Long-term Effects of Severe Sepsis on Dendritic Cell Function

by

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To my beloved daughter Sonia S. Wen

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Chapter 1

Introduction

Clinical and Experimental Sepsis

Review of clinical and experimental sepsis

Sepsis, caused by gram-negative (G^-) and gram-positive (G^+) bacteria, fungi, viruses, and parasites has become increasingly significant over the past decades, as it affects approximately 700,000 people annually and accounts for about 210,000 deaths per year in the US (1-5). Despite technical developments in intensive care units (ICUs) and advanced supportive treatment, the incidence of sepsis is rising at rates between 1.5% and 8% per year (4, 5). These rates may be caused by the increased usage of catheters and other invasive instruments, chemotherapy for cancer patients, and immunosuppression in patients with organ transplants or inflammatory diseases. Sepsis represents a major burden to the US health care system, with costs of approximately $16.7 billion per year, due in part to the extended hospitalization of septic patients (4). Interestingly, the contribution of G^+ bacteria to sepsis has dramatically increased during the past thirty years (5), with *Staphylococcus aureus* and *S. epidermidis* being responsible for more than half of these cases of sepsis (6, 7). In addition, the rate of fungal infections is reported to have increased more than 200% (5). The clinical definition of sepsis has been continuously modified based on additional clinical symptoms and laboratory findings.

Table 1.1 Sepsis diagnostic criteria

General changes	Hyperthermia (> 38.3°C) or hypothermia (< 36°C) Heart rate > 90/min or > 2 SD above normal value for age) Breath rate > 30/min) Changes in mental state Edema or positive fluid balance (> 20ml/kg over 24 h) Plasma glucose > 120mg/dl or > 7.7 mM
Inflammatory changes	WBC > 12×10^9/L or < 4×10^9/L Normal WBC count with > 10% immature band forms Plasma C reactive protein > 2 SD above normal Plasma procalcitonin > 2 SD above normal
Hemodynamic changes	Arterial hypotension (SBP < 90 mmHg, MAP < 70, or a SBP decrease > 40 in adults or < 2 SD below the normal for age) SvO_2 > 70% Cardiac index > 3.5 L/min/M^{-23}
Organ dysfunction	Arterial hypoxemia (PaO_2/FiO_2 < 300) Acute oliguria (urine output < 0.5 ml/kg/h or < 45 mM for at least 2 h) Creatinine increase > 0.5 mg/dl Coagulation abnormalities (INR > 1.5 or aPPT > 60 s) Ileus (absent bowel sounds) Platelet count < 100,000/μL Plasma total bilirubin > 4 mg/dl or > 70 mM
Tissue perfusion	Serum lactate level > 1mM Decreased capillary refill

WBC, white blood cell; SBP, systolic blood pressure; MAP, mean arterial pressure; SvO_2, mixed venous oxygen saturation; PaO_2, arterial partial pressure of oxygen; FiO_2, fraction of inspired oxygen; INR, international normalized ratio; aPPT, activated partial thromboplastin time.

Discussion at the most recent consensus conference led to the establishment of the extended definition of sepsis, which is outlined in table 1.1 (8-10).

During the initiation and progression of sepsis, two dynamic stages are present; a systemic inflammatory response syndrome (SIRS) in the acute phase and a compensatory anti-inflammatory response syndrome (CARS) in the later phase (9). The hallmark of SIRS/CARS is an exacerbated production of pro-and anti-inflammatory cytokines/chemokines, leading to the so-called “cytokine storm”. While these inflammatory mediators are essential in providing an effective host defense, their

overzealous production can be deleterious, resulting in a "double-edge sword" property of reducing pathogens at the expense of organ injury (11, 12). In the host, toll-like receptors (TLRs) and intracellular pattern recognition receptors (PRRs) [e.g. nucleotide-binding oligomerization domain (NOD)-leuine-rich repeat (LRR) family (NOD-LRR)] act as sensors for invading pathogens or their components, resulting in the release of an array of proinflammatory mediators from both leukocytes and structural cells. These mediators include, but are not limited to, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, CXCL8 (IL-8), IL-18, CCL2 (MCP-1), CCL3 (MIP-1α), CXCL10 (IP-10) (10, 11, 13, 14), prostaglandins, lipid mediators and reactive oxygen species (7). The effects of these host-derived factors include vasodilatation and upregulation of adhesion molecules, resulting in the extravasation of leukocytes and the activation of these cells along with epithelial and endothelial cells. Coagulation is an additional vascular event that is dictated directly by pathogen components and indirectly by pathogen-induced cytokines. This physiological response can be more severe and result in disseminated intravascular coagulation (DIC), causing hypoperfusion, hypoxia and death. Collectively, the tissue damage caused by activated immune cells and pathological coagulation resulting in DIC can lead to multiple organ failure (MOF), involving the lungs (acute respiratory distress syndrome), liver, and kidneys (2, 15-17). Based on the understanding of molecular mechanisms underlying sepsis, new clinical therapies have been designed to neutralize several key molecules involved in the initiation and progression of sepsis, including lipopolysaccharide (LPS), a core component of G^- bacteria cell wall, IL-1 and TNF-α. Although experimental studies using animal models of sepsis has documented a beneficial effect of these therapies (18-22), subsequent clinical trials were disappointing

as they showed no substantial benefits (23-27). So far, recombinant human activated protein C that targets coagulation remains the only effective new therapy for clinical severe sepsis (28). These observations support the concept that severe sepsis is a highly dynamic and complex disorder and efficient treatment strategies remain elusive.

In order to understand the mechanisms of clinical sepsis and develop effective therapeutic modalities, there is a need to use effective experimental models that faithfully replicate what occurs in patients with sepsis. Several models, including intravenous endotoxin challenge, injection of live organisms into the peritoneal cavity, establishment of abscesses in the extremities, and the induction of polymicrobial peritonitis via cecal ligation and puncture (CLP) have been used to study sepsis (29-31). The latter experimental model, CLP, has been proposed to more closely replicate the nature and course of clinical sepsis, as compared to other models (32, 33). The benefits of the CLP model are its reproducibility and the potential to alter the severity of sepsis by controlling needle size, number of cecal punctures and antibiotics utilization (34). The following surgical conditions have been successfully utilized to induce severe sepsis in mice: a 21G needle was used to puncture the cecum 9 times, followed by antibiotics treatment and saline resuscitation for 3 days. The genetic background of the animals is also important. In contrast to T helper (Th) 2-dominant BALB/c mice, C57BL/6 mice are Th1-dominant and more resistant to trauma-induced sepsis (35) and Th1-eliciting pathological infections, such as *Leishmania major* (36, 37). Thus, female C57BL/6 mice were used extensively as an animal of choice for inducing experimental sepsis. On average, this nine-puncture CLP procedure results in approximately 40% mortality in the acute phase of sepsis (38-40). Following the induction of CLP-induced peritonitis, inflammatory cytokines/

chemokines such as IL-12p70, IL-10, TNF-α, CCL2, CCL3 and CXCL10 are rapidly induced in the peritoneal cavity (local response) (Fig 1.1A), blood (Fig 1.1B) and peripheral organs (systemic response) (data not shown) within 4 hours and peak at 24 hours. Three days later, the local and systemic levels of inflammatory cytokines/chemokines mostly return to baseline levels, indicating the end of the acute phase of sepsis. However, a variety of functions in the host response are altered after day 3.

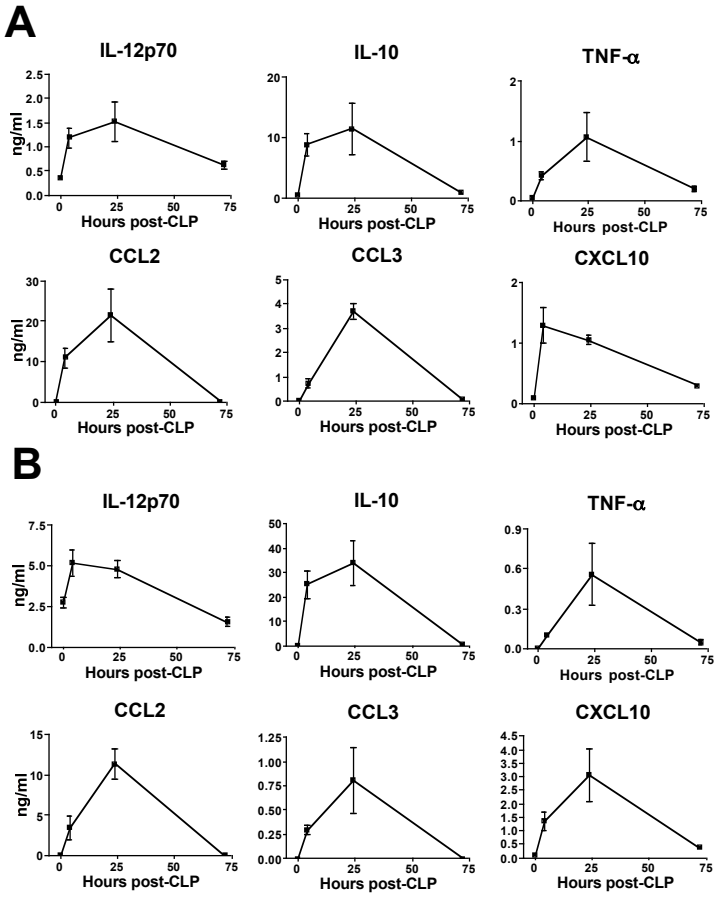

Figure 1.1 Cytokine profile following experimental peritonitis. At different time point (4, 24 and 72 hours) after cecal ligation and puncture (CLP) procedure, peritoneal lavage fluid (A) and blood samples (B) were collected. Levels of inflammatory cytokines were determined by ELISA.

Long-term immunosuppression following sepsis

A high mortality rate is often found during the acute phase of severe clinical sepsis, which may be associated with the development of a sustained anti-inflammatory

or immunosuppressive state. This response has been termed "immunoparalysis" and often follows the initial hyper-inflammatory response. The immunoparalysis is manifested by an inability to eradicate a primary infection and/or the development of new secondary infections (41-46). Many of the pathogens responsible for the secondary, hospital-acquired infections are not particularly pathological in patients with competent immune systems, indicating the immunosuppressive state of patients with sepsis. Interestingly, this particular population of septic patients is at significant risk of dying from various complications up to 8 years after initially being hospitalized for sepsis (47, 48). Thus, the original episode of severe sepsis, characterized by a dysregulated inflammatory response, appears to lead to long-lasting host defense complications.

Although the mechanism(s) that is responsible for severe sepsis-induced long-term immunosuppression is still under investigation, it has been proposed that depletion of immune cells via apoptosis following sepsis is an initiating factor involved in this process (49, 50). In patients who die of sepsis, extensive apoptosis of lymphocytes and gastrointestinal epithelial cells have been observed, as compared with patients who die of non-septic etiologies (49). Further studies revealed that most of the apoptotic lymphocytes are B cells and $CD4^+$ T cells (51) with a significant loss of circulating lymphocytes in septic patients compared with non-septic patients in the ICU (52, 53). In addition, dendritic cells (DCs) are essential immune cells that are reported to be dramatically depleted in septic patients (54). Similar observations have been made in experimental animal models of sepsis, which have documented significant apoptosis of lymphocytes and DCs (55-59). We have monitored the presence of $CD11c^+CD11b^+$ $MHCII^{hi}$ myeloid DCs (mDCs) in both lung and spleen at different time points following

the induction of experimental CLP-induced peritonitis. At early time points after CLP, myeloid DCs were significantly decreased in both the lungs and spleens from septic mice compared with sham control, which is consistent with the above studies. The proportion of splenic and lung mDCs gradually returned to the levels found in sham mice 4 weeks and 10 weeks after CLP, respectively (Fig 1.2). $CD11c^{+}B220^{+}$ plasmacytoid DCs (pDCs), another subset of DCs, showed a similar dynamics after severe sepsis-initial depletion followed by gradual recovery (data not shown). Thus, severe sepsis causes the depletion of DCs, the most potent antigen-presenting cells (APCs), which compromises both the innate and adaptive immune response. The profound decrease in the numbers of T and B cells further impairs the adaptive immune response (50).

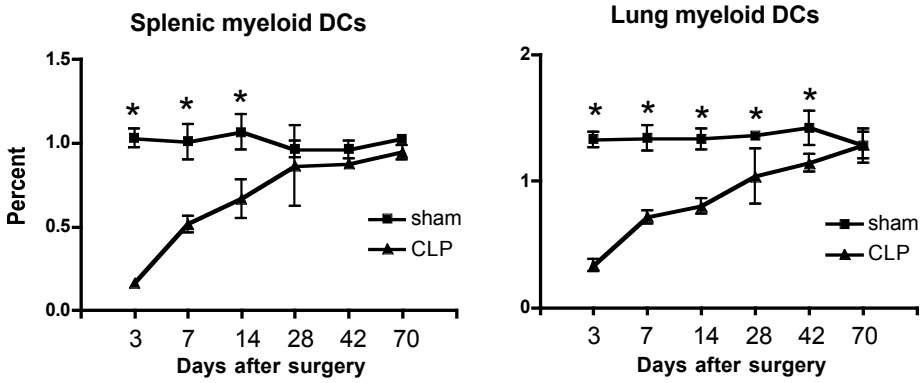

Figure 1.2 Alteration in DC percentages in spleen and lung following experimental peritonitis. At different time point after cecal ligation and puncture (CLP) procedure, spleen (A) and lung (B) were collected and dispersed. The cells were stained with FITC-anti-CD11c, PerCP-Cy5.5-anti-CD11b and PE-anti-I-A^{b}. Myeloid dendritic cells (mDCs) were characterized as $CD11c^{+}CD11b^{+}MHCII^{hi}$. *, $p < 0.05$ compared with mDC numbers observed in sham mice.

Based on the long-term susceptibility of septic patients to secondary pathological infections (48), and our observation of a gradual restoration of DCs after severe sepsis (Fig 1.2), it is likely that immune cell depletion alone is not the only mechanism

responsible for the long-term immunosuppression found in post-septic patients and animals. Dysfunction of re-established immune cells may be another potentially important mechanism. However, It is worth noting that the apoptosis and dysfunction of immune cells are not exclusive to each other. It has been reported that apoptotic cells induce anergy and Th2 response in surviving immune cells (50, 60, 61), promote the production of anti-inflammatory cytokines by DCs and macrophage (62), and inhibit the release of pro-inflammatory cytokines (63, 64). While convincing data have identified sepsis-induced depletion of immune cells as a mechanism leading to sepsis-induced immunosuppression, few investigations have focused on alteration in immune cell function after immune cells have been restored to depleted tissues. Previously, using 9-puncture CLP procedure, our group has characterized the immunosuppressed status of the survivors of severe experimental sepsis. These post-septic mice were susceptible to a pulmonary challenge of *Aspergillus fumigatus*, a relatively innocuous microbe, compared with sham mice (38, 46). This study highlighted an impaired innate pulmonary immune response in post-septic mice, since *A. fumigatus* normally poses few problems to an intact pulmonary immune system. Furthermore, adoptive transfer of bone marrow-derived DCs (BMDC) from sham mice restored the protective immune response of post-septic mice to *A. fumigatus* challenge. This observation, together with the consideration of the essential role of DC in both innate and adaptive immunity, led us to hypothesize that severe sepsis-induced DC dysfunction is an important mechanism of long-term immunosuppression after the acute phase of sepsis.

Dendritic cell biology

Dendritic cell subtypes and precursors

The DCs were first described by Ralph Steinman as a novel cell population in the mouse spleen (65-67). Since then, the properties and functions of this unique immune cell have been extensively studied. DCs comprise a heterogeneous group of cells with particular locations and divergent effects on the immune response (68, 69). Traditionally, DCs are categorized according to the expression of a series of surface markers, which differ in such immune functions as cytokine production, antigen uptake and presentation (Table 1.2) (70, 71). At least six subtypes of DCs under steady state have been characterized (68, 72), and their individual properties are briefly summarized in table 1.2. It is interesting to note that novel DC populations that are not found in the steady state have been characterized as a consequence of infection or inflammation (69). Several studies have reported the existence of these inflammatory DCs; namely tumor necrosis factor and inducible nitric oxide synthase-producing DCs (Tip DCs), DCs produced in vivo when pDCs are stimulated by the influenza virus (73) and DCs that appear after challenging mice with either *Listeria monocytogenes* (74) or *Leishmania major* (75).

DCs originate as haematopoietic stem cells (HSCs) in the bone marrow (BM) giving rise to a series of downstream precursor cells, including the common myeloid progenitor and the common lymphoid progenitor (76, 77). Although it was initially considered that DCs solely originated from myeloid-lineage precursors, subsequent studies revealed that both myeloid and lymphoid precursor cells can generate DC subtypes (77-82). A study using limiting-dilution approach has shown that both a single common myeloid progenitor and a single common lymphoid progenitor could generate

Table 1.2 Murine DC subsets and important properties

	Conventional DC					Plasmacytoid DC
Surface markers	$CD8^+$ DC	$CD4^+$ DC	DN DC	Langerhans' cell	Dermal DC	
CD11c	+	+	+	+	+	Low
MHCII	+					
CD8α	+	-	-	**-/low**	-	**±**
CD4	-	+	-	-	-	**±**
CD205	+	-	-	**High**	+	-
CD11b	-	+	+	+	**±**	-
CD45RA	-	-	-	-	-	+
IL-12 production	+	**±**	**±**	**?**	**?**	-
IFN-γ production	+	-	-	**?**	**?**	-
IFN-α production	-	-	-	**?**	**?**	**high**
Ag-presenting	+	+	+	+	+	**?**
CD4 T-cell priming	+	+	+	+	+	+

DN, double negative;

both conventional DCs (cDCs) and pDCs (81). These observations imply the developmental flexibility of DC lineages at the early precursor stage. Thus, it seems to be more appropriate to ask which early precursor population contributes to DC generation *in vivo*, instead of asking which population, either myeloid or lymphoid precursor cells, has DC developmental potential in an *in vitro* assay system (69). Since myeloid precursors are numerically dominant in BM, it is possible that most DCs in peripheral sites and the secondary lymphoid organs such as the spleen or lymph nodes should be of myeloid origin, whereas in the thymus, where lymphoid precursors are dominant, most DCs may be of lymphoid origin. The extent to which these precursors contribute to DC commitment needs further investigation (69). Interestingly, several studies revealed a common progenitor population for DCs and macrophage (83, 84). These CX_3CR1^+c-kit^+Lin^- clonogenic precursor cells identified in the bone marrow (BM) could give rise to DCs and macrophages, but not polymorphonuclear cells (PMNs), B or NK cells (84).

Furthermore, CX_3CR1^+c-kit$^+$Lin$^-$ cells exist within the c-kit$^+$ Sca1$^-$IL-7Rα^- CMPs populations (77), indicating that the crucial events in DC differentiation and the development of the separate DC subtypes are downstream of CLPs and CMPs (69).

In addition to the complexity and controversy of the commitment and development of DCs under steady state, it has been well characterized that inflammatory $CCR2^+$ blood monocytes can differentiate into DCs under conditions of inflammation (75, 85-87). The differences between monocyte-derived inflammatory DCs and steady-state splenic cDCs include the expression levels of CD11c, CD11b, MAC3 (a glycoprotein also found on activated macrophages) and the presence or absence of CD4 or CD8 expression (69, 88). Since these inflammatory DCs are absent under steady state (75, 85, 88), they are considered to be transient cells of an inflammatory response and further highlight the developmental flexibility of DCs under steady state.

Role of Dendritic cells in innate and adaptive immune response

DCs are unique immune cells in that they function in both the innate and the adaptive immune response and bridge these two branches of host immunity (72, 89-91). In the innate immune response, DCs recognize pathogen-associated molecular patterns (PAMPs) via the functions of a series of germ-line-encoded receptors known as pattern recognition receptors (PRR), including DEC-205, the mannose receptor, the scavenger receptors and toll-like receptors (TLR) (92, 93).

TLR family are the best-characterized class of PRR molecules and are localized either on the cell surface (TLRs 1, 2, 4, 5, 6) or in intracellular compartments (TLRs 3, 7, 8, 9), detecting a multitude of PAMPs from a wide variety of pathogens (94, 95). For example, TLR4 in complex with myeloid differentiation protein-2 (MD-2), an accessory

Table 1.3 TLR expressions on Murine DC subsets and signaling pathways

	DC subtypes				MyD88	MyD88
	$CD8^+$ DC	$CD4^+$ DC	DN DC	pDC	dependent	independent
TLR1[a]	+	+	+	+		
TLR2	+	+	+	+	+	-
TLR3	+	+	+	+	-	+
TLR4	+	+	+	+	+	+
TLR5	+	-	+	+	+	-
TLR6[a]	+	+	+	+		
TLR7	+	-	+	+	+	-
TLR8	+	+	+	+	+	-
TLR9	+	+	+	+	+	-

MyD88, myeloid differential factor 88; [a] Either TLR1 or TLR6 functions in a complex with TLR2.

protein, recognizes LPS from G^- bacteria (96, 97); TLR2 detects peptidoglycans (PGN) from G^+ bacteria (98); TLR3 recognizes double-stranded RNA (dsRNA) from double-stranded and negative-strand viruses (99); TLR 7 and 8 recognize single-strand viral RNA (100, 101); TLR9 detects unmethylated CpG motifs present in viral and bacterial DNA (102, 103); TLR5 detects bacterial flagellins (104); TLR11 detects protozoan profilin-like proteins (105). In addition to ligand specificity, individual TLRs differ in their expression patterns on DC subtypes and the signal transduction pathways they utilite, including myeloid differentiation factor 88 (MyD88)-dependent or –independent pathways (Table 1.3) (106). The activation of DCs via TLRs plays a pivotal role in shaping the outcome of primary immune responses. This is due to the multiple functions of DCs, including their ability to generate numerous cytokines and chemokines, enhanced phagocytosis and killing of invading microbes, regulation of recruitment of the inflammatory immune cells, activation of both leukocytes (NK cells, mast cells) and

stromal cells (92, 106, 107) and the inhibition of anti-inflammatory function of regulatory T cells (Treg) (108).

Besides its important role in innate immunity, DCs are the most potent antigen-presenting cells (APC) and responsible for the initiation of adaptive immune responses (72, 89). In the peripheral tissues, DCs take up antigens and process them into optimal peptides in lysosomes (109, 110). The engagement of TLRs with their appropriate ligands activate and induce DC migration to draining lymphoid tissues to present these antigenic peptides on the relevant major histocompatibility complex (MHC) molecules. The entire process involves phagocytosis and processing of antigen, the upregulation of costimulatory and MHC molecules, a switch in chemokine receptor expression, the secretion of cytokines and chemokines, and the presentation of antigens by DCs to T-cell receptor (TCR) specific naïve T cells (72, 106). The activated T cells undergo an initial clonal expansion that generates effector T-cells, followed by clonal contraction. Finally, memory T cells are generated and maintained for an extended period (111, 112). Depending on the properties of antigens, expression of costimulatory molecules on DCs and T cells, and the cytokine environment, the so-called "3 signals" (113), diverse Th1, Th2, Th17 and Treg responses can be induced to defend the host against a myriad of pathogens (114). DCs play a key role in all of the following lymphocyte-mediated responses. In response to infections with intracellular microbes or viruses, $CD4^+$ T cells differentiate into Th1 cells that produce IFN-γ and enhance the killing function of $CD8^+$ cytotoxic T cells (CTLs) targeting infected cells. In contrast, in response to parasite infections, Th2 cells are induced and produce Th2 cytokines (IL-4, IL-5, and IL-13), induce IgE production by B cells and enhance eosinophil-mediated destruction of the

pathogen. Th17 cells have recently been characterized by the production of IL-17 and are key cells to the immunopathology associated with multiple autoimmune diseases (115). Furthermore, bacterial and viral infections promote the generation of Treg and enhance their functions, which subsequently suppress the proliferation and differentiation of Th or CTLs and limit the potential immunopathology that might be caused by an overexuberant immune response (116, 117).

One of the inflammatory cytokines generated by activated DCs, IL-12, is extremely important to the progression of immune reactivity as this cytokine can induce interferon-γ production, direct the differentiation of Th1 cells, and form a link between innate and adaptive immune response (118). IL-12 is a heterodimer composed of a 35-kDa light chain (p35) and 40-kDa heavy chain (p40) (119), encoded by p35 and p40 gene located in different chromosomes both in mouse and human: p35 (human chromosome 3; mouse chromosome 3) and p40 (human chromosome 5; mouse chromosome 11). Thus, the efficient production of biologically active IL-12p70 requires the coordinate transcription and translation of both genes in the same cell (118, 120). The upregulation of both subunit genes can be induced by external stimuli such as TLR agonists (121). The transcriptional factors essential for gene expression are not identical but rather overlapping between the two genes (122). IL-12p70 signals through IL-12 receptor composed of two chains IL-12Rβ1 and IL-12Rβ2, which subsequently activate the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) signal transduction pathway. STAT4 is especially responsible for IL-12 cellular effects. Activated/phosphorylated STAT4 is relocated into nucleus, binds to the promoter regions of target genes and initiates IL-12-mediated gene transcription together with other

essential transcription machinery (123, 124). Activation of T cells through the TCR promotes IL-12R expression on the cell surface and further induces T cell responsiveness to IL-12. IL-12R expression is also induced by IL-12 itself, IFN-α, IFN-γ, TNF-α and via co-stimulation through CD28 (125, 126).

IL-12 is a pro-inflammatory cytokine in the context of pathological infections and is produced by a series of activated inflammatory cells, including DCs, monocytes, macrophages, neutrophil and microglia (118). IL-12 production is controlled by both positive and negative mechanisms. While TLR agonists and IFN-γ are strong inducers of IL-12 production, IL-10 and transforming growth factor-β (TGF-β) are potent inhibitors of IL-12 production (127-129). Interestingly, the balance between IL-10 and IL-12 has been observed in specific pathological conditions, such as tumors and autoimmune diseases, indicating that there might be a reciprocal regulatory mechanism(s) of these two important genes (130, 131). Th1 differentiation and IFN-γ production require the presence of IL-12 (132). Although IL-12 production is not limited to DCs, DC-derived IL-12 is essential for the initiation of Th1 immune response against intracellular pathogens *in vivo*, since DCs are the most potent APCs and function to contact and activate TCR-specific naïve T cells (72). IL-12 also enhances the generation and functions of CTLs and NK cells in part by inducing the transcription of genes that encode cytotoxic granule-associated molecules such as perforin and granzymes and by upregulating the expression of adhesion molecules (118). In summary, DCs are essential immune cells for host resistance against infectious agents and DC-derived IL-12 is the key cytokine involved in initiating a Th1 immune response.

In addition to TLRs, signals through chemokine/chemokine receptors also mediate DC activation and migration both in homeostasis and in pathological conditions (133). Chemokines represent a large superfamily of 8- to 15-kd proteins that possess diverse biological functions, such as the maintenance of homeostasis, angiogenesis/angiostasis, wound healing, tumor growth and metastasis, cellular differentiation and activation, lymphocyte development and trafficking, lymphoid organ development, and influencing the type 1/type 2 balance of an immune response (133, 134). Chemokines are divided into four different groups based upon a conserved cysteine arrangement: CC, CXC, C and CX_3C. Chemokine receptors belong to a large family of G protein-coupled receptors (GPCRs) that are characterized by seven-transmembrane domains and utilize heterotrimeric G proteins for downstream signal transduction. The promiscuous interactions between chemokines and chemokine receptors complicate their roles in the immune system. Immature and mature DCs express a distinct repertoire of chemokine receptors that mediate their migration either into infected peripheral sites (inflammatory), or into draining lymph nodes (homing). Among inflammatory chemokine receptors, CCR6 is unique in that it has only one chemokine ligand, CCL20/MIP-3α (135). CCL20 plays a key role for the homing of DC to mucosal surfaces, and CCR6/CCL20-dependent immature DC migration is an essential mechanism for CD8+ T cell activation and host immunity against invasive pathogens in the skin and GI tract (87, 136). $CCR6^{-/-}$ mice have a defect in both leukocyte homeostasis and in the humoral response to an oral antigen challenge (137, 138). Despite an established role of CCL20/CCR6 in adaptive immunity, little is know about their role in the innate host response.

Role of Dendritic cells in granulomatous response

Granulomas are specific immune sequestration responses of mononuclear phagocytes that function to "wall off" pathogens that cannot be phagocytosed or destroyed by surrounding immune cells in order to prevent their dissemination to healthy tissue (139). Thus, the granulomatous response is considered to be a compromised strategy of the host to deal with specific pathogens that they fail to completely destroy. Granulomatous responses have been characterized in response to different agents, such as bacteria, fungi or helminthes that induce immune hypersensitivity granulomas, and other agents that induce non-immune foreign-body granulomas. Hypersensitivity granulomas can be further classified as either type-1 or type-2, depending upon whether a predominant Th1 or Th2 cytokine immune response. Examples of Th1 or Th2-type granulomas are those induced by tuberculosis and schistosomiasis, respectively.

The granulomatous response induced by i.v. administration of live *Schistosoma mansoni* eggs has been well characterized as a cell-mediated, Th2-dominant immune response. This granulomatous response has been accepted as a valuable model to study Th2 polarization, the function of individual immune cells that are involved in the initiation and development of the granulomas (DCs, monocytes/macrophages, eosinophils, lymphocytes, *et al*), and function of important molecules responsible for cell activation and migration (139-142). Either a single i.v. injection of live *S. mansoni* eggs (primary granuloma), or sensitization of animals with *S. mansoni* egg antigen (SEA) followed by i.v. injection of *S. mansoni* live eggs (secondary granuloma) can be used to experimentally induce granuloma formation in the lung. In contrast to secondary *S. mansoni* granulomas that are dominated by a Th2 response, there is transient Th0

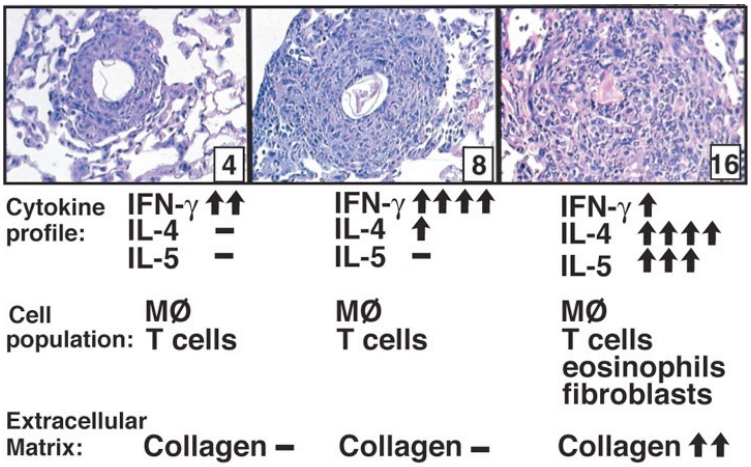

Figure 1.3 Primary lung granulomas induced by *Schistosoma mansoni* eggs. Left panel, 4 days after egg injection; middle panel, 8 days after egg injection; right panel, 16 days after egg injection.

response in the very early time point (day 2) after the induction of primary granulomas, i.e. the upregulation of both Th1 cytokine IFN-γ and Th2 cytokines IL-4, IL-5, IL-13 (143). As the granulomas develop in response to the stimuli, Th2 cells rapidly dominate over Th1 cells, evidenced by the further increase in levels of Th2 cytokines. In the later stage (day 16), eosinophil recruitment and tissue fibrosis are evident in the granulomatous lesions, events primarily mediated by IL-5 and IL-13, respectively (144, 145) (Fig 1.3)

As immune cells that link innate and adaptive immunity, DCs have been studied in the context of Th1 or Th2 granulomas. They are recognized as an essential component of the granulomas and play a role in both the generation and regulation of the granulomatous response (142). The formation and development of both Th1 and Th2 granulomas are accompanied by an increase in DC migration and activation, further indicating that DCs participate in the granulomatous response (139, 141). Moreover, DCs themselves can be activated and "educated" to possess Th1-inducing capacity in response to Th1-eliciting antigens when they are adoptively transferred into recipients (139). Since the ablation of $CD11c^+$ DCs is possible by treating CD11c-diphtheria toxin

receptor (DTR)-green fluoresent protein (GFP) transgenic mice with low dose of DT (146), it will be of interest to study the granulomatous response in the absence of $CD11c^+$ DCs. Those experiments will offer the direct evidence of the role of DCs on the granulomatous response and the effect of DCs on other immune cells that contribute to granuloma formation.

The activation of DCs by the Th2-inducing antigen SEA is an enigma. Our group and others have observed that in contrast to well-recognized TLR agonists, SEA fails to induce the upregulation of DC activation-associated molecules, such as CD40, CD80, CD86 and MHCII, and promote the production of several classical pro-inflammatory cytokines (H.W. and S.L.K., unpublished data) (147-149). More importantly, SEA fails to promote IL-4 or IL-10 production by DCs. The classical "DC maturation" model states that "immature" DCs, characterized by low expression of MHCII, costimulatory molecules (CD80 and CD86) and CD40, are efficient in antigen uptake and processing, but poor in T cell priming. In contrast, "mature" DCs are potent in antigen presentation to TCR specific T cells and result in protective immunity (150, 151). Thus, these observations are somewhat conflicting against the classical "DC maturation" model, as SEA-stimulated "immature" DCs (low expression of MHCII, costimulatory molecules and CD40) proved to be efficient activators of naïve T cells and induce a definitive SEA-specific Th2 response after transfer into naïve recipient mice (147, 152). Confounding this model is that "mature" DCs are not always immunogenic and have been reported to cause T cell depletion, anergy, or regulate and maintain peripheral tolerance in some circumstances (153). The apparent discrepancy suggests that "phenotypically mature"

DCs do not necessarily equal "functionally mature" DCs (113); other molecular and cellular mechanisms may influence DC: T cell interaction and needs further investigation.

IL-12 based vaccination has been shown to attenuate granulomatous response and tissue fibrosis induced by *S. mansoni* eggs, supporting the classical Th1/Th2 paradigm (154, 155). Experiments examining the interactions between DC and pathogens raised the possibility that IL-10 may balance IL-12 in deciding between the Th1 and Th2 fate (156). The effects of IL-10 have been associated with the promotion of a Th2 phenotype (157) and DC-derived IL-10 can limit Th1 expansion both through its autocrine inhibition of IL-12 (158) and by its ability to stimulate IL-10 release from T cells later in the immune response (159). Thus, the granulomatous response induced by *S. mansoni* eggs may be an ideal model to study IL-12/IL-10 balance, as well as DCs functions.

Epigenetic regulation of gene expression

General concept of epigenetic mechanism of gene regulation

Epigenetics is the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence. These changes include DNA methylation, histone modification, chromatin remodeling, and histone variant incorporation (160). In the last decade, chromatin biologists have achieved convincing data suggesting that the regulation of chromatin structure or epigenetic regulation confers heritable stabilization of gene expression patterns and thereby plays an essential role both in basic physiological processes and in diseases (161, 162). In addition to the interactions between transcription factors (TFs) and DNA sequences in promoters and other regulatory regions, gene expression in eukaryotic cells is also

influenced by epigenetic changes in chromatin structure that control the access of DNA-binding proteins, such as TFs and RNA polymerase II, to their conserved binding sites during the initiation period of transcription. Conversely, TFs dictate epigenetic changes by recruiting histone-modifying enzymes and chromatin-remodeling complexes.

In eukaryotic cells, DNA is packed into a high-order nuclear structure called chromatin with the assistance of chromosomal proteins such as histones. The basic unit of chromatin is the nucleosome, which consists of 146 base pairs of double-strand DNA wrapped around a histone octamer protein core (two molecules each of H2A, H2B, H3 and H4) (163, 164). Histone modifications, including acetylation, phosphorylation, methylation, and ubiquitylation, are under intensive investigation and have been considered to be an important epigenetic mechanism regulating gene expression. These modifications, catalyzed by several families of histone-modifying enzymes, form a "histone code" that constitutes an important epigenetic determinant of the transcriptional state (165, 166). Individual histone modifications can be simply categorized as "permissive" or "repressive", in reference to gene transcriptional activity (Table 1.4). For example, histone acetylation is generally associated with gene activation, partially due to neutralization of the positive charge of the ε-amino group of lysine and a consequent lowered stability of interactions between histones and the negatively charged DNA (167). Acetyl histones can also provide a specific binding site for bromodomian-containing proteins, such as ATP-dependent chromatin remodeling complexes, which help to unravel chromatin structure and recruit essential TFs (168, 169). Two classes of acetylation-modifying enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), mediate an increase and decrease in lysine acetylation,

Table 1.4 Histone modifications

Chromatin modifications	Relevant histone sites	Transcriptional role
Acetylation (lysine)	H3 (9,14,18,56), H4 (5,8,13,16) H2A, H2B	Activation
Methylation (lysine)	H3 (4,36,79) H3 (9,27), H4 (20)	Activation Repression
Methylation (Arginine)	H3 (17,23), H4 (3)	Activation
Phosphorylation (Serine/Theorine)	H3 (3,10,28), H2A, H2B	Activation
Ubiquitylation (lysine)	H2A (119) H2B (123/120)	Repression Activation
Sumoylation (lysine)	H2A (126), H2B (6/7)	Repression
Isomerization (Proline)	H3 (30-38)	Activation/Repression

respectively, and the balance between them determines lysine acetylation status. It is worth noting that HATs and HDACs can also regulate gene transcription by modifying other non-histone proteins such as BCL6, Runx and p53, and thereby altering their stability and/or transcriptional potential (170-174). Although histone acetylation is an essential step during initial gene transcription, it has been suggested that histone acetylation is dynamically regulated by HATs and HDACs and is not stable; therefore, histone acetylation cannot be inherited to progeny cells (170, 175). Histone acetylation is not likely an initiator of epigenetic changes influencing gene activation, but probably it is a responder to other more stable epigenetic changes such as histone methylation.

Histone methylation is now recognized as an important modification linked to inherited changes in both transcriptional activation and repression (165). The landmark discovery of the molecular identity of the first histone methyltransferase (HMT),

SUV39H1, by Jenuwein *et al*. demonstrates that histone methylation is able to regulate RNA synthesis (176). Histones contain numerous lysine and arginine amino acids and can be modified on both of the amino acids with up to three methyl groups. Methylation of lysine residues at six sites (H3K4, -9, -27, -36, -79 and H4K20) has been linked to transcriptional regulation (165, 177), which in conjunction with various methylation levels (mono-, di- or tri-methylation) provides remarkable regulatory potentials for chromatin modification and gene regulation.

Lysine methylation is mediated by so-called SET domains, which originally was characterized as a common motif in three main classes of HMTs in *Drosophila*: SU(VAR)3-9 (178), the *Polycomb*-group protein E(Z) (179) and trithorax protein (180). They are specifically responsible for the methylation of H3K9, H3K27 and H3K4, respectively. H3K4 methylation (H3K4me) generally correlates with gene activation and is therefore considered a permissive modification (Fig 1.4). H3K4me3 has been found to be associated with the promoter and 5'-coding regions of active genes in yeast and higher eukaryotes (165, 181). H3K4me2 exhibits a much broader genomic distribution, including transcriptionally active genes, inactive genes that are kept in a transcriptionally permissive state, and regulatory elements such as distal enhancers and insulators (182). Thus, it seems that H3K4me3 functions specifically in the initiation of gene transcription, presumably by maintaining permissive chromatin structure and assisting the recruitment of TFs and RNA polymerase machinery. The first H3K4 methyltransferase to be identified was the *S. Cerevisias* Set1 complex (183). Mixed-Lineage Leukemia (MLL), a mammalian homologue to the *Drosophila* trithorax protein, is one of the best-characterized members of the H3K4 HMT family (184), and was isolated as a common

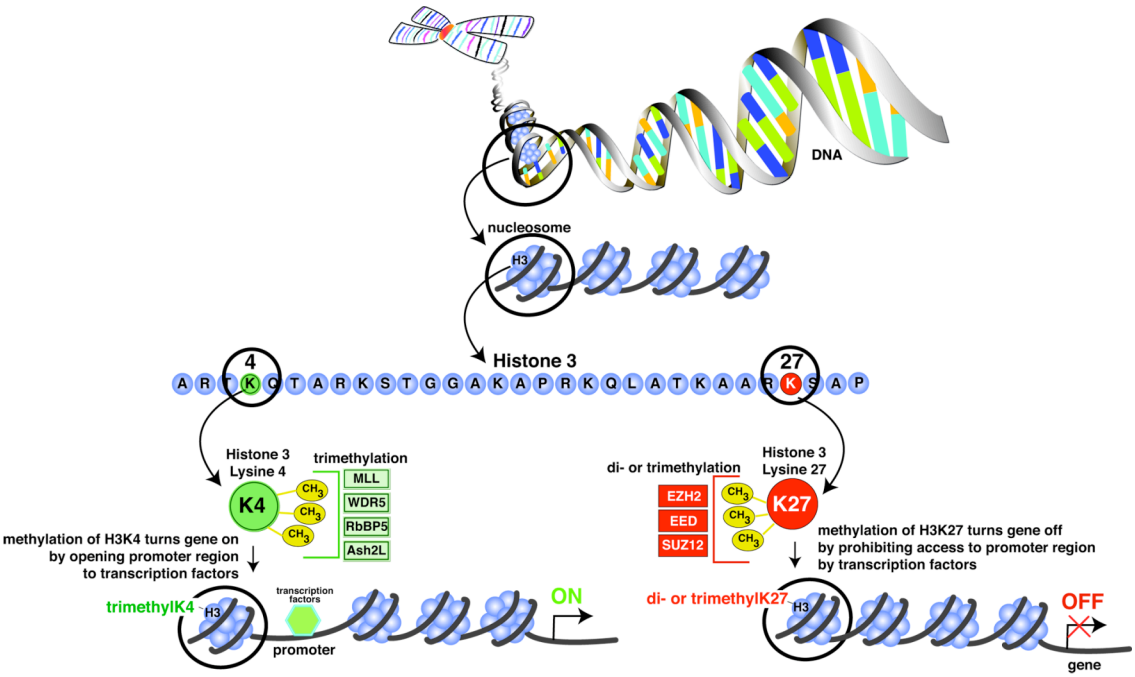

Figure 1.4 Diagram of the biological effects of methylation at histone K3 lysine-4 (H3K4) and H3K27. H3K27 methylation maintains the chromatin in a conformation that does not allow transcription factors access to a promoter and the gene is silenced. When H3K4 is methylated the conformation of the chromatin is relaxed and the transcription factors now have access to the promoter, resulting in gene expression. MLL complex containing core components MLL, WDR5, RbBP5 and Ash2L possesses methyl transferase enzyme activity specific for H3k4, while PRC2 complex containing EED, EZH2 and SUZ12 specifically mediates H3K27 methylation.

target of chromosomal translocations observed in human acute leukemia (185). MLL forms a HMT complex, in conjunction with other core component proteins, including the WD40-repeat protein WDR5, RbBP5 and Ash2L (186, 187) (Fig 1.4). A deficiency of each of these subunits impairs MLL methyltransferase enzyme activity (186, 187). H3K27 trimethylation and H3K9 di- and trimethylation contribute to the maintenance of repressive chromatin structure and correlate with gene silencing (177, 188, 189). Methyl H3K9 serves as a binding site for the chromodomain-containing protein heterochromatin protein HP1, which is essential for the establishment and maintenance of silent chromatin

(heterochromatin) (190, 191). Methyl H3K27 offers a binding site for a chromodomain-containing protein Polycomb, a core component of Polycomb repressive complex 1 (PRC1), which together with PRC2 has a pivotal role in silencing the developmentally important *Hox* gene and X-chromosome inactivation (177). PRC2 (also known as EED-EZH2 complex) possesses several core components-Enhancer of Zeste 2 (EZH2), Suppressor of Zeste 12 (SUZ12), and embryonic ectoderm development (EED). EZH2 has a SET domain and possesses an intrinsic HMT enzyme activity, and methylation of H3K27 by EZH2 subsequently recruits PRC1, which exhibits a ubiquitin E3 ligase activity and influences gene transcription via the ubiquitylation of histone H2A (177). Interestingly, it has been observed that both H3K4me3 and H3K27me3 are present within regions enriched for genes encoding developmentally important TFs, which are proposed as "bivalent " chromatin structure (192). This observation suggests that bivalent domains, established by incorporation between permissive (H3K4me3) and repressive (H3K27me3) modifications, silence developmental genes in embryonic stem (ES) cells while keeping them poised for activation (192). Thus, it has been argued that the balance between multiple chromatin modifications decides chromatin structure and gene transcription fate.

An expanding body of evidence has suggested that regulation by histone modification is dynamic, and that the presence of certain modifications may not necessarily correlate with the predicted regulatory status, i.e. turning genes "on" or "off". It is the entire chromatin context containing multiple enzyme complexes with either cooperative or opposing effects that determine the overall outcome (160). In support of this theory, there are some exceptions for the general concept of permissive and repressive histone modifications. For example, methylation of H3K9, characteristic of

the silenced heterochromatin, was recently also shown to be associated with transcriptional elongation within active genes (193). In contrast, permissive modification of H3K4me2 has been associated with silenced chromatin of centromeres in combination with H3K9 and H4K20 methylation. Moreover, methyl H3K4 not only offers a binding site for permissive chromatin modifying-complexes, such as HAT-containing enzyme complex (194) and ATP-dependent nucleosome remodeling factor (NURF) (195), but also can be recognized and bound by repressive chromatin modifying factors, such as HDAC1-containing complex (196). One explanation of these seemingly contradictory findings is that these modifying complexes with opposing effects may function at different stages of gene transcription, such as initiation or elongation of transcription, thereby regulating gene expression from both positive and negative sides (160). In summary, individual modification interacts with neighboring positive-acting or negative-acting modifications. The overall outcome of these modifications results in the dynamic regulation of chromatin structure and subsequently determines the fate of gene transcription.

Epigenetic regulation of gene expression in immune system

Considering the property of inheritance and stability, the epigenetic mechanisms may be involved in the long-term maintenance of gene expression pattern in a particular cell. It has been convincingly documented that the maintenance of "memory" for genes expressed by Th1 and Th2 cells depends on chromatin modifications including histone acetylation, methylation and DNA methylation. The Th2 locus containing *Il5, Rad50, Il13 and Il4* gene has been well characterized to exhibit distinct combinations of individual chromatin modifications, which correlate with different patterns of gene

expression (182). Three stages are recognized during the development of a Th2 cell phenotype, including the initiation of Th2 differentiation, reinforcement of Th2 phenotype, and maintenance of Th2 fate. Multiple key TFs and signaling pathways are known to be essential for the first two stages, such as IL-4/IL-4R/STAT6 pathway, nuclear factor of activated T cells (NFAT), GATA3, *et al* (197, 198). Deficiency of these molecules inhibits normal Th2 development both *in vitro* and *in vivo* (182, 197, 198). However, GATA3, a Th2-specific TF, is not absolutely required for IL-4 production during the maintenance stage of Th2 cells, since conditional depletion of GATA3 in differentiated Th2 cells only results in a minor decrease in IL-4 production without affecting the total number of cells capable of producing IL-4 (199). This observation strongly suggests that differentiated Th2 cells have acquired "cellular memory" of Th2 cytokine production without the further requirement of polarizing signals, which are essential for the initial establishment of this memory. Indeed, subsequent studies of chromatin modifications in Th2 locus during Th1/Th2 development have revealed that Th2 memory was conferred by permissive histone modifications, while repressive modifications were associated with silencing of Th2 locus in differentiated Th1 cells (182). Compared with naïve T cells, Th2 cells exhibit a striking increase in histone lysine acetylation (H3K4/9Ac) and H3K4me2 through Th2 locus, which are general indicators of gene activation (see above General Concept). In contrast, Th1 cells show enhanced H3K27me3 in the Th2 locus, an indicator of repressive chromatin structure and associated with gene silencing, in which EZH2 plays an essential role (182, 200). Moreover, experiments exploring epigenetic changes in *Ifng* locus during Th1/Th2 differentiation have shown that Th1 cells acquire permissive histone-acetylation marks

that are dependent on both STAT4 and T-bet (201), whereas Th2 cells exhibit enhanced H3K27me2 dependent on STAT6 and GATA3 (202). Recent investigations of the role of MLL on immune system are consistent with these findings and reveal that memory Th2 cells with heterozygous disruption of *Mll* gene ($Mll^{+/-}$) show a deficient Th2 cytokine (IL-4, IL-5 and IL-13) production. This was associated with decreased GATA3 expression, presumably through the effect of impaired *Gata3* locus histone modifications (203). Together, these data show that the long-term maintenance of Th1/Th2, or "Th1/Th2 memory" correlates with distinct histone modifications.

In addition to the Th1/Th2 paradigm, the newly discovered effector T cell lineage, Th17, has also been linked to epigenetic changes in *Il17* locus (204). Compared with either Th1 or Th2 cells, Th17 cells exhibit an enhanced histone acetylation and H3K4me3 at the *Il17* promoter region, which is consistent with the general regulatory concept discussed above. A recent study from the Medzhitov group has expanded our understanding of epigenetic regulation and maintenance of gene expression in immune system to macrophages (205). Restimulation of macrophages with LPS resulted in an attenuated inflammatory response (LPS tolerance), but maintained an intact antimicrobial response, which are represented by two kinds of genes, tolerizeable and non-tolerizeable, respectively. Tolerizeable genes lose the permissive histone modifications, H4Ac and H3K4me3, at their promoter regions during a secondary challenge with LPS, and treatment with chemicals enhancing these modifications rescues the impaired gene production. In contrast, non-tolerizeable genes are still able to mount the permissive histone modifications, H4Ac and H3K4me3, in response to a secondary LPS challenge, which correlate with intact capacity to transcribe specific genes. Thus, this short-term

cellular memory of tolerized macrophages is at least in part due to the regulation of the gene expression pattern by epigenetic changes at gene-specific loci (206). This study highlights the fact that analogues to adaptive immune cells, the cells of innate immune system are also capable of mounting epigenetic-influenced cell memory in response to specific external stimuli. Regarding the essential role of the innate immune response in pathological conditions, including infections, cancer and autoimmune diseases, the epigenetic mechanism-mediated innate immune cell memory has significant clinical relevance. Epigenetic memory in innate immune cells may potentially explain long-term maintenance of some disease conditions, presumably through the effects of aberrant cell phenotype and the maintenance of inappropriately expressed genes, leading to immunoparalysis induced by severe sepsis, or macrophage polarization (M1/M2) in tumorgenesis (131). Further investigations are required to characterize the molecular mechanism(s) responsible for epigenetic changes and the establishment of cell memory.

In summary, to explore the long-term alterations in DC function after severe sepsis, my graduate research work has addressed the following issues:

1. Role of CCR6 in severe sepsis.
2. Long-term effects of severe sepsis on DC functions, including cytokine production, Ag-presenting function, and Th1/Th2 polarizing capacity.
3. Contribution of epigenetic changes to the regulation of cytokine genes in post-septic DC in the context of long-term immunoparalysis after severe sepsis.
4. Immunological consequences of severe sepsis on a subsequent immune response induced by *S. mansoni* eggs.

Chapter 2

The chemokine receptor CCR6 is an important component of the innate immune response

Summary

In our initial studies we found that naïve CCR6-deficient ($CCR6^{-/-}$) C57BL/6 mice possessed significantly lower numbers of both $F4/80^{+}$ macrophages and DCs, but higher numbers of B cells in the peritoneal cavity, as compared to naïve WT controls. Furthermore, peritoneal macrophages isolated from $CCR6^{-/-}$ mice expressed significantly lower levels of inflammatory cytokines and NO following LPS stimulation, as compared to WT macrophages. In a severe experimental peritonitis model induced by cecal ligation and puncture (CLP), $CCR6^{-/-}$ mice were protected when compared with WT controls. At 24 hours following the induction of peritonitis, $CCR6^{-/-}$ mice exhibited significantly lower levels of inflammatory cytokines/chemokines in both the peritoneal cavity and blood. Interestingly, DC recruitment into the peritoneal cavity was impaired in $CCR6^{-/-}$ mice during the evolution of CLP-induced peritonitis. Peritoneal macrophages isolated from surviving $CCR6^{-/-}$ mice 3 days after CLP-induced peritonitis exhibited an enhanced LPS-response compared with similarly treated WT peritoneal macrophages. These data illustrate that CCR6 deficiency alters the innate response by attenuating the hyperactive local and systemic inflammatory response during CLP-induced peritonitis.

Introduction

Despite advances in intensive care unit interventions and the use of specific antibiotics, sepsis with concomitant multiple organ failure is the most common cause of death in many acute care units. In this setting, approximately one-third of 750,000 people annually affected by sepsis die (4, 5, 207, 208). Sepsis is characterized by an initial overwhelming systemic inflammatory response syndrome (SIRS) (also known as a cytokine storm), which is followed by a compensatory anti-inflammatory response syndrome (CARS) (9). Innate immune cells, including macrophage, PMNs and DCs are the main inflammatory cell types responsible for excessive cytokine production during the evolution of this response. These cells both produce and respond to proinflammatory cytokines and chemokines, suggesting that they might be appropriate targets for the treatment of severe sepsis. A number of novel therapeutics, specifically developed to prevent sepsis-induced mortality have failed, with the exception of recombinant activated protein C (28), supporting the concept that severe sepsis is dynamic and complex disease.

Chemokines are a family of structurally related chemotactic proteins, whose functions have been well studied on many fundamental aspects of immunology, including the development, homeostasis and host inflammatory response. The effects of specific chemokines/chemokine receptors in the initiation and the progression of severe experimental sepsis have been previously reported, due to divergent and varied mechanisms (209-215). However, the role of CCR6 in severe sepsis has not been previously investigated.

Interestingly, unlike most of the chemokine family members, CCR6 has only one chemokine ligand, CCL20, although β-defensin has been reported to bind CCR6 with a

lower affinity (216). CCR6 has been shown to be expressed on immature DCs (135, 216, 217), memory T cells (218) and B cells (219), suggesting that CCR6 plays an important role in adaptive immunity. This notion is further exemplified by CCR6/CCL20-dependent immature DC migration, which is an essential mechanism for CD8+ T cell activation and host immunity against invasive pathogens in the skin and GI tract, respectively (87, 136). CCR6/CCL20 has also been reported to be involved in lymphocyte-mediated chronic inflammation, such as asthma (217) and chronic obstructive pulmonary disease (COPD) (220). Little is known about the role of CCR6 in innate immunity, except that CCL20 has been shown to direct the recruitment of NK cells (221).

The aim of the present study is to evaluate the contribution of CCR6 to the systemic inflammatory response and *in vivo* outcome in an experimental sepsis model induced by cecal ligation and puncture (CLP). We demonstrate that deficiency of CCR6 ($CCR6^{-/-}$) alters peritoneal cellularity in naïve mice and renders mice resistance to CLP-induced mortality. In the latter situation, the absence of CCR6 was associated with dramatically lower peritoneal and systemic cytokine levels. The number of DCs was significantly decreased in naïve $CCR6^{-/-}$ mice and the recruitment of DCs was impaired during severe sepsis, which supports the concept that CCR6 is involved in DC recruitment to inflammatory sites. Furthermore, $CCR6^{-/-}$ peritoneal macrophages show an attenuated response to TLR agonist stimulation compared with similarly treated WT control. The latter information may provide the mechanism for the lower levels of inflammatory cytokines and the survival advantage in $CCR6^{-/-}$ mice. Thus, this study demonstrates that CCR6 deficiency modulates the initial local immune response and

alters subsequent inflammatory cascades normally associated with systemic immune reactivity.

Materials and Methods

Mice

Specific pathogen-free female C57BL/6 WT mice were purchased from Taconic Farms, Inc. (Germantown, NY). Female CCR6-deficient ($CCR6^{-/-}$) mice were originally generated in Dr. Sergio A. Lira's laboratory and were subsequently backcrossed eight generations onto a C57BL/6 background (137, 217). Mice were housed in the animal care facility at the University of Michigan. The University Committee on Use and Care of Animal (UCUCA) at the University of Michigan approved all experimental procedures.

Experimental sepsis induced by CLP

CLP surgery was performed on mice as previously described (222). In brief, mice were anesthetized with an i.p. injection of 2.25 mg of ketamine HCL (Abbott Laboratories, Chicago, IL) and 150 μg of xylazine (Lloyd Laboratories, Shenandoah, IA). Under sterile surgical conditions, a 1-cm midline incision was made to the ventral surface of the abdomen, and the cecum was exposed. The cecum was partially ligated at its base with a 3.0 silk suture and punctured nine times with 21-gauge needle. The cecum was returned to the peritoneal cavity, and the abdominal incision was closed using surgical staples. Mice were rehydrated with 1 ml saline s.c. and placed on a heating pad until they recovered from anesthetic. At 4, 24 and 72 h after surgery, CLP mice were anesthetized and bled. Peritoneal lavage was performed with 2 ml of cold sterile saline. Serum and cell-free peritoneal fluid were collected for chemokine/cytokine protein analyses. RBCs

were lyzed in ammonium chloride buffer (150 mM NH_4CL, 10 mM NaHCO3, 1mM EDTA- tetrasodium), and the total cell numbers were determined using a hemocytometer. Cytospins (Shandon Inc., Waltham, MA) were prepared and stained with Diff-Quik solutions (Dade Behring, Dudingen, Switzerland). The leukocyte composition was analyzed, and the percentage of PMNs and mononuclear cells was multiplied by the total cell count to determine their absolute number.

Survival studies following CLP

The first set of survival studies was performed to determine the effect of the presence of CCR6 on survival in CLP-induced sepsis animal model. WT and $CCR6^{-/-}$ mice were subjected to CLP surgery in the presence or absence of the treatment with antibiotic INVANZ (Ertapenem) i.p. injected at 75mg/kg (Merck & Co. Inc., Whitehouse station, NJ) 6 h after surgery and every 24 h until day 3 post-CLP. Survival was monitored for 6 days following surgery. The second set of survival studies focused on the role of endogenous CCL20 following induction of acute septic peritonitis. WT mice were i.p. injected with 20 μg/per mouse of anti-mouse CCL20 monoclonal antibody (R&D systems, Rochester, MN) 2 h before CLP and every day following surgery to block endogenous CCL20. Rat IgG_1 was used as a control. Survival was monitored for 6 days following surgery.

Flow cytometry analysis

Total peritoneal cells were harvested by peritoneal lavage with 10 ml cold sterile saline for 2 times from naïve WT or $CCR6^{-/-}$ mice or at indicated time points following CLP. After lysing RBCs with ammonium chloride buffer, total cell numbers were determined using a hemocytometer. Fc binding was blocked via a 10 min incubation with purified

rat anti-mouse CD16/CD32 (FcγIII/II receptor). Then the cells were stained with the following monoclonal antibodies to identify series of mouse cell types: PE-conjugated anti-CD11b in combination with FITC-conjugated anti-F4/80 (macrophage), FITC-anti-CD19 (B cell), PE-anti-I-A^b in combination with FITC-anti-CD11c (DC), PE-anti-NK1.1 (NK cell), PE-anti-CD4 in combination with FITC-anti-CD8 ($CD4^+$ and $CD8^+$ T cell). The appropriate IgG isotypes were used as controls. All antibodies and IgG isotypes were purchased from BD PharMingen (San Diego, CA), except for anti-F4/80 (Serotec, Raleigh, NC). The cells were fixed in 1% paraformaldehyde and kept in the dark at 4°C until analysis with a FACSCaliber (CELLQuest™ software; Becton and Dickinson, Mountain View, CA).

Peritoneal macrophage collection and in vitro stimulation

Total peritoneal cells were harvested by peritoneal lavage with 10 ml cold sterile saline performed twice for WT or $CCR6^{-/-}$ mice in naive status or at day 3 post-CLP. Lavage was pooled for mice in the same group. RBCs were lyzed in ammonium chloride buffer. The remaining cells were thoroughly washed with saline and counted in a hemocytometer. Cytospins were prepared and stained with Diff-Quik solutions, and the number of peritoneal macrophages was determined. Cells were resuspended at a concentration of 10^6 macrophages/ml in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 5% FCS, 2mM L-glutamine, 100 U/ml penicillin, and 100 μg streptomycin. Macrophages were plated in plastic 48-well cell culture plate ($4X10^5$/well) (Corning Inc., Acton, MA) and incubated 2 h at 37°C in 5% CO_2. Nonadherent cells were removed, and adherent cells were washed with complete RPMI 1640 medium, followed by the stimulation with 1 μg/ml LPS (Sigma-Aldrich, St. Louis, MO). Four hours later, total

RNA was isolated from cultured peritoneal macrophages using Trizol reagent and quantitative Real-time PCR (TaqMan) was performed to measure cytokine/chemokine gene expression. Twenty-four hours later, cell-free supernatant from each sample was collected and stored in -80°C until cytokine/chemokine protein assay by ELISA. For the LPS rechallenge experiments, isolated peritoneal macrophages were pretreated with 10 ng/ml LPS for 24 hours, washed twice and then exposed to a second challenge with 1 μg/ml LPS. Twenty-four hours later, the supernatants were collected to measure cytokine protein levels by ELISA.

RNA isolation and real-time PCR (TaqMan)

Total RNA was isolated from cultured peritoneal macrophages using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. A total of 2.0 μg of RNA was reverse transcribed to yield cDNA in a 25μl reaction mixture containing 1X first strand (Life Technologies, Gaitherburg, MD), 250 ng of oligo $(dT)_{12-18}$ primer, 1.6 mM dNTPs (Invitrogen), 5 U of RNase inhibitor (Invitrogen), and 100 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen). Complementary DNA was then analyzed by quantitative, real-time PCR using a TaqMan 7500 sequence detection system (Applied Biosystems). GAPDH was analyzed as an internal control. The fold differences in mRNA expression between treatment groups were determined by the Sequence Detection Systems software (Applied Biosystems).

ELISA

Concentrations of IL-10, IL-12p70, TNF-α, CCL2, CCL3, CXCL10 were measured in cell-free peritoneal lavage fluid, serum, and cell culture supernatants using a standardized sandwich ELISA technique as previously described in detail (217, 223). Briefly, C96

Maxisorp Nunc-Immuno plates (Fisher Scientific) were coated with 1-5 μg/ml capture Abs in coating buffer overnight at 4°C and washed with PBS containing 0.05% Tween 20. All Abs used for ELISA were purchased from R&D Systems. Nonspecific binding sites were blocked with 2% BSA in PBS for 90 min at 37°C. Plates were rinsed four times with wash buffer and cell-free supernatants were loaded and incubated for 1 h at 37°C. After four washings, a biotinylated detection polyclonal Ab was added for 45 min at 37°C. The plates were washed again and peroxidase-conjugated streptavidin (Bio-Rad) was added to the well for 30min at 37°C. Plates were washed, and after the addition of chromogen substrate (Bio-Rad), OD readings were measured at 490 nm using an ELISA plate reader. Recombinant murine cytokines were used to generate the standard curves from which the concentrations present in the samples were calculated. The limit of detection of the assays was 50 pg/ml.

Nitrite production

Nitrite (NO_2^-) levels were determined using the Griess method as previously described (224). The measurement of this parameter is widely accepted as indicative of NO production. Briefly, naive peritoneal macrophages were plated at 4 x 10^5/well of a 96-well cell culture plate (Corning) and rested overnight. Cells were treated with fresh complete RPMI 1640 medium alone, 20 U/ml IFN-γ (PeproTech), 100 ng/ml LPS, or both IFN-γ and LPS. After 48 h at 37°C in 5% CO2, 50 μl of cell-free supernatants was transferred to a flat-bottom 96-well plate and treated with 100 μl of 0.5% sulfanilamide (Sigma) and 0.05% naphthylethylenediamine dihydrochloride (Sigma) in 2.5% phosphoric acid (H_3PO4). The absorbance was read at 550 nm in a microplate reader. A

standard curve was generated using known concentrations of $NaNO_2$ (Sigma). Similar results were shown in three independent experiments.

Statistics

Statistical analysis was carried out with Prism 4 for Macintosh. In survival studies a log-rank test was used to test for significance. For all other studies results are presented as the mean±SEM, and unpaired Student's *t* test was applied to evaluate significance. Values of $p<0.05$ were considered statistically significant.

Results

Depletion of CCR6 alters inflammatory cell populations in naïve mice

Given that CCR6 has been reported to regulate DC localization in Peyer's patches (137, 138), we tested whether the depletion of CCR6 alter the localization of resident cell populations in the peritoneal cavity of naïve mice. Flow cytometric analysis was used to compare the numbers of several resident peritoneal cell types between WT C57BL/6 ($CCR6^{+/+}$) and C57BL/6 $CCR6^{-/-}$ mice. Whereas there was no difference in the number of total peritoneal cells between two groups, we found $CCR6^{-/-}$ mice had significant fewer $CD11b^{+}F4/80^{+}$ macrophages ($50.4\pm7.7\times10^{4}$ vs $72.4\pm8.4\times10^{4}$/per mouse, n=5) and $CD11c^{+}MHCII^{+}$ DCs ($0.8\pm0.1\times10^{4}$ vs $1.5\pm0.2\times10^{4}$/per mouse, n=5) compared with WT controls (Table 2.1). In contrast, there were significantly more $CD19^{+}$ B cells in the peritoneal cavities of $CCR6^{-/-}$ mice compared with controls ($1.5\pm0.25\times10^{6}$ vs $1.2\pm0.3\times10^{6}$/mouse, n=5) (Table 2.1). No difference was found in the number of peritoneal $CD4^{+}$ and $CD8^{+}$ T cells, as well as NK cells between the $CCR6^{-/-}$ and WT groups. Therefore, CCR6 depletion alters the normal composition of peritoneal cells and results in a

Table 2.1 Peritoneal leukocyte subsets in naive mice [a]

Group	$CCR6^{+/+}$	$CCR6^{-/-}$
Total cells	293.5±46	305.5±48.8
CD11b+ F4/80+ [b]	72.4±8.4	50.4±7.7
CD19+ [b]	115.5±27.6	151.6±25.4
CD4+	15.3±0.4	18.7±0.4
CD8+	4.8±0.3	5.9±0.3
NK1.1+	3.1±0.4	2.7±0.4
CD11c+ MHCII+ [b]	1.5±0.2	0.8±0.1

[a] All values are ($X10^4$)/per mouse
[b] Significant difference between the two groups

decrease in the cell types of myeloid origin, but an increase in B cell number.

Naïve $CCR6^{-/-}$ peritoneal macrophages display an attenuated LPS-response

Macrophages are major participants in innate immunity, because of their ability to phagocytose pathogens, generate cytotoxic oxygen and nitrogen intermediates, and express chemokines and cytokines that either attract or activate other immune cells. However, the overproduction of inflammatory factors during host defense leads to serious deleterious effects in normal tissues if left uncontrolled. We next sought to determine whether the presence of CCR6 affected the production of peritoneal macrophage-derived inflammatory mediators. Peritoneal macrophages from WT and $CCR6^{-/-}$ mice were harvested by peritoneal lavage and stimulated with 1μg/ml LPS. After 4 h, $CCR6^{-/-}$ peritoneal macrophages contained significantly lower mRNA levels of IL-12p35, IL-12p40, IL-10, TNF-α, CCL2, CCL3 and CXCL10 (Fig 2.1A). Furthermore, after 24 h of

stimulation with LPS, CCR6$^{-/-}$ peritoneal macrophages produced significantly lower protein levels of IL-12p70, IL-10, TNF-α, CCL2, CCL3 and CXCL10 (Fig 2.1B), which is consistent with their mRNA levels after 4 h of LPS stimulation.

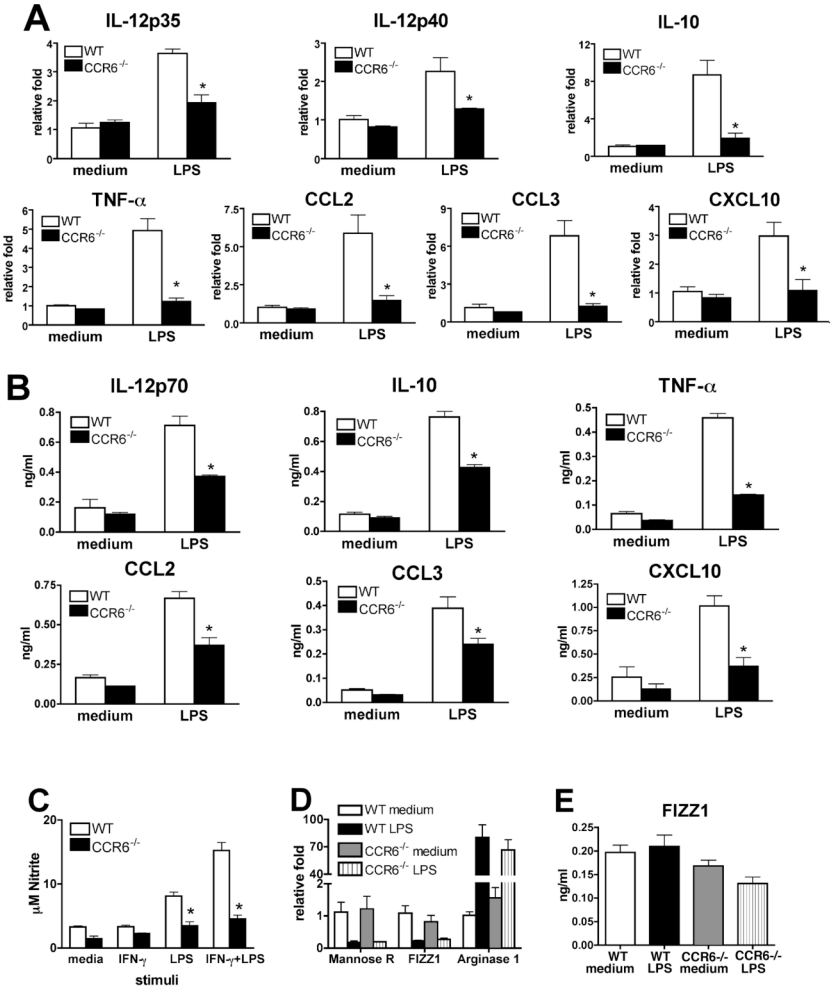

Figure 2.1 Cytokine production by peritoneal macrophages from naive WT and CCR6$^{-/-}$ mice. Peritoneal macrophages were collected by peritoneal lavage and stimulated with 1 μg/ml LPS. Messenger RNA levels (A) and protein production (B) of inflammatory cytokines were measured by Taqman and ELISA, respectively. (C) Nitrite production was measured following the activation of peritoneal macrophages with 20 U/ml IFN-γ, 100 ng/ml LPS, or both IFN-γ and LPS. (D) Messenger RNA levels of mannose receptor, FIZZ1 and arginase 1 were measured by Taqman. (E) Protein level of FIZZ1 was measured by ELISA. The results shown are representative of 5 experiments and are expressed as mean ± SEM. * P≤0.05 compared with cytokine levels measured in WT peritoneal macrophages, n=5.

Naïve CCR6$^{-/-}$ peritoneal macrophages were also assessed for the levels of nitrite, which reflect the expression of NO, one of the major macrophage-derived effector molecules that is cytotoxic (225) and can induce host tissue injury (226). Peritoneal macrophages isolated from CCR6$^{-/-}$ mice generated significantly lower level of nitrite in response to either a 100 ng/ml LPS challenge (3.7 ± 1.0 vs 8.3 ± 1.1 μM, n=5), or challenged with LPS plus 20 U/ml IFN-γ (4.7 ± 0.98 vs 15.3 ± 2.2 μM, n=5), respectively, as compared to WT peritoneal macrophages (Fig 2.1C). Collectively, our data indicated that the absence of CCR6 resulted in an attenuated LPS-response by naïve peritoneal macrophages.

Previously, our group has shown that CCR4$^{-/-}$ peritoneal macrophages exhibited a constitutive phenotype of alternative activation (227). Since we observed that CCR6$^{-/-}$ peritoneal macrophages showed an attenuated LPS-response, we further tested if CCR6$^{-/-}$ macrophages were also skewed to alternative activation status. Alternative activated macrophages has been tightly linked to the upregulation of several molecules, including mannose receptor (MR), arginase 1 and the found in inflammatory zone 1 (FIZZ1) (227). There was no difference in mRNA levels of MR, arginase 1 and FIZZ1 between CCR6$^{-/-}$ and WT peritoneal macrophages in response to either medium alone or LPS (Fig 2.1D). Both WT and CCR6$^{-/-}$ peritoneal macrophages generated similar amount of FIZZ1 protein in *in vitro* culture system (Fig 2.1E). These data suggest that CCR6$^{-/-}$ peritoneal macrophages are not skewed to alternative activation.

CCR6$^{-/-}$ mice are resistant to CLP-induced peritonitis

The difference in LPS-response between WT and CCR6$^{-/-}$ peritoneal macrophages prompted us to test the role of CCR6 in an experimental model of severe sepsis induced

by CLP. In initial studies, 100% mortality was observed in WT mice without antibiotic treatment as early as 3 days post-CLP (Fig 2.2A). However, CCR6$^{-/-}$ mice were significantly protected from CLP-induced lethality (p=0.011), and showed a 25% mortality rate at day 2 and 38% mortality rate at day 6; these latter mice were long-term survivors. Clearly, the absence of CCR6 provided a survival benefit compared with CCR6 competent mice. Antibiotic treatment for 3 days following CLP attenuated the severity of CLP and increased survival rates both in the WT and CCR6$^{-/-}$ groups (Fig 2.2B). However, CCR6$^{-/-}$ mice were more resistant to CLP-induced mortality compared to WT controls (p=0.001). Specifically, none of CCR6$^{-/-}$ mice died at day 3 after CLP. At day 6 after CLP, 88% of the CCR6$^{-/-}$ mice were alive compared with 25% of the WT mice.

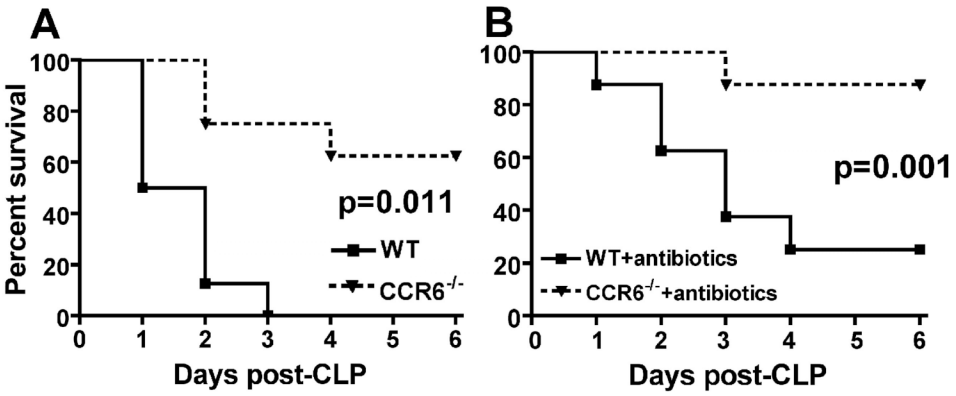

Figure 2.2 Survival study during CLP-induced severe sepsis. WT and CCR6$^{-/-}$ mice were subjected to CLP surgery, either in the absence (A) or presence (B) of antibiotics treatment. Survival was monitored for 6 days following surgery. Each group: n=10.

CCR6$^{-/-}$ mice express an attenuated cytokine profile after CLP-induced peritonitis

The polymicrobial peritonitis induced by CLP is associated with an amplified inflammatory response, in which the local and systemic expression of many cytokines

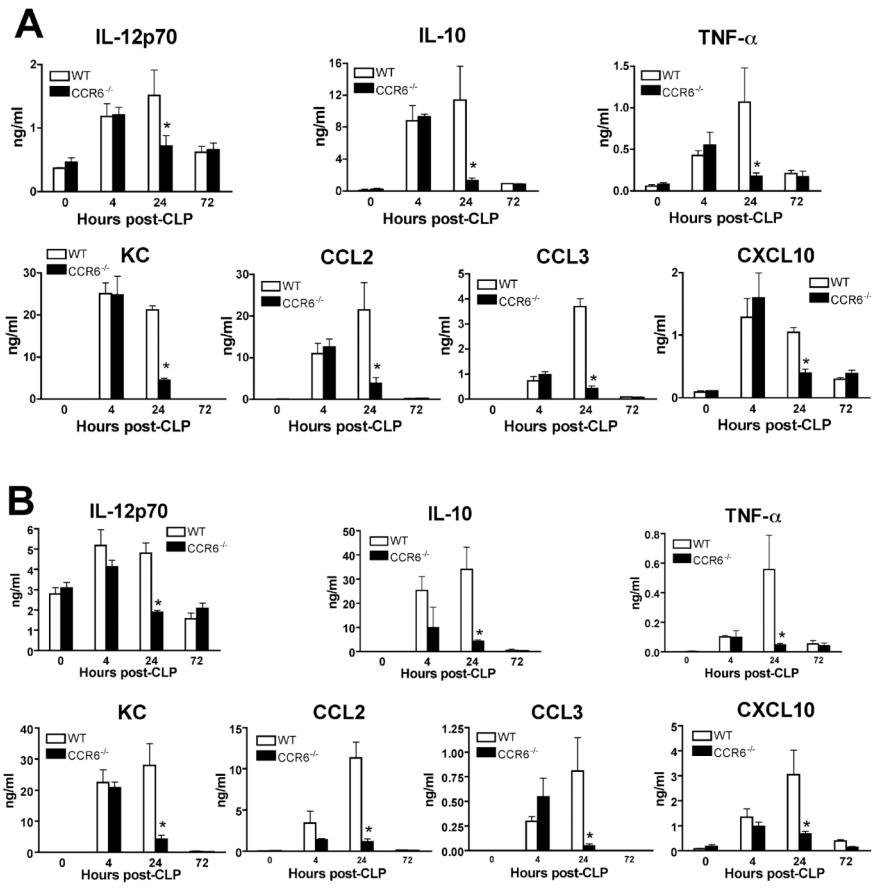

Figure 2.3 Cytokine profile in WT and CCR6$^{-/-}$ mice following CLP operation. To compare the production of inflammatory cytokines in peritoneal cavities (A) and blood (B) between WT and CCR6$^{-/-}$ mice after CLP, ELISA was performed to measured protein levels of IL-12p70, IL-10, TNF-α, KC, CCL2, CCL3 and CxCl10 in peritoneal lavage fluid and serum. * P≤0.05 compared with cytokine protein levels measured in WT mice 24 h after CLP, n=5. The results shown are representative of 3 individual experiments.

and chemokines are augmented (228). We compared the cytokine profiles in peritoneal lavage fluid and blood between WT and CCR6$^{-/-}$ mice before and after CLP-induced peritonitis. Compared to WT mice, CCR6$^{-/-}$ mice produced a similar amount of inflammatory cytokines, including IL-12p70, IL-10, TNF-α, KC, CCL2, CCL3 and CXCL10 in peritoneal cavity and blood at 4 h after surgery. However, CCR6$^{-/-}$ mice showed significantly lower levels of inflammatory cytokines both in the peritoneal cavity

(Fig 2.3A) and in blood (Fig 2.3B) at 24 h after CLP-induced peritonitis. We also observed that CCR6$^{-/-}$ mice had significantly lower levels of inflammatory cytokines in both the lung and liver, as compared to WT controls (data not shown).

Leukocyte recruitment into the peritoneal cavity post CLP-induced peritonitis

Based on the observation that CCR6$^{-/-}$ displayed a decreased cytokine production in mice, we designed a set of investigations to determine if the alterations were due to changes in leukocyte recruitment into the peritoneal cavity following CLP-induced peritonitis. Two methods were used to assess leukocyte peritoneal infiltration in WT and CCR6$^{-/-}$ mice at various times after CLP (Fig 2.4). Cytospins were prepared and the leukocyte composition, including PMNs and mononuclear cells, was determined by microscopic differential counting. No difference in total numbers of leukocytes (Fig 2.4A), mononuclear cells (Fig 2.4B) and PMNs (Fig 2.4C) in the peritoneal cavity was observed between the two groups of mice after CLP-induced peritonitis. Our earlier observations demonstrated a difference in cellular composition in the peritoneal cavity between naïve WT and CCR6$^{-/-}$ mice and other investigations showed alterations in the recruitment of immature DCs during inflammatory responses (87, 136). Thus, we investigated changes in DC population in CCR6$^{-/-}$ mice and found significantly fewer MHCII^{+}CD11c^{+} DCs in the peritoneal cavity before and 4 and 24 hours after CLP-induced peritonitis, as compared to WT mice (Fig 2.4D). However, no difference was observed in the number of CD11b^{+}F4/80^{+}macrophage (data not shown).

CCL20 showed no effect on peritoneal macrophages and CLP-induced mortality

CCL20, the only chemokine ligand for CCR6, is upregulated during inflammation (229, 230) and by specific proinflammatory cytokines (231). An increase in CCL20 level

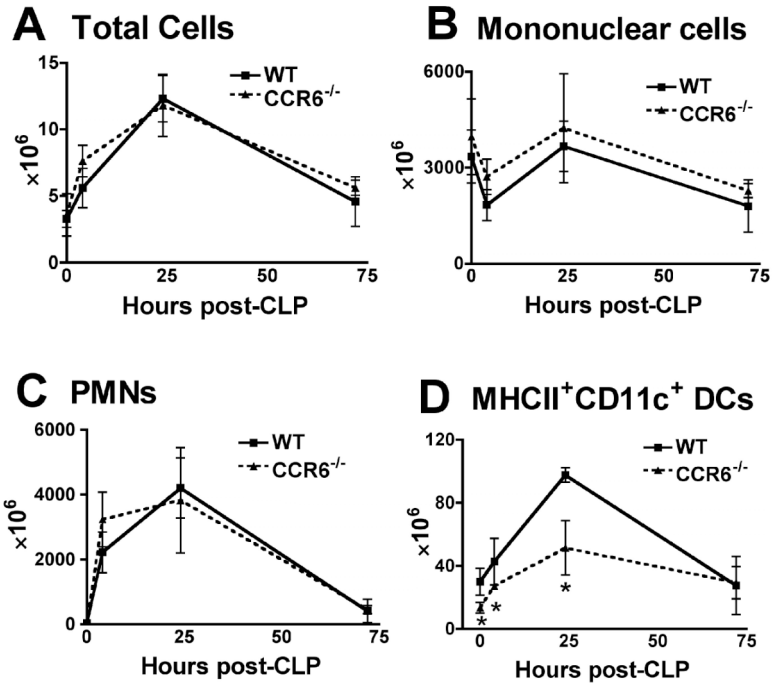

Figure 2.4 Leukocyte recruitment into the peritoneal cavity in WT and CCR6$^{-/-}$ mice during CLP-induced sepsis. WT and CCR6$^{-/-}$ mice (n=5/group/time point) were sacrificed at 4 h, days 1 and 3 after CLP surgery. Naive mice were used as baseline values. Peritoneal lavage was performed to collect total peritoneal cells. (A) Total peritoneal cell numbers were determined using a hemocytometer. (B, C) Cytospins were prepared. The absolute numbers of mononuclear cells and PMNs were determined by multiplying the percentage of cells by the total cell count. (D) Peritoneal cells were stained with PE-anti-I-A^{b} in combination with FITC-anti-CD11c, followed by flow cytometry analysis. The results shown are representative of two experiments and are expressed as mean ± SEM. * P≤0.05 compared with the number of MHCII^{+}CD11c^{+} DCs in peritoneal cavity of WT mice in naïve status, or 4 and 24 h after CLP.

in the peritoneal cavity was observed in WT mice with CLP-induced peritonitis with a peak at 24 h (Fig 2.5A). CCR6$^{-/-}$ mice showed a significantly lower level of CCL20 in the peritoneal cavity 24 hours after CLP-induced peritonitis, as compared to WT mice, which was consistent with other inflammatory cytokines. This might be due to an attenuated inflammatory response in CCR6$^{-/-}$ mice. We next sought to determine whether the presence of CCL20 exacerbated the dysregulated immune response and promoted CLP-induced mortality in WT mice. When endogenous CCL20 was passively neutralized

with anti-CCL20 2 h prior to CLP-induced peritonitis and each following day, survival was not altered, as compared to mice treated with IgG control (Fig 2.5B). These data suggested that the interaction between CCL20 and CCR6 was not involved in the survival of CCR6$^{-/-}$ mice after CLP-induced peritonitis. We also tested whether CCL20 altered the LPS-response of WT peritoneal macrophages. LPS induced inflammatory cytokine production by WT peritoneal macrophages, which was not altered by the presence of CCL20 in either low (1ng/ml) or high (100 ng/ml) concentrations (Fig 2.5C).

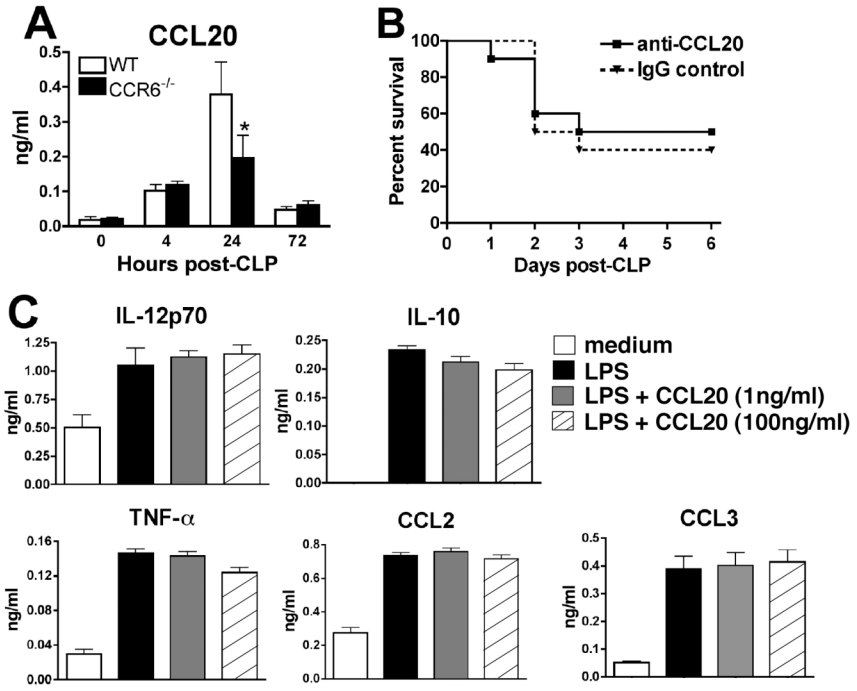

Figure 2.5 Effects of CCL20 in CLP-induced mortality and LPS-response of WT peritoneal macrophages. (A) WT and CCR6$^{-/-}$ mice (n=5/group/time point) were subjected to CLP operation. Peritoneal lavage fluid was collected from naïve WT and CCR6$^{-/-}$ mice and at 4, 24, 72 hours post-CLP. ELISA was performed to measure CCL20 level. * P≤0.05 compared with CCL20 level in peritoneal lavage fluid from WT mice at 24 hours post-. (B) WT mice were subjected to CLP surgery in the presence of antibiotics treatment. Endogenous CCL20 was blocked by anti-CCL20. Rat IgG_1 was used as a control. Survival was monitored for 6 days following surgery. Each group: n=10. (C) Peritoneal macrophages were collected from WT mice and stimulated with LPS in the absence or presence with either 1ng/ml or 100 ng/ml recombinant murine CCL20 protein. Twenty-four hours later, cell culture supernatants were collected and levels of cytokines were measured by ELISA.

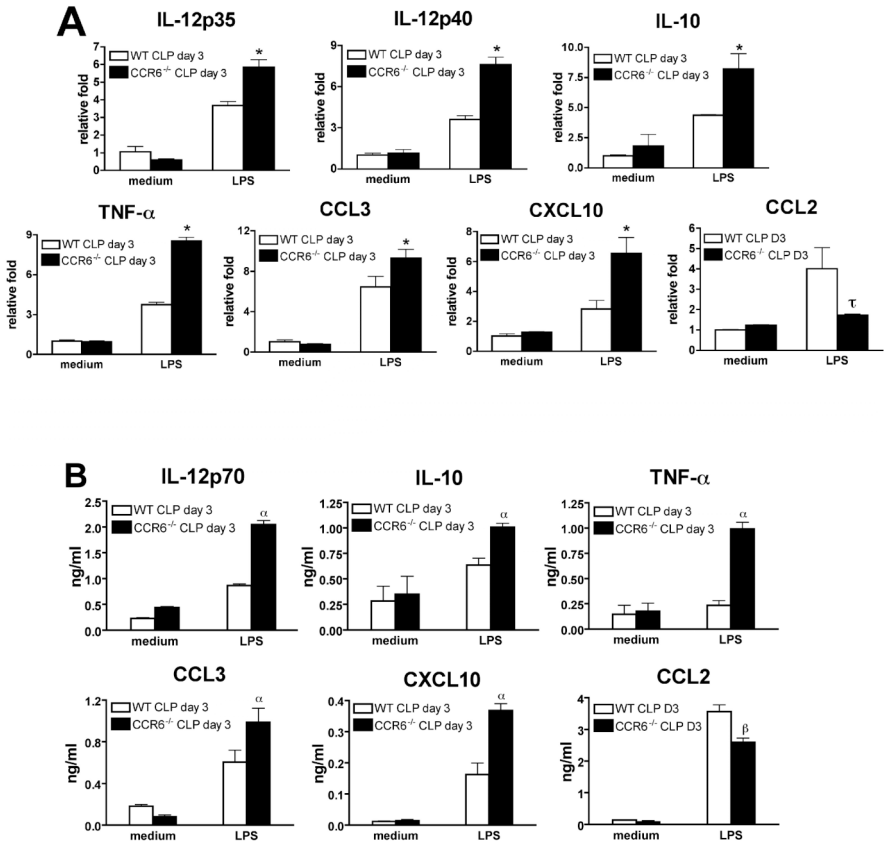

Figure 2.6 Cytokine production by peritoneal macrophages from WT and CCR6$^{-/-}$ mice day 3 post-CLP. WT and CCR6$^{-/-}$ mice (n=5) were sacrificed at day 3 after CLP surgery. Peritoneal macrophages were collected by peritoneal lavage and exposed to 1 μg/ml LPS. (A) Four hours after LPS stimulation, mRNA levels of inflammatory cytokines in peritoneal macrophages were measured by Taqman. *, τ P≤0.05 compared with mRNA levels of inflammatory cytokines in WT peritoneal macrophages. (B) Twenty-four hours after LPS stimulation, protein levels of inflammatory cytokines in culture supernatants were measured by ELISA. α, β P≤0.05 compared with protein levels of inflammatory cytokines produced by WT peritoneal macrophages.

CCR6$^{-/-}$ macrophages show enhanced LPS-responsiveness after CLP-induced peritonitis

It has been well established that macrophages develop a unresponsiveness to LPS challenge after LPS pretreatment, a process referred to as endotoxin tolerance (232). The mechanism for this tolerance response is not fully clear. Since we observed that CCR6$^{-/-}$

mice had an attenuated inflammatory immune response and higher survival rate during CLP-induced severe sepsis, we investigated whether the septic response induced LPS tolerance in CCR6$^{-/-}$ peritoneal macrophages. We initially focused on cytokine production in response to LPS stimulation. Three days after CLP, the levels of inflammatory cytokines generally decreased to the base line, indicating the end of the acute phase of severe sepsis. In contrast to naïve macrophages, CCR6$^{-/-}$ peritoneal macrophages, at day 3 after CLP-induced peritonitis, had significantly higher LPS-induced mRNA levels of IL-12p35, IL-12p40, IL-10, TNF-α, CCL3 and CXCL10, but lower CCL2 mRNA level compared with WT peritoneal macrophages isolated at the same time after CLP (Fig 2.6A). Consistently, after 24 h of stimulation with LPS, CCR6$^{-/-}$ peritoneal macrophages produced significantly higher protein levels of IL-12p70, IL-10, TNF-α, CCL3 and CXCL10, but less CCL2 (Fig 2.6B). These data suggest that CCR6$^{-/-}$ peritoneal macrophages do not exhibit classical LPS-induced tolerance.

To test if the greater LPS-responsiveness of CCR6$^{-/-}$ macrophages day 3 after CLP-induced peritonitis is an intrinsic property of CCR6$^{-/-}$ macrophages, or it is because of an attenuated cytokine storm affecting CCR6$^{-/-}$ peritoneal macrophages during CLP-induced peritonitis (Fig 2.3A), we next performed a classical LPS rechallenge experiment using *ex vivo* peritoneal macrophage isolated from naïve WT and CCR6$^{-/-}$ mice. Naïve CCR6$^{-/-}$ peritoneal macrophages produced significantly lower protein levels of IL-12p70, IL-10, TNF-α, CCL2, CCL3 and CXCL10 in response to one-time LPS challenge compared to similarly treated WT control, which we have previously observed (Fig 2.1). Both WT and CCR6$^{-/-}$ peritoneal macrophages, which have been pretreated with 10 ng/ml LPS, showed unresponsiveness to a second challenge with 1μg/ml LPS, indicating that CCR6$^{-/-}$

peritoneal macrophages possessed a similar unresponsiveness to LPS challenge after LPS pretreatment as WT controls (Fig 2.7).

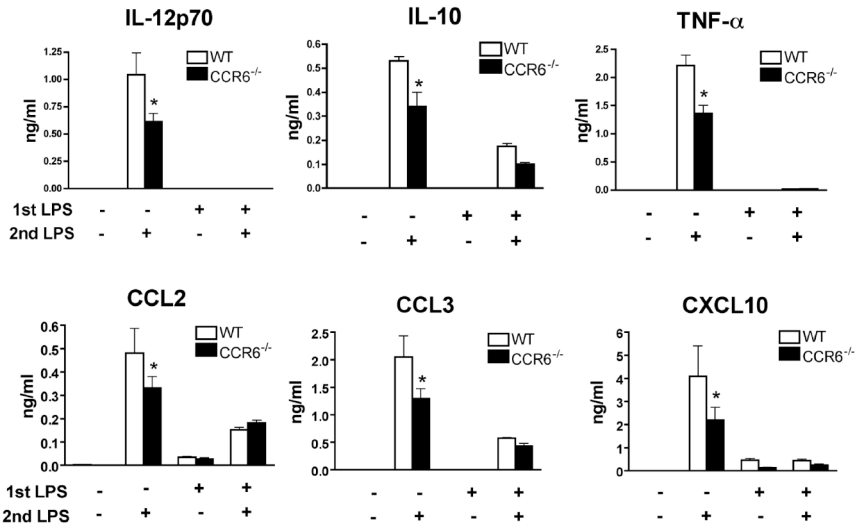

Figure 2.7 LPS rechallenge of peritoneal macrophages from naive WT and CCR6$^{-/-}$ mice. Peritoneal macrophages were collected by peritoneal lavage and pretreated with 10ng/ml LPS for 24 hours. After 2 times wash, these cells were stimulated with 1 μg/ml LPS for 24 hours. Supernatants were collected to measure cytokine protein levels by ELISA. * P≤0.05 compared with cytokine levels measured in WT peritoneal macrophages, n=5.

Since severe sepsis induces a long-term impairment in IL-12 production by DCs (chapter 3 and 4) and CCR6 deficiency alters cytokine production of peritoneal macrophages, we wondered if CCR6 has a role in severe sepsis-induced IL-12 downregulation in DCs. However, we observed a similar decrease in IL-12 production by splenic DCs following severe sepsis between WT and CCR6$^{-/-}$ mice (data now shown), which indicated that CCR6 is not involved in IL-12 downregulation in post-septic DCs. Further investigations have been carried out to study the molecular mechanisms of severe sepsis-induced IL-12 downregulation (chapter 4).

Discussion

In the present study, the role of CCR6 during experimental severe sepsis was investigated. We found that mice lacking CCR6 exhibited a constitutive decrease in resident peritoneal macrophage populations, as well as DC numbers, as compared to WT mice. Although cytokine production by pulmonary DCs and bone marrow–derived DCs in response to TLR agonist stimulation was comparable between WT and $CCR6^{-/-}$ mice (data not shown), we observed that naïve $CCR6^{-/-}$ peritoneal macrophage exibited a decreased production of inflammatory cytokines and NO in response to LPS stimulation compared with similarly treated WT controls. $CCR6^{-/-}$ mice were significantly protected from CLP-induced lethality and showed an attenuated localized and systemic inflammatory cytokine production compared with WT mice. Surprisingly, CCL20, the only chemokine ligand of CCR6, did not appear to play a role in the pathology of experimental sepsis, since localized neutralization of endogenous CCL20 had no effect on survival in septic WT mice, and CCL20 failed to influence the LPS-responsiveness of WT peritoneal macrophages.

Chemokines and chemokine receptors were originally described to be essential mediators directing homeostatic cell trafficking and cellular recruitment during inflammatory responses. However, the biological activities of chemokines are more than chemotactic factors alone. They are now known to play important roles in a variety of activities including maintenance of homeostasis, angiogenesis/angiostasis, wound healing, tumor growth and metastasis, cellular differentiation and activation, lymphocyte development and trafficking, lymphoid organ development, and influencing the type 1/type 2 balance of immune responses (133, 134). Monocyte/macrophage trafficking has

been shown to depend on the interactions between chemokines and chemokine receptors. For example, CCR2 mediated homeostatic monocyte emigration from bone marrow into circulation, and inflammation-induced monocyte recruitment from bone marrow to peripheral infected tissue also requires CCR2 signaling (233). Although it has been reported that CCR2 was not involved in tissue entry of monocyte from the circulation (233), new data has shown that CCR2 is necessary for efficient monocyte recruitment from the blood into inflamed tissue (234). $CCR2^{-/-}$ mice also show an impaired recruitment of macrophage into the peritoneal cavity in response to thioglycollate stimulation (235). Thus, $CCR2^{-/-}$ mice exhibit deficient monocyte recruitment into inflected sites in response to a broad range of stimuli (236, 237).

While we were not surprised to observe a decrease in peritoneal DC population in naïve status and impaired DC recruitment in inflammatory responses in $CCR6^{-/-}$ mice, which was consistent with other studies (137, 138, 217, 220), it was not predicted that the population of peritoneal macrophages also was decreased. In contrast to numerous reports showing monocyte/macropahge chemotactic property of chemokines and chemokine receptors, little is know about the influence of chemokine signaling on monocyte/macrophage lineage development and normal function. The chemokine receptor CXCR4 has been reported to be expressed on hematopoietic stem cells (HSCs) and the interaction of CXCR4 with its cognate ligand, CXCL12, was essential for the colonization of bone marrow by HSCs during development (238). CXCR4 was also involved in HSCs homing to bone marrow (239), as well as their survival and proliferation (240). Thus, it is highly possible that chemokine signaling has a role in directing normal hematopoiesis, including the development of monocyte/ macrophage

lineage. Our data showed that CCR6 controlled the localization of macrophages and DCs in the peritoneal cavity and altered the phenotype of naïve peritoneal macrophages, suggesting that CCR6 signaling may be involved in the development of monocyte/macrophage lineage. However, the molecular mechanism whereby CCR6 influences the development and function of peritoneal macrophages is not clear. The possibilities are that CCR6 functions either during the development of monocyte/ macrophage lineage from progenitor cells in bone marrow, or influences migration of monocyte/ macrophage to peripheral tissues. Thus, the role of CCR6 in the development of monocyte/macrophage lineage is worthy of further investigation.

A number of *in vitro* and *in vivo* studies have underscored the importance of chemokines/chemokine receptors in the initiation of the immune response to sepsis, as well as a direct correlation with tissue pathology, organ dysfunction and failure, and lethality. Immunoneutralization of CXCR2 with specific antibody decreased PMN recruitment and CLP-induced lethality (212, 241). $CCR1^{-/-}$ mice had accelerated cytokine production and were protected against the deleterious effects of sepsis, due in part to the fact that $CCR1^{-/-}$ peritoneal macrophages displayed both an early and enhanced cytokine/chemokine expression profile and anti-bacterial response. CCR4 gene knockout has been shown to protect mice from systemic challenge with either LPS (214) or other TLR agonists, as well as protect mice against bacterial peritonitis (211). $CCR4^{-/-}$ peritoneal macrophages were demonstrated to possess a constitutive phenotype of an alternatively activated macrophage and exhibit features associated with alternative activation, including enhanced production of type-2 cytokines, CCL2, CCL17 and the found in inflammatory zone 1 (FIZZ1) protein (211). $CCR8^{-/-}$ mice were also resistant to

CLP-induced lethality, which mechanistically may be related to the phenotype of $CCR8^{-/-}$ peritoneal macrophages. These inflammatory cells were highly activated, exhibited enhanced bactericidal activities, and generated high levels of superoxide, lysosomal enzymes and NO in response to LPS stimulation (210, 213). Collectively, our data in the present study and the above reports suggests that chemokines/chemokine receptors have a role in the development and maintenance of normal function of peritoneal macrophage, since chemokine receptor deficiencies alter the functions of peritoneal macrophages, including cytokine production in response to TLR agonists.

Although CCL20 is the main ligand for CCR6, β-defensin has also been shown to bind CCR6 with a lower affinity (216). Defensins, a family of arginine-rich cationic peptide, have direct antimicrobial effect and are considered to be an important effector molecule in innate immune response (242, 243). Furthermore, defensins were also shown to chemoattract immune cells, including monocyte/ macrophages (244, 245), mast cells (246) and DCs (216). This suggests that defensins contribute either directly or indirectly to the mobilization of host immune defense in response to pathological infections. In the present study, we observed an attenuated inflammatory cytokine production and survival advantage in $CCR6^{-/-}$ mice during severe sepsis, which was not related to CCL20 function. Thus, this finding raised the question whether β-defensin was involved in exacerbating dysregulated immune response and cytokine production in WT mice. Interestingly, it has been shown that human α-defensin promoted the expression and production of CXCL5 and CXCL8 by bronchial epithelial cells (247) and increased the production of TNF-α and IL-1 by monocytes (248). Thus, it is possible that, during CLP-induced bacterial peritonitis, β-defensin promoted the inflammatory cytokine

production by innate immune cells, including peritoneal macrophages and DCs in a CCR6-dependent mechanism.

In summary, we have shown that genetic depletion of CCR6 decreased mononuclear cells of myeloid origin including macrophage and DCs in peritoneal cavity. Peritoneal macrophages from $CCR6^{-/-}$ generated significantly lower amounts of inflammatory cytokines and NO compared with similarly treated WT controls. Furthermore, CCR6 depletion increased survival rate during CLP-induced severe sepsis, which was associated with an attenuated cytokine storm, an indicator of severity of sepsis. Moreover, peritoneal macrophages isolated from day 3 post-CLP $CCR6^{-/-}$ mice showed an enhanced LPS-response compared with WT control, indicating that these cells did not develop tolerance to bacteria-derived factors such as LPS, which is one of the key features associated with immunosuppression in patients who survive severe sepsis. Thus, these data suggested that CCR6 is a key mediator in modulating the innate inflammatory response during a septic insult and is a potential target for immunotherapy in septic patients.

Chapter 3

Severe sepsis exacerbates cell-mediated immunity in the lung due to an altered dendritic cell cytokine profile

Summary

Severe sepsis leads to long-term alterations in the immune response of surviving individuals. We have modeled this alteration in host immunity by studying the survivors of severe experimental sepsis (murine cecal ligation and puncture-CLP), who were subsequently challenged with lung granuloma-inducing *Schistosoma mansoni* eggs. This granulomatous response is a well-studied cell-mediated immune reaction, which is characterized by elevated levels of type-2 cytokines. Pulmonary granulomas induced by *S. mansoni* eggs in CLP survivors were significantly larger and contained more eosinophils than granulomas in sham-operated mice. Significantly lower IL-12p40 mRNA and IL-12p70 protein levels were observed in the lungs of post-septic mice with developing granulomas, compared to controls. Post-septic mice had significantly fewer dendritic cells (DCs) in the lungs during the granulomatous response. Isolated lung DCs from post-septic mice at days 8 and 16 after *S. mansoni* egg challenge exhibited defective IL-12 synthesis, but enhanced IL-10 synthesis, following TLR agonist challenge. Pulmonary transfection with an IL-12 expressing adenovirus in post-septic mice reversed the skewing of the pulmonary cytokine profile and normalized the lung granulomatous response. Our data indicates that severe sepsis shifts the pulmonary cytokine

environment, presumably via effects on pulmonary DCs, which in turn alters the lung cell-mediated immune response.

Introduction

Sepsis is the most common cause of death in intensive care units, afflicting 750,000 people and killing 210,000 people annually (4, 5, 208). The initiation and maintenance of sepsis and acute lung injury are triggered by an initially exaggerated innate immune response leading to a cytokine storm, which has been extensively studied (9). In contrast, the long-term consequence of severe sepsis on the host immune response has been poorly characterized. During severe sepsis, dysregulation of the systemic immune response may result in either rapid death due to multiple organ failure (MOF) or susceptibility to secondary infections with bacteria, viruses or fungi due to long-term immunosuppression (38, 249). The dramatic loss of DCs in septic patients and in animals with severe sepsis has been reported, which may significantly impair B and T cell function and contribute to the long-term defective adaptive immunity after the acute phase of sepsis (54, 57). The respiratory system is most susceptible to secondary infections after severe sepsis and pulmonary complications often lead to mortality in this group of patients (47).

Previously, our lab has shown that mice that have recovered from cecal ligation and puncture (CLP) were susceptible to a pulmonary challenge of *Aspergillus fumigatus* whereas sham-operated mice were not (38, 39). This previous study suggested that post-septic mice had impaired innate pulmonary immunity, since *A. fumigatus* normally poses few problems to an intact pulmonary immune system (250). To further investigate the

influence of severe sepsis on the pulmonary adaptive immune response, we used granuloma-inducing *Schistosoma mansoni* eggs as a pulmonary challenge in survivors of severe sepsis induced by CLP surgery.

Granulomatous immune responses induced by *S. mansoni* eggs are unique inflammatory sites and offer a rewarding model to study the evolving immune response (251). The granulomatous response in the lung is not lethal to the host and thereby allows for the long-term study of the immune response. During the pathological development of *S. mansoni* egg-induced granulomas, the host initiates a Th0 immune response, which subsequently switches to a type-2 immune response driven by alternatively activated macrophages, T-lymphocytes and eosinophils (143). In addition, it has been shown that $CD11c^+$ DCs are a notable component of *S. mansoni* granulomas and are involved in directing the pulmonary cytokine response (139, 141).

The purpose of this study was to investigate the long-term influence of severe sepsis on the adaptive immune response reproducibly elicited by *S. mansoni* eggs. We demonstrate that mice, which survived CLP-induced severe sepsis, developed significantly larger granulomas. The greater granulomatous response was associated with a selectively impaired type-1 and enhanced type-2 cytokine profile in the lung. Study of lung DCs revealed a decrease in their recruitment. In addition, pulmonary DCs showed the defective production of IL-12 and the enhanced production of IL-10. Following the introduction of an IL-12-producing adenovirus vector, the skewed cytokine profile in the post-septic lung was reversed and the granulomatous response was normalized, but DC recruitment was not influenced. Thus, this study highlights the adverse consequences of severe sepsis on the adaptive immune response in the lung. IL-12 gene transfer in the

post-septic lung appeared to rebalance the adaptive immune response and diminish the remodeling pathology associated with pulmonary granulomatous responses.

Materials and Methods

Mice

Female C57BL/6 mice (6-8 wks; Taconic Company, Germantown, NY) were housed under specific pathogen-free conditions at The Unit for Laboratory Animal Medicine (ULAM) of the University of Michigan. The animal ethical committee approved the experiments.

Survivors of CLP subsequently challenged by *S. mansoni* eggs

Sham and CLP surgery was performed as described in Chapter 2. At day 3 post-surgery, both surviving CLP mice and sham-operated mice were intravenously injected with 5000 viable *S. mansoni* eggs via the tail vein. Live *S. mansoni* eggs were purified from the livers of *S. mansoni* cercariae-infected Swiss-Webster mice, which were kindly provided by Dr. Fred Lewis (Biomedical Research Laboratory, Rockville, MD).

Morphometric Analysis of pulmonary granuloma and eosinophil count

The left lung lobe from both sham and CLP groups at days 1, 2, 4, 6, 8, 16, and 30 after the intravenous *S. mansoni* egg challenge was fully inflated with 10% formalin, dissected, and placed in fresh formalin for an additional 24 hours. Routine histological techniques were used to paraffin-embed lung tissue, and 5-μm sections were stained with hematoxylin and eosin (HE). The structural alternations and eosinophil infiltration were examined around individual (ie. containing a single egg) pulmonary granulomas using light microscopy at a magnification of 200X and 1000X, respectively. Morphometric

analysis of egg granuloma size was performed using the Scion Image 1.63 software program (Scion Corporation, Frederick, MD). The number of eosinophils recruited to the granuloma were counted and normalized to the granuloma size. A minimum of 15 granulomas per lung section was analyzed for granuloma size and 25 granulomas per mouse were analyzed for the eosinophil infiltrate. The same protocol was applied for the mice transfected with IL-12 expressing adenovirus.

Cytokine and cytokines ELISA Analysis

IL-4, IL-5, IL-10, IL-12p70 and IL-13 were measured in 50-µl samples from cell-free supernatants either from lower right lobes homogenates or from tissue culture plates using a standardized sandwich ELISA technique as described in Chapter 2.

Quantitative Real-time PCR (Taqman)

Analysis of transcripts with Taqman was performed as described in Chapter 2.

Flow cytometry analysis

Whole lungs were dispersed in 0.2 % collagenase (Sigma-Aldrich) in RPMI 1640 (Mediateck, Inc.) + 5% fetal bovine serum (Atlas, Fort Collins, CO) at 37°C for 45 min. After lysing red blood cells with ammonium chloride buffer (4.01 g NH4Cl, 0.42 g NaHCO3, and 0.185 g tetrasodium EDTA in 500 ml dH2O), Fc binding was blocked via a 10-min incubation with purified rat anti-mouse CD16/CD32 (FcγIII/II receptor). Then the cells were stained with either PE-labeled anti-CD11c (HL3) and FITC-anti-CD11b (M1/70) or PE-anti-CD11c and FITC-anti-CD45R/B220 (RA3-6B2) in Dulbeco's PBS + 0.2% BSA + 0.1% NaN_3 for 30 min at 4°C in the dark. The appropriate IgG isotypes were used as controls. All antibodies and IgG isotypes were purchased from BD PharMingen (San Diego, CA). The cells were fixed in 1% paraformaldehyde and kept in

the dark at 4°C until analysis with a FACSCaliber (CELLQuest™ software; Becton and Dickinson, Mountain View, CA).

Pulmonary dendritic cell isolation and in vitro stimulation

Dendritic cells were isolated from the pooled lungs from sham and CLP groups (n=5 mice/group) at days 8 and 16 after the *S. mansoni* egg challenge, as described in the flow cytometry analysis section. Then, cell suspensions were enriched with anti-CD11c magnetic beads and positive selection MS^+ columns according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Briefly, the cells, re-suspended in 10-ml RPMI 1640, were incubated in 200mm cell culture dish (Corning Inc., Acton, MA) for 1 hour at 37°C to remove the adherent macrophages. Non-adherent cells were collected and resuspended in 400μl of buffer (1XPBS/0.5% BSA) containing 100μl of CD11c microbeads. After a 15-min incubation at 4°C, free beads were washed away and the cells conjugated with beads were passed through MS^+ columns for positive selection. The viability of the purified DCs was estimated by trypan blue staining. The purity of these DCs was determined by either cell morphology using cytospin (Shandon Inc.) followed by hematoxylin-eosin (HE) staining or stained by PE-labeled CD11c and FITC-labeled-MHCII followed by flow cytometry analysis. Then the purified DCs were counted on a hemocytometer and subsequently diluted at $5X10^6$/ml. The aliquots of 100μl containing these cells were added to 96-well plates. A group of stimuli were added to the plated DCs including 100μg/ml *S. mansoni* egg antigen (SEA), 1μg/ml lipopolysaccharide (LPS), or 2.5μg/ml Pam3cys. After 6 hrs of stimulation, total RNA was isolated using Trizol reagent and quantitative Real-time PCR (Taqman) was performed to measure IL-10 and IL-12 gene expression. After 48 hrs of stimulation, cell

free supernatant from each sample was collected and stored in -80°C until the measurement of IL-12 and IL-10 protein levels by ELISA.

IL-12 expressing adenovirus (AdmIL-12) intrapulmonary infection

A recombinant human adenovirus expressing a functional heterodimeric mouse IL-12 protein was generated as previously described (252). The AdmIL-12 construct contains the p35 and p40 cDNA fragment inserted into the E1 and E3 regions of human type-5 adenoviral genome. Expression of both p35 and p40 cDNAs is driven by the human CMV immediate early promoter and terminated by the SV40 polyadenylation signal. Transfection with this replication-defective construct results in the expression of active IL-12 both in vitro and in vivo (252). As a control, a previously described replication-deficient, E1-deleted Ad70 construct was used (253). At day 3 after CLP or sham surgery, mice were anesthetized with a combination of ketamine and xylazine, as described for the CLP procedure (see above). The tracheas of mice were exposed by making 0.5-cm midline incision on the ventral surface of the neck. Mice were intratracheally injected with 30μl saline containing $3X10^8$ plaque-forming units of either AdmIL-12 or Ad70 and then immediately injected intravenously with 5000 *S. mansoni* eggs. The adenoviral vector results in very efficient infectivity of the airway epithelium and produces high levels of IL-12 mRNA and protein in lung (254). At 6, 8 and 16 days, the mice were sacrificed and the whole lungs were collected for histology, gene expression analysis (Taqman) and protein measurement (ELISA) (see above).

Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). The means between sham and CLP groups at different time points were compared by two-way

ANOVA. Individual differences were further analyzed using the Bonferroni post-tests. For the in vitro studies with lung DCs, the Mann-Whitney U test was used to compare the means between sham and CLP group. $P<0.05$ was considered statistically significant.

Results

Severe sepsis augmented the pulmonary granulomatous response

In order to investigate the long-term consequence of severe sepsis on the adaptive immune response in the lung, mice that survived three days following severe experimental sepsis induced by CLP were subsequently challenged intravenously with *S. mansoni* eggs. Sham-operated mice were used as controls. The formation and development of pulmonary granulomas induced by *S. mansoni* eggs have been previously well characterized in non-surgically manipulated mice (255, 256), thereby providing a paradigm for the study of this response in post-septic mice. Histological examination of lung tissue revealed that *S. mansoni* egg-induced granulomas were similar in size in both sham and post-septic mice up to day 8 after egg injection (Fig 3.1A). However, at day 16, post-septic mice exhibited significantly ($p<0.001$) larger granulomas (28498.3±1152.6 μm^2, n=5) compared with the sham mice (18882.2±814.1 μm^2, n=5). This difference was also present at day 30 after egg challenge (28232.7±1607.7 μm^2, n=4 Vs. 14310.6±644.7 μm^2, n=5; $p<0.001$) (Fig 3.1B). Quantification of eosinophil content in lung granulomas revealed that eosinophil number in post-septic mice at day 8 (0.525±0.026/100 μm^2) and day 16 (0.999±0.052/100 μm^2) were significantly greater than those in the sham mice at day 8 (0.370±0.025/100 μm^2; $p<0.01$) and day 16 (0.612±0.040/100 μm^2; $p<0.001$), respectively (Fig 3.1C).

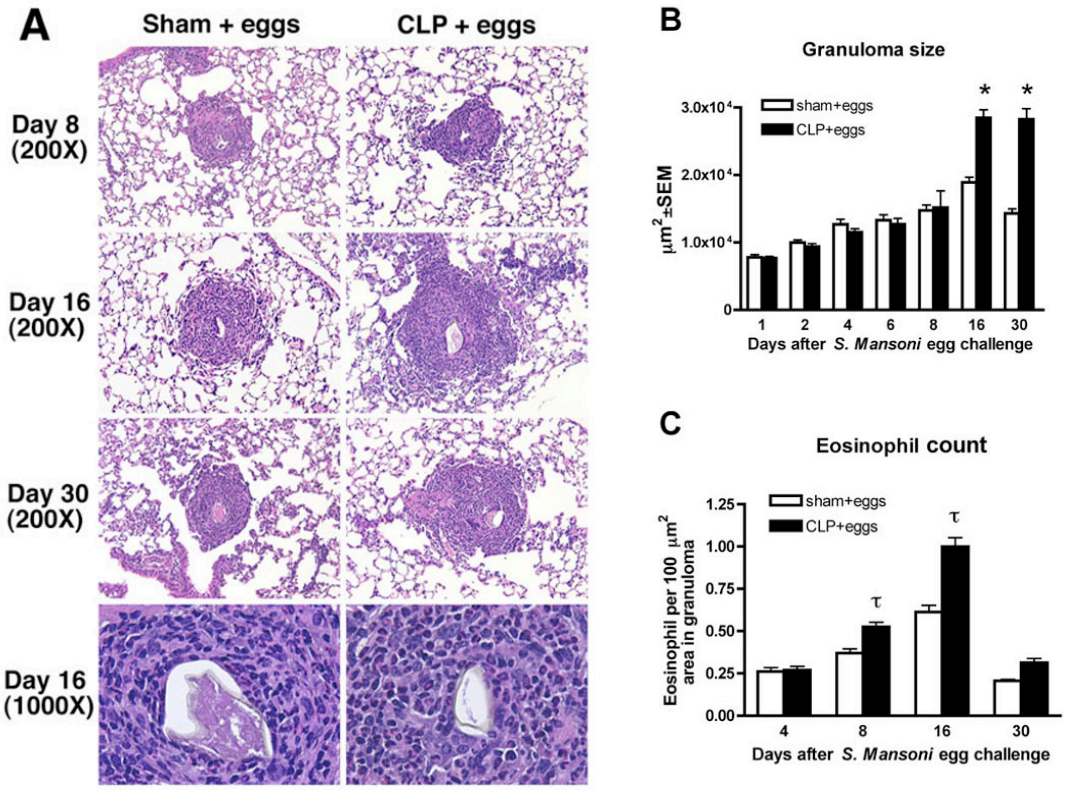

Figure 3.1 The histology of pulmonary granulomas induced by *S. mansoni* eggs. (A) Sham and CLP groups were challenged with 5000 live *S. Mansoni* eggs at day-3 post-surgery. The left lung lobe from both groups was collected at different time points for histological analysis. H&E-stained lung sections from sham or CLP mice are shown. (B) Quantification of granuloma size at a series of indicated time point after egg challenge. * $P \leq 0.05$ compared with granuloma size measured in sham mice. (C) Quantification of eosinophil number in pulmonary granulomas. Each group: n=5~6/time point. The results shown are representative of three experiments and are expressed as mean ± SEM. τ $P \leq 0.05$ compared with eosinophil number in the pulmonary granulomas from sham mice.

Severe sepsis altered the cytokine profile in lung tissue

The changes in pulmonary granuloma size and cellular content in post-septic mice suggested that an altered immune status was present at this tissue site, therefore we hypothesized that this was associated with an altered cytokine profile. The expression of a series of cytokines in lung tissue was measured both at the transcript level by

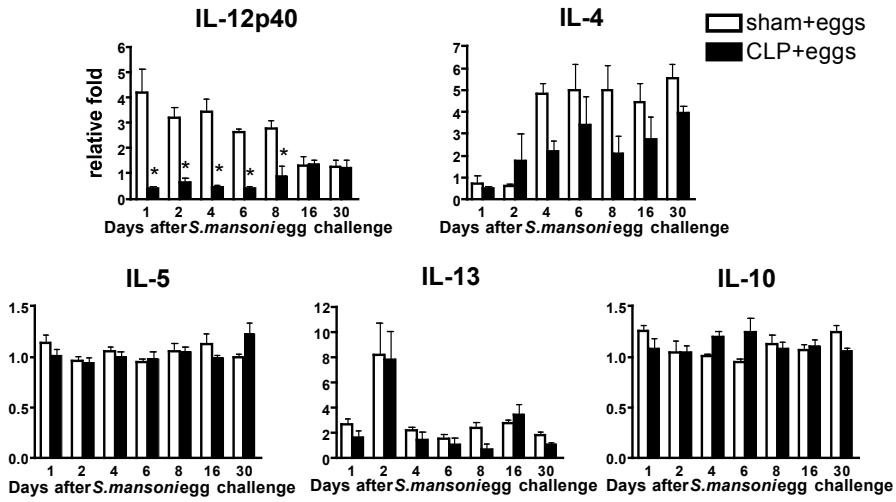

Figure 3.2 Cytokine transcript expression in the lungs from sham and CLP mice. After egg challenge at day-3 post-surgery, mice from the sham or CLP groups were anesthetized and the upper right lobe was collected to investigate the gene expression level of IL-4, IL-5, IL-10, IL-12p40 and IL-13 in the whole lungs at days 1, 2, 4, 6, 8, 16 and 30 post-egg challenge. Total RNA was isolated and reverse transcribed to cDNA. Quantitative real-time PCR (Taqman) was performed to measure the transcript level of each cytokine. The mRNA level of each sample was normalized by mRNA level in naïve mice and the data is shown as the fold of increase [$2^{(-\Delta\Delta CT)}$]. * $P \leq 0.05$ compared with IL-12p40 mRNA level measured in the lungs of sham mice, n=4~6.

quantitative real-time PCR (Taqman) and at the protein level by ELISA. IL-12p70 is a heterodimeric cytokine, which consists of covalently bound p40 and p35 subunits and has a central role in type-1 immune response (118). In the present study, post-septic mice had significantly lower whole lung transcript level of IL-12p40 (Fig 3.2) and protein level of IL-12p70 (Fig 3.3) compared with sham mice at several time points up to day 8 after *S. mansoni* egg challenge. For the type-2 related cytokines, no differences were observed at the transcript level for IL-4, IL-5, IL-10 and IL-13 between two groups (Fig 3.2). However, post-septic mice exhibited significantly higher whole lung IL-4 and IL-13 protein levels at day 1 and IL-10 protein level at day 2 after egg challenge compared with sham group (Fig 3.3).

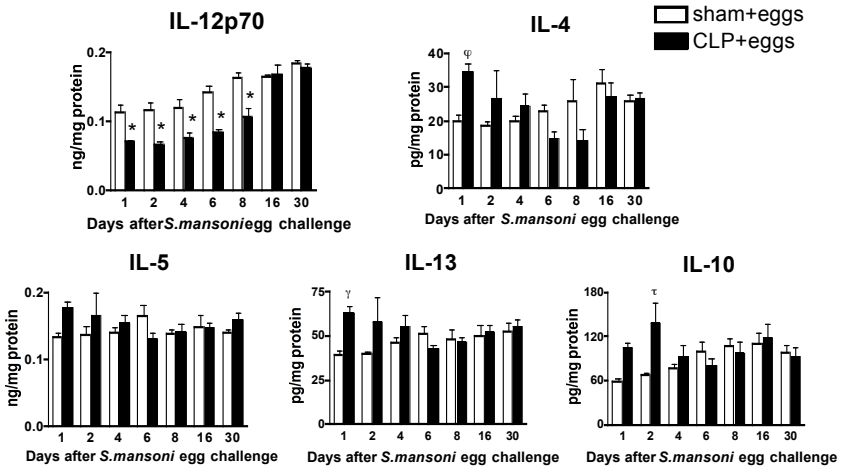

Figure 3.3 Cytokine protein levels in the whole lungs from mice of sham and CLP group. To investigate the production of IL-4, IL-5, IL-10, IL-12p40 and IL-13 in the whole lungs of sham and CLP mice, the lower right lobes from sham or CLP mice were analyzed by ELISA and normalized to the total protein level. * P≤0.05 compared with IL-12p70 protein level in the lungs of sham mice. φ P≤0.05 compared with IL-4 level in the lungs of sham mice at day 1; τ P≤0.05 compared with IL-10 level in the lungs of sham mice at day 2; γ P≤0.05 compared with IL-13 level in the lungs of sham mice at day 1.

S. mansoni eggs-induced DC recruitment to lung was impaired in post-septic mice

Given its accessory role during immune responses, DCs are recognized as one of the key initiators of adaptive immunity and have a pivotal role in directing the type-1/type-2 cytokine response (72, 257-259). Since the cytokine profile in the lung revealed an altered adaptive immune response in post-septic mice, we used flow cytometry to study the influence of severe sepsis on the presence of DC subpopulations in the lungs. In sham mice, peak percentages of two subpopulations of DCs, namely $CD11c^+CD11b^+$ DCs and $CD11c^+B220^+$ DCs, were observed in the lung at day 2 after egg challenge (Fig 3.4). However, lungs from post-septic mice contained significantly lower proportions of these two subpopulations of DCs at day 2 and day 8 after egg challenge when compared

to similar samples from sham mice. At day 16 after egg challenge, DC percentages were similar in both groups of mice.

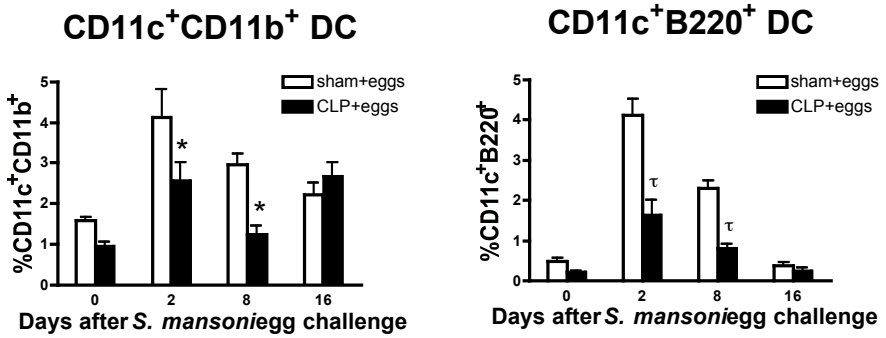

Figure 3.4 Severe sepsis impairs DC recruitment to pulmonary granulomas induced by *S. mansoni* eggs. Mice from sham or CLP group (n=5~7/group/time point) were sacrificed at days 2, 8 and 16. Mice killed at day 0 did not receive eggs and were used as baseline values. Lungs were digested by collagenase IV and the dispersed cells were stained as follows: $CD11c^+CD11b^+$ and $CD11c^+B220^+$. These cell surface markers were assessed by flow cytometry analysis. The results shown are representative of two experiments and are expressed as mean ± SEM. *** τ,** $P \leq 0.05$ compared with $CD11c^+CD11b^+$ DCs and $CD11c^+B220^+$ DCs in the lungs of sham mice at days 2 and 8, respectively.

Alteration in the cytokine production in pulmonary DCs induced by severe sepsis

The cytokine profile in the whole lung samples revealed a significant decrease in IL-12 level in post-septic mice compared with sham-operated mice. These findings indicated that pulmonary type-1 immune response might have been impaired by severe sepsis. Given the importance of DCs in the initiation of the adaptive immune response and polarizing type-1 and type-2 response, we hypothesized that, in addition to impairing the recruitment of DCs into the lungs, severe sepsis also altered cytokine production by lung DCs. After positive selection by CD11c microbeads, 90% of the purified DCs were alive established by trypan blue staining. Given that lung macrophages have been removed by 1 hour incubation at 37°C, HE staining revealed the purified cells

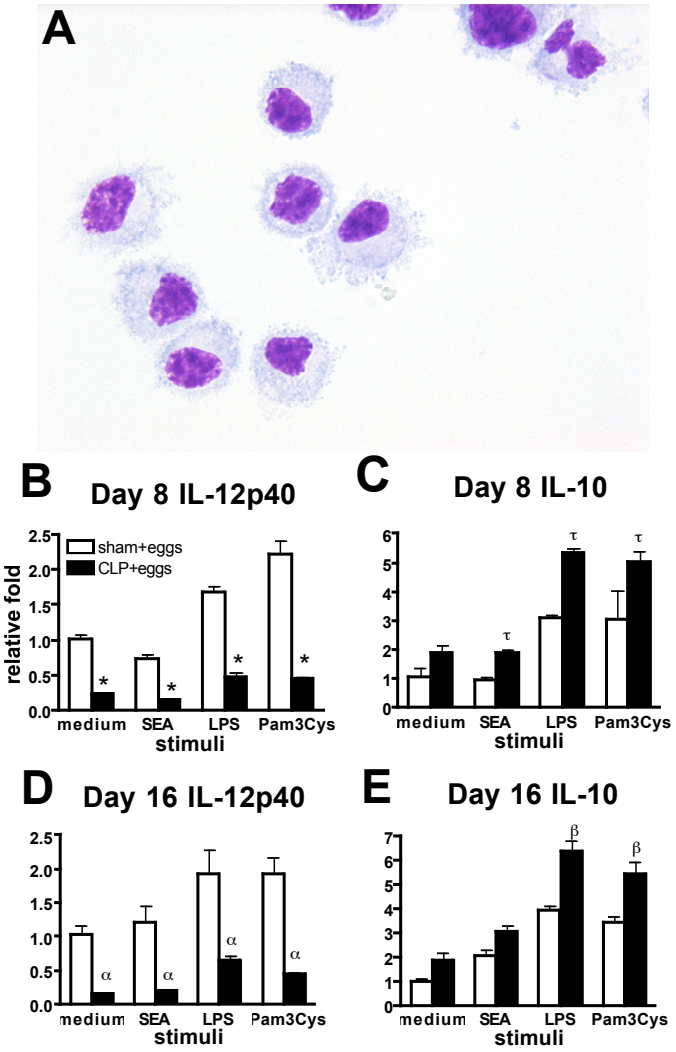

Figure 3.5 Alteration of gene expression of IL-12p40 and IL-10 in DCs from post-septic mice. Sham and CLP-operated mice (n=6-8/group/time point) were subsequently intravenously challenged with 5,000 live *S. mansoni* eggs. Eight and 16 days later, mice were sacrificed and lung DCs were purified by positive selection with $CD11c^+$ microbeads. After overnight culture, purified DCs (as shown in A) were stimulated with medium, 80μg/ml SEA (TLR2 agonist), 1μg/ml LPS (TLR4 agonist) or 2.5μg/ml Pam3cys (TLR2 agonist). Quantitative real-time PCR was performed to measure the mRNA levels of IL-10 and IL-12p40 in cultured DCs after 6 hrs of stimulation. * α, P≤0.05 compared with IL-12p40 mRNA level in the DCs from sham mice at days 8 and 16, respectively. τ β, P≤0.05 compared with IL-10 mRNA level in the DCs from sham mice at days 8 and 16, respectively, after egg challenge.

homogenously matched DC morphology (Fig 3.5A). Ninety percent of these cells were $CD11c^+MHCII^+$ (data not shown). When compared with DCs from sham mice, lung DCs from post-septic mice showed decreases of 4- and 7-fold in the baseline mRNA level of IL-12p40 at day 8 and day 16, respectively (Fig 3.5B and D medium). In response to the stimulation with different Toll-like receptor (TLR) agonists [SEA (TLR2), LPS (TLR4) and Pam3Cys (TLR2)], lung DCs from post-septic mice contained significantly lower IL-

12p40 mRNA level compared with DCs from sham mice (Fig 3.5B and D). Conversely, lung DCs from post-septic mice exhibited a 2-fold increase in baseline mRNA level of IL-10 at both time points after egg challenge (Fig 3.5C and E medium). Significantly higher IL-10 mRNA level in lung DCs was observed in post-septic mice compared with sham mice in response to LPS and Pam3Cys at both time points (Fig 3.5C and E). The stimulation with SEA only induced a significant increase in IL-10 mRNA level in DCs from post-septic mice at day 8 post egg challenge.

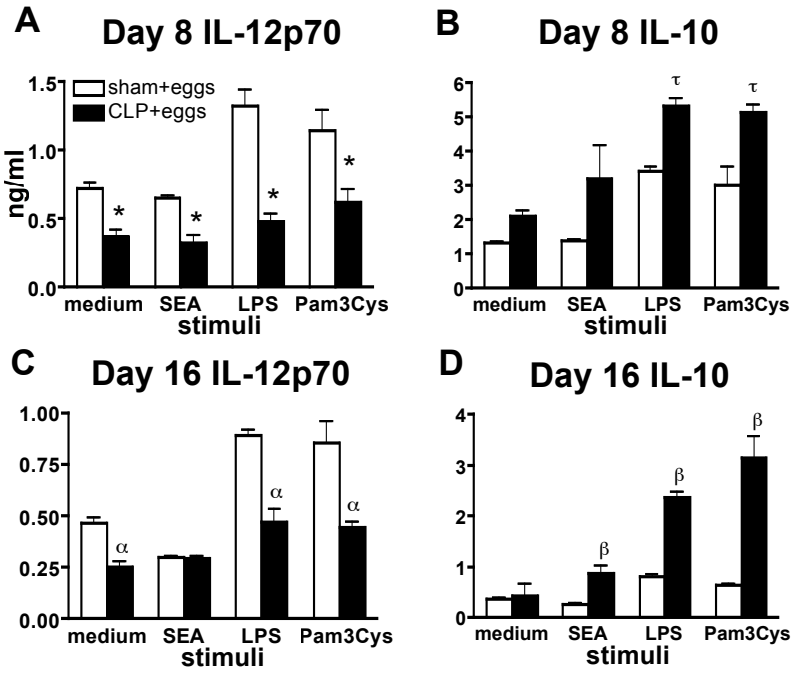

Figure 3.6 DCs from post-septic mice show the defective IL-12p70 production and enhanced IL-10 production. Sham and CLP-operated mice were sacrificed at days 8 and 16 post egg challenge. Lung DCs were purified by positive selection with $CD11c^+$ microbeads from each animal. After overnight culture, purified DCs were stimulated with medium, 80μg/ml SEA (TLR2 agonist), 1μg/ml LPS (TLR4 agonist) or 2.5μg/ml Pam3cys (TLR2 agonist). ELISA was performed to measure the protein levels of IL-10 and IL-12p70 in the supernatant of cultured DCs after 48 hrs of stimulation. * α, P≤0.05 compared with IL-12p70 level in the DCs from sham mice at days 8 and 16, respectively. τ β P≤0.05 compared with IL-10 level in the DCs from sham mice at days 8 and 16, respectively.

The protein levels of IL-12p70 and IL-10 in DC cultures were consistent with mRNA levels measured in DCs from sham and post-septic mice. Specifically, DCs from post-septic mice produced lower amounts of IL-12p70 and higher amounts of IL-10 constitutively and following TLR activation compared with DCs from sham mice (Fig 3.6).

IL-12 adenoviral vector restored the type-1 cytokine profile in post-septic mice

Having observed that post-septic mice had larger pulmonary granulomas and greater eosinophil recruitment when challenged with *S. mansoni* eggs, we next sought to determine whether this amplified granulomatous response could be altered by exogenous IL-12. To increase IL-12 expression level in the lung, we utilized a transient IL-12 transgene expression system whereby mice were administrated intratracheally a adenoviral vector containing the p35 and p40 cDNAs inserted into the E1 and E3 region of the viral genome, respectively. Given that the E1 region of the viral genome has been deleted, this recombinant viral vector is replication deficient, resulting in transient expression of IL-12 in lung. The peak concentration of IL-12 within the bronchoalveolar lavage fluid (BALF) was observed at day 1 after intratracheal administration of AdmIL-12, followed by a gradual decrease through day 7 (254). As shown in figure 3.7, there were lower IL-12p-70 protein level in the whole lungs from post-septic mice compared with sham mice in the control virus group at days 6 and 8, which is consistent with the observation shown in figure 3.3. However, compared with Ad70 group, post-septic mice receiving the IL-12 vector showed a significant increase in the expression of IL-12p70 at day 6 (0.272 ± 0.008 vs. 0.5 ± 0.029 ng/mg total protein, $n=4$; $p<0.001$) and day 8 (0.274 ± 0.029 vs. 0.556 ± 0.021 ng/mg total protein, $n=6$; $p<0.001$). Although the IL-12

vector did not affect all the type-2 related cytokines, it did reduce IL-4 protein levels significantly at day 6 (0.141±0.009 vs. 0.032±0.004 ng/mg total protein, n=4; p<0.001). No changes in IL-5, IL-10 and IL-13 expression were observed in post-septic mice that received the IL-12 vector.

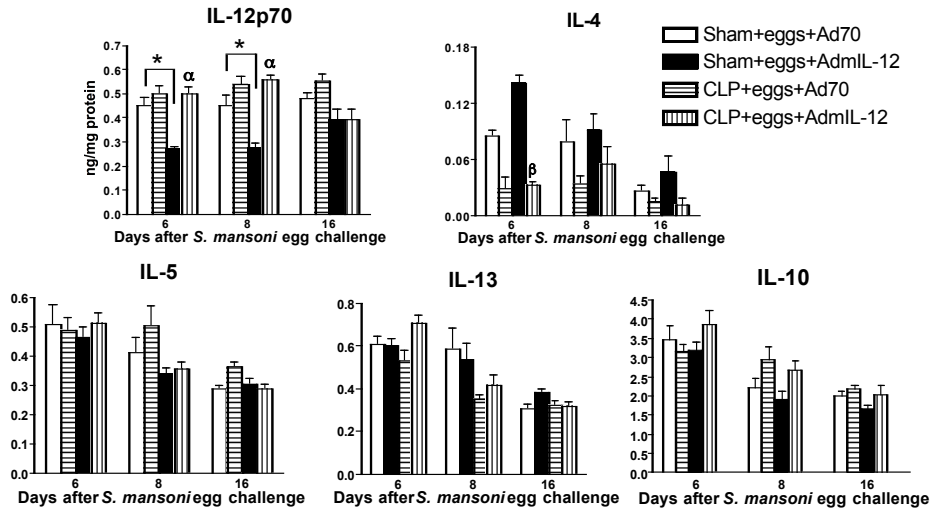

Figure 3.7 Introduction of IL-12-expressing adenovirus reverses the type-2 biased cytokine profile in the post-septic lung. Sham or post-septic mice (n=4-6/group/time point) were intratracheally administrated with $3X10^8$ PFU IL-12 expressing adenovirus (AdmIL-12) or control adenovirus (Ad70) immediately followed by 5,000 *S. mansoni* eggs given intravenously. At 6, 8 and 16 days after virus and egg challenge, mice were killed and the lower right lobes were collected from each mouse for cytokine analysis by ELISA. The results shown are representative of two experiments and are expressed as mean ± SEM. * P≤0.05 compared with IL-12p70 level in the lungs of sham mice receiving control virus at days 6 and 8. α P≤0.05 compared with IL-12p70 level in the lungs of CLP mice receiving Ad70 at days 6 and 8. β P≤0.05 compared with IL-4 protein level in the lungs of CLP mice receiving control virus at day 6.

IL-12 adenoviral vector restored the altered pulmonary granulomatous response in post-septic mice, but did not influence DC recruitment to lung

We sought to determine whether the compartmentalized overexpression of IL-12 influenced the alteration in granuloma size and eosinophil content in post-septic mice. Histological analysis showed that in Ad70 control groups, post-septic mice exhibited

significantly larger pulmonary granulomas than sham mice (30838.0±1166.4 μm^2 vs. 20713.6±1125.9 μm^2, n=5; p<0.001) at day 16. IL-12 vector significantly (p<0.001) decreased the granuloma size in post-septic mice compared with post-septic mice receiving Ad70 control virus (21792.3±1133.0 μm^2 vs. 30838.0±1166.4 μm^2, n=5; p<0.001) (Fig 3.8A). Interestingly, the significant reduction in granuloma size in post-septic mice induced by the IL-12 vector was also observed at days 6 and 8. Quantification of eosinophils showed that the IL-12 vector also significantly reduced eosinophil recruitment to post-septic lung granulomas at day 8 (0.322±0.023 vs. 0.488±0.027/100 μm^2, n=25; p<0.001) and day 16 (0.610±0.025 vs. 0.909±0.036/100 μm^2, n=25; p<0.001) (Fig 3.8B).

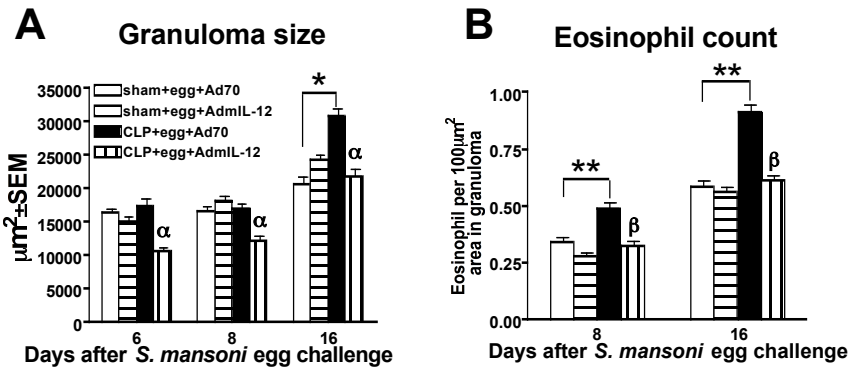

Figure 3.8 Introduction of IL-12-expressing adenovirus attenuates pulmonary granuloma pathology. Sham or post-septic mice (n=4-6/group/time point) were intratracheally administrated with $3X10^8$ PFU IL-12 expressing adenovirus (AdmIL-12) or control adenovirus (Ad70) immediately followed by 5,000 *S. mansoni* eggs given intravenously. At 6, 8 and 16 days after virus and egg challenge, mice were killed and the left lobe was collected from each mouse for histological analysis. (A) Quantification of granuloma size. * P≤0.05 compared with granuloma size measured in sham mice receiving Ad70 at day 16 after egg challenge. α P≤0.05 compared with CLP mice receiving Ad70 at all of three time points. (B) Eosinophil number. ** P≤0.05 compared with intragranulomatous eosinophil count in sham mice receiving Ad70 at days 8 and 16 after egg challenge. β P≤0.05 compared with intragranulomatous eosinophil count in CLP mice receiving Ad70.

Finally, to determine whether the attenuation of granulomatous response induced by the administration of AdmIL-12 was due to alteration in DC recruitment into the lungs of post-septic mice, we investigated the influence of IL-12 overexpression on the pulmonary DC component. Compared with post-septic mice receiving Ad70, post-septic mice receiving AdmIL-12 contained similar percentages of both DC subpopulations in the lung at day 8 post egg challenge, indicating that IL-12 overexpression did not influence the recruitment of DCs to lung of post-septic mice (Fig 3.9).

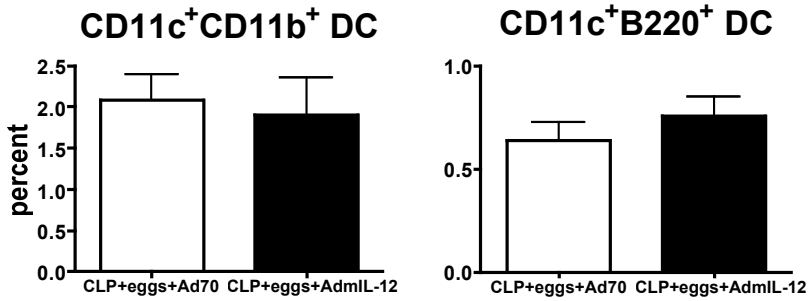

Figure 3.9 Introduction of IL-12-expressing adenovirus fails to enhance DC recruitment to post-septic lung. Post-septic mice (n=5/group) were intratracheally administrated with $3X10^8$ PFU AdmIL-12 or Ad70 immediately followed by 5,000 *S. mansoni* eggs given intravenously. Eight days later, lungs were harvested from both group and digested by collagenase IV. The dispersed lung cells were stained as follows: $CD11c^+$ in combination with $CD11b^+$ or $B220^+$, followed by flow cytometry analysis.

Discussion

Although the diagnosis and treatment of sepsis during the acute phase of this disease remain clinically challenging, recent attention has been directed towards studying the long-term consequence of severe sepsis on the host immune activity. It has been shown that patients who survive severe sepsis are at a higher risk for secondary infections, cancer, and other complications for up to 8 years after the septic episode (47, 48). This

suggests that the initial sepsis episode results in a profound alteration to the host innate and adaptive immune responses. Previously, our lab observed that post-septic mice were more susceptible to a pulmonary infection initiated by the fungal pathogen *A. fumigatus* (38). In the present study, we explored the pulmonary adaptive immune response following severe sepsis using freshly isolated *S. mansoni* eggs to induce a pulmonary granulomatous response. *S. mansoni* eggs promote the development of a type-2 biased pulmonary granuloma (144, 260), but this response is not lethal, even under an immunodeficient condition. In the present study, post-septic mice exhibited significantly larger granulomas with a higher eosinophil content. These histological findings in post-septic mice coincided with significantly decreased whole lung IL-12 levels and significantly increased whole lung levels of IL-4, IL-10 and IL-13 compared with sham mice during the formation and maintenance of the granulomatous response. Since DCs play a central role during adaptive immunity and can polarize a type-1 immune response through the production of IL-12 (132, 261), we hypothesized that the skewed adaptive response might have been a consequence of alterations in DC recruitment and/or DC phenotype. Post-septic mice had lower percentages of two DC subpopulations: $CD11c^{+}CD11b^{+}$ DCs and $CD11c^{+}B220^{+}$ DCs in the lungs. Also associated with changes in DC proportions, lung DC production of IL-12 was decreased whereas these cells produced much greater IL-10 in post-septic mice compared with DCs from sham mice. The importance of IL-12 to the pulmonary immune response was highlighted in subsequent experiments in which an IL-12 expressing adenovirus modulated the pulmonary granulomatous response to that typically observed in non-surgically manipulated mice. Furthermore, the mechanism whereby IL-12 overexpression

attenuated the granulomatous response in lung seemed to be downstream of DC function rather than DC recruitment, since the impaired DC recruitment induced by severe sepsis was not reversed by AdmIL-12 administration.

The polymicrobial peritonitis induced by CLP surgery closely mimics the clinical course of abdominal sepsis, resulting in a systemic inflammatory response syndrome (SIRS) (222). While the severity of sepsis depends on the number of punctures and the gauge (size) of needle, the CLP operation may lead to the formation of an intra-abdominal abscess (32), which is considered to be a protective "walling-off" response of the host against bacterial (262). In our system, we found that the localized inflammatory response in the peritoneal cavity was significantly decreased after three days post-CLP, evidenced by the normalized levels of inflammatory cytokines/chemokines (data not shown). This suggested the termination of the acute phase of the peritonitis; however, there is the possibility of a chronic inflammatory response continuing at the original puncture lesion.

S. mansoni egg-induced granulomas require the efficient and sequential recruitment of a series of immune effecter cells, including macrophages (263), $CD4^+$ T cells (264) and eosinophils (265). These specific immune cells form a strong cellular barrier and sequester the *S. mansoni* eggs from the surrounding healthy tissue. We and others have shown that the cytokine/chemokine network plays a central role in the recruitment and activation of immune cells around the eggs (255, 266). IL-4 operates as the key cytokine driving the type-2 response (144, 267). IL-5 induces the recruitment of eosinophils (144). IL-13 is a key mediator in the fibrotic process during schistosomiasis (145). Conversely, mice receiving exogenous IL-12 protein develop smaller pulmonary

and hepatic granulomas and exhibit less severe tissue fibrosis (154, 155). In the present study, the defect in IL-12 expression in the lungs of post-septic mice was associated with the development of a larger pulmonary granuloma characterized by significantly greater eosinophil numbers. In contrast, the administration of an IL-12 expressing gene vector attenuated the aggressive eosinophilic granulomatous response in post-septic mice. This supports the concept that IL-12 is a negative regulator of granulomatous responses induced by *S. mansoni* eggs, probably by altering the type-1/type2 cytokine profile, as shown in the present study. Although the increased expression of type-2 related cytokines IL-4, IL-5, IL-10, IL-13 in post-septic mice was observed early and not later during the granulomatous response, it appears that this early alteration in the cytokine profile has profound effects in progression of the granulomatous response. The development of a type-1 immune response requires the dominance of type-1 over type-2 cytokines, however the persistent impairment in IL-12 production in post-septic lungs appears to have resulted in the imbalance between the type-1 and type-2 response.

Several studies have focused on the recruitment and function of DCs in the granulomatous response, since the DC is a critical cell type linking the innate and adaptive immune response and the DC has the capacity to modulate type-1 or type-2 immune responses (139-141). Immature DCs capture antigen in the peripheral tissue and migrate to the draining lymph nodes (DLN), where they activate T-cells, and initiate the type-1 or type-2 immune response depending on the presence of IL-12 (132, 261) or IL-4 (132), respectively. Controversy surrounds the origin of DCs, as well as subsets and functional differences between subsets, despite considerable recent focus on DC biology. In the present study, two general DC subsets, myeloid ($CD11c^{+}CD11b^{+}$) and

plasmacytoid ($CD11c^{+}B220^{+}$) DCs were differentially measured (268), which have been reported to differ in TLR expression (269) and cytokine production in response to viral or bacterial infection (268). We observed the decreased recruitment of both DC subpopulations, and a defect in IL-12 production by a mixed population of DCs. With the development of pulmonary granulomas, local immunity typically proceeds to a type-2 biased response from a Th0 lung environment (143). However, in the post-septic lungs, the alteration in the recruitment of DCs combined with the alteration in their cytokine generation appeared to have accelerated the appearance and magnitude of the type-2 cytokine profile. This may be explained in part by the enhanced IL-10 production and impaired IL-12 production in pulmonary DCs from post-septic mice. IL-10 is a pleiotropic immunomodulatory cytokine that regulates the development and function of immune cells. IL-10 induces anergy in T cells by inhibiting the proliferation and cytokine production of these cells (270) and promotes the development of regulatory T cells (271). In addition to its effect on suppressing inflammatory and type-1 response, IL-10 strongly inhibits DC maturation and down-regulates MHC class II expression and the capacity to produce IL-12 (272-274). Also DC-derived IL-10 was required for the optimal development of type-2 cells in a DC transfer study (156). The ability of IL-10 to inhibit the type-1 response and IL-12 production is well established, although the manner in which IL-10 achieves its regulatory function has not been identified yet (118).

Several studies have reported the loss of DCs in the spleen and lymph nodes of septic patients or animals with severe sepsis (57)4, (54)7(58). In the present study, we found a similar loss of DCs in the lung, supporting the concept that severe sepsis results in the systemic loss of DCs, probably due to detrimental influence on DC development or

increased DC apoptosis. We observed an altered cytokine production profile by lung DCs in post-septic mice, which was directly related to the exacerbation of *S. mansoni* egg-induced granulomatous response. Although we did not investigate other functions of lung DCs, it is highly possible that severe sepsis also impairs antigen presentation by DCs. The present study suggested that the altered cytokine production in pulmonary DCs induced by severe sepsis was due to an alteration in DC phenotype rather than differential recruitment of different DC subtypes (275), but further study of the role of DC subsets in the altered adaptive immune response in the post-septic lung is warranted. Accordingly, the modulation of DC recruitment and/or function could be a potential therapeutic modality to restore normal adaptive immune response in post-septic hosts.

In summary, we examined the alteration in pulmonary adaptive immunity after a severe innate immune response (due to sepsis). Following sepsis, a type-2 biased cytokine environment was observed in the lung leading to an accelerated granulomatous response characterized by augmented eosinophilia. Severe sepsis resulted in decreased DC recruitment and alterations in IL-12 and IL-10 synthesis by these cells. Interestingly this response was reversed by the artificial elevation of IL-12 in the post-septic lung. This study supports the concept that a biased type-2 cytokine profile in the lung is a major consequence of severe sepsis, which may account for the altered host immunity seen in post-septic patients. Thus, restoring the cytokine balance in the post-septic lung may be a novel therapy in this highly susceptible group of patients.

Chapter 4

Epigenetic regulation of dendritic cell-derived interleukin-12 production facilitates immunosuppression following severe sepsis

Summary

A variety of alterations are known to occur in both clinical and experimental severe sepsis, including long-term immunosuppression. We have identified that the production of interleukin (IL)-12 by dendritic cells (DCs) is chronically suppressed during experimental severe sepsis induced by cecal ligation and puncture (CLP), which persisted six weeks after CLP. Using chromatin immunoprecipitation (ChIP) techniques, we have demonstrated that the deficiency in IL-12 production by post-septic DCs was accompanied by reciprocal changes in histone H3 lysine-4 trimethylation (H3K4me3) and histone H3 lysine-27 dimethylation (H3K27me2), as well as changes in cognate histone methyltransferase (HMT) complexes on both the *Il12p35* and *Il12p40* promoters. Interestingly, hyperacetylation of histone H3 on the *Il12p35* promoter was also observed in post-septic splenic DCs and correlated with transcription repression. The treatment with trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, significantly decreased IL-12 production by bone marrow-derived DCs generated from naïve mice. Furthermore, TSA *in vivo* treatment enhanced animal survival following CLP-induced severe sepsis. Collectively, these data implicate histone modification enzymes in

suppressing DC-derived IL-12, which may provide one of the mechanisms of long-term immunosuppression subsequent to the septic response.

Introduction

Severe sepsis is often characterized by an initially overwhelming systemic immune response syndrome in response to various etiologies, followed by a compensatory anti-inflammatory response syndrome (208). One of the sequels to septic response is immunosuppression that is responsible for severe sepsis-induced morbidity and mortality. Clinical records have clearly documented that patients who survive sepsis are more susceptible to secondary infections, cancer, and other complications long after being discharged from the intensive care unit (44). Interestingly, an epidemiologic study found that 8 years after surviving severe sepsis only 20% of this cohort were still alive (48), suggesting that the initial septic response resulted in a profound alteration in the host's innate and/or adaptive immune response. Recent experimental investigations have been directed towards studying the immunoregulation following severe sepsis and have identified alterations in dendritic cell (DC) numbers and functions as a potential mechanism responsible for the long-term reduction of the host's immune response (59). Furthermore, we have characterized DC-derived IL-12 as a key cytokine, which is significantly downregulated in post-septic animals. This severe sepsis-dependent alteration in DC-derived IL-12 subsequently renders these animals susceptible to a secondary microbial challenge and induces an exacerbated Th2 cytokine response in response to an acquired immune event (39, 40).

A growing body of evidence demonstrates that an epigenetic mechanism is involved in the regulation of gene expression during normal embryonic development (161, 165). Mis-regulation of histone modifying enzymes is commonly found in many diseases such as cancer and autoimmune diseases (276, 277). Among various histone modifications, methylation of histone H3 lysine-4 (H3K4) and H3K27 mediated by distinct histone methylmetransferase (HMT) complexes is highly correlated with transcription activation (278, 279) and repression (189), respectively. Furthermore, histone acetylation generally leads to chromatin opening up and gene transcription (168), although there are data showing that several histone deacetylase (HDAC) inhibitors decrease the production of proinflammatory cytokines (280-282). Both histone methylation and acetylation have been implicated in the development of immune system, especially in the differentiation and maintenance of Th1/Th2 memory cells (182). These data indicate that histone modifications play pivotal roles in immune reactivity. However, since most of this evidence has been derived from investigations assessing T cell activity, it remains elusive as to whether these essential epigenetic regulators are involved in modulating DC function, given an essential role of DCs both in innate and adaptive immune response (72). Using an experimental severe sepsis model induced by cecal ligation and puncture (CLP), we demonstrate that changes in histone methylation and acetylation correlate with long-term reduction in IL-12 production in splenic DCs. *In vitro* treatment with trichostatin A (TSA), a HDAC inhibitor, decreased IL-12 production by bone marrow-derived DCs (BMDCs). *In vivo* TSA treatment resulted in an enhanced animal survival following CLP-induced peritonitis. Thus, these epigenetic changes are

potentially important for the establishment of certain cellular memory, which is essential for long-term suppression of host immune system following severe sepsis.

Materials and Methods

Mice

Female C57BL/6 mice and female *Il10*$^{-/-}$ (C57BL/6-*Il10*^{tm1Cgn}) were purchased from Taconic Farms (Germantown, NY) and The Jackson Laboratory (Bar Harbor, ME), respectively. These mice were housed under specific pathogen-free conditions at The Unit for Laboratory Animal Medicine of the University of Michigan and treated in accordance with the guidelines of the animal ethical committee.

CLP

Sham and CLP surgery was performed as described in Chapter 2.

Isolation of splenic DCs

Spleens were collected from sham or CLP groups day 11 or 6 weeks after surgery. A sterile insert from 10 ml syringe was used to grind spleen tissue through 40 μM nylon membrane (BD Biosciences, San Jose, CA). After lysing red blood cells (RBCs) with ammonium chloride buffer (150mM NH_4Cl, 10mM NaHCO3, 1mM tetrasodium EDTA), cell suspensions were enriched with anti-CD11c magnetic beads (Miltenyi Biotek, Auburn, CA). Briefly, the cells were resuspended in 400μl of buffer (1×PBS/0.5% BSA) containing 100μl of CD11c microbeads. After a 15-min incubation at 4°C, free beads were washed away and the cells conjugated with beads were passed through MS^+ columns for positive selection. Purified DCs were counted on a hemocytometer and subsequently diluted at $2X10^6$/ml. The aliquots of 200μl containing these cells were

added to 96-well plates, and stimulated with a group of TLR agonists: 2.5μg/ml Pam3cys (EMC microcollections, Tubingen, Germany), 1μg/ml lipopolysaccharide (LPS from *Escherichia coli* 0111:B4; Sigma-Aldrich, St. Louis, MO), or 2μM mouse CpG-DNA (HyCult Biotechnololgy, Canton, MA). After 6 hrs of stimulation, total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and quantitative Real-time PCR (Taqman) was performed to measure gene expression of inflammatory cytokines (Applied Biosystems, Foster City, CA). After 24 hrs of stimulation, cell free supernatant from each sample was subject to ELISA to measure protein levels of inflammatory cytokines. In the experiments neutralizing IL-10, 1 or 10 μg/ml polyclonal neutralizing anti-10 antibody (R&D systems, Rochester, MN) was used with goat IgG as control.

Generation of mouse BMDCs

BMDCs were generated as descried previously with modification (39). Briefly, BM cells were flushed out from the femurs and tibias of naïve female C57BL/6 mice, and RBCs were lysed with ammonium chloride buffer. Whole BM cells were then cultured in RPMI 1640 (Mediateck, Chestnut Hill, MA) supplemented with 10% fetal calf serum (Atlas Biologicals, Fort Collins, CO), 2 mM L-glutamine (Mediateck), 100 U/ml penicillin, 100 μg/ml Streptomycin (Gibco, Carlsbad, CA), 50 μg/ml gentamicin (Gibco), 1 mM sodium pyruvate (HyClone, Logan, UT), 1X nonessential amino acids (Mediateck), 50 μM 2-mercaptoethanol (Sigma) and 20 ng/ml recombinant murine granulocyte-macrophage colony-stimulating factor (rmGM-CSF) (R&D systems). Three days later, 5 ml culture medium containing 10ng/ml rmGM-CSF was added into culture flask. Non-adherent cells were collected at day 6 of culture and then subject to positive selection with anti-CD11c magnetic microbeads (see above). The cells were stimulated with

1μg/ml LPS in the absence or presence of TSA (Sigma) at a series of increasing concentrations.

Messenger RNA decay assay

BMDCs from C57BL/6 mice were stimulated with 1 μg/ml LPS alone. Four hours later, 5 μg/ml Actinomycin D (ActD from Sigma) was added into culture for 1 h to stop RNA synthesis, followed by further incubation for 0-3 h with TSA (10 ng/ml). The remaining IL-12p35 mRNA level was measured by Taqman RT-PCR at different time points after incubation with ActD (0, 1, 2 and 3 h).

***In vivo* TSA treatment**

TSA was dissolved in DMSO to prepare stock solution of 1 mg/ml, which was further diluted 10 times with saline in the time of *in vivo* injection. Ten-week-old female C57BL/6 mice were treated with *s.c.* injection of TSA at 1mg/kg body weight daily for 1 week. The dose of TSA was based on published results with modification, which demonstrated significant inhibition of tumor growth and proinflammatory cytokine production (280, 283). Control mice were injected with saline-diluted DMSO alone. There were no differences between TSA-treated and vehicle-treated mice in weight, food and water consumption. CLP surgery with either 9 punctures or through and through puncture was performed and survival was monitored for 7 days following surgery.

Quantitative Real-time PCR (Taqman)

Analysis of transcripts with Taqman was performed as described in Chapter 2.

Measurement of cytokine protein levels

Concentrations of IL-10, IL-12p40 and IL-12p70 were measured in BMDCs and isolated splenic DCs cell culture supernatants using a Luminex Bio-Plex 200 system (Bio-Rad

Laboratories, Hercules, CA), according to manufacturer's protocol. Briefly, 96-well multiplex assay plate was coated with anti-mouse cytokine 3-plex conjugated beads (IL-10, IL-12p40 and IL-12p70). Plates were rinsed 2 times with wash buffer A and cell-free supernatants, as well as series diluted standard cytokine, were loaded and incubated for 30 min in room temperature with gentle vortex. After 3 washings, the biotinylated mouse cytokine 3-plex detection antibody was added for 30 min in room temperature with gentle vortex. The plates were washed again and PE-conjugated streptavidin was added for 10 min in room temperature with gentle vortex. Plates were washed and read using luminx Bio-Plex 200 system plate reader. Murine stock cytokines of known concentrations, which came together with kit, were used to generate the standard curves from which the cytokine concentrations present in the samples were calculated. The threshold of each cytokine is 5 pg/ml.

Chromatin immunoprecipitation (ChIP) assay

The ChIP procedure was performed using an assay kit (Upstate, Charlottesville, VA) according to the manufacturer's instructions. Briefly, $1X10^6$ isolated splenic DCs or BMDCs were stimulated with 1 μg/ml LPS for 6 hrs in the absence or presence of TSA at a series of increasing concentrations. DNA-protein structure was then cross-linked by 1% (v/v) formaldehyde for 10 min at 37°C. Cells were collected and lyzed in 400 μl SDS lysis buffer. The resulting lysates was sonicated to obtain DNA fragments ranging from 200–1,000 bp using a Branson Sonifier 450 (VWR Scientific, West Chester, PA) under the following condition: 6 times for periods of 15 s each. After centrifuge, the supernatant containing chromatin was diluted from which an aliquot (2% volume) was saved to indicate the input DNA in each sample. The remaining chromatin fractions were

precleared with salmon sperm DNA/protein A agarose beads followed by immunoprecipitation with the following antibodies: anti–acetyl histone H3 (06-599; Upstate), anti–H3K4me3 (ab8580; Abcam, Cambridge, MA), anti–H3K27me2 (07-452; Upstate), anti–MLL, anti-WDR5 and anti-EED (Dr. Yali Dou) (279), anti-RbBP5 (A300-109A; Bethyl Laboratories, Monttgomery, TX), or anti-SUZ12 (ab12073; Abcam) overnight at 4°C with gentle rotation. Cross-linking was reversed for 4 h at 65°C and was followed by proteinase K digestion. DNA was purified by standard phenol/ chloroform and ethanol precipitation, and subjected to real-time PCR. Primers for mouse *Il12p35* promoters: forward, 5'- GGGACGGTCCCGAATCTC; reverse, 5'- GGTGGCGCTTTCGAATTAAC, primers for mouse *Il12p40* promoter: forward, 5'- TT CCCCCAGAATGTTTTGACA; reverse 5'- TGATGGAAACCCAAAGTAGAAACTG.

Statistical analysis

Results were expressed as means ± SEM. Student's *t* test and one-way ANOVA were used to detect statistical significance.

Results

Deficiency in IL-12 production by post-septic splenic DCs

DCs serve as sentinel at portals of pathogen entry and orchestra host innate and adaptive immunity against invading pathogens by the production of inflammatory cytokines and the subsequent presentation of antigen to naïve T cells in the draining lymph nodes (72). One of the key cytokines that facilitates these important immune responses is IL-12, which is a heterodimeric inflammatory cytokine composed of an IL-12p35 and IL-12p40 subunit (118). Since IL-12 generated by DCs is the key molecule to

direct a type-1 immune response (132), we sought to determine whether severe sepsis altered the capacity of DCs to produce IL-12. The polymicrobial peritonitis induced by cecal ligation and puncture (CLP) procedure closely mimics the clinical course of abdominal sepsis (32). At day 11 after surgery, splenic DCs were purified from sham or CLP mice and stimulated with a series of toll-like receptor (TLR) agonists: Pam3Cys (TLR2), LPS (TLR4) or mouse CpG-DNA (TLR9). Real-time PCR showed that post-septic splenic DCs had significant lower mRNA levels of both *Il12p35* and *Il12p40*, but higher mRNA level of regulatory cytokine *Il10* compared with similarly treated sham splenic DCs (Fig 4.1A). Consistently, post-septic DCs generated significantly lower protein level of IL-12p70, but higher protein level of IL-10 in response to the stimulation with TLR agonists in *in vitro* culture systems (Fig 4.1B). Lung DCs isolated from day 11 after CLP showed a similar deficiency in IL-12 production compared with lung DCs from sham mice (data not shown).

As a regulatory cytokine, IL-10 has been reported to directly inhibit IL-12 production both *in vivo* and *in vitro* (129, 284). To test the hypothesis that the deficiency in IL-12 production by post-septic DCs was due to excessive IL-10 production in culture system, two concentrations of specific anti-IL-10 antibody (1 and 10 μg/ml) were used to block IL-10 production by both sham and post-septic splenic DCs in response to LPS stimulation (Fig 4.1C left panel). Although IL-12 production by sham DCs was increased in the presence of anti-IL-10 in a dose-dependent manner, IL-12 production by post-septic DCs was not rescued by anti-IL-10 (Fig 4.1C right panel), which suggested that the deficiency in IL-12 production by post-septic DCs was not due to excessive IL-10 production.

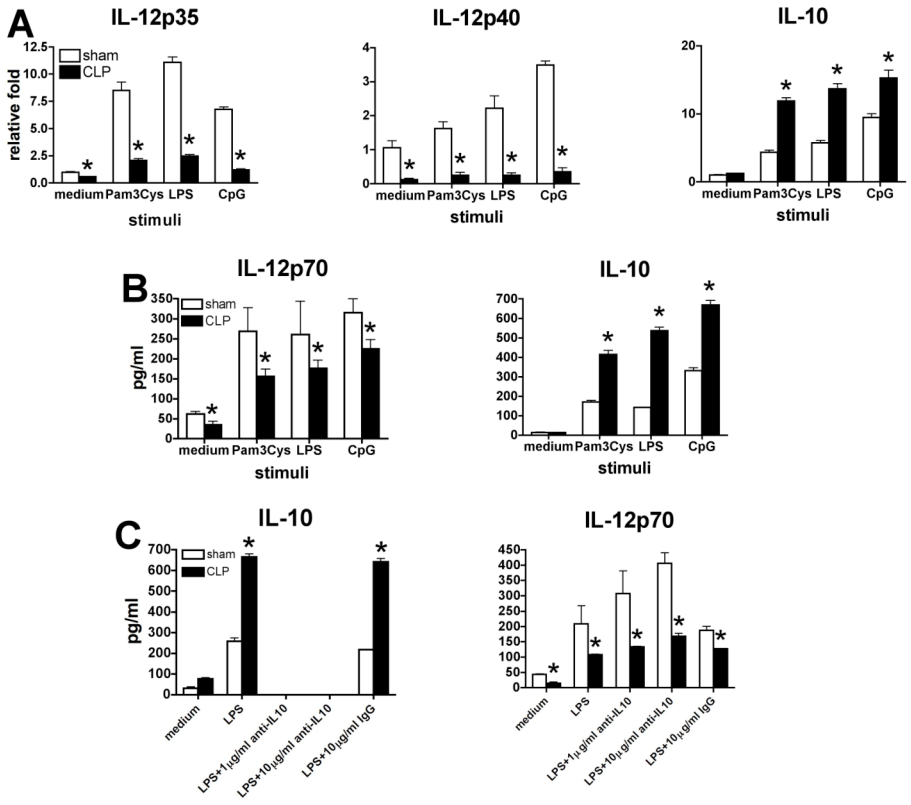

Figure 3.1 Impaired IL-12 production by splenic DCs day 11 following experimental peritonitis. (A) Purified splenic DCs from septic or control mice day 11 after surgery were stimulated by a series of TLR agonists. Messenger RNA levels of the two IL-12 subunits and IL-10 were determined by Taqman. (B) Protein levels of IL-12p70 and IL-10 were measured by Bio-plex. (C) Purified splenic DCs from septic or control mice day 11 after surgery were stimulated by LPS, in the absence or presence of anti-IL-10 antibody. Protein levels of IL-12p70 and IL-10 were determined by Bio-plex. Results in A, B and C represent three to five independent experiments and are expressed as mean ± SEM. *, $P \leq 0.05$ compared with cytokine levels measured in splenic DCs from sham mice.

We next determined whether the deficient phenotype in IL-12 production exhibited by post-septic DCs was preserved over time in animals surviving experimental sepsis. In this set of studies, cytokine production by splenic DCs was determined 6 weeks after sham and CLP procedures. No difference in weight or behavior was observed between the two groups of mice at this time point. However, splenic DCs recovered from post-septic mice showed significantly lower mRNA levels of *Il12p35* and

Il12p40 and produced lower IL-12p70 protein level in response to TLR agonist stimulation compared with similarly treated control splenic DCs (Fig 4.2A and B). No difference in IL-10 production was observed between sham and post-septic splenic DCs, which suggested that the deficiency in IL-12 production by post-septic DCs, at this time point, was not due to regulatory effects of IL-10.

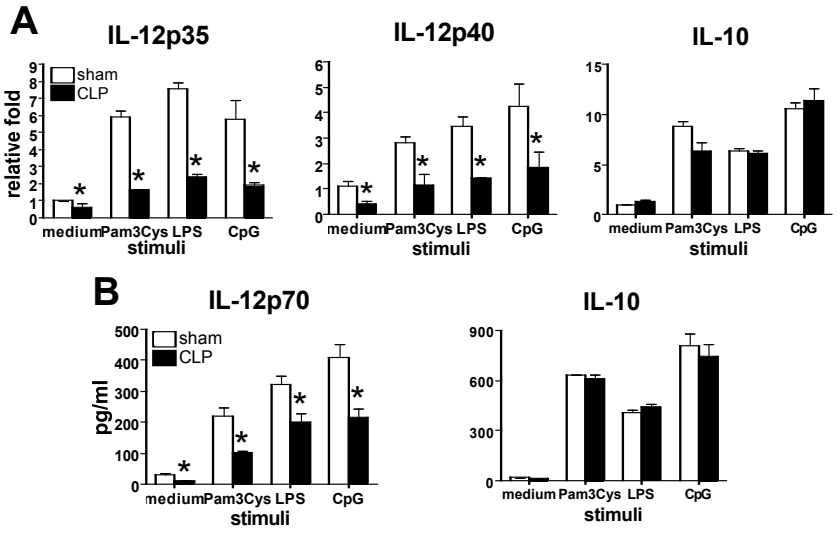

Figure 4.2 Long-term deficiency in IL-12 production by splenic DCs following experimental peritonitis. (A) Purified splenic DCs from septic or control mice 6 weeks after surgery were stimulated by a series of TLR agonists. Messenger RNA levels of the two IL-12 subunits and IL-10 were determined by Taqman. (B) Protein levels of IL-12p70 and IL-10 were measured by Bio-plex. *, $P \leq 0.05$ compared with cytokine levels measured in splenic DCs from sham mice.

Altered histone modifications on *Il12* promoters in post-septic splenic DCs

Histone H3K4 and K27 methylations are required for the establishment and maintenance of gene regulation status (177, 285). Specifically, methylation of H3K4, mediated by MLL family histone methyltransferases (HMTs), is correlated with transcription activation while H3K27, mediated by EZH2, is correlated with gene

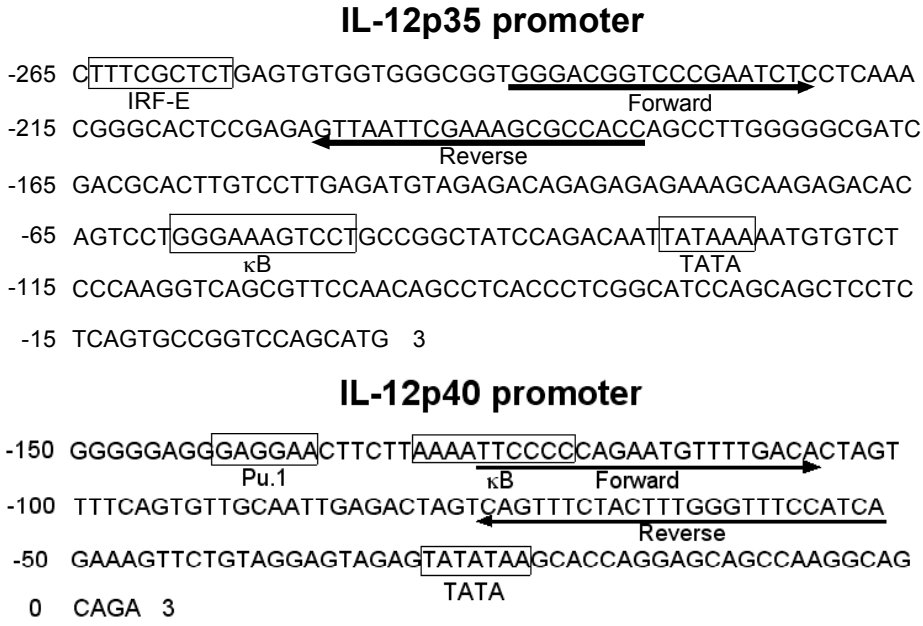

Figure 4.3 Promoter sequence of *Il12p35* and *Il12p40* with consensus motifs for key transcription factors. The positions of Taqman primers around promoter regions of *Il12p35* and *Il12p40* during ChIP experiments are indicated.

silencing (182). The long-term maintenance of the deficiency in IL-12 production prompted us to hypothesize that an epigenetic mechanism might be involved in this process. We tested this hypothesis by chromatin immunoprecipitation (ChIP) assay using anti-H3K4 trimethylation (H3K4me3) and anti-H3K27 dimethylation (H3K27me2) antibodies. Taqman primers were designed at the promoter regions of *Il12p35* and *Il12p40* around the essential binding sites for several key transcriptional factors, such as Pu.1, IFN Regulatory Factor (IRF) and κB (Fig 4.3). We found that splenic DCs from control mice showed a relative high H3K4me3 and low H3K27me2 at both *Il12p35* and *Il12p40* promoters, indicating that both of these genes are surrounded by permissive chromatin structure and are poised for expression upon exposure to stimulus. Conversely, splenic DCs at day 11 after CLP showed significantly decreased H3K4me3 and increased

H3K27me2 at *Il12p35* and *Il12p40* promoters, suggesting that the chromatin status was skewed towards a more repressive status after the CLP procedure (Fig 4.4A). This is a long-term effect as the repressive chromatin environment is present at 6 weeks after CLP; significantly decreased H3K4me3 and increased H3K27me2 at both *Il12p35* and *Il12p40* promoters were observed in post-septic splenic DCs (Fig 4.4B). Thus, the altered status of H3K4 and H3K27 methylations at *Il12p35* and *Il12p40* promoters correlated well with the decreased gene expression.

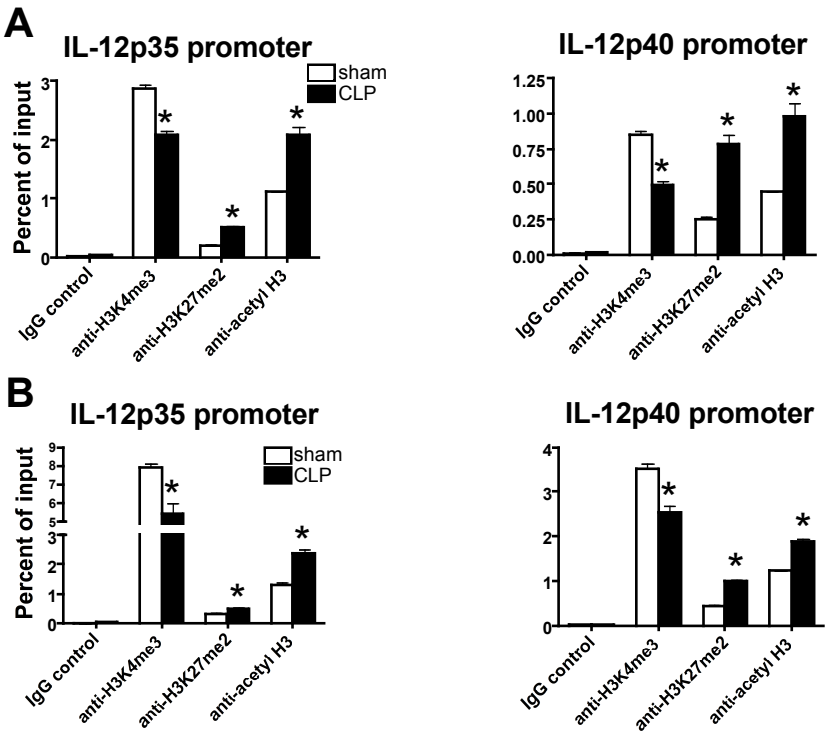

Figure 4.4 Alterations in histone H3 methylation and acetylation on *Il12* promoters in post-septic splenic DCs. Splenic DCs were isolated from sham and CLP mice at either day 11 (A) or 6 weeks (B) after surgery. ChIP assay was performed to determine the histone H3 methyaltion and acetylation status at the promoter regions of *Il12p35* and *Il12p40*, respectively. The results shown are representative of two to three independent experiments and are expressed as mean ± SEM. * P≤0.05 compared with splenic DCs from sham mice.

Differential recruitment of core components of HMT complexes on *Il12* promoters

We next explored the molecular mechanism whereby post-septic splenic DCs exhibited decreased H3K4me3 and increased H3K27me2 on *Il12* promoters. Methylations at H3K4 and H3K27 sites are mainly mediated by MLL family H3K4 methyltransferases and PRC2 complex, respectively (189, 278). MLL family HMTs share common structures, which include the catalytic subunit, as well as several structural proteins including the WD40-repeat protein WDR5, RbBP5 and Ash2L (186, 187). PRC2 complex consists of the core components EZH2, Suppressor of Zeste 12 (SUZ12), and embryonic ectoderm development (EED) (189). The activities of both MLL family HMTs and PRC2 require the interaction of the catalytic subunits with other components. To assess whether the alterations in histone methylation were the result of the altered recruitment or the complex integrity of MLL complex and/or PRC2 complex at *Il12* promoters, respectively, we performed ChIP assays using a series of antibodies directed against the core components of these two complexes. Although there was no difference in MLL1 binding on H3K4 at *Il12* promoters between post-septic splenic DCs and control DCs, the recruitment of WDR5 and RbBP5 on *Il12* promoters were significantly decreased in post-septic DCs compared with sham DCs (Fig 4.5). In contrast, the recruitment of EED and SUZ12 were significantly increased in post-septic DCs compared with sham DCs (Fig 4.5A). EZH2 binding was not investigated, because of the lack of ChIP-grade antibody. Thus, our data suggests that alterations in the levels of H3K4me3 and H3K27me2 are related to the inactivation of the corresponding complexes on *Il12* promoters. The same observations were made in mice 6 weeks after CLP (Fig 4.5B).

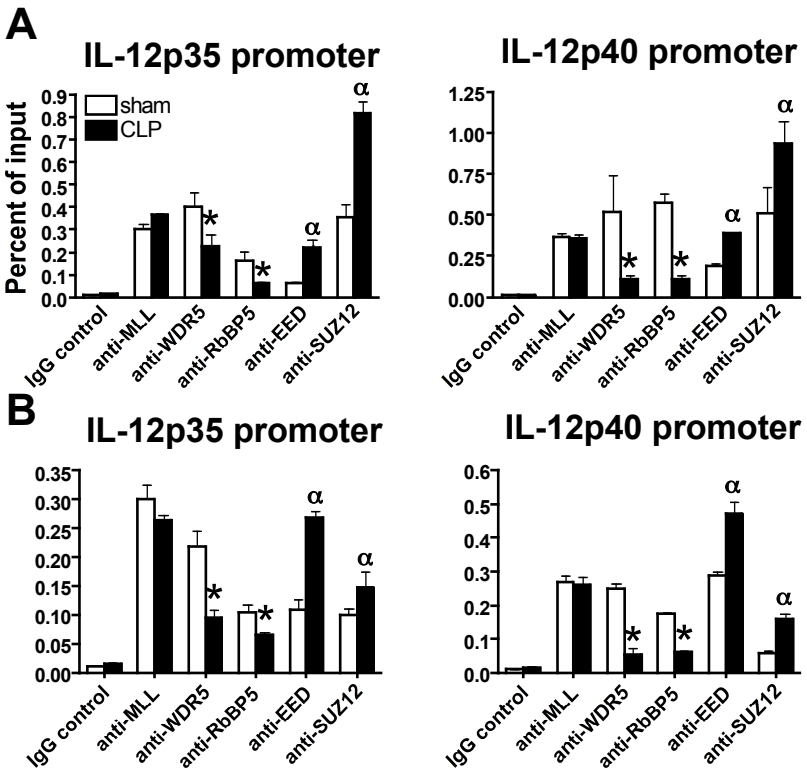

Figure 4.5 Altered recruitment of HMT complexes at Il12 promoters in post-septic splenic DCs. Splenic DCs were isolated from sham and CLP mice at either day 11 (A) or 6 weeks (B) after surgery. Using appropriate antibodies directed against the core components of the MLL and PRC2 complexes, recruitment of the core components to the promoter regions of *Il12p35* and *Il12p40* was determined by ChIP assay. The results shown are representative of two to three independent experiments and are expressed as mean ± SEM. * P≤0.05 compared with splenic DCs from sham mice.

HDAC inhibitor downregulates *Il12p35* gene transcription and IL-12p70 production

In addition to methylation, we also assessed the histone acetylation level in post-septic DCs. To our surprise, splenic DCs at either day 11 or 6 weeks after CLP exhibited enhanced histone acetylation on *Il12* promoters compared with sham splenic DCs (Fig 4.4), which was accompanied with the downregulation of the genes. This result suggests that histone acetylation is associated with decreased IL-12 production by post-septic DCs, which contrasts with the general assumption that histone acetylation is associated with

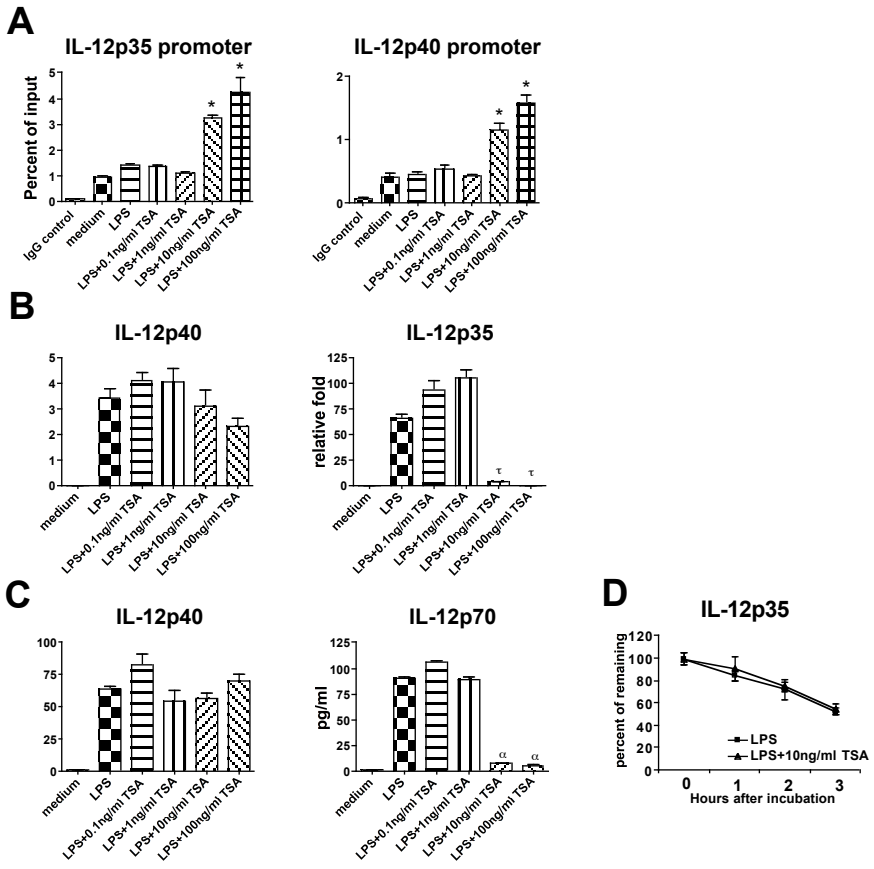

Figure 4.6 TSA-induced hyperacetylation of histone H3 is associated with downregulation of *Il12p35* transcription and IL-12p70 production. (A) Naïve BMDCs were stimulated with LPS, in the absence or presence of a series of increasing concentrations of TSA. * P≤0.05 compared with H3 acetylation at *Il12* promoters in LPS-stimulated BMDCs. (B) Messenger RNA levels of *Il12p35* and *Il12p40* were measured by Taqman in LPS-stimulated BMDCs in the absence or presence of increasing concentrations of TSA. τ P≤0.05 compared with *Il12p35* mRNA level in LPS-stimulated BMDCs. (C) Protein production of IL-12p70 and IL-12p40 by LPS-stimulated BMDCs were measured by Bio-plex ELISA, in the absence or presence of a series of increasing concentrations of TSA. α P≤0.05 compared with IL-12p70 produced by LPS-stimulated BMDCs. (D) BMDCs were stimulated with 1 μg/ml LPS for 4 h, then 5 μg/ml ActD was added into culture for 1 h to stop RNA synthesis, followed by further incubation for 0-3 h with TSA (10 ng/ml). The remaining IL-12p35 mRNA level was measured by Taqman RT-PCR at 0, 1, 2 and 3 h after incubation with ActD. The results shown represent three to five independent experiments and are expressed as mean ± SEM.

transcription activation. A similar observation was made showing that HDAC inhibitors (HDIs) downregulated IL-12 production both *in vitro* (286) and *in vivo* (280). To further

understand the role of histone acetylation in IL-12 repression, we investigated the effects of an HDAC inhibitor on the transcription of two subunits of IL-12 in bone marrow-derived DCs (BMDCs) generated from naïve mice. Trichostatin A (TSA) is a broadly used HDI and induces hyperacetylation of histones. A series of increasing concentrations of TSA were used to increase acetylation of histones in BMDCs in the presence of LPS stimulation. We found that high concentrations of TSA (10 and 100 ng/ml), which did not alter cell viability, significantly increased histone H3 acetylation at both *Il12p35* and *Il12p40* promoters (Fig 4.6A); meanwhile, they efficiently abolished the LPS-induced increase in *Il12p35* transcript and IL-12p70 protein production, but not *Il12p40* gene transcription and translation (Fig 4.6B and C). Messenger decay assay rules out the possibility that TSA-induced decrease in *Il12p35* mRNA level in the presence of LPS stimulation was due to an increased mRNA degradation, since 10 ng/ml TSA did not alter the degradation rate of *Il12p35* mRNA after the incubation of actinomycin D (ActD) (Fig 4.6D). In contrast, low concentrations of TSA (0.1 and 1 ng/ml) altered neither histone acetylation on *Il12* promoters nor LPS-induced IL-12p40 and IL-12p70 protein production. Interestingly, we observed a slight increase in *Il12p35* transcript level in the presence of low concentrations of TSA (0.1 and 1 ng/ml) in a dose-dependent manner (Fig 4.6B). We hypothesized that this was due to the downregulation of LPS-induced IL-10 production by TSA in a dose-dependent manner (Fig 4.7A and B). BMDCs generated from naïve WT or IL-10 deficient ($Il10^{-/-}$) mice were stimulated with LPS in the absence or presence of a series of increasing concentrations of TSA. Again, LPS-induced *Il12p35* mRNA level was slightly increased in the presence of low concentrations of TSA (0.1 and 1 ng/ml). However, this slight increase was completely abolished in similarly treated

Il10$^{-/-}$ BMDCs (Fig 4.7C), suggesting that slight increase in *Il12p35* mRNA level in the presence of low concentrations of TSA was due to the downregulation of LPS-induced IL-10 production by TSA. Thus, our data indicated that histone hyperacetylation on *Il12p35* promoter induced by TSA decreased *Il12p35* transcription and IL-12p70 production. The transcription and translation of *Il12p40* were not influenced by histone hyperacetylation on its promoter.

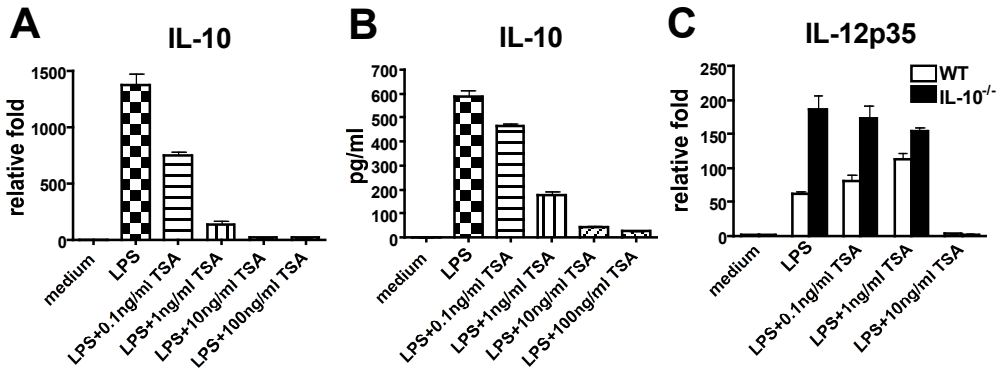

Figure 4.7 Effects of TSA on LPS-induced *Il12p35* transcription and IL-10 production by BMDCs. (A) Naïve BMDCs were stimulated with LPS, in the absence or presence of a series of increasing concentrations of TSA. Messenger RNA levels of *Il10* were measured by Taqman RT-PCR. (B) Protein levels of IL-10 were measured by Bio-plex. (C) BMDCs generated from naïve WT and IL-10$^{-/-}$ mice were stimulated with LPS, in the absence or presence of a series of increasing concentrations of TSA. Messenger RNA levels of *Il12p35* were measured by Taqman RT-PCR.

***In vivo* TSA treatment enhances animal survival in CLP-induced moderate sepsis**

The hallmark of acute phase of sepsis is an exacerbated production of inflammatory cytokines/chemokines, the so-called "cytokine storm", resulting in multiple organ dysfunction and death (11). Our observation that TSA treatment inhibits the production of proinflammatory cytokine IL-12, in conjunction with other reports with similar observations (280, 286), leads us to hypothesize that TSA treatment may rescue animals from CLP-induced mortality. For a long time our group has observed that the severity of

sepsis could be controlled by needle size, number of punctures and antibiotics utilization. While CLP with 9 punctures results in severe sepsis, CLP with through and through puncture leads to moderate sepsis (data not shown). Although TSA *in vivo* treatment failed to influence animal survival in severe sepsis model induced by CLP with 9 punctures, animal survival rate in moderate sepsis induced by CLP with through and through puncture were significantly increased from 56% to 100% by TSA treatment (Fig 4.8). This observation suggests that TSA treatment cannot completely reverse cytokine storm in severe sepsis, and it requires other synergetic interventions to achieve clinical efficiency.

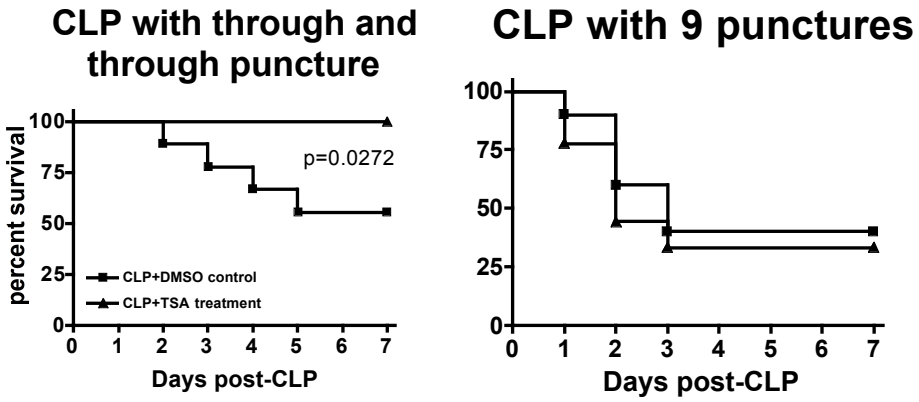

Figure 4.8 In vivo TSA treatment enhances animal survival in moderate sepsis. Mice were *s.c*. injected with TSA at 1mg/kg body weight daily for 1 week, and subjected to CLP surgery. Similarly diluted DMSO was used as control. CLP with 9 punctures was used to induce severe sepsis, and CLP with through and through puncture was used to induce moderate sepsis. Animal survival was monitored for 7 days after surgery.

Discussion

The initiation and maintenance of the septic response is dependent on ill-understand cellular and molecular mechanisms. In addition to a significant challenge of the diagnosis and treatment of sepsis during the acute phase of this disease, clinical

records have clearly documented a sustained anti-inflammatory or immunosuppressive state in patients who survive severe sepsis following the initial hyper-inflammatory response. The "immunoparalysis" in septic patients is manifested by an inability to eradicate the primary infection and/or the development of new secondary infections (44). Thus, the initial episode of severe sepsis, characterized by a dysregulated inflammatory response, leads to long-lasting complications regarding how the host responds to and deals with subsequent challenges. Although the depletion of immune cells such as DCs and lymphocytes due to extensive apoptosis has been related to long-term immunosuppression (50), it seems unlikely that the decrease in immune cell numbers is the only mechanism whereby septic hosts fail to mount an appropriate immune response, since we have observed a gradual restoration of immune cells after the initial apoptosis-induced immune cell depletion. Therefore, dysfunction of immune cells, probably delivered by to a long-term maintenance of abnormal gene expression pattern, is a potential mechanism worthy of extensive investigation.

Epigenetic mechanism of gene regulation has been well established to play an essential role in the maintenance of gene expression pattern during embryogenesis and cancer (161, 276). The combination of individual histone modifications, catalyzed by several families of histone-modifying enzymes, form a "histone code" that constitutes an important epigenetic determinant of the transcriptional state (165, 166). It has been recently proposed that histone modifications regulate gene expression pattern in immune system (182). The chromatin remodeling events have been documented on the *IFNγ* promoter, *Il4* locus (including *Il5*, *Rad50*, *Il13* and *Il4*) and *Il17* promoter during the differentiation of Th1, Th2 and Th17 memory T cells (182, 204). LPS-tolerant

macrophages have been shown to exhibit the "repressive" histone modifications at the promoters of tolerizeable class of genes (205). MLL, a HMT enzyme specific for H3K4 methylation has been reported to be essential for the maintenance of memory Th2 cell response (203), while EZH2, a core component of polycomb repressive complex 2 (PRC2), has been identified as an important molecule for the continuance of a Th1 cytokine phenotype via silencing of the cytokines *Il4* and *Il13* (182, 200). In contrast to this clear evidence showing epigenetic alterations in some specific conditions in the immune system, the signaling pathways leading to those epigenetic changes remain largely unexplored. In memory T cells, the candidate pathways include T cell receptor and cytokine/cytokine receptor signaling pathways, whereas the signaling pathways or key molecules that mediate the long-term histone modifications on *Il12* promoters in splenic DCs after the induction of severe sepsis remain to be elucidated. Based on the fact that overwhelmed pathological infection and dysregulated cytokine storm are hallmarks of severe sepsis, it is reasonable to hypothesize that toll-like receptor signaling and cytokine/cytokine receptor signaling pathways are potential contributors to the epigenetic changes described herein, and these pathways are worthy of further investigation.

In the present study, we report for the first time an epigenetic mechanism of gene regulation via histone methylation and acetylation in DCs. We characterized several histone modifications on various *Il12* promoter regions as important long-term regulators for IL-12 production by splenic DCs after the induction of severe sepsis. We postulate that these alterations might contribute to immunosuppression observed in post-septic animals and patients. We have observed both H3K4me3 and H3K27me2 at *Il12*

promoters. The existence of bivalent domains of H3K4me3 and H3K27me2 has recently been reported in embryonic stem cells (192) and in terminally differentiated T cells (287). It suggests that the relative ratio between these two marks determines transcription outcomes. Consistently, a decreased ratio between H3K4me3 and H3K27me2 on the promoter regions of *Il12p35* and *Il12p40* was associated with downregulation of gene expression in post-septic splenic DCs in the present study. Furthermore, the decreased ratio of H3K4me3 and H3K27me2 was a result of altered binding of cognate HMT complexes. In addition to the changes in histone methylation, we also found that post-septic DCs exhibited enhanced histone acetylation at *Il12* promoters. Although histone hyperacetylation mostly correlates with gene activation, our data showed that pharmacological induction of histone hyperacetylations on the *Il12p35* promoter resulted in decreased *Il12p35* transcription and IL-12p70 production in BMDCs. Given a recent report that H3K4me3 can be recognized by both HDAC and histone acetyltransferase (HAT), the observed hyperacetylation during gene activation is likely due to the decreased recruitment of HDACs upon H3K4 trimethylation (196). The mechanisms that link H3K4 methylation to histone deacetylation at *Il12* promoters will be an area of future studies.

Chapter 5

Severe sepsis-induced dysfunction in DC: T cell interactions

Summary

Severe sepsis has been defined as a systemic inflammatory disorder represented by an initial uncontrolled inflammatory response. Clinical and experimental studies have shown that the acute phase of severe sepsis is followed by a chronic immunosuppression phase. While all the mechanisms that contribute to the long-lasting immunosuppression are not clear, it is known that tissue dendritic cells (DCs) are initially depleted during the early stage of severe sepsis via apoptotic mechanism, but are subsequently repopulated. Interestingly, the repopulated DCs appear to have significant alterations that contribute to the persistent state of immunosuppressioin. We now show that in addition to aberrant cytokine production (chapter 3 and 4), post-septic DCs exhibit an impaired antigen-presenting capacity in co-culture with T cells. T cells cultured with DCs recovered from surviving septic animals generate significantly higher levels of T helper 2 (Th2) cytokines, but lower level of interferon-γ (IFN-γ) compared to sham DCs, indicating that post-septic DCs have a Th2-polarized capacity. Using microarray analysis followed by Taqman, we identified a dramatic upregulation of a cysteine proteases inhibitor (stefin A1) in bone marrow, lung and splenic DCs from animals that survive severe sepsis, which potentially may contribute to the sepsis-induced DC defect in antigen-presenting function. These data further indicate that severe sepsis results in an additional DC

dysfunction, which leads to altered interactions with T cells and an impaired host immune response.

Introduction

The clinical manifestations of sepsis are usually the initial consequence of a dysregulated host immune response to either a known or uncharacterized insult (9, 45). Despite advances in intensive care technology and mechanical ventilator support, efficacious pharmacologic options are limited (17). In addition, epidemiologic studies have demonstrated that patients who survive severe sepsis have a significant risk of dying that exceeded normal predictions for several years after recovering from severe sepsis (47, 48). These patients were shown to be more susceptible to secondary infections, cancer, and other complications long after being discharged from the intensive care unit (47, 48). Therefore, these investigations suggest that the initial episode of severe sepsis results in a profound alteration in the host's innate and/or adaptive immune response.

Recent experimental investigations studying the immunoregulation following a severe innate immune response due to sepsis have identified alterations in DC number and function as potential mechanisms for the long-term alteration in the host's immune response (59). We have previously characterized DC-derived IL-12 as a key host defense cytokine that is significantly downregulated even 6 weeks after the original septic event, which was associated with epigenetic changes at the *Il12* promoters (chapter 4). This sepsis-dependent alteration in DC-derived IL-12 has been shown to render animals susceptible to a secondary microbial challenge and induces an exacerbated Th2 cytokine response in response to an acquired immune event (39, 40). However, in the context of

severe sepsis, the antigen (Ag)-presenting function of DCs, which is essential for the initiation of host adaptive immune response, has not been thoroughly investigated.

Adaptive immune responses are initiated in the T cell-zones of secondary lymphoid organs, where naïve T cells encounter antigen-bearing mature DCs (72). In order to present antigen to T cell receptor (TCR)-specific naïve T cells, the DCs phagocytose and degrade the antigen into optimal peptides (antigenic epitopes), but complete destruction of the antigen by endosomal/phagosomal processing enzymes is avoided (288). The efficient formation of MHCII-peptide complex in late endosome requires endosomal/phagosomal proteolysis both to generate suitable peptides and to remove the dedicated class II MHC chaperone invariant chain (Ii) (289). These processes are tightly regulated by a family of lysosomal cysteine proteases (cathepsins) (290). Within this set of enzymes, cathepsin S appears to be the major protease involved in Ii degradation and is one of the major endoproteases involved in Ag-processing outside of the thymus (291, 292). Meanwhile, the enzyme activity of cathepsin S is regulated by its endogenous inhibitor stefin A (*Stfa*), which belongs to a large superfamily of highly conserved proteins that act as potent inhibitors of endosomal cathepsins (293) and has been genetically linked to day 3 thymectomy (D3Tx)-induced antoimmune ovarian dysgenesis (AOD) (294). Interestingly, cathepsin S has been reported to be involved in D3Tx-induced antoimmune disease of the salivary and lacrimal glands (295). Therefore, the abnormal expression and effects of cathepsins or stefin A may contribute to the alteration in the host adaptive immune response, resulting in either immunoamplification or immunodeficiency.

In the present study, we observed that after severe sepsis, splenic DCs exhibited an impaired Ag-presenting function and Th2-polarized capacity during the interaction with TCR-specific T cells. Interestingly, dramatic and long-term upregulation of stefin A1 was observed in bone marrow, splenic DCs and lung DCs after animals had survived severe sepsis. This correlation suggests that overexpression of stefin A1 in tissue DCs induced by severe sepsis enhances its inhibitory effect on cathepsin S and DC Ag-presenting function, which subsequently contributes to the immnosupression observed in septic patients and experimental septic animals.

Materials and Methods

Mice

Female C57BL/6 mice (6-8 wks; Taconic Company, Germantown, NY) and OVA-specific TCR-transgenic OT II mice (The Jackson Laboratory, Bar Harbor, ME) were housed under specific pathogen-free conditions at The Unit for Laboratory Animal Medicine (ULAM) of the University of Michigan. The University Committee for the Using and Care of Animals (UCUCA) approved the experiments.

Cecal ligation and puncture (CLP)

Sham and CLP surgery was performed as described in Chapter 2.

Isolation of lung and splenic DCs

Isolation of lung DCs and splenic DCs were performed as described in Chapter 3 and 4.

cDNA Microarray

DNA microarray analysis of gene expression in total BM cells was done as previously described (296). Briefly, BM cells were flushed from the femurs and tibias and total

RNA was isolated using Trizol Reagent. Isolated RNA (20 μg) was used as a template for cDNA generation using reverse transcriptase (Invitrogen, Carlsbad, CA) in the present of amino allyl-dUTP (Sigma, St. Louis, MO) that allow for subsequent fluorescent labeling of cDNA using Cy3 or Cy5 NHS ester dyes (Amersham Pharmacia Biotech Ltd., Buckinghamshire, UK). The cDNA from each time point-matched control sample was labeled with fluorescence dye Cy3 and the experimental sample with Cy5. The labeled probes were then hybridized to mouse cDNA microarray (Mouse M22.4K1; University Health Network Microarray Center, Toronto, Canada) at 65°C overnight. RNA samples from a total of three mice at each time point were analyzed separately by three individual chips (one for each rat sample). Fluorescent images were obtained using a GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA). Primary analysis was performed by using the GenePix software package (Axon Instruments). Cy3 to Cy5 ratios were determined for the individual genes along with various other quality control parameters, i.e., intensity over local background. The average median ratio values for all spots were normalized to 1.0. The data set was imported into Cluster Analysis of Gene Expression Dynamics (CAGED). CAGED v1.0 software is designed to perform Bayesian model-based clustering on temporal gene expression data (297).

Quantitative Real-time PCR (Taqman)

For quantitative mRNA analysis, total RNA was isolated from total BM cells, purified lung DCs and splenic DCs. Primer Express 2.0 software (Applied Biosystems, Foster City, CA) was used to design Taqman primers for stefin A1 (*Stfa1*), which were then purchased from Sigma-Genosys (The Woodlands, TX). Primers for stefin A1 were as follows: forward, 5′- AACCTGCCACACCAGAAATCC; reverse, 5′-

TTCTTATTGGTTTTTGCTTCAAGCT. GAPDH was analyzed as an internal control. Analysis of transcripts with Taqman was performed as described in Chapter 2.

Antigen-presenting and Th1/Th2-polarizing capacity assay

Splenic DCs were isolated from sham or CLP mice at day 11 after surgical operation (described above), and pulsed with 5 μg/ml $OVA_{323-339}$ peptides (Peptides International, Inc., Louisville, KY) for 4 hours. After removing soluble $OVA_{323-339}$ peptide in the medium, $CD4^+$ T cells isolated from spleens of naïve OT II mice with anti-CD4 magnetic beads (Miltenyi Biotech, Auburn, CA) were added to the DC culture. After 24 hours, supernatants were collected and stored in -80°C until measurement of protein levels of IL-4, IL-5, IL-13 and IFN-γ by ELISA. In a parallel experiment, three days after co-culture between $OVA_{323-339}$-pulsed DC and $OVA_{323-339}$-specific $CD4^+$ T cells, T cell proliferation was measured by the incorporation of [^{3}H]-thymidine with a multi-purpose scintillation counter (Beckman Coulter, Fullerton, CA).

In another set of experiments using *Schistosoma mansoni* egg antigen (SEA), splenic DCs isolated from sham or CLP mice day 11 after surgical operation were pulsed with 10 μg/ml SEA for 4 hours (40). Splenic $CD4^+$ T cells were isolated with anti-CD4 magnetic beads (Miltenyi Biotech) from mice that had been immunized with 3,000 live *S. mansoni* eggs for 2 weeks, and added to the SEA-pulsed DC culture. After 24 hours, supernatants were collected for the measurement of protein levels of IL-4, IL-5, IL-13 and IFN-γ by ELISA. After 3 days, T cell proliferation was measured by the incorporation of [^{3}H]-thymidine.

Flow cytometry analysis

At day 3 or day 11 after CLP, spleens were collected from sham and CLP mice and dispersed in 0.2 % collagenase (Sigma-Aldrich, St. Louis, MO) in RPMI 1640 (Mediateck, Inc., Herndon, VA) at 37°C for 30 min. After lysing red blood cells, Fc binding was blocked via a 10-min incubation with purified rat anti-mouse CD16/CD32 (FcγIII/II receptor). Then the cells were stained with FITC-labeled anti-CD11c in combination with each of the following antibodies: PE-anti-CD40, PE-anti-CD80, PE-anti-CD86, or PE-anti-I-A^b in Dulbeco's PBS + 0.2% BSA + 0.1% NaN_3 for 30 min at 4°C in the dark. The appropriate IgG isotypes were used as controls. All antibodies and IgG isotypes were purchased from BD PharMingen (San Diego, CA). The cells were fixed in 1% paraformaldehyde and kept in the dark at 4°C until analysis with a FACSCaliber (CELLQuest™ software; Becton and Dickinson, Mountain View, CA).

Statistical analysis

Student's *t* test and one-way ANOVA were used to detect statistical significance.

Results and Discussion

Impaired antigen-presenting function of post-septic splenic DCs

DCs have an essential role in both the innate and adaptive immune response via their unique functions, including cytokine production and Ag-presenting activities (72, 89). Our previous data showed that severe sepsis resulted in a long-term deficiency in IL-12 production by DCs, which was associated with epigenetic alterations (chapter 4). This observation is consistent with the susceptibility of post-septic mice to a secondary challenge with *Aspergillus fumigatus*, as an intact host innate immune response with a

robust IL-12 production is required to clear this normally innocuous fungus challenge (38, 39). This data underscores the notion that defective cytokine production by DCs impairs the host innate immunity. Furthermore, we have shown that post-septic mice exhibited an altered granulomatous response to a secondary challenge with *S. mansoni* eggs, which is known to induce a cell-mediated Th2-skewed immune response (chapter 3); thus, we hypothesized that severe sepsis impairs DC Ag-presenting function. OVA peptide-specific $CD4^+$ T cells isolated from TCR transgenic OT II mice were employed to study DC Ag-presenting function following experimental peritonitis. At day 11 after CLP, $CD4^+$ T cells were observed to proliferate at a significantly lower rate in co-culture with $OVA_{323-339}$-pulsed splenic DCs isoloated from post-septic mice, as compared with $CD4^+$ T cells in co-culture with similar treated splenic DCs from sham mice, indicating that severe sepsis results in an impaired DC Ag-presenting function (Fig 5.1A).

Although SEA fails to induce the "classical" maturation of DCs, including the upregulation of CD40, CD80, CD86 and MHCII molecule, DCs have been shown to be able to present SEA to naïve T cells and induce SEA-specific Th2 response (147, 152). To confirm the observation from the above Ag-presenting assay using OVA peptide, we also utilized SEA-pulsed DCs to induce the proliferation of $CD4^+$ T cells obtained from mice sensitized with SEA. At day 11 after CLP, $CD4^+$ T cells proliferated with a significantly lower rate in co-culture with SEA-pulsed post-septic splenic DCs, as compared with $CD4^+$ T cells in co-culture with similar treated sham splenic DCs (Fig 5.1B). Thus, data from experiments using different antigens suggest that severe sepsis results in an impaired DC Ag-presenting function.

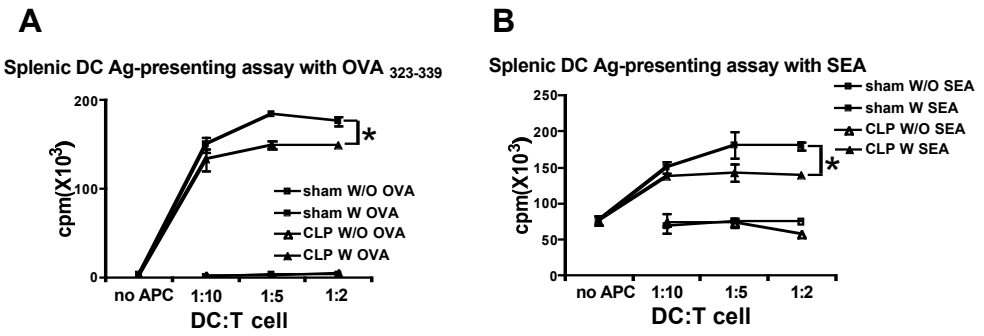

Figure 4.1 Impaired antigen-presenting function of post-septic splenic DCs. (A) Purified splenic DCs from post-septic or control mice day 11 after surgery were pulsed with 5 μg/ml $OVA_{323-339}$ peptide, and co-cultured with splenic $CD4^+$ T cells isolated from naïve TCR transgenic OT II mice. Seventy-two hours later, 1 μCi $[^3H]$-thymidine was added to the DC: T cells co-culture for 6 hrs. T cell proliferation rate was measured by a multi-purpose scintillation counter. (B) Purified splenic DCs from sham or CLP day 11 after surgery were pulsed with 10 μg/ml SEA, and co-cultured with splenic $CD4^+$ T cells isolated from mice sensitized with 3,000 live *S. mansoni* eggs. T cell proliferation was measured by the incorporation of $[^3H]$-thymidine. *, $P \leq 0.05$ compared to $CD4^+$ T cell $[^3H]$-thymidine incorporation in co-culture with sham splenic DCs.

It has been well established that DC Ag-presenting function is determined by three signals, including MHCII-peptide complex, co-stimulatory molecules expressed on the cell surface of DC and T cells, and the cytokine environment (113). To determine if there is any alteration in co-stimulatory molecule expression that might influence DC Ag-presenting capacity in septic mice, we measured the expression levels of co-stimulatory molecules, including CD40, CD80 and CD86, as well as MHCII molecule, on DC cell surface following experimental peritonitis. At day 11 after CLP, there was no difference in the expression of any of these molecules, indicating that the impaired Ag-presenting function of post-septic DCs was not due to the effects of co-stimulatory molecules (Fig 5.2B). Similar observations were made when we measured the expression levels of co-stimulatory and MHCII molecules on DC cell surface at 6 weeks after CLP (data now shown). Interestingly, we observed increased expression of CD40, CD80 and CD86 on

splenic DC cell surface at day 3 after CLP, presumably reflecting the activated DC phenotype associated with systemic inflammatory immune response in the acute phase of severe sepsis (59) (Fig 5.2A).

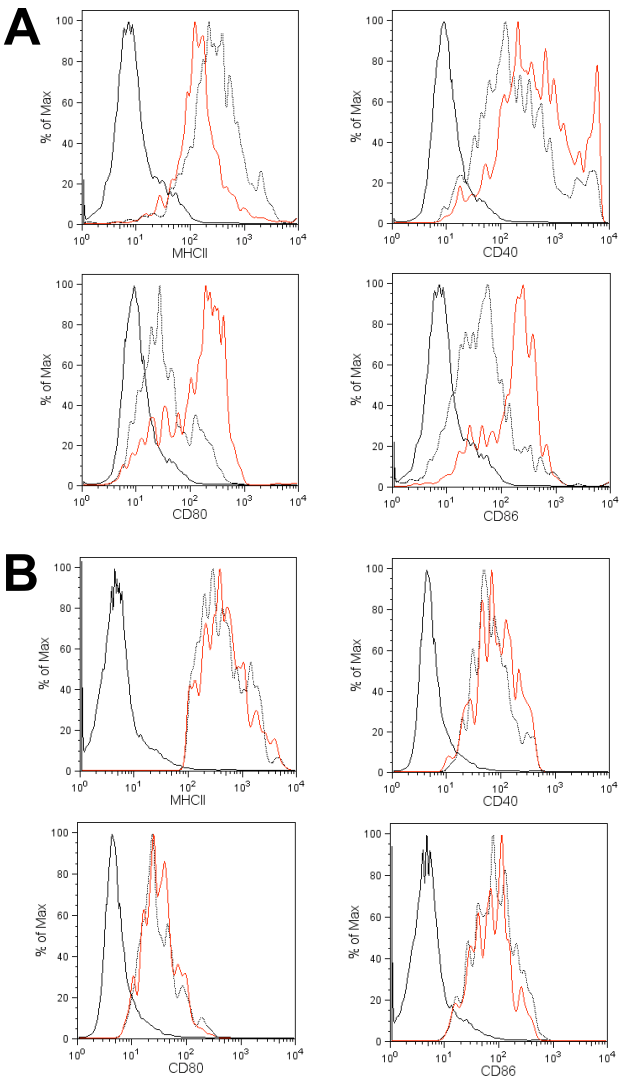

Figure 5.2 Expression of costimulatory and MHCII molecules on DC cell surface following severe sepsis. Spleens were collected and dispersed from sham and CLP mice at day 3 (A) or day 11 (B) after CLP. Expression of CD40, CD80, CD86 and MHCII were investigated on cell surface of $CD11c^{+}$ DCs. Black line, isotype control; dotted line, sham group; red line, CLP group.

Dramatic increase in stefin A1 expression in bone marrow and tissue DCs following severe sepsis

When we compared the global gene expression pattern in total BM cells between sham and septic mice using microarray analysis, we identified a novel gene called stefin

A1 (*Stfa1*) that was dramatically upregulated in septic mice at day 11 after CLP. Three individual hybridizations showed that there was a 4-fold increase in *Stfa1* transcript level in post-septic BM cells, as compared to sham BM cells (Fig 5.3A). Stefin A1 has been identified to be an intracellular inhibitor of cysteine proteinases (cathepsins) (293), including cathepsin S (294). Cathepsin S is the major protease involved in Ii degradation and therefore plays an essential role in the formation of MHCII-peptide complex during MHCII-restricted Ag-presentation process in DCs (289). Thus, stefin A1 appears to inhibit DC Ag-presenting function via its inhibitory effect on cathepsin S. We hypothesized that the upregulation of stefin A1 induced by severe sepsis could compromise DC Ag-presenting function. We utilized RT-PCR to measure *Stfa1* transcript level in tissue DCs following severe sepsis. At day 11 after CLP, post-septic splenic DCs had significantly higher mRNA level of *Stfa1* in response to either medium or series of TLR agonists (Pam3Cys for TLR2, LPS for TLR4, CpG for TLR9) (Fig 5.3C). The upregulation of *Stfa1* is a long-term consequence of severe sepsis, since post-septic splenic DCs maintained a higher *Stfa1* mRNA level six weeks after CLP (Fig 5.3D). Interestingly, total BM cells also contained significantly higher *Stfa1* mRNA level out to 19 days post CLP (Fig 5.3B). Furthermore, *Stfa1* mRNA levels were dramatically induced in lung DCs from post-septic mice at day 11 or 6 weeks after CLP, although there was no detectable *Stfa1* mRNA in lung DCs from sham mice, indicating severe sepsis-induced stefin A1 upregulation is common to tissue DCs. Our observations suggest that severe sepsis promotes the expression of stefin A1 in tissue DCs, which may subsequently enhanced its inhibitory effect on cathepsins and Ag-presenting function of post-septic DCs. Further investigations are required to study the role of stefin A1 on DC

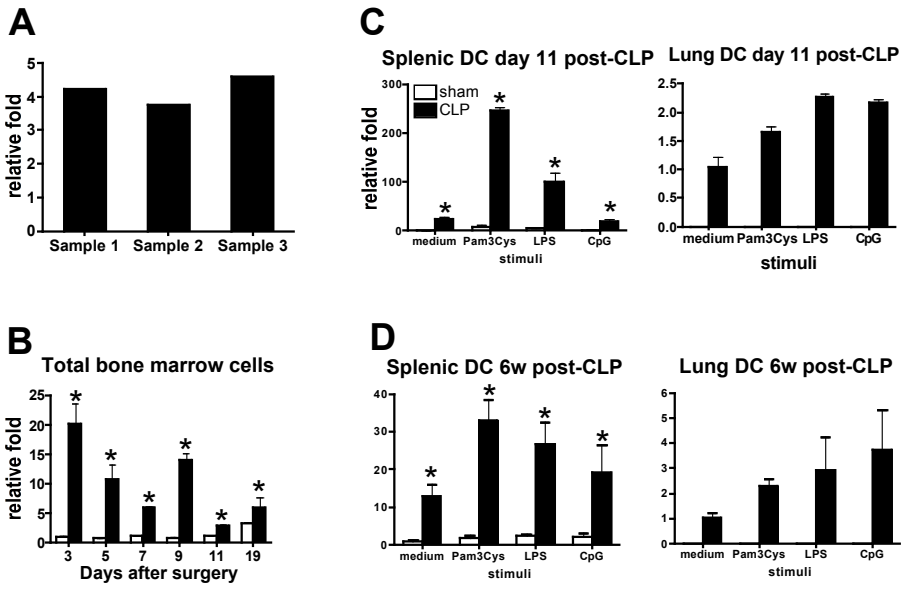

Figure 5.3 Stefin A1 expression in bone marrow and tissue DCs following severe sepsis. (A) Total BM cells were flushed from femors and tibias day 11 after CLP. Upregulation of stefin A1 in BM cells following severe sepsis was identified by cDNA microarray analysis. (B) Messenger RNA levels of *Stfa1* in total BM cells at different time points after CLP were determined by Taqman. (C) Purified splenic DCs from septic or control mice day 11 after surgery were stimulated by series of TLR agonists. Messenger RNA levels of *Stfa1* in splenic DCs were determined by Taqman. (D) Purified splenic DCs from septic or control mice 6 weeks after CLP were stimulated by series of TLR agonists and mRNA levels of *Stfa1* were determined by Taqman. Results in B, C and D represent two to four independent experiments and are expressed as mean ± SEM. *, $P \leq 0.05$ compared with *Stfa1* transcript levels measured in sham mice.

Ag-presentation in the context of severe sepsis and may provide insight into the development of new therapeutic strategies for dysfunctional DCs and immunosuppression observed in septic patients.

Th2-polarized capacity of post-septic splenic DCs

When challenged with *S. mansoni* eggs, post-septic mice showed an enhanced Th2 cytokine profile and an exacerbated Th2 cells-mediated granulomatous response (chapter 3). Therefore, we hypothesized that after severe sepsis, Ag-loaded DCs directs naïve T cells towards a Th2-baised response. Splenic DCs recovered from day 11 post-

septic mice were pulsed with OVA peptide and co-cultured with OVA peptide-specific $CD4^+$ T cells isolated from TCR transgenic OT II mice. These T cells generated significantly higher levels of Th2 cytokines such as IL-4, IL5 and IL-13, but lower level of Th1 cytokine IFN-γ, as compared to similar co-culture using splenic DCs from sham mice (Fig 5.4A).

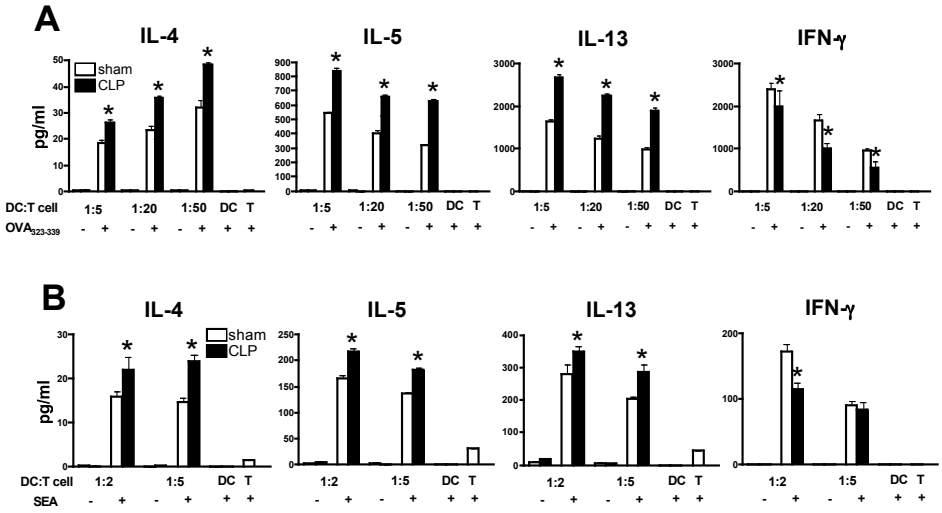

Figure 5.4 Th2-polarized capacity of post-septic splenic DCs. (A) Purified splenic DCs from post-septic or control mice day 11 after surgery were pulsed with 5 μg/ml $OVA_{323-339}$ peptide, and co-cultured with splenic $CD4^+$ T cells isolated from naïve TCR transgenic OT II mice. Twenty-four hours later, the supernatants were collected to measure cytokine protein levels by Bio-Plex. (B) Purified splenic DCs from sham or CLP day 11 after surgery were pulsed with 10 μg/ml SEA, and co-cultured with splenic $CD4^+$ T cells isolated from mice sensitized with 3,000 live *S. mansoni* eggs. Twenty-four hours later, the supernatants were collected to measure cytokine protein levels by Bio-Plex. Results represent 3 independent experiments and are expressed as mean ± SEM. *, $P \leq 0.05$ compared to cytokine protein levels in the supernatants of co-culture between $CD4^+$ T cells and sham splenic DCs.

We further studied Th1/Th2 polarizing function of post-septic DCs pulsed with SEA and co-cultured with $CD4^+$ T cells that were obtained from the mice sensitized with SEA for 2 weeks. These $CD4^+$ T cells also generated significantly higher levels of Th2 cytokines such as IL-4, IL-5 and IL-13, but lower level of Th1 cytokine IFN-γ, as

compared with in co-culture with similar treated sham splenic DCs (Fig 5.4B). Taken together, our data suggest that post-septic DCs induce a Th2-polarized effect on T cells, which may provide a cytokine environment that renders the host susceptibility to a secondary pathological challenge that would require an intact Th1 immune response.

In addition to aberrant cytokine production, DCs also demonstrate an impaired Ag-presenting function and capacity to polarize T cells to a Th2 phenotype following severe sepsis. These deficiencies likely impair both the innate and adaptive immune response of the host, which is manifested by clinical reports showing that patients who survive sepsis are at a significant risk of dying due to secondary pathological infections and cancer for years after the initial severe septic event (44, 45, 47, 48). Since we characterized epigenetic mechanisms involved in long-term deficiency in IL-12 production by post-septic mice, one intriguing hypothesis is that the impaired Ag-presenting function and Th2-polarized capacity of post-septic DC are mediated by long-term aberrant expression of some key molecules, which also involves epigenetic alterations. Stefin A1 is a candidate molecule, since we observed that upregulation of *Stfa1* transcript was maintained long-term and stefin A1 is a potential regulator of DC Ag-presenting function. Therefore, genome-wide analysis of epigenetic changes at promoter regions in DCs following severe sepsis by ChIP-chip technique, in conjunction with global comparison of gene expression pattern between sham and post-septic DCs by microarray will improve our understanding of molecular mechanisms of DC dysfunction and immunosuppression, and facilitate the development of new therapeutic strategies for this under-studied disorder.

Chapter 6

Conclusions and future directions

The general consensus of recent investigations is that the acute inflammatory response is tightly linked to innate immunity, which usually occurs over a relatively short time period. During this limited time frame, a functional acute inflammatory response is supported by the temporal expression of a set of inflammatory mediators and activation of specific leukocyte subpopulations. For example, within the first hour of an *in vivo* LPS challenge, mRNA expression for early response cytokines such as TNF-α can be detected in mononuclear phagocytic cells followed by the expression of secondary response cytokines and chemokines (298, 299). Interestingly, the quantity and complexity of the initial antigen or pathogen determine the severity and duration of acute inflammatory responses (249, 300). An additional factor that determines the clinical course of an acute inflammatory response is the rigor of the host's immune response to the inciting agent (48, 301). An interesting inverse clinical correlation exists between the severity of the initial acute inflammatory response and the ability of the host to deal with a subsequent immune challenge months later, indicating an important role of the initial acute response in shaping the host's response to a subsequent challenge (5, 47, 48).

The chronic consequences linked to severe acute inflammation have been found in many severe acute human diseases, including the acute ischemia/reperfusion injury post organ transplant (302), severe respiratory syncial viral infection in neonates (303,

304), and severe burn and trauma injury (305, 306). Many of these clinical cases appear to be governed by severe, life-threatening inflammatory events, followed by a long lasting improper or immunosuppressed immune response. One of the best examples of the chronic effects of severe acute inflammation is severe sepsis, both in patients and in experimental animal models. Several studies have noted that the septic population has an increased risk of dying for many years after the initial hospitalization (47, 48). Following the initial hyperinflammatory response, septic patients develop a sustained anti-inflammatory or immunosuppressive state that has been termed "immunoparalysis", which is manifested by an inability to eradicate the primary infection and/or the development of new secondary infections (41-46). Many of the pathogens responsible for the secondary, hospital-acquired infections are not particularly pathological in patients with competent immune systems, indicating the immunosuppressive state of patients with severe sepsis. Thus, the initial episode of severe sepsis, characterized by a dysregulated inflammatory response, appears to lead to long-lasting complications regarding the manner in which the host responds to and deals with subsequent challenges. In the present study, we modeled the long-term immunosuppression after severe sepsis using a CLP sepsis animal model and characterized the DC alterations in cytokine production and Ag-presenting functions. Our studies generated 3 additional questions that will be discussed below.

There is little doubt that DCs, although small in number, play a large role in maintaining an appropriate and protective innate and acquired host response. Therefore, DCs are logical participants in the initial severe infection and dysregulated cytokine storm in the early stage of severe sepsis. Indeed, DCs undergo a rapid depletion in septic

patients (54) and in experimental sepsis animal models (55-59). We observed an initial dramatic depletion of both mDC and pDC in peripheral organs at the early stage of severe sepsis (Fig 1.2), which was consistent with previous reports. In addition, DC populations are gradually reseeded to the distant organs, as their numbers gradually return to normal levels after a significant recovery period (Fig 1.2). The first question we would like to address in future studies is: Where does the generation of DCs occur post sepsis? It is possible that after sepsis DCs are newly generated in BM, and migrate to peripheral sites via the action of chemokines (233). Evidence supporting this hypothesis is from the study showing that experimental peritonitis induced by colon ascendens stent peritonitis (CASP), another common procedure to induce severe sepsis (32), significantly enhances cell proliferation in BM and promotes colony formation by granulocytic-monocytic precursors (307). An alternative possibility is that DCs are generated in peripheral sites from precursor cells, which have been recruited into the periphery during the acute inflammatory response. It is important to answer these questions, since normal hematopoiesis is influenced by the surrounding tissue microenvironment (hematopoietic niche) (308, 309). The tissue location in which DCs are generated will definitely contribute to the final DC phenotype and direct the properties of the newly generated DCs. Future studies, monitoring the migration of HSCs between BM and peripheral sites following severe sepsis will reveal the locations of DC generation.

Our observation of a gradual restoration of DC numbers after severe sepsis indicates that sepsis-induced DC depletion may not be the only mechanism responsible for the long-term immunosuppression and that DC functionality can also be altered after severe sepsis. Our findings show that post-septic DCs exhibit a long-term impairment in

IL-12 expression, which subsequently compromises both the innate and adaptive immune response to a secondary pathological challenge. Based on our studies, we propose that the long-term maintenance of DC alterations may be due to epigenetic regulation of expression patterns of key effector molecules. Our data show that the long-term maintenance of aberrant gene expression is associated with epigenetic changes at *Il12* promoters in post-septic DCs, including histone acetylation and methylation at distinct lysine sites of histone proteins. Furthermore, these epigenetic alterations are mediated by the altered recruitment of HMT complexes that specifically methylate individual lysine sites. These observations led to the second question: What are the key molecules/signals that mediate the epigenetic alterations in DCs post sepsis? Recent data generated from experiments studying LPS-stimulated macrophages may shed some light on this question (205). LPS-TLR4 signaling was shown to induce a short-term LPS unresponsiveness in macrophages, which was mediated by alterations in histone modifications. This study indicated that TLR signaling is a potential contributor to epigenetic changes; however, signaling through TLR4 alone is not sufficient to build a long-term immune cell memory for the expression of certain genes. In memory T cells, the candidate pathways leading to epigenetic alterations include T cell receptor and cytokine/cytokine receptor signaling pathways. Based on the fact that an overwhelming pathological infection followed by a cytokine storm are the hallmarks of severe sepsis, it is reasonable to hypothesize that toll-like receptor signaling and cytokine/cytokine receptor signaling pathways are potential contributors to the epigenetic changes. It is also highly possible that synergy between individual TLR signaling pathways exists that results in downstream epigenetic changes, since synergy between different TLR signaling pathways has been shown to promote the

Th1-polarizing function of DCs (310). Investigations of the role of the epigenetic alterations, including DNA methylation and histone modifications, in long-term DC dysfunction and immunosuppression following a severe acute inflammatory response will likely provide mechanistic insight into the longer-term sequela of sepsis and acute lung injury survivors. These future studies will be important in laying the groundwork for the development of potentially novel cell and mediator-based therapeutic interventions.

Our data show that splenic DC dysfunction and epigenetic changes induced by severe sepsis persist for 6 weeks after the induction of peritonitis, by which time DC numbers have returned to normal levels. This observation indicates that re-populated tissue DCs maintain aberrant cytokine production patterns, which are mediated, in part, by epigenetic changes. The third remaining question is: What is the mechanism leading to abnormal DC differentiation from precursor cells, given that epigenetic changes such as histone methylation are inherited during cell division (165, 311). Further investigation is warranted to address the hypothesis that the intrinsic alterations in HSCs or precursor cells induced by severe sepsis lead to the generation of phenotypically altered DCs. Alternatively, severe sepsis might generate a long-lasting aberrant BM niche, which may continuously compromise normal DC differentiation. Still another possibility is that after the acute episode of severe sepsis, the microenvironment in peripheral organs is permanently altered by the presence of abnormal structural cells or non-self antigens, which subsequently alter the phenotype of newly recruited DC. To address this question, cross-transfer of sham or CLP BM cells into irradiated sham or CLP recipients followed by the investigation of peripheral DC phenotype will be performed. Co-transfer of $CD45.1^+$ sham and $CD45.2^+$ CLP BM cells of equal number into an irradiated naïve

mouse followed by functional comparasion between peripheral CD45.1^{+} and CD45.2^{+} DC will further indicate whether severe sepsis-induced intrinsic alterations of HSCs is responsible for long-term maintenance of altered DC phenotype.

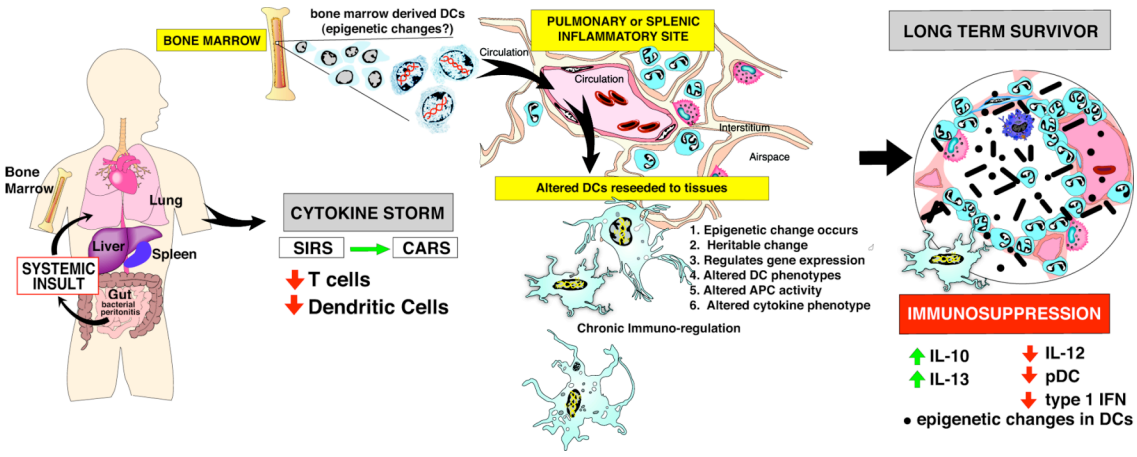

Figure 6.1 Schematic of the long-term consequences of severe sepsis on the host's immune status. There is a depletion of immune cells in the distal tissues and reduction of bone marrow cell populations. When the tissues are re-seeded from the bone marrow profound changes have occurred, some induced by epigenetic alterations, which render the immune cells (DCs) unable to function normally in host responses and immune surveillance.

Figure 6.1 outlines our evolving hypothesis of the cellular and molecular mechanism by which an initial overwhelming innate immune response leads to a long-term immunosuppressive state, such that the affected host loses the immune capacity to mount an appropriate defense response against a secondary pathological challenge. Severe sepsis results in a rapid depletion of immune cells such as DCs and T cells due to massive apoptosis at the early stage of this disorder; meanwhile, the subsequent repopulation of DCs, presumably through enhanced DC differentiation and proliferation from precursor cells has been initiated. Although DC numbers can return to pre-septic levels after a long-term recovery, the newly generated BM-derived DCs are phenotypically altered, including aberrant cytokine expression pattern and APC function,

which are associated in part with epigenetic changes. The long-term maintenance of dysfunctional DCs in peripheral tissue sites appears to compromise the host defense to a secondary pathological challenge. The key aspects of this hypothesis include long-term abnormal DC differentiation from precursor cells and the maintenance of DC dysfunction mediated by an epigenetic mechanism, which can profoundly control the transcription of immune-specific genes. Given that transplantation of DCs that have been manipulated *in vitro*, such as by challenge with tumor antigen, has become a successful strategy in cancer therapy (312-314), transplantation of immuno-competent DCs to patients who survive severe sepsis is a potential therapeutic strategy to rescue sepsis-associated immunosuppression. The broader window of treatment timing of this approach bestows potential clinical benefits when compared to treatments aimed at the acute phase of sepsis during which time transition from SIRS to CARS occurs so rapidly that treatment strategies often fail (9, 17). Although chemical manipulation of histone modifications is not yet available, this approach may present a future therapeutic strategy to reverse the long-term epigenetic changes induced by severe sepsis. Collectively, our studies assessing novel mechanisms that dictate the long-lived immunosuppression associated with severe sepsis survivors provide insight into the development of new therapeutic strategies for an under-studied syndrome with significant clinical and financial implications to our health care system.

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