokine activity mediated by HMGB1 was revealed from studies of the biological response of monocyte/macrophage cultures to HMGB1 (Andersson *et al.*, 2000). Addition of HMGB1 to macrophage cultures significantly stimulates the release of TNF, IL-1, IL-6 and nitric oxide (Andersson *et al.*, 2000). The stimulation of macrophages occurs at extremely low concentrations of HMGB1, indicating that HMGB1 is the most potent endogenous stimulator of TNF release yet described. Addition of highly purified recombinant HMGB1 to either human monocytes/macrophages, primary murine macrophages or macrophages derived from C3H/HeJ mice leads to significant increases in TNF release over a 12 h interval. Maximal increases in TNF mRNA are not achieved until 8 h, compared with endotoxin stimulation in which TNF mRNA levels are typically increased within 30 min. This uniquely delayed kinetic attribute of HMGB1 as a proinflammatory stimulus suggests that it occupies a unique position in the cytokine cascade and can function in a feed-forward mechanism to promote downstream inflammation. HMGB1-mediated signal transduction in monocytes/macrophages appears to be partially dependent on interaction with RAGE, a membrane receptor for advanced glycation end-products, S100, β-amyloid fibrils and HMGB1. Activation of RAGE by ligand is associated with stimulation of signal transduction through p21ras, NF-κB, Cdc42, Rac and MAP kinases (Bucciarelli *et al.*, 2002).

HMGB1 has shown proinflammatory activities in other cells also. Addition of HMGB1 to neutrophils activates the nuclear translocation of NF-κB and stimulates the release of proinflammatory cytokines (Park *et al.*, 2003). HMGB1 signal transduction in human neutrophils activates the p38 MAP kinase, phosphatidylinositol 3-kinase/Akt and ERK1/2 pathways. The kinetics of neutrophil activation by HMGB1 differs significantly to that by endotoxin: neutrophils release TNF within 60 min after addition of HMGB1, whereas maximal TNF mRNA levels in endotoxin-stimulated neutrophils occur 4 h after addition of LPS (Park *et al.*, 2003). Thus, early neutrophil responses to HMGB1 stand in stark contrast to the monocyte/macrophage response to HMGB1. In the former case, HMGB1 mediates an acute or early proinflammatory response, whereas in the latter case, HMGB1 signaling occurs significantly later or downstream compared with classic endotoxin-induced responses.

The proinflammatory activity of HMGB1 on epithelial cells was revealed by adding recombinant HMGB1 to cultured human enterocytic monolayers (Sappington *et al.*, 2002). HMGB1 mediates an increase in epithelial permeability of monolayers that is dependent on increased inducible nitric oxide synthase (iNOS) expression. Inhibition of signaling through RAGE abrogates approximately 50% of the HMGB1-mediated barrier dysfunction in these epithelial cells, suggesting that HMGB1/RAGE signaling contributes at least partially to the epithelial responses to HMGB1. Inhibition of nitric oxide synthase activity, or scavenging of peroxynitrite, significantly inhibits HMGB1-induced epithelial barrier failure (Sappington *et al*., 2002). Systemic administration of HMGB1 to mice significantly increases mucosal permeability and bacterial translocation to mesenteric lymph nodes, but mice deficient in iNOS are protected from HMGB1-mediated barrier dysfunction, indicating that HMGB1 mediates epithelial gut barrier failure through increased nitric oxide production.

HMGB1 activates cytoskeleton reorganization in rat smooth-muscle cells, stimulates chemotaxis and increases chemokinesis (Degryse *et al.*, 2001). Smooth-muscle cells exposed to recombinant HMGB1 develop a polarized morphology that is typical of mobile cells. Smooth-muscle cell migration mediated by HMGB1 is abrogated by antibodies against RAGE, and is also inhibited by addition of either pertussis toxin or the MAP kinase inhibitor PD9805922. In smooth-muscle cells, the HMGB1 A box and the HMGB1 B box each stimulated cell migration, suggesting that the

proinflammatory and anti-inflammatory activities of these protein domains can be disassociated from their effects on stimulating chemotaxis. Significant binding of HMGB1 to the surface of smooth-muscle cells occurs in association with expression of RAGE on the plasma membrane.

Endothelial cells, which also express RAGE, respond to recombinant HMGB1 and exhibit a time- and dose-dependent increase in expression of vascular cell adhesion molecule-1 (VCAM-1), RAGE and intercellular adhesion molecule-1 (ICAM-1) (Fiuza *et al.*, 2003). Other proinflammatory cellular endothelial responses to HMGB1 include increased release of TNF, IL-1, IL-8, monocyte chemottractant protein 1 (MCP-1), plasminogen activator inhibitor-1 and tissue plasminogen activator. Anti-TNF antibodies partially inhibit the HMGB1-mediated stimulation of endothelial cells. HMGB1-mediated signal transduction in these cells is in part dependent on activation of MAP kinases, extracellular signal related kinases and Jun N-terminal kinases (Fiuza *et al.*, 2003). When considered with the previously discussed data indicating that HMGB1 can activate neutrophils, monocytes and smooth-muscle cells, it now appears that the release of HMGB1 into the extracellular milieu can drive a proinflammatory endothelial immune cell response.

1.2 SEPSIS

Sepsis syndrome has been differently defined over the years and in order to better characterise the pathology, various definitions are used.

The definitions currently used are as follows:

- Infection is a pathologic process, occurs when pathogenic or potentially pathogenic microorganisms invade normally sterile tissue, body cavity or fluid.
- Sepsis is said to occur when suspected or documented infection is associated with systemic inflammatory syndrome signs (any one).
- Severe Sepsis is said to have occurred when sepsis is complicated by pre-defined organ dysfunction.

- Septic Shock is said to have occurred when sepsis induced ACF (acute circulatory failure) is characterized by persistent arterial hypotension despite adequate volume resuscitation and not explained by causes other than sepsis.

Organ dysfunction can be defined as acute lung injury; coagulation abnormalities; thrombocytopenia; altered mental status; liver, renal or cardiac failure; or hypoperfusion with lactic acidosis.

Sepsis is an aggressive and multifactorial disease state that has been ranked as the tenth-leading cause of death in the US (Hoyert *et al.*, 2001). Morbidity and mortality remain unacceptably high despite increasing knowledge about the pathophysiological pathways and processes involved in sepsis. Mortality rates from severe sepsis are on a similar scale to breast, lung and colon cancer, and it is one of the leading causes of death in the intensive care unit (ICU) (Bone *et al.*, 1992; Kanji *et al.*, 2001). Due to its multifactorial and aggressive nature, sepsis is a rapid killer. The mortality rates of septic shock could be as high as 50% or up to 75% on longer follow-ups, even with the best treatment available (Angus *et al.*, 2001; Angus *et al.*, 2006; Balk, 2000; Quartin *et al.*, 1997; Rangel-Frausto *et al.*, 1995; Silva *et al.*, 2004). Death is common among sepsis patients, with around 30% of patients dying within the first month of diagnosis and 50% dying within 6 months (Bernard *et al.*, 2001; Natanson *et al.*, 1998; Rivers *et al.*, 2001).

1.2.1 Endotoxin and Sepsis: Connection

Circulating endotoxin appears to be present in most patients who meet classical clinical criteria for sepsis (Marshall *et al.*, 2004; Opal *et al.*, 1999), however not all (Bates *et al.*, 1998; Danner *et al.*, 1991). This discrepancy reflects, in part, the well-recognized limitations of the Limulus amebocyte lysate assay for endotoxin in protein-containing specimens, but also an important clinical reality: endotoxin is present in some, but not all patients with sepsis, as well as in many patients with acute life-threatening illnesses that would not meet the criteria for sepsis. Moreover, it does not necessarily follow that the simple presence of circulating endotoxin constitutes an adequate rationale for its elimination.

Endotoxemia has been demonstrated in a variety of clinical settings, including following cardiopulmonary bypass (Riddington *et al.*, 1996), in patients with congestive heart failure (Niebauer *et al.*, 1999), in chronic renal failure (Goncalves *et al.*, 2006), in cirrhosis (Lin *et al.*, 2007) and in patients with a ruptured abdominal aortic aneurysm (Roumen *et al.*, 1993). While endotoxemia is prominent in critically ill patients with sepsis, it is also detectable in more than half of all ICU patients on the day of ICU admission, even though the majority of these patients do not meet sepsis criteria (Marshall *et al.*, 2004).

Endotoxemia is a purely pathologic state, as shown by antiendotoxin therapies, which showed reproducible evidence of benefit when they are employed in disease processes such as sepsis in which endotoxemia is common. Early studies supported this hypothesis. Ziegler and colleagues (Ziegler *et al.*, 1982), for example, showed that neutralizing endotoxin with an antiserum resulted in improved survival for patients with Gram-negative infections, and particularly for those in shock. A monoclonal antibody directed against endotoxin from a mutant strain of Escherichia coli showed similar promise of efficacy in a multicenter study of 543 patients (Ziegler *et al.*, 1991), an effect, however, that was not replicated in a subsequent larger study (McCloskey *et al.*, 1994). Similarly the extracorporeal removal of endotoxin using a polymyxin B column has shown evidence of efficacy in pooled data from a number of small trials that recruited an heterogeneous population of patients (Cruz *et al.*, 2007), as well as in a study of patients with severe intra-abdominal infections (Cruz *et al.*, 2009). There is, however, evidence that neutralization of endotoxemia may not always be beneficial. The lack of efficacy apparent in a number of recent studies using a variety of approaches to neutralize endotoxin in patients with sepsis (Angus *et al.*, 2000; Dellinger *et al.*, 2009) may be explained by shortcomings of the intervention, suboptimal dosing or a low prevalence of endotoxemia in the target population. Elevated levels of circulating endotoxin can cause a syndrome that bears most of the features of clinical sepsis , and the acute administration of a large amount can result in organ dysfunction. But endotoxemia, rather than sepsis, is the specific therapeutic target, and the unanswered challenge remains to determine in which patients with endotoxemia will intervention be beneficial.

1.2.2. LPS and other sepsis triggers

Sepsis can be caused by infection with Gram-positive bacteria, Gram-negative bacteria, viruses fungi, or (and particularly *Candida*). Sepsis may also occur in the absence of any detectable bacterial invasion, and in these cases the sepsis is initiated and mediated by microbial toxins, particularly Gram-negative bacteria endotoxin (lipopolysaccharide), and endogenous cytokine production. Although activation of the immune system during microbial invasion is generally protective and to curb infection, development of septic shock in a significant number of patients is a consequence of a poorly regulated immune response to the offending organism.

The driving force of the immune system is the need to recognize danger, while the goal is to respond to the dangers threatening the organism. Anything which causes tissue stress or damage is defined as danger by the immune system. PAMPs (Pathogen-associated molecular patterns) like LPS, being exogenous molecules derived from micro-organisms can activate immune cells. Besides pathogen-associated molecular patterns, endogenous alarmins can also activate cells like macrophages and neutrophils (immune competent cells). Alarmins are molecules produced in stressed or damaged tissues in connection with trauma, burn, ischaemia, haemorrhage, or other conditions of altered homeostasis like (i.e. high mobility group box-1, heat shock proteins, S100 proteins, hyaluran etc.). Danger-associated molecular patterns (DAMPs) include both PAMPs and alarmins.

Inflammation is the primary response to DAMP. The activation of immune cells is a prerequisite for initiation of inflammation, which includes inflammation inducers, sensors, mediators and effectors. Pattern-recognition receptors (PRR) recognize both alarmins and PAMP which are conserved motifs expressed by pathogens, but are absent in humans. PRR trigger the production of inflammatory mediators which may alter the function of tissues and organs. Alarmins induce SIRS but sepsis involves suspected or confirmed infection.

A vigorous innate immune response is now recognized as a double-edged sword, with a crucial role in defending the host through activation of antimicrobial defences, and yet, if left unchecked, the same system contributes to systemic inflammation, tissue injury, intravascular coagulation, and death caused by severe sepsis.

The initiation of the host response during sepsis or tissue injury involves three families of pattern recognition receptors (PRRs): 1) toll-like receptors (TLRs); 2) nucleotide-oligomerization domain leucine-rich repeat (NOD-LRR) proteins; and 3) cytoplasmic caspase activation and recruiting domain helicases such as retinoic-acid-inducible gene I (RIG-I)-like helicases (RLHs) (Creagh and O'Neill, 2006; Uematsu and Akira, 2007). These receptors initiate the regulate the adaptive immune response and innate immune response to tissue injury or infection. Gram-positive and Gram-negative bacteria, parasites, viruses, and fungi all possess a limited number of unique cellular constituents, which are not found in vertebrate animals. These elements are now referred to as pathogen activated molecular patterns (PAMPs), or more appropriately microbial associated molecular patterns, as these molecules are also common in commensal and nonpathogenic bacteria (Granucci *et al.*, 2005). pathogen activated molecular patterns (PAMPs) bind to pathogen recognition receptors (PRRs), such as TLRs which are expressed on the surface of host cells (almost all). Cytoplasmic pathogen recognition receptors (PRRs) detect invasive intracellular pathogens (Liew *et al.*, 2005). The NOD proteins recognize common fragments of bacterial peptidoglycan, muramyl dipeptide from peptidoglycan is the ligand for NOD2 and diamino-pimelate from Gram-negative bacteria is the ligand for NOD1 in the cytosol. The PRRs also recognized damage signals from the release of endogenous peptides and glycosaminoglycans from apoptotic or necrotic host cells (Akira *et al.*, 2006; Bianchi, 2007; Mollen *et al.*, 2006).

TLR expression is significantly upregulated in experimental models of sepsis and in patients with sepsis (Armstrong *et al.*, 2004; Tsujimoto *et al.*, 2004; Tsujimoto *et al.*, 2005; Williams *et al.*, 2003). Trauma including thermal injury generates danger-associated molecular patterns (DAMPs) that augment TLR expression like PAMPs. It also primes or initiates the innate immune system for enhanced TLR reactivity, resulting in excess LPS-induced mortality (Paterson *et al.*, 2003). Multiple positive feedback loops between PAMPs and danger associated molecular patterns (DAMps), and their overlapping receptors PRRs, temporally and spatially drive these processes. It may also represent the molecular basis for the observation that infections, and also nonspecific stress factors, can trigger excessive systemic inflammatory response.

1.2.3. LPS Signaling

LPS is an important structural component of the membrane of Gram-negative bacteria (outer membrane). The interaction of LPS with immune cells leads to the formation and release of a large spectrum of inflammatory mediators which are essential for the early innate and subsequent adaptive anti-bacterial defense. However, it can also lead to a fatal septic syndrome if the inflammatory response is amplified and uncontrolled (Beutler and Rietschel, 2003).

LPS consists of three parts: core oligosaccharide, lipid A and O side chain. Lipid A moiety of LPS is the minimal fragment that triggers the cellular response, so called as 'endotoxin principle'. Lipid A is composed of phosphorylated (1,6 linked) D-glucosamine disaccharide that carries upto six acyl residues. Although structurally less variable but variations exists in length, position and number of fatty acids. Minimal requirement for bioactivity is lipid A of E.coli having six fatty acids, two Gluco-configurated hexosamine residues and two phosphoryl groups. Deficiency of any of the components results in decrease of the activity. However the requirement differs from species and also depends on the subtype of Toll-like receptor 4.

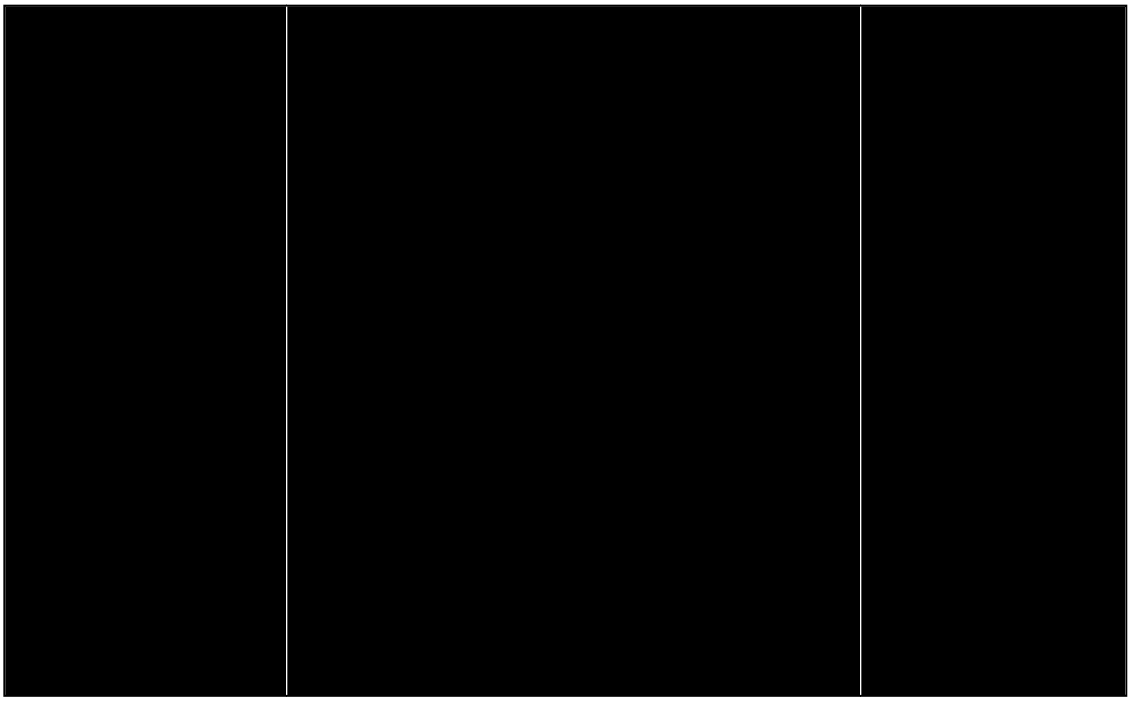


Figure 1.3: Recognition of LPS by CD14 and MD-2. LPS initially recognized by LBP, eventually leads to formation of TLR4-MD-2-LPS complex via CD14 on membrane, resulting in dimerization of TLR4 subunits triggering TLR4 pathway.

LPS is bipolar macromolecule that contains both hydrophobic and hydophillic elements. Lipid A core structure contains hydrophobic and repeating polysaccharide surface components contain hydrophilic elements. LPS because of its bipolar nature forms microaggregates in blood and then interacts with a variety of serum or membrane bound lipophilic proteins. Receptors of LPS recognized in human cells are CD14-MD2-TLR4 molecules (soluble or membrane-bound), CD11/CD18 molecules and scavenger receptors for lipid molecules. Even small quantities of LPS show a significant response because of soluble and membrane bound CD14 which potentiate the host response (Hoffmann *et al.*, 1999). In human plasma and other body fluids, LPS trafficking is facilitated by a hepatically derived, acute phase plasma protein known as LBP (LPS-binding protein) (Opal *et al.*, 1994). LBP performs a shuttle service picking up polymeric LPS aggregates and transferring LPS monomers to CD14. LPS competes with another neutrophil-derived LPS-binding molecule known as bactericidal/permeability-increasing protein (BPI). Despite 45% primary amino acid sequence homology of BPI with LBP, BPI specifically antagonizes the actions of BPI. LBP assists in the delivery of LPS to immune effector cells while BPI inhibits LPS delivery to CD14. The relative concentrations of these two LPS-binding proteins primarily determine the net effect of LPS release (Levin *et al.*, 2000; Opal *et al.*, 1994). CD14 is a glycosyl phosphatidylinositol-linked protein found primarily on the cell surfaces of myeloid cells. After docking to membrane-bound CD14, LPS is delivered to an essential extracellular adaptor protein known as MD2 (Akira *et al.*, 2006; Lynn *et al.*, 2003; Nagai *et al.*, 2002). This LPS–MD2 complex is then presented to the extracellular leucine-rich domain of TLR4 where multimers of this complex aggregate on lipid rafts on the cell surface. This series of events then triggers a signal to the intracellular space, subsequently activating Lipolpolysaccharide-responsive genes. CD14 also binds to bacterial lipopeptides and peptidoglycan and delivers these microbial ligands to TLR2 for intracellular signaling.

LPS induces dimerization of TLR4/MD-2 complex however the mechanism is not so clear because TLRs have been refractory to crystallographic analysis. However recently *Kim et al* proposed a model for LPS induced TLR4/MD-2 dimerization. The crystal structure (using hybrid LRR technique) of TLR4 as TLR4-MD-2 and TLR4-MD-2-eritoran complex showed that othe two TLR4 molecules form a ‘‘m’’-shaped complex with binding MD-2 in the two hooks. MD-2-eritoran complex failed to activate the dimerization of TLR4 but eritoran showed no contact with TLR4. Although TLR4-MD-2 still remains to be cocrystallized with its agonist ligand LPS, mutational studies however have shown that residues of MD-2 that are not involved in direct contact with either LPS or TLR4 play a key role in homodimerization of the TLR4-MD-2 complex in the presence of LPS. Lipolpolysaccharide, is thus proposed to induce conformational changes in MD-2 that promotes interaction between MD-2 and the central and/or C-terminal domain of the second TLR4. This model is supported by recent studies.

Once TLR4 binds to its LPS ligand two possible pathways of cellular activation can occur through either the myeloid differentiation factor 88 (MyD88) or the TLR domain adaptor inducing interferon-β, (TRIF) pathway (Beutler and Rietschel, 2003). A series of signaling events occur with sequential activation of specific tyrosine and threonine/ serine kinases. This signaling cascade ultimately leads to ubiquitination, phosphorylation, and degradation of inhibitory κB (IκB) along with other transcriptional activators. IκB degradation releases nuclear factor κB (NF-κB) to translocate into the nucleus. Clotting elements, complement, other acute phase proteins, cytokines, chemokines, and nitric oxide synthase genes have NFκB-binding sites at their promoter regions. The uncontrolled release of inflammatory cytokines and other inflammatory mediators after LPS exposure contributes to generalized inflammation (Calvano *et al.*, 2005; Reitsma *et al.*, 2003; Suntharalingam *et al.*, 2006).

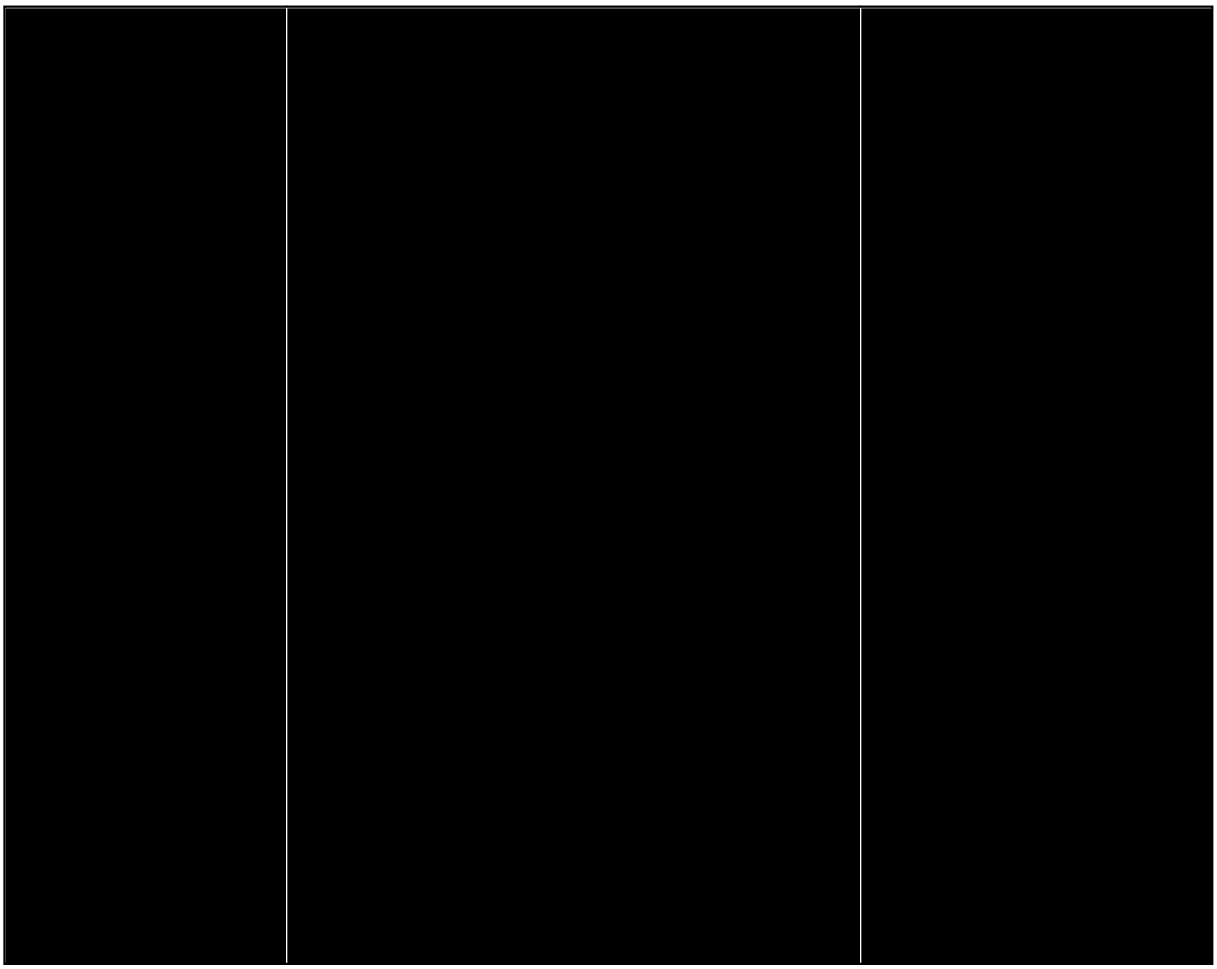


Figure 1.4: TLR4 Pathway. Dimerization of TLR4 leads to cascade of pathway leading to activation of NF-κB, AP-1 and IRF3 via MyD88 dependent and TRIF dependent pathway.

1.2.4. Pathophysiology of sepsis

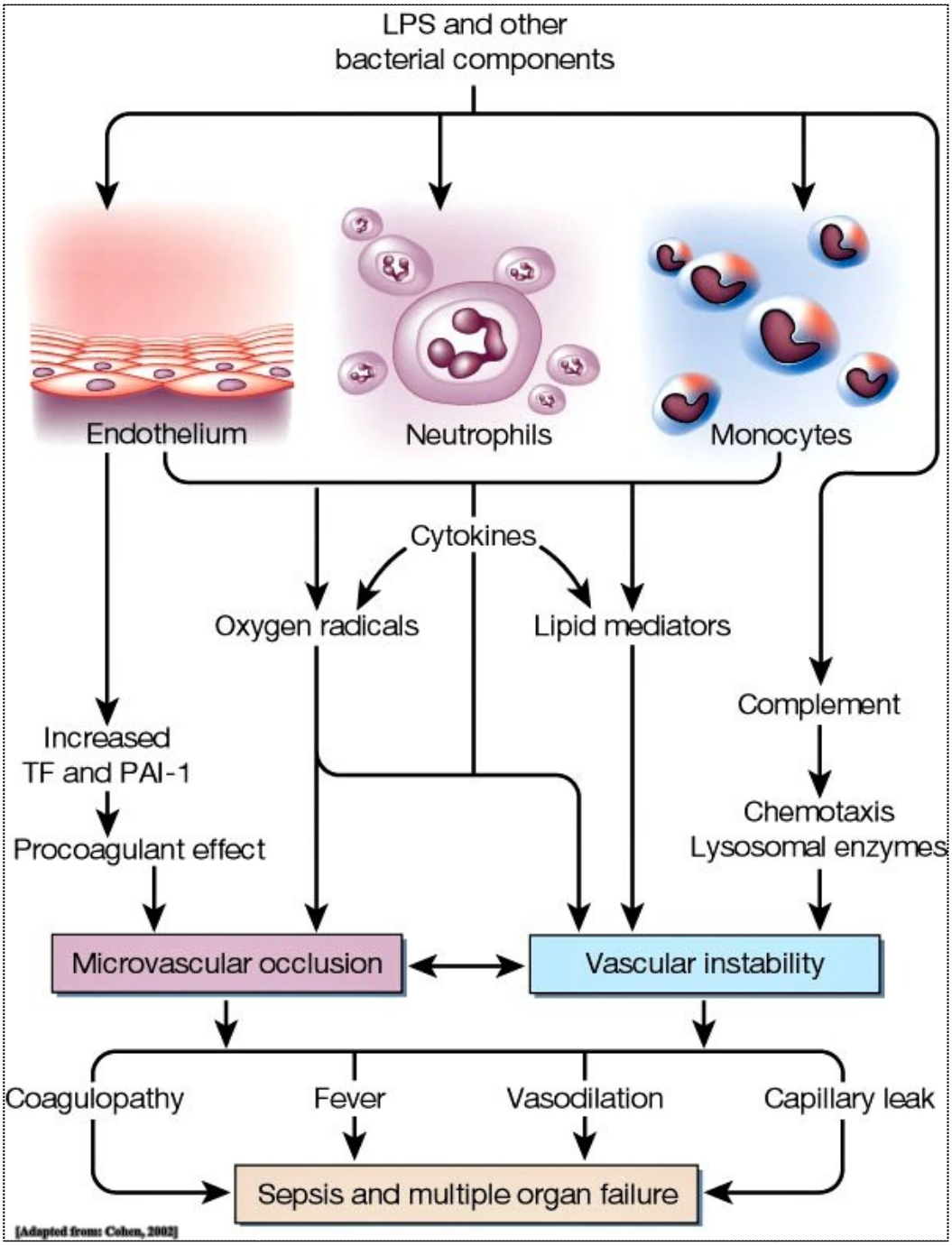


Figure 1.5: Pathophysiology of Sepsis/shock. Lipopolysaccharide (LPS) and other PAMPs simultaneously activate multiple parallel cascades that contribute to the pathophysiology of Sepsis/shock. The combination of impaired peripheral vascular tone , poor myocardial contractility, and microvascular occlusion leads to inadequate oxygenation, and tissue hypoperfusion and thus to organ failure/dysfunction.

1.2.4.1. Role of Cytokines

Cytokines are a key element in the inflammatory response that characterizes sepsis and septic shock. They are immunoregulatory peptides with a potent inflammatory or anti-inflammatory action, mediating the immune/metabolic response to an external noxious stimulus and fueling the transition from sepsis to septic shock, multiple organ dysfunction syndrome, and/or multiple organ failure. Synergistic interactions between cytokines can cause or attenuate tissue injury (Calandra *et al.*, 2002; Casey, 2000). Cytokines may be divided into pro-inflammatory (such as tumor necrosis factor [TNF-α], IL-1 and IL-8) and anti-inflammatory (such as IL-10). An infectious or inflammatory trigger, such as a microbial toxin, stimulates the macrophages that produce in response large amounts of TNF-α, IL-1, and IL-6. TNF-α is one of the most important cytokines involved in the pathophysiology of sepsis and is released early in the process of sepsis. TNF-α induced tissue injury is largely mediated through neutrophils, that respond by producing superoxide ion, hydrogen peroxide, elastase, leukotriene B4, sPLA2, platelet-activating factor (PAF), and thromboxane A2 (Aldridge, 2002). IL-1β stimulates the synthesis and release of prostaglandins (PGs), collagenases and, elastases promotes transendothelial migration of neutrophils and activates endothelial microvascular cells, which respond by releasing PAF and IL-8 (both of which are powerful neutrophil-stimulating agents). TNF-α and IL-1 are synergistic and share many biological effects in sepsis. Their inhibition improves survival and organ function in animal models of sepsis (Herbertson *et al.*, 1995). The controversial role of IL-6 is proved by various studies. Some consider IL-6 as an anti-inflammatory cytokine; however, some studies show that IL-6 augments the cytotoxic potential of neutrophils via selective increase of elastase release (Johnson *et al.*, 1998). IL-6 formation is attenuated by the production of either TNF-α or IL-1. It is possible that IL-6 is toxic only when it is produced with other cytokines (synergistic action of cytokines) (Casey, 2000). IL-8, a neutrophil chemotactic cytokine, is involved in the process of tissue inflammation (Baggiolini *et al.*, 1994), it along with other neutrophil chemotaxins (chemokines), causes an activation of the motile apparatus of neutrophils, directional migration, and expression of surface adhesion molecules (Baggiolini, 1995; Baggiolini *et al.*, 1994). During the course of inflammation, an equal anti-inflammatory response develops. This anti-

inflammatory response is associated with the production of anti-inflammatory cytokines, IL-4, IL-10, and IL-13 which leads to immune depression, and may increase the susceptibility to infections(Cavaillon and Annane, 2006). Because of the changing roles of cytokines, the pro-inflammatory versus anti-inflammatory dichotomy may be an oversimplification of the inflammatory response involved in sepsis.

However, migration inhibitory factor (MIF) and high-mobility group box (HMGB)-1 protein are seeming as two central cytokines in critical illness induced by sepsis. MIF, which was one of the first cytokines to be discovered, has a pivotal role in regulating local and systemic inflammatory responses (Calandra and Roger, 2003). Bacterial exo- and endotoxins, and pro-inflammatory mediators such as IFNγ, tumour-necrosis factor (TNF-α), and C5a are strong inducers of MIF secretion by leukocytes. MIF functions as a classical pro-inflammatory cytokine and promotes innate and adaptive immune responses by activating macrophages and T cells (Calandra and Roger, 2003). HMGB1 which is called late mediator of sepsis lethality is dicussed later

1.2.4.2. Role of ROS and RNS

Reactive oxygen species (ROS) are highly reactive and partly reduced derivatives of molecular oxygen. This family of substances includes superoxide radical anion, hydroxyl radical, hydrogen peroxide, and peroxynitrite (Fink, 2002). Reactive nitrogen species (RNS) are derivatives of nitrogen and includes nitric oxide and its derivatives. RNS and ROS exert several beneficial physiologic functions, such as intracellular signaling for growth factors and several cytokines, secondary messengers for hormones and redox regulation. Despite their importance and vital role as a defense mechanism against invading pathogens, an overwhelming production of RNS and ROS or a deficit in antioxidant defenses and oxidant scavenger result in oxidative/nitrosative stress, a key step in the deleterious processes in sepsis (Macdonald *et al.*, 2003; Matejovic *et al.*, 2007). Stimulated neutrophils produce ROS and RNS through the nicotinamide adenine dinucleotide phosphate oxidase complex, xanthine oxidoreductase & myeloperoxidase and represent a defense mechanism against invading microorganisms (Fialkow *et al.*, 2007).

Lipopolysaccharide and other proinflammatory mediators activate nicotinamide adenine dinucleotide phosphate oxidase to produce superoxide radical (O_2). In aqueous environments, superoxide radical is rapidly catalyzed by superoxide dismutase to hydrogen peroxide (H_2O_2) and hydroxyl radicals. Myeloperoxidase is present in neutrophil azurophilic granules produces hypochlorous acid from hydrogen peroxide (H_2O_2) and chloride anion (Cl^-) during respiratory burst. These free radicals are highly cytotoxic and are used by neutrophils to kill fungi, bacteria and other pathogens. superoxide radical (O_2^-) in the presence of nitric oxide, generates peroxynitrite ($ONOO^-$), which is a key player in the pathogenesis of sepsis induced organ dysfunction. peroxynitrite ($ONOO^-$) can cause DNA strand breakage, which initiates the activation of DNA repair enzymes like poly (adenine dinucleotide phosphate-ribose) polymerase. Poly (adenine dinucleotide phosphate ribose) polymerase inhibitors protect against nitrosative and oxidative stress induced organ dysfunction in endotoxemia (Cinel *et al.*, 2002; Ozdulger *et al.*, 2002; Taner *et al.*, 2001). Recently, the potential role of poly (adenine dinucleotide phosphate ribose) polymerase activation has been implicated in the pathogenesis of myocardial contractile dysfunction associated with septic shock (Soriano *et al.*, 2006).

Nitric oxide plays an essential role in the pathophysiology of septic shock. Full expression of inducible nitric oxide synthase is complex requiring TNF-α, IL-1, LPS, and probably other regulatory elements. Nitric oxide is the major endothelial-derived relaxing factor responsible for the vasodilation and systemic hypotension observed in septic shock. Within minutes of administration of an inhibitor of nitric oxide synthesis, blood pressure in hypotensive patients in septic shock returns toward normal levels (Lopez *et al.*, 2004). Nitric oxide also, inhibits a variety of key enzymes in the glycolytic pathway, the tricarboxylic acid pathway, DNA repair systems, energy-exchange pathways and electron transport pathways. Nitric oxide alters the function of many metalloenzymes, carrier proteins, and structural proteins. Like many other components of the host inflammatory response, nitric oxide may have both positive and negative effects in sepsis. Nitric oxide regulates microcirculation to vital organs and contributes to intracellular killing of microbial pathogens. However, excessive and prolonged release of nitric oxide results in

generalized vasodilatation and refractory septic shock. Nitric oxide continues to be a target for therapeutic manipulation in sepsis. Non-selective inhibitors of nitric oxide synthase have been shown to improve the hemodynamics of septic patients but increased myocardial work loads and worsened the outcome in a placebo-controlled trial in patients with septic shock (Lopez *et al.*, 2004).

1.2.5. Multiple organ dysfunctions

Organ dysfunction is a hallmark of severe sepsis. There is a close relationship between the severity of organ dysfunction on admission to an ICU and the probability of survival and the risk of death and between the numbers of organs failing. The mechanism of organ dysfunction involve widespread fibrin deposition that causes microvascular occlusion or blockade. The development of tissue exudates that further compromise and decrease appropriate oxygenation and disorders of microvascular homeostasis that result from the increase in levels of vasoactive substances such as histamine, PAF and prostanoids. Cellular infiltrates, particularly that of neutrophils, cause wide spread damage of tissues directly by lysosomal enzymes and superoxide-derived free radicals released from these cells. TNF-α, IL-1 and other cytokines increase the expression of inducible nitric oxide synthase (iNOS), and increased production of nitric oxide causes further vascular instability, occlusion and may also contribute to the direct myocardial depression that occurs in sepsis (Hotchkiss *et al.*, 1999a; Hotchkiss *et al.*, 1999b; Hotchkiss *et al.*, 2001).

The tissue hypoxia that develops in sepsis is reflected in the oxygen debt or deficiency, i.e., the difference between oxygen delivery and oxygen requirements. Studies show close association between extent of the oxygen debt is and the outcome from sepsis, and also seen that strategies designed to optimize oxygen delivery to the tissues can improve survival (Nakagawa *et al.*, 2006). In addition to hypoxia, cells are also seen to be dysoxic (unable to properly utilize available oxygen) in sepsis. This may be another consequence of excess NO production because impaired mitochondrial respiration have been seen in skeletal muscle biopsies from septic patients , which is inhibited by nitric oxide and suggested recently (Docke *et al.*, 1997). Cross-talk between neurohormones and

cytokines is the cornerstone of restoration of homoeostasis during stress. Release and production of vasopressin and corticotropin-releasing hormone are enhanced by circulating TNF-α and interleukins-1, -2 and -6 by locally expressed NO and interleukin 1 and by afferent vagal fibers. Also, cortisol synthesis is modulated and changed by locally expressed TNF-α and interleukin-6. These up-regulated hormones help maintain cellular metabolism, cardiovascular homoeostasis and contain foci of inflammation. Impaired endocrine responses to sepsis might result from neuronal apoptosis, cytokines, metabolic and ischemic derangements in the hypothalamic-pituitary and drug administration or adrenal glands. Deficiencies or abnormalities in adrenal gland function and vasopressin production occur in almost 1/2 and 1/3 of septic shock cases, and contribute to hypotension and/or death.

1.3. HMGB1 in endotoxemia/sepsis

Studies show extracellular HMGB1 contributes to a variety of pathophysiological process, including diabetes (Han *et al.*, 2008), antibacterial activity (Zetterstrom *et al.*, 2002), smooth-muscle cell chemotaxis (Degryse *et al.*, 2001), cell differentiation (Huttunen *et al.*, 2002b; Sparatore *et al.*, 2001), myocardial regeneration (Limana *et al.*, 2005), angiogenesis (Mitola *et al.*, 2006), tissue repair (Palumbo *et al.*, 2004) and cancer (Huttunen *et al.*, 2002a). In addition, HMGB1 is a potent proinflammatory cytokine and associates with a variety of inflammatory diseases, especially sepsis (Kokkola *et al.*, 2002; Wang *et al.*, 1999a).

1.3.1 Role of HMGB1 in endotoxin lethality

Knowledge of HMGB1 biology as a delayed mediator of systemic inflammation has altered our understanding about mechanisms of lethality from Gram-negative endotoxemia. Administration of lethal doses of endotoxin to mammals activates a biphasic cytokine response that can be divided into early and late kinetic profiles. The early response include classical proinflammatory cytokine response, with peak levels of TNFα, IL-1β and macrophage-inhibiting factor macrophage migration inhibitory factor (MIF) occurring within 6 h (or less). HMGB1 release occurs significantly later; levels of this late mediator reach a prolonged plateau beginning 18/24 h after the onset of endotoxemia (Wang *et al.*, 1999a). This late activity of HMGB1 is a unique kinetic response for a proinflammatory cytokine, with significant implications for

understanding the lag between the onset of endotoxemia and death of the host, which occurs significantly later.

Figure 1.6: Delayed release of HMGB1. HMGB1 is released after a significant lag by innate cells in infection, placing it downstream of an early TNF response.

Administration of HMGB1 to animals in doses that reach the serum levels observed during endotoxemia is lethal (Li *et al.*, 2003; Wang *et al.*, 1999a). Animals exposed to high levels of recombinant HMGB1 develop a sickness syndrome characterized by piloerection, decreased mobility, increased somnolence, weight loss and fever. Elevated HMGB1 levels significantly increase the sensitivity of animals to endotoxin, such that the administration of sublethal doses of HMGB1 concomitantly with sublethal doses of endotoxin is lethal (Wang *et al.*, 1999a). Histopathological examination of animals succumbing to HMGB1 toxicity reveals little necrosis or inflammation in the tissues of major organs. The necropsy results are rather bland, an observation that is quite similar to the necropsy results seen in patients succumbing to sepsis (Li *et al.*, 2003). A plausible mechanism for the cause of death from HMGB1 poisoning is that HMGB1 induces diffuse epithelial dysfunction (Sappington *et al.*, 2002). This is consistent with the pathological evidence, because failure of epithelial cells to maintain tight junctions would be difficult to discern histologically. Death would occur from fluid and electrolyte shifts secondary to failed maintenance of concentration and energy gradients between cells.

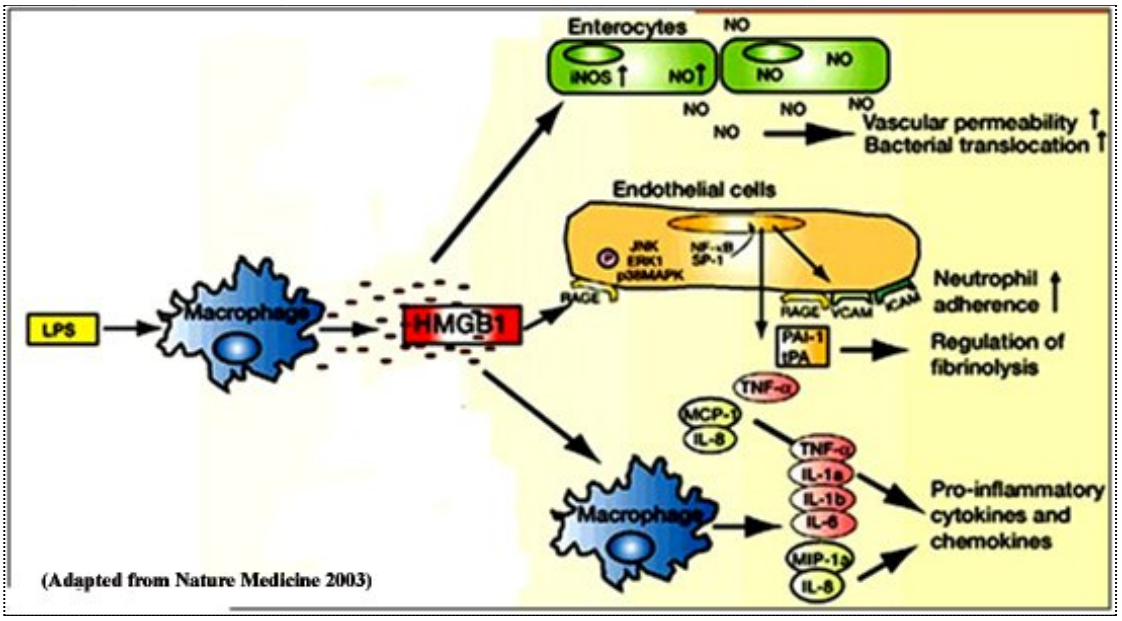


Figure 1.7: HMGB1 in sepsis/endotoxemia. Effect of HMGB1 on different cells in sepsis or endotoxemia. HMGB1 activates macrophages, endothelial cells and enterocytes hours after insult or infection

1.3.2 Role of HMGB1 in sepsis

The recent focus on the biology of HMGB1 as a mediator of sepsis has provided some pathogenic insights. In a standard animal model of sepsis caused by surgical perforation of the cecum in rodents (CLP), leading to severe peritonitis and a syndrome of lethal sepsis, HMGB1 levels increase in the serum over a 24/48 h period after cecal perforation (Yang *et al.*, 2004).

Clinical studies reveal that HMGB1 levels are significantly increased in patients with severe sepsis (Wang *et al.*, 1999a). The range of HMGB1 serum levels observed in the patients with severe sepsis extends up to 150 ng/ml, a range that is similar to the pathological range of HMGB1 levels observed in murine sepsis. An important question in the field of HMGB1 biology is whether the circulating form of HMGB1 identified in the serum of patients with sepsis is complexed to carrier proteins or to neutralizing receptor fragments. Thus far, reported serum HMGB1 measurements have been achieved using Western immunoblotting methods performed under denaturing conditions to reveal a 30 kDa band. It is not yet known whether the biological activity of HMGB1 in the patients with severe sepsis correlates to long-term survival or outcome. The role of HMGB1 in the pathogenesis of human sepsis

will have to be defined on the basis of clinical trials using neutralizing anti-HMGB1 antibodies in humans with severe sepsis. Clinical trials of neutralizing HMGB1 in the treatment of sepsis may be in the offing.

1.4. Targeting HMGB1 in sepsis

1.4.1 Sepsis therapy

Septic shock and sepsis involves entire team of health care, accounting for a huge burden on the system. Also sepsis remained a source of morbidity and mortality so numerous attempts were done to find a better drug, however little success have been achieved so far. Various immunomodulatory drugs showed good results in animal models. Despite promising preclinical results, the trails showed little success in clinical settings (Angus *et al.*, 2001; Annane *et al.*, 2003; Martin *et al.*, 2003).

Clinical management usually begins with determination of the probable infection site, prompt recognition, early administration directed therapy (EGDT) which involves crystalloid infusions, vasodilators or vasopressors, transfusion of packed red blood cells, and dobutamine (Balk, 2000; Balk, 2004). But this involves the whole-hearted involvement of the entire healthcare team which cannot be stressed enough. So the researchers are in search of better therapeutic agents to curb this aggressive, complex, and increasingly prevalent condition. Corticosteroids once seen as target drugs in septic shock are under controversies and are now suggested only for some patients sepsis whose blood pressure is poorly responsive to fluids and vasopressor therapy (Dellinger *et al.*, 2008).

Another effort which created big hopes in area of sepsis therapy was development of drotrecogin alfa (activated),which is a recombinant version of activated protein C (APC). It was developed by Eli Lilly and Company and is marketed under the brand name Xigris. APC has profound anti-inflammatory properties and anti-apoptotic, in addition to its anticoagulant activity. The APC was seen as “magic bullet” against sepsis menace, as was reported in a landmark report published in 1987 by Taylor and colleagues (Taylor *et al.*, 1987), who showed that infusing protein C (derived from a human plasma product and activated with human thrombin) could protect baboons from mortality caused by injecting the animals with an otherwise lethal dose of viable Escherichia coli. The support to theory was given by Bernard et al. who showed in PROWESS study (Recombinant Human Activated Protein C (rhAPC) Worldwide Evaluation) that mortality of septic patients decreased when treated by rhAPC. However later, Eli Lilly and Company carried out a second (post marketing) clinical

trial of drotrecogin alfa (activated) to evaluate the efficacy of Xigris known as ADDRESS study (Administration of Drotrecogin alfa (activated) in Early Stage Severe Sepsis). The study was prematurely terminated because of the futility for reaching the primary study goal. The rhAPC was seen beneficial only in selective patients. Thus the search is still on for identifying better target and drugs for sepsis.

1.4.2 HMGB1 as therapeutic target

HMGB1-specific antibodies have been shown to protect mice against endotoxin and sepsis lethality. Anti-HMGB1 antibodies administered to endotoxemic animals confer significant protection in them from lethality (Wang *et al.*, 1999a). Anti-HMGB1 antibodies can be administered several hours after the early cytokine response has resolved, indicating that this late mediator of lethal endotoxemia can be therapeutically targeted through a wider window than previously described for early proinflammatory cytokines. These observations describe a new secondary inflammatory response that occurs downstream of the classical early response of cytokines such as IL-1 and TNF. Each of these responses can mediate lethality, but are distinct and can be separately inhibited to improve survival in animal models. TNF, the prototypical early cytokine mediator of lethal endotoxemia, causes shock, tissue injury and widespread necrosis in vital organs. Lethal quantities of TNF are produced during lethal endotoxemia and administration of similar quantities of TNF to normal animals causes pathological changes that are indistinguishable from over-whelming endotoxemia, endotoxemic shock or septic shock syndrome (Tracey *et al.*, 1986). Inhibiting the early TNF response during endotoxemia or acute septic shock can prevent (Tracey *et al.*, 1986; Tracey *et al.*, 1987), but this scenario is dissimilar from the protracted time-course that characterizes systemic inflammation in humans without shock. HMGB1 lethality is attributable to epithelial dysfunction without the development of shock and tissue injury (Li *et al.*, 2003; Sappington *et al.*, 2002; Wang *et al.*, 1999a).

Administration of anti-HMGB1 antibodies to animals with established sepsis significantly reverses the signs of sepsis and prevents lethality in animals with established infection. Three separate approaches have been reported to inhibit HMGB1 in rodent sepsis and each of these improves survival significantly.

Neutralizing anti-HMGB1 antibodies have been produced and are defined based on their ability to inhibit the macrophage stimulating activity of recombinant HMGB1. Neutralizing antibodies significantly improve survival from sepsis even when the first dose of antibodies is administered to animals 24 h after the onset of infection (Yang *et al.*, 2004). This is the widest therapeutic window described for any cytokine in this standardized model. Anti-TNF antibodies worsen survival from sepsis in this model and anti-MIF antibodies are no longer effective when administered after a 6 h window from the time of sepsis onset (Calandra *et al.*, 2000; Eskandari *et al.*, 1992).

Another strategy to inhibit the activity of HMGB1 in sepsis is to administer recombinant HMGB1 A box protein. Repeated administration of HMGB1 A box protein beginning 24h after cecal perforation significantly improves survival in animals with established sepsis (Yang *et al.*, 2004). The response to A box protein is time and dose dependent, with maximal responses seen when A box is first administered either 12 or 24h after the onset of infection. Administration of A box 36h after the onset of sepsis does not reverse mortality in this highly lethal model, suggesting that a clinically relevant therapeutic window for inhibiting HMGB1 may exist within the first 24h of the onset of sepsis.

Humanized anti-HMGB1 monoclonal antibodies could therefore find applications in both acute and chronic inflammatory diseases. Blockage of the RAGE signaling pathways could also result in attenuation of the proinflammatory effects of HMGB1. Several strategies, such as the administration of soluble forms of RAGE or the blocking of the Fab fragment derived from anti-RAGE and/or anti-HMGB1 IgG, have been reported (Hanford *et al.*, 2004; Lutterloh *et al.*, 2007).

Considered together with the results of anti-HMGB1 antibodies and the A box protein, it now appears that neutralizing HMGB1 can significantly improve survival from established sepsis in standardized animal models. Although an antibacterial activity of HMGB1 has been described, inhibiting HMGB1 with either antibodies or A box protein did not accelerate bacterial proliferation in these models. It remains to be seen whether neutralizing antibodies to HMGB1 will be associated with the development of immunosuppression or toxicity, but results to date of treating animals

with either endotoxemia or sepsis have not revealed either toxicity or secondary infections.

Thrombomodulin has recently been shown to bind to HMGB1 so that thrombin–thrombomodulin complexes can effectively degrade HMGB1 into a less proinflammatory form (Abeyama *et al.*, 2005; Esmon, 2005; Ito *et al.*, 2008). This means that recombinant thrombomodulin can promote the degradation of HMGB1 and suppress the proinflammatory effects of HMGB1 (Ito *et al.*, 2008). Thrombomodulin can also suppress coagulatory responses; therefore, recombinant thrombomodulin should be a promising therapeutic option against DIC or sepsis (Ito *et al.*, 2008).

Several small-molecule chemical compounds have been used to inhibit HMGB1 proinflammatory activities in vivo. These pharmacological agents belong to the class of cytokine-release inhibitory drugs (CRIDs) and include ethyl pyruvate, the cholinergic agonists nicotine and acetylcholine, and steroid-like pigment tanshinone IIA (Chen *et al.*, 2005; Li *et al.*, 2007; Ulloa *et al.*, 2002; Wang *et al.*, 2004). These agents were found to interfere specifically with HMGB1 release from the nucleus into the extracellular space, without affecting HMGB1 mRNA or protein levels (Ulloa *et al.*, 2002; Wang *et al.*, 2004). In contrast, many other steroidal drugs and nonsteroidal anti-inflammatory drugs failed to significantly inhibit HMGB1 extracellular release (Li *et al.*, 2007). The HMGB1 inhibiting molecules have shown impressive efficacy in animal models of lethal endotoxemia and sepsis, with protective effects at therapeutically achievable, safe doses, supporting the therapeutic potential of these inhibitors in HMGB1- mediated human inflammatory diseases (Chen *et al.*, 2005; Li *et al.*, 2007; Ulloa *et al.*, 2002; Wang *et al.*, 2004).

Ethyl pyruvate, an anti-inflammatory agent developed as an antioxidant, cytoprotectant in tissue ischemia, was recently discovered to be an effective inhibitor of HMGB1 synthesis. Administration of ethyl pyruvate to animals with established sepsis significantly reduced serum HMGB1 levels and significantly improved survival (Ulloa *et al.*, 2002).

Another molecule, glycyrrhizin, inhibits the chemotactic and mitogenic activities of HMGB1 (Mollica *et al.*, 2007). Unlike CRIDs, glycyrrhizin does not interfere with

the release of HMGB1, but directly inhibits its extracellular cytokine activities (Mollica *et al.*, 2007). This means that glycyrrhizin can inhibit not only actively released HMGB1 but also passively released HMGB1. However, the affinity of glycyrrhizin for HMGB1 is relatively modest and will need to be improved for any therapeutic application (Mollica *et al.*, 2007). Several other commercially available drugs, such as sivelestat, nafamostat, antithrombin III, and γ-globulin, have also been suggested to modulate inflammatory response through HMGB1-related mechanisms (Hagiwara *et al.*, 2008a; Hagiwara *et al.*, 2007; Hagiwara *et al.*, 2008b; Suda *et al.*, 2007).

1.5. Animal Models of Sepsis

Thousands of failed preclinical trials performed over more than five decades have prompted a rethinking of both the pathophysiological mechanisms of sepsis and the use and interpretation of animal models of sepsis. On the basis of the causative agent, sepsis models can be divided into three categories: exogenous administration of a toxin (such as lipopolysaccharide (LPS), endotoxins or zymosan); exogenous administration of a viable pathogen (such as bacteria); or alteration of the animal's endogenous protective barrier (inducing colonic permeability, allowing bacterial translocation). Although models have contributed significantly to our understanding of host defence mechanisms during infection, but there are many examples of controversy, when considering the application of animal models to the development of sepsis therapeutics. Although several models are used to study mechanisms of sepsis pathophysiology but the correct model to use in the development of drug therapy is one that most closely mimics the course of human disease. Unfortunately, there are significant differences between each of the current animal sepsis models that prevent any single one from emerging as the perfect vehicle for sepsis drug discovery.

Some animal models which replicate the signs and laboratory findings seen in human sepsis include intravascular infusion of endotoxin (Brackett *et al.*, 1985; Johnston *et al.*, 1989; Lindsey *et al.*, 1991; Nomura *et al.*, 1993; Pittet *et al.*, 2000; Talke *et al.*, 1985; Taylor, 2001; Traber *et al.*, 1988) or live bacteria (Di Giantomasso *et al.*, 2003; Lagoa *et al.*, 2004; Silverstein *et al.*, 2000; Taylor, 2001), bacterial

peritonitis(Ahrenholz and Simmons, 1980; Fink *et al.*, 1984; Goldfarb *et al.*, 2002; Lang *et al.*, 1983; Mathiak *et al.*, 2000; Natanson *et al.*, 1986; Quezado *et al.*, 1993; Stamme *et al.*, 1999), cecal ligation and perforation (Ebong *et al.*, 1999; Richmond *et al.*, 1985; Wichterman *et al.*, 1980), soft tissue infection (Durkot and Wolfe, 1989), pneumonia model (Karzai *et al.*, 2003; Murakami *et al.*, 2002), and meningitis model (Ribes *et al.*, 2003).

1.5.1 Endotoxicosis models

Endotoxin or lipopolysaccharide (LPS), stimulates various cells to release inflammatory mediators which are responsible for initiating sepsis (Opal and Cohen, 1999). LPS is a stable in its pure form and can be stored easily when in lyophilized form. An accurate dose can be measured according to body weight and may be administered as a bolus or infusion (Traber *et al.*, 1988). In endotoxicosis, activation of TLR4 through bolus injection of LPS leads to an overwhelming innate immune response, with inflammatory cytokines such as TNF representing crucial mediators (Murakami *et al.*, 2002; Piper *et al.*, 1996; Silverstein *et al.*, 2000). This has formed the basis for the simplest sepsis model. Endotoxin is commonly used in animal models of sepsis, there are considerable differences between species in sensitivity to endotoxin. Humans and animals like rabbits, sheep and nonhuman primates are relatively more sensitive than rodents, cats and dogs (Fink and Heard, 1990; Michie, 1998; Piper *et al.*, 1996).

Endotoxin, when administered to human subjects mimics many features of sepsis. In critically ill patients, increased concentrations of serum endotoxin have been associated with the development of sepsis, disease severity, and mortality (Opal and Cohen, 1999; Piper *et al.*, 1996; Traber *et al.*, 1988). The plausibility of the hypothesis that endotoxin plays a significant role in the pathogenesis of sepsis is supported by many studies that show that antibiotic administration may lead to a sudden release of massive amounts of endotoxin from dead bacteria and an acute hemodynamics worsening (Hollenberg *et al.*, 2001; Lepper *et al.*, 2002; Opal and Cohen, 1999; Piper *et al.*, 1996; Silverstein *et al.*, 2000).

Inspite of various evidences (animal models) that endotoxin may play an important role in the pathogenesis of sepsis, several authors have expressed concerns that the infusion of endotoxin is not a suitable model to study sepsis.

1.5.2 Bacterial Infusion

Bacterial infection provide important insights into mechanisms of the host response to pathogens, however they do not recapitulate many important features of human sepsis. Inoculation of animals with pure or mixed bacterial flora has been a common tool for studying sepsis mechanisms (Deitch, 1998; Fink and Heard, 1990; Wichterman *et al.*, 1980). However, high doses of bacteria commonly administered do not typically colonize and replicate within the host, often due to rapid lysis by complement (Cross *et al.*, 1993). This leads to a potential model of intoxication with endotoxins rather than a true model of infection (Cross *et al.*, 1993).

1.5.3 Cecal Ligation and Puncture (CLP) model

The CLP model is regarded as the gold standard for sepsis research (Remick *et al.*, 2002; Wichterman *et al.*, 1980). The CLP model mimics the human diseases of ruptured appendicitis or perforated diverticulitis. The technique involves midline laparotomy, exteriorization of the caecum, ligation of the caecum distal to the ileocaecal valve and puncture of the ligated caecum. This process creates a bowel perforation with leakage of faecal contents into the peritoneum, which establishes an infection with mixed bacterial flora and provides an inflammatory source of necrotic tissue (Ayala *et al.*, 2000; Wichterman *et al.*, 1980). The severity of disease, as assessed by mortality, can be adjusted by increasing the needle puncture size or the number of punctures. The severity of CLP can be adjusted such that mortality evolves rapidly over hours to days, or more slowly over 28 days (Remick *et al.*, 2005).

A further advantage of the model is that it can identify an irreversible stage of sepsis, such that excision of the necrotic tissue is unable to improve survival (Latifi *et al.*, 2002; Remick *et al.*, 2002). The CLP technique has achieved its popularity because of its general reproducibility and similarity to human disease progression. Most notably, the CLP model recreates the hemodynamic and metabolic phases of human sepsis (Wichterman *et al.*, 1980). Subsequently, apoptosis of selected cell types and host

immune responses seem to mimic the course of human disease, adding further clinical validity to this model (Ayala and Chaudry, 1996; Hotchkiss *et al.*, 2003). Unlike endotoxic shock, which produces severe hypotension and low tissue perfusion immediately after the administration of a large dose of endotoxin, CLP is characterized by a biphasic hemodynamic response, i.e., an early, hyperdynamic stage followed by a late hypodynamic stage of sepsis. The CLP model of sepsis, however, produces persistent and not episodic bacteremia, which occurs under certain clinical situations. The animal model of single hit sepsis utilizes healthy animals whereas in the clinical situation, the patient becomes septic usually following a traumatic injury or disease. The single hit model of sepsis may not be analogous to the patient in ICU with the sepsis syndrome since animals are initially healthy.

2.1 *In-Vitro* study

2.1.1 Cell culture

Murine macrophage-like RAW 264.7 cells were obtained from the National Institute of Immunology (NII Delhi) and cultured in DMEM (Sigma Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma Aldrich), 2 mM glutamine (Sigma Aldrich), and penicillin/streptomycin/gentamycin (Sigma Aldrich) in a humidified incubator with 5% CO_2. The cells were sub-cultured in 6, 12, 96-well plates and used at 70% confluence.

2.1.2 Peritoneal macrophage isolation and culture

The peritoneal macrophages (PMphs) were isolated from C57BL/6 by following procedure:

a) Mouse was euthanized and sprayed with 70% ethanol. Mouse was then mounted on the styrofoam block on its back.
b) Using a scissors and forceps, the outer skin of the peritoneum was cut and gently pulled back to expose the inner skin lining of the peritoneal cavity.
c) 5 ml of ice cold PBS (with 3% FBS) was injected into the peritoneal cavity using a 27G needle. The needle was inserted slowly in the peritoneum being careful not to puncture any organs.
d) After injection, the peritoneum was gently massaged to dislodge any attached cells into the PBS solution.
e) Another 25G needle attached to a 5 ml syringe was inserted in the peritoneum and fluid was collected while moving the tip of the needle gently to avoid clogging by the fat tissue or other organs. Maximum possible fluid was collected in tubes and the cell suspension was kept on ice.
f) The sample having any visible blood contamination was discarded.
g) The collected suspension was centrifuged at 2000 RPM for 5-10 min at room temperature.
h) The supernatant was discarded and 25 ml media (RPMI) was added. The cells were resuspended and centrifuged again at 1000 RPM for 20 min.

i) The pellet was then resuspended in about <3ml media. The cells were count on hemocytometer after appropriate dilutions.

j) The cells were resuspended in appropriate volume of media with RPMI + 10% FBS + 1x Pen/Strep + glutamine. The cells were seeded with higher density than normal cells in 96-well plates.

k) The cells were allowed to adhere for 3 hrs, and then washed twice (with PBS) to remove non-adherents cells. The cells were incubated overnight and used next day.

2.1.3 Compounds used and their preparation

a) Aloe-emodin preparation. Aloe-emodin (AE), purchased from Sigma-Aldrich (Milan, Italy), was dissolved in DMSO and stored at -20°C. Aloe-emodin was diluted in appropriate media immediately before use.

b) Psychosine preparation. Psychosine, purchased from Sigma-Aldrich (Milan, Italy), was first dissolved in DMSO and then diluted in appropriate media immediately before use.

c) Emodin preparation. Emodin, purchased from Sigma-Aldrich (Milan, Italy), was dissolved in DMSO and stored at -20°C. Emodin was diluted in appropriate media immediately before use.

d) Rosuvastatin preparation: Rosuvastatin, purchased locally (Novastat), was dissolved in PBS and stored at 4ºC.

e) Safranal preparation: Safranal purchased from Sigma-Aldrich (Milan, Italy), was dissolved in methanol and stored at 4ºC.

2.1.4. Cell Viability assay

Cell viability was monitored by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay. MTT is a pale yellow substrate that is reduced by the living cells to yield a dark blue formazan product. This process requires mitochondria (active) and even freshly dead cells do not reduce significant amounts of MTT. RAW 264.7 mouse macrophage cells were cultured

in a 96-well flat-bottom plate for 12 h and subsequently treated with various concentrations of test compounds for 24 h. Thereafter, the culture medium was aspirated and 100 µl of MTT dye (1 mg/ml in PBS) was added into each well and incubated at 37ºC for 4 h. Acidified isopropanol (0.1 N HCl)was added to each well to solubilize the blue formazan crystals. Absorbance was read at 570nm in microplate reader (Epoc Biotech). The index of cell viability was determined by measuring the OD of color produced by MTTdye reduction, compared with that of the untreated control cells [(OD of test compound-treated cells/OD of solvent-treated cells) ×100].

2.1.5. Nitric oxide assay

Nitrite, a stable NO metabolite, was determined by the method of Griess Assay. 100µl of supernatant was mixed with 100µl of Griess reagent prepared by mixing equal volumes of N-(1-naphthyl)ethylenediamine (NED) and sulfanilic acid and incubated for 15 min at room temp. The absorbance was read at 550nm in a microplate reader. Sodium nitrite (1.56-100uM) was used as a standard. .

2.1.6. Western blotting analysis

Proteins were run on (8-12%) SDS-PAGE gel and transferred on PVDF membranes using wet blotting procedure at 70V constant current. Membranes were washed three times with PBS for 10 minutes each and blocked for 2hr with ODYSSEY blocking buffer (LI-COR biotechnologies USA). Primary antibodies were diluted 1:1500 (anti-iNOS; sigma), 1:500 (anti-HO-1; Sigma), 1: 1000 (anti-HMGB1; abcam, USA), 1:1000 (anti-beta tubulin; Sigma) in 0.5 % blocking solution and incubated overnight at 4°C on a rocking plate. Thereafter, membranes were washed three times with PBS-T (PBS with 0.05% Tween 20), followed by an incubation for 1 hr at RT with a 1:10,000 dilution of Infrared dye conjugated goat anti-rabbit secondary antibody (800CW) or 1:10000 dilution of Infrared dye conjugated goat anti-mouse secondary antibody (680CW) or 1:3000 dilution of Alkaline phosphate conjugated goat anti-rabbit secondary antibody. After subsequent washes with PBS-T, antibody binding was visualized by imaging and quantified on LICOR ODYSSEY infrared system or densitometry (Biorad). The

bands in western blots were analysed by LICOR-ODYSSEY software or Biorad densitometer software.

2.1.8 HMGB1 Detection

2.1.8.1 HMGB1 precipitation and western blot

HMGB1 present in cell supernatant was precipitated by TCA/acetone method. The lysate was mixed with acetone and TCA(100%) in the ratio of (1:8:1) and allowed to precipitate at -20 °C overnight. The mixture was centrifuged at 12K rpm for 45 min at 4 °C in a microcentrifuge. The pellet obtained was dried (but not over dried) and dissolved in 1× SDS-PAGE loading dye. The concentrated protein present in supernatant was then analysed for HMGB1 level by western blot (as above).

2.1.8.2 ELISA

An in–house ELISA was developed for detection of HMGB1 levels in small volumes of cell supernatant (as in peritoneal macrophages). Sandwich ELISA was done using two antibodies corresponding to different epitopes of HMGB1, i.e., anti-HMGB1 corresponding to N-terminal i.e., amino acid 2-17 (Sigma) as capture antibody and anti-HMGB1 corresponding to C-terminal i.e., 150 to the C-terminus (abcam) as detection antibody. The HRP conjugated or ALP conjugated goat anti-rabbit secondary antibody was used for colour development with TMB or PNPP as substrate.

2.1.8.3 ELISPOT assay

HMGB1 was detected by in-house developed Elispot assay which allows detecting HMGB1 release from individual cells. 96-well plates (PVDF membrane bottomed) were coated with polyclonal HMGB1 antibody (specific for N-terminal, corresponding to amino acid 2-17 of human HMGB1; Sigma), at 4°C overnight. Following washing, cells were added (1000 –2000/well). The plates were incubated in a humidified 37°C, 5% CO_2 incubator for about 3hrs and then stimulated as described. After appropriate time, plates were washed and second anti-HMGB1 antibody was added (specific for C-terminal, corresponding to amino

acid 150 to the C-terminus: abcam) and the plates were incubated overnight at 4°C. There after followed incubation with anti-rabbit alkaline phosphatase antibody (sigma). Colour development was via 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt/nitroblue tetrazolium chloride chromogen liquide substrate (BCIP/NBT) . The reaction was stopped with water, and the plates were dried at room temperature overnight. Images of wells were acquired using a compound microscope.

2.1.9 Fluorescence Immunostaining

RAW 264.7 cells were grown to subconfluence, and stimulated with LPS in the absence or presence of test-compound for various period of time. Subsequently, cells were fixed with 2% formalin for 10 min, and permeabilized with 0.1%Triton X-100 in PBS (1 min, room temperature). After extensive washing with PBS, cells were incubated sequentially with rabbit anti-HMGB1 antibodies, and goat anti-rabbit secondary antibodies conjugated with FITC (Santa Cruz). Nuclear morphology was analyzed with the fluorescent dye, Propidium Iodide. Images were acquired using a fluorescence microscope (**Lieca**).

2.2 In-Vivo study

2.2.1 Animals

Male C57/B6 mice (15–20 wks old, 25-30g) were purchased from The Indian Institute of Integrated Medicine, Canal Road Jammu, India. Mice were bred and maintained under a 12-hr light/dark cycle at a controlled temperature (21 ± 2°C). Animals received pelleted rodent feed (Ashirwad Industries, Chandigarh, India) and water *ad libitum*. Animal care followed the Institutional Animal Ethics Committee, University of Kashmir, and the experiments were performed in adherence to the Indian National Science Academy, New Delhi (1992) guidelines for the treatment of animals and ethical animal research.

2.2.2 Induction of Endotoxemia

To induce endotoxemia, C57BL/6 mice were injected i.p. with different amounts (100- 400 μg/mouse) of LPS (from Salmonella enteridis; Sigma-Aldrich) and observed for 24hrs for mortality and clinical signs of endotoxemia like piloerection, huddling, diarrhoea, weight loss Also TNF-α levels were checked for analysis of development of endotoxin induced cytokine storm. It was observed that lower doses (>200μg/mouse) were not able to induce high levels of TNF-α and/or observations associated with endotoxemia.

2.2.3 Aloe-emodin treatment protocol

Animals received 0.1ml of PBS (with 0.2% DMSO) or a similar volume of aloe-emodin prepared in PBS (with 0.2% DMSO). A single dose was injected intraperitoneally at 1hour before LPS infusion.

2.2.4 Study design

To induce endotoxemia, C57BL/6 mice were injected i.p. with sublethal (200 μg/mouse) and lethal doses (400 μg/mouse) of LPS. Animals were treated i.p. with medium (controls, PBS) or with different concentrations (0.9μmol/mouse and/or 1.3μmol/mouse as mentioned) of aloe-emodin (Sigma-Aldrich), 1hour before challenge with LPS. Animals were monitored after every 3 hours for survival and other clinical signs including piloerection, lethargy, appearance of diarrhea, and body weight loss. Blood samples were collected by orbital sinus for small volumes and cardiac puncture for terminal collection and liver, lungs, and kidney were collected. The blood samples were clotted for 1h at room temperature and serum was obtained after centrifugation for determination of cytokines. Tissue specimens were immediately stored in 10% formalin for histological studies or frozen in liquid nitrogen for protein extraction and cytokine determination, and MPO activity measurement.

All animal experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee of the University of Kashmir, Srinagar, J&K, India.

2.2.5 Cytokine estimation

Supernatant of cells, mice serum and tissue homogenate was collected and frozen at -80ºC for cytokine analysis. Levels of various cytokines [TNF-α, IL-1β, and IL-6] were measured using ELISA kits from eBioscience according to the manufacturer's instructions.

2.2.6 Blood chemistries

Serum levels of alkaline phosphate (ALP), alanine transaminase (ALT), blood urea nitrogen (BUN) and creatinine were measured using an autoanalyzer (Biolyzer 600, Blooming implex, Germany).

2.2.8 Cytokine determination

For cytokine determination in lung tissues, homogenisation of equally weighed tissue peices (50 mg tissue/ml) was done in phosphate buffer (50 mM, pH 7) with 0.5% triton, and 10 μg/ml of a cocktail of proteinase inhibitors containing phenyl methyl sulfonyl fluoride, pepstatin, and leupeptin by Teflon homogenizer. Samples were centrifuged at 15,000 rpm for 45 min and stored at −80°C until cytokine determination.

2.2.9 Histopathology

The lung, liver and kidney tissues were fixed in 10% formalin for about week and then dehydrated with different gradients of alcohol for about 3hrs. The tissues were then paraffin embedded and sections prepared under microtome were stained with hematoxylin/eosin after deparrafinization. Damage was defined on various patterns present in sepsis/endotoxemia. The degree of damage was estimated at 400× magnification using six to nine fields.

2.2.10 Myeloperoxidase assay

Neutrophil infiltration in the lung tissue was monitored by measuring MPO activity using 3,3',5,5'-tetramethylbenzidine (TMB) as a reducing substrate. TMB reacts with peroxidase enzymes such as MPO and forms a blue product. In brief,

tissue specimens were homogenized at 50 mg/ml in phosphate buffer (50 mM, pH 6.0).The homogenate was 10×diluted in acetate buffer (pH 5.4). 100µl of TMB reagent (2.4mM TMB, 0.3mM H_2O_2, 300mM NaAc (pH5.4)) was added to 100µl homogenate in 96-well plate. The resulting colour change was read in a microplate reader at a wavelength of 630 nm.

2.2.11 Statistical analysis

Values in figures and tables are expressed as mean±S.E.M of n observations, unless otherwise noted. All treatments were compared with relevant negative control (vehicle treated) and positive control (LPS treated) groups separately. The statistical analysis was done by Graph pad Prism 5. The comparisons were done by ANOVA (in case of multiple comparisons) and t-test. The p values were derived by comparing separate groups individually by Beneforrini multiple comparison test. The survival assay was done by Gehan-Breslow-Wilcoxon Test.

2.2.11 Procurement of chemicals:

All chemicals (unless otherwise mentioned) were purchased from Sigma Aldrich Germany.

Part-I: Screening

Our strategy was to look for compounds which can inhibit HMGB1 (high mobility group box 1) release in activated macrophages and check effect of screened compound on endotoxemic mice. Anti-inflammatory molecules capable of attenuating HMGB1 release hold great potential in the prevention and treatment of inflammatory disease like sepsis/endotoxemia. So we first screened compounds on the basis of their nitric oxide (NO) scavenging potential in activated macrophages. NO has a fundamental contribution in development septic shock, its metabolites nitrite and nitrate rise progressively in various animal models of sepsis. The NO mediates hypotension leading to severe hypoxia in peripheral vital organs, resulting in progressive organ failure may also directly contribute to tissue and organ injury by its direct, peroxynitrite-mediated cytotoxic effects.

In macrophages, LPS (lipopolysaccharide) stimulation leads to very high levels of NO which remain sustained to long periods because of high level expression of iNOS. NO has very short life span so the production of nitrite, the stable metabolite of NO was used as an indicator of NO production. The nitrite level can be detected in serum or cell culture media by Griess assay which is a simple colorimetric assay.

3.1 Assessment of non-toxic concentration of test compounds in RAW 264.7 cells.

Effect of different compounds on RAW 264.7 cell viability was assessed by MTT assay. Murine macrophage-like RAW 264.7 cells were plated in 96-well plate and after 12hr, various concentrations of different compounds were added. At 24hr after treatment, MTT assay was done. Rosuvastatin and safranal showed no significant effect on viability, however psychosine, emodin and aloe-emodin showed decrease in viability at higher concentrations. The compounds were used below the concentrations showing any change in viability.

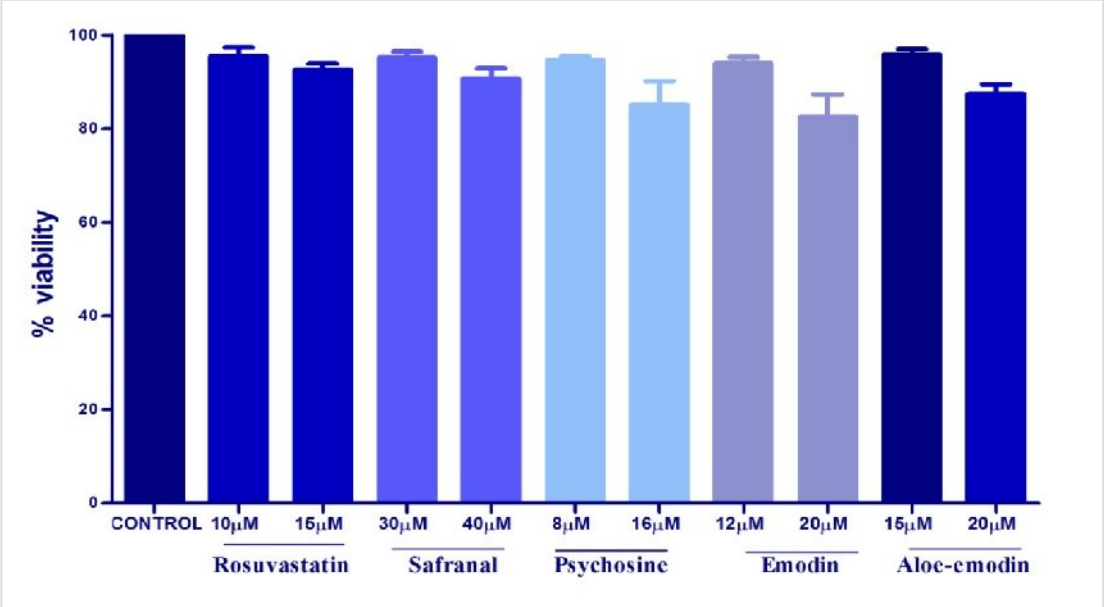


Figure 3.1: Effect of different test compounds on RAW 264.7 cell viability. The cells were treated with test compounds (at concentrations mentioned in the figure) and at 24hr after treatment cell viability was determined by MTT assay, and expressed as the mean (±SEM) of four experiments in duplicate.

3.2 LPS induced nitric oxide (Dose and Time dependence)

LPS induced NO in RAW 264.7 cells rises to detectable range just after 6hr, but maximum levels are attained from 18 to 24hr. Murine macrophage-like RAW 264.7 cells were plated in 24-well plates at $4x10^6$ cell density. After 12hr, various concentrations of LPS (50ng/ml, 100ng/ml, 1µg/ml, 10µg/ml) were added. At 24hr after LPS stimulation, supernatant was collected and nitric oxide levels were measured. Maximum activation was seen at 1µg/ml and 10µg/ml. Low concentrations of nitric oxide were detected at 100ng/ml (**Fig 3.2a**).

LPS induced nitric oxide level rise to detectable range after 6hrs, however the level increase with time till 24hrs. Nitric oxide was measured at different intervals of time after LPS stimulation. RAW 264.7 cells plated in 24-well plate, were stimulated with LPS (1µg/ml) and NO levels were measured in supernatant collected at 6, 12, 18 24 and 30hr after stimulation (**Fig 3.2b**).

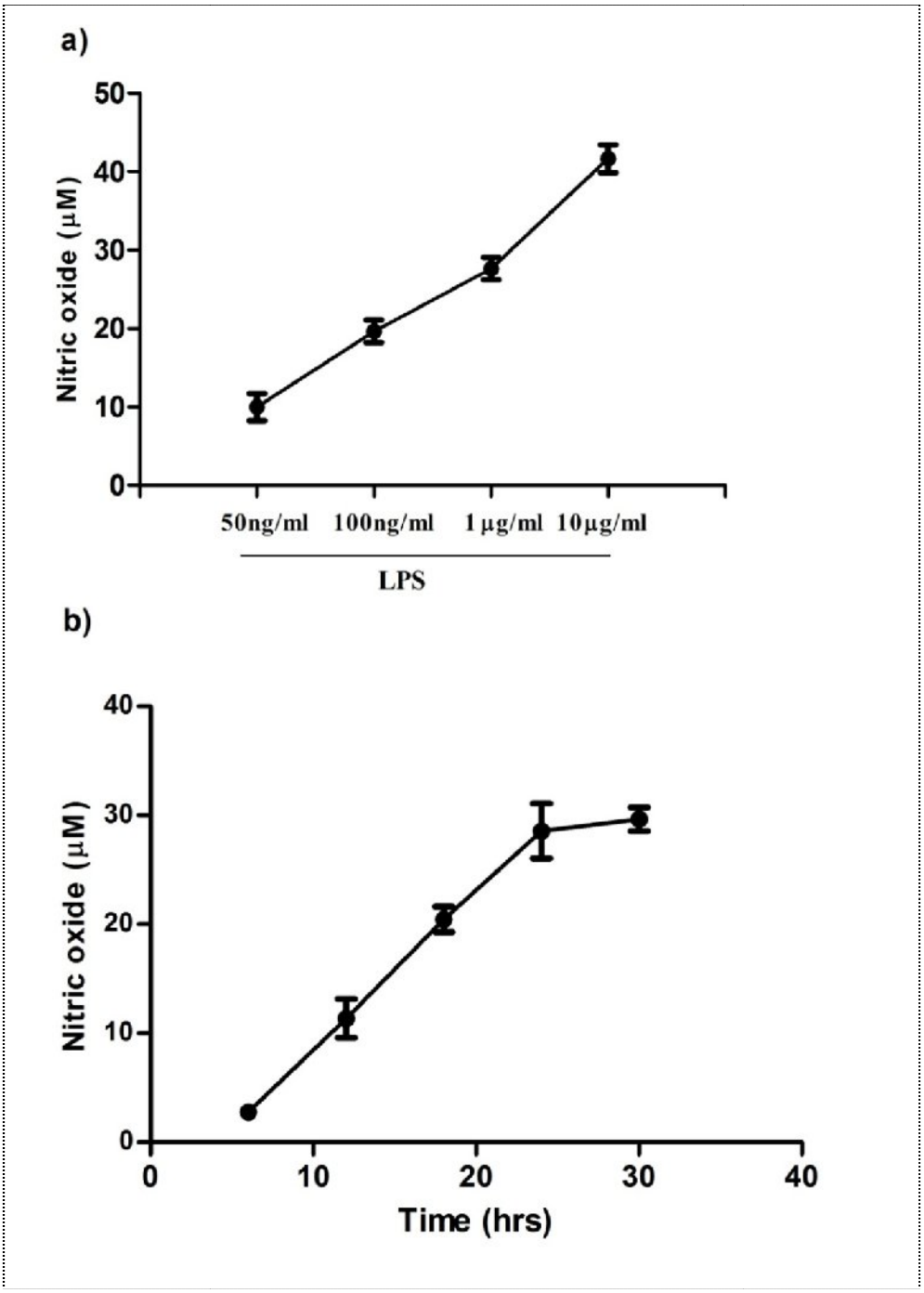


Figure 3.2: **LPS induced Nitric oxide**. a) Dose-dependent curve. RAW 264.7 cells were stimulated with different concentrations of LPS and nitric oxide levels were measured in cell supernatant at 24hr after stimulation. b) Time dependent curve. RAW 264.7 cells were stimulated with 1µg/ml LPS and nitric oxide levels were measured in cell supernatant at various time points.

3.3 Effect of Rosuvastatin on LPS induced Nitric oxide levels.

The statins are a class of lipid-lowering drugs which inhibit the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, an early rate-limiting step in cholesterol biosynthesis. Statins show pleiotropic actions, affecting many pathways that are involved in the pathogenesis of sepsis. Various studies have been done on their role in sepsis and some are proving promising. Rosuvastatin a synthetic statin, is a competitive inhibitor of the enzyme HMG-CoA reductase, with mechanism of action similar to that of other statins.

To assess the role of rosuvastain in LPS stimulated inflammation in macrophages, RAW 264.7 cells were challenged with LPS along with rosuvastatin simultaneously. RAW 264.7 cells were plated and activated cells were treated with different concentrations of rosuvastatin (5µM 10µM and 15µM) along with LPS (1µg/ml). Although rosuvastatin at 5uM and 10µM concentration showed decrease in nitric oxide but this decrease was somehow reverted back at 15µM concentration. Thus results obtained with rosuvastatin were sort of tricky to be followed.

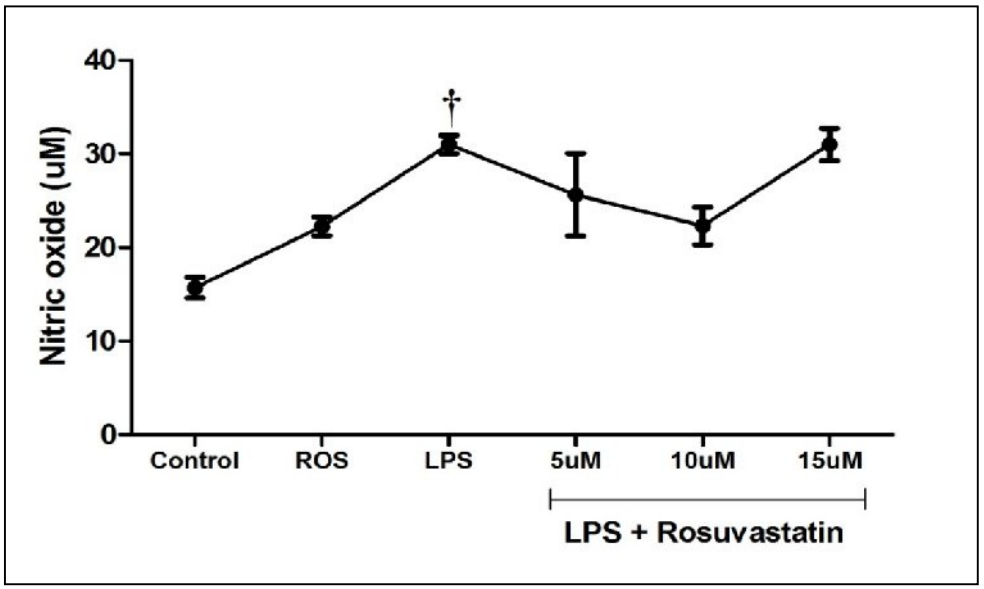


Figure 3.3: **Effect of Rosuvastatin on LPS induced nitric oxide accumulation**. Murine macrophage-like RAW 264.7 cells were stimulated with LPS (1µg/ml) and different concentrations (5, 10 & 15uM0 of rosuvastatin simultaneously. Nitric oxide level was measured in cell supernatant at 24hr after stimulation. Data represent the mean ± SEM of three independent experiments (n= 3). †, $p< 0.001$ when compared with control (vehicle only).

3.4 Effect of Safranal on LPS-induced Nitric oxide levels.

Safranal is an organic compound isolated from *Crocus sativus* L. (Iridaceae). Studies have showed high antioxidant and free radical scavenging activity of safarnal. Also because of anti-inflammatory actions of crocus extracts, it seems interesting to study its effect on LPS induced oxidative stress in RAW 264.7 cells.

To determine the effect of safranal on nitric oxide release by macrophages, murine macrophage line RAW 264.7 cells were stimulated with LPS in the presence of safranal and nitric oxide levels checked. RAW 264.7 cells were plated and then treated with safranal (20, 30, 40μM) in combination with LPS (1μg/ml), 24hr later the supernatant was collected and nitric oxide levels measured by Griess assay. The safranal seemed to change the nitric oxide levels (as per Griess assay), however there was a slight decrease in nitric oxide level even in drug control, additionally the colour of medium changed significantly, interfering the assay.

In order to check the actual effect of safranal on NO, the expression of iNOS (which is responsible for high level of NO release in macrophages) was checked, The effect on iNOS protein expression was studied by immunoblot, equal amounts of protein (50μg) were resolved in SDS-PAGE and then transferred to a PVDF membrane and the expression of iNOS was then detected using specific antibodies. The detection of β-actin was also performed as an internal control. The intensity of protein bands were analyzed using blot analysis software, which showed no change in iNOS levels by safranal treatment thereby providing supporting evidence that safranal has insignificant effect on LPS induced nitric oxide levels.

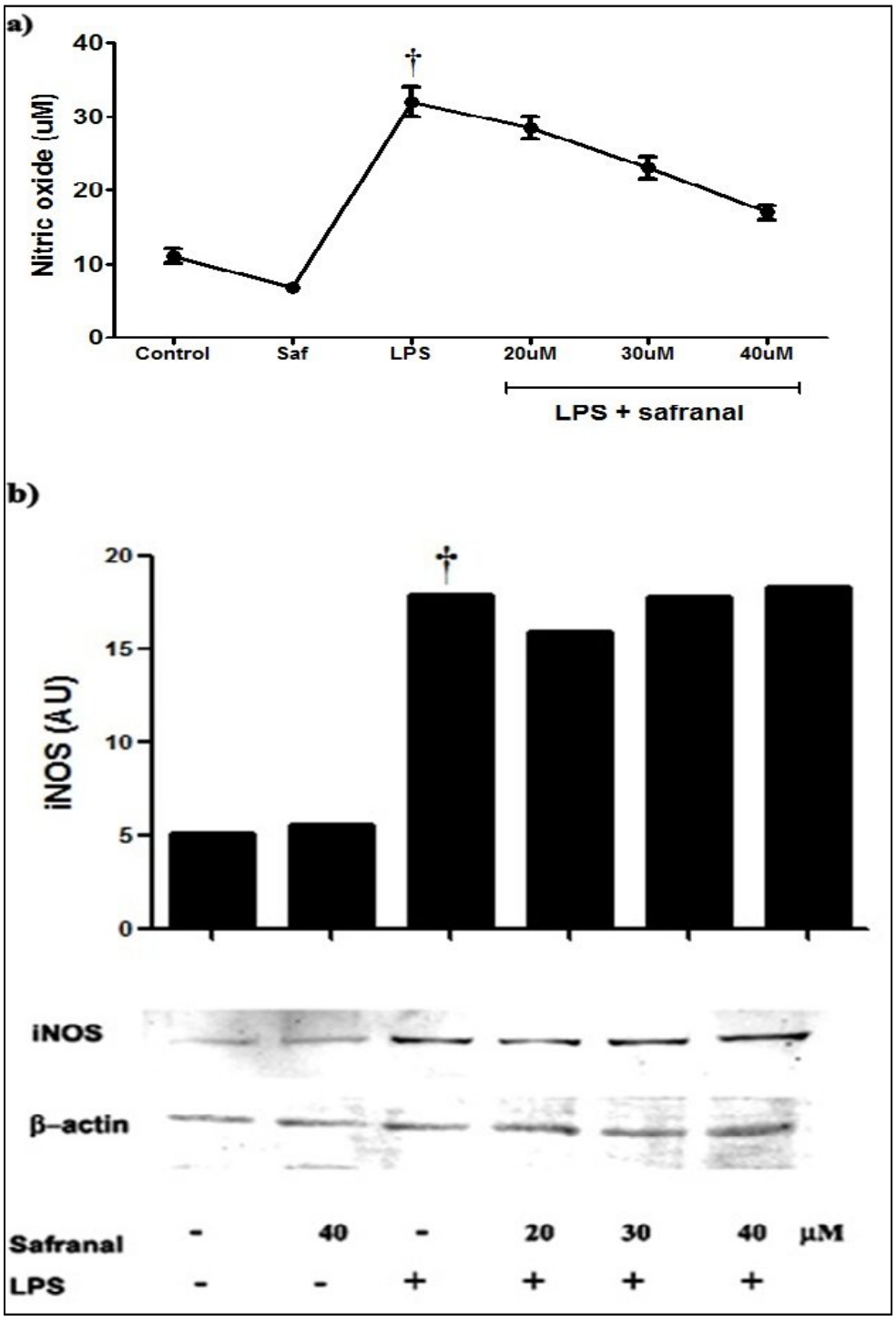


Figure 3.4: **Effect of Safranal on LPS induced nitric oxide and iNOS**. Murine macrophage-like RAW 264.7 cells were stimulated with LPS (1µg/ml) and different concentrations of safranal; a) At 24hr after stimulation, nitric oxide level was measured in supernatant by Griess assay; b) At 24hr after stimulation, iNOS levels in RAW 264.7 cells. Data represent the mean ± SEM of three independent experiments (n = 3). †, $p < 0.001$ when compared with control (vehicle only).

3.5 Effect of psychosine on nitric oxide levels

Psychosine is the trivial name for a monoglycosylsphingolipid, which is the lyso or non-acylated form of a cerebroside, e.g. galactosylsphingosine. To analyse anti-inflammatory potential of psychosine in RAW 264.7 macrophage cells, cells were treated with two different concentrations of psychosine (4 & 8μM) simultaneously with LPS (1μg/ml). After 24hr the supernatant was collected. The effects of psychosine on LPS-induced nitric oxide levels in supernatant were examined at 24hr after stimulation. Psychosine at lower concentration (4μM) induced comparatively less reduction in the levels of nitric oxide level, however there was a significant ($p<0.05$) decrease in nitric oxide levels at higher concentration of psychosine (8μM).

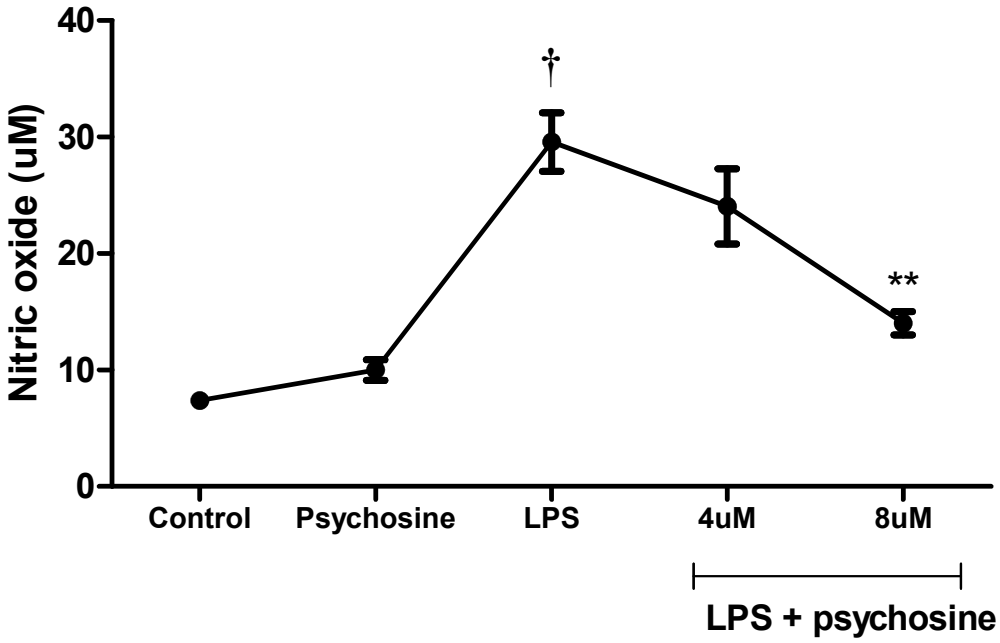


Figure 3.5: **Effect of psychosine on LPS induced nitric oxide**. Murine macrophage-like RAW 264.7 cells were stimulated with LPS (1μg/ml) and different concentrations of psychosine (4 and 8μM). At 24hr after stimulation, nitric oxide level was measured in supernatant by Griess assay. Data represent the mean ± SEM of three independent experiments (n = 3).†, $p< 0.001$ when compared with control (vehicle only) **$p < 0.05$ when compared with the group treated with LPS (1 μg/ml) alone ; p-values were calculated by Bonferroni non-parametric ANOVA test.

3.6 Effect of emodin on nitric oxide levels in RAW 264.7 cells

Emodin is seen in various plants of medicinal importance. In order to investigate the effect of emodin on LPS-induced nitric oxide levels in cell supernatant, RAW 264.7 macrophage cells were stimulated with LPS (1 µg/ml) along with two concentrations of emodin (6 & 12µM). 24hr later cell supernatant was obtained for nitric oxide measurement. The emodin showed significant inhibitory effect on nitric oxide levels at both 6 and 12µM concentrations.

By the time of our experiments, some researchers showed that emodin inhibited HMGB1 release in activated RAW 264.7 cells (Chen et al 2010).

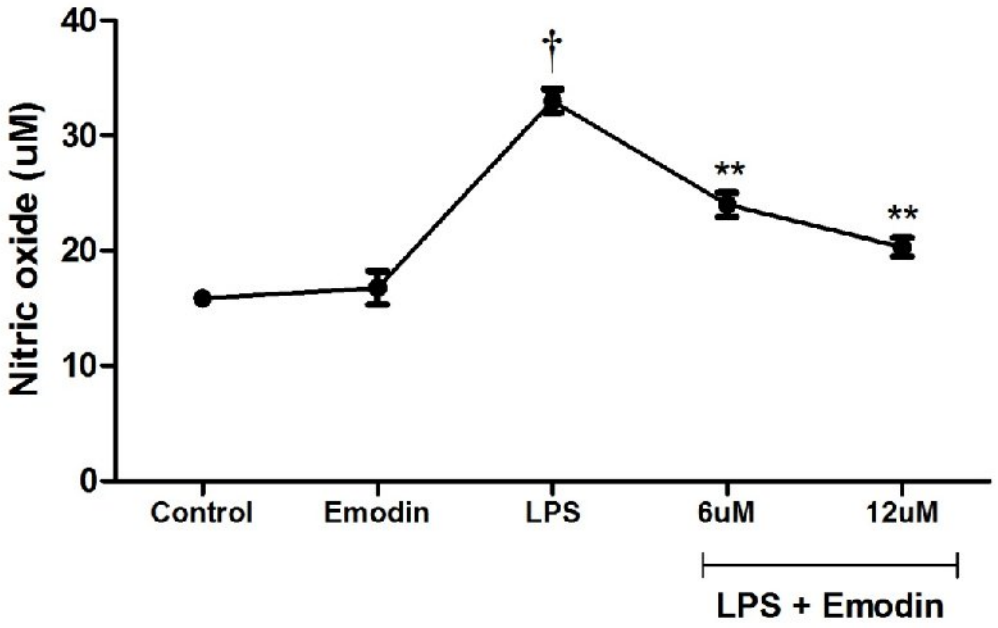


Figure 3.6: Effect of emodin on LPS induced nitric oxide. Murine macrophage-like RAW 264.7 cells were stimulated with LPS (1µg/ml) and different concentrations of emodin. At 24hr after stimulation, nitric oxide level was measured in supernatant by Griess assay. Data represent the mean ± SEM of three independent experiments (n = 3). †,$p < 0.001$ when compared with control (vehicle only), **$p < 0.01$ and ***$p<0.005$ when compared with the group treated with LPS only; p-values were calculated by Bonferroni non-parametric ANOVA test.

3.7 Effect of aloe-emodin on nitric oxide in RAW 264.7 macrophages

Recent research has shown beneficial effects of aloe-emodin in treating cancer, however not much is known about its effect in inflammation. To investigate the effect of aleo emodin (AE) on nitric oxide production, RAW 264.7 macrophage cells were treated with AE (5, 10 & 15µM) along with LPS (1µg/ml). And 24hr after LPS induction, NO concentrations were measured in the culture supernatants by Griess assaay. Aloe-emodin significantly inhibited LPS-induced NO production in a dose-dependent manner with 15µM inhibiting the most.

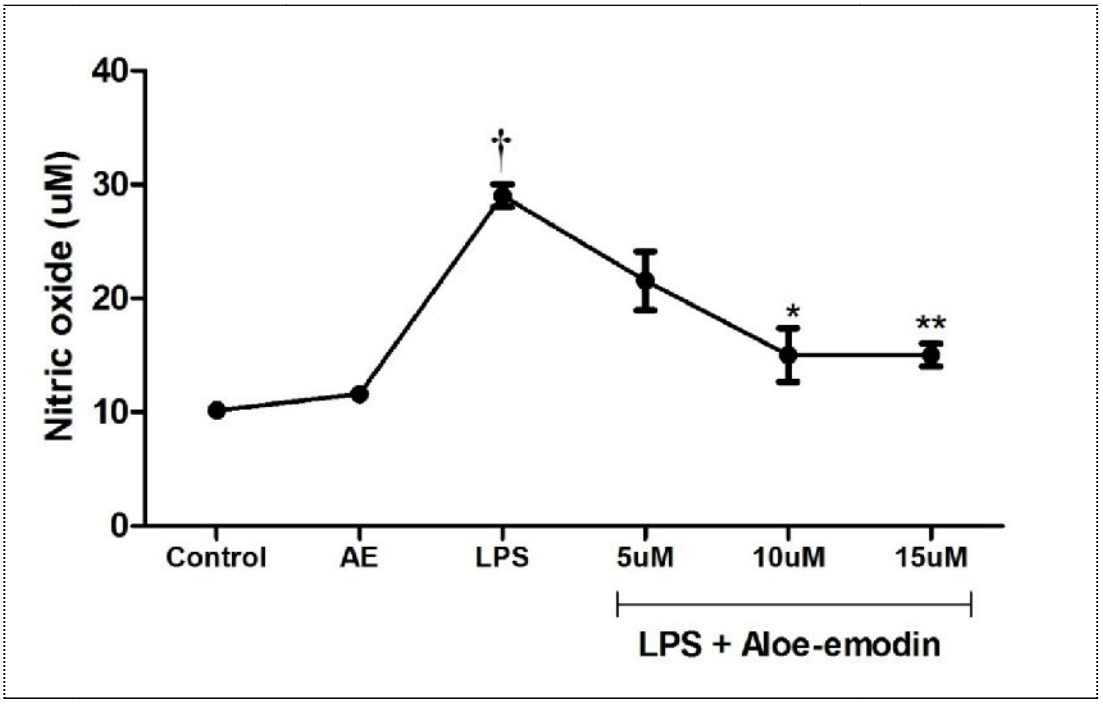


Figure 3.7: **Effect of aloe-emodin on LPS induced nitric oxide**. Murine macrophage-like RAW 264.7 cells were treated with different concentrations of AE and LPS (1µg/ml). At 24hr after stimulation, nitric oxide level was measured in supernatant by Griess assay. Data represent the mean ± SEM of three independent experiments (n = 3) done in duplicate. †,$p<0.001$ when compared with control (vehicle only), **$p < 0.01$ and ***$p<0.005$ when compared with the group treated with LPS only; p-values were calculated by Bonferroni non-parametric ANOVA test.

Part II- Psychosine

3.8 Effect of psychosine on Nitric oxide release

Psychosine is the trivial name for a monoglycosylsphingolipid, which is the lyso or non-acylated form of a cerebroside, e.g. β-galactosylsphingosine. It is a minor intermediate in the catabolism of monoglycosylceramides, and is normally present in brain tissues at very low concentrations. In Krabbe disease, deficiency of galactosyl ceramidase results in accumulation of psychosine. Psychosine mediates apoptosis in oligodendrocytes and the debris activates microglial cells (resident macrophages in the brain), which are the primary mediators of neuroinflammation. However the effect of psychosine on peripheral cells is still unknown.

We examine the possibility of NO scavenging property in LPS stimulated murine macrophage-like RAW 264.7 cells.

RAW 264.7 macrophage cells were treated with LPS in the presence of two different concentrations of psychosine (4μM and 8μM) and nitric oxide level in cell supernatant was examined after 24hr. Psychosine treatment showed a concentration dependent effect with lower concentration (4μM) inducing decrease in the levels of nitric oxide level and a significant (**$p<0.05$**) decrease in nitric oxide levels at higher concentrations (8μM) (**Fig 3.8a**).

It was recently observed that psychosine effect varies from individual cell lines to that of mixed primary cell cultures. So assessing the psychosine effects in primary murine peritoneal macrophages (primary cell culture) was essential. Primary murine peritoneal macrophages (PMphs) were isolated from C57BL/6 and incubated overnight in RPMI. PMphs were then stimulated with LPS (1μg/ml) along with different concentrations of psychosine. At 24hr, after stimulation NO levels were checked in cell supernatant by Griess assay. In PMphs, LPS induced significant increase in nitric oxide in comparison to control however the psychosine treatment in stimulated cells slightly increased the nitric oxide levels although not significant enough (**$p>0.05$**). The treatment of psychosine alone also showed slight increase in nitric oxide levels.

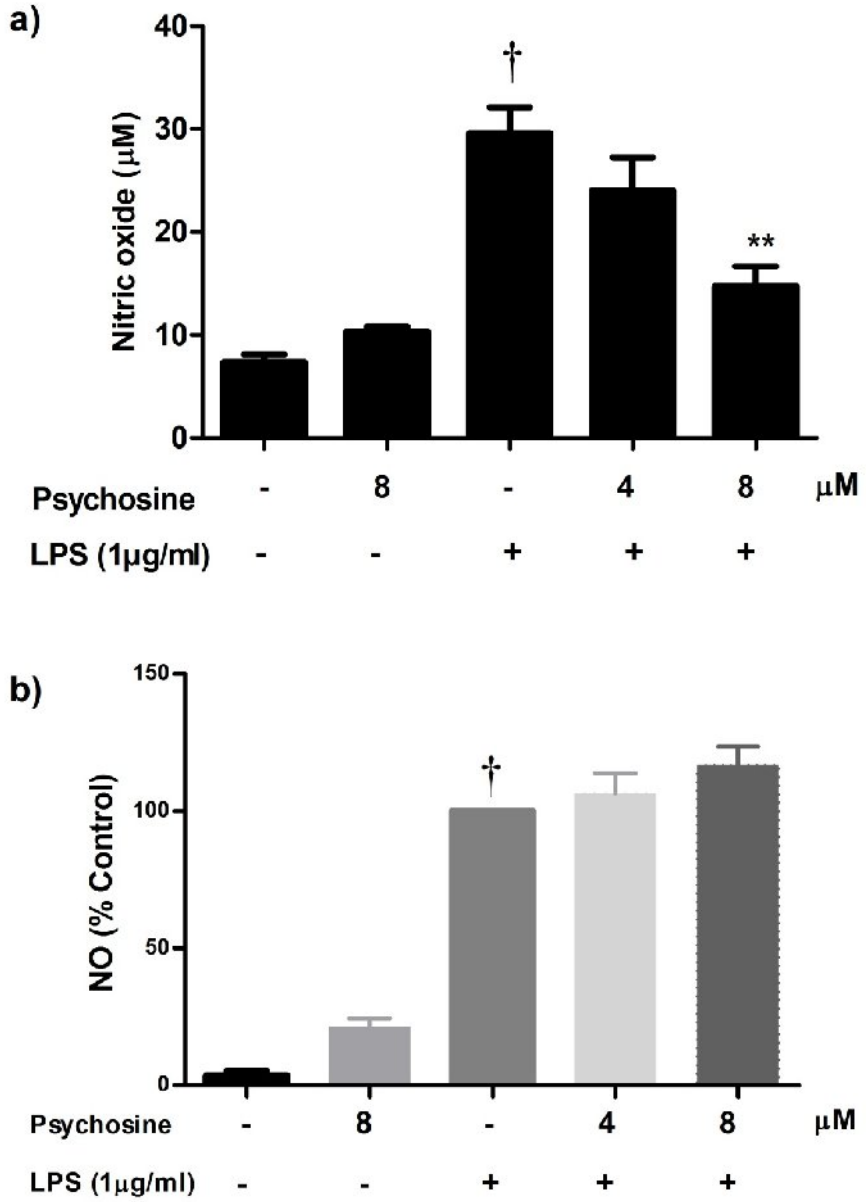


Figure 3.8. Effect of psychosine on LPS-induced nitric oxide release a) RAW 264.7 cells b) Primary murine peritoneal macrophages. The nitric oxide levels in supernatant were checked at 24hr after LPS stimulation in the presence of pychosine. Data from at least three experiments were normalized by denoting the NO levels in supernatant of LPS stimulated cells as 100% and subsequently calculating the effect achieved by addition of psychosine. Data represent the mean ± SEM of three independent experiments (N = 3). †$p < 0.001$ when compared with control (vehicle only), *$p < 0.05$ and **$p < 0.01$when compared with the group treated with LPS (1 μg/ml) alone ; p-values were calculated by Bonferroni non-parametric ANOVA test.

3.9 Effect of psychosine on LPS induced HMGB1 release

Psychosine showed different effect on LPS induced nitric oxide in RAW cells and primary PMphs. Since our main target was HMGB1, so we went ahead to check the effect of psychosine on LPS induced HMGB1 release. HMGB1 which is released actively by LPS or TNF-α stimulated macrophages, is released quite late than early mediators (TNF, IL-1), it is thus known as later mediator of sepsis and is detectable after 18hr.

To study the effect of psychosine on LPS-induced HMGB1 release, cells were treated with different concentrations of psychosine (4μM & 8μM) and HMGB1 release was checked by immunoblot. RAW 264.7 macrophage cells were pretreated with psychosine and then stimulated with LPS (1μg/ml). After 24hr of stimulation, supernatant was collected and total protein concentrated by precipitation was resolved in SDS-PAGE gel and then transferred to a PVDF membrane. The blot was first stained with ponceau S for checking equal protein load and then using specific antibody for HMGB1, expression was analysed. It was seen that, psychosine at both concentrations had no significant effect on the LPS-induced release of HMGB1 (**Fig 3.9a**).

Since psychosine showed different effects on NO levels in RAW 264.7 cells and primary PMphs, so we analysed if psychosine has any effect on HMGB1 release in different cellular environment. PMphs were isolated from C57BL/6 and incubated overnight in RPMI. PMphs were then incubated with psychosine (4 & 8μM) and stimulated with LPS (1μg/ml). Supernatant was collected at 24hr after LPS stimulation, and HMGB1 levels were checked by in-house ELISA. In combination with LPS, psychosine increased the release of HMGB1, with higher concentration (8μM) increasing most, psychosine alone also showed small but significant increase in HMGB1 levels.

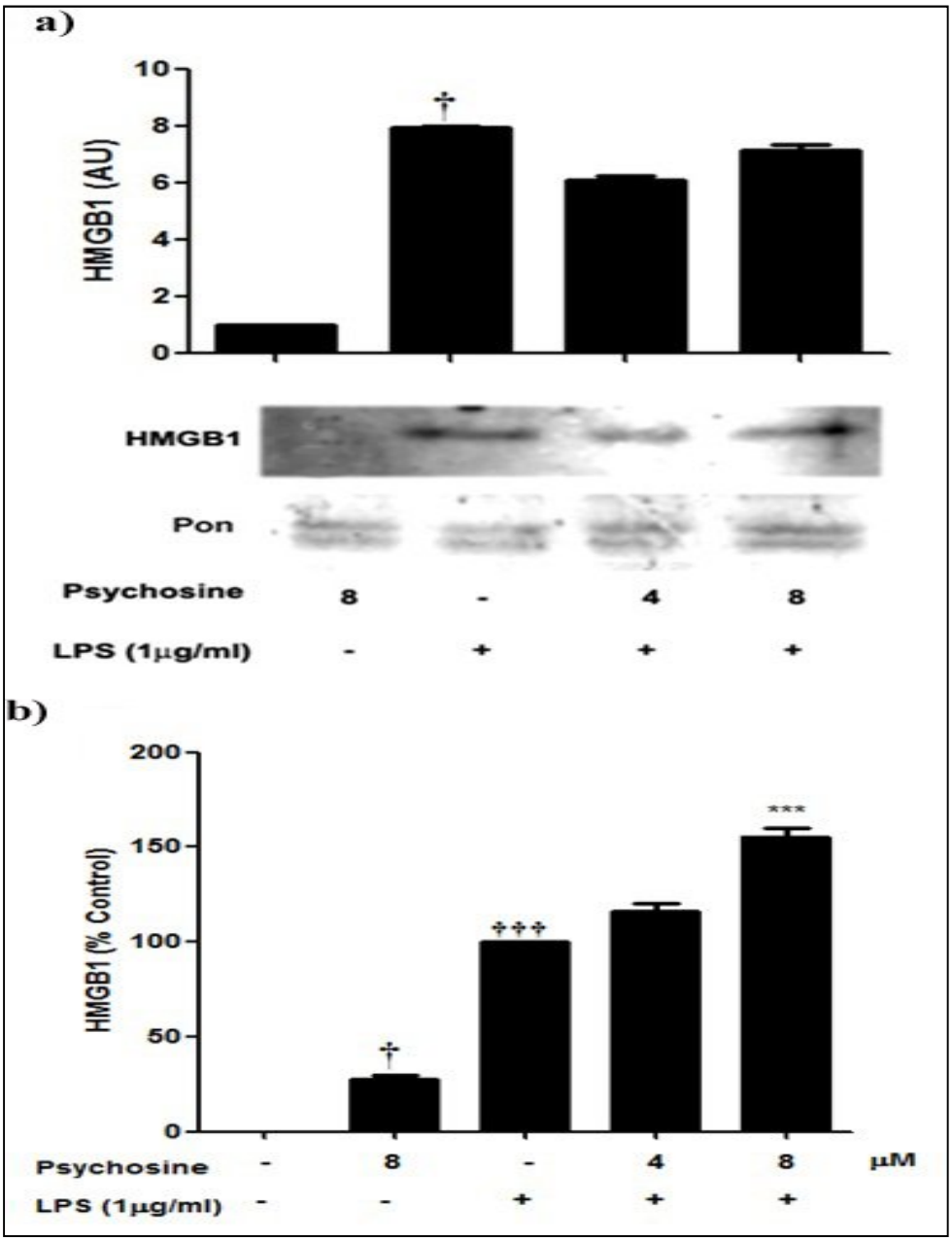


Figure 3.9: **Effect of psychosine on LPS induced HMGB1 release** a) The HMGB1 expression in RAW 264.7 cell supernatant was seen by western blot at 24hr after LPS stimulation. Density of the protein bands were expressed (in arbitrary units (AU)) as mean ± SEM of 2 independent experiments (Ponceau S staining was done for checking equal load of protein) b) HMGB1 release in PMphs cell supernatants was detected by in-house ELISA. Data from at least three experiments were normalized by denoting the HMGB1 release from LPS stimulated cells as 100% and subsequently calculating the effect achieved by addition of psychosine. The values are expressed in mean (±SEM) of two experiments in duplicate. †p< 0.05 and †††p< 0.001 when compared with control and ***p<0.001 when compared with LPS group; p-values were calculated by Bonferroni non-parametric ANOVA test.

3.10 Effect of psychosine on LPS induced TNF-α

Psychosine increased HMGB1 release in primary PMphs without effecting its release in RAW 264.7 cell levels. As HMGB1 release is dependent partly on TNF-α stimulation so it was interesting to check the effect of psychosine on TNF-α levels in two cellular milieu. Murine macrophage-like RAW 264.7 cells were stimulated with LPS and treated with different concentrations of psychosine. At 12hr after stimulation, TNF-α level was measured in cell supernatant by ELISA. It was observed that psychosine at high concentrations (8μM) attenuated the rise in levels of TNF-α significantly compared to LPS stimulated group. At lower concentrations (4μM) psychosine decreased the TNF-α level but the decrease was not significant enough.

Parallel experiments as above were done in PMphs, which were stimulated with LPS (1μg/ml) along with different concentrations of psychosine. The TNF-α levels were measured in cell supernatant at 12hr after stimulation. Psychosine in contrast to RAW 264.7 cells increased the TNF-α level in combination with LPS. Psychosine alone slightly increased the TNF-α level compared to control (not significant, $p>0.05$), however when PMphs were treated in combination with psychosine the TNF-α levels were raised in concentration-dependent manner with higher concentration (8μM) showing significant increase than lower concentrations.

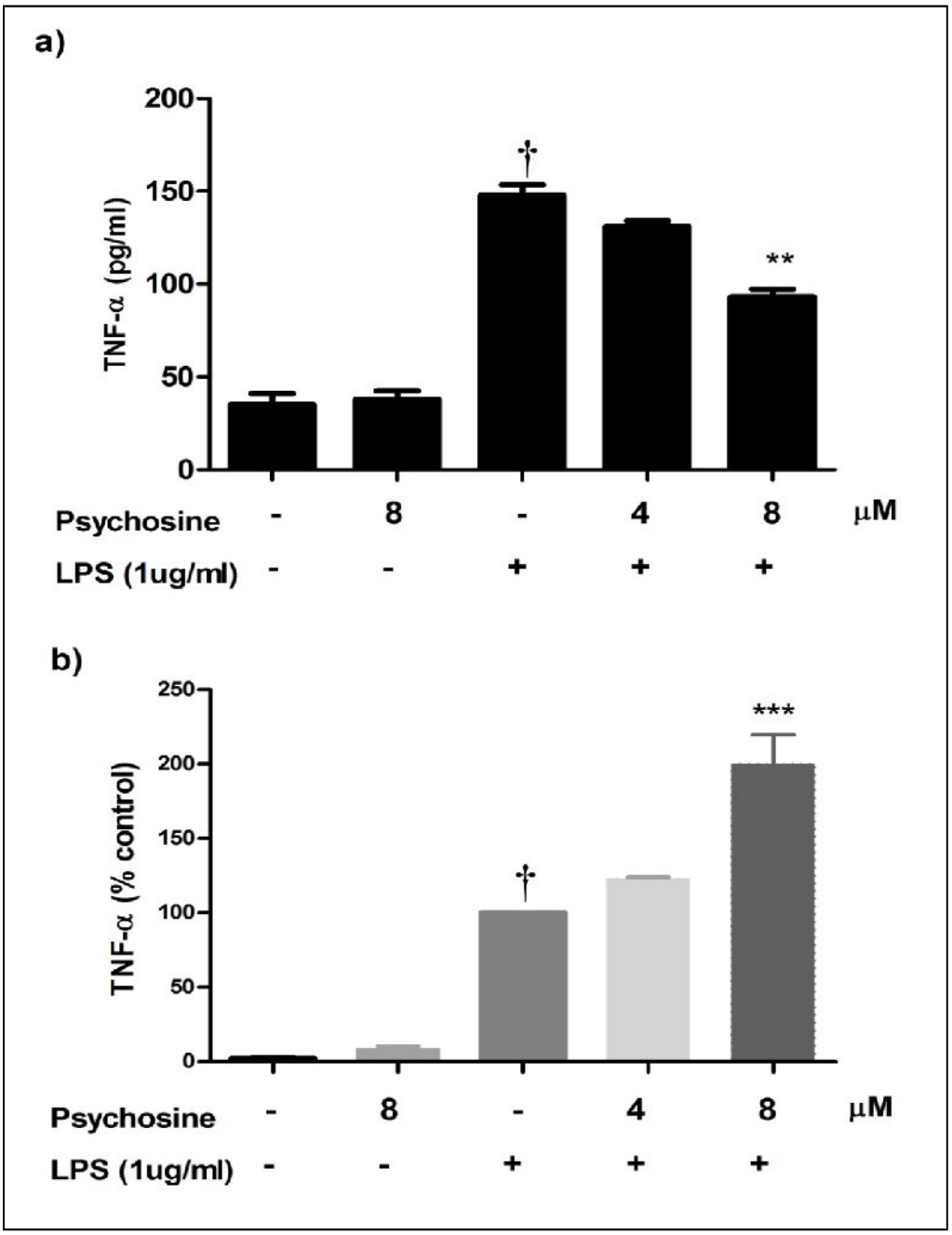


Figure 3.10: **Effect of psychosine on LPS induced TNF-α levels.** a) RAW 264.7 b) PMphs. The TNF-α levels were checked at 12hr after LPS stimulation in the presence of psychosine using ELISA. Data from at least three experiments were normalized by denoting the TNF-α release from LPS stimulated cells as 100% and subsequently calculating the effect achieved by addition of psychosine. The values are expressed in mean (±SEM) of three experiments in duplicate. †$p < 0.001$ when compared with control; **$p < 0.01$ and ***$p<0.001$ when compared with the group treated with LPS (1 μg/ml) alone; p-values were calculated by Bonferroni non-parametric ANOVA test.

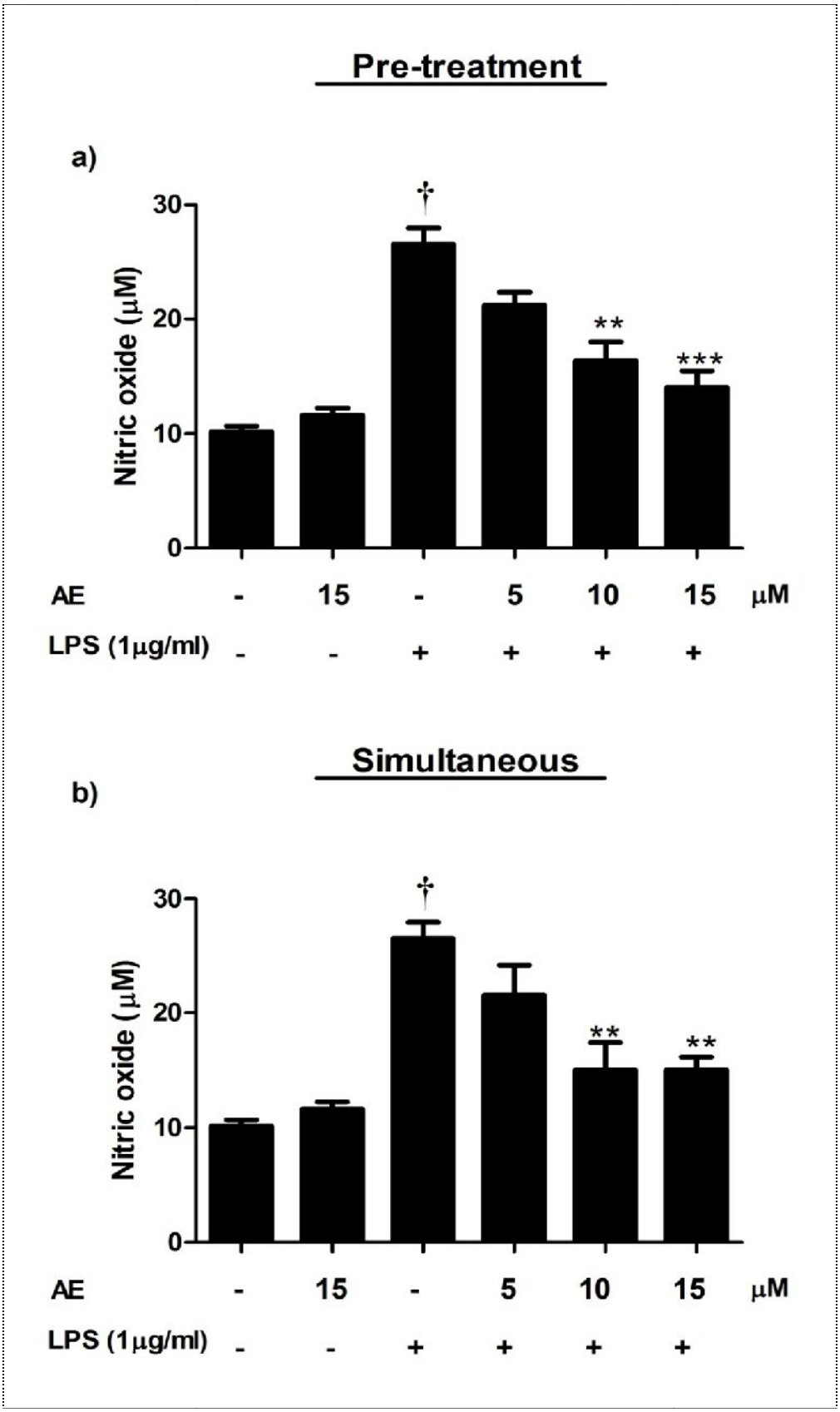
Part III- Aleo-Emodin
(In-vitro study)

Aloe emodin is present in various plants of medicinal importance. It belongs to a family of compounds called anthraquinones, which are reported to possess anti-inflammatory and anticancer effects. As reported in screening aloe-emodin showed a significant decrease in LPS-induced nitric oxide level, so we now had to optimise the timing of aloe-emodin(AE) treatment relative to LPS treatment on the basis of NO levels.

3.11 Effect of Aloe-emodin on LPS induced NO (optimisation of timing)

LPS was used to stimulate NO production in RAW 264.7 mouse macrophage cells. The cells were treated with aloe-emodin (5, 10 & 15μM) but at different time points relative to LPS treatment i.e., pre-treatment (1h before LPS treatment), simultaneous (along with LPS), post-treatment (1h after LPS treatment). After 24hr, nitrite concentration (μM) was determined in cell supernatants. The nitrite production in RAW 264.7 mouse macrophages was inhibited significantly at 10μM and 15μM concentrations of AE, however pre-treatment with LPS further increased its significance and consistency (**Fig 3.11**). Keeping in view the results, aloe-emodin treatment was done 1hr before LPS stimulation in all experiments.

The NO scavenging potential of aloe-emodin was also checked in LPS induced primary murine peritoneal macrophages (PMphs). PMphs, isolated from C57BL/6, plated at equal density in 96-well plate and incubated overnight in RPMI. PMphs were pretreated with different concentrations of aloe-emodin (5, 10 & 15μM) for 1hr and then stimulated with LPS (1μg/ml). 24h after LPS stimulation, NO was estimated in supernatant by Griess assay. It was observed that aloe-emodin at 10 μM and 15μM concentration decreased LPS induced NO levels in concentration dependent manner.


Pre-treatment
a)
Nitric oxide (μM)
30
20
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AE - 15 - 5 10 15 μM
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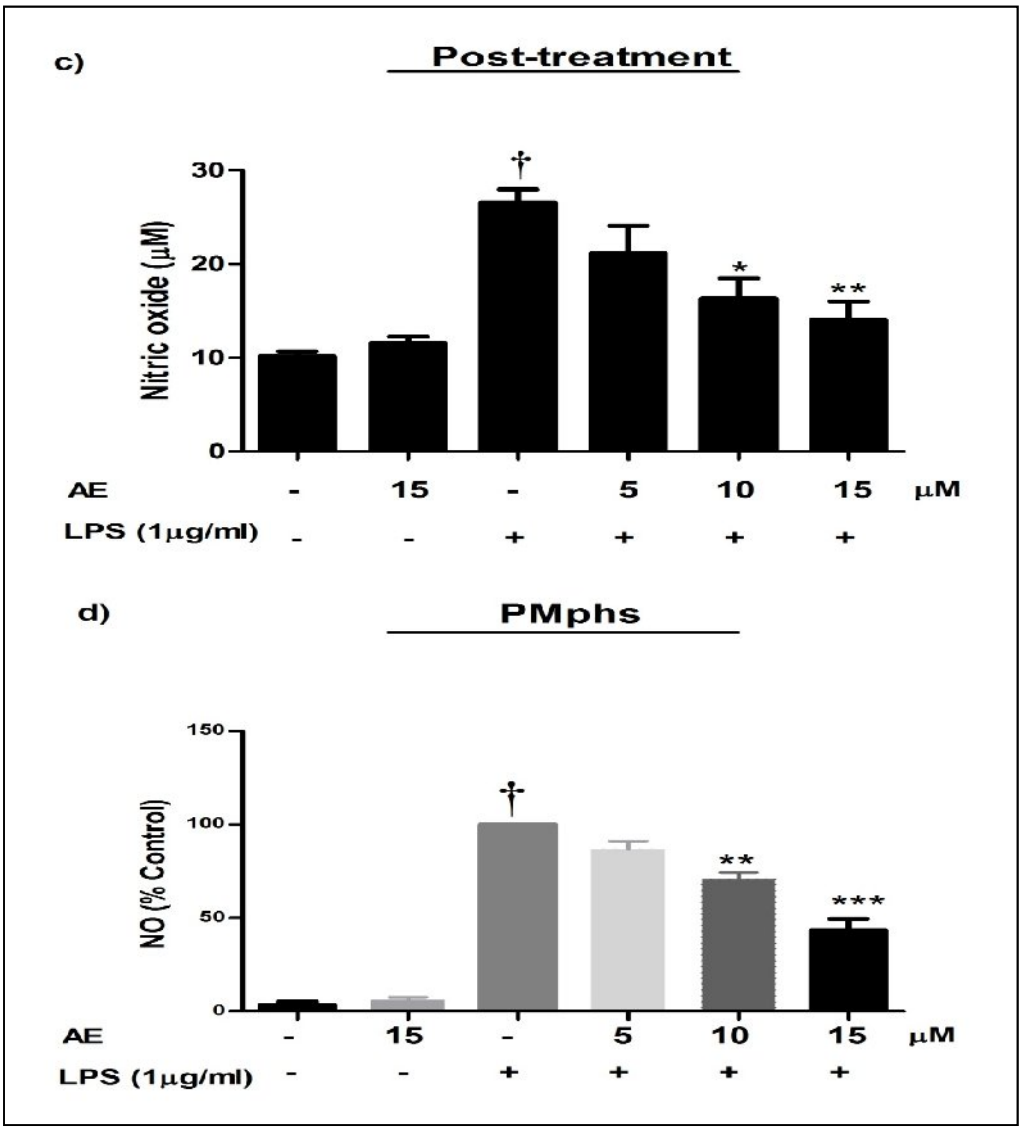


Figure 3.11: Effect of aloe-emodin treatment (timing) on LPS-induced nitric oxide release. RAW 264.7 cells were treated with aloe-emodin at different timings relative to LPS treatment i.e., a) pre-treatment for 1hr b) simultaneous treatment c) post- treatment for 1h d) PMphs isolated from C57BL/6 and treated with aloe-emodin (pre-treated for 1hr) and LPS (1µg/ml). NO levels checked 24h after LPS treatment. Data from at least three experiments were normalized by denoting the NO levels in supernatant of LPS stimulated cells as 100% and subsequently calculating the effect achieved by addition of aloe-emodin. Data represent the mean ± SEM of three independent experiments (N = 3). †$p < 0.001$ when compared with control (vehicle), *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$when compared with the group treated with LPS (1 µg/ml) alone ; p-values were calculated using Bonferroni non-parametric ANOVA test.

3.12 Effect of Aloe-emodin on iNOS expression

In order to investigate whether the aloe-emodin mediate inhibition of NO production was due to a decreased protein expression of iNOS, the effect on iNOS protein expression was studied by immunoblot. Equal amounts of protein (50μg) were resolved in SDS-PAGE and then transferred to a PVDF membrane and the expression of iNOS was then detected using specific antibodies. The results showed that incubation with aloe-emodin (15 μM) after 24 h inhibited iNOS protein expression in RAW 264.7 mouse macrophage cells. The detection of β-actin was also performed as an internal control. The intensity of protein bands were analyzed using blot analysis software, showing an average of 60% down-regulation of iNOS protein after treatment with aloe-emodin (15μM) compared to positive control (LPS) (**Fig 3.12**).

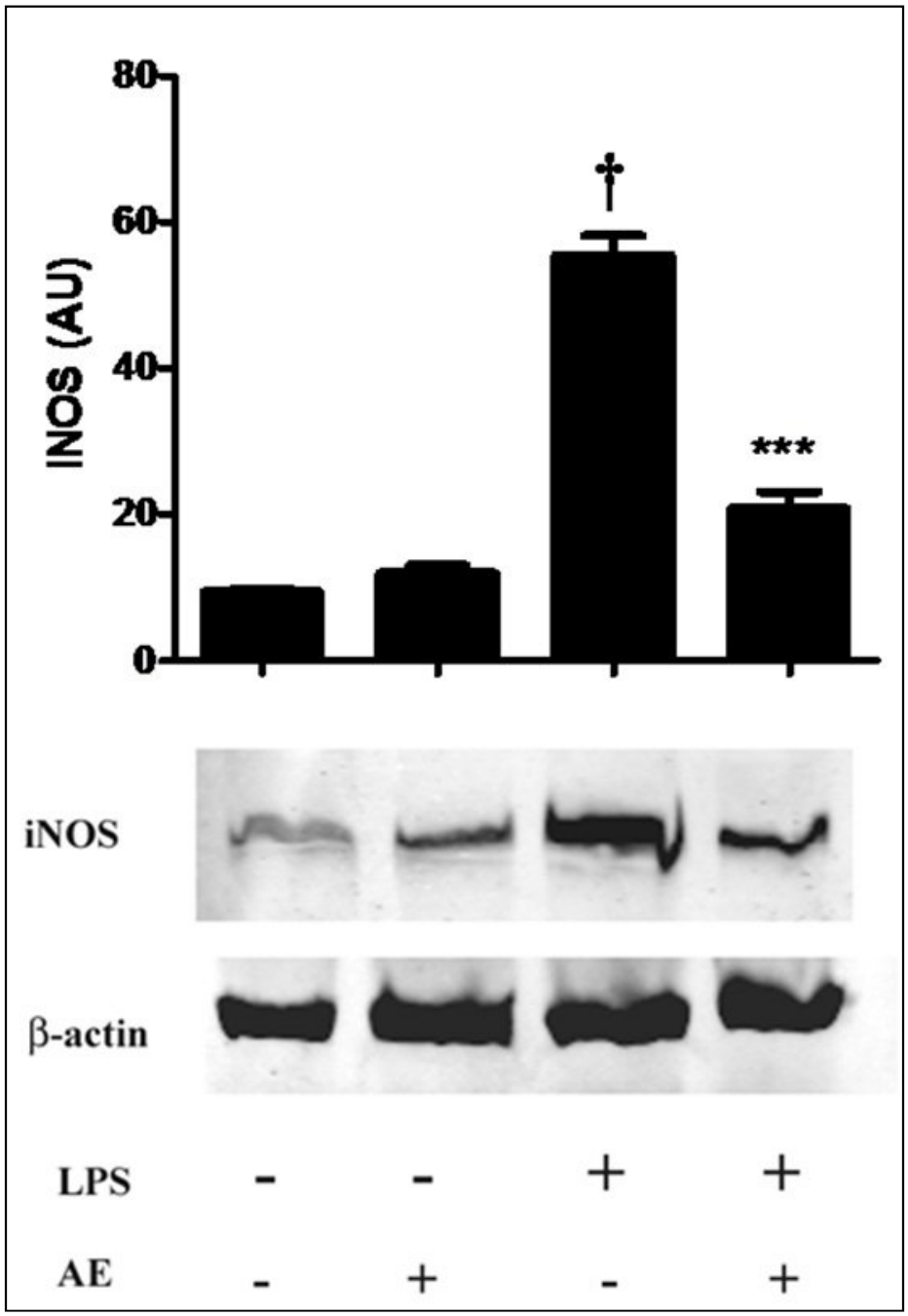


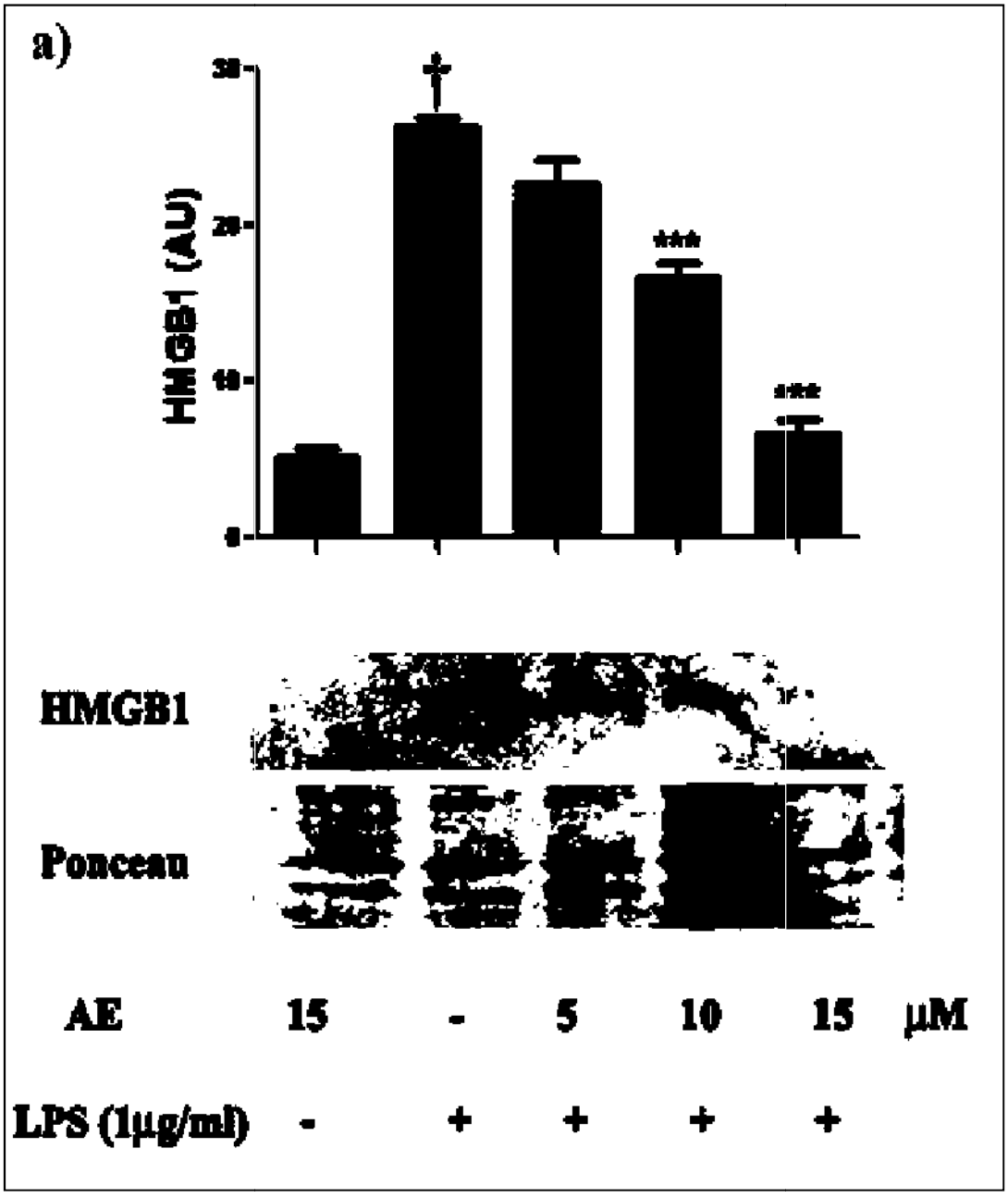
Figure 3.12: Effect of aloe-emodin on LPS-induced iNOS. RAW 264.7 cells were treated with 1 μg/mL of LPS alone (positive control) or with aloe-emodin (15 μM) for 24 h. The iNOS expression was analysed by western blot with β-actin as loading control and density of the protein bands were expressed (in arbitrary units (AU)) as mean ± SEM of 2 independent experiments. The values are expressed in mean (±SEM) of two experiments in duplicate. †$p < 0.001$ when compared with control and ***$p<0.001$ when compared with LPS group; p-values were calculated by Bonferroni non-parametric ANOVA test.

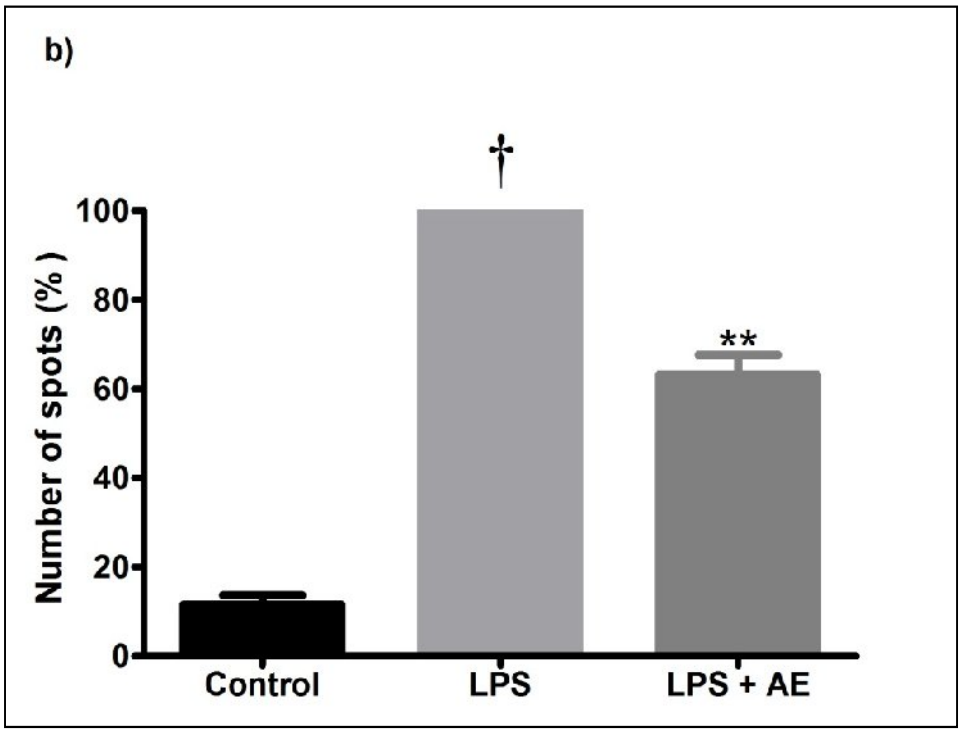
3.13 Effect of Aloe-emodin on LPS induced HMGB1 release

In response to stimulation with lipopolysaccharide (LPS) or endogenous proinflammatory cytokines [such as tumour necrosis factor (TNF), interleukin (IL)-1β, interferon (IFN)-γ], cultures of macrophages/monocytes actively release HMGB1. HMGB1 is time-dependently released from endotoxin-stimulated macrophage cultures, with significant HMGB1 accumulation first detectable at 8hr after stimulation.

To study the effect of aloe-emodin (AE) on LPS-induced HMGB1 release, stimulated cells were treated with different concentrations of aloe-emodin (5μM, 10μM, 15μM) and HMGB1 release was checked by immunoblot. RAW 264.7 macrophage cells were pretreated with aloe-emodin for 1hr and then stimulated with LPS (1μg/ml). After 24hr of stimulation, supernatant was collected and total protein concentrated by precipitation was resolved in SDS-PAGE gel and then transferred to a PVDF membrane. The blot was first stained with Ponceau S for checking equal protein load and then HMGB1 expression was done using specific antibody for it, expression was analysed. It was observed that aloe-emodin inhibited the LPS induced release in concentration-dependent manner, with 15μM inhibiting the release most (**Fig 3.13a**).

The HMGB1 inhibiting potential of aloe-emodin was also checked by in-house developed ELISPOT assay. Around 2000 cells were added per well (96-well coated with PVDF membrane) and after overnight incubation the cells were treated with aloe-emodin (15μM) for 1 h and then stimulated with LPS (1μg/ml). After 24h the plate was processed and HMGB1 release was detected by spots on membrane. Spots indicates the HMGB1 release and comparing spots it was seen that aloe-emodin treatment significantly reduced the LPS induced release in RAW 264.7 cells (**Fig 3.13b**).


a)
HMGB1 (AU)
HMGB1
Ponceau
AE 15 - 5 10 15 µM
LPS (1µg/ml) - + + + +



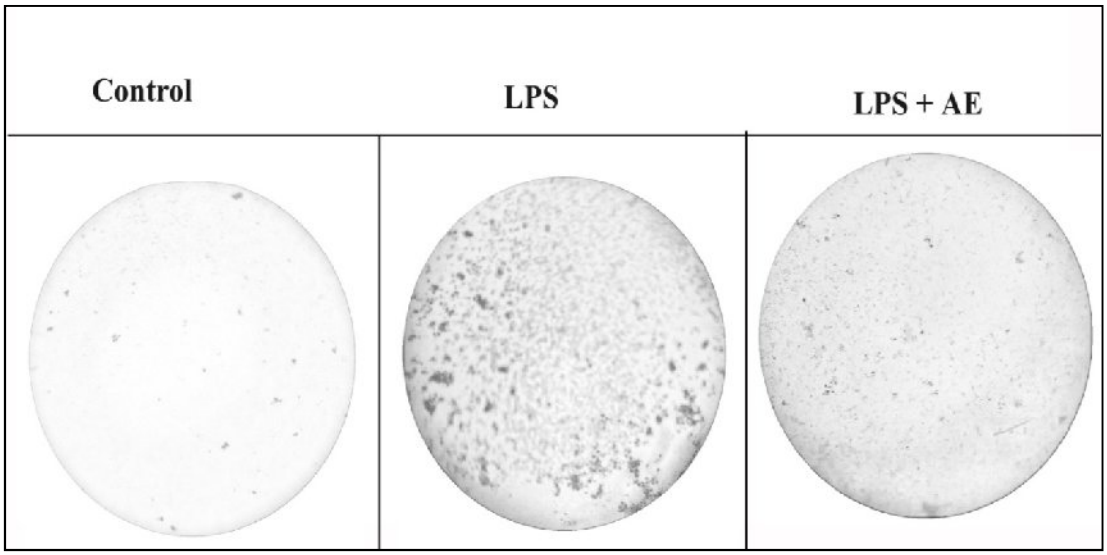


Figure 3.13: Effect of aloe-emodin on LPS-induced HMGB1 release. RAW 264.7 cells were pre-treated with aloe-emodin for 1hr before stimulation with LPS (1µg/ml). a) The HMGB1 expression in culture medium was seen by western blot at 24hr after LPS stimulation. The blot was stained with Ponceau S for checking equal loading and density of the protein bands were expressed (in arbitrary units (AU)) as mean ± SEM of 2 independent experiments b) Secretion of HMGB1 was also detected by ELISPOT. Data from at least three experiments were normalized by denoting the number of spots from LPS stimulated cells as 100% and subsequently calculating the effect achieved by addition of the drug.

We further confirmed aloe-emodin inhibition on HMGB1 release using primary murine peritoneal macrophages (PMphs). PMphs were isolated from C57BL/6 and incubated overnight in RPMI. PMphs were then pretreated with different concentrations of AE (5, 10 & 15μM) for 1hr and stimulated with LPS (1μg/ml). Supernatant was collected 24hr after LPS stimulation, and HMGB1 levels were checked by in-house ELISA. The results were interpreted in comparison to positive control (LPS). It was seen that HMGB1 release is decreased in primary PMphs in concentration-dependent manner with 15μM decreasing the HMGB1 release most (**Fig 3.14**).

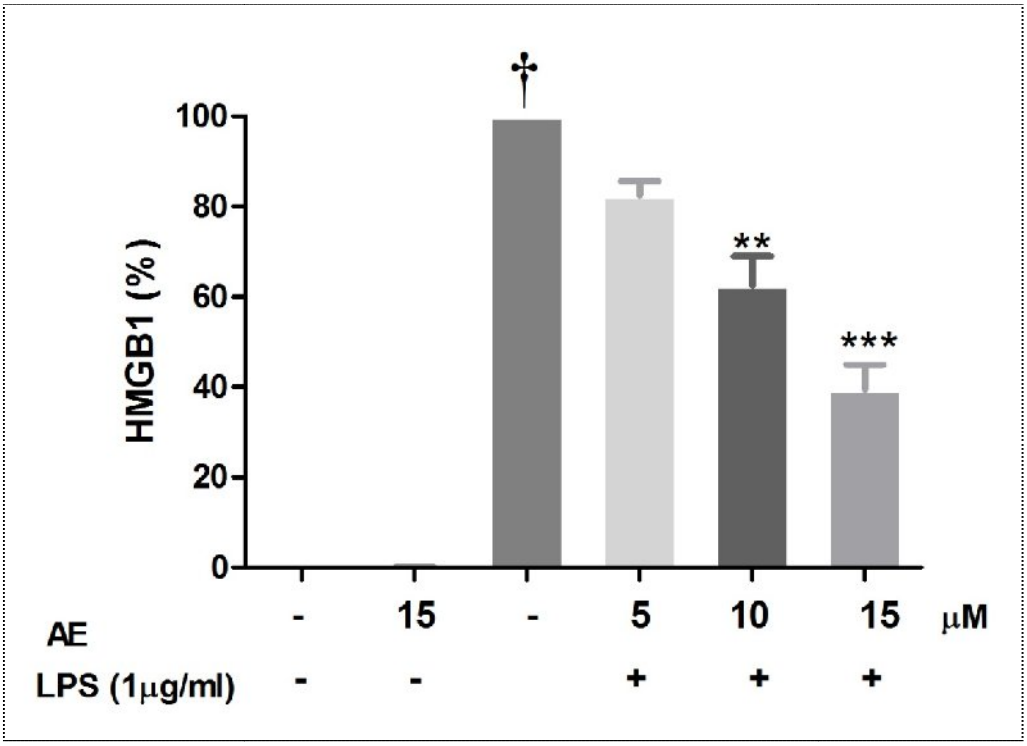


Figure 3.14: Effect of aloe-emodin on LPS-induced HMGB1 release in primary peritoneal macrophages. Primary PMphs stimulated with LPS (1μg/ml) after 1hr pretreatment with different doses of aloe-emodin. HMGB1 release in culture supernatants was detected by in-house ELISA. Data from at least three experiments were normalized by denoting the HMGB1 release from LPS stimulated cells as 100% and subsequently calculating the effect achieved by addition of aloe-emodin. The values are expressed in mean (±SEM) of three experiments in duplicate. †$p<0.001$ when compared with control and **$p<0.01$ and ***$p<0.001$ when compared with LPS group; p-values were calculated by Bonferroni non-parametric ANOVA test.

3.14 Effect of Aloe-emodin on HMGB1 translocation

HMGB1 release by activated macrophages is not due to cell death, but dependent on active translocation of HMGB1 from the nucleus to the cytoplasm and then to extra-cellular space. HMGB1, which is predominantly localized in the nucleus of macrophages, is released to the extracellular space by macrophages on stimulation with LPS. As aloe-emodin was seen to inhibit the HMGB1 release outside cell so we next checked if aloe-emodin treatment effected the nuclear cytoplasmic distribution in activated macrophages.

The LPS induced translocation from nucleus to cytoplasm in LPS-stimulated RAW 264.7 cells was checked by immunofluorescence. It was seen after 16h, HMGB1 which is localized predominantly in nucleus in control cells, is significantly translocated to cytoplasm by LPS treatment. However aloe-emodin treatment markedly inhibited LPS-induced HMGB1 cytoplasmic translocation and preserved its nuclear localization (**Fig 3.15**).

HMGB1 is constitutively expressed in normal and stimulated cells and stimulation with LPS or TNF, induces the nuclear HMGB1 translocation to cytoplasm without effecting the gene expression. To check effect of aloe-emodin or LPS or both on HMGB1 expression, total levels of HMGB1 was checked in macrophages. RAW 264.7 cells were treated with aloe-emodin for 1hr and at 16hr after LPS stimulation, cells were lysed. Equal amounts of protein (100μg) were resolved in SDS-PAGE and then transferred to a PVDF membrane. The expression of HMGB1 and β-actin (loading control) was then detected using specific antibodies (**Fig 3.16a**). To check HMGB1 release by LPS (alone or in comibation with aloe-emodin) in stimulated RAW 264.7 cells is not due to cell death we observed the effect of LPS alone/combination on cell viability (**Fig 3.16**).

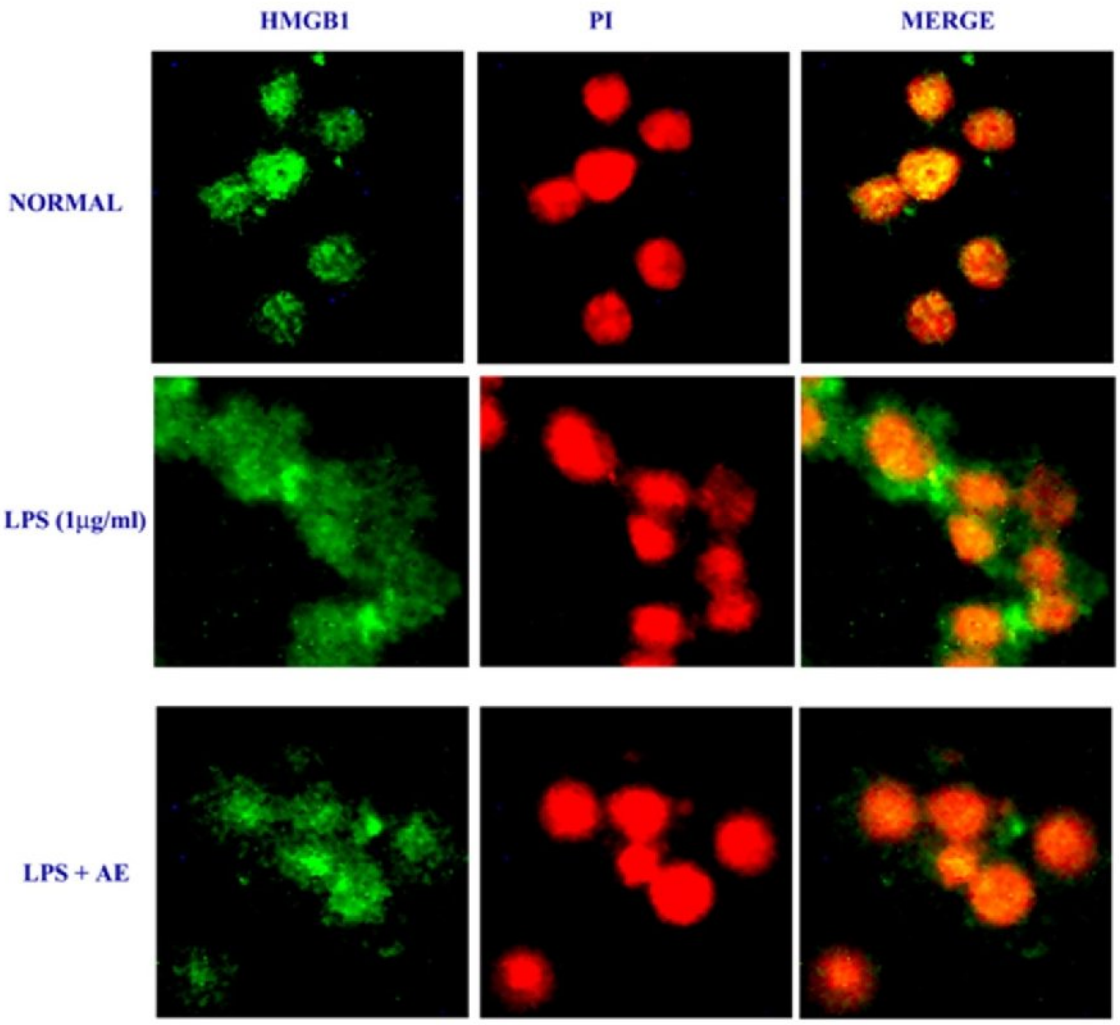


Figure 3.15: Effect of aloe-emodin on LPS-induced HMGB1 translocation. RAW 264.7 cells were cultured for 24 hours without exogenous stimulus (Normal), or with LPS (1µg/ml) or with LPS+AE (15µM). The cells were then fixed and stained by immunofluorescence to identify HMGB1 (green) or cell nuclei (red, Propidium Iodide). HMGB1 was dominantly expressed intranuclearly in unstimulated cells, Cells activated with LPS demonstrated HMGB1 presence both in the nucleus and cytoplasm compared to cells in control. Cells activated by LPS in the presence of AE (15µM) expressed the strong nuclear intensity compared to LPS treated cells.

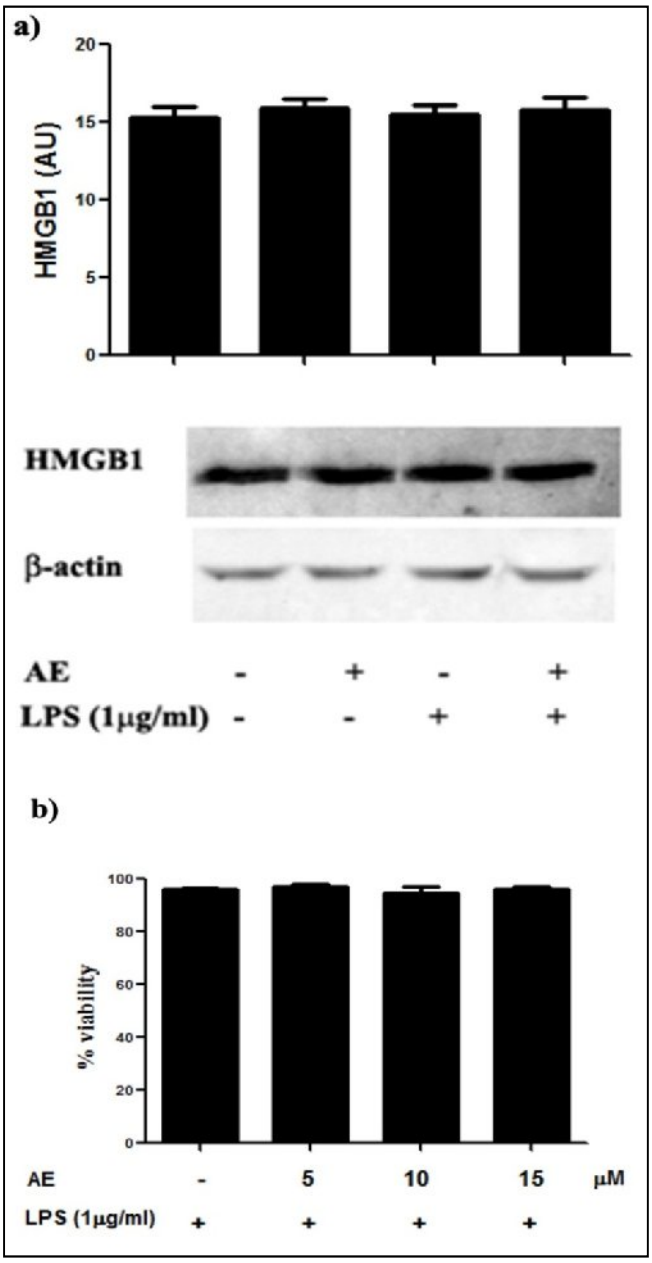


Figure 3.16: Effect of aloe-emodin on HMGB1 expression and cell viability. RAW cells were stimulated with AE (15μM) and LPS (1μg/ml) a) After 16hr, western blot of total cell lysate was done using β-actin as loading control. Density of the protein bands were analyzed and expressed (in arbitrary units (AU)) as mean ± SEM of 2 independent experiments. b) Cell viability assay (MTT assay) was done after 24hr. Data from at least three experiments were normalized by denoting the cell viability from unstimulated/untreated cells as 100% and subsequently calculating the effect achieved by addition of AE (15μM) and or LPS (1μg/ml). The values are expressed in mean (±SEM) of three experiments in duplicate.

3.15 Effect of Aloe-emodin on pro-inflammatory cytokines in activated macrophages.

LPS induced macrophages/monocytes to sequentially release early (TNF-α and IL-1β) and late (HMGB1) proinflammatory cytokines. Early cytokines are released initially to protect from damage but high levels result in dysregulated inflammation and tissue injury. Aloe-emodin decreased the release of late mediator HMGB1, so we now analysed its effect on early mediators.

TNF-α

TNF-α is regarded as a pro-inflammatory cytokine that is produced in response to injury, exerting a large number of important roles in the immune system and during inflammatory responses. In response to LPS, macrophages releases TNF-α in few hours which then enhance the LPS action by inducing release of other pro-inflammatory cytokines.

To determine the effect of aloe-emodin on LPS induced pro-inflammatory cytokine release, experiments on RAW 264.7 cells and primary PMphs were done.

RAW 264.7 macrophage cells pre-treated with AE for 1hr with three different concentrations, were stimulated with LPS. After 12h of stimulation, the TNF-α levels were measured in cell supernatant by ELISA. It was seen that LPS stimulation increased the TNF-α levels from 41.75±2.32 pg/ml (control) to 233.9± 17.02 pg/ml in 12 h. The AE treatment decreased the TNF-α levels in concentration-dependent manner, with 5μM (202.6±8.727 pg/ml) showing no significant change in levels, however higher concentrations decreasing TNF-α significantly (10μM concentration, 159.3±13.27 pg/ml: 15μM concentration, 84.64±6.91 pg/ml) (**Fig 3.17a**).

We further confirmed the effect of AE on TNF-α using primary murine peritoneal macrophages (PMphs). PMphs were isolated from C57BL/6 mice and incubated overnight in RPMI. PMphs were then pretreated with different concentrations of aloe-emodin (5, 10 & 15μM) for 1hr and stimulated with LPS (1μg/ml). Supernatant was collected at 12 h after LPS stimulation, and TNF-α levels were checked by ELISA. It was observed that TNF-α levels increased significantly after LPS treatment and aloe-

emodin inhibited LPS induced increase in TNF-α levels significantly ($p<0.001$) in concentration-dependent manner (**Fig 3.17b)**.

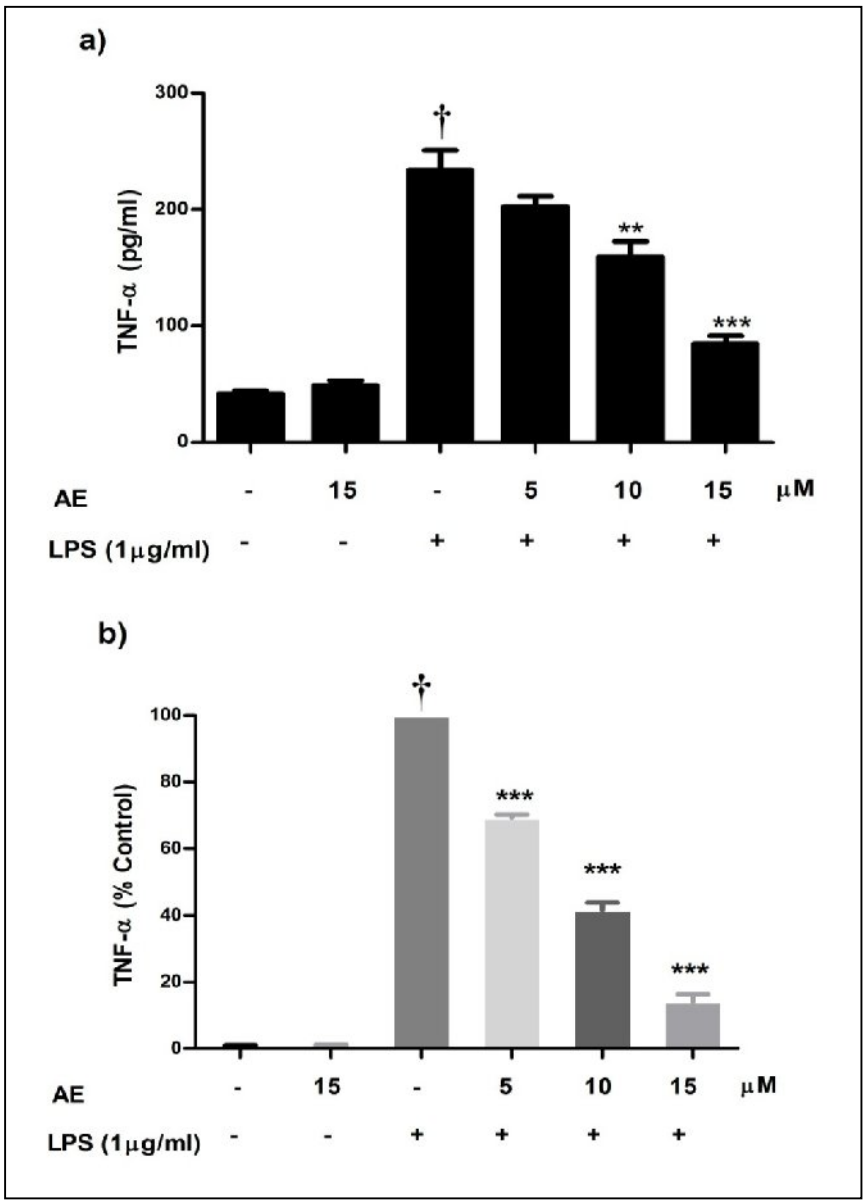


Figure 3.17: Effect of aloe-emodin on TNF-α release in activated macrophages a) RAW 264.7 cells b) PMphs, isolated from C57BL/6 mice. Cells were treated with AE for 1hr and followed by stimulation with LPS (1μg/ml). The TNF–α levels were checked at 12hr after LPS stimulation. Data from at least three experiments were normalized by denoting the TNF-α release from LPS stimulated cells as 100% and subsequently calculating the effect achieved by addition of aloe-emodin. The values are expressed in mean (±SEM) of three experiments in duplicate. †$p< 0.001$ when compared with control; **$p < 0.01$ and ***$p<0.001$ when compared with the group treated with LPS (1 μg/ml) alone; p-values were calculated by Bonferroni non-parametric ANOVA test.

IL-1β

IL-1 is synthesized by mononuclear phagocytes, polymorphonuclear leucocytes and other cell types and affects a wide variety of tissues. IL-1β is the predominant form of this mediator produced by endotoxin-stimulated human monocytes and detected in the plasma of septic animals.

To determine the effect of aloe-emodin on LPS induced IL-1β release, experiments on RAW 264.7 cells and primary PMphs were done.

RAW 264.7 macrophage cells pre-treated with aloe-emodin, for 1hr with three different concentrations, were stimulated with LPS. After 12h of stimulation, the IL-1β levels were measured in supernatant by ELISA. It was seen that LPS stimulation increased the IL-1β levels from 10.55±1.381 pg/ml (control) to 66.56±11.35 pg/ml in 12 h. The aloe-emodin treatment decreased the IL-1β levels in concentration-dependent manner, with 5μM (51.20±3.975 pg/ml) showing no significant change in levels, however higher concentrations decreasing IL-1β significantly (10μM concentration, 36.32±3.777 pg/ml: 15μM concentration, 39.24±1.505 pg/ml) (**Fig 3.18a)**.

We further confirmed the effect of aloe-emodin on IL-1β release in LPS stimulated primary murine peritoneal macrophages (PMphs). PMphs were isolated from C57BL/6 and incubated overnight in RPMI. PMphs were then pretreated with different concentrations of aloe-emodin (5, 10 & 15μM) for 1hr and stimulated with LPS (1μg/ml). Supernatant was collected at 12hr after LPS stimulation, and IL-1β levels were checked by ELISA. It was observed that IL-1β levels increased significantly after LPS treatment and aloe-emodin inhibited the increase in IL-1β levels significantly ($p<0.001$) in concentration-dependent manner (**Fig 3.17b)**.

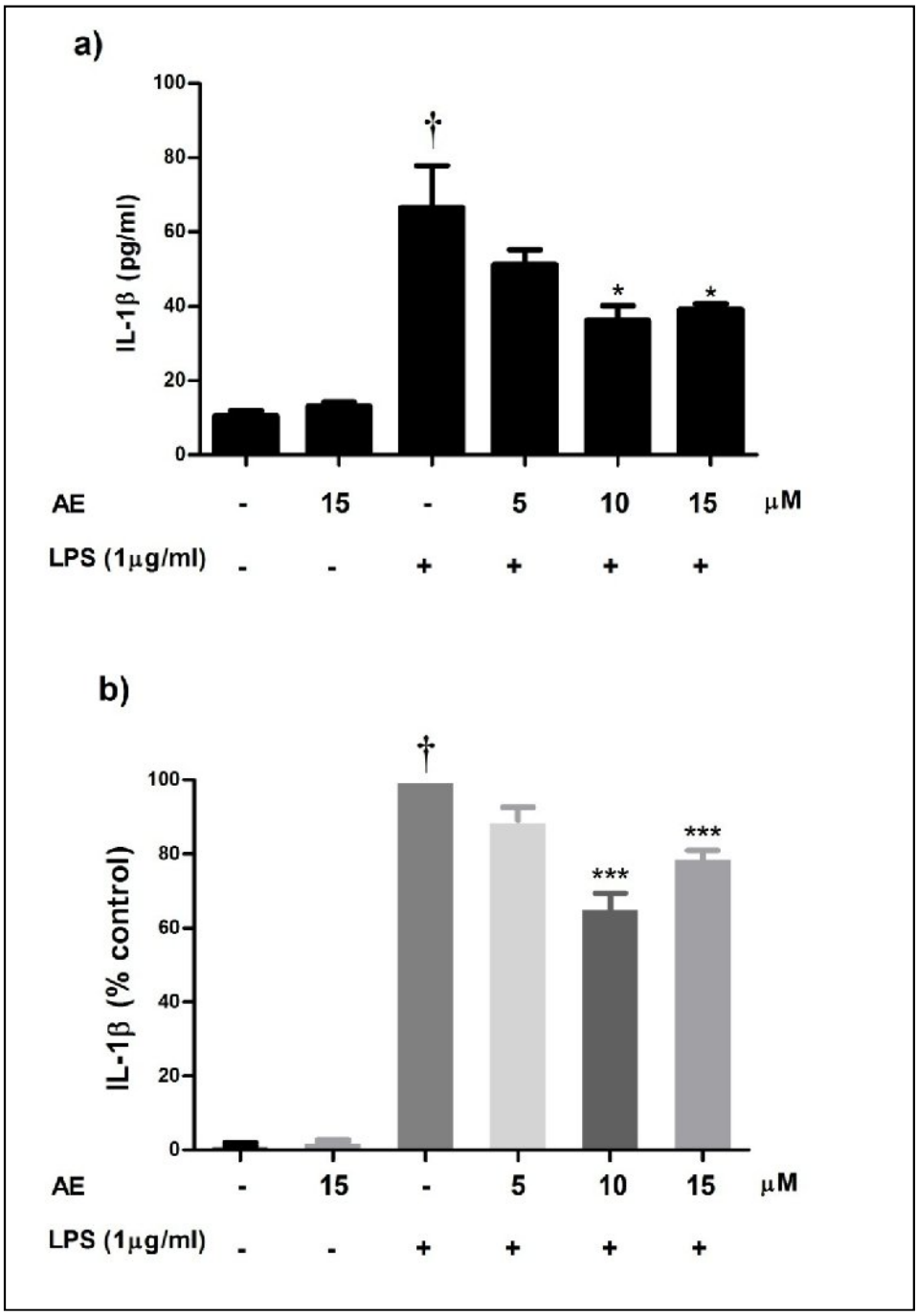


Figure 3.18: Effect of aloe-emodin on IL-1β release in activated macrophages a) RAW 264.7 cells. b) PMphs cells isolated from C57BL/6. The IL-1β levels were checked at 12hr after LPS stimulation. Data from at least three experiments were normalized by denoting the IL-1β release from LPS stimulated cells as 100% and subsequently calculating the effect achieved by addition of aloe-emodin. The values are expressed in mean (±SEM) of three experiments in duplicate. †$p < 0.001$ when compared with control; **$p < 0.01$ and ***$p<0.001$ when compared with the group treated with LPS (1 μg/ml) alone; p-values were calculated by Bonferroni non-parametric ANOVA test.

3.16 Effect of Aloe-emodin on Heme-oxygenase 1 expression

Heme-oxygenase (HO-1) catalyzes the conversion of heme into free iron, carbon monoxide (CO) and bilirubin (a metabolite of biliverdin), which mediate its actions. The HO-1 system exerts anti-oxidant, anti-apoptotic, and immunomodulatory functions in various situations and the productions of HO-1 ameliorate the injury in endotoxin-induced organ dysfunction in animal models. Up-regulation of HO-1 is seen to protect mice from the lethal effect of LPS- and CLP-induced sepsis, paralleled by a decrease in the systemic levels of HMGB1.

RAW 264.7 cells were pre-treated with aloe-emodin for 1h and then stimulated with LPS. At 12 h after LPS stimulation, HO-1 expression was analysed by western blot. Equal amounts of protein were resolved in SDS-PAGE gel and transferred to PVDF membrane. The expression of HO-1 and β-actin (loading control) was then detected using specific antibodies. The blot showed HO-1 induction with LPS and aloe-emodin treatment increased the HO-1 expression in stimulated macrophages, expression was also seen in cells treated with aloe-emodin only (**Fig 3.19**).

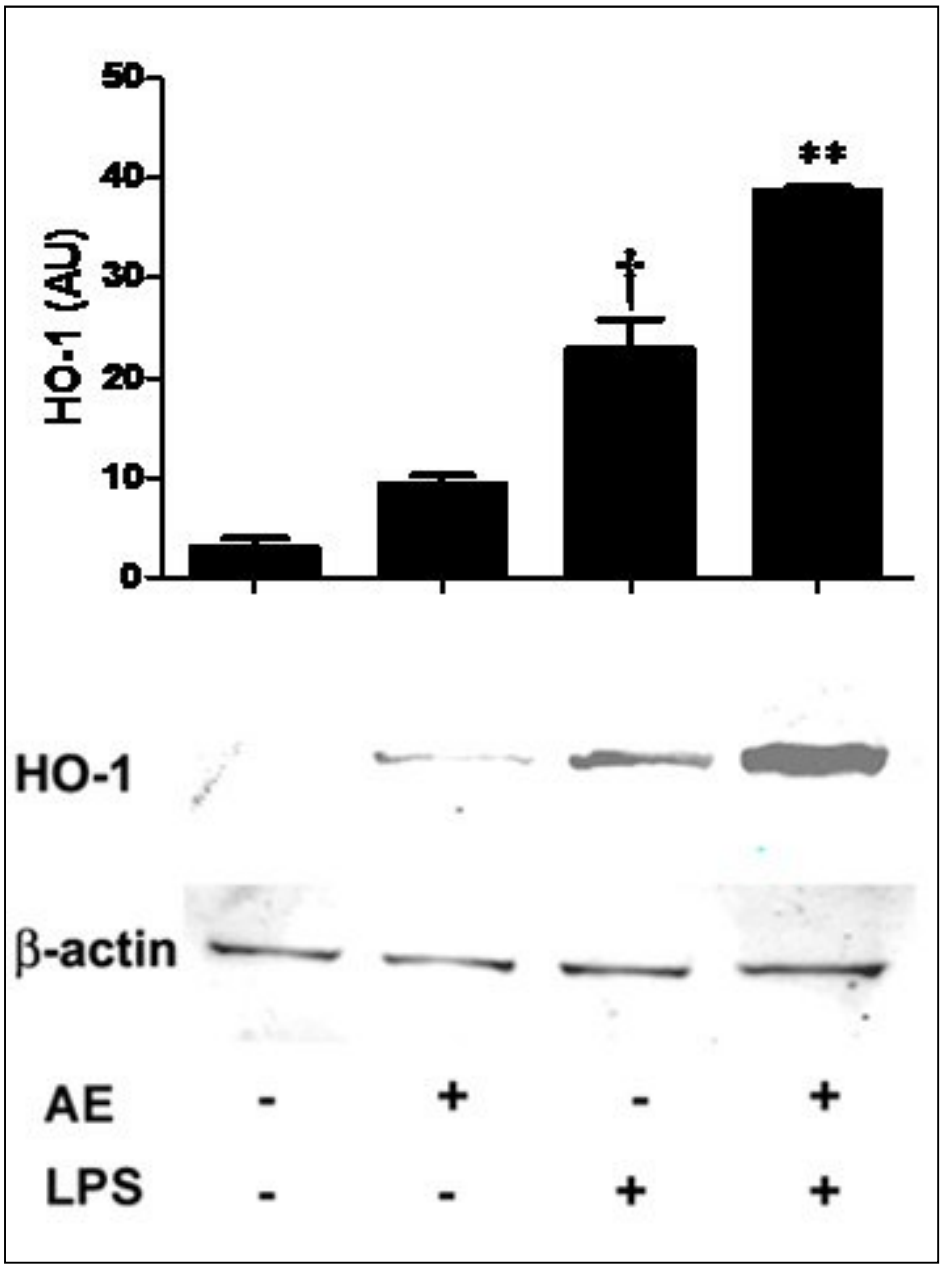


Figure 3.19: Effect of aloe-emodin on HO-1 induction. RAW 264.7 cells were treated with 1 μg/mL of LPS alone (positive control) and or with aloe-emodin (15 μM) for 12 h. The HO-1 expression was analysed by western blot with β-actin as loading control and density of the protein bands was expressed (in arbitrary units (AU)) as mean ± SEM of 2 independent experiments. The values are expressed in mean (±SEM) of two experiments in duplicate. †p< 0.001 when compared with control and **p<0.01 when compared with LPS group; p-values were calculated by Bonferroni non-parametric ANOVA test.

PART III- ALEO-EMODIN

(In-vivo study)

Endotoxin or LPS is the principal component of the gram-negative bacterial cell wall, stimulates the release of various pro-inflammatory mediators from various cell types, responsible for initiating the process of septic shock or sepsis. LPS is a stable, relatively pure compound that can be stored in lyophilized form. Bolus administration or infusion results in simplest sepsis model commonly called as endotoxemia. Endotoxin is commonly used in animal models of sepsis, as it is simplest and mimics most of features, predominantly early features of sepsis.

3.17 Standardization of mice model of endotoxemia

Infusion of bolus injection of LPS (i.p., or i.v.) results in sepsis like condition known as endotoxemia. Although various studies have standardised the optimum dose of LPS for onset of endotoxemia in C57BL/6 mice, however the dose varies as per the conditions. Mice were randomly grouped and were injected with different doses of LPS (i.p.). The onset of endotoxemia was confirmed by the levels of TNF-α in early stages i.e., at 3-4h after LPS administration. After testing various doses, two doses were selected, sublethal i.e. 200µg/mouse and lethal i.e. 400µg/mouse.

3.18 Effect of Aloe-emodin pretreatment in mice model of endotoxemia

Mice were pretreated with two doses of aloe-emodin (AE) (0.9µmol/mouse and 1.3µmol/mouse, i.p) for 1hr and then sub lethal dose of LPS (200µg/mouse) was administered (i.p.). The survival of animal was monitored for 60hr. It was seen, aloe-emodin at 0.9µmol/mouse increased the survival rate significantly than at higher dose (1.3µmol/mouse) (**$p<0.05$**) (**Fig 3.21a**). We then explored efficacy of aloe-emodin (0.9µmol/mouse) in animal model of lethal endotoxemia induced by intra peritoneal administration of higher dose of LPS (400µg/mouse). Aloe-emodin (0.9µmole/mouse) pretreatment for 1hr showed survival rate of 71% compared to 0% in LPS treated group (**Fig 3.21b)**. Aloe-emodin pretreatment also attenuated the clinical manifestations of endotoxemia including huddling, lethargy, diarrhea, piloerection, and malaise developed within few hours after induction.

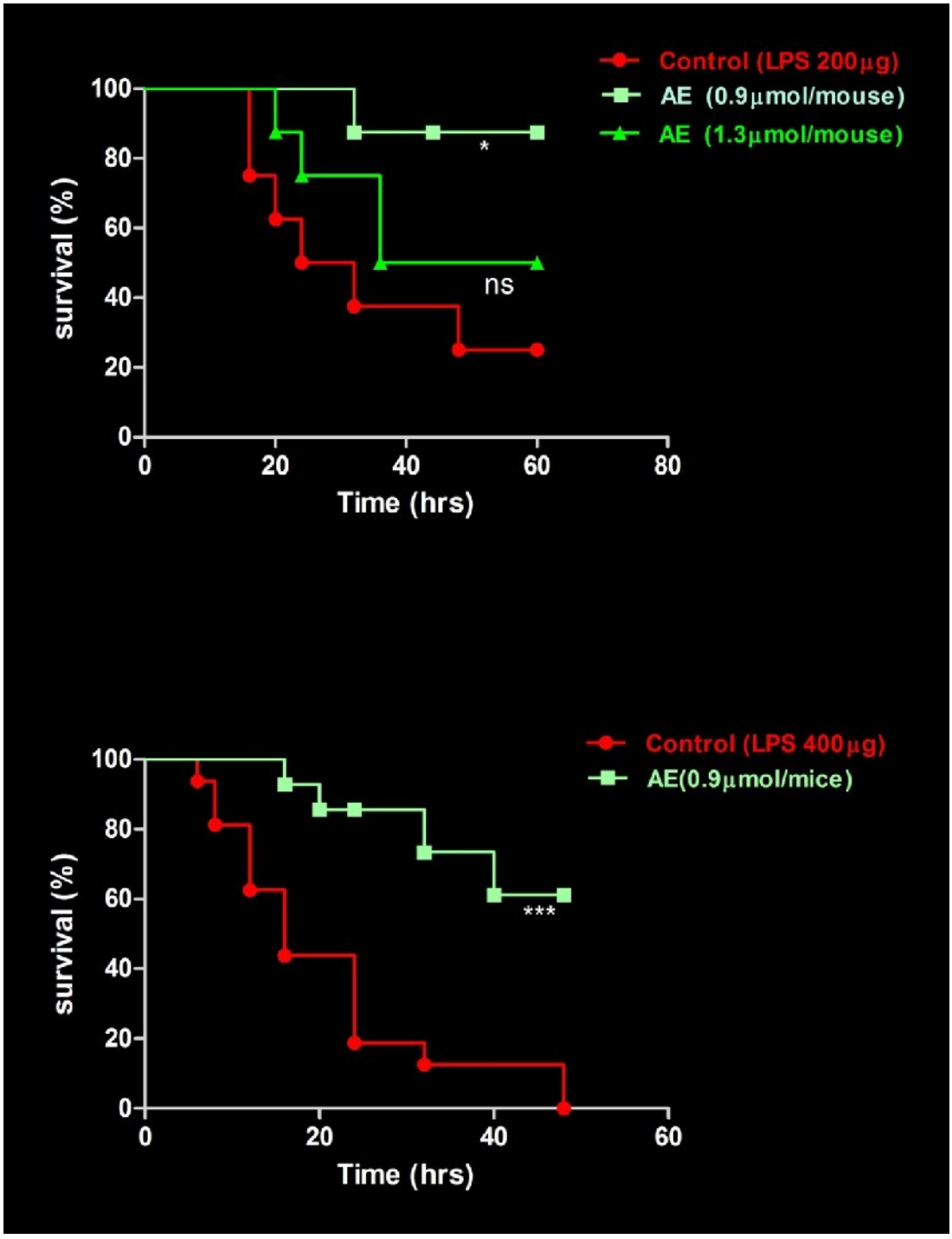


Figure 3.20: Effect of aloe-emodin on survival of endotoxemic mice. a) C57BL/6 mice were injected intra peritoneally with 0.9µmol and 1.3µmol/mouse for 1hr before LPS (200µg/mouse) injection. The mortality rate was monitored for 60hr. b) In similar experiment, mice were pretreated with aloe emodin (0.9µmol/mouse) for 1h and then injected with LPS (400µg/mouse). Survival was monitored for 60hr. n = 10–12 mice/group. ***,$p<0.001$, *,$p < 0.005$ versus control mice, survival analysis done by Gehan-Breslow-Wilcoxon Test.

3.19 Effect of Aloe-emodin on cytokine levels in endotoxemic mice

Cytokines are main mediators of pathology of sepsis/endotoxemia as they are elevated within hours of onset of disease. Administration of recombinant TNF-α or IL-1β to animals mimics the features of septic shock, and treatment with anti TNF-α antibodies or blocking the effects of IL-1 with IL-1receptor antagonist (IL-1Ra) protect animals from lethal endotoxemia and gram-negative sepsis. TNF-α and IL-1β concentrations have been widely evaluated in septic patients, and found to be increased, correlating with the severity of sepsis. From survival analysis curve, it was seen that aloe-emodin showed better survival rates at 0.9µmol/mouse than at 1.3µmol/mouse.

TNF-α

TNF-α is a primary mediator of inflammation, and has been implicated in a large number of infectious and non-infectious inflammatory diseases. Elevated levels of TNF-α are seen in both septic patients and animal models of sepsis. Also antibodies targeting TNF-α have been observed to have beneficial effects in septic shock.

We checked the effect of aloe-emodin (AE) on TNF-α at two doses of LPS (sub lethal and lethal dose of LPS). It was seen that of aloe-emodin pretreatment, decreased the elevation levels of TNF-α in endotoxemic mice. In control group, the levels ranged between 6.46±1.847 pg/ml in both the groups. The serum TNF-α level increased within an hour after LPS administration and after 4hr it was seen that TNF-α level reached upto 1097±98.46 pg/ml (LPS- 400µg/mouse) and 481.1±9.468 pg/ml (LPS-200µg/mouse). Pre-treatment of aloe-emodin for 1hr induced marked suppression in the increase of serum level of TNF-α at 4hr after LPS stimulation. In low dose LPS model, two doses of aloe-emodin were used (0.9µmol/mouse and 1.3µmol/mouse), both decreasing the TNF-α levels in serum significantly with 1.3µmol/mouse (162.1±3.704 pg/ml) decreasing more than 0.9µmol/mouse (280.5±27.19 pg/ml). In other model of endotoxemia (induced by lethal dose of LPS), 0.9µmol/mouse decreased the serum TNF-α levels significantly to 454.5±33.49 pg/ml.

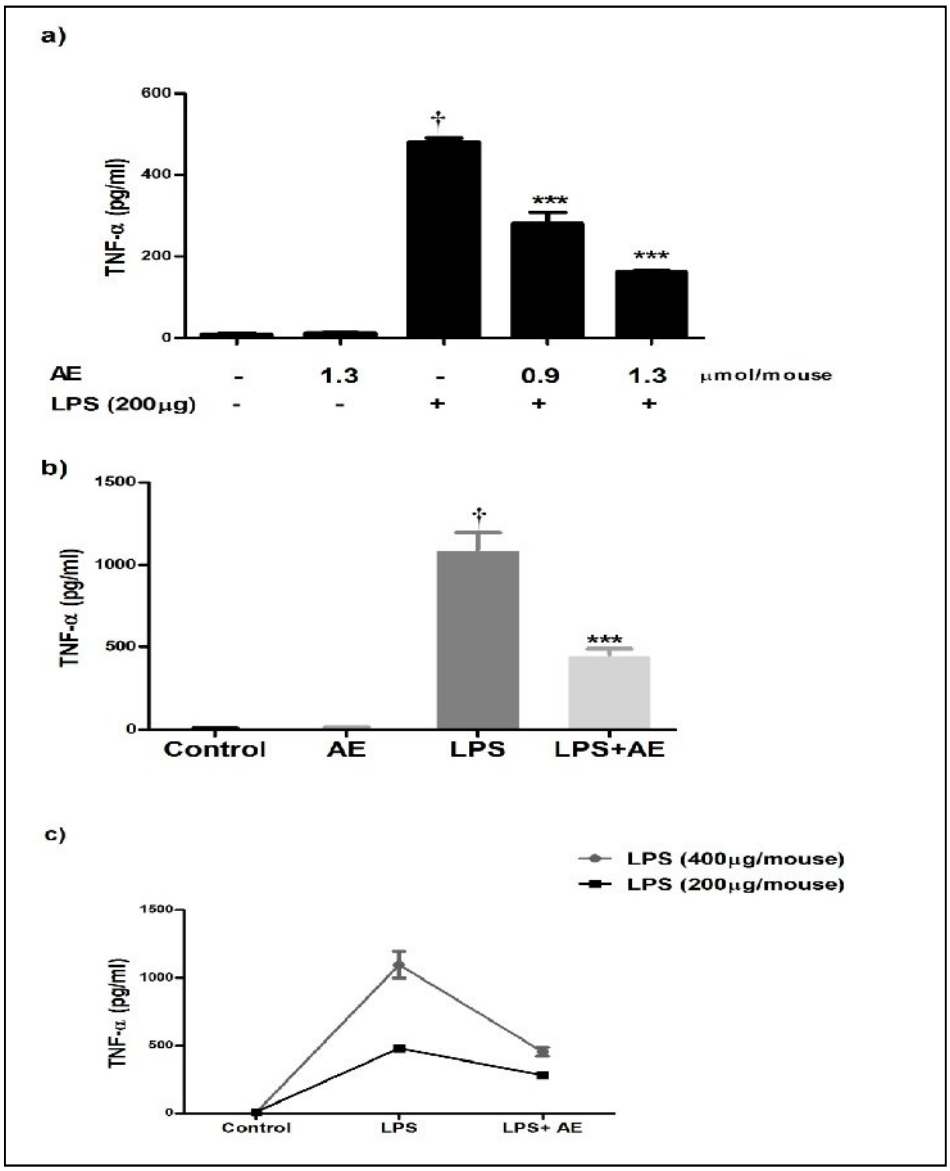


Figure 3.21: Effect of aloe-emodin on TNF-α levels in endotoxemia. a) C57BL/6 mice were pretreated (i.p. injection) with two doses of AE(0.9µmol/mouse and 1.3 µmol/mouse) for 1hr and endotoxemia was induced by sub-lethal doses of LPS (200ug/mouse) (i.p.), serum was collected after 4hr and used for cytokine analysis **b)** C57BL/6 mice were pretreated (i.p. injection) with AE(0.9µmol/mouse) for 1hr and endotoxemia was induced by high doses of LPS (400ug/mouse) (i.p.), serum was collected after 4hr and used for cytokine analysis c) comparative effect of AE (0.9µmol/mouse) on TNF-α levels in two different endotoxemic mice. The values are expressed in mean (±SEM) †, $p < 0.001$ when compared with control, *$p < 0.05$ and **$p < 0.01$ when compared with the groups treated with endotoxemic mice, n=5 in each group; p-values were calculated by Bonferroni non-parametric ANOVA test.

IL-1 β

IL-1 is synthesized by mononuclear phagocytes, polymorphonuclear leucocytes and other cell types and affects a wide variety of tissues. IL-1β is the predominant form of this mediator produced by endotoxin-stimulated human monocytes and detected in the plasma of septic animals. IL-1β is increased in humans after infusion of endotoxin, although at lower concentrations than TNF-α. Like TNF-α, IL- 1β activates the production of other cytokines, including IL-6, IL-8 and TNF-α. Some studies have shown a direct correlation of survival rates of septic patients with serum IL-1β levels.

IL-1β levels peak at 4hr in septic models. We checked the effect of aloe-emodin on serum levels of IL-1β at this peak concentration. IL-1β levels in serum were low in both vehicle control (11.40±1.134 pg/ml) and drug control (13.04±1.617 pg/ml). At 4hr after LPS (200 μg) infusion, serum IL-1β increased to 341.1±21.13 pg/ml significantly compared to controls. Pretreatment of aloe-emodin for 1hr attenuated the rise in IL-1β levels significantly, however 0.9μmol/mouse (163.2 ±10.39 pg/ml) decreased the levels more than 1.3μmol/mouse (272.9±17.24 pg/ml). In other LPS induced endotoxemic mice model (400μg), IL-1β showed significant increase (456.6±39.91 pg/ml) compared to control groups and aloe-emodin (0.9μmol/mouse) pretreatment induced marked suppression in this increase (209.3±35.32 pg/ml).

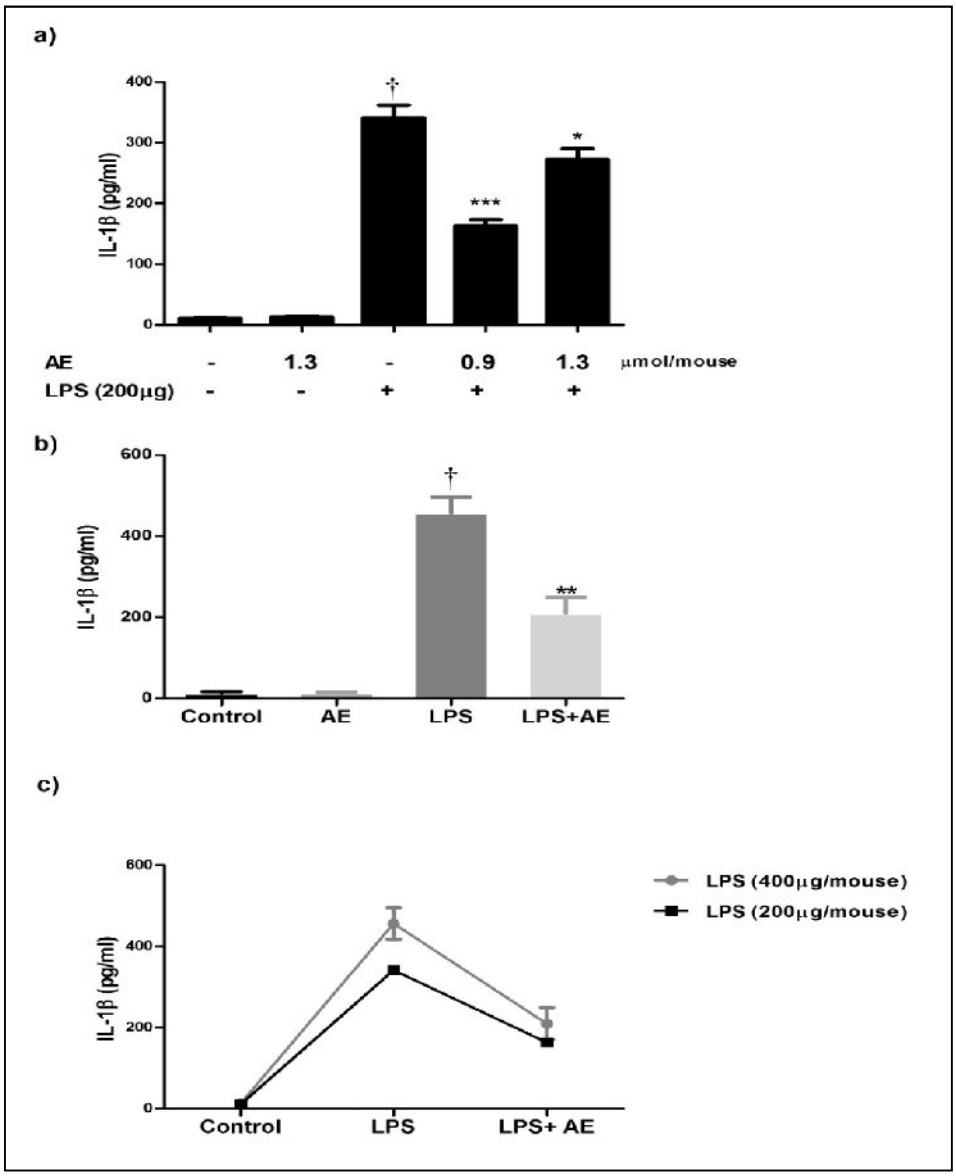


Figure 3.22: Effect of aloe-emodin on IL-1β levels in endotoxemia. a) C57BL/6 mice were pretreated (i.p. injection) with two doses of AE(0.9µmol/mouse and 1.3 µmol/mouse) for 1hr and endotoxemia was induced by sub-lethal doses of LPS (200ug/mouse) (i.p.), **b)** C57BL/6 mice were pretreated (i.p. injection) with AE(0.9µmol/mouse) for 1hr and endotoxemia was induced by high doses of LPS (400ug/mouse) (i.p.). Serum was collected after 4hr and used for cytokine analysis c) comparative effect of AE (0.9µmol/mouse) on IL-1β levels in two different endotoxemic mice. The values are expressed in mean (±SEM) †, $p < 0.001$ when compared with control, *$p < 0.05$ and **$p < 0.01$ when compared with the groups treated with endotoxemic mice, n=5 in each group; p-values were calculated by Bonferroni non-parametric ANOVA test.

IL-6

IL-6 is a 21-kDa glycoprotein produced by many cell types, including lymphocytes, fibroblasts and monocytes. IL-6 has a variety of biological effects, including activation of B- and T-lymphocytes, induction of acute phase protein production in the liver, and modulation of haematopoiesis. In addition, IL-6 can activate the coagulation system and function as a pyrogen. In vitro, IL-6 suppresses the production of TNF-α and IL-1β. The exact role of IL-6 in sepsis is uncertain.

We checked the effect of aloe-emodin on IL-6 levels in endotoxemic mice. It was seen after 4hr, IL-6 levels were raised (183.4±6.139 pg/ml) significantly compared to control group (6.141±2.268 pg/ml), however pretreatment with aloe-emodin for 1hr did not affect the levels significantly (166.3±6.885 pg/ml).

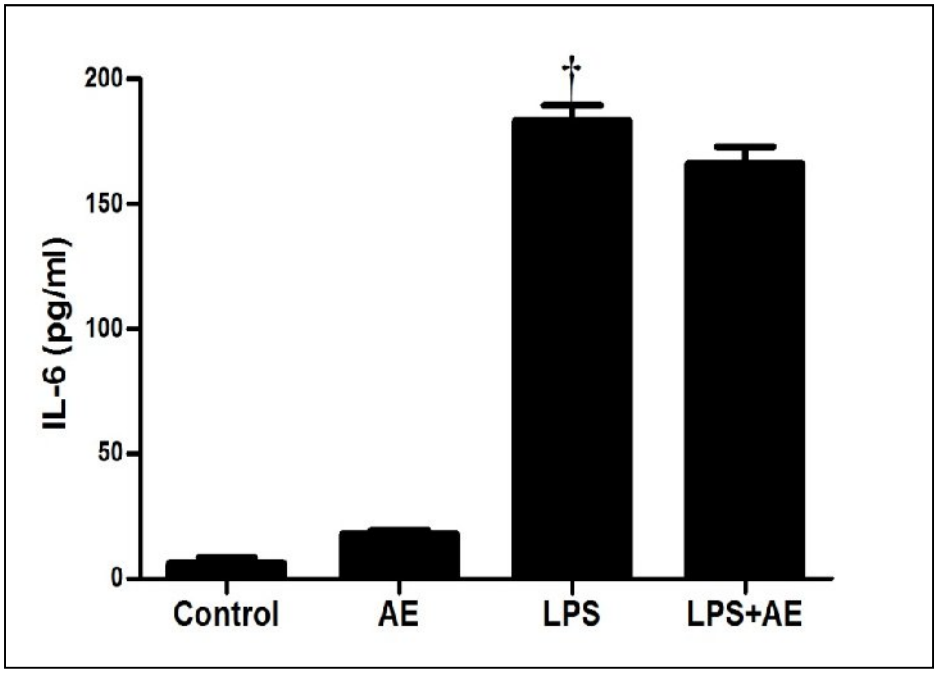


Figure 3.23: Effect of aloe-emodin on IL-6 levels in endotoxemia. C57BL/6 mice were pretreated (i.p. injection) with AE(0.9µmol/mouse) for 1hr and lethal endotoxemia was induced by high doses of LPS (400ug/mouse) (i.p.), serum was collected after 4hr and used for cytokine analysis. The values are expressed in mean (±SEM) †, $p< 0.001$ when compared with control, *$p < 0.05$ and **$p < 0.01$ when compared with the groups treated with endotoxemic mice, n=5 in each group; p-values were calculated by Bonferroni non-parametric ANOVA test.

Multiple organ dysfunction

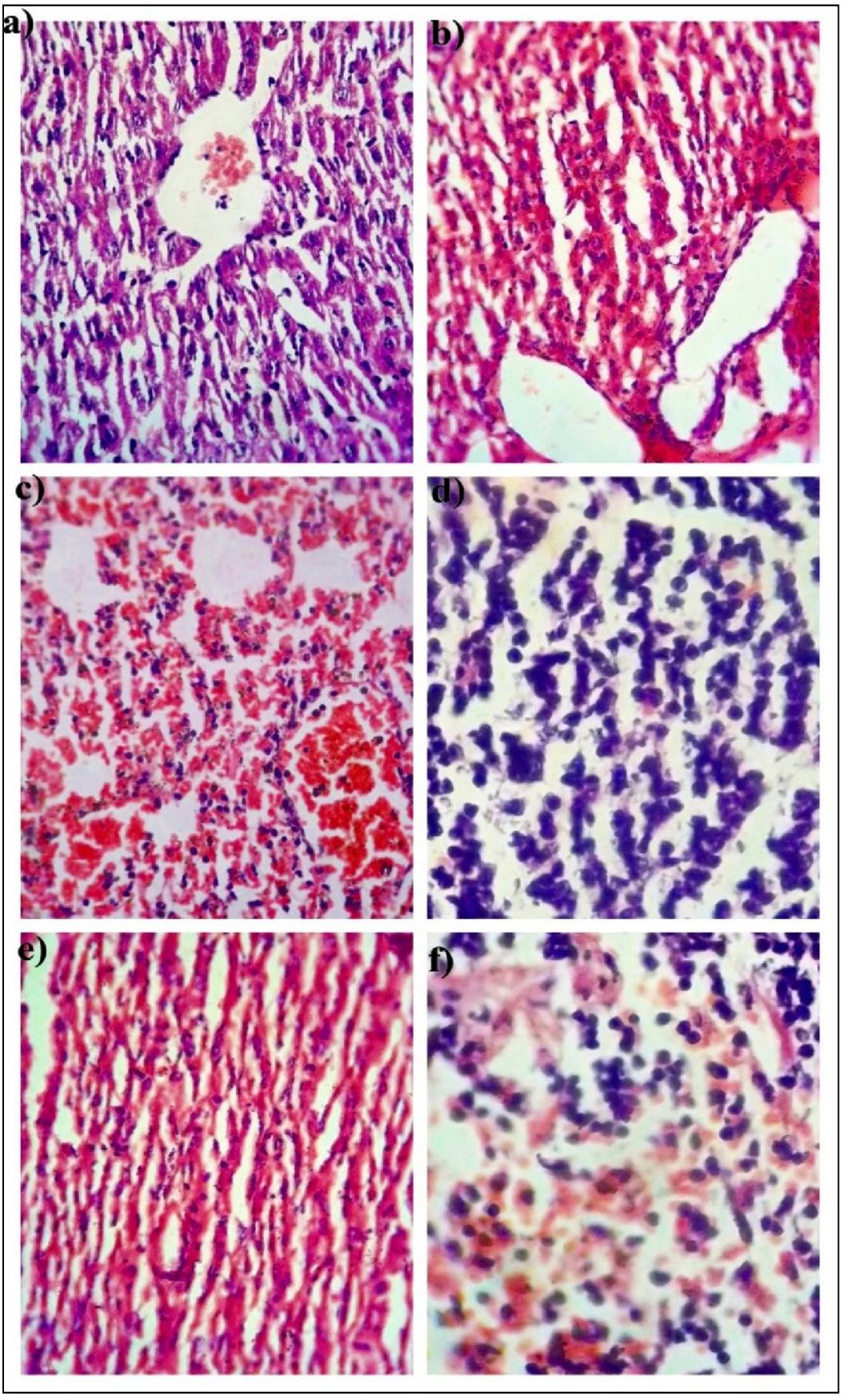
Organ dysfunction is a hallmark of severe sepsis. There is a close relationship between the severity of organ dysfunction on admission to an ICU and the probability of survival and between the numbers of organs failing and the risk of death. To analyse the effect of aloe-emodin on liver, kidney, lung dysfunction in endotoxemia, lethal dose of LPS (400μg/mouse) was administred (i.p.) 1hour after aloe-emodin (0.9μmol/mouse) treatment.

3.20 Effect of aloe-emodin on liver injury in endotoxemia

The liver is a central regulator of the systemic immune response following acute traumatic or surgical insult. It is the primary site for clearance of bacterial endotoxin, so is also subject to injury and dysfunction during sepsis. Liver involvement in sepsis is common and characterized by either hepatitis like injury or a mixed, hepatic and cholestatic, pattern of injury. In endotoxemia and sepsis patients have also been seen to have abnormal liver biochemistry. To assess the effect of aloe-emodin on liver injury, histopathological studies and serum profile for liver injury were checked.

3.20.1 Liver Histopathology

Mice from control, endotoxemic and treated groups were sacrificed after 18h of LPS administration. Histological evaluation was done of liver tissue sections from all three groups. Histological evaluation revealed endotoxemia-induced liver injury with parenchymal necrosis, parenchymal infarction, sinusoidal dilatation, perivenular inflammation, ductular cholestasis and parenchymal infilitration by inflammatory cells in endotoxemic mice. No apparent changes were observed in tissue sections obtained from vehicle control and drug control mice. Pre-treatment of aloe-emodin was seen to decrease the severity of liver injury significantly, as revealed by hematoxylin & eosin staining (overall score) ($p<0.05$) (**Fig 3.24**).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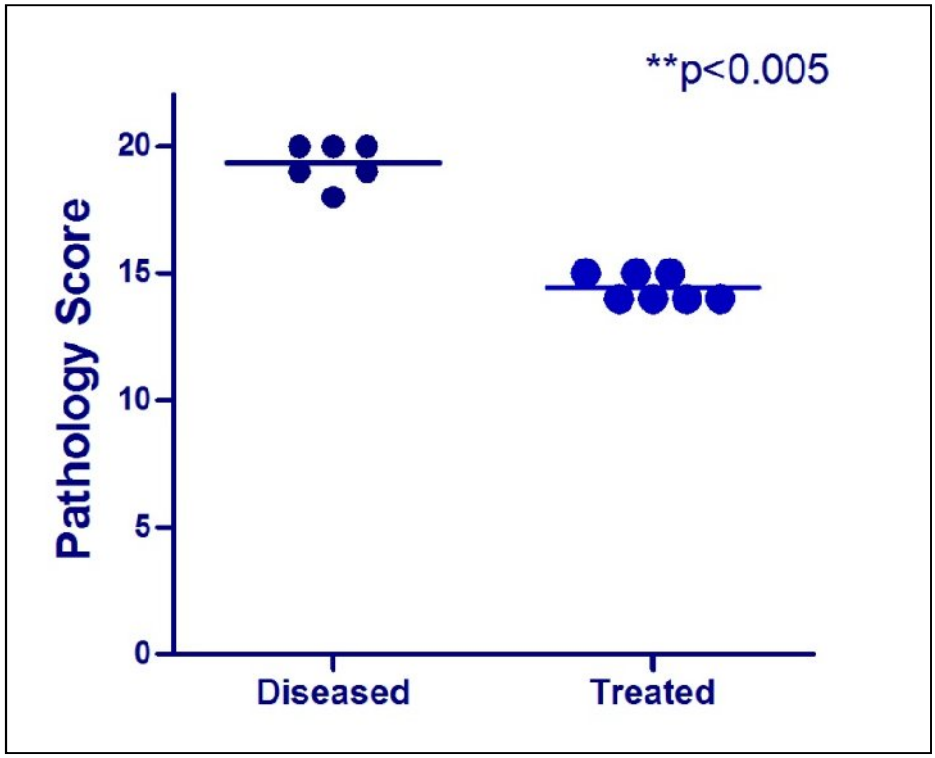


Figure 3.24: Effect of aloe-emodin on liver histopathology in endotoxemia. Mice were pretreated (i.p. injection) with AE (0.9µmol/mouse) for 1hr and lethal endotoxemia was induced by lethal doses of LPS (i.p.). Mice were sacrificed after 18hr and liver from all three groups were harvested and stained with H/E [Normal (a & b), endotoxemic (c & d), treated (e & f)]. The overall pathology score sum of six fields (g) showed improvement (left,*p<0.05).

3.20.2 Clinical Data

Liver-related biochemical patterns were checked in all the groups of mice at 18hr after LPS treatment. Endotoxemic mice showed a significant rise in liver damage markers i.e., alkaline phosphate (ALP) and alanine amino-transferase (ALT) compared to control group and pretreatment with aloe-emodin resulted in significant decrease in ALT levels but not ALP (serum ALT: vehicle control, 59±13.40 U/L; AE only, 61.67±21.11 U/L; LPS only, 273±41.24 U/L; LPS+AE, ± 135±21.39 U/L: serum ALP: vehicle control, 12±0.91 U/L; AE only, 20±8.83 U/L; LPS only, 120.8±15.02 U/L; LPS+AE, 70.67±41.70 U/L). Decrease in ALT levels, suggest that aloe-emodin decreases the hepato-cellular damage in liver.

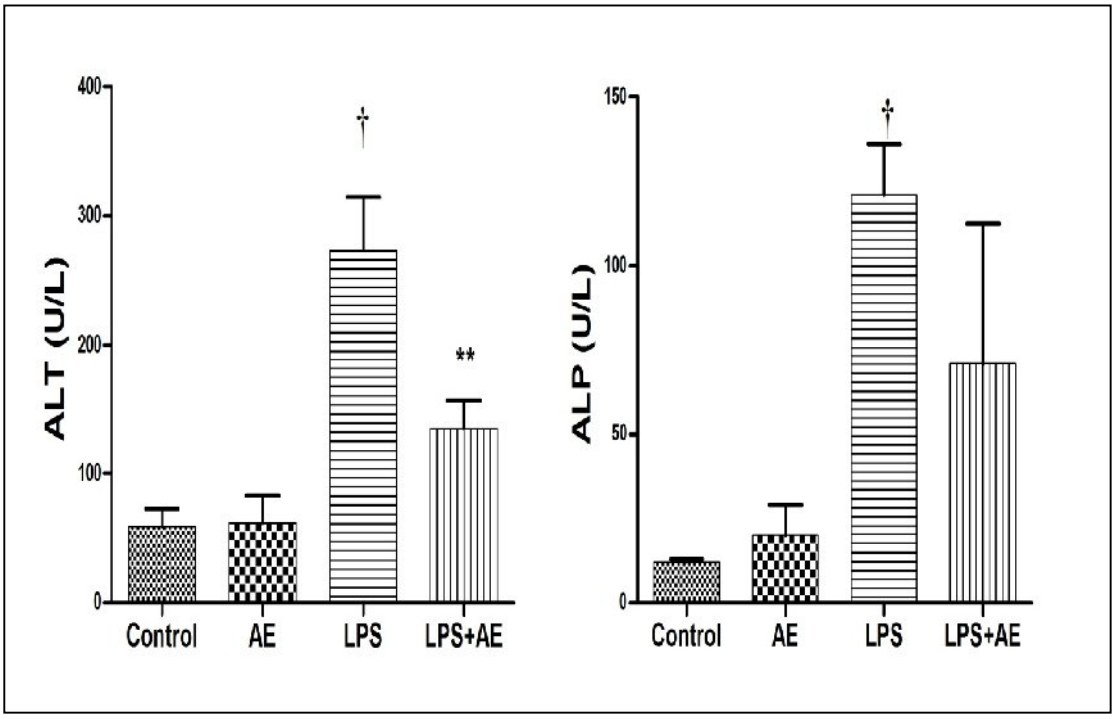


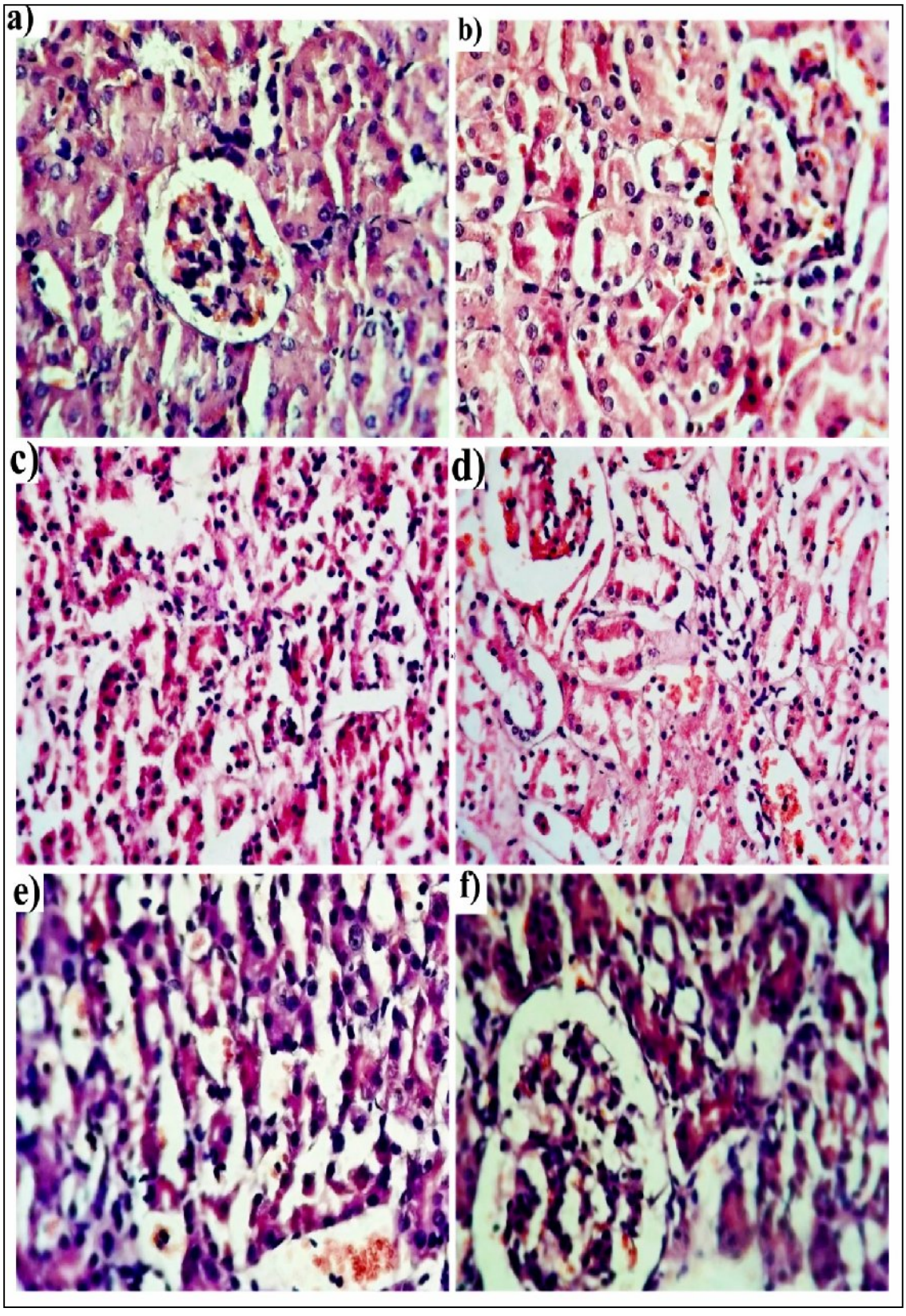
Figure 3.25: Effect of aloe-emodin on liver function in endotoxemia. Mice were pretreated (i.p. injection) with AE (0.9µmol/mouse) for 1hr and lethal endotoxemia was induced by high doses of LPS (400µg/mouse) (i.p.), serum was collected 18hr after LPS injection and assayed for ALT and ALP. The values are expressed as mean (±SEM) †, $p < 0.001$ when compared with control, *$p < 0.05$ and **$p < 0.01$ when compared with the groups treated with endotoxemic mice, n=5 in each group; p-values were calculated by Bonferroni non-parametric ANOVA test.

3.21 Effect of aloe-emodin on kidney injury in endotoxemia

Development of acute renal failure during sepsis syndrome is common and portends a poor outcome. The interplay between systemic host responses, local insults in the kidney, immune system and vascular bed, all play a role in the development of sepsis-induced acute renal failure. Despite recent advances in critical care, mortality rates in septic patients remained high for sepsis-associated acute renal failure.

3.21.1 Histopathology

Histological evaluation revealed endotoxemia-induced kidney injury with interstitial oedema with separation of tubules, tubular cell swelling**,** shedding of tubule cells, interstitial inflammation, perivascular/interstitial lymphoid and myeloid cells, vascular Congestion, glomerular infilitration in endotoxemic mice. No apparent changes were observed in tissue sections obtained from vehicle control and drug control mice. Pre-treatment with aloe-emodin decreased the overall kidney injury as implicated by total histopathology score compared to endotoxemic mice ($p<0.005$) and significant attenuation was seen in inflammation induced swelling, accumulation of perivascular/interstitial lymphoid and myeloid cells and also glomerular infilitration (**Fig 3.26**).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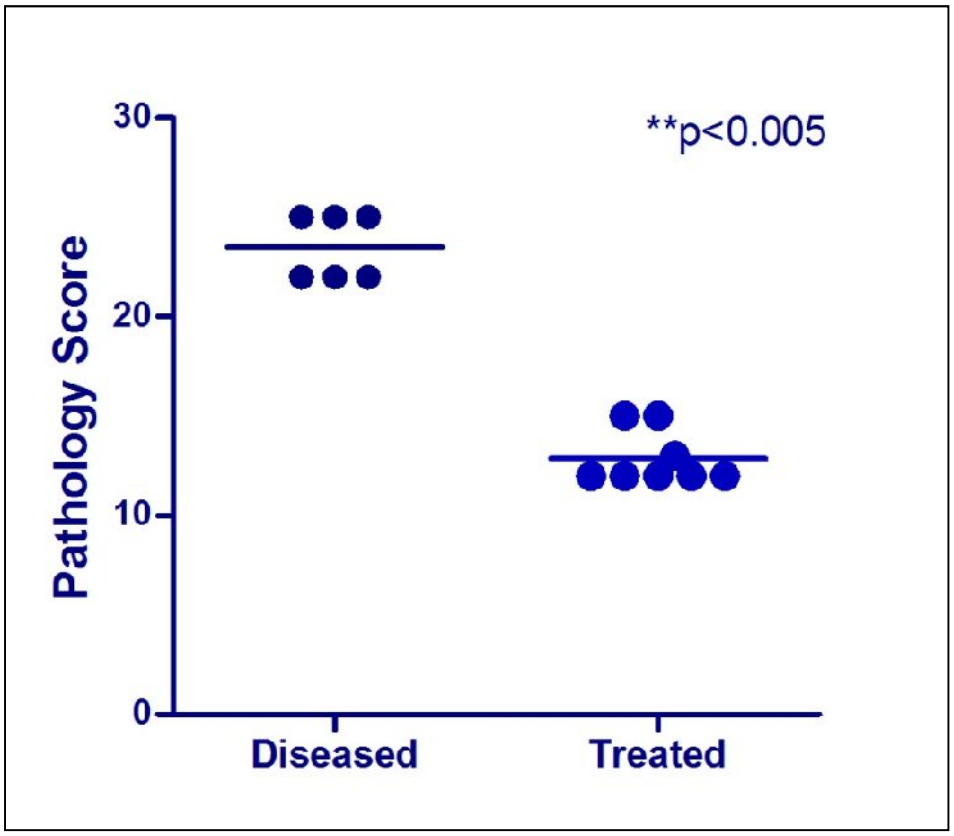


Figure 3.26: Effect of aloe-emodin on kidney histopathology in endotoxemia. Mice were pretreated (i.p. injection) with AE (0.9µmol/mouse) for 1hr and lethal endotoxemia was induced by lethal doses of LPS (i.p.). Mice were sacrificed after 18hr and kidneys from all groups were harvested and stained with H&E [Normal (a & b), endotoxemic (c & d), treated (e & f)]. The overall pathology score sum of six fields (g) showed improvement (left,*p<0.05).

3.21.2 Clinical Data

Following LPS infusion mice developed a comparable acute renal failure (ARF) with elevated levels of BUN and creatinine compared to control mice after 18hr. Pretreatment with aloe-emodin resulted in significant decrease in both these parameters (serum creatinine: vehicle control, 0.107±0.027 mg/dl; AE only, 0.107±0.024 mg/dl; LPS only, 0.513±0.031 mg/dl; LPS+AE, ± 0.143±0.073 mg/dl: serum BUN: vehicle control, 113±11.69 mg/dl; AE only, 123.1±15.07 mg/dl; LPS only, 578±14.88 mg/dl; LPS+AE, 328.1±59.84 mg/dl).

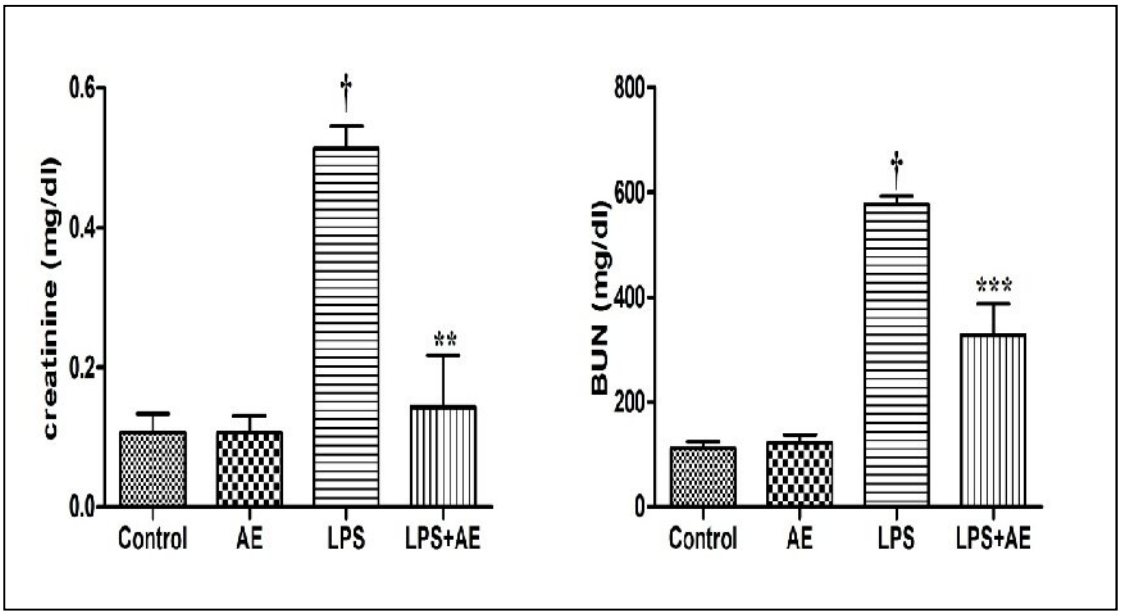


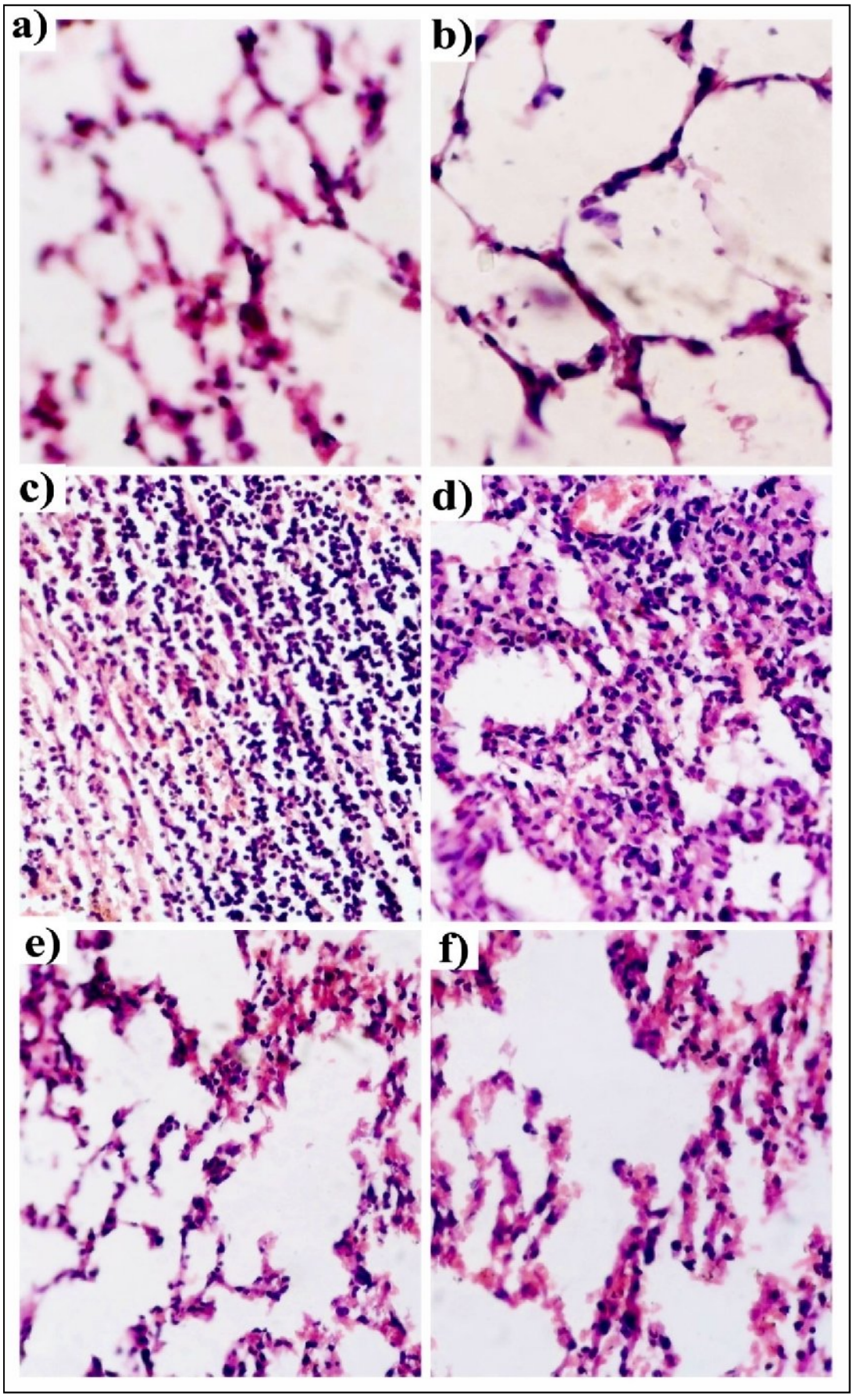
Figure 3.27: Effect of aloe-emodin on kidney function in endotoxemia. Mice were pretreated (i.p. injection) with aloe-emodin (0.9µmol/mouse) for 1hr and lethal endotoxemia was induced by high doses of LPS (400µg/mouse) (i.p.), serum was collected at 18hr after LPS injection and assayed for BUN and Creatinine. The values are expressed in mean (±SEM) †, $p< 0.001$ when compared with control, *$p < 0.05$ and **$p < 0.01$ when compared with the groups treated with endotoxemic mice, n=5 in each group; p-values were calculated by Bonferroni non-parametric ANOVA test.

3.22 Effect of aloe-emodin treatment on ALI in endotoxemia

The lung is the most frequently failing organ in sepsis as the increased metabolic rate associated with sepsis necessitates a high minute volume requirement. Inflammatory and oxidative damage to alveolar epithelial cell and microvascular endothelium leads to changes in pulmonary structure and function that characterize acute lung injury (ALI).

3.22.1 Histopathology

LPS injection (leading to endotoxemia) resulted in severe lung injury as observed in tissue sections of mice which were sacrificed after 18hrs. Endotoxemic mice tissues showed alveolar collapse, hemorrhage, and edema, neutrophil accumulation, intra-septal lymphocytic inflammation, intra-alveolar inflammation, peribronchi/ perivascular inflammation. The aloe-emodin pretreatment significantly decreased the overall score in mice lungs compared to endotoxemic mice, decreasing the inflammation mediated alveolar collapse, intra-septal and intra-alveolar inflammation and also peribronchi/perivascular inflammation.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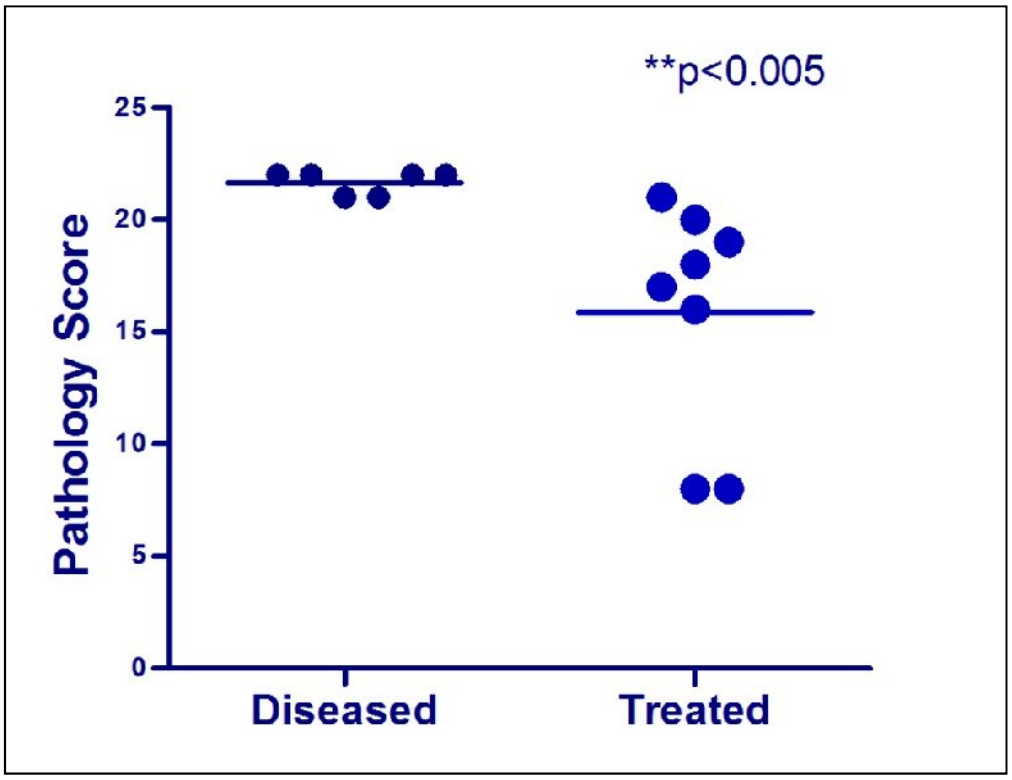

Figure 3.28: Effect of aloe-emodin on lung histopathology in endotoxemia. Mice were pretreated (i.p. injection) with aloe-emodin (0.9µmol/mouse) for 1hr and lethal endotoxemia was induced by lethal doses of LPS (i.p.). Mice were sacrificed after 18hr and lungs from all groups were harvested and stained with H/E [Normal (a & b), endotoxemic (c & d), treated (e & f)]. The overall pathology score sum of six fields (g) showed improvement (left,*p<0.05).

3.22.2 Neutrophil Infiltration

Endotoxin administration or sepsis produce acute inflammatory lung injury (ALI). ALI is characterized by the accumulation of large numbers of neutrophils into the lungs and a pulmonary inflammatory response in which there is increased production of immunoregulatory cytokines. Neutrophil infiltration in lungs of endotoxemic mice was confirmed by myeloperoxidase (MPO) assay, which is an indirect marker for neutrophil presence. As compared with control mice, endotoxemic mice tissues had a significantly greater level of lung MPO and aloe-emodin treatment decreased the levels significantly (**$p<0.05$**).

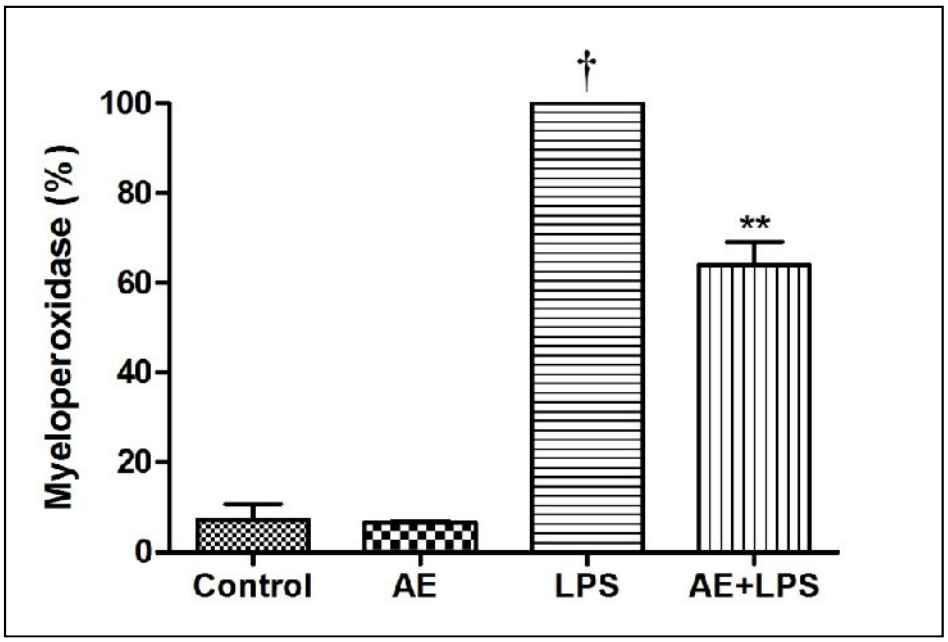

Figure 3.29: Effect of aloe-emodin on myeloperoxidase levels in lung tissues. Mice were pretreated (i.p. injection) with aloe-emodin (0.9µmol/mouse) for 1hr and lethal endotoxemia was induced by high doses of LPS (400µg/mouse) (i.p.). Mice were sacrificed after 18 h and lungs from all groups harvested and tissue homogenate assayed for MPO. The assay was expressed in percentage with LPS group referring to 100%. The values are expressed as mean (±SEM) †, $p< 0.001$ when compared with control, **$p < 0.01$ when compared with the groups treated with endotoxemic mice, n=5 in each group; p-values were calculated by Bonferroni non-parametric ANOVA test.

T**NF-α**

Accumulation of neutrophils in lung tissues results in acute inflammation in lung tissues. Neutrophils express proinflammatory cytokines, including IL-1α, IL-1β, IL-8, and TNF-α. TNF-α, which is said to be main mediator of organ damage, was analysed in lung tissues from different mice groups at 18hr after LPS injection. Compared with lung tissues from control group, TNF-α level were markedly high in endotoxemic mice (vehicle control, 6.88±2.2 pg/ml: AE only, 10.06±0.7492 pg/ml: LPS, 101.7±17.46 pg/ml), however the aloe-emodin treatment significantly attenuated this increase (LPS +AE, 40.05±3.36 pg/ml).

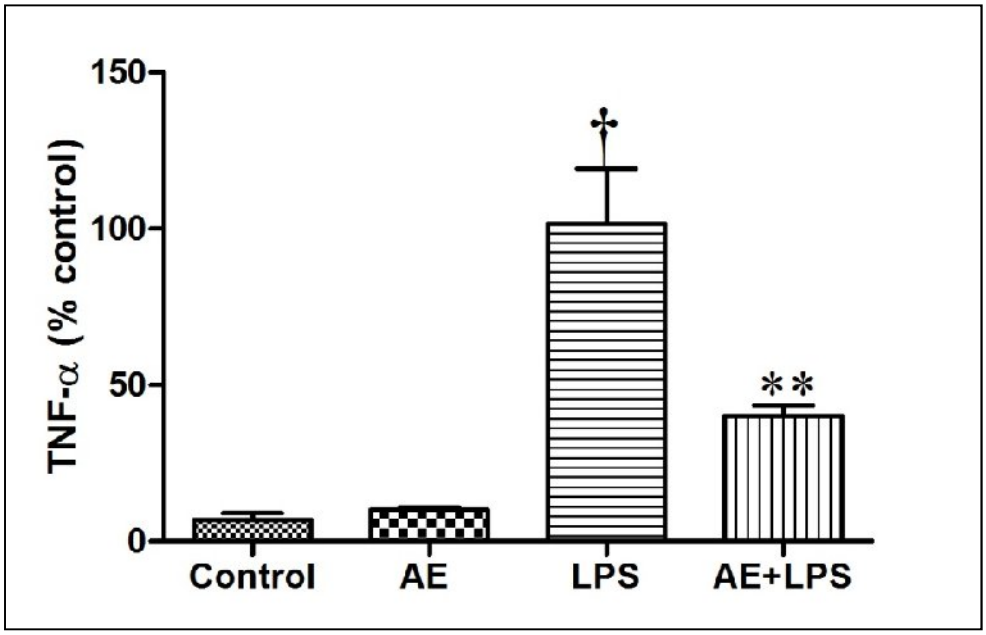

Figure 3.30: Effect of aloe-emodin on TNF-α levels in lung tissues. Mice were pretreated (i.p. injection) with AE (0.9µmol/mouse) for 1hr and lethal endotoxemia was induced by high doses of LPS (400µg/mouse) (i.p.). Mice were sacrificed after 18 h and lungs from all groups harvested and tissue homogenate assayed by ELISA. The values are expressed in mean (±SEM) †, $p < 0.001$ when compared with control, **$p < 0.01$ when compared with the groups treated with endotoxemic mice, n=5 in each group; p-values were calculated by Bonferroni non-parametric ANOVA test.

Sepsis is the tenth leading cause of death. Most of sepsis cases (40 percent) are fatal, and in most cases, the resulting multi organ failure and not the basic underlying infection, is the primary or main cause of death. Sepsis describes a complex clinical syndrome that develops when initial, appropriate host response to an infection becomes amplified and then dysregulated. Sepsis can be caused by infection with Gram-negative bacteria, Gram-positive bacteria, fungi, or viruses. Sepsis may also occur in the absence of detectable bacterial invasion, which include, microbial toxins (exo or endo), particularly Gram-negative bacterial endotoxin (LPS) and endogenous cytokine production have been implicated as mediators and initiators (Hardaway, 2000; Wheeler and Bernard, 1999).

Many features of local and systemic inflammation can be mimicked by administration of LPS i.e., endotoxemia. LPS is capable of selective activation of a class of macrophages through an elaborate series of events (Aderem and Ulevitch, 2000; Martins *et al.*, 2006; Wright *et al.*, 1990). Toll like receptors (TLRs) initiate a casacade of intracellular signaling activating various kinases e.g. PKC, MAPK, SRC resulting in the activation of pro-inflammatory transcription factors such as activator protein-1 (AP-1), nuclear factor kappa B (NF-κB), and interferon response factor-3 (IRF-3), leading to the production of pro-inflammatory chemokines, cytokines and nitric oxide (Beutler, 2004).

Cytokines are key elements in the inflammatory response that characterizes sepsis and septic shock. Two types of cytokines are released: proinflammatory (which include early such as tumor necrosis factor-α [TNF-α], IL-1β and IL-8 and late HMGB1) and anti-inflammatory (such as IL-10).TNF-α is one of the most important cytokines involved in the pathophysiology of sepsis and is released early in the process of sepsis. TNF-α induced tissue injury is largely mediated through neutrophils that respond by producing elastase, superoxide ion, sPLA2, hydrogen peroxide, platelet-activating factor (PAF), thromboxane A2 and leukotriene B4,. In addition, TNF-α amplifies inflammatory cascades in an autocrine and paracrine manner by activating macrophages/monocytes to secrete other pro-inflammatory cytokines (Calandra *et al.*, 2002; Herbertson *et al.*, 1995). IL-1β stimulates the synthesis and release of prostaglandins, elastases, and collagenases. It promotes transendothelial migration of neutrophils, and activates endothelial microvascular cells, which respond

by releasing PAF and IL-8 (both of which are powerful neutrophil-stimulating agents) (Curfs *et al.*, 1997). TNF and other proinflammatory cytokines (like IFN- γ) stimulate macrophages to release HMGB1 (Ivanov *et al.*, 2007; Wang *et al.*, 1999). HMGB1 is also released by macrophages when stimulated directly by exogenous bacterial products (such as endotoxin or CpG-DNA) (Rendon-Mitchell *et al.*, 2003; Tang *et al.*, 2007). HMGB1 is now perceived to be a major mediator of prolonged and sustained inflammation in sepsis, since it is released about 16hrs after early cytokines and its level remain high for several days (Chen *et al.*, 2004; Jiang *et al.*, 2007; Tang *et al.*, 2005). Animals exposed to high levels of recombinant HMGB1 develop a sickness syndrome characterized by piloerection, decreased mobility, increased somnolence, weight loss and fever similar to sepsis syndrome.

Despite the extraordinary developments in understanding the immunopathology and pathobiology of sepsis, therapeutic advances have been drastically slow. Currently, available strategies for the management of sepsis patients include: rapid identification of causative organisms; timely patient identification and diagnosis; improved ventilatory techniques (low-pressure ventilation); appropriate, timely antimicrobial therapy; appropriate (goal-directed) haemodynamic support; targeted pharmacological therapies (recombinant activated protein C) immunological therapy and glycaemic control (intensive insulin therapy); effective supportive therapies (prophylaxis against stress ulcers, administration of anticoagulants and dialysis); appropriate nutrition; and patient management by highly qualified clinicians, techniques and nursing staff (Bollaert *et al.*, 1998; Bone *et al.*, 1992; Briegel *et al.*, 1994; Ibrahim *et al.*, 2008; Martins *et al.*, 2006; Natanson *et al.*, 1998; Rivers *et al.*, 2001; Wheeler and Bernard, 1999; Zhang *et al.*, 1995). These strategies have helped to reduce the support failing organs, incidence of infections, and prevent complications (Rivers *et al.*, 2001). But these measures require whole-hearted involvement of the entire healthcare team and the provision of strong support in achieving these objectives cannot be stressed enough. So presently the focus is to find better drugs to combat disease and curb it at initial stages.

Development of drotrecogin alfa (activated), which is a recombinant version of activated protein C (APC), for sepsis therapy created big hopes. It was developed by Eli Lilly and Company and is marketed under the brand name Xigris. Activated

protein C has profound anti-apoptotic & anti-inflammatory properties, in addition to its anticoagulant activity. However APC was found to have its own complications in ADDRESS (Administration of Drotrecogin alfa (activated) in Early Stage Severe Sepsis) trial.

A series of antilipopolysaccharide treatment strategies for sepsis have been done for more than 20 years, including antiendotoxin antibodies, lipid A (harboring the LPS biological activity) antagonists, polymixinB, extracorporeal endotoxin absorber, bactericidal /permeability-increasing protein, cathelicidins, limulus antilipopolysaccharide factor and lactoferrin. These therapeutic agents have demonstrated efficacy in animal research, however, numerous attempts to neutralize LPS in clinical trials in septic patients have proven ineffective (Nahra and Dellinger, 2008). TLR4 antagonists (like E5564 and TAK-242) were also developed to curb sepsis menace, however they too provided no respite (Leon et al., 2008).

HMGB1 levels in serum remains high days after actual insult giving a wide therapeutic window for therapy compared to other cytokines. It is speculated that agents capable of decreasing HMGB1 release might prove as potential candidates for sepsis therapy (Sappington *et al.*, 2003). This study was undertaken to identify novel compound targeting HMGB1 and further elucidating its role in sepsis/ endotoxemia.

Since HMGB1 is released in both processes i.e., apoptosis and necrosis (Raucci *et al.*, 2007; Tang *et al.*, 2010) so we needed to assess non toxic doses of test compounds for macrophage cells. Any kind of stress would change the distribution of cellular HMGB1. Non toxic doses of all the test compounds were used keeping this in mind same is depicted in the results under viability assay (**Fig 3.1**).

Screening of test compounds

Based on HMGB1 release it would be cumbersome to screen many compound so we screened compounds on the basis of their nitric oxide (NO) scavenging potential in activated macrophages. During the course of sepsis, increased amounts of nitric oxide levels are produced, and elevated levels of nitric oxide metabolites in patients with sepsis have been correlated with LPS or endotoxin levels and with organ-failure scores (Gomez-Jimenez *et al.*, 1995; Groeneveld *et al.*, 1996).

Statins are reported to greatly reduce the leucocytes migration and leucocytes recruitment induced by lipopolysaccharide (LPS) (Diomede *et al.*, 2001; Pruefer *et al.*, 2002). Statins reduce leucocytes adhesion to endothelium by down-regulating surface expression of endothelial cell adhesion molecule (ECAMs): P-selectin, CD11b, and CD18 (Weber *et al.*, 1997; Yoshida *et al.*, 2001). Statins have also seen to affect the production of many acute phase reactants, such as TNF-α, IL-8, IL-6, monocyte chemoattractant protein-1 (MCP–1), and C-reactive protein (CRP) (Albert *et al.*, 2001; Arnaud *et al.*, 2005; Musial *et al.*, 2001). Many statins are reported to have beneficial role in sepsis, we studied effect of rosuvastatin (a synthetic statin) in endotoxin induced inflammation in RAW 264.7 macrophages cells. Rosuvastatin did not showed any significant downregulation on LPS induced NO in RAW 264.7 cells (**Fig 3.3**).

Crocus extracts (*Crocus sativus* L. (Iridaceae) are reported to process anti-inflammatory and immunomodulatory actions (Hosseinzadeh and Sadeghnia, 2005; Kianbakht and Ghazavi, 2011). One of the main components of crocus extracts is safranal which is shown to have high antioxidant and free radical scavenging activity (Hosseinzadeh and Sadeghnia, 2005). Safranal did not show any effect on LPS induced NO generation, which was confirmed by iNOS expression (**Fig 3.4**).

Emodin (3-methyl-1,6,8-trihydroxyanthraquinone) is an anthraquinone derivative from the rhizome of Rheum palmatum, an herb widely used as a laxative in traditional Chinese medicine (Shi et al., 2001). It has been reported that emodin possesses a variety of biological activities, such as vasorelaxative (Huang et al., 1991), immunosuppressive (Kuo et al., 2001), hepatoprotective (Lin et al., 1996), and anti-tumor activity (Shi et al., 2001; Chang et al., 1996). Treatment of RAW 264.7 macrophages with emodin (20 μg/ml) inhibited the expression of a panel of inflammatory-associated genes, including TNF-α, iNOS, interleukin-10 (IL-10), cytosolic inhibitor of κB (IκB)α, IκB kinase (IKK)-α and IKK-γ, and the nuclear translocation of nuclear factor-κB (NF-κB) (Li et al., 2005). Our study showed significant inhibition of LPS induced NO by emodin **(Fig 3.4).** Since effect of emodin on HMGB1 in macrophages were reported during the course of study, so we did not pursue its use further (Chen et al., 2010).

Effect of psychosine on inflammatory mediators in RAW 264.7 cells and peritoneal macrophages

Galactosyl sphingosine (Psychosine) is abnormally increased in brain in a disease known as Krabbe disease. Psychosine accumulation is believed to be the primary cause of the rapid degeneration of the myelin-forming cells and consequent demyelination that is seen in this disease (Miyatake and Suzuki, 1972). The mechanisms by which psychosine mediates cell death is unclear yet. In Krabbe disease the microglial cells are activated which become phagocytic and secrete a variety of cytokines, including the proinflammatory cytokines, TNF-α, interleukin 1-beta and interferon-gamma. These cytokines perpetuate and augment microgliosis as well as induce glial cells and astrocytes to become hypertrophic and undergo reactive astrocytosis (Merrill and Benveniste, 1996). The role of psychosine outside neuronal system is poorly studied. In addition effect of psychosine on different cell types varies considerably, however effect on RAW 264.7 cells has not been seen yet.

We observed that psychosine showed different response in RAW 264.7 cells and primary peritoneal macrophages. The results were in concordance with earlier reports of psychosine showing different response in different cells depending upon origin of cell line (glial cell line vs primary mixed gial cells) (Bashir and Haq, 2011). The primary peritoneal macrophages is mixed in nature including primarily macrophages and specific B cell subsets, thus probably explaining the variation in response of psychosine in two.

In LPS stimulated RAW 264.7 cells, psychosine showed decrease in NO and TNF-α level but no change in levels of HMGB1 was seen. On the other side, in activated peritoneal macrophages, psychosine showed a significant increase in LPS-induced NO, TNF-α, along with HMGB1. The psychosine induced significant release of HMGB1 levels in primary peritoneal macrophages even in the absence of LPS. It has already been reported that psychosine potentiates the LPS-induced expression of iNOS and the production of proinflammatory cytokines (IL-1β, TNF-α, and IL-6) production in rat primary astrocytes (Giri *et al.*, 2002). Psychosine is said to cause apoptotic mediated death of oligodendrocytes (Haq *et al.*, 2003; Zaka and Wenger, 2004). As HMGB1 is released in apoptotic death and also mediates the inflammatory

response in later stages. Thus increase in HMGB1 release by psychosine might be one of mediators of psychosine induced lethality. However we used low doses of psychosine which had no effect on RAW 264.7 cell viability (**Fig 3.1**) so it is proposed that psychosine mediated HMGB1 release in peritoneal macrophages is not because of apopotosis or necrosis but a different pathway may be involved. Based on the known literature, it can be proposed that, psychosine at low levels may result in activation of cells releasing HMGB1 which surge inflammation and later mediate inflammation and apoptosis (Galbiati *et al.*, 2007; Galbiati *et al.*, 2009). However, further studies need to check the actual role of HMGB1 in Krabbe disease.

Effect of aloe-emodin on inflammatory mediators in RAW 264.7 cells and peritoneal macrophages

Aloe-emodin (AE), a hydroxyanthraquinone naturally present in the leaves of Aloe vera (Dutta *et al.*, 2007), has antiviral, antimicrobial and hepatoprotective activities (Arosio *et al.*, 2000; Eshun and He, 2004) and anticancer activity in neuroectodermal tumors (Pecere *et al.*, 2000), lung squamous cell carcinoma (Lee, 2001), hepatoma cells (Kuo *et al.*, 2002) and in glial cell line (Acevedo-Duncan *et al.*, 2004). Studies have shown aloe-emodin as antioxidant under various stress. However aloe-emodin role in LPS induced inflammation has not yet been studied.

During this study we found aloe-emodin decreased LPS induced NO production and iNOS expression dose dependently (**Fig 3.7)**. Similar dose dependent effect was seen on LPS induced extra-cellular release of HMGB1 in RAW 264.7 macrophages and primary peritoneal macrophage cells (**Fig 3.13 & 3.14)**. On LPS stimulation HMGB1 is first translocated from nucleus to cytoplasm and then outside the cells. Aloe-emodin was seen to partially decrease the nuclear to cytoplasmic translocation of HMGB1 (**Fig 3.15)**.

The pro-inflammatory cytokines i.e., TNF-α, IL-1β are released much early than HMGB1 and mediate early inflammatory damage on stimulation with LPS. The drugs which inhibit pro-inflammatory cytokines both early cytokines (TNF, IL-1) and late cytokines (HMGB1) are seen as good candidate for sepsis/endotoxemia therapy. The aloe-emodin treatment inhibited both TNF-α, IL-1β in dose dependent manner in RAW 264.7 cells with 15μM decreasing the levels most (**Fig 3.17 & 3.18)**. However

in peritoneal macrophages the pattern of inhibition of TNF-α and IL-1β is different. Aloe-emodin inhibited the TNF dose dependently in peritoneal macrophages, but IL-1β inhibition showed different pattern with maximum inhibition at 10μM.

In response to oxidative stress, macrophages also induce heme-oxygenase 1 (HO-1), which has protective effect in innate immune cells and exerts anti-inflammatory effects that limit the damaging consequences of inflammation and immunity (Otterbein *et al.*, 2003; Wagener *et al.*, 2003). Aloe-emodin increased the LPS induced HO-1 expression in RAW 264.7 cells (**Fig 3.19**). Recent studies proved a close relationship between HO-1 and HMGB1 in inflammatory conditions (Tsoyi *et al.*, 2009). HO-1 has been reported to down regulate HMGB1 release via carbon monoxide in activated macrophages (Tsoyi *et al.*, 2009). Up-regulation of HO-1 is seen to protect mice from the lethal effect of LPS- and CLP-induced sepsis, paralleled by a decrease in the systemic levels of HMGB1. Thus HO-1 may be involved in aloe-emodin mediated HMGB1 inhibition.

Drugs inhibiting HMGB1 are seen as good targets for sepsis therapy. Efficacy of aloe-emodin against sepsis was tested in mice model of endotoxemia. It was observed that aloe-emodin protected the mice in both sub-lethal and lethal model of endotoxemia. The survival was seen more in mice administered with aloe-emodin at the dosage of 0.9μg/mouse (**Fig 3.20)**.

Proinflammatory cytokines are major players in sepsis lethality and among the cytokines involved in endotoxic shock, TNF-α appears to play a central role. Indeed, increased serum TNF-α levels appear during endotoxemia and TNF-α injection induces shock, tissue damage, and death (Tracey *et al.*, 1986). During endotoxic shock, TNFα shows a large spectrum of harmful effects. These effects include increase in procoagulant activity of vascular endothelial cells, activation of macrophages and neutrophils, and increase in combination with IFNγ in the expression of adherent molecules resulting in increased neutrophil/monocyte adherence to endothelial cells and tissue infiltration. Aloe-emodin decreased the levels of proinflammatory cytokines i.e., TNF-α and IL-1β (**Fig 3.21 &3.22**). The pattern of inhibition however varies in case of TNF-α and IL-1β indicating pleiotropic

actions of aloe-emodin. Aloe-emodin treatment at both high and low doses showed no effect on IL-6 levels in endotoxemic mice (**Fig 3.23)**.

TNFα in early stages and HMGB1 in later stages mediates neutrophil and macrophage accumulation which damages the organs, thereby causing multi-organ dysfunction syndrome (MODS). Treatment with aloe-emodin protected endotoxemic mice from organ dysfunction as seen by serum biochemistries (LFT and KFT) checked 18hrs after LPS administration (**Fig 3.25 & 3.27**). Protective effect of aloe-emodin was also observed in histopathology study of liver, kidney and lung sections analysed by H&E staining. Treatment with aloe-emodin shows improved organ histology compared to endotoxemic mice (**Fig 3.24, 3.26 & 3.28**). Histopathology showed significant decrease in neutrophil infilitration in lung tissue sections compared to endotoxemic mice. Neutrophil infiltration and high level of cytokine in lungs leads to acute lung injury (ALI), which is an early characteristic of multiple organ dysfunction and is responsible for high mortality and poor prognosis in patients with sepsis [Ware, 2000]. In this present study, the levels of both TNF and neutrophils increased in endotoxemic mice even after 18hrs of LPS administration. Aloe-emodin was observed to inhibit both release of TNF and levels of neutrophils in lung tissues compared to endotoxemic mice (**Fig 3.29 & 3.30**).

This study can serve as a model to evaluate the mechanism of aloe-emodin mediating inhibition of HMGB1 and its effect in other septic models like cecal ligation and puncture (CLP).

Thus, the results of present study clearly reveal that the aleo-emodin has potential to ameliorate HMGB1 release under inflammatory stress and also to decrease the level of proinflammatory cytokines and oxidative stress markers. Aloe-emodin showed good immunotherapeutic potential in LPS induced endotoxemic murine model of sepsis. The present study provides the basis for the future evaluation of aloe-emodin as a potential adjunct to the already existing sepsis therapy.

- Aloe-emodin inhibited HMGB1 release in activated macrophage cells, by inhibiting LPS induced HMGB1 translocation from nucleus to cytoplasm.

- Aloe-emodin decreased the release of LPS-induced pro-inflammatory cytokines (TNF-α, IL-1β) in activated macrophages.

- Aloe-emodin decreased the acute inflammation induced stress markers NO, iNOS, HO-1 which may be a mechanism of HMGB1 regulation.

- Aloe-emodin protected mice from endotoxemic lethality.

- Aloe-emodin decreased the LPS induced proinflammatory cytokines (TNF-α, IL-1β) in mice at early hours of onset of disease.

- Aloe-emodin decreased the organ dysfunction (liver, kidney, lung) as seen by serum markers and histopathology study.

- Aloe-emodin decreased neutrophil infiltration and proinflammatory cytokine (TNF-α) in lungs.

- Psychosine showed different effect on LPS-induced inflammatory mediators depending on nature of cell.

- Effects on primary peritoneal macrophages resemble the in-vivo pathobiology of psychosine which hints a possibility of major role of HMGB1 in psychosine induced Krabbe disease.

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