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 sepsis. 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.
Figure 1.1: 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 particularly, 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
Introduction, Background & Rationale

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.
Introduction, Background & Rationale

- 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 diaminopimelate 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 up to six acyl residues. Although structurally less variable but variations exist 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.
LPS is bipolar macromolecule that contains both hydrophobic and hydrophilic 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 heptatically 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 Lipopolysaccharide-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 the 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. Lipopolysaccharide, 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

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.

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 discussed 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-$\alpha$, 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
Introduction, Background & Rationale

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.
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 overwhelming 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
Introduction, Background & Rationale

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 (Ebbing 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).
In spite 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.