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THE ROLE OF SCAVENGER RECEPTOR CLASS B TYPE I- REGULATED INDUCIBLE GLUCOCORTICOID IN SEPSIS

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THE ROLE OF SCAVENGER RECEPTOR CLASS B TYPE I-REGULATED
INDUCIBLE GLUCOCORTICOIDS IN SEPSIS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By

Junting Ai

Lexington, Kentucky

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ABSTRACT OF DISSERTATION

THE ROLE OF SCAVENGER RECEPTOR CLASS B TYPE I-REGULATED INDUCIBLE GLUCOCORTICOID IN SEPSIS

Sepsis claims over 215,000 lives in the US annually. Inducible glucocorticoids (iGC) is produced during sepsis. However, the precise effects of iGC in sepsis remain unclear due to a lack of appropriate animal models. Glucocorticoid (GC) insufficiency is associated with a marked increase in mortality and occurs in 60% of severe septic patients. Yet the conclusion of GC therapy on septic patients is still controversial.

Scavenger receptor class B type I (SR-BI) in the adrenal mediates the selective uptake of cholesteryl ester from lipoproteins for GC synthesis. SR-BI^{-/-} mice completely lack iGC during sepsis and are highly susceptible to septic death, which presents SR-BI^{-/-} mice as a GC insufficient model. However, SR-BI^{-/-} mice display multiple defects contributing to septic death, making it difficult to study iGC by using these mice. Therefore, we utilized adrenal-specific SR-BI^{-/-} mice (ADR-T SR-BI^{-/-}) generated by adrenal transplantation. As expected, the ADR-T SR-BI^{-/-} mice failed to generate iGC under cecal ligation and puncture (CLP)-induced sepsis and showed a significantly higher mortality than the control mice, demonstrating that iGC is essential for preventing septic death. High blood urea nitrogen (BUN) was observed in the ADR-T SR-BI^{-/-} mice but not in the control mice in CLP, indicating that iGC protects kidney injury in sepsis. Plasma IL-6 was remarkably higher in the ADR-T SR-BI^{-/-} mice than the control mice, demonstrating an anti-inflammatory effect of iGC in sepsis. The ADR-T SR-BI^{-/-} mice also displayed significantly lower phagocytic activity of monocytes and neutrophils in the blood and lower activation of T cells in the spleen compared to the control mice in CLP, suggesting that iGC is immunomodulatory in sepsis. Low-dose GC supplementation significantly improved the survival of SR-BI^{-/-} mice in CLP, but did not increase the survival rate of SR-BI^{+/+} mice in CLP, indicating that GC supplementation improves the survival specifically in mice with adrenal insufficiency.

Overall, we revealed that iGC is essential for sepsis survival. iGC prevents kidney damage, modulates inflammatory responses and exerts immunomodulatory functions in sepsis. GC supplementation specifically improves survival of individuals with adrenal insufficiency in sepsis.

Keywords: scavenger receptor class B type I, inducible glucocorticoids, adrenal insufficiency, sepsis.

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THE ROLE OF SCAVENGER RECEPTOR CLASS B TYPE I-REGULATED
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Chapter 1 Introduction

1.1 Sepsis

1.1.1 History of sepsis

The word “sepsis”, deriving from the Greek word “σήψις”, was first introduced by Hippocrates (ca. 460-370 BC) in reference to decomposition or putrefaction. Sepsis was described as a form of tissue breakdown; it described the processes of putrefaction and decay and was associated with death and disease. The concept of sepsis introduced in classical antiquity was used until the 19th century. At the beginning of the 19th century, Justus von Liebig expanded the theory by claiming that the contact between wounds and oxygen was responsible for the development of sepsis. Later, Semmelweis deduced that the puerperal fever, a form of sepsis, was caused by “decomposed animal matter that entered the blood system” and succeeded in lowering the mortality rate by introducing hand washing with a chlorinated lime solution before every gynaecological examination. Louis Pasteur (1822-1895) discovered that tiny single-cell organisms that we now call bacteria caused putrefaction and correctly deduced that these microbes could be causing disease. In 1914, Hugo Schottmüller (1867-1936) paved the way for a modern definition of sepsis: "Sepsis is present if a focus has developed from which pathogenic bacteria, constantly or periodically, invade the blood stream in such a way that this causes subjective and objective symptoms." Thus, for the first time, the source of

infection as a cause of sepsis came into focus. In pre-antibiotic time, a number of patients developed sepsis and the death rate was very high. These patients often showed a very low blood pressure, a condition called septic shock. With the introduction of antibiotics, the death rate of sepsis was reduced. With technological progress, intensive care medicine started to develop, and sepsis patients soon became the main patient fraction in intensive care units (ICU). In the 1980s, it was discovered that the inflammatory reaction in sepsis patients was in the whole body. Hence it became clear that the onset of sepsis did not derive from an infectious focus alone, but that the host response against infection must in some way play a role. In 1989, Roger C. Bone (1941-1997) established a sepsis definition that is still valid today: "Sepsis is defined as an invasion of microorganisms and/or their toxins into the bloodstream, along with the organism's reaction against this invasion." [1, 2]

1.1.2 Epidemiology of sepsis

Contemporarily, despite advances in critical care and anti-microbial therapy, sepsis remains a leading cause of death in intensive care units (ICUs).[3] Approximately 20-35% of people with severe sepsis and 30-70% of people with septic shock die.[4] Sepsis causes millions of deaths globally each year.[5] It is estimated that in the United States, sepsis is diagnosed in three per 1,000 people each year and leads to 215,000 deaths annually.[6] According to the data from the National Hospital Discharge Survey, the number and rate per 10,000 people hospitalized for septicemia or sepsis was more than doubled from 2000 through

2008 and 17 % of septicemia or sepsis hospitalizations ended in death, whereas only 2 % of other hospitalizations did.[7]

The incidence of sepsis varies among populations. Males appear to be at greater risk of developing sepsis than females.[3, 8] Racial and ethnic origin also matters as sepsis appears to be highest among African-American males.[9] Elderly people are more susceptible to sepsis. In the United States, patients ≥ 65 years of age account for nearly 60 percent of all episodes of severe sepsis, and this is likely to increase over the next 20 years.[7, 9, 10]

Sepsis can be triggered by infections in any part of the body, among which respiratory infection is the most common cause of sepsis and is associated with the highest mortality, followed by abdominal infection and urinary tract infection.[11] The contribution of various infectious organisms to the burden of sepsis has changed over time.[12, 13] Gram-positive bacteria as a cause of sepsis have increased in frequency over time, such that they are now more common than gram-negative infection, although the number of cases of gram-negative sepsis remains substantial.[3, 14, 15] The incidence of fungal sepsis has also increased over the past decade, but remains lower than bacterial sepsis.[16] Infection of parasites such as falciparum malaria may also cause sepsis and requires management in the intensive care unit.[17, 18]

1.1.3 Definitions for sepsis

The sepsis syndrome is a continuum of clinical events with increasing severity and mortality. In the past, the terms bacteremia, septicemia, sepsis,

sepsis syndrome, and septic shock were used interchangeably, which lead to an imprecise understanding of sepsis. In 1991, the American College of Chest Physicians (ACCP) and Society of Critical Care Medicine (SCCM) published consensus definitions for sepsis.[19] These definitions were reconsidered in 2001 during an International Sepsis Definitions Conference and again in 2012.[12, 20] Table 1.1 shows the current definitions for sepsis.

The sepsis consensus definitions recognize a series of progressive stages in sepsis, including the systemic inflammatory syndrome (SIRS), sepsis, severe sepsis and septic shock. SIRS is a reference for a range of clinical symptoms that result from a systemic activation of the innate responses, regardless of the cause. Thus SIRS may also arise from non-infectious insults. Sepsis is defined as the SIRS resulting from infection. Uncomplicated sepsis, such as that caused by flu or urinary infection, is common and may not need hospital treatment. Severe sepsis arises when sepsis occurs in combination with multiple organ dysfunction syndrome (MODS). Because of problems with their vital organs, people with severe sepsis are more likely to die (mortality $\approx 30\%$) than those with uncomplicated sepsis. Septic shock occurs when sepsis is complicated by shock. Patients with septic shock are very ill and need rapid emergency admission to the hospital intensive care unit (ICU). Despite active treatment in the ICU, the death rate of septic shock patients is around 50 %.

Table 1.1 Clinical definitions for sepsis.

SIRS	<p>The systemic inflammatory response to a variety of severe clinical insults. The response is manifested by two or more of the following conditions:</p> <p>(1) temperature >38°C or <36°C; (2) heart rate >90 beats per minute; (3) respiratory rate >20 breaths per minute or PaCO₂ <32 mm Hg; (4) white blood cell count >12,000/cu mm, <4,000/cu mm, or >10% immature (band) forms.</p>
Sepsis	<p>The systemic response to infection, manifested by two or more of the following conditions mentioned under SIRS.</p>
Severe sepsis	<p>Sepsis associated with organ dysfunction, hypoperfusion, or hypotension. Hypoperfusion and perfusion abnormalities may include, but are not limited to lactic acidosis, oliguria, or an acute alteration in mental status.</p>
Septic shock	<p>Sepsis-induced hypotension (a systolic blood pressure <90 mm Hg or a reduction of ≥40 mm Hg from baseline) despite adequate fluid resuscitation along with the presence of perfusion abnormalities that may include, but are not limited to, lactic acidosis, oliguria, or an acute alteration in mental status. Patients who are receiving inotropic or vasopressor agents may not be hypotensive at the time that perfusion abnormalities are measured.</p>

1.1.4 Sepsis pathophysiology

Sepsis begins as the inflammatory response to an infection. The inflammatory response to an infection is initiated by the recognition of the microorganisms and the activation of the innate immune system. Activation of the innate immune system involves the production of oxygen radicals, the release of cytokines and inflammatory mediators and the activation of complement system. Meanwhile, coagulation is activated due to endothelial injuries and is enhanced by pro-inflammatory cytokines. In a typical inflammatory response, the oxygen radicals, cytokines and activated complement are generated to defense and kill invasive pathogens, which can be controlled by the host. However, when the host is unable to successfully constrain an infection, a complex dysregulation of inflammation arise and sepsis occurs. The factors underlie a typical inflammatory response all contribute to the pathophysiology of sepsis (Figure 1.1).

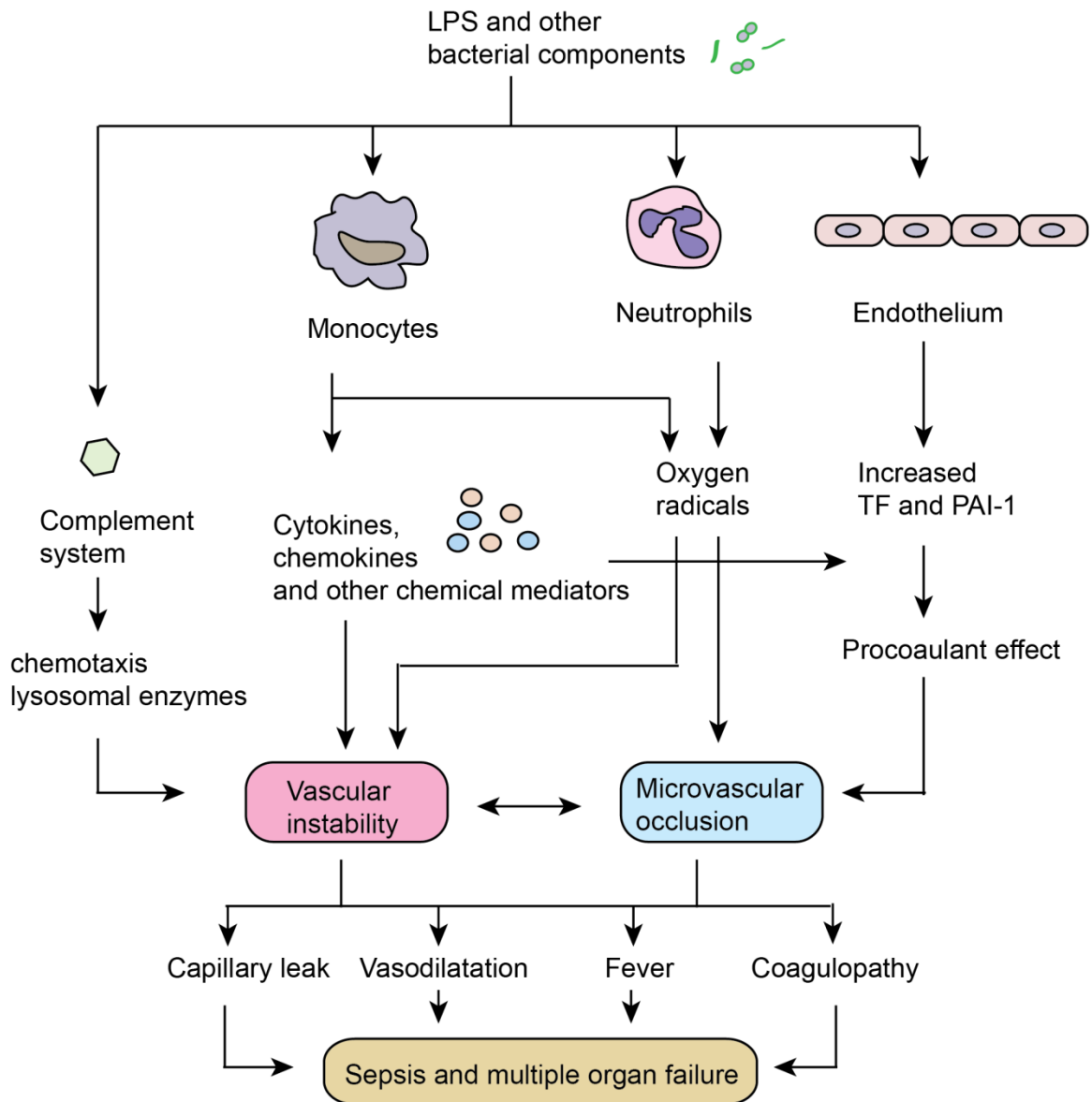


Figure 1.1 Pathogenic networks during sepsis.

LPS and other microbial products simultaneously activate the production of cytokine and inflammatory mediators, the complement system and the coagulation cascade that contributes to the pathophysiology of sepsis and septic shock. Reduced vascular stability and microvascular occlusion leads to coagulation, fever, vasodilatation, and capillary leakage, provoking hypoperfusion and inadequate oxygenation and thus organ failure.[21]

1.1.4.1 Initiation of the inflammatory response to an infection

The inflammatory response to an invading pathogen is initiated by the recognition of the microorganisms and the activation of the innate immune system. After entering tissues, many pathogens are recognized, ingested, and killed by phagocytes. Two main types of phagocytes are mononuclear phagocytes, or macrophages, which reside in the tissues and the neutrophils. In most cases, the tissue macrophages are the first cells to encounter most pathogens, and are soon reinforced by the recruitment of large numbers of neutrophils to the site of infection. Macrophages and neutrophils recognize pathogens by means of cell surface receptors, such as the macrophage mannose receptor and scavenger receptor. In addition, the repeating patterns of molecular structures on the surface of microorganisms, or called pathogen associated molecular patterns (PAMPs), can be recognized by specific pathogen recognition receptors (PRRs). Typical PAMPs include lipopolysaccharides (LPS) from gram-negative bacteria, lipoteichoic acid (LTA) and peptidoglycan from gram-positive bacteria as well as CpG DNA (bacterial DNA rich in cytosine-phosphate diester guanosine), bacterial flagellins and double-stranded RNAs (dsRNA) from viruses. Correspondingly, these PAMPs are recognized by three families of PRRs: toll-like receptors (TLRs), intracellular nucleotide-binding oligomerisation domain (NOD) proteins and peptidoglycan recognition proteins.[22, 23] These PRRs distinguish different types of pathogens and signal to direct appropriate immune responses to the pathogens.

In many cases, binding of a pathogen to the cell-surface receptors in phagocytes leads to phagocytosis, in which the pathogens are killed in acidified phagosomes or killed in phagolysosomes formed by the fusion of phagosomes and lysosomes. Macrophages and neutrophils also produce a variety of toxic products to help kill the engulfed microorganism. These involve the anti-microbial peptides such as defensins and cationic proteins, nitric oxide (NO), and reactive oxygen species (ROS) including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot HO$). In sepsis, however, there is a massive production, which leads to the systemic damage of vascular endothelium.[24, 25]

1.1.4.2 Production of cytokines, chemokines and other chemical mediators

Another important effect of the interaction between pathogen and tissue macrophages is the activation of macrophages to release cytokines, chemokines and other chemical mediators that attract neutrophils and plasma proteins to the infection site. The binding of a microbial molecule to its specific TLR or NOD results in the signal transmission that leads to the activation of nuclear transcription factor NF- κ B, which drives the transcription of a large range of important pro-inflammatory cytokine and chemokine genes.[26-29] Pro-inflammatory cytokines (i.e. tumor necrosis factor α [TNF- α], interleukin 1 [IL-1], IL-6, IL-12 and high-mobility group box protein 1 [HMGB1]), chemokines (e.g. monocyte chemoattractant protein-1 [MCP-1] and IL-8) as well as lipid mediators (e.g. platelet-activating factor [PAF], prostaglandins [PGs] and leukotrienes) are released. This secretion of inflammatory cytokines provokes an activation of surrounding innate immune cells and later activation of adaptive immune cells.

Chemokines mainly function as chemoattractants for leucocytes, recruiting monocytes, neutrophils, and other effector cells from the blood to the sites of infection in order to enhance the defense against the pathogens. In favorable situation, the infection-induced immune response is tightly controlled by different mechanisms that are triggered by PRRs themselves to control the process, including the inhibition of the TLR-related intracellular signaling, production of soluble receptors and antagonists that neutralize extracellular pro-inflammatory cytokine and production of anti-inflammatory cytokines (e.g. IL-10).[30, 31] However, in sepsis, this inflammatory response is dysregulated and overwhelming levels of pro-inflammatory cytokines are produced, which contributes to the endothelial damage and promotes coagulation in sepsis.[32, 33] In particular, the systemic release of TNF- α causes vasodilation, which leads to a drop in blood pressure and increased vascular permeability.[34]

Another activated enzyme of major importance is inducible nitric oxide synthase (iNOS), which is induced by NF- κ B. Sepsis is accompanied by the production of excess NO.[35, 36] Excess NO in sepsis provokes a systemic vasodilation, which contributes to septic shock and can also induce hepatocyte damage, increase gut epithelial permeability [37], impair neutrophil migration to infection site [38-40], and exert cytotoxic effects through the formation of ONOO-, resulting in damage to DNA, membrane phospholipids and mitochondria.[41]

1.1.4.3 Activation of the complement system

Microorganisms and endotoxins also turn on the humoral innate immune responses. The complement system can be activated by the binding of

complement component C1q to an antibody complexed with antigen, by the direct binding of C1q to the pathogen surface, or by the binding of C1q to the C-reactive protein bound to the pathogen. Activation of the complement system initiates a series of cleavage reactions that culminates in the formation of C3 convertase, which cleaves complement component C3 into C3b and C3a. C3b can mediate the opsonization of pathogens that facilitates phagocytosis. In addition, binding of C3b to C3 convertase further forms a C5 convertase which cleaves C5 to C5a and C5b. C3a and C5a are peptide mediators of inflammation. They are potent leukocyte chemoattractants and can also induce degranulation of basophilic granulocytes and mast cells, and thereby lead to a release of histamine, serotonin, and leukotriene B4 (LTB₄). These molecules further increase vasodilatation. Sepsis is accompanied by the excess production of C5a and C3a. A high level of plasma C5a in septic patients correlates with poor outcome.[42]

1.1.4.4 Activation of the coagulation cascade

The activation of coagulation at the site of infection is important to block small blood vessels and therefore to prevent further spreading of pathogens by the formation of fibrin. However, in sepsis, coagulation is increased and the mechanisms that favor fibrinolysis (fibrin breakdown by plasmin) are reduced. The endothelial injury at the site of infection, microbial components as well as the pro-inflammatory cytokines initiate and promote coagulation pathways leading to the increased expression of tissue factor (TF) on polymorphonuclear leukocytes (PMNs) and endothelial cells. Exposure of the coagulation system to TF

activates a series of proteolytic cascades, which result in the conversion of prothrombin to thrombin. Thrombin in turn catalyzes the conversion of fibrinogen to fibrin. Meanwhile, there is an increase in plasminogen activator inhibitor-1 (PAI-1) that inhibits fibrinolysis by preventing transformation of plasminogen into active plasmin. The net result is enhanced production and reduced removal of fibrin. This disorder of coagulation leads to the deposition of fibrin clots in small blood vessels and inadequate tissue perfusion.[21]

1.1.4.5 Adaptive immunity in sepsis

Adaptive immunity can be activated when the infection is not constrained in the local site. Unlike innate immunity which plays a major role in the earliest phases of the infection, adaptive immunity needs several days for clonal expansion and differentiation of naive lymphocytes into effector T cells and antibody-secreting B cells that, in most cases, effectively target the pathogen for elimination. In the adaptive immunity, the antigen presenting cells (APCs), including dendritic cells, B cells and macrophages, present peptide fragments of phagocytosed micro-organisms to CD4⁺ T cells by their major histocompatibility complex II (MHCII) and initiate the antigen specific immune response. CD4⁺ T cells then clonally expand with the actions of IL-2 induced by the activation of T cell receptor (TCR)-associated CD3 complex and the actions of co-stimulatory molecules induced on the APCs during their interaction with microorganisms. CD4⁺ T cells then differentiate to effector cells, which can migrate to the site of infection and further differentiate into a variety of T helper cell subsets, including Th1, Th2 and Th17 cells.

Adaptive immune cells classically are considered as bystanders in the inflammatory responses in sepsis; however, recent studies have shown that both T cells and B cells play a protective role in sepsis. Hotchkiss, *et al.* have demonstrated that Rag1^{-/-} mice that lack mature T and B lymphocytes have increased mortality after bacterial sepsis.[43, 44] Although a later study by Bosmann M *et al.* showed no difference in the survival rate between Rag1^{-/-} mice and wild type mice in cecal ligation and puncture (CLP)-induced sepsis[45], Kelly-Scumpia, K.M. *et al.* recently have demonstrated a protective role of IFN- γ -activated B cells in sepsis by enhancing early innate immune response.[46] In addition, Inoue, M *et al.* have demonstrated that T cells can interact with macrophages in the spleen and down-regulate TNF-mediated inflammation in LPS-induced endotoxemia.[47]

1.1.5 Multiple organ damage in sepsis

In sepsis, organ damage is frequently observed in vital organs such as the kidneys, liver, heart, and lungs that are quickly compromised by the failure of blood perfusion. Multiple organ failure contributes to an increased mortality in septic patients.[3] The pathogenesis of organ damage, however, is incompletely understood. Tissue hypoperfusion and hypoxia are important reasons for the organ damage. The mechanisms may involve the decreased microperfusion secondary to thrombi formation, reduced red blood cell deformability, blood maldistribution, and tissue edema caused by increased capillary permeability. In addition, NO-induced impairment in mitochondrial respiration impacts the utilization of available oxygen by the cells.[48] Infiltration of neutrophils and

lymphocytes can also damage tissue directly by releasing lysosomal enzymes and superoxide-derived free radicals.[49]

1.1.6 Immunosuppression in sepsis and “compensatory anti-inflammatory response syndrome”

Traditionally, an exacerbated production of pro-inflammatory mediators as illustrated by the so-called “cytokine storm” was considered to be the cause of sepsis.[50] However, recent studies have shown that anti-inflammatory responses occur early and simultaneously in sepsis as well.[51] Therefore, the host immune response to sepsis is currently considered to be characterized by two established hemodynamic phases that may overlap. The initial phase, or the hyper-dynamic phase, is defined as a massive production of pro-inflammatory cytokines, chemokines and reactive oxygen species by macrophages and neutrophils that affects vascular permeability (leading to hypotension) and cardiac function and induces metabolic changes culminating in tissue necrosis as well as organ failure. The second phase, or the hypo-dynamic phase, is an anti-inflammatory process involving altered monocyte antigen presentation, decreased lymphocyte proliferation and responsiveness and promoted lymphocyte apoptosis and anergy.[52, 53] The latter state, also known as immunosuppression or immune depression, sharply increases the risk of nosocomial infections and ultimately, death.[54] Figure 1.2 shows a simplified paradigm of the host response to sepsis.

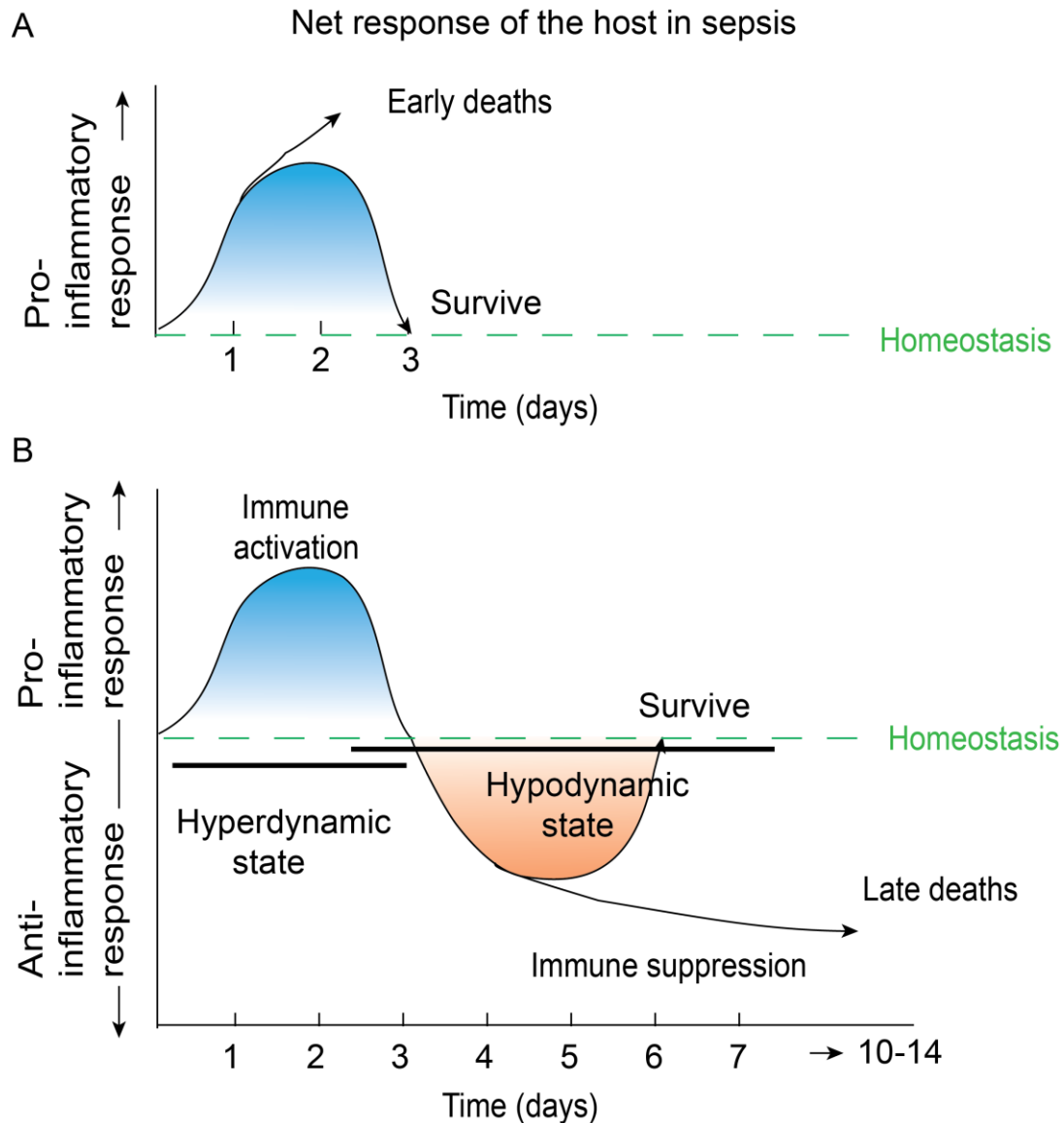


Figure 1.2 Simplified paradigm of the host response to sepsis.

Progression of sepsis is complex, nonlinear, and varies from individual patient to another.

(A) The initial response in previously healthy patients with severe sepsis is typified by an overwhelming hyper-inflammatory phase. The hyper-inflammation contributes to early deaths in sepsis. (B) Patients who survived the first hyper-inflammatory phase may develop immunosuppression due to the alterations in immunity during sepsis. This immunosuppressive status makes them susceptible to nosocomial infection, which also causes death. [55]

In clinic, the release of anti-inflammatory mediators appears to be exacerbated in septic patients, which can be illustrated by the strong relationship between high levels of plasma anti-inflammatory mediators and poor outcome.[56] In addition, the plasma of sepsis patients has the capacity to inhibit leukocyte functions and is considered an immunosuppressive milieu.[57] Furthermore, the fact that intensive care patients are highly susceptible to nosocomial infection indicates an alteration of their immune status. Thus, words such as anergy [58], immunodepression [59] and immunoparalysis [60] have been employed to define such immune status. In 1997, Roger Bone coined a new acronym CARS for “compensatory anti-inflammatory response syndrome” to describe the properties of immune status in patients with sepsis.[61, 62]

The immunosuppression in sepsis involves the alterations in both innate immunity and adaptive immunity. In innate immunity, sepsis can reduce the phagocytic activity of neutrophils. Phagocytosis by neutrophils of septic patients was reported to be lower than that of neutrophils from control patients, probably due to the immaturity of neutrophils in septic patients.[63] The impaired phagocytic activity of neutrophils in septic patients is associated with a high mortality sepsis.[64] However, no difference has been observed in the phagocytic activity of monocytes in the septic patients.[64] In adaptive immunity, sepsis-induced lymphocyte apoptosis may play an important role in inducing immunosuppression. Prevention of lymphocyte cell death in experimental sepsis can improve the survival of mice.[65] Profound progressive loss of B and CD4 T

helper cells was observed in septic patients, which is likely mediated by both death receptor and mitochondrial-mediated apoptotic pathway.[66, 67]

A major consequence of CARS is the increased susceptibility to nosocomial infections. It has been indicated that the severity of the immunosuppression is associated with an increased probability of developing severe sepsis.[68] In line, “two hit” animal models have suggested that an enhanced susceptibility to a sublethal infection occurs following a first septic insult.[69-71] A first insult, usually a peritonitis which is experimentally induced by CLP, renders the animal more susceptible to a secondary bacterial or viral lung infection.[69-71] In fact, most of the patients with sepsis or septic shock undergoing an immunosuppression do not die from the initial systemic infection, but from a second or third hit, that they acquire in hospital. [54, 72]

Thus, a model of immune disorders in sepsis is proposed. In the beginning of sepsis, the pro-inflammatory mediators (referred to as the SIRS) and the anti-inflammatory mediators (referred to as the CARS) are balanced. Inflammation activates the SIRS mediators, which activates the host immune-inflammatory system and can be de-activated through the expression of CARS mediators; or once CARS mediators are more abundant. As sepsis develops, the expression of SIRS and CARS is out of control, resulting in an exaggerated and dysfunctional inflammatory response.[73] The dysregulation of inflammatory and immune responses may be different from individual to individual. This heterogeneity of septic patients makes it difficult to treat sepsis clinically.[74] (Figure 1.3)

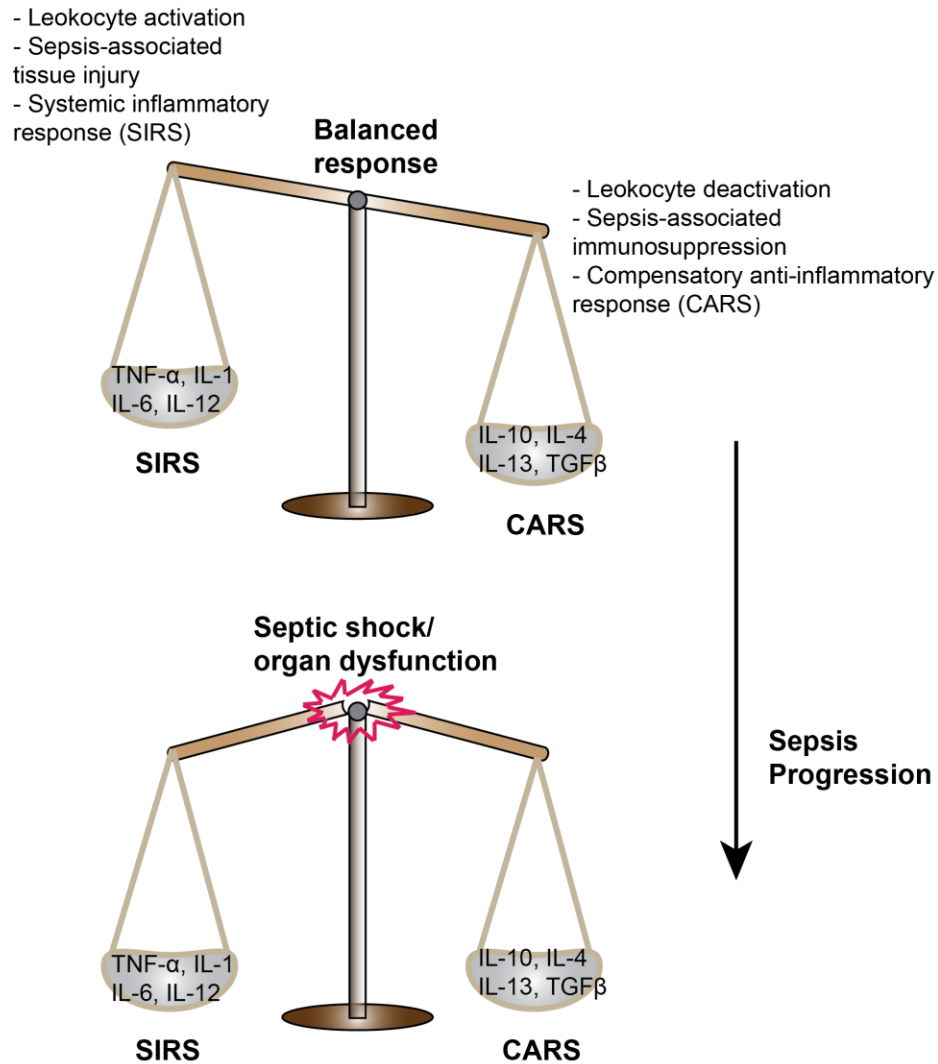


Figure 1.3 Sepsis and CARS

The host inflammatory response during inflammation can be viewed as a balanced response between pro-inflammatory mediators (referred to as the SIRS) and anti-inflammatory mediators (referred to as the CARS). Inflammation activates the SIRS mediators such as TNF- α , IL-1, IL-6 and IL-12, which activate the host immune-inflammatory system and can be de-activated through the expression of CARS mediators, including TGF- β , IL-4, IL-10 and IL-13; or once CARS mediators are more abundant. During the development of sepsis, the expression of SIRS and CARS is out of control, resulting in an exaggerated and dysfunctional inflammatory response.[73]

1.2 Glucocorticoids (GC)

1.2.1 Synthesis of GC

Glucocorticoids (GC) is a class of steroid hormones. The name glucocorticoid derives from its role in the regulation of the metabolism of glucose, its synthesis in adrenal cortex and its steroidal structure. Adrenal glands are the main source of GC in circulation. Each gland is made of two parts: the medulla and the cortex. The medulla belongs to the sympathetic system and produces catecholamine (epinephrine, norepinephrine and dopamine). The cortex has three zonas. From the exterior to the interior is the zona glomerulosa which secretes mineralocorticoids (aldosterone and desoxycorticosterone); the zona fasciculata which produces GC (mainly cortisol in human and corticosterone in rodents); and the zona reticularis which produces androstenedione, a metabolic intermediate in the biosynthesis of the androgen and estrogen sex steroids. The hormones secreted from adrenal cortex all derive from cholesterol following a cascade of enzymatic reactions, as shown in Figure 1.4.

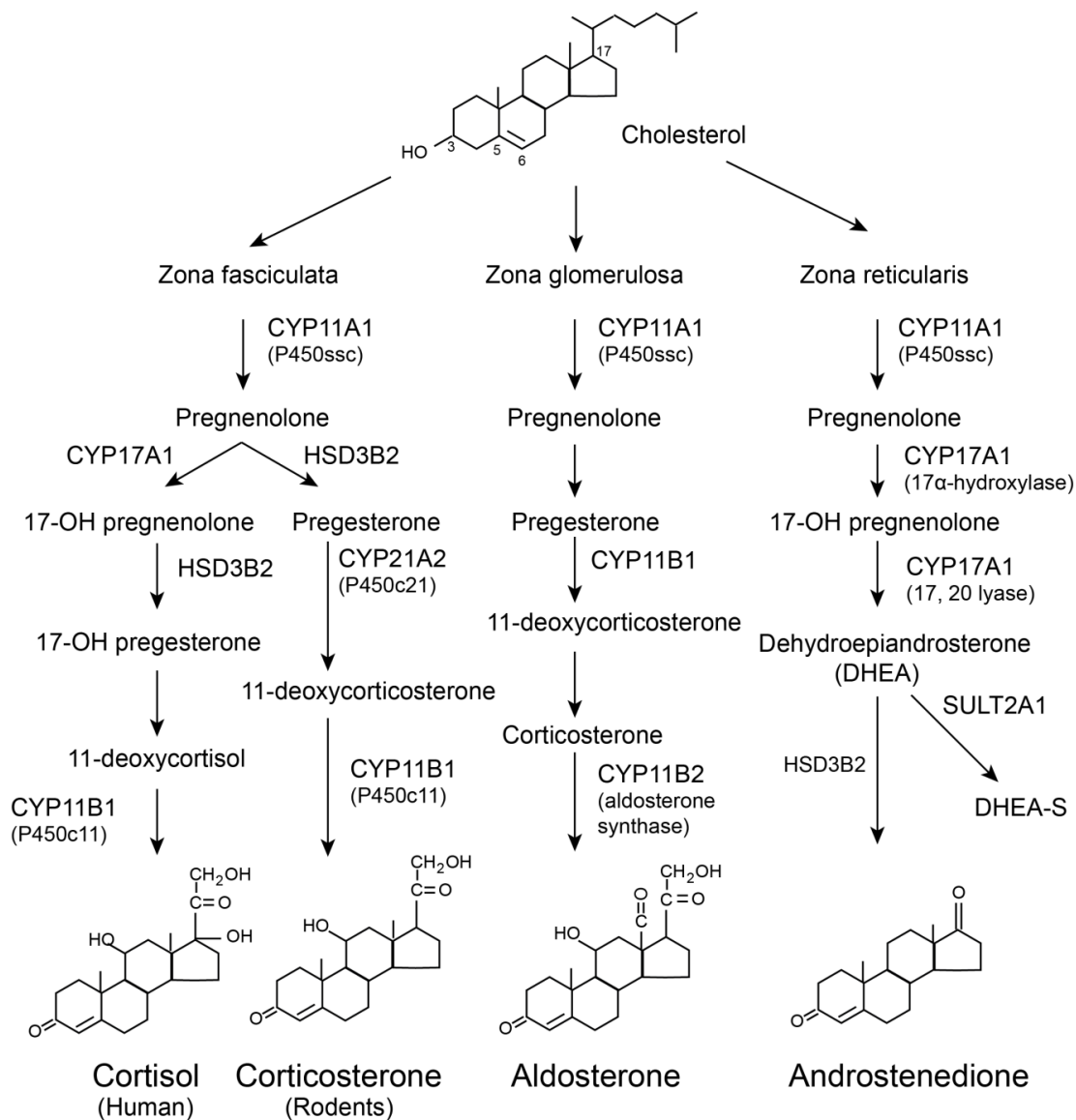


Figure 1.4 Synthesis of GC in adrenal glands.

Steroid hormones are synthesized in the cortex of adrenal glands which contain zona glomerulosa, zona fasciculata and zona reticularis. Each zona is distinguished for the synthesis of different kinds of steroid hormones through specific enzymes. Cholesterol is the common substrate for steroid hormones. GC, mainly cortisol in human and corticosterone in rodents, are synthesized in zona fasciculata. Mineralocorticoids, mainly aldosterone, are synthesized from zona glomerulosa. Androstenedione, a common precursor for sex hormone, is produced from zona reticularis.

Free cholesterol is the substrate for the glucocorticoid synthesis. In steroidogenic cells, about 80% cholesterol required for steroidogenesis comes from exogenous sources, namely circulating lipoproteins, and about 20% of the cholesterol comes from local *de novo* synthesis from acetate (acetyl CoA).[75] Exogenous cholesterol esters from lipoproteins can be delivered by either receptor-mediated endocytic uptake or “selective” cellular uptake. Low-density lipoprotein (LDL) receptor mediates the endocytic uptake of LDL, or other apolipoprotein B-or apolipoprotein E-containing lipoproteins, where the intact lipoprotein is internalized and degraded in lysosomes.[76] The cholesterol esters delivered via this pathway are hydrolyzed to free cholesterol in lysosomes. Scavenger receptor B1 (SR-BI) mediates the “selective” uptake of lipoprotein cholesterol esters in which SR-BI selectively delivers the cholesteryl esters into cells without internalizing the lipoprotein particle itself. Selectively-delivered cholesteryl esters are hydrolyzed by neutral cholesteryl ester hydrolase (hormone-sensitive lipase, HSL).[77] Unesterified cholesterol is also derived from the hydrolysis of cholesteryl esters (CE) stored in lipid droplets through the actions of HSL or via endogenous synthesis from acetyl CoA via the rate limiting enzyme hydroxymethylglutaryl coenzyme A reductase (HMG-CoA reductase). Unesterified cholesterol can be esterified for storage in lipid droplets by acyl-coenzyme A:cholesterol acyltransferase (ACAT) or can be transported into mitochondria via steroidogenic acute regulatory protein (StAR) for metabolism by cholesterol side chain cleavage enzyme (CYP11A1). (Figure 1.5)

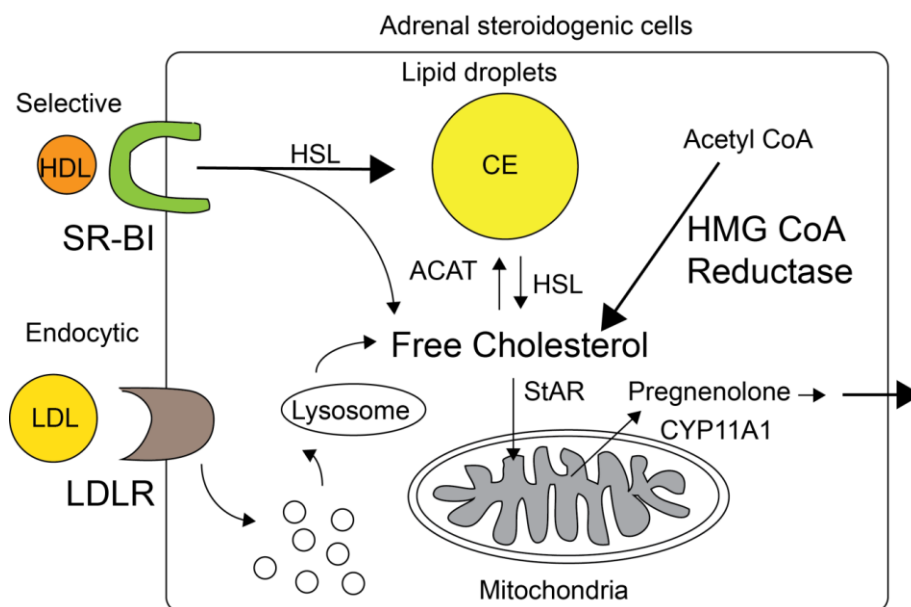


Figure 1.5 Sources of cholesterol for the synthesis of GC in adrenal steroidogenic cells.

Cholesterol is the substrate for the synthesis of GC. Uptake of cholesterol from lipoproteins involves selective uptake of high-density lipoprotein (HDL) cholesteryl esters via SR-BI and the endocytic uptake of low-density lipoprotein (LDL) cholesteryl esters via LDL receptor. Selectively delivered cholesteryl esters are hydrolyzed by hormone-sensitive lipase (HSL), whereas cholesteryl esters delivered by receptor-mediated endocytosis are hydrolyzed to free cholesterol in lysosomes. Free cholesterol is also derived from the hydrolysis of cholesteryl esters (CE) stored in lipid droplets through the actions of HSL or via endogenous synthesis from acetyl CoA via the rate limiting enzyme hydroxymethylglutaryl coenzyme A reductase (HMG CoA Red). For steroidogenesis, free cholesterol is transported into mitochondria via steroidogenic acute regulatory protein (StAR) for metabolism by cholesterol side chain cleavage enzyme (CYP11A1).

[78]

1.2.2 Metabolism of GC

The major site of GC metabolism is the liver, where GC is reduced, oxidized, or hydroxylated. The products of these modifications are made water soluble by conjugation with sulfate or glucuronic acid to facilitate their excretion in urine. Many enzymes are involved in these processes of GC metabolism, such as 5 α -reductases, 5 β -reductases, and 3 α -hydroxysteroid dehydrogenases in the reduction, the CYP3A4 enzyme in the hydroxylation, uridine diphosphoglucouronosyl transferases [79] and sulfotransferases [80] in the conjugation. 11 β -hydroxysteroid dehydrogenase (11- β -HSD1) is also a key enzyme in glucocorticoid metabolism, with both dehydrogenase and reductase activities.[81]

1.2.3 Basal GC and inducible GC (iGC)

Under normal conditions, the synthesis and release of GC is regulated by the hypothalamic-pituitary-adrenal (HPA) axis in a feedback manner. The paraventricular hypothalamus secretes corticotropin releasing hormone (CRH) into the hypothalamic-pituitary venous system and enhances the pituitary production of adrenocorticotrophic hormone (ACTH) yielded from proopiomelanocortin (POMC). ACTH in circulation applies a trophic action on the adrenal glands, increasing the production and releasing of GC. GC feeds back on the HPA axis negatively, reducing the production of CRH and ACTH. Basal plasma GC concentrations display a circadian rhythm reaching the zenith between 6-8 am and nadir at midnight.[82] Circulating basal GC is important for maintaining organismal homeostasis and is thought to have permissive effects: at

basal levels, glucocorticoids help the organism prepare for subsequent energetic challenges and potentially enhance the initial response to future stressors.[83]

Under stressed conditions such as sepsis, the rhythm and feedback of basal GC are lost. Circulating and locally expressed pro-inflammatory mediators, especially cytokine TNF- α , IL-1 β and IL-6 activate the HPA axis independently and synergistically. Afferent vagal fibers at local tissue also stimulate the HPA axis after sensing the inflammatory threat.[84] In addition, the production of cortisol binding globulin (CBG) decreases at the same time, which further increases the plasma concentration of free GC. Consequently, the level of circulating GC goes up. Our study has been focused on the GC produced upon sepsis, which I call inducible GC (iGC). (Figure 1.6)

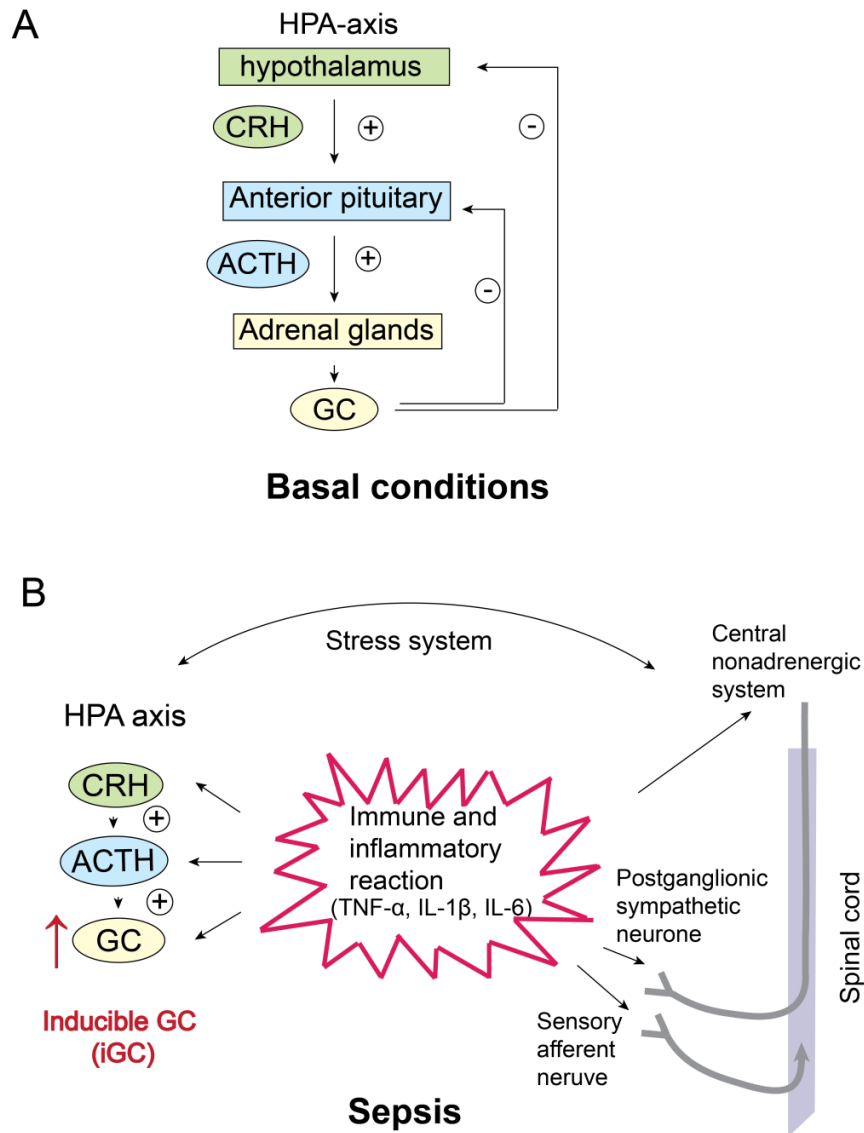


Figure 1.6 Regulation of GC under basal conditions and in sepsis.

Under basal conditions, the production of GC is tightly regulated by the hypothalamic-pituitary-adrenal (HPA) axis in a feedback pattern. In sepsis, the immune and inflammatory reaction produces local and systemic inflammatory cytokines and mediators, which activate the HPA axis directly and indirectly through the activation of stress system. In this case, the feedback is lost and the inducible GC (iGC) is produced. CRH, corticotropin releasing hormone; ACTH, adrenocorticotrophic hormone

1.2.4 Synthetic GC

Most of research on the function of GC is conducted using synthetic GC. In humans, these include prednisolone and methylprednisolone; in animal models and *in vitro*, dexamethasone is most commonly used. However, because synthetic GC has different properties from endogenous GC, studies of synthetic GC may have provided an over-simplified view of the actions of GC.

Modification of the natural GC yields synthetic GC that may have more potent glucocorticoid activity as well as an increase in mineral corticoid potency. In contrast to natural GC with high affinity for CBG, synthetic steroids other than prednisolone either bind weakly to albumin (two-thirds) or circulate as free steroid (one-third). Exogenous GC is subject to the same reduction, oxidation, hydroxylation, and conjugation reactions as endogenous steroids. However, the plasma half-lives of synthetic GC are generally longer than those of endogenous GC.[85-87] GC that are fluorinated at the 6- α or 9- α position (dexamethasone, fludrocortisone, betamethasone) or methylated at the 6- α position (methylprednisolone), or methyloxazoline at position 16,17 (deflazacort), are protected from oxidation inactivation by the type 2 11- β -hydroxysteroid dehydrogenase (11 β -HSD2) [88], which may explain their longer activity than cortisol. Overall, the variation in the susceptibility to metabolism by 11- β -hydroxysteroid dehydrogenase and in the binding affinities for GR and MR leads to the diversity of synthetic GC in ant-inflammatory or mineralocorticoid potency and duration of action.[89] (Table 1.2)

Table 1.2 GC in clinic.

		Potency relative to Hydrocortisone		Half-Life	
	Equivalent Glucocorticoid Dose (mg)	Anti-Inflammatory	Mineral-Corticoid	Plasma (minutes)	Duration of Action (hours)
<i>Short Acting</i>					
Hydrocortisone (Cortisol)	20	1	1	90	8-12
Cortisone Acetate	25	0.8	0.8	30	8-12
<i>Intermediate Acting</i>					
Prednisone	5	4	0.8	60	12-36
Prednisolone	5	4	0.8	200	12-36
Triamcinolone	4	5	0	300	12-36
Methylprednisolone	4	5	0.5	180	12-36
<i>Long Acting</i>					
Dexamethasone	0.75	30	0	200	36-54
Betamethasone	0.6	30	0	300	36-54
<i>Mineralocorticoid</i>					
Fludrocortisone	0	15	150	240	24-36
Aldosterone	0	0	400+	20	- -

Modification of the natural GC yields synthetic GC that may have more potent glucocorticoid activity as well as an increase in mineral corticoid potency. The resistance to the oxidation inactivation by the 11 β -HSD2 contributes to a longer half-life of synthetic GC than that of endogenous GC.

1.2.5 Molecular mechanisms of GC action

Free GC is the biologically active form of GC. However, under normal conditions, 90-95 % of plasma cortisol, the main GC in human, is bound to albumin and cortisol binding globulin (CBG). The CBG-bound cortisol has restricted access to target cells.[90, 91] At inflammatory sites, CBG acts as a substrate for elastase produced by neutrophils, allowing localized delivery of cortisol.[91] Cortisol then can freely cross the cell's membrane, or it may interact with specific membrane binding sites. Alternatively, cortisol is inactivated by the action of type 2 11- β -hydroxysteroid dehydrogenase (11- β HSD2), which converts cortisol to cortisone, the inactivate form. The 11- β HSD2 is found mainly in mineralocorticoid target tissues (kidney, colon, salivary glands) and in the placenta. Conversely, cortisone can be converted into cortisol by type 1 11 β -hydroxysteroid dehydrogenase (11- β -HSD1) converts cortisone into cortisol, which is expressed in many glucocorticoid target tissues.[92, 93]

GC exerts their effects mainly by binding to corticosteroid receptors. There are two types of corticosteroid receptors: the type I receptor, which is also referred to as the mineralocorticoid receptor (MR) and the type II receptor, which is commonly known as the glucocorticoid receptor (GR). In classical mineralocorticoid target tissues involved in electrolyte and volume regulation, the MR is co-localized with 11- β -HSD2 and thus inactivates GC. (Figure 1.7)

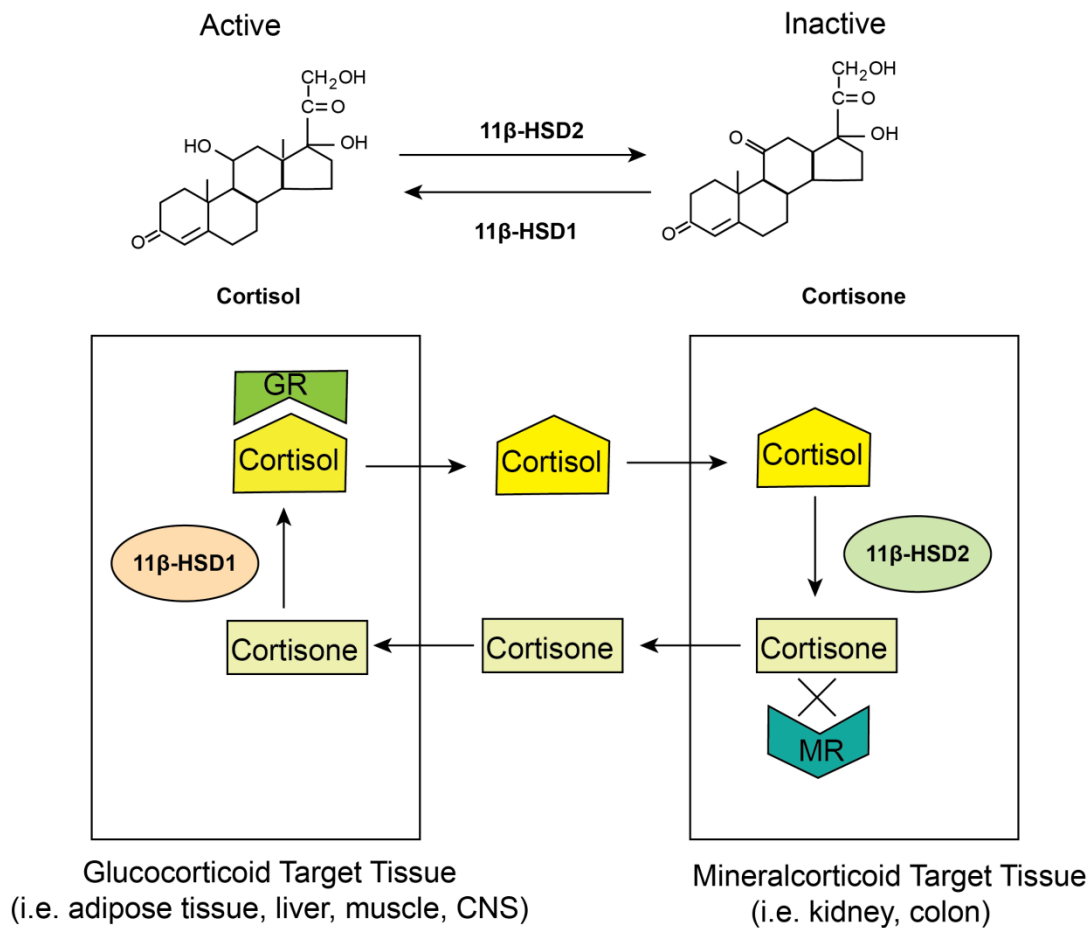


Figure 1.7 11β-Hydroxysteroid dehydrogenases modulate GC action.

11β-Hydroxysteroid dehydrogenases (11β-HSDs) modulate the activation and inactivation of GC. 11-β HSD2 inactivates GC and 11-β-HSD1, conversely converts the inactive form into cortisol. In mineralcorticoid target tissues, the mineralcorticoid receptor co-localizes with 11-β HSD2 and inactivates GC. In contrast, glucocorticoid target tissues express 11-β-HSD1, which allows GC to exert its functions. CNS, central nervous system

Glucocorticoid receptor (GR) is a 94 kDa heterodimeric protein, which belongs to the nuclear receptor family. The human glucocorticoid receptor gene (GR) is located on chromosome 5 and is responsible for the expression of α and β subunits. Like the other steroid receptors[94], GR has three major distinct functional domains- the N-terminal or immunogenic domain, the DNA binding domain (DBD), and the ligand-binding domain (LBD).[95] GR α in unbound state is located primarily in the cytoplasm as part of a hetero-oligomeric complex that contains heat shock proteins (HSPs) 90, 70, 50 and 20 and, possibly other proteins as well.[96] The free cortisol can enter into the cell and bind to the intracellular glucocorticoid receptor type α (GR α). The GR isoform β (GR β) does not itself bind corticosteroids and resides constitutively in the nucleus of cells.[96-100].

Most of the actions of GC are attributable to the transcriptional effects of GR.[101] After binding to an agonist ligand, GR undergoes conformational changes, dissociates from the HSPs and other chaperone proteins, and translocates into the nucleus as a monomer or dimer by means of an active ATP-dependent process mediated by its nuclear localization signals. Once in the nucleus, ligand-activated GR dimers interact directly with glucocorticoid-responsive elements (GREs) in the promoter regions of target genes. Ligand-activated GR monomers also interact with other transcription factors at the cytoplasmic level and thus indirectly regulate the activity of the latter on their own target genes [96]. The ability of GR to inhibit the activity of crucial transcriptional regulator of pro-inflammatory genes including NF- κ B and activating protein-1

(AP-1) is believed as an important mechanism for the anti-inflammatory action of GC. In addition, GC can also signal through membrane-associated receptors (non-genomic pathways) through a GR-dependent or independent manner, which contributes to the quick actions of GC.[102, 103]

GC has been considered as an anti-inflammatory and immunosuppressive agent [101] due to its inhibitory effects on the pro-inflammatory cytokines and suppressive actions on immune cells, such as inducing the apoptosis of lymphocytes [104] and inhibiting the function of T lymphocytes [105]. However, some recent studies challenged this conventional concept. *In vitro* culture of macrophages with the treatment of corticosterone showed that corticosterone exerts opposing effects on macrophage functions in a concentration-dependent manner. Low-corticosterone treatment can enhance the macrophage production of NO, chemotaxis of macrophages and the phagocytosis of *E. coli* by macrophages, whereas high-corticosterone treatment represses these macrophages functions.[106, 107] In addition, it has been demonstrated that GC can regulate genes involved in the phagocytosis of monocytes and leads to a significant increase in the phagocytosis of latex beads and live parasites by the cultured human monocytes.[108] These findings led us to ask the question that whether or not iGC has actions to promote immunity in sepsis.

1.3 SR-BI

1.3.1 Basics of SR-BI

Scavenger receptor class B, type I (SR-BI, gene name SCARB1) is a 75kDa cell surface glycoprotein that belongs to the CD36 superfamily and has the common structural features of this family, including membrane-associated hydrophobic regions near the N- and C-termini, a large extracellular loop that comprises the portion of the peptide sequence between the two membrane-bound end regions, and several sites of N-linked glycosylation.[109-111]

Although SR-BI is expressed in many mammalian tissues and cell types, it is most highly expressed in tissues that are dependent on HDL cholesterol for bile acid or hormone synthesis, including liver, ovary, testes and adrenal glands.[112, 113]

SR-BI binds to a variety of ligands including native and modified lipoproteins, modified serum proteins such as maleylated serum albumin, lipid vesicles containing anionic phospholipids, apoptotic cells and bacterial cell surface components such as LPS.[114, 115] Correspondingly, SR-BI has multiple functions.

1.3.2 SR-BI as a multi-functional protein

The best-known function of SR-BI is to serve as the HDL receptor.[116] Studies of knockout and transgenic mice have revealed that SR-BI plays a critical role in HDL metabolism, being a very efficient receptor for cholesterol and cholesteryl ester transport between cells and HDL.[116-119] Deficiency of SR-BI

leads to a disruption of cholesterol metabolism that is characterized by abnormal HDLs which are larger in size and enriched with free cholesterol. Thus, SR-BI-deficient mice develop hypercholesterolemia with a remarkable increase in plasma free cholesterol and a high ratio of plasma unesterified cholesterol (UC)-to-total cholesterol (TC, unesterified plus esterified cholesterol). The increase in plasma UC-to-TC ratio in SR-BI-deficient mice leads to the increase of free cholesterol in red blood cells [120] and platelets in circulation [121]. Deposition of free cholesterol in red blood cells in SR-BI-deficient mice contributes to their phenotypes of reticulocytosis (an increase in reticulocytes in blood) and impairs the development of erythrocytes.[120, 122] The high unesterified cholesterol in platelets in SR-BI-deficient mice also contributes to their abnormal morphologies, elevated rates of clearance of platelets from circulation and defects in ADP-induced aggregation.[123-125] These abnormalities in platelets in mice are associated with an increased susceptibility to thrombosis [126]. In addition, as the HDL receptor, SR-BI mediates the activation of endothelial nitric oxide synthase (eNOS) by HDL, which leads to increased production of NO [127-130] and can induce a ligand-independent apoptotic pathway that is regulated by eNOS and HDL [131].

SR-BI is also important in immunity. In innate immunity, SR-BI mediates the uptake of gram-negative bacteria, gram-positive bacteria, LPS and LTA in macrophages, thus it facilitates the clearance of bacteria and the alleviation of inflammation.[132, 133] Recently, studies have revealed that SR-BI also mediates HCV entry [134-136], which is very likely attributed to the lipid transfer

function of SR-BI. [137] Paradoxically, SR-BI may also be important in initiating the host defense against HCV, as dendritic cells depend on SR-BI to uptake HCV.[138] In adaptive immunity, SR-BI is critical in maintaining lymphocyte homeostasis, as indicated by an imbalanced lymphocyte expansion in the spleen and the development of autoimmune disorders in SR-BI-deficient mice.[139] The adaptive immune defects in SR-BI-deficient mice are at least partly attributed to their abnormal HDL particles which are dysfunctional in inhibiting anti-CD3-stimulated T cell proliferation and LPS-induced B cell proliferation.[139]

1.3.3 SR-BI and GC synthesis

While both SR-BI and LDL receptor participate in the uptake of cholesterol from circulating lipoproteins, human studies support that the SR-BI-mediated pathway, rather than the other is more important for the steroidogenic function of adrenal glands. In homozygous familial hypercholesterolemia patients who have complete LDL-receptor deficiency, the adrenal cortex can respond to a single injection of ACTH with a normal increase in corticosteroid production [140] and only present a mildly reduced function upon a prolonged stimulation (24 to 36 h) with ACTH [141]. Additionally, a 50 % reduction in the number of high affinity LDL receptors in heterozygous familial hypercholesterolemia patients does not result in any impairment in the delivery of cholesterol to the adrenal cortex even during conditions of maximal corticosteroid production (36 h i.v. infusion of ACTH).[119] In contrast, carriers of the P297S mutation, a functional mutation of SR-BI, have attenuated adrenal steroidogenesis, as evidenced by decreased urinary excretion of sterol metabolites, a higher level of cortisol-binding globulin in the

plasma, a decreased response to corticotropin stimulation, and symptoms of diminished adrenal function such as fatigue and dizziness.[142]

In rodents, the SR-BI-mediated selective uptake of HDL cholesterol provides a major supply of cholesterol for GC production under stress.[118, 143-147] A normal maximum corticosterone production upon ACTH stimulation can be observed in the LDL receptor knockout mice indicating that the LDL receptor is not necessary for acute adrenal steroidogenesis.[148, 149] In contrast, mice deficient in ApoA-I, the major HDL ligand for SR-BI, show a blunted corticosterone response to ACTH stimulation [112] and lecithin-cholesterol acyltransferase (LCAT) deficient mice which have decreased plasma HDL levels also present a diminished corticosterone response to ACTH injection or LPS exposure[150]. These studies demonstrate the importance of HDL as cholesterol donor for the synthesis of GC in adrenal under stress. In this case, SR-BI, as the only receptor that mediates the adrenal uptake of cholesteryl esters from HDL in mice [151], is critical for GC synthesis under stress.

1.3.4 SR-BI and sepsis

SR-BI is important in preventing septic death. Our group first reported a protective role of SR-BI in sepsis induced by LPS [152] or by cecal ligation and puncture (CLP) [110]. SR-BI-deficient mice display an increased susceptibility to death in LPS-induced endotoxemia and CLP-induced sepsis. A sublethal-dose of LPS in wild type mice can cause a mortality of 90 % in SR-BI-deficient mice.[152] CLP that causes a 20 % fatality in wild type mice can lead to a 100 % fatality in SR-BI-deficient mice.[110] SR-BI can protect against septic death through many

mechanisms. A significant suppression of tyrosine-nitrated protein formation by SR-BI which prevents NO-induced cytotoxicity may contribute to the ability of SR-BI to protect against LPS-induced death.[152] SR-BI in the adrenal mediates the uptake of cholesterol for the production of iGC in sepsis, which is likely to play an essential role in preventing septic death given the potent anti-inflammatory effects of GC.[109, 110] SR-BI-deficient mice present no iGC production in endotoxemia or CLP. Supplementation of corticosterone by drinking water restored survival of SR-BI-deficient mice in LPS-induced endotoxemia [109], though supplementation of corticosterone in the same way failed to increase the survival rate of SR-BI-deficient mice in CLP-induced sepsis [110]. In addition, macrophage SR-BI is important for controlling the inflammatory response. Macrophage SR-BI can suppress the macrophage production of pro-inflammatory cytokines by inhibiting TLR4-mediated NF- κ B activation. [110] The regulation of macrophage pro-inflammatory production by SR-BI also involves JNK and p38 cell signaling pathways.[153] This inhibitory effect of SR-BI protects against septic death as indicated by that over-expression of SR-BIC323G (a mutant SR-BI lacking activity for GC production but capable of suppressing TLR4-NF- κ B signaling) in SR-BI-deficient mice significantly improves their survival in CLP.[110] SR-BI in the liver is also a critical protective factor in sepsis by mediating LPS clearance. *Scarb1*^{I179N} mice, a mouse model specifically deficient in hepatic SR-BI, show a higher mortality than the wild type mice.[154] Therefore, SR-BI protects the animals from septic death through multiple mechanisms as shown in Figure 1.8.

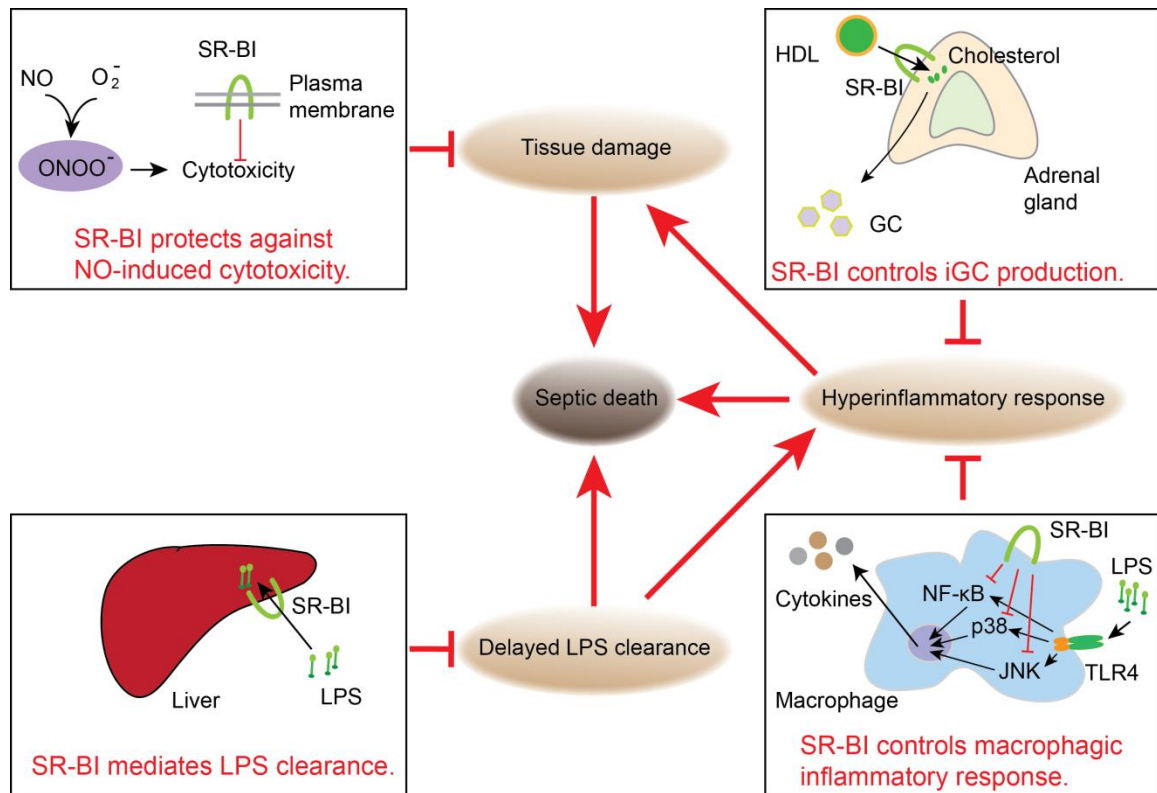


Figure 1.8 SR-BI plays multiple functions in preventing septic death.

SR-BI knockout mice show increased susceptibility to septic death. A number of possible mechanisms may contribute to the protective effects of SR-BI in sepsis: (1) SR-BI protects against NO-induced cytotoxicity (upper left), which prevents tissue damage; (2) Adrenal SR-BI mediates the uptake of HDL cholesterol ester to provide cholesterol for the synthesis of iGC, which keeps inflammatory responses under control; (3) Macrophage SR-BI inhibits LPS-induced inflammatory signaling involving TLR4, JNK, p38 and NF- κ B pathways (bottom right), which alleviates inflammatory response. (4) Hepatic SR-BI mediates the clearance of LPS and thus attenuates the endotoxemia in sepsis.[155]

1.4 Statement of the problem

1.4.1 Adrenal insufficiency in sepsis

Adrenal insufficiency (AI) is indicated by the incapability of adrenal to produce cortisol and has been reported in association with septic shock.[156-158] The secretion failure of the adrenal glands was first implicated as a factor in the pathogenesis of circulatory shock associated with infection after the original report of Waterhouse and Friderichsen.[159] These observations emphasized that the functional integrity of the HPA axis is essential to survival in severe insults. Several decades later, variations in adrenocortical responsiveness in septic patients were observed as indicated by the different plasma cortisol response to synthetic ACTH.[156] In a study of 32 septic shock patients, the patients with poor plasma cortisol response to ACTH all died.[158]

However, the definition of adrenal insufficiency in sepsis has not been well defined. The reported range of serum cortisol in sepsis has varied widely, which was probably due to different degree of stress that the enrolled subjects had, the type of infection, length of time in shock, and the blood pressure at the time of blood sampling.[156, 160-162] Of note, the increase in cortisol levels not only is due to the activation of the HPA axis but also partly results from decreased cortisol extraction from the blood, decreased binding to CBG, and an increase in the half-life of cortisol.[163, 164] Some reports stated that absolute AI is uncommon in critically ill patients, but incidence depends on criteria used to define it.[158, 163, 165, 166] In addition, the optimal range for serum cortisol levels in severe sepsis remains unclear. Therefore, the concept of relative AI

then emerged. In relative AI, the cortisol level, despite being normal or high, is still considered to be inadequate for the current physiologic stress, and the patient may be unable to respond to any additional stress.[158, 167] Thus, relative AI is currently defined as an inadequate response to exogenous ACTH. Unfortunately, the methods and criteria to diagnose AI or relative AI in critical illness or sepsis have not been well defined or standardized. Investigators have used a lack of increase in plasma cortisol in response to ACTH [158, 165, 167], or an inappropriately low cortisol level during critical illness/septic shock [160, 168], or both [156, 169] to diagnose AI. Meanwhile, the lower end of serum cortisol used to diagnose AI in critical illness has ranged from less than 12.5 µg/ml to 25 µg/ml.[160, 165, 168-171] These factors overall led to the reported incidence of AI varying from 1.5% to 54%.[156, 158, 160, 167-169]

1.4.2 The use of GC in sepsis

The use of GC in the therapy of sepsis has been controversial for many decades.[172] Early in 1970s, the safety and efficacy of GC in the treatment of septic shock was investigated, finding that administration of dexamethasone or methylprednisolone decreased the mortality of septic patients.[173, 174] After this study, a short course of high-dose GC became an accepted therapy. However, subsequent studies did not confirm a survival benefit with this regimen and suggested an increase in mortality which is related to secondary infection.[175-179] In the 1990s, interest was renewed with the observation of HPA axis dysfunction in patients with septic shock.[165, 169, 180] Later, the investigators used lower doses of hydrocortisone (200 to 300 mg per day) and

found that the low-dose GC therapy decreased the duration of shock in septic patients.[181, 182] These studies prompted subsequent larger randomized trials. In a multicenter double-blind French trial, Annane *et al.* revealed that 7-d administration of hydrocortisone (50 mg intravenously every six hours) plus fludrocortisone (50 mcg enterally once a day) within eight hours of the onset of septic shock reduced 28-day mortality by 10% in patients with inadequate adrenal reserve (non-responders, maximum cortisol increase of ≤ 9 mcg/dl upon a high-dose (250 mcg) ACTH stimulation test), but made no difference to the survival of septic patients with adequate adrenal reserve (responders).[183] Following this study, the association between GC therapy and improved mortality in sepsis was supported by two subsequent meta-analyses.[184, 185] Then the use of low-dose corticosteroids was incorporated into the 2004 surviving Sepsis Campaign guidelines, recommending the use of GC for patients with septic shock who require vasopressor treatment despite adequate fluid resuscitation. Yet, the Corticosteroid Therapy of Septic Shock (CORTICUS), a large multicenter double-blind trial, revealed that hydrocortisone (50 mg) every six hours for five days can lead to a significantly faster reversal of shock (3.3 versus 5.8 days in the placebo group) but an increased incidence of new infection and non-improved 28-day mortality (35 versus 32 percent in the placebo group) in both the responders and the non-responders to the ACTH stimulation.[186] In comparing CORTICUS with the French trial, there are important methodological differences, especially the characterization of patients including the sources of infection, sepsis severity and administration time, etc. [187, 188], which may in

part explain the contradiction between their findings. (Table 1.3) At least, the time to initiate GC supplementation seems significantly matters the outcome. A study of 178 septic shock patients showed that the mortality rates increased significantly with increasing quintiles of time to initiation of low-dose corticosteroid therapy, as the early therapy group (administered within 6 hours after the onset of septic shock, n = 66) had a 37% lower mortality rate than the late therapy group (administered more than 6 hours after the onset of septic shock, n = 112; 32% versus 51%, p= 0.013).[189]

Table 1.3 Comparison of French trial and CORTICUS

	French trial	CORTICUS
Characteristics of patients		
SAPS II mean (Placebo/treatment)	57/60	49/50
Control group mortality	60%	32%
Corticotropin non-responders	77%	47%
Admission category-medical	60%	35%
Hospital-acquired infection	21%	47%
Post-surgical infection	16%	61%
Abdominal infection	16%	19%
GC therapy		
Time	within 8 hours after the onset of shock	within 72 hours after the onset of shock
GC type	hydrocortisone and fludrocortisone	hydrocortisone

SAPS II: Simplified Acute Physiology Score

The different results in the relative adrenal insufficiency subgroups between the French trial and CORTICUS resulted in new recommendations for steroid use in the more recent Surviving Sepsis Campaign guidelines that suggest steroid use only in adult patients in septic shock who are poorly responsive to fluid resuscitation and vasopressor therapy. However, trials following these recommendations have not shown benefits of steroid use in sepsis. The Surviving Sepsis Campaign included a large observational database of 17,847 patients who met eligibility criteria for low-dose systemic corticosteroids (hydrocortisone 50 mg intravenously every six hours or 100 mg every eight hours) following the guideline. The results from this campaign showed that the adjusted hospital mortality was significantly higher in patients who received low-dose steroids compared to those who did not.[190] A prospective, multicenter observational study reevaluated the Survival Sepsis Campaign guidelines recommendations in steroid use and reported that low-dose steroid had no benefits on the hospital mortality.[191] More recent international PROGRESS (PROmoting Global Research Excellence in Severe Sepsis) cohort study of 10,925 severe sepsis patients revealed that the hospital mortality in the low dose corticosteroid (LDC, equivalent or lesser potency to hydrocortisone 50 mg/6 hourly plus 50 µg 9-alpha-fludrocortisone) group was higher than the control group without LDC treatment.[192] Thus, it can be seen that the use of GC in sepsis is still controversial and a better understanding of its function during sepsis is needed to use GC more effectively in the treatment of sepsis.

1.4.3 Animal models to study GC in sepsis

1.4.3.1 Previous approaches to study GC function are not feasible to study iGC in sepsis.

Before the current study, two different approaches have been used to investigate GC function: removal of endogenous GC (by adrenalectomy or GC-receptor antagonists) or administration of exogenous GC. However, these approaches are not feasible to study iGC in sepsis.

The approaches of adrenalectomy and GC-receptor antagonist, which remove both basal GC and iGC, are not feasible to specifically investigate the role of iGC in sepsis. Previous studies have revealed that endogenous basal GC has a role in regulating the homeostasis of metabolism, immune functions and behavior under basal condition.[193, 194] Our preliminary data have shown that survival sepsis is accompanied by the maintenance of basal GC (Figure 1.9). Therefore, considering the importance of basal GC in organismal homeostasis and a potential role in sepsis, an approach that specifically eliminates the production of iGC is needed to study the functions of iGC in sepsis.

Also, exogenous GC administration is not feasible to study iGC in sepsis. For one thing, synthetic GC is different from endogenous GC in terms of the half-life and potency [195]. For another, administration of natural GC does not restore the function of endogenous GC in GC-eliminated animals [196], indicating a different action of endogenous natural GC and exogenously supplemented natural GC. Therefore, GC supplementation is not a good approach to investigate the role of iGC in sepsis.

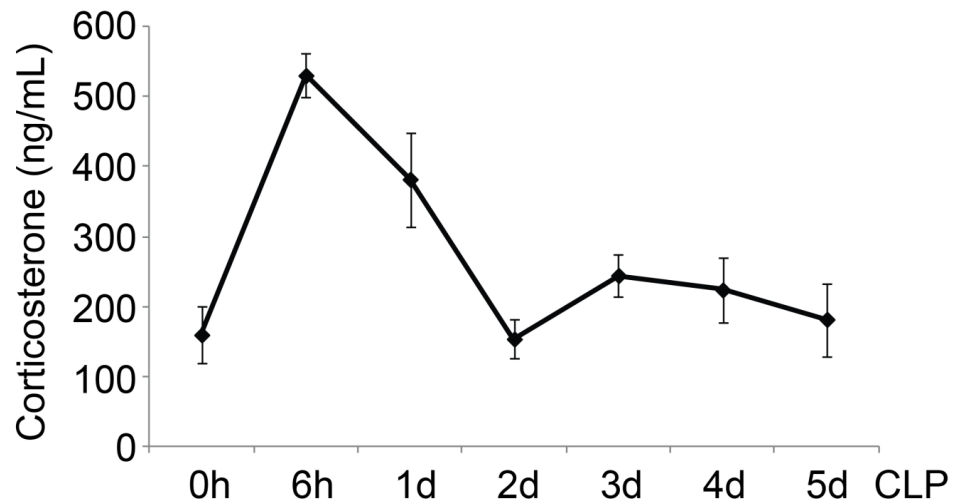


Figure 1.9 Plasma baseline GC is maintained in survival sepsis in mice.

B6 mice were subjected to a sublethal insult of CLP (21-gauge, half ligation). Plasma corticosterone concentrations were evaluated at designated time points. Data represent mean \pm SEM. (n=7)

1.4.3.2 SR-BI^{-/-} mice as a model of adrenal insufficiency

Due to the importance of SR-BI-mediated delivery of HDL cholesterol in iGC production, SR-BI knockout (SR-BI^{-/-}) mice display adrenal insufficiency under stress. Overnight fasting (approximately 16 h) stimulates the plasma level of corticosterone by 2-fold in wild-type mice but has no effect on plasma corticosterone levels in SR-BI^{-/-} mice.[197] SR-BI^{-/-} mice also cannot generate iGC in LPS-induced endotoxemia [109] or in cecal ligation and puncture (CLP)-induced sepsis [110]. Importantly, the plasma corticosterone levels under the basal conditions in SR-BI^{-/-} mice are in normal range.[110] Therefore, SR-BI^{-/-} mice can be used as an animal model of adrenal insufficiency.

1.4.3.3 Adrenal-specific SR-BI^{-/-} mice as a model to study iGC in sepsis

Although the SR-BI^{-/-} mouse is a good model of adrenal insufficiency, it is not perfect for the investigation of iGC in sepsis due to its abnormalities in other aspects such as dyslipidemia and impaired macrophage function.[155] In this regard, the adrenal-specific SR-BI-deficient (adrenal-specific SR-BI^{-/-}) mouse model is preferred to investigate the role of iGC in sepsis. Compared to previous approaches, this model has a normal range of basal plasma GC concentration. Compared to SR-BI^{-/-} mice, this model has normal SR-BI expression in other tissue and organs and is free of SR-BI deficiency-induced abnormalities other than the lack of iGC. The adrenal-specific SR-BI^{-/-} mouse model has been reported previously. Using the technique of adrenal transplantation, Hoekstra M *et al.* generated adrenal-specific SR-BI^{-/-} mice to study the role of GC in lipid

metabolism.[198] They have shown that the adrenal-specific deficiency of SR-BI leads to an impaired iGC production under a mild stress, such as fasting.

1.5 Hypothesis and experimental design

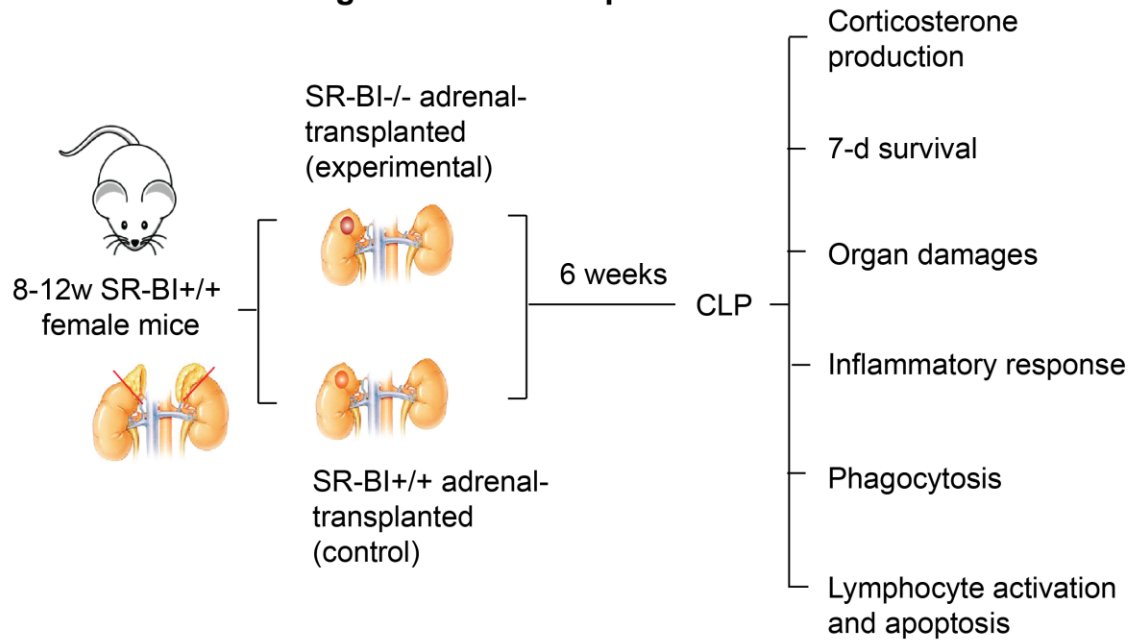
In the current project, we aim to elucidate two issues (1) the role of iGC in sepsis and (2) the effects of GC supplementation on survival in sepsis. We hypothesized that (1) the SR-BI-regulated endogenous iGC is essential for survival in sepsis and (2) GC supplementation benefits the individuals with adrenal insufficiency in sepsis but not those without adrenal insufficiency.

To study the role of iGC in sepsis, we first characterized the phenotypes of the adrenal-specific SR-BI^{-/-} mice in terms of the production of corticosterone after CLP, the lipoprotein profile and the complete blood count and verified that the adrenal-specific SR-BI^{-/-} mice can serve as a good model to study iGC in sepsis. Next, in order to elucidate the importance of iGC in sepsis, we analyzed the survival rate, the organ damages of the liver, lung and kidneys and the production of TNF- α , IL-6, NOx and IL-10 in the adrenal-specific SR-BI^{-/-} mice under CLP. In addition, to see if iGC exerts immunosuppressive effects in sepsis, we analyzed the phagocytic activity of phagocytes in the blood and spleen as well as the activation and apoptosis of lymphocytes in the spleen in the adrenal-specific SR-BI^{-/-} mice in CLP. (Figure 1.10A)

To study the effects of GC supplementation on survival in sepsis, we first transplanted SR-BI^{+/+} adrenal to SR-BI^{-/-} mice to see if supplementation of endogenous GC can improve the survival rate of adrenal insufficient mice in CLP-induced sepsis. Then we exogenously supplemented low-dose GC to SR-

BI^{-/-} mice after CLP to see if exogenous GC can benefit the survival of adrenal insufficient mice in sepsis. We also supplemented low-dose GC to SR-BI^{+/+} mice after CLP to test our hypothesis that GC supplementation is not beneficial to the mice without adrenal insufficiency in sepsis. In addition, we used a “two-hit” model of secondary infection in sepsis to study if GC supplementation also benefits the survival of adrenal insufficient mice in the secondary infection in sepsis. (Figure1.10B)

A. The role of SR-BI-regulated iGC in sepsis



B. The effects of GC supplementation on survival in sepsis

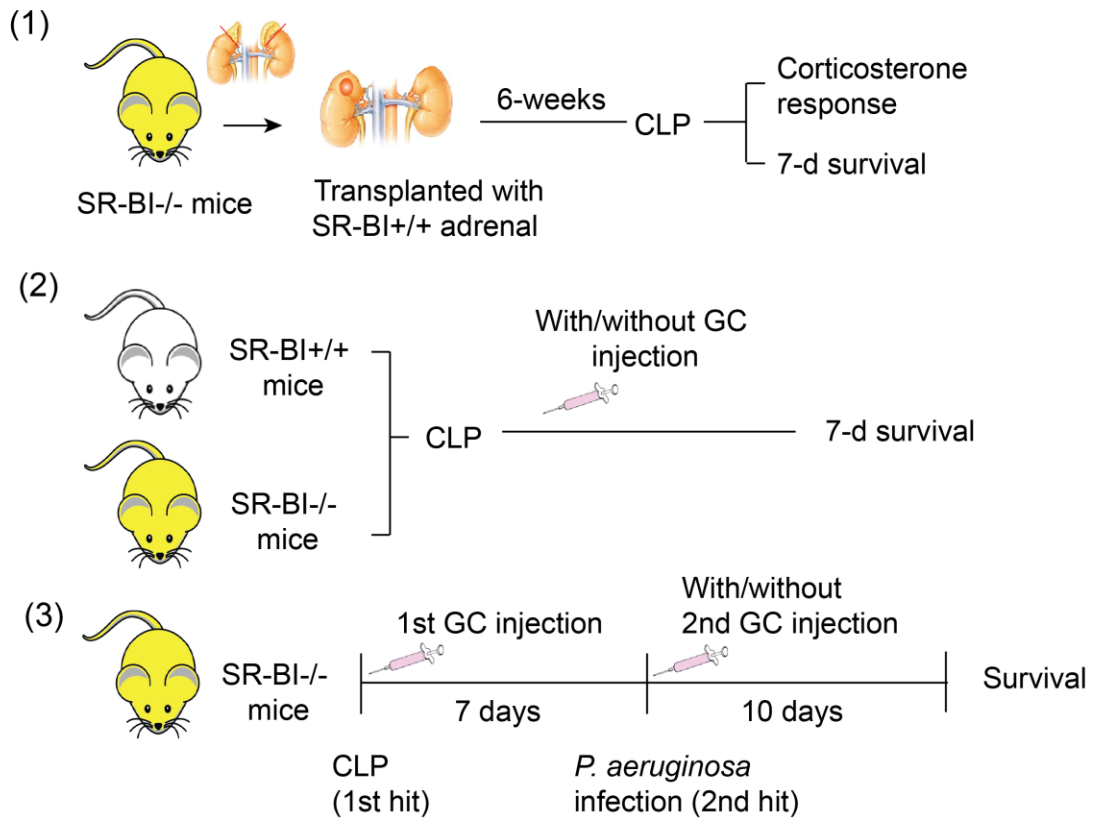


Figure 1.10 Experimental design for this project.

(A) The role of iGC in sepsis was investigated using the adrenal-specific SR-BI^{-/-} mice which was generated by adrenal transplantation. Sepsis was induced by cecal ligation and puncture. The GC production, survival rate, organ damages, cytokine levels, phagocytosis and lymphocyte activation and apoptosis in CLP were evaluated. (B) The effects of GC supplementation on survival in CLP-induced sepsis were studied by (1) supplementing endogenous GC through transplanting SR-BI^{+/+} adrenal to SR-BI^{-/-} mice and by (2) administrating exogenous a GC cocktail to SR-BI^{-/-} mice. SR-BI^{+/+} mice were also administrated with the GC cocktail to demonstrate the effects of GC supplementation on the survival of individuals without adrenal insufficiency in sepsis. (3) A “two-hit” model was used to evaluate the effects of GC supplementation on the survival of SR-BI^{-/-} mice in the secondary infection in sepsis.

Chapter 2 Materials and methods

2.1 Material

2.1.1 Mice

SR-BI^{+/-} (B6;129S2-Srb1^{tm1Kri/J}) mice were from the Jackson Laboratory. SR-BI^{-/-} mice were generated by breeding SR-BI^{+/-} mice and using the littermates. Mice were kept and bred in a temperature-controlled room with 12 h light and 12 h dark diurnal cycle at the animal facility of the University of Kentucky. They were housed in filter-topped cages and were fed a standard laboratory chow and water *ad libitum*. Animal care and experiments were approved by the Institutional Animal Care and Use Committee of the University of Kentucky. All procedures involving with this strain of mice followed the IACUC protocol # 2012-0975.

2.1.2 Reagents, kits and materials

The reagents, kits and materials used in this project are listed in Table 2.1.

Table 2.1 List of reagents, kits and materials and their providers

Chemical/Reagent/Kit	Provider (Cat#)
UltraPure™ Agarose	Invitrogen
BSA (bovine serum albumin)	Sigma-Aldrich
Corticosterone EIA kit	Cayman Chemical
Corticosterone: HBC complex	Sigma-Aldrich
Cholesterol E	Wako Diagnostics
Ready-SET-Go!® ELISA sets	eBioscience

Continued Table 2.1

Dulbecco's Phosphosphate Buffered Saline (DPBS)	Gibco
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EDTA (ethylenediaminetetraacetic acid)	Fisher
Ethanol	Fisher
Escherichia coli (K-12 strain) BioParticles®	Invitrogen
Fludrocortisone acetate	Sigma-Aldrich
Formaldehyde	Fisher
Free Cholesterol E	Wako Diagnostics
Hydrocortisone- water soluble	Sigma-Aldrich
Hypodermic disposable needles 27G ½"	EXEL INT®
Insulin syringe U100 29G ½"	BD Biosciences
In Situ Cell Death Detection Kit	Roche
Isopropyl alcohol	Fisher
6δ- methylprednisolone	Sigma-Aldrich
Micro-Hematocrit Capillary Tubes	Fisherbrand®
MyTaq™ DNA Polymerase 2x	Bioline
NaCl (sodium chloride)	Sigma
Needles (21- 27 guage)	BD Biosciences
Nitrate/Nitrite Colorimetric Assay Kit	Cayman Chemical
NP-40 (nonyl phenoxy polyethoxy ethanol)	Calbiochem
Nuclei lysis solution	Promega Corporation
Protein Precipitation Solution	Promega Corporation
Proteinase K	Invitrogen (25530015)
RPMI	GIBCO®
Rotating Tail Injector	Braintree scientific, Inc.
Sterile cell strainer (100 µm)	Fisherbrand®
Syringes (3-5 ml)	BD
Tris Base	Fisher

2.1.3 Antibodies

The antibodies used for flow cytometry in this project are listed in Table 2.2.

Table 2.2 List of antibodies and their sources and providers

Antibody	Clone	Provider (Cat#)
Anti-B220-APC	RA3-6B2	Biolegend (103212)
Anti-CD11b-PerCP-cy5.5	M1/70	BD (550993)
Anti-CD11c-APC	HL3	BD (550261)
Anti-CD115- PE	AFS98	Biolegend (135506)
Anti-CD16/32	93	Biolegend (101302)
Anti-CD19-APC	6D5	Biolegend (115512)
Anti-CD3-APC-cy7	17A2	Biolegend (100222)
Anti-CD3-FITC	17A2	BD (553138)
Anti-CD3-PE-cy5	17A2	BD (555276)
Anti-anti-CD45-APC-cy7	30-F11	Biolegend (103116)
Anti-anti-CD69-PE	H1.2F3	BD (553237)
Anti-F4/80-PE	BM8	eBioscience (12-4801-82)
Anti-Gr1-APC	RB6-8C5	BD (553129)
Anti-Ly6C-APC	HK 1.4	Biolegend (128016)

2.1.4 Equipment and software

The equipments used in this project are listed in Table 2.3.

Table 2.3 List of antibodies instruments, software and their providers

Instruments & Software	Provider
Allegra™ 25R Centrifuge	Beckman Counter™
Anesthesia Machine	Survivert®
Centrifuge	Eppendorf
C24 Incubator shaker	New Brunswick Scientific
FlowJo software	Tree Star, Inc.
GenoAmp® PCR System	Applied Biosystems
Image Reader LAS-4000	FUJIFILM
Microscope	Nikon
Mettler Toledo AB analytical balance	MonoBloc Weighing Technology
Multi Gauge V3.0 software	FUJIFILM
SoftMax® Pro software	Molecular Devices' Industry
Spectramax® microplate reader	Molecular Devices' Industry
SPSS Statistics	IBM
Stomacher	Seward
Thermalert TH-5	Physitemp
Utility oven	VWR

2.2 Methods

2.2.1 Mouse work

2.2.1.1 Genotyping of mice

Genotypes of mice were determined by PCR using tail genomic DNA before weaning the pups and were double-checked using genomic DNA from the liver tissue after sacrificing the mice.

Tail tips were used to genotype the pups before weaning. To prepare the genomic DNA, each tail-tip (~0.5 cm) was digested with the digestion buffer (100 µl lysis buffer + 2 µl 20 mg/ml proteinase K solution, see appendix) in a 55 °C water bath for 16-18 h. The digested tails were then centrifuged at a speed of 2,000 rpm for 5 min at room temperature, and then heated at 98 °C for 10 minutes to deactivate the proteinase K. 400 µl ddH₂O were added to dilute the solution. The tubes were then centrifuged 2,000 rpm for 5 min at 4 °C to sediment the undigested materials and cooled down on ice. 1 µl supernatant (Template) was used for PCR.

The liver tissue was used to check the genotypes. About 30 mg liver tissue was harvested from each mouse. To isolate the genomic DNA from the liver, the tissue was cut into small pieces with small scissors, mixed well with 600 µl digestion buffer (see appendix) by vortex, and digested in a 55 °C water bath for 18-20 h. After digestion, 200 µl Protein Precipitation Solution was added to each tube. And the tubes were vortexed and then put on ice for 5 min to allow the protein to precipitate and then centrifuged 12,000 rpm for 5 min at 4 °C. The supernatant was mixed with isopropanol at a ratio of 1:1. After DNA was

separated out, the tubes were centrifuged 12,000 rpm, 1 min at 4 °C and the supernatant was discarded. The DNA pellet was washed with 70 % ethanol and finally dissolved in 400 µl ddH₂O. 1 µl DNA template was used for PCR.

The primers and reaction system are shown below:

Primers [199]:

{ AB2: 5'-GAT GGG ACA TGG GAC ACG AAG CCA TTC T-3'
AB3: 5'-TCT GTC TCC GTC TCC TTC AGG TCC TGA-3'

Reaction system:

{ ddH₂O 7 µl
Taq Red Mix 10 µl
10 µM AB2 primer 1 µl
10 µM AB3 primer 1 µl
Template 1 µl

Setup of genotyping PCR reaction

Enzyme activation		95 °C	5 min
Denaturation		94 °C	30 s
Annealing	35 cycles	58 °C	30 s
Extension		72 °C	2 min
Elongation		72 °C	10 min

PCR products were analyzed on 1.5 % agarose gels, 100 voltages for 30 min. Pictures were taken using the LAS-4000 Imaging System and the Multi Gauge V3.0 software. (Figure 2.1)

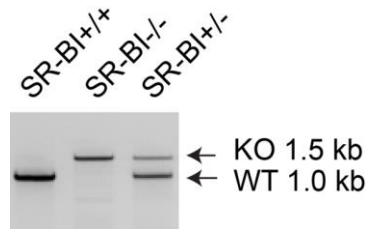


Figure 2.1 Genotyping for SR-BI alleles.

PCR system for SR-BI genotyping was set up as described in 2.2.1. 15 μ l PCR products were resolved in a 1.5% agarose gel under 100 voltages for 30 min.

2.2.1.2 Adrenal transplantation (ADR-T) Surgery

Adrenalectomy and subsequent adrenal transplantation were performed as previously described by Karpac *et al.*[200] At postnatal day 8, the pups from SR-BI^{+/-} breeders were genotyped using genomic DNA isolated from tails. At day 9, the pups were euthanized by CO₂ to harvest their adrenal glands right before the transplantation.

Immediately after the donor adrenal glands were isolated, recipient mice (8-10 weeks) were anesthetized by 5 % isoflurane inhalation and maintained with 2.5% isoflurane during the surgery. After anesthetization, the mouse was bilaterally adrenalectomized by a pair of surgery tweezers through a dorsal midline skin incision and lateral retroperitoneal incisions. The left incision was sutured right after removal of the left adrenal gland. Then the right kidney was

exposed, and one donor adrenal gland was put under the right kidney capsule through a slit in the kidney capsule made by the tweezers. Then the right kidney was returned; and the right incision and the skin were sutured. The mouse was kept warm on a heater mat until it waked up from narcosis. The mice were given 0.9% NaCl and normal water *ad libitum*. Then they were housed separately for one week and four mice per cage in the following weeks. The mice were used for experiments 6 weeks after the surgery. (Figure 2.2)

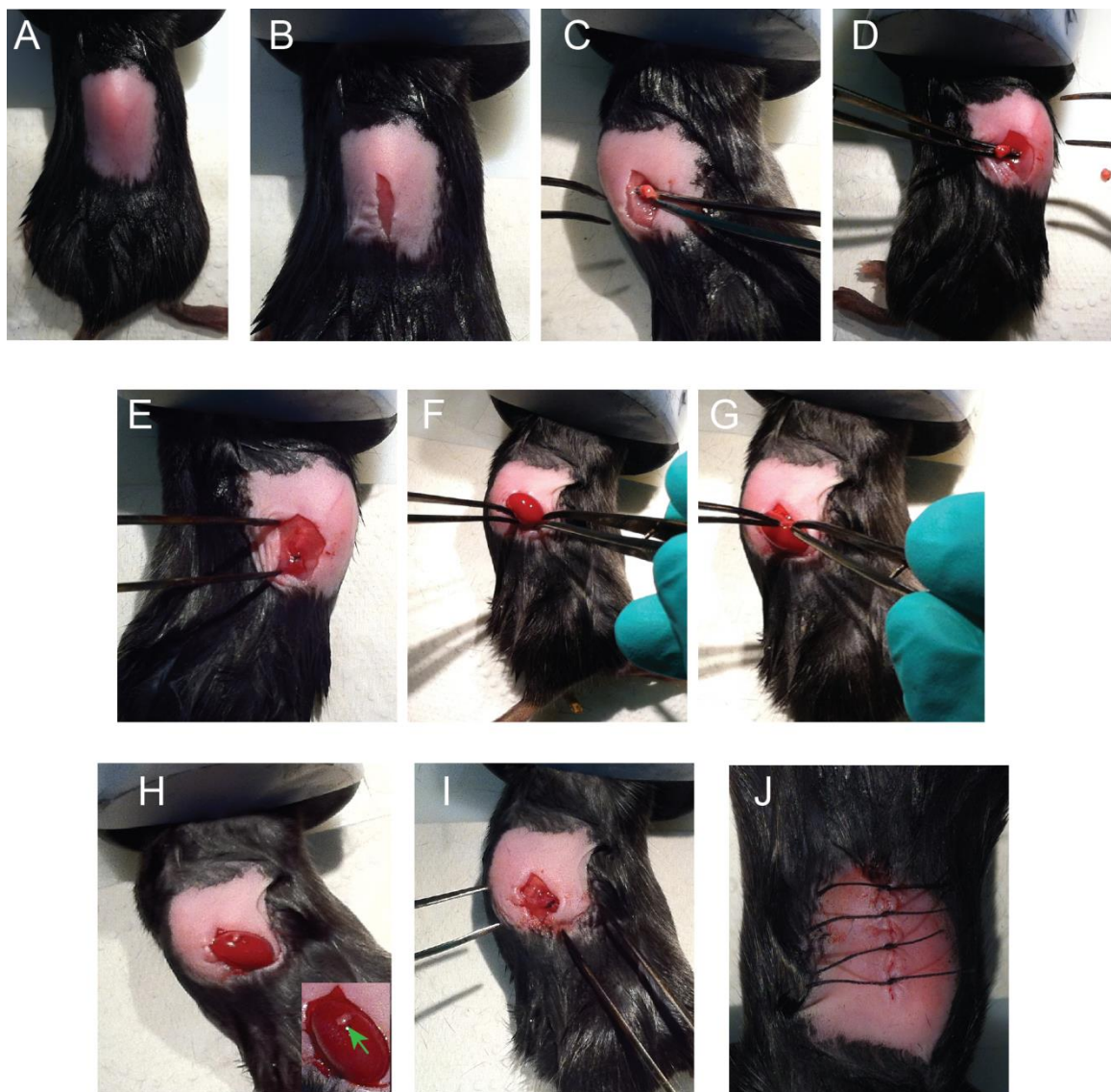


Figure 2.2 The adrenal transplantation surgery

(A) After anesthetization under isoflurane inhalation, the surgery area is shaved, cleaned and disinfected with iodine and 70 % ethanol. Then (B) a dorsal midline skin incision is made and (C and D) bilateral adrenal glands are removed by lateral retroperitoneal incisions. (E) The left incision is sutured right after the removal of left adrenal gland. Then (F) the right kidney is exposed. Subsequently, (G) a slit in the adrenal capsule is made by tweezers and (H) one donor adrenal gland from a 9-d pup is put under the right kidney capsule. (I) The kidney with the transplanted adrenal is put back and the right incision is sutured. Finally, (J) the dorsal skin incision is sutured. The mice are kept warm on a heater mat until they wake up from narcosis.

2.2.1.3 Cecal Ligation and Puncture (CLP) sepsis model

The cecal ligation and puncture (CLP) was used to induce septic conditions in mice. This model has many pathophysiological similarities to the clinical situation where bowel perforation-induced peritonitis results from an infection that is caused by mixed intestinal flora. The CLP model is one of the most widely used models of sepsis and is considered as a golden model for sepsis study. This model fulfills the human condition that is clinically relevant. Like in humans, mice that undergo CLP with fluid resuscitation show the first (early) hyperdynamic phase that in time progresses to the second (late) hypodynamic phase.[201] In addition, the CLP-induced sepsis shows a cytokine profile similar to human sepsis.[202, 203]

For the CLP surgery, the mouse was anesthetized by isoflurane inhalation. The abdominal hairs were shaved, and the area was disinfected with iodine followed by 70% ethanol. The abdominal cavity was opened via a midline laparotomy incision of about 1 cm in an aseptic fashion and the cecum was exposed. The cecum was ligated at a designated distance from the ileocecal valve. The ligated cecum was punctured twice with a needle. Next, sufficient pressure was applied to the cecum to extrude a single droplet of fecal material from each puncture site. The abdomen was closed, and mouse was resuscitated with 1 ml of PBS by i.p. injection. The mouse was kept warm on a heater mat until it woke up from narcosis. (Figure 2.3)

In the CLP model, the grade of severity has a direct impact on the percentage of survival. The severity of sepsis can be modified by the length of

the ligation, the thickness of the needle and the number of punctures, which could be controlled by the animal surgeon.[204, 205] A substantial variability in the CLP results could be induced by the mouse strain [206, 207], sex [208] and age [209] of the animal as well. Thus, we used sex and age-matched mice and adjusted the severity with different ligation and puncture conditions in our study.

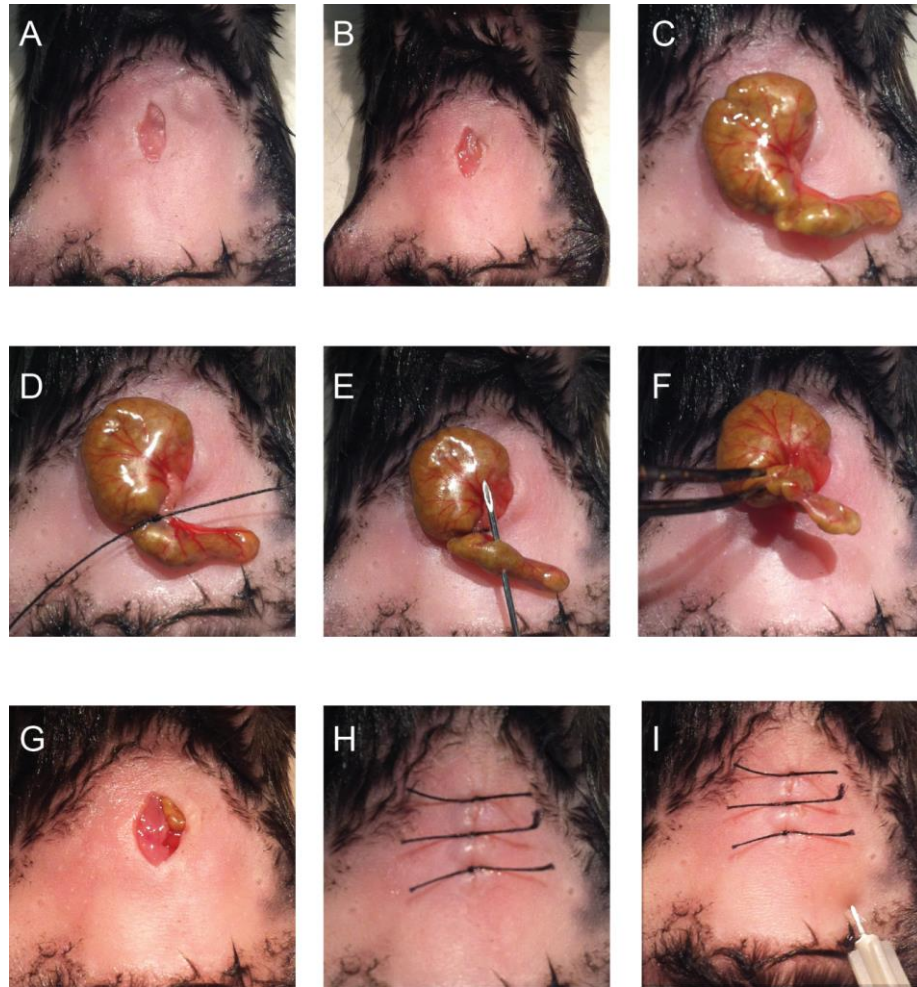


Figure 2.3 The cecal ligation and puncture sepsis model.

(A) After anesthetization, the abdominal hairs are shaved and the area is disinfected. (B) The abdominal cavity is opened via a midline incision of about 1 cm in an aseptic fashion. Then (C) The cecum is exposed and (D) is ligated at a designated distance to the ileocecal valve, carefully not to disturb the ileocecal flow. (E) The ligated cecum is punctured once “through-and-through” with a needle and (F) sufficient pressure is applied to the cecum to extrude a single droplet of fecal material from each puncture site. At last, (G) the cecum is put back in to the abdomen cavity and (H) the abdomen is closed. (I) the mouse is given 1 ml of PBS by i.p. injection for fluid resuscitation and is monitored regularly.

2.2.1.4 *In vivo* delivery of bacteria

FITC labeled-*Escherichia coli* (E. coli, K-12 strain) were used for the *in vivo* study of bacteriae delivery. FITC-E.coli powder was reconstituted in a vial at 20 mg/ml in sterile PBS with 2 mM sodium azide as the stock. To prepare the bacteria for injection, the designated amount of FITC-E.coli were pipetted out to a new sterile 1.5 ml EP tube, washed with sterile 1ml PBS by centrifuging at a speed of 8,000 rpm at 4 °C for 5 min and suspended in sterile PBS to a final concentration of 1×10^9 / ml. Then the bacteria were killed by heating at 75 °C for 20 min. 100 µl final E.coli solution was injected to the mouse by tail vein injection.

2.2.1.5 GC supplementation

For GC supplementation to CLP mice, a cocktail containing 100 µg hydrocortisone, 25 ng fludrocortisone acetate and 20 µg 6δ-methylprednisolone was injected to the mouse right after the surgery. Water-soluble hydrocortisone was dissolved in PBS at a concentration of 9.2 mg/ml (equivalent of 1 mg/ml hydrocortisone), and 100 µl/mouse hydrocortisone solution was injected intraperitoneally. Fludrocortisone acetate was dissolved in oil at a concentration of 2.5 mg/ml and diluted to a final concentration of 500 ng/ml. 6δ-methylprednisolone was dissolved in oil at a concentration of 2 mg/ml and diluted to a final concentration of 400 µg/ml. A mix of fludrocortisone and methyprodinisolone was injected 50 µl/ mouse subcutaneously. For corticosterone administration, corticosterone: HBS was dissolved in PBS and 200 µg/mouse corticosterone was injected intraperitoneally. For GC supplementation to mice with secondary infection, a cocktail containing a half dose (50 µg

hydrocortisone, 12.5 ng fludrocortisone acetate and 10 µg 6δ-methylprednisolone) was injected after inoculation by the same approach.

2.2.1.6 Body temperature measurement

Body temperature was measured in the adrenal-specific SR-BI^{-/-} mice and the control mice via rectum. Briefly, the mice were held vertically and the rectal temperature was monitored by the TH-5 Thermalert Monitoring Instrument. The rectal probe was cleaned by 75% ethanol between measurements.

2.2.1.7 Blood collection

Blood was collected through abdominal arterial puncture or tail bleeding. For the abdominal arterial puncture, the mice were anesthetized by isoflurane inhalation to collect blood through abdominal arterial puncture. 3 ml syringe and 27-gauge needles were rinsed with 0.5 M EDTA and 20 µl 0.5 M EDTA was added to each 1.5 ml EP tube for blood collection to prevent coagulation. For the tail bleeding, the blood was collected through a tail cut using a microhematocrit capillary tube and pipetted out to a collecting EP tube. The collected blood was centrifuged immediately at 12,000 rpm, 4 °C for 10 min. The plasma was pipetted out and stored at -80 °C for use.

2.2.1.8 Lung wet-to-dry (W/D) ratio

Lung W/D ratios were detected as a parameter of pulmonary edema. Immediately after the mice were killed, the lungs were carefully dissected from large airways, heart and mediastinal structures. Excess fluid was absorbed with soft tissue paper. The lungs were weighed immediately after being isolated (wet

weight) and again after 96 h at 65°C in a drying oven (dry weight) for computation of the W/D ratio dividing the wet weight by the dry weight.[210]

2.2.2 Histology

Immediately after mice were sacrificed, the kidneys with or without the transplanted adrenal glands were carefully removed and fixed in 10% formalin in PBS for 24 h. Specimens were then embedded in paraffin. A series of 5- μ m-thick sections were cut and stained with hematoxylin and eosin (H&E) by the Histology Core in the Markey Cancer Center. Pictures were taken using the Nikon Microscope and Nikon capture software.

2.2.3 Biochemistry and molecular biology

2.2.3.1 Corticosterone assay

The concentrations of plasma corticosterone were measured using the Corticosterone EIA kit (Cayman Chemicals) according to the manufacturer's instructions. This assay is a competition based assay in which corticosterone competes with corticosterone-acetylcholinesterase (AChE) conjugate (Corticosterone Tracer) for binding to a limited number of corticosterone-specific sheep antiserum binding sites. AChE hydrolyzes acetylcholine, producing thiocholine. The reaction of thiocholine with the detection reagent 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) produces 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm. Samples were diluted 1/200 to 1/400.

2.2.3.2 Cholesterol assay

Plasma total and free cholesterol concentrations were measured by kits from the Wako Diagnostics. Briefly, it is an enzymatic method involving the oxidation of free cholesterol by cholesterol oxidase to Δ^4 -cholestenone and the simultaneous production of hydrogen peroxide. The total amount of cholesterol in the test sample is determined by measurement of the absorbance at 600 nm.

2.2.3.3 Cytokine assay

The concentrations of plasma TNF- α , IL-6 and IL-10 were measured by ELISA kits from the eBioscience. For the TNF- α assay, all samples were diluted 1/5. For the IL-6 assay, samples were diluted 1/10 to 1/40. For the IL-10 assay, samples were diluted 1/5 to 1/10.

2.2.3.4 Nitric oxide assay

Measurement of NO *in vivo* is difficult because of its short half-life, but plasma nitrate/nitrite (NOx), the stable products of NO oxidation, can be measured as markers of NO activity.[211, 212] Therefore, the concentrations of plasma NOx were measured by the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical). This assay is involved with a conversion of nitrate to nitrite by nitrate reductase and a further conversion of nitrite into a deep purple azo compound, which has a strong absorbance at 540 nm or 550 nm. Samples were diluted 1/4 to 1/8.

2.2.3.5 Urea nitrogen assay

Blood urea nitrogen (BUN) raises if kidneys fail to remove urea from the body and has been used a marker for the kidney injury.[213] The concentrations of BUN were determined by measuring the urea nitrogen in the plasma using the QuantiChrom™ Urea Assay Kit with a quantitative colormetric method.

2.2.3.6 Alanine aminotransferase assay

Alanine aminotransferase (ALT) is mainly in the liver and normally kept in a low level in the blood. When the liver is damaged, ALT is released into the blood stream and therefore increased ALT in the blood is considered as a marker for liver damage. To evaluate the liver damage in CLP mice, the concentrations of plasma ALT were measured by the ALT reagent from BQ kits. It is a colorimetric method. The ALT catalyzes L-alanine and α -ketoglutarate to form pyruvate and glutamate. The pyruvate then reacts with 2, 4-dinitrophenylhydrazine (2, 4-DNPH-one), which can be measured at 505 nm after being dissolved by the addition of sodium hydroxide.

2.2.3.7 Lipoprotein profile analysis

Plasma lipoprotein profile was analyzed by FPLC as described previously.[214] Briefly, plasma (50 μ l) was resolved by gel filtration chromatography using an FPLC system equipped with a Superose 6 column (GE Healthcare). The column was eluted at a flow rate of 0.5 ml/min in buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), and 0.01% sodium azide, and 0.5 ml/fraction was collected. 100 μ l of sample was mixed with an equal

volume of 2x assay reagent (Wako Chemicals) to determine the cholesterol content of fractions.

2.2.4 Primary cell analysis

2.2.4.1 Preparation of single cell suspensions

Single cell suspensions were prepared from freshly dissected spleens following standard procedures. Briefly, spleens were weighed immediately after dissection and 70-100 mg of a spleen was used. The spleen tissue for homogenization was put into a stomacher bag (Seward) containing 10 ml RPMI 1640-5 (see appendix) and disrupted using the Stomacher 80 (Seward) at the high speed for 2 min. The cell suspension was transferred to a 50 ml centrifuge tube (BD Biosciences) through a 100 µm cell strainer (BD Biosciences) by a 10 ml pipette. 5 ml of RPMI 1640-5 was added to wash the stomacher bag in order to transfer the cells thoroughly. Then the cell suspension was centrifuged at 1,500 rpm for 5 min at 4 °C. The supernatant was discarded and the cells were incubated with 5 ml of ACK lysis buffer (see appendix) at room temperature for 5 min to deplete erythrocytes. The lysis of erythrocytes was ceased by adding 10 ml of Dulbecco's Phosphate Buffered Saline (DPBS). 100 µl of the cell suspension after lysis was diluted 1/10 with RPMI 1640-5 and counted using a counting chamber. The cells were centrifuged at 1,500 rpm for 5 min at 4 °C, washed again with DPBS and finally suspended in 5 ml RPMI 1640-5.

Blood was collected from mouse by abdominal arterial puncture as described earlier. 300 µl of anti-clotted blood was added into 5 ml of ACK lysis

buffer in a 50 ml centrifuge tube to remove erythrocytes. After incubation for 5 min, 45 ml of DPBS was added to cease lysis and the cells were centrifuged at 1,500 rpm for 5 min at 4°C. The pellet was resuspended with 1 ml of RPMI 1640-5. 10 µl of the cell suspension was directly counted using a counting chamber.

2.2.4.2 Fluorescence-activated cell sorting (FACS) analysis

5x10⁵ cells were used for the surface marker antibody staining. The single cell solution was centrifuged at 2,000 rpm for 5 min at 4°C and the supernatant medium was removed. The cells were resuspended with 30 µl FACS staining buffer (see appendix) containing 0.25 µg of anti-mouse CD16/CD32 and incubated for 10 min at 4°C to block Fc receptors. Then 20 µl FACS staining buffer containing fluorescence-labeled antibodies (0.125 µg per antibody) were added and the cells were further incubated at 4°C for 30 min. After the incubation, the cells were washed twice with 500 µl of FACS staining buffer and suspended in 250 µl FACS staining buffer. Finally the stained cells were analyzed with FACSCalibur or LSRII flow cytometer (BD Biosciences). The generated data were analyzed with FlowJo software.

2.2.4.3 TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is a widely used method for cell apoptosis analysis by detecting DNA fragmentation.[215] The rationale for this assay is that the terminal deoxynucleotidyl transferase (TdT) can identify the nicks in DNA and thus catalyze the addition of dUTPs that are secondarily labeled. In this study, TUNEL incorporation detection was conducted by using the *in situ* cell death detection kit

following the manufacturer's protocol (Roche). Briefly, cells were stained with cell surface marker antibodies as described in 2.2.4.2, and then incubated with 100 µl of BD Cytofix/Cytoperm solution (BD Biosciences) for 20 min at room temperature. After the incubation, the cells were washed twice with 1x BD Perm/Wash buffer (BD Biosciences) and incubated with 50 µl of TUNEL reaction mixture (Roche) for 1 hour at 37°C. Finally the cells were washed twice with FACS staining buffer, suspended in 500 µl of FACS staining buffer and analyzed with LSRII flow cytometer.

2.2.5 Microbiology

The strain *Pseudomonas aeruginosa* (ATCC 27853) was utilized in the secondary infection model. All procedures involving with this strain of bacteria followed the biosafety protocol #B11-1792-M3.

2.2.5.1 Preparation of bacteria stock

Bacteria were propagated immediately following the instructions of ATCC after arriving. Briefly, the bacteria were rehydrated with 1 ml sterile tryptic soy broth (TSB) and then aseptically transferred into 5 ml sterile TSB broth and several drops of the suspension were used to inoculate a sterile TSB agar plate. After incubation at 37 °C for 24 h, a single colony was used for further propagation in a flask containing 400 ml sterile broth by incubating at 37 °C for 24 h with shaking. Then the bacteria in the broth were then divided into 40 ml/ tube and centrifuged at 5,000 rpm, 4°C for 20 min. The supernatant was discarded and the bacteriae were resuspended with sterile 7 ml PBS. Then 3 ml sterile 50 % glycerol was added to each tube. The bacteria suspension was

finally mixed thoroughly and divided into sterile 1.5 ml EP tubes with 1 ml/ tube. A drip of the suspension was used to inoculate the agar plates to count the colony-forming unit (CFU).

2.2.5.2 Inoculation of animals

Stock bacterial solution was warmed at 37 °C in water bath. After thoroughly melted, the bacteria were washed once by 1 ml sterile PBS by centrifuging at 8,000 rpm, 4°C for 5 min and resuspended in 1 ml sterile PBS. The bacterial suspension was diluted to a concentration of 1×10^9 CFU/ ml for inoculation. For inoculation, the mice were anesthetized under isoflurane inhalation and held vertically in a “heads-up” position. Using an Eppendorf pipette that was calibrated, 50 µl of the bacteria suspension (1×10^9 CFU/ ml) was slowly injected intranasally and observed to be aspirated on inhalation. After the inoculation, the mice were monitored in a BSL-2 animal room for survival study.

2.3 Data Analysis

All the statistical analyses were performed with SPSS Statistics, version 21 software. For comparison of means, differences in quantitative variables in the same group were analyzed by 2-tailed Student's t test. Data from multiple groups were analyzed with one-way ANOVA with Tukey post hoc test. All groups analyzed conformed to the constraints of parametric analysis. Data were presented as means \pm S.E.M. Differences in survival rate and survival time were analyzed by Kaplan-Meier method with log-rank χ^2 test. Correlations between variables were evaluated by two-tailed Pearson's correlation coefficient value.

Data were presented as Pearson's coefficient (r). P value of <0.05 was considered significant.

Chapter 3 The role of SR-BI-regulated iGC in sepsis

Section 1 Adrenal-specific SR-BI-deficient mice are a good iGC-deficient animal model.

3.1.1 Introduction

Despite new developments in critical care and sepsis therapy, severe sepsis and septic shock are still major healthcare problems.[3, 6, 8] The initial inflammatory response to sepsis stimulates the production and release of iGC from the adrenal cortex.[92] However, the exact function of iGC in sepsis remains unclear due to a lack of good animal model.

Previous approaches are not feasible to study the role of iGC in sepsis. The approaches of adrenalectomy and GC-receptor antagonist, which remove both basal GC and iGC, are not feasible to investigate the role of iGC in sepsis considering that basal GC is important in regulating the organismal homeostasis [193, 194] and is maintained in survival sepsis (preliminary data). In addition, studies of exogenous GC may over-simplify the action of endogenous GC.[195] Thus a better animal model is needed to investigate the function of endogenous iGC in sepsis.

Mouse specifically deficient in iGC may serve as a good animal model to study endogenous iGC in sepsis. SR-BI^{-/-} mice have normal plasma corticosterone under basal conditions but cannot produce iGC in sepsis [109,

110, 197, 216, 217], therefore they may serve as an adrenal insufficient mouse model. However, mice that are specifically deficient in adrenal SR-BI will be a more suitable model than SR-BI^{-/-} mice to study iGC in sepsis, because SR-BI^{-/-} mice also display abnormalities in other aspects such as dyslipidemia, impaired functions of macrophages and attenuated liver uptake of LPS. [155].

In this section, we characterized adrenal-specific SR-BI^{-/-} mice as a good model of iGC deficiency to study the role of iGC in sepsis. We verified that the adrenal-specific SR-BI^{-/-} mice have normal basal GC concentration in plasma but cannot generate iGC in CLP. We also showed that the transplanted SR-BI^{-/-} adrenal grafts can grow with normal structure. In addition, we showed that the adrenal-specific SR-BI^{-/-} mice have no difference in the plasma lipoprotein profile and blood cell counts compared to the control mice, demonstrating that adrenal-specific SR-BI^{-/-} mice are a good model to study the role of iGC in sepsis.

3.1.2 Results

3.1.2.1 Adrenal-specific deficiency of SR-BI does not impair basal GC but leads to iGC deficiency in CLP-induced sepsis.

To generate the mouse specifically lack SR-BI in adrenal, we transplanted a donor adrenal gland from 9-day SR-BI^{-/-} pups to 8-10-week-old female SR-BI^{+/+} mice after their own adrenal glands were removed. The control mice were generated by transplanting a donor adrenal gland from age-matched SR-BI^{+/+} littermate pups following the same procedure. To verify the success of this model, we conducted CLP to these mice and measured their plasma corticosterone

levels at the points of CLP 0 h, 4 h and 18 h. As shown in Figure 3.1, at CLP 0 h, the adrenal-specific SR-BI^{-/-} mice did not show significant difference in the plasma corticosterone concentration compared to the control mice (113.2 ± 21.0 ng/ml vs. 173.5 ± 36.6 ng/ml, $p=0.192$), indicating that adrenal-specific deficiency of SR-BI does not impair basal GC. At CLP 4 h, the control mice had a 2.4-fold increase in plasma corticosterone (Figure 3.1) compared to their basal levels, indicating a production of iGC upon CLP. In contrast, the adrenal-specific SR-BI^{-/-} mice displayed a 2.3-fold decrease in plasma corticosterone at CLP 4 h compared to their basal levels, which was significantly lower than the level in the control mice at this point. At CLP 18 h, the plasma corticosterone in the control mice was still 80 % higher than their basal level, whereas the plasma corticosterone in the adrenal-specific SR-BI^{-/-} mice remained at the basal level and was significantly lower than the control mice (114 ± 14.9 ng/ml vs. 311 ± 26.2 ng/ml, $p<0.001$). These data demonstrate that the adrenal-specific SR-BI^{-/-} mice are completely deficient in iGC in CLP-induced sepsis.

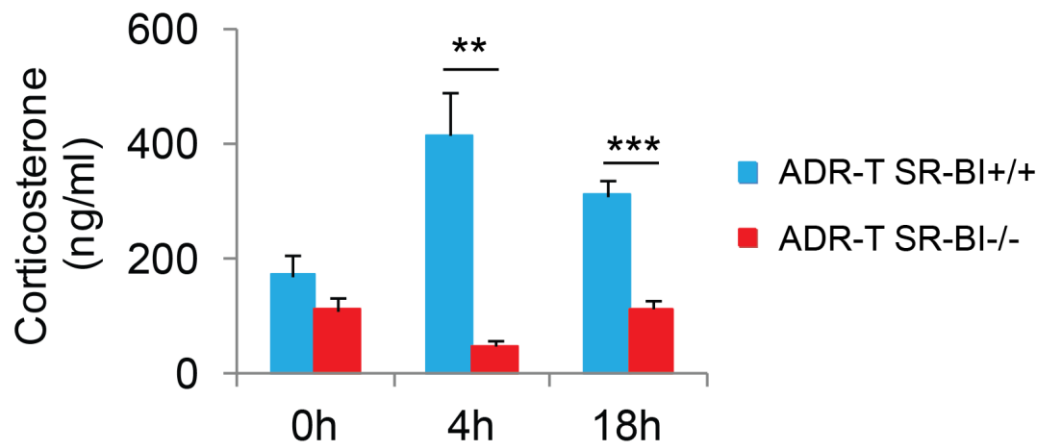


Figure 3.1 Adrenal-specific deficiency of SR-BI leads to iGC deficiency in CLP-induced sepsis.

Adrenal-transplanted mice were subjected to CLP surgery (21-gauge, half ligation) and the plasma was taken at designated time for plasma corticosterone assay. Data represent mean \pm SEM. (n=6-8 per group). ** $p < 0.01$, *** $p < 0.001$

3.1.2.2 Adrenal-specific deficiency of SR-BI does not influence the growth of transplanted adrenal grafts.

On euthanization of the mice, we harvested the kidneys and adrenal transplants from the recipient mice for further gross morphological examination and histological analysis. As shown in Figure 3.2, both SR-BI^{+/+} and SR-BI^{-/-} adrenal grafts grew under the kidney capsule. Further histological analysis showed that both SR-BI^{+/+} and SR-BI^{-/-} adrenal transplants consisted of adrenal cortex surrounding the medulla and the adrenal cortex has clear zona glomerulosa, zona fasciculata and zona reticularis (Figure 3.2). These data indicate that adrenal-specific deficiency of SR-BI does not influence the growth of transplanted adrenal grafts in the recipients.

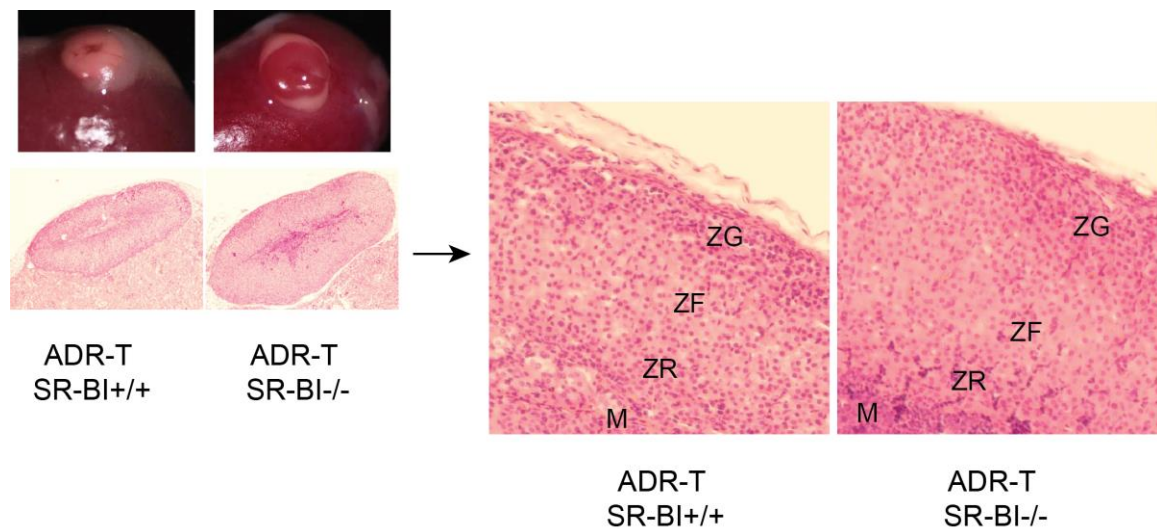


Figure 3.2 Transplanted adrenal glands show normal structure.

Six weeks after adrenal transplantation, the mice were sacrificed and the right kidneys with transplanted adrenal glands were harvested for the pictures and histology analysis. The left panel shows the appearance of adrenal gland under the kidney capsule (upper) and the HE staining of the adrenal glands (lower). The right panel shows the amplified HE staining pictures of the transplanted adrenal grafts. ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis; M, medullary region

3.1.2.3 Adrenal-specific deficiency of SR-BI does not influence plasma cholesterol concentrations or lipoprotein profiles under basal conditions.

Since plasma lipoproteins serve as the major source of cholesterol for GC synthesis in the adrenal [147, 218] and atherogenic diet-fed adrenal-specific SR-BI-deficient mice display decreased plasma very-low-density and low-density lipoprotein levels [198], we determined the plasma cholesterol concentrations and lipoprotein profiles to see whether or not the chow diet-fed adrenal-specific SR-BI^{-/-} mice display changes in the lipoproteins. As shown in Figure 3.3A, the adrenal-specific SR-BI^{-/-} mice showed comparable concentrations of plasma total cholesterol (83.8 ± 1.8 mg/dl vs. 89.3 ± 3.7 mg/dl, $p=0.227$) and free cholesterol (19.4 ± 0.7 mg/dl vs. 20.2 ± 1.6 mg/dl, $p=0.661$) with the control mice. The FPLC analysis also showed no difference in the lipoprotein profile between the adrenal-specific SR-BI^{-/-} mice and the control mice (Figure 3.3B). These data indicate that the adrenal-specific deficiency of SR-BI does not influence the plasma lipoprotein levels.

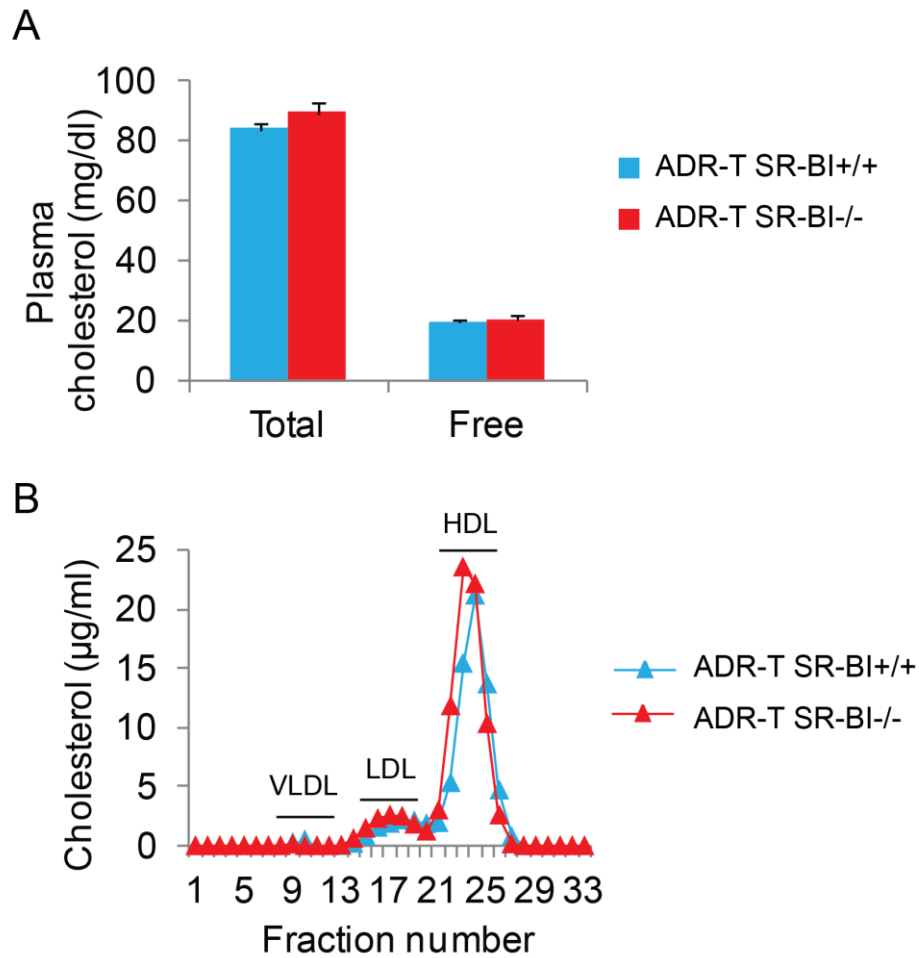


Figure 3.3 Adrenal-specific deficiency of SR-BI does not influence plasma cholesterol concentrations or lipoprotein profiles.

Six weeks after adrenal transplantation, the plasma was taken from untreated adrenal-transplanted mice. (A) Plasma total and free cholesterol concentrations were measured. Data present mean \pm S.E.M. (n=6) (B) Plasma lipoprotein profile was analyzed by FPLC. Data show representative lipoprotein profiles. VLDL, very-low density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein

3.2.1.4 Adrenal-specific deficiency of SR-BI does not influence the blood cell counts under basal conditions.

It has been reported that adrenalectomized mice have a significant increase in the number of mononuclear cells in the blood [219], thus we analyzed the complete blood counts in the adrenal-transplanted mice to see if adrenal-specific SR-BI deficiency also impacts those cells. As indicated in the Table 3.1, the adrenal-specific SR-BI-deficient mice showed a normal blood cell profile and no significant difference in terms of the number of white blood cells, neutrophils, lymphocytes, monocytes and red blood cells compared to the control mice. These data demonstrate that the adrenal-specific deficiency of SR-BI does not influence the blood cell counts.

Table 3.1 Adrenal-specific deficiency of SR-BI does not influence the blood cell counts under basal conditions.

	ADR-T SR-BI+/+	ADR-T SR-BI-/-	P value
WBC (K/ μ l)	3.49 \pm 0.91	3.72 \pm 0.33	p>0.05
NE (K/ μ l)	0.70 \pm 0.09	1.00 \pm 0.14	p>0.05
LY (K/ μ l)	2.61 \pm 0.82	2.26 \pm 0.21	p>0.05
MO(K/ μ l)	0.17 \pm 0.05	0.20 \pm 0.04	p>0.05
NE (%)	22.28 \pm 3.29	26.69 \pm 2.32	p>0.05
LY (%)	72.45 \pm 3.29	61.75 \pm 5.61	p>0.05
MO (%)	4.94 \pm 0.26	5.77 \pm 1.38	p>0.05
RBC (M/ μ l)	8.45 \pm 0.19	8.85 \pm 0.26	p>0.05
Hb (g/dL)	12.35 \pm 0.38	12.78 \pm 0.19	p>0.05
PLT (K/ μ l)	663.5 \pm 60.42	639.8 \pm 14.9	p>0.05

Anticoagulant blood harvested from the artery puncture was used for complete blood counting. WBC, white blood cell; NE, neutrophil; LY, lymphocyte; MO, monocytes; RBC, red blood cell; Hb, hemoglobin; PLT, platelet. n=4 vs. 5

3.2.3 Summary

In this section, we checked the phenotypes of adrenal-specific SR-BI^{-/-} mice generated by adrenal transplantation and verified these mice as a good animal model to study the role of iGC in sepsis. First, we conducted CLP to the adrenal-transplanted mice, showing that the adrenal-specific SR-BI^{-/-} mice have a normal level of plasma corticosterone under basal conditions but totally lack iGC upon CLP, which indicates that adrenal-specific SR-BI deficiency leads to iGC deficiency in sepsis. Then we showed that both SR-BI^{+/+} and SR-BI^{-/-} adrenal grafts grow well and have normal adrenal structure. In addition, because changes of plasma lipoproteins have been reported in the atherogenic diet-fed adrenal-specific SR-BI^{-/-} mice [198], we measured the plasma cholesterol concentrations and lipoprotein profiles to see if adrenal-specific SR-BI deficiency influences plasma lipoprotein on a chow-diet feeding. We showed that the adrenal-specific SR-BI^{-/-} mice have normal plasma cholesterol concentrations and lipoprotein profiles, demonstrating that adrenal-specific deficiency of SR-BI does not influence the plasma lipoproteins. Finally, since adrenalectomized mice have a significant increase in the number of mononuclear cells in the blood [219], we analyzed the complete blood counts in the adrenal-specific SR-BI^{-/-} mice and observed no difference in the blood counts between the adrenal-specific SR-BI^{-/-} mice and the control mice. Overall, this section demonstrates that adrenal-specific SR-BI^{-/-} mice are a good model to investigate iGC in sepsis.

Section 2 Role of SR-BI-regulated iGC in CLP-induced sepsis.

3.2.1 Introduction

The exact function of iGC in sepsis remains unclear due to the lack of a good animal model. After characterizing adrenal-specific SR-BI^{-/-} mice as a good animal model to investigate iGC in sepsis, we used this model to test our hypothesis that iGC is essential for survival in sepsis and investigated the functions of iGC in sepsis.

First, we observed a significantly higher mortality in the adrenal-specific SR-BI^{-/-} mice in CLP-induced sepsis compared to the control mice, demonstrating that iGC is essential for survival in sepsis. We also detected a higher occurrence of low body temperature, an indicator of septic shock, in the adrenal-specific SR-BI^{-/-} mice than the control mice, indicating that iGC participates in preventing the development of septic shock. Then, because multiple organ damage contributes to a high mortality in sepsis, we measured the plasma alanine amino transferase (ALT) level, the lung wet-to-dry (W/D) ratio and blood urea nitrogen (BUN), which respectively reflect the damage of liver, lung and kidneys in the adrenal-specific SR-BI^{-/-} mice to see if iGC protects organ damage in sepsis. We found that iGC has no effect on the liver or lung damage but prevents the kidney injury in sepsis. To further understand the mechanism underlying the protective effect of iGC in sepsis, we analyzed the production of pro-inflammatory cytokine TNF- α and IL-6, pro-inflammatory mediator NO and anti-inflammatory cytokine IL-10 in the adrenal-specific SR-BI^{-/-} mice to determine if iGC benefits sepsis survival through its anti-inflammatory action. We found that iGC regulates the

pro-inflammatory cytokines, particularly exerting an inhibitory effect on IL-6. Finally, since GC has been considered as an immunosuppressive agent [101] and immunosuppression in sepsis is associated with an increased mortality in the late phase of sepsis[52], we tested whether or not iGC is immunosuppressive in sepsis. To assess the effects of iGC on innate immunity, we measured the phagocytic activity of phagocytes in the blood and spleen by analyzing the phagocytosis of injected fluorescence-labeled *E. coli*. We found that iGC in sepsis increases the phagocytic activity of neutrophils and monocytes in the blood. To assess the effects of iGC on adaptive immunity, we quantified the activation and apoptosis of lymphocytes in the spleen by staining CD69 and TUNEL in the CLP adrenal-specific SR-BI^{-/-} mice and showed that iGC in sepsis enhances the activation of T lymphocytes and does not induce more apoptotic cells in the spleen. In all, our data in this section reveal that iGC is essential for survival in sepsis. It prevents the kidney injury, regulates cytokine production and exerts immunomodulatory action in sepsis.

3.2.2 Results

3.2.2.1 Inducible GC protects against CLP-induced septic death.

After characterizing adrenal-specific SR-BI^{-/-} mice as an iGC-deficient mouse model, we used this model to investigate the effects of iGC in sepsis. First, we determined the influence of iGC deficiency on the mortality in sepsis by looking at the survival rate of the adrenal-specific SR-BI^{-/-} mice in CLP. As shown

in Figure 3.4, CLP induced a 46% fatality in the adrenal-specific SR-BI^{-/-} mice as against no fatality in the control mice at CLP 24 h. There was a 62% fatality in the adrenal-specific SR-BI^{-/-} mice compared to a 17% fatality in the control mice at CLP 48 h. After CLP 48 h, no more death occurred in the control mice whereas the fatality in the adrenal-specific SR-BI^{-/-} mice increased to 71% at CLP 72 h and 87% at CLP 120 h. Overall, a 7-day survival rate reached a significant difference between these two groups ($p < 0.001$). These data demonstrate that iGC is critical for survival in sepsis.

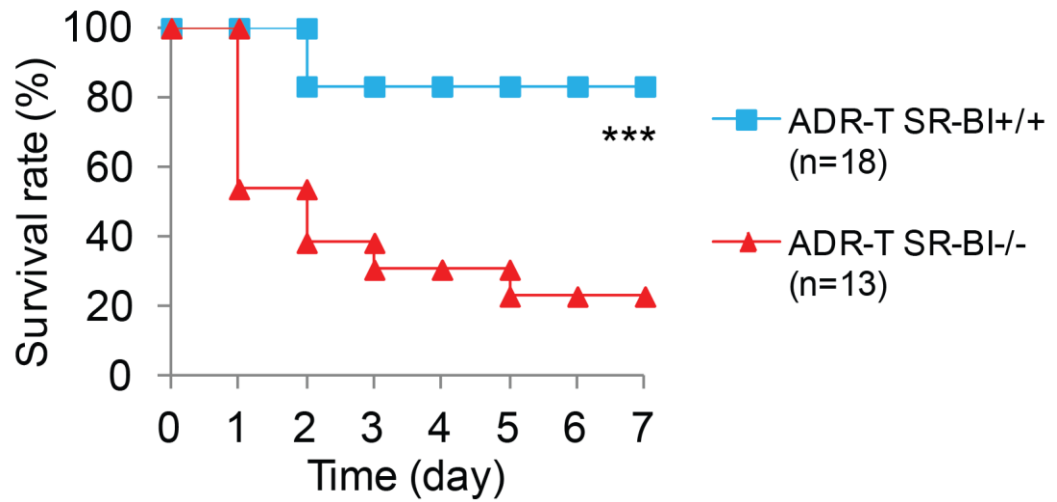


Figure 3.4 Inducible GC protects against CLP-induced septic death.

The adrenalectomized SR-BI^{+/+} mice transplanted with one SR-BI^{+/+} or SR-BI^{-/-} adrenal gland were subjected to CLP (21-gauge, half ligation) 6 weeks after adrenal transplantation. The survival was monitored for 7 days. Data are expressed as the percentage of mice surviving at the indicated time and analyzed by the log-rank χ^2 test.

*** $p < 0.001$

3.2.2.2 Inducible GC prevents the development of septic shock.

Low body temperature ($<30^{\circ}\text{C}$) is an indicator of septic shock.[220] As shown in Figure 3.5, low body temperature was present in 9/10 adrenal-specific SR-BI^{-/-} mice but only in 1/6 of the control mice at CLP 18 h. The average body temperature in the adrenal-specific SR-BI^{-/-} mice was significantly lower than the control mice ($24.9 \pm 1.6^{\circ}\text{C}$ vs. $34.0 \pm 2.2^{\circ}\text{C}$, $p=0.004$; Figure 3.5). These data suggest that iGC protects the mice from septic shock.

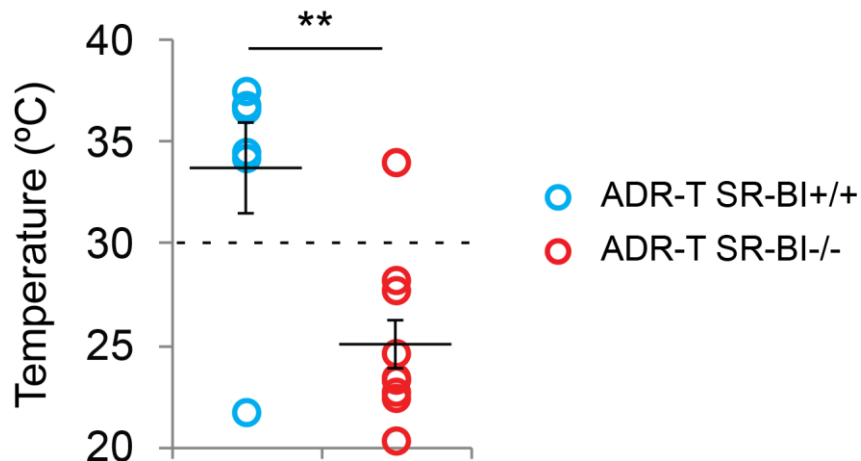


Figure 3.5 Inducible GC prevents the development of septic shock.

The SR-BI^{+/+} mice transplanted with SR-BI^{+/+} or SR-BI^{-/-} adrenal glands were subjected to CLP (21-gauge, half ligation) 6 weeks after adrenal transplantation. The body temperature was measured at CLP 18 h through rectum. Data represent mean \pm S.E.M. $n=6$ vs. 10 in control versus adrenal specific SR-BI^{-/-} mice respectively, ** $p<0.01$

3.2.2.3 Inducible GC protects against CLP-induced kidney injury in sepsis.

Multiple organ dysfunctions are a hallmark of severe sepsis, which contributes to an increased mortality in sepsis.[3] To understand the mechanism by which SR-BI protects against septic death, next we investigated the impact of iGC in organ functions by assessing liver, lung and kidney damages in CLP. As shown in Figure 3.6A and 3.6B, compared to the control mice, the adrenal-specific SR-BI^{-/-} mice displayed no difference in the plasma ALT level and the lung W/D ratio after CLP, indicating that iGC may not protect liver or lung injury in sepsis. However, measurements of plasma BUN concentrations showed a significant difference between the adrenal-specific SR-BI^{-/-} mice and the control group. As shown in Figure 3.6C, BUN increased only transiently at CLP 4 h and decreased to the basal level at CLP 18 h in the control mice. In contrast, in the adrenal-specific SR-BI^{-/-} mice, although BUN only increased slightly at CLP 4 h, it continued to increase by 2.4-fold at CLP 18 h (68.5 ± 9.0 mg/dl vs. 28.8 ± 2.6 mg/dl, $p=0.002$, ANOVA), leading to a significantly higher BUN level than that in the control mice at CLP 18 h (68.5 ± 9.0 mg/dl vs. 27.9 ± 6.1 mg/dl, $p=0.036$, t test). These data suggest that iGC prevents kidney injury in sepsis.

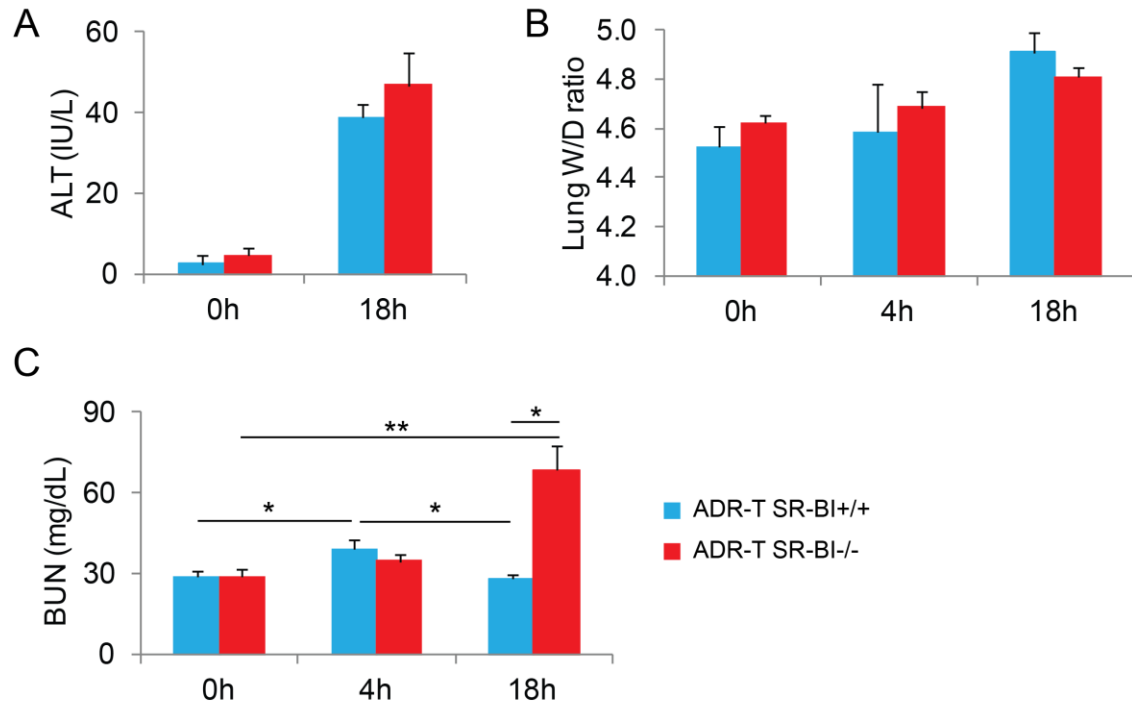


Figure 3.6 Inducible GC prevents kidney injury in CLP.

CLP (21-gauge needle, half ligation) surgery was conducted on mice 6 weeks after adrenal transplantation. Samples were harvested from CLP 0 h, 4 h and 18 h mice for assays. (A) Liver injury was indicated by an elevation of plasma alanine aminotransferase (ALT). (B) Lung edema was indicated by an increase in lung W/D ratio. (C) Kidney injury was evaluated by detecting the levels of blood urea nitrogen (BUN).

Data represent mean \pm SEM. (n=6-8), * p<0.05, ** p<0.01

3.2.2.4 Inducible GC regulates the inflammatory response in sepsis.

GC is known as a potent anti-inflammatory agent.[221] To investigate whether the decreased survival rate in adrenal-specific SR-BI^{-/-} mice is due to an expansion of inflammatory cytokines, we assessed the plasma cytokine concentrations in the adrenal-specific SR-BI^{-/-} mice in CLP. As shown in Figure 3.7A and 3.7B, the adrenal-specific SR-BI^{-/-} mice had the same levels of basal plasma TNF- α and IL-6 to the control mice, indicating that SR-BI deficiency in adrenal does not affect inflammatory cytokines under basal conditions. At CLP 4 h, the control mice exhibited a 6.6-fold increase in TNF- α ($p < 0.001$, ANOVA) and a 50-fold increase in IL-6 (11.6 ± 2.6 ng/ml vs. 0.23 ± 0.19 ng/ml, $p = 0.044$, ANOVA). The adrenal-specific SR-BI^{-/-} mice also displayed elevations in TNF- α and IL-6 at CLP 4 h. However, they exhibited slightly higher TNF- α (300.8 ± 44.9 pg/ml vs. 240.9 ± 48.5 pg/ml, $p = 0.382$, t test) and over 2-fold higher IL-6 (26.8 ± 4.3 ng/ml vs. 11.6 ± 2.6 ng/ml, $p = 0.012$, t test) compared to the control mice at the same time point. At CLP 18 h, the TNF- α and IL-6 levels in the control mice were markedly decreased compared to their levels at CLP 4 h. Interestingly, the TNF- α in the adrenal-specific SR-BI^{-/-} mice also decreased significantly at CLP 18 h compared to its level at CLP 4 h (137.6 ± 37.7 pg/ml vs. 300.7 ± 44.9 pg/ml, $p = 0.028$, ANOVA), although at this time point, the TNF- α in the adrenal-specific SR-BI^{-/-} mice was 2.5-fold higher than that in the control mice (137.6 ± 46.1 pg/ml vs. 54.4 ± 16.9 pg/ml, $p = 0.15$, t test). More importantly, the IL-6 in the adrenal-specific SR-BI^{-/-} mice remained at a high level at CLP 18 h, which was 5.4-fold higher than that in the control mice at CLP 18 h (22.1 ± 6.8 ng/ml vs. 4.1 ± 2.3

ng/ml, $p=0.03$, t test). These data indicate that iGC inhibits the production of pro-inflammatory cytokines.

NO is an important pro-inflammatory mediator to defend invasive bacteria during sepsis. However, excess NO damages tissues.[35, 36] Next, we measured the plasma nitrite and nitrate (NOx) levels in the adrenal-specific SR-BI^{-/-} mice to determine whether iGC affects NO production in sepsis. As shown in Figure 3.7C, though the NOx concentrations were elevated in both groups by CLP, the adrenal-specific SR-BI^{-/-} mice displayed a 2.3-fold higher plasma NOx level than the control mice at CLP 4 h ($11.48 \pm 3.28 \mu\text{M}$ vs. $5.09 \pm 1.21 \mu\text{M}$, $p=0.08$, t test). At CLP 18 h, the adrenal-specific SR-BI^{-/-} mice exhibited a similar plasma NOx level to the control mice ($14.76 \pm 2.79 \mu\text{M}$ vs. $12.19 \pm 4.08 \mu\text{M}$, $p=0.63$, t test). These data indicate that iGC may inhibit NOx production in early sepsis.

We also evaluated the effect of iGC on the production of anti-inflammatory cytokine IL-10 during sepsis. As shown in Figure 3.7D, in the control mice, the plasma IL-10 increased significantly at CLP 4 h ($2908.7 \pm 785.3 \text{ pg/ml}$ vs. $488.4 \pm 141.4 \text{ pg/ml}$, $p=0.014$, ANOVA) and increased further at CLP 18 h ($4034.2 \pm 467.2 \text{ pg/ml}$) compared to the basal level. In the adrenal-specific SR-BI^{-/-} mice, the plasma IL-10 also increased at CLP 4 h compared to their basal level ($2976.0 \pm 848.9 \text{ pg/ml}$ vs. $792.2 \pm 386.3 \text{ pg/ml}$, $p=0.082$, ANOVA) but was not higher at CLP 18 h compared to the level at CLP 4 h ($2885.3 \pm 585.8 \text{ pg/ml}$). These data indicate that iGC is not responsible for the early production of IL-10 and may have a small role in the production of IL-10 cytokine in a later stage of sepsis.

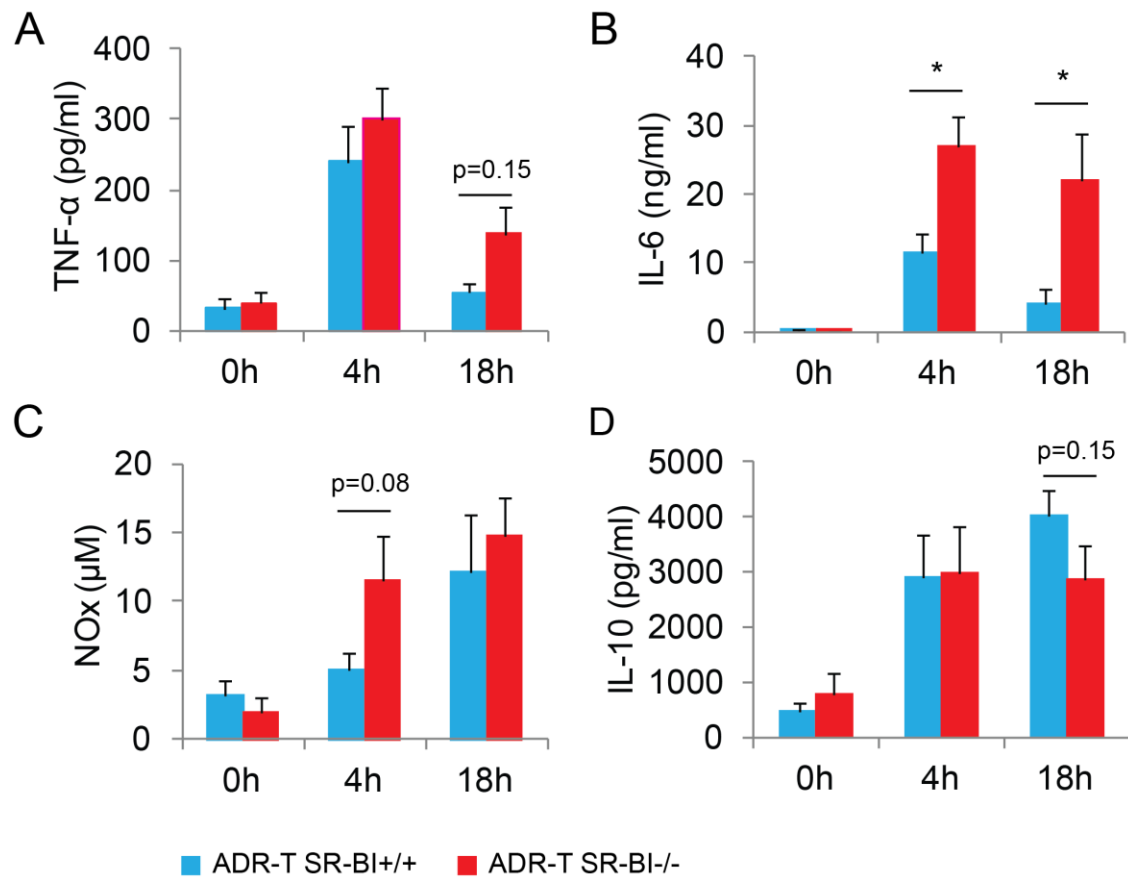


Figure 3.7 Inducible GC regulates the inflammatory cytokine production in sepsis.

The adrenal-specific SR-BI^{-/-} mice and the control mice were subjected to CLP for 0 h, 4 h or 18 h, and the serum concentrations of TNF-α (A), IL-6 (B), IL-10 (C) and NOx (D) were quantified. Data represent mean ± SEM. (n=6-8), * p<0.05

Next, we analyzed the correlation between the plasma corticosterone concentration and cytokine concentrations. As shown in Table 3.2, the plasma corticosterone showed a significant correlation with the plasma IL-6, but not with TNF- α , suggesting that iGC particularly exerts a strong inhibitory effect on IL-6.

Overall, these results demonstrate that iGC regulates the production of cytokines and inflammatory mediators and prevents the overwhelming pro-inflammatory response during CLP.

Table 3.2 Correlation between the plasma corticosterone concentration and cytokine production in CLP

	TNF- α	IL-6	NOx	IL-10
CLP 4 h	-0.308 (p=0.246)	-0.551 (p=0.027)	-0.346 (p=0.190)	-0.261 (p=0.347)
CLP 18 h	-0.412 (p=0.113)	-0.541 (p=0.025)	-0.494 (p=0.073)	0.407 (p=0.118)
CLP 4 and 18 h	-0.249 (p=0.169)	-0.468 (p=0.006)	-0.343 (p=0.064)	-0.087 (p=0.641)

Data from Figure 2.1 and Figure 3.6 were used for the correlation analysis. Data from the adrenal-specific SR-BI^{-/-} mice and the control mice were analyzed together. Pearson correlation analysis of the plasma corticosterone concentration and cytokine levels was done by SPSS. Data show the correlation efficient (r) and p value (two-tailed). Statistical correlations are considered significant at p < 0.05.

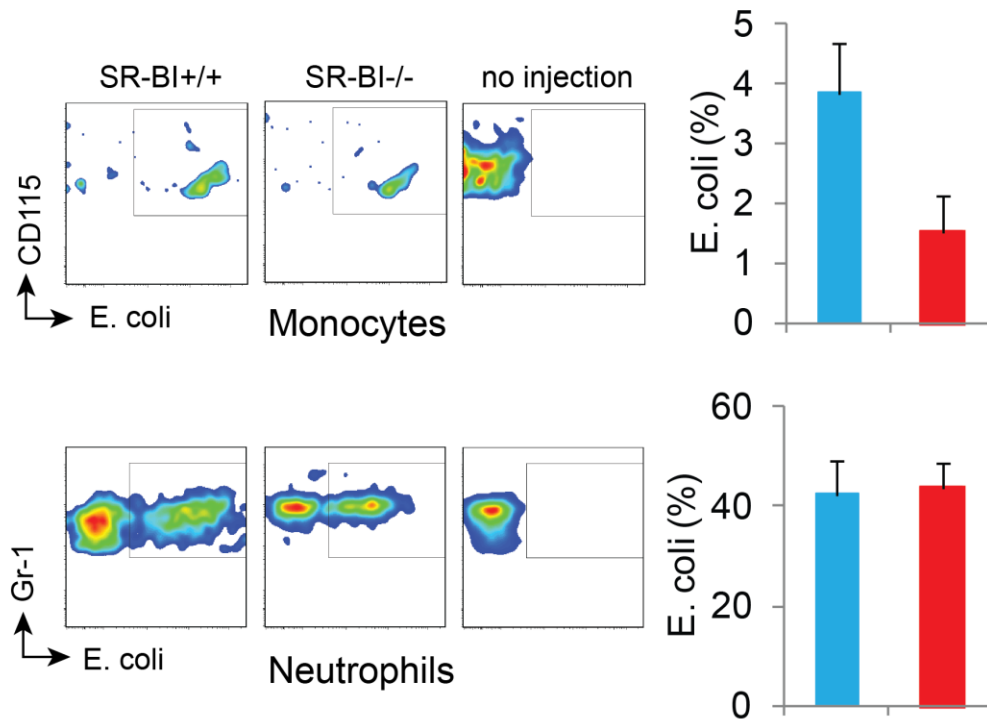
3.2.2.5 Inducible GC in sepsis increases the phagocytic activity of phagocytes in sepsis.

GC has been considered as an immunosuppressive agent.[101] However, the immunosuppression in sepsis is associated with an increased mortality in the late phase of sepsis.[52] The phagocytic activity of neutrophils has been reported impaired in septic patients [63] and is associated with a high mortality sepsis.[64] Therefore, we evaluated the phagocytic activity of phagocytes in the adrenal-specific SR-BI^{-/-} mice in CLP *in vivo* [222] to see whether or not iGC suppresses phagocytosis in sepsis. The adrenal-specific SR-BI^{-/-} mice and the control mice were intravenously injected with 1×10^8 heat-killed fluorescein isothiocyanate (FITC)-labeled *E. coli* at CLP 17 h. An hour later, the mice were killed for flow cytometry. The phagocytosis of injected *E. coli* by monocytes (CD115⁺ CD11b⁺ Ly6C⁺) and neutrophils (Gr-1⁺ CD11b⁺) in the blood and macrophages (F4/80⁺), neutrophils (Gr1⁺ CD11b⁺) and dendritic cells (CD3-CD11c⁺) in the spleen was analyzed.

As shown in Figure 3.8A, compared to the control mice, the adrenal-specific SR-BI^{-/-} mice had a 60% lower percentage of the phagocytic monocytes (1.53 ± 0.61 % vs. 3.85 ± 0.85 %, $p=0.064$) and an unchanged percentage of the phagocytic neutrophils (43.78 ± 4.86 % vs. 42.36 ± 7.10 %, $p=0.873$) in the blood. The capability of phagocytosis was evaluated by the mean fluorescence intensity (MFI) of *E. coli* in the phagocytes. We found that the adrenal-specific SR-BI^{-/-} mice displayed a 39 % lower MFI of *E. coli* in monocytes ($19.3 \pm 3.1 \times 10^3$ vs. $31.6 \pm 3.3 \times 10^3$, $p=0.027$) and a 55 % lower MFI of *E. coli* in neutrophils ($9.2 \pm$

2.7×10^3 vs. $20.1 \pm 1.5 \times 10^3$, $p=0.011$) than the control mice (Figure 3.8B), demonstrating that iGC can increase the phagocytic capability of monocytes and neutrophils in the blood from the CLP-adrenal-specific SR-BI^{-/-} mice.

A



B

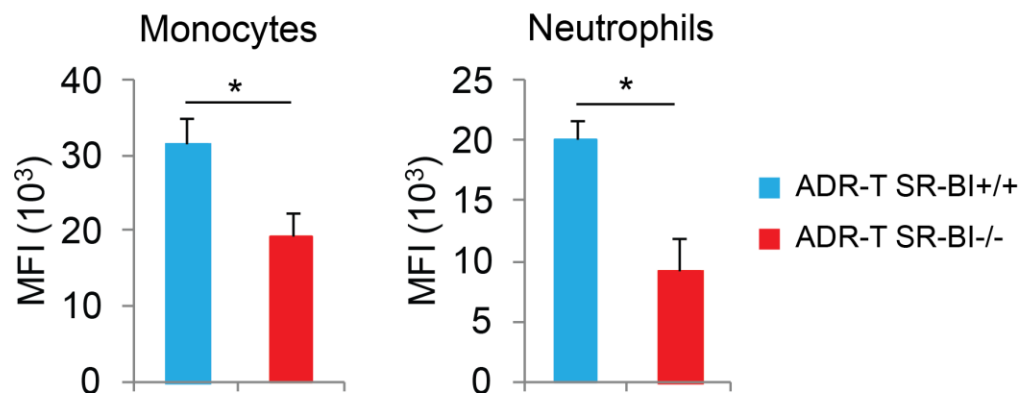


Figure 3.8 Inducible GC in sepsis increases the phagocytic activity of monocytes and neutrophils in the blood.

At CLP 17 h, the adrenal-specific SR-BI^{-/-} mice and the control mice were i.v. injected with 1×10^8 heat-killed FITC-E. coli, and the phagocytosis capability of phagocytes in the blood was analyzed by flow cytometry. (A) The percentage of phagocytic monocytes and neutrophils in the blood. The left panel shows representative gating pictures for the E.coli+ monocytes (upper) and E. coli+ neutrophils (lower). The right panel shows the average percentage of E. coli+ monocytes (upper) and neutrophils (lower) in the blood. (B) The average of mean fluorescence intensity (MFI) of E.coli in monocytes (left) and neutrophils (right) in the blood. Data represent mean \pm SEM. (n>4), * p<0.05

In the spleen, compared to the control mice, the adrenal-specific SR-BI^{-/-} mice had a lower percentage of phagocytic cells, as indicated by a 54 % lower percentage of the E. coli+ macrophages (7.19 ± 1.21 % vs. 15.57 ± 4.41 %, $p=0.131$), a 40 % lower percentage of E. coli+ neutrophils (15.54 ± 1.15 % vs. 25.78 ± 4.83 %, $p=0.101$) and a 25 % lower percentage of E. coli+ dendritic cells (3.92 ± 0.82 % vs. 5.28 ± 0.68 %, $p=0.254$; Figure 3.9A). In addition, the phagocytic capability of macrophages and neutrophils in the spleen also slightly decreased in the adrenal-specific SR-BI^{-/-} mice. As shown in Figure 3.9B, the adrenal-specific SR-BI^{-/-} mice displayed a 25% decrease in the MFI of E.coli in the macrophages (11.4 ± 2.8 vs. 15.2 ± 2.1 , $p=0.280$) and a 32 % decrease in the MFI of E.coli in the neutrophils (6.6 ± 1.8 vs. 9.7 ± 2.2 , $p=0.240$), compared to the control mice. The MFI of E. coli in the dendritic cells in the spleen from the adrenal-specific SR-BI^{-/-} mice was similar to that from the control mice (8.6 ± 1.8 vs. 7.1 ± 0.9 , $p=0.457$). These results, together with the data from the phagocytes in the blood, demonstrate that iGC in sepsis increases the phagocytic activity.

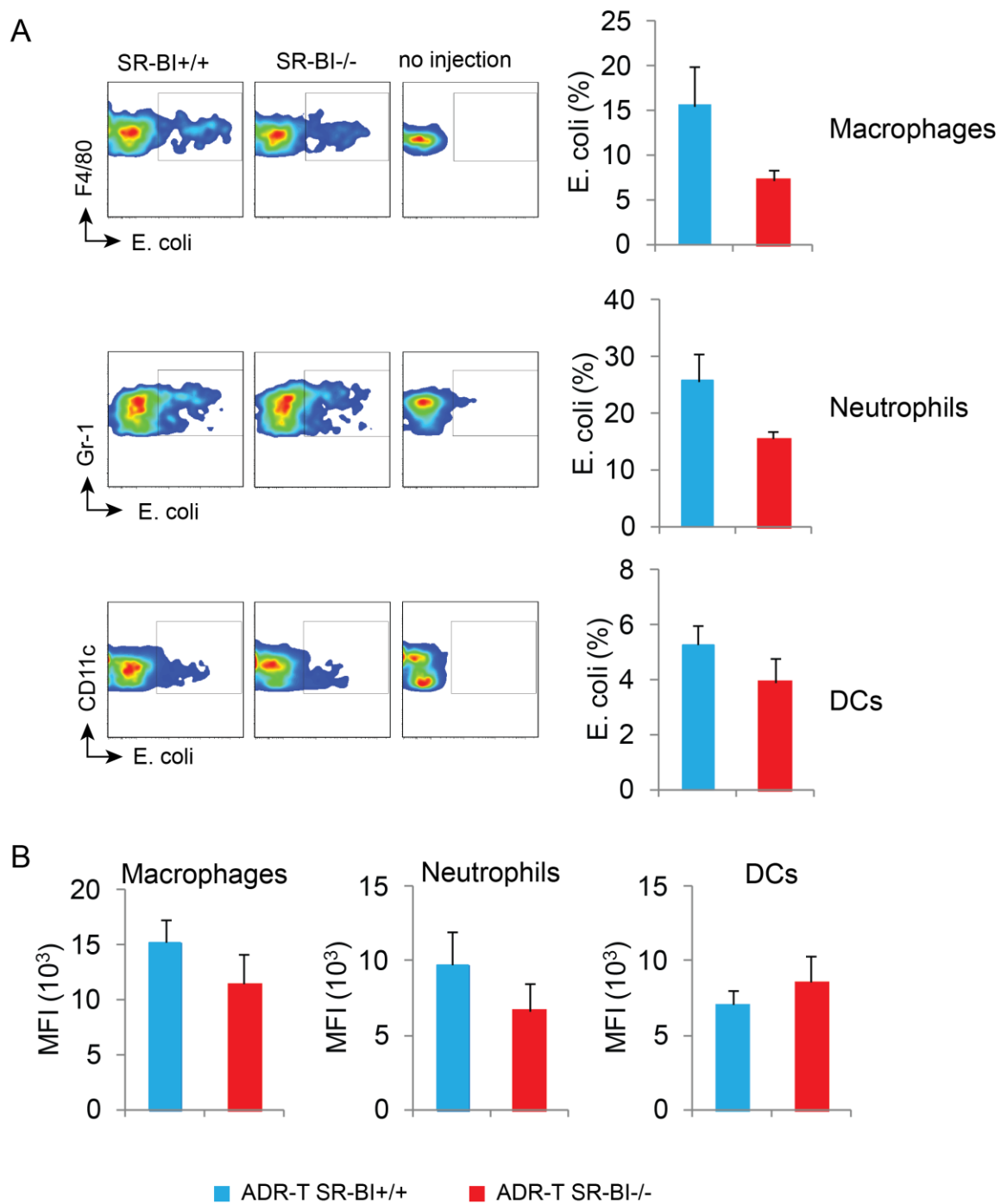


Figure 3.9 Inducible GC in sepsis trends to increase the phagocytic activity of macrophages and neutrophils in the spleen.

At CLP 17 h, the adrenal-specific SR-BI^{-/-} mice and the control mice were i.v. injected with 1×10^8 heat-killed FITC-E. coli, and the phagocytic capability of macrophages, neutrophils and dendritic cells in the spleen was analyzed by flow cytometry. (A) The left panel shows representative gating pictures for the E.coli+ macrophages (upper), E. coli+ neutrophils (middle) and E. coli+ dendritic cells (lower). The right panel shows the average percentage of phagocytic macrophages, neutrophils and dendritic cells in the spleen. (B) The average of mean fluorescence index (MFI) of E.coli in macrophage (left), neutrophils (middle) and dendritic cells (right) in the spleen. Data represent mean \pm SEM. (n > 4); DCs, dendritic cells

3.2.2.6 Inducible GC in sepsis does not lead more apoptosis but induces higher activation of the lymphocytes in the spleen.

It is known that GC can induce the apoptosis of lymphocytes[223], and the apoptosis-induced loss of lymphocytes is considered as a potential factor in the immunosuppression of sepsis.[67] To investigate if iGC has such actions in sepsis, we evaluated the apoptosis and the activation of lymphocytes in the spleen at CLP 18 h. As shown in Figure 3.10A and 3.10B, there was no difference between the adrenal-specific SR-BI^{-/-} mice and the control mice in the number of spleen cells, the percentage of lymphocytes in spleen cells ($66.51 \pm 1.81\%$ vs. $68.16 \pm 1.61\%$, $p=0.507$) and the percentage of T cells (CD3⁺, $29.61 \pm 1.54\%$ vs. $27.32 \pm 1.56\%$, $p=0.315$) or B cells (CD19⁺, $58.90 \pm 1.35\%$ vs. $61.72 \pm 1.48\%$, $p=0.186$) in lymphocytes in the spleen, indicating that the lack of iGC does not affect lymphocyte numbers in the spleen. We then analyzed the apoptosis of lymphocytes in the spleen by TUNEL staining. As shown in Figure 3.10C, we did not observe any difference in the percentage of TUNEL⁺ cells in lymphocytes ($3.87 \pm 0.81\%$ vs. $3.45 \pm 0.29\%$, $p=0.644$), T cells ($2.31 \pm 0.59\%$ vs. $2.08 \pm 0.28\%$, $p=0.740$) and B cells ($1.42 \pm 0.53\%$ vs. $0.86 \pm 0.11\%$, $p=0.334$) between the adrenal-specific SR-BI^{-/-} mice and the control mice, indicating that iGC is not associated with the apoptosis-induced loss of lymphocytes in sepsis. Using CD69 as an activation marker, we analyzed the activation of lymphocytes in the spleen from the adrenal-specific SR-BI^{-/-} mice. Interestingly, we observed that the adrenal-specific SR-BI^{-/-} mice had a significantly lower percentage of activated T cells (CD69⁺CD3⁺) in total

lymphocytes compared to the control mice (7.21 ± 0.43 % vs. 5.69 ± 0.35 %, $p=0.017$; Figure 3.10D). Also, the adrenal-specific SR-BI^{-/-} mice had a slightly lower percentage of activated B cells (CD69⁺CD19⁺) in total lymphocytes than the control mice (6.24 ± 0.49 % vs. 4.90 ± 0.63 %, $p=0.124$; Figure 3.10D). These data demonstrate that iGC is not necessarily immunosuppressive but may enhance the activation of lymphocytes in sepsis.

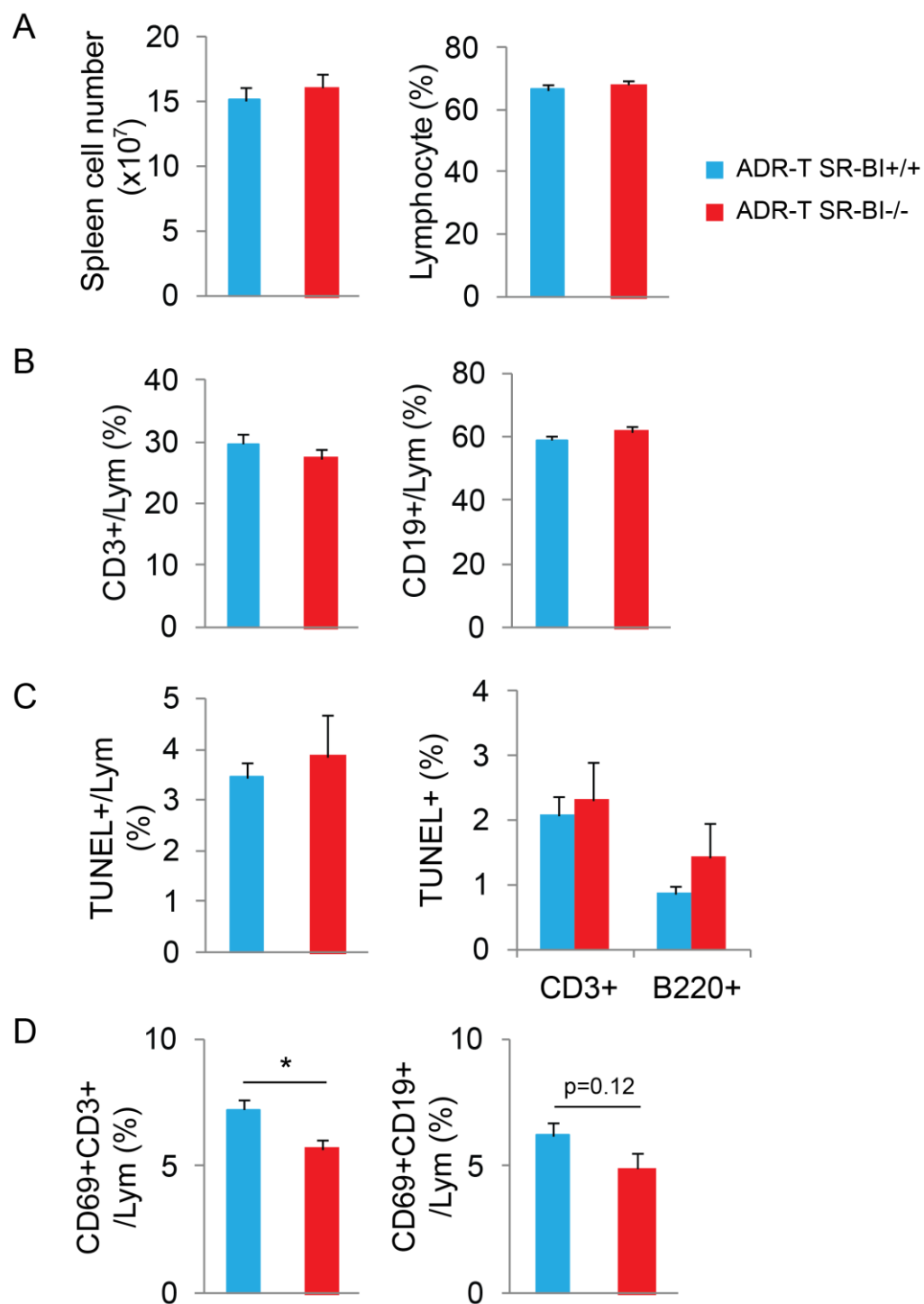


Figure 3.10 Inducible GC does not lead to more apoptosis but induces higher activation of the lymphocytes in the spleen.

The spleens were harvested from the adrenal-specific SR-BI^{-/-} mice and the control mice at CLP 18 h for the flow cytometry. Data show (A) the spleen cell number and the lymphocyte percentage in the spleen cells; (B) the percentage of T cells (CD3+) and B cells (CD19+) in lymphocytes; (C) the percentages of apoptotic (TUNEL+) cells in total lymphocytes, T cells, or B cells; and (D) the activation of T cell and B cells marked by CD69. Data represent mean \pm SEM. (n=7-9), * p<0.05

3.2.3 Summary

In this section, the important role of iGC in protecting against septic death is investigated using the adrenal-specific SR-BI^{-/-} mouse as an iGC deficient model. The adrenal-specific SR-BI^{-/-} mice had a high mortality of 87 % in CLP-induced sepsis whereas only 17 % of the control mice died under the same treatment, demonstrating that iGC is important in preventing the sepsis-induced death. Compared to the control mice, the adrenal-specific SR-BI^{-/-} mice had a high BUN level in the plasma at CLP 18 h, demonstrating an essential role of iGC to maintain kidney function during sepsis. Cytokine analysis showed that the adrenal-specific SR-BI^{-/-} mice displayed significantly higher levels of plasma IL-6 at CLP 4 h and CLP 18 h compared to the control mice, a marginally higher level of the plasma TNF- α at CLP 18 h and a 2.3-fold higher plasma NOx at CLP 4 h, indicating that iGC inhibits the pro-inflammatory cytokines and mediator in sepsis. In addition, a strong correlation between the plasma corticosterone and IL-6 was observed, indicating that iGC exerts strong inhibitory effects on IL-6 in sepsis. *In vivo* phagocytosis analysis showed that the adrenal-specific SR-BI^{-/-} mice presented a lower percentage of phagocytic monocytes in the blood and lower percentages of phagocytic macrophages, neutrophils and dendritic cells in the spleen at CLP 18 h. In addition, the monocytes and neutrophils from the adrenal-specific SR-BI^{-/-} mice displayed a reduced phagocytic capability compared to the cells from the control mice. These data demonstrate that iGC enhances the phagocytic activity of these phagocytes in sepsis. The activation of T cells in the spleen from the adrenal-specific SR-BI^{-/-} mice at CLP 18 h was significantly lower

than that in the control mice, demonstrating that iGC induces a higher activation of lymphocytes in sepsis. The data in this section overall reveal that iGC is essential for survival in sepsis and exerts actions of (1) inhibiting the pro-inflammatory cytokine and mediators, (2) preventing the kidney injury, (3) increasing the phagocytic activity of monocytes and neutrophils in the blood and (4) enhancing the activation of lymphocytes. (Figure 3.11)

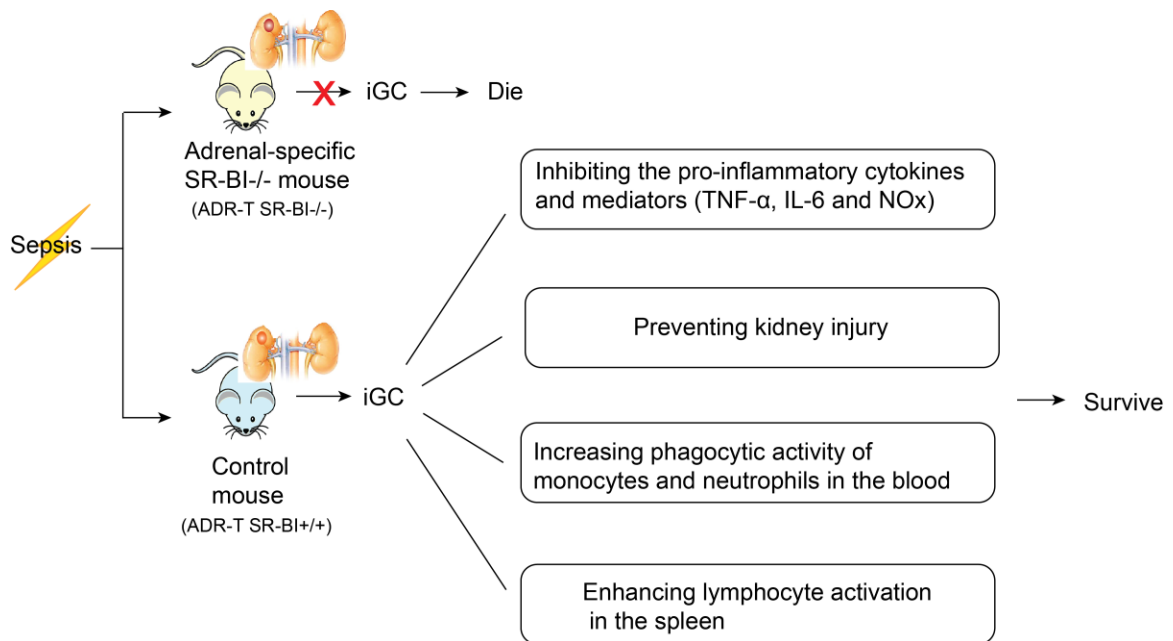


Figure 3.11 The role of SR-BI-regulated iGC in sepsis.

iGC is produced in sepsis by adrenal glands, which is important for the survival in sepsis. Due to the lack of iGC, the adrenal-specific SR-BI^{-/-} mice are very susceptible to death in sepsis. In contrast, the control mice survive well in the same CLP treatment. Our data revealed that during the sepsis, iGC can (1) inhibit the production pro-inflammatory cytokines and mediators, (2) prevent the kidney injury, (3) increase the phagocytic activity of monocytes and neutrophils and (4) enhance lymphocyte activation in the spleen.

Chapter 4 GC supplementation and survival in sepsis

Section 1 GC supplementation and survival in the CLP-induced septic death.

4.1.1 Introduction

GC has been given to septic patients for more than five decades, yet GC treatment in septic patients got controversial results on survival. Annane. *et al* have shown that low-dose GC supplementation can significantly increase the survival rate of septic shock patients who have adrenal insufficiency but cannot improve the survival rate of those who do not have adrenal insufficiency.[183] However, the Survival Sepsis Campaign used GC in “septic shock patients who require vasopressor treatment despite adequate fluid resuscitation” and showed no difference in the survival rate between the GC group and the placebo group.[190] Using the criteria from the Survival Sepsis Campaign, several other trials also revealed no benefit of GC use in sepsis.[191, 192] The reason why GC failed to improve sepsis survival in these trials remains unknown.

We suspected that distinguishing adrenal insufficient patients or not may have made a difference in the results of previous trials. We hypothesized that GC supplementation improves survival of adrenal insufficient individuals in sepsis but not that of individuals without adrenal insufficiency. To test this hypothesis, we first investigated whether GC supplementation is beneficial for SR-BI^{-/-} mice, which are adrenal insufficient in sepsis. We used two approaches to supplement GC to SR-BI^{-/-} mice. The first approach was by transplanting a SR-BI^{+/+} adrenal gland to adrenalectomized SR-BI^{-/-} mice; the second was by injecting GC to the

SR-BI^{-/-} mice. We found that GC injections increased the survival rate of SR-BI^{-/-} mice in CLP. Then we also tested if GC supplementation benefits individuals without adrenal insufficiency by injecting the same GC to SR-BI^{+/+} mice in CLP. We found that GC supplementation that improved survival of SR-BI^{-/-} mice failed to increase the survival rate of SR-BI^{+/+} mice in CLP. Altogether, the results from this section support our hypothesis that GC supplementation improves survival of adrenal insufficient individuals in sepsis but not that of individuals without adrenal insufficiency.

4.1.2 Results

4.1.2.1 Survival in sepsis is positively associated with the strength of corticosterone response.

To test our hypothesis, we first sought to restore iGC of SR-BI^{-/-} mice by transplanting SR-BI^{+/+} adrenal to these mice. By comparing the corticosterone concentration of the SR-BI^{+/+} → SR-BI^{-/-} mice with that of SR-BI^{+/+} → SR-BI^{+/+} mice post CLP, we observed a significantly lower production of iGC in the SR-BI^{+/+} → SR-BI^{-/-} mice compared to the SR-BI^{+/+} → SR-BI^{+/+} mice at CLP 4 h (Figure 4.1A). Nevertheless, the plasma corticosterone concentration at CLP 4 h showed a significant correlation with the survival time in the SR-BI^{+/+} → SR-BI^{-/-} mice (Figure 4.1B) and the average plasma corticosterone concentration in the survivors was significantly higher than that in the non-survivors (Figure 4.1C) in these mice. When we divided these mice into responders (C>200 ng/ml) and

non-responders ($C \leq 200$ ng/ml) by their plasma corticosterone at CLP 4 h, we found that the responders had a significantly improved survival compared to the non-responders ($p=0.049$, Figure 4.1D). These data demonstrate that survival in sepsis is positively correlated with the strength of corticosterone response.

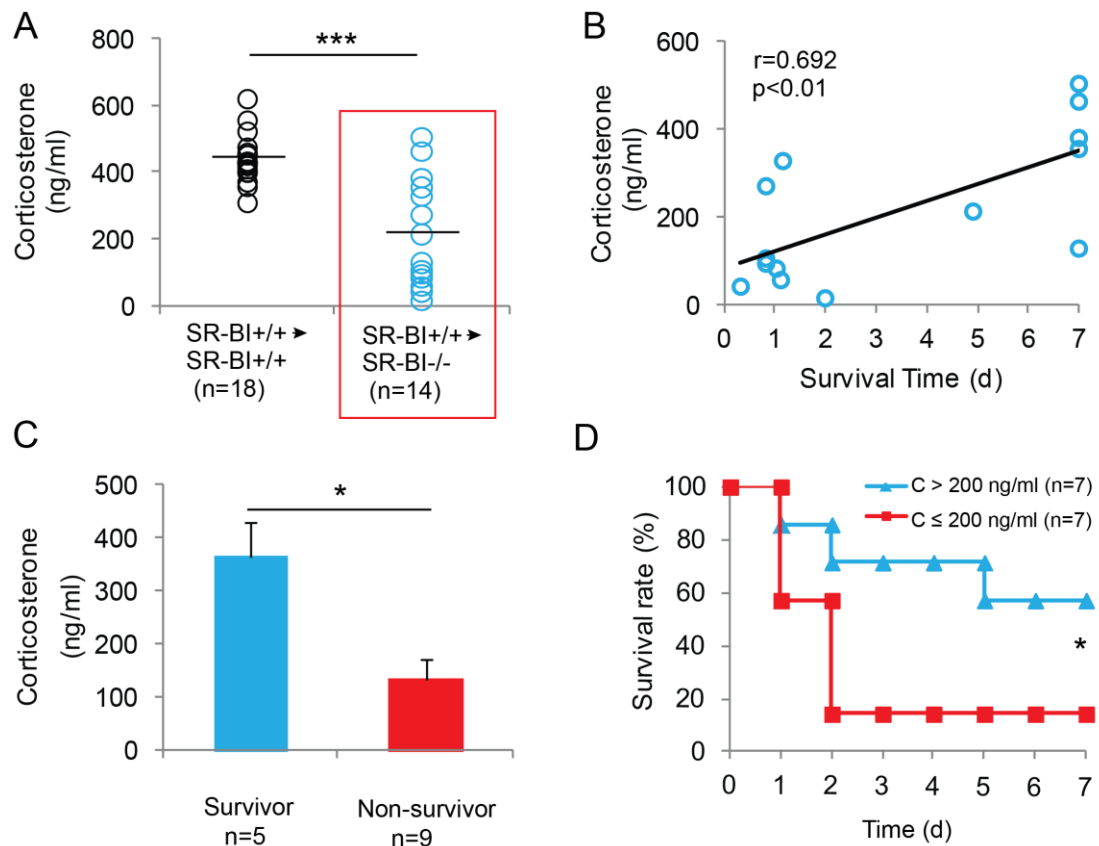


Figure 4.1 The strength of corticosterone response is positively correlated with the survival time in CLP-induced sepsis.

SR-BI^{-/-} mice were transplanted with SR-BI^{+/+} adrenal gland (SR-BI^{+/+}→SR-BI^{-/-}) and subjected to CLP surgery 6 weeks after the transplantation. Plasma was harvested through tail bleeding 4 hours after CLP. (A) The plasma corticosterone level at CLP 4 h in the SR-BI^{+/+}→SR-BI^{-/-} mice was compared with that in the SR-BI^{+/+}→SR-BI^{+/+} mice. The line shows the mean. *** p<0.001 (B), (C) and (D) show further analysis of data from the SR-BI^{+/+}→SR-BI^{-/-} group. (B) The level of plasma corticosterone concentrations 4 h post CLP is positively related with the survival time (r=0.692, p=0.006, Pearson correlation). (C) The average plasma corticosterone level from non-survivors was significantly lower than that in survivors. Data represent mean ± SEM. * p<0.05 (D) The SR-BI^{+/+}→SR-BI^{-/-} mice were divided into a high corticosterone group (C>200ng/ml) and a low corticosterone group (C≤200ng/ml) according to the CLP 4 h plasma corticosterone concentration. The low corticosterone group had a mortality of 85% while the high corticosterone group had a mortality of 42% (p=0.049, log-rank χ^2 test). * p<0.05

4.1.2.2 GC supplementation improves the survival of adrenal insufficient mice in CLP-induced sepsis.

Compared to adrenal transplantation, GC infusion is a more feasible strategy to be used in clinical patients; therefore we applied GC injections to further study the benefits of GC supplementation in adrenal insufficient mice. In our earlier studies, we used corticosterone supplementation through drinking water [110] and dexamethasone and fludrocortisones supplementation through i.p. injections [224]; however, both approaches failed to improve survival of SR-BI^{-/-} mice in CLP-induced sepsis. Considering that the dose and kind of supplemented GC may affect the results in survival study, in this study, we used a corticosteroid cocktail containing 100 µg hydrocortisone, 25 ng fludrocortisone acetate and 20 µg methylprednisolone, which was modified from previous publication.[225] As shown in Figure 4.2A, administration of the GC cocktail increased the survival rate in the SR-BI^{-/-} mice from 40% to 92% ($p < 0.001$). Then, because hydrocortisone is recommended in the 2012 surviving sepsis guideline, we studied the effects of hydrocortisone supplementation to the survival of the SR-BI^{-/-} mice in CLP. Supplementation of 100 µg hydrocortisone alone increased the survival rate of SR-BI^{-/-} mice to 70% ($p = 0.07$; Figure 4.2B). Finally, we also supplemented the natural GC, corticosterone, to the mice. Supplementation of 200 µg corticosterone increased the survival rate of SR-BI^{-/-} mice to 80% ($p = 0.067$; Figure 4.2C). These data demonstrate that GC supplementation is beneficial for the survival of adrenal insufficient mice in sepsis.

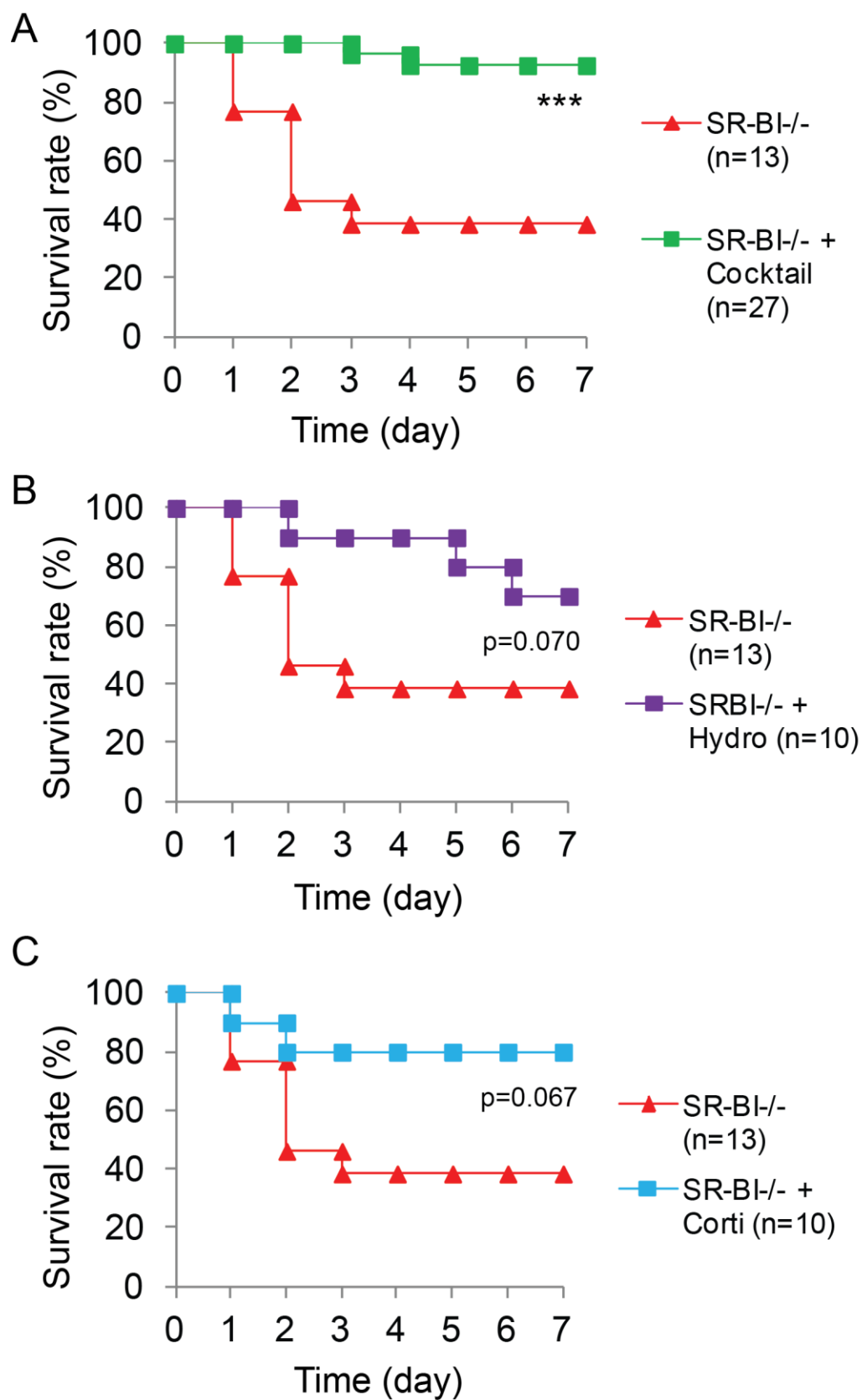


Figure 4.2 GC supplementation increases the survival rate of SR-BI^{-/-} mice in CLP.

CLP (23-gauge needle, half ligation) was conducted on SR-BI^{-/-} mice. For GC supplementation, the mice were administrated with (A) a GC cocktail containing 100 µg hydrocortisone, 25 ng fludrocortisone acetate and 20 µg 6δ-methylprednisolone (+ Cocktail), (B) 100 µg hydrocortisone only (+ Hydro) or (C) 200 µg corticosterone (+ Cort). The control mice received no GC supplementation. Survival was observed for 7 days. Data are expressed as the percentage of mice surviving at the indicated time points and analyzed by the log-rank χ^2 test. *** $p < 0.001$

4.1.2.3 GC supplementation is unhelpful for the survival of wild type mice in CLP-induced sepsis.

Based on the above results, it seems that GC supplementation is beneficial to adrenal insufficient mice in CLP-induced sepsis. However, this did not explain why clinical trials on the use of GC in septic patients got controversial results. We hypothesized that GC supplementation may only benefit adrenal insufficient individuals in sepsis but not those without adrenal insufficiency. We conducted CLP in SR-BI^{+/+} mice using the same condition as that in SR-BI^{-/-} mice. CLP which caused a 60 % mortality in the SR-BI^{-/-} mice (Figure 4.2) only induced an 20% mortality in the SR-BI^{+/+} mice (Figure 4.3A), which was consistent with former reports [110]. Nevertheless, GC supplementation failed to increase the survival rate in the SR-BI^{+/+} mice in CLP, indicating that GC supplementation does not benefit survival of SR-BI^{+/+} mice in sepsis (Figure 4.3A). When we conducted a stronger CLP to SR-BI^{+/+} mice and injected 200µg/mouse corticosterone to the mice, the SR-BI^{+/+} mice with GC supplementation showed a lower survival rate than the SR-BI^{+/+} mice without GC supplementation (80 % vs. 40 %, p=0.013; Figure 4.3B). These data demonstrate that GC supplementation is unhelpful for survival of mice without adrenal insufficiency in sepsis.

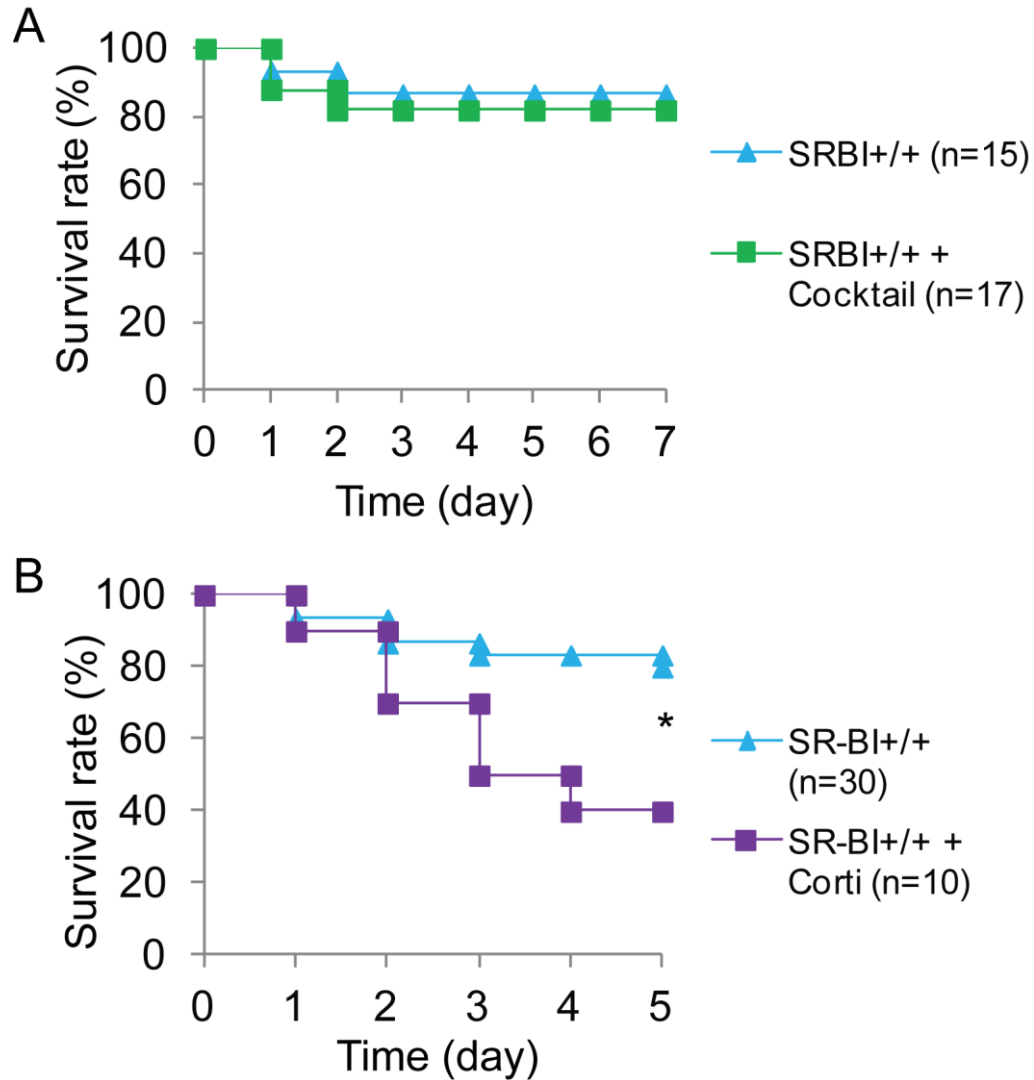


Figure 4.3 GC supplementation is unhelpful for survival of SR-BI^{+/+} mice in CLP-induced sepsis.

(A) SR-BI^{+/+} mice were treated with CLP (23-gauge needle, half ligation) and were supplemented with or without a GC cocktail containing 100 µg hydrocortisone, 25 ng fludrocortisone acetate and 20 µg 6δ-methylprednisolone after CLP. Survival was observed for 7 days. (B) SR-BI^{+/+} mice were treated with CLP (22-gauge needle, full ligation) and were supplemented with or without 200 µg corticosterone after CLP. Survival was observed for 5 days. Data are expressed as the percentage of mice surviving at the indicated time points and analyzed by the log-rank χ^2 test. *, $p < 0.05$

4.1.3 Summary

The study in this section investigated the effects of GC supplementation on survival in CLP-induced septic death. We hypothesized that GC supplementation is beneficial for the individuals with adrenal insufficiency but not for those without adrenal insufficiency in sepsis. First, by transplanting SR-BI^{+/+} adrenal glands to the adrenalectomized SR-BI^{-/-} mice, we observed that plasma corticosterone level at CLP 4 h was strongly correlated with the survival time of the SR-BI^{+/+} → SR-BI^{-/-} mice in CLP-induced sepsis. Second, we injected a GC cocktail containing 100 µg hydrocortisone, 25 ng fludrocortisone acetate and 20 µg 6δ-methylprednisolone to the SR-BI^{-/-} mice, which increased the survival rate of SR-BI^{-/-} mice from 40 % to 92 %. Supplementation of 100 µg hydrocortisone or 200 µg corticosterone can also increase the survival of SR-BI^{-/-} mice to 70 % or 80 %, respectively. These data demonstrate that GC supplementation can improve the survival of adrenal insufficient mice. In SR-BI^{+/+} mice, injections of GC cocktail or corticosterone did not increase their survival rates in CLP, demonstrating that GC supplementation is unhelpful for the individuals without adrenal insufficiency in sepsis.

Section 2 GC supplementation and survival in the “two-hit” model of secondary infection-induced death in sepsis.

4.2.1 Introduction

Whereas some patients rapidly succumb to the overwhelming pro-inflammatory cytokine-driven inflammation and died at an early time after the initiation of sepsis, more than 60 % of deaths in sepsis are estimated to occur after the first 3 days of the disorder.[54] Thus an investigation on the effects of GC on the survival in the late stage of sepsis is also needed. A “two-hit” model has been used to study the late stage of CLP-induced sepsis in mice.[71] The rationale of this model is based on the fact that nosocomial infection of septic patients contributes to the late death of septic patients.[55] In the “two-hit” model, the CLP is used as the “first hit” and an infection of sublethal-dose *Pseudomonas aeruginosa* or *Streptococcus pneumonia* is used to induce pneumonia in mice after CLP as the “second hit”. *P. aeruginosa* is a gram-negative bacterium that is one of the most common causes of nosocomial pneumonia and therefore has been used in our study.[226] In this section, we used *P. aeruginosa*-induced secondary infection in CLP mice to address the importance of iGC in secondary infection in sepsis.

4.2.2 Results

4.2.2.1 The “two-hit” model of secondary infection in sepsis was generated by infection with sublethal-dose *P. aeruginosa* after CLP.

After identifying the benefit of GC supplementation in adrenal insufficient individuals in the “first insult” in the sepsis, we used a “two-hit” model to evaluate the importance of iGC and the effects of GC supplementation in the secondary infection in sepsis. According to previous studies using the “two-hit” model, a decrease in survival in secondary pneumonia infection can be observed in mice that were infected 3 days or 4 days after CLP when innate immune responses are impaired, but not in mice that were infected 7 days after CLP when the innate immunity is recovered. [226, 227] In our study, a sublethal dose of 5×10^7 CFU/mouse was identified as an appropriate dose of *P. aeruginosa* for the secondary infection in SR-BI^{+/+} mice. At this dosage, the non-CLP SR-BI^{+/+} mice all survived the secondary infection, whereas the CLP-3 d SR-BI^{+/+} mice only had a survival rate of 25 % ($p < 0.001$; Figure 4.4A). The CLP-7 d SR-BI^{+/+} mice had a high survival rate which is not significantly different from the survival in non-CLP group (Figure 4.4B). This result is consistent with earlier studies on the “two-hit” model in sepsis [226, 227]; therefore we used 5×10^7 CFU/mouse *P. aeruginosa* for secondary infection in the following experiments.

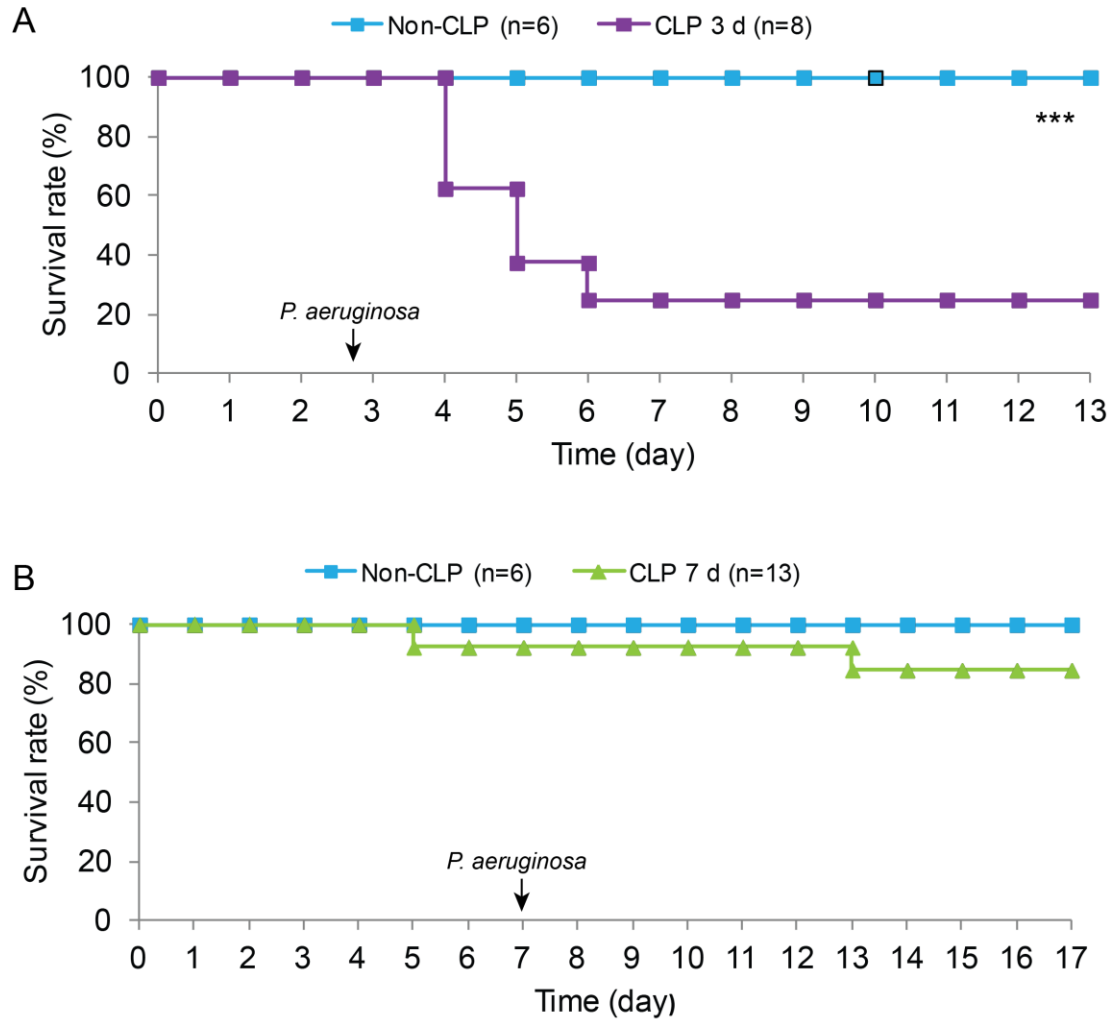


Figure 4.4 A “two-hit” model was generated by a secondary infection of *P. aeruginosa* after CLP.

A “two-hit” model was generated by a secondary infection of *P. aeruginosa* in CLP (23-gauge, half ligation) SR-BI^{+/+} mice. Mice were challenged with an intranasal administration with 5×10^7 CFU *P. aeruginosa* without CLP, 3 days after CLP or 7 days after CLP. Survival was observed for 10 days after the infection. Data are expressed as the percentage of mice surviving at the indicated time points and analyzed by the log-rank χ^2 test. *** $p < 0.001$

4.2.2.2 CLP-7 d SR-BI-deficient mice have impaired GC production in secondary infection in sepsis.

Next, we investigated the iGC production in the secondary infection model. As shown in Figure 4.6B, compared to the non-CLP SR-BI^{+/+} mice, the CLP-7 d SR-BI^{+/+} mice displayed a lower plasma corticosterone concentration at 4 hours post infection (265.3 ± 53.8 $\mu\text{g/ml}$ vs. 160.6 ± 27.3 $\mu\text{g/ml}$), indicating that less iGC is needed for survival in secondary infection than CLP. However, the CLP-7 d SR-BI^{-/-} mice still showed a significantly lower plasma corticosterone level at 4 hours post infection compared to the CLP-7 d SR-BI^{+/+} mice (160.6 ± 27.3 $\mu\text{g/ml}$ vs. 23.0 ± 9.0 $\mu\text{g/ml}$, $p < 0.001$, t test), indicating that CLP-7 d SR-BI^{-/-} mice have iGC insufficiency in secondary infection.

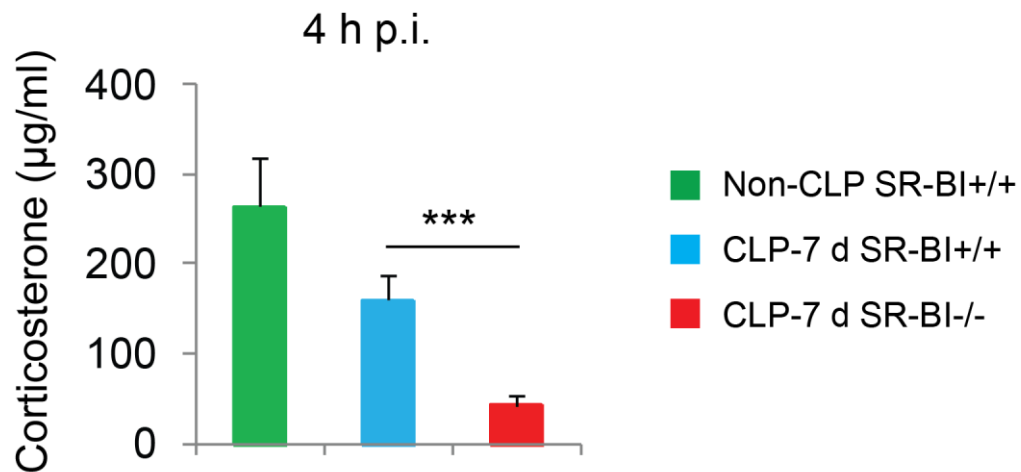


Figure 4.5 CLP-7 d SR-BI-deficient mice have adrenal insufficiency in secondary infection.

For non-CLP SR-BI^{+/+} mice, the mice were directly treated with intranasal infection of 5×10^7 CFU/mouse *P. aeruginosa*. For CLP-7 d SR-BI^{+/+} and SR-BI^{-/-} mice, the mice were first subjected to CLP (23-gauge, half ligation). Seven days after CLP, the survived mice were infected with *P. aeruginosa* 5×10^7 CFU/mouse intranasally. To maintain a high survival rate of the SR-BI^{-/-} mice in CLP, the SR-BI^{-/-} mice were injected the GC cocktail after CLP. The plasma were harvested through tail bleeding 4 hours after the bacterial infection and used for corticosterone assay. Data present mean \pm SEM. n=6 for non-CLP SR-BI^{+/+} mice, n=12 for CLP-7 d SR-BI^{+/+} mice and CLP-7 d SR-BI^{-/-} mice, *** p<0.001

4.2.2.3 CLP-7 d SR-BI-deficient mice are susceptible to the secondary infection-induced death in sepsis.

Since SR-BI^{-/-} mice presented a deficiency of iGC in secondary infection, we then used this mice to investigate whether the lack of iGC is associated with an increased susceptibility to secondary infection-induced death in sepsis. As shown in Figure 4.6A, CLP-7 d SR-BI^{-/-} mice displayed a mortality of ~85 % in secondary infection, while CLP-7d SR-BI^{+/+} mice displayed a mortality of only 8 % ($p < 0.001$). This result indicates that iGC insufficiency is associated with an increased mortality in secondary infection during sepsis.

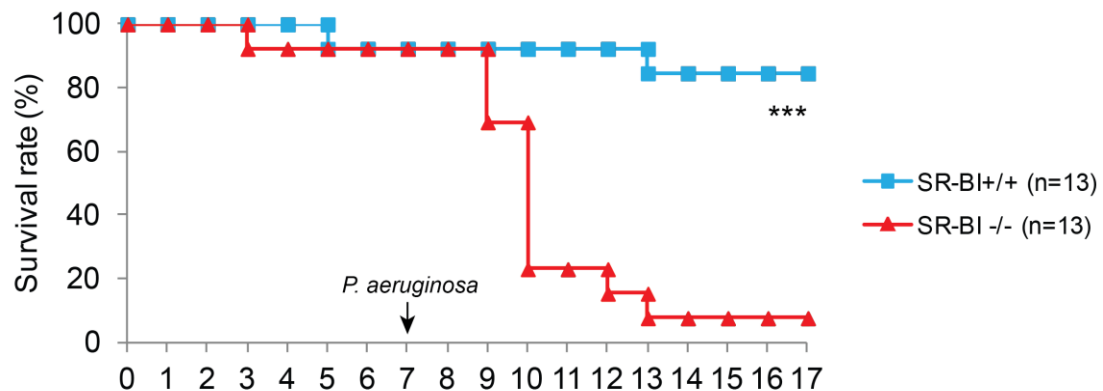


Figure 4.6 CLP-7 d SR-BI-deficient mice are susceptible to secondary infection-induced death in sepsis.

SR-BI^{+/+} and SR-BI^{-/-} mice were subjected to CLP (23-gauge, half ligation). SR-BI^{-/-} mice were injected the GC cocktail after CLP. Seven days after CLP, the survived mice were infected with *P. aeruginosa* 5×10^7 CFU/mouse intranasally. Survival was observed for 10 days after the infection. Data are expressed as the percentage of mice surviving at the indicated time points and analyzed by the log-rank χ^2 test. *** $p < 0.001$

4.2.2.4 GC injection trends to increase the survival time of SR-BI-deficient mice in the second infection after CLP.

We then tried to rescue SR-BI^{-/-} mice in the secondary infection by GC supplementation. Since a lower plasma corticosterone concentration had been observed in the CLP-7 d SR-BI^{+/+} mice than the non-CLP SR-BI^{+/+} mice, we used a half dose of GC cocktail (50µg hydrocortisone, 12.5 ng fludrocortisone acetate and 10 µg 6δ-methylprednisolone) for the CLP-7 d SR-BI^{-/-} mice in secondary infection. The SR-BI^{-/-} mice were subjected to CLP and received the GC cocktail (100 µg hydrocortisone, 25 ng fludrocortisone acetate and 20 µg 6δ-methylprednisolone) after CLP to maintain a high survival rate. Seven days after CLP, the survived SR-BI^{-/-} mice were intranasally administered *P. aeruginosa* with or without the injection of a half-dose GC cocktail after the infection (Figure 4.7A). As shown in Figure 4.7B, one day after the infection, a 28 % fatality in the SR-BI^{-/-} mice with the 2nd GC injection was induced, as controlled by a 23 % fatality in those who did not receive the 2nd GC injection. At two days post the infection, the fatality of the no 2nd GC injection group increased to 69% while the fatality of 2nd GC injection group only elevated to 36%. During the observation period, only 8 % mice without the 2nd GC injection finally survived the secondary infection challenge, whereas the survival rate increased to 23 % by a 2nd GC injection (p=0.282).

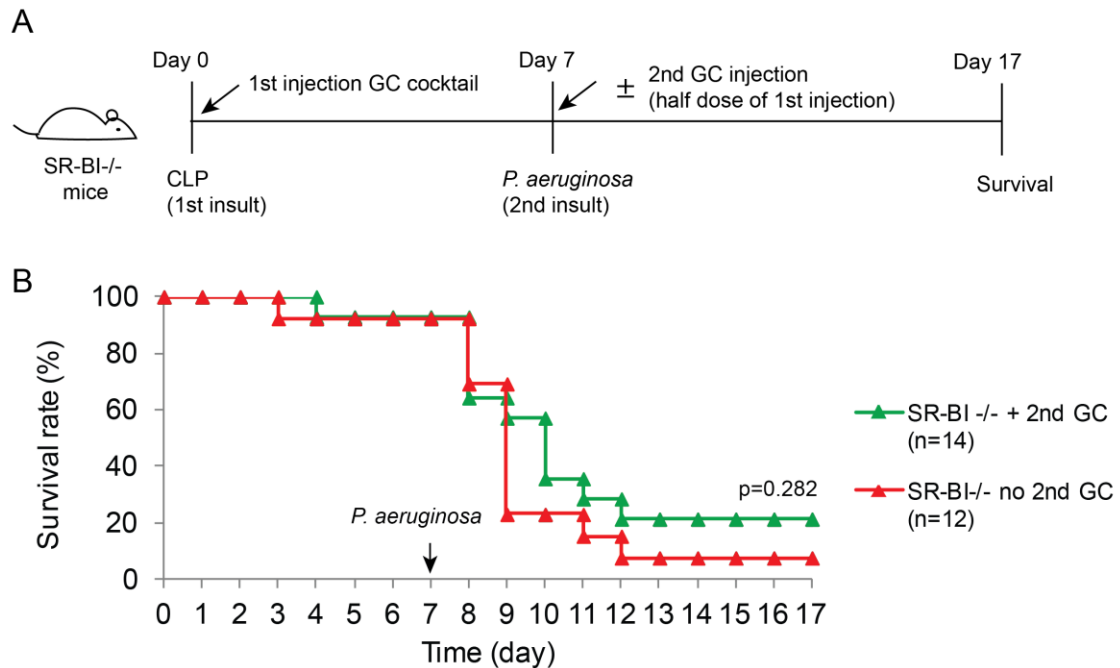


Figure 4.7 GC injection increases the survival time of SR-BI-deficient mice in second infection after CLP.

(A) The strategy of GC supplementation in secondary infection model. SR-BI^{-/-} mice were subjected to CLP (23-gauge, half ligation) and injected with the GC cocktail after CLP surgery. Seven days after CLP, mice were intranasally administered 5×10^7 CFU *P. aeruginosa* with or without the injection of a half-dose GC cocktail after the infection. (B) Survival was observed for 10 days after the infection. Data are expressed as the percentage of mice surviving at the indicated time and analyzed by the log-rank χ^2 test.

4.2.3 Summary

In this section, the effect of GC supplementation on survival in secondary infection in sepsis was investigated in a “two-hit” model induced by *P. aeruginosa* infection after CLP. First, we figured out the proper dosage of *P. aeruginosa* for the CLP condition and mouse strain in our study. With the infection of 5×10^7 *P. aeruginosa*, the CLP-3 d SR-BI^{+/+} mice displayed a survival rate of 25 % and the CLP-7 d SR-BI^{+/+} mice displayed a high survival rate that is non-significantly different from the survival in non-CLP group. This result is consistent with previous studies on the “two-hit” model, thus we used 5×10^7 *P. aeruginosa* to induce the secondary infection the following experiments. Next, we showed that at 4 hours post infection, the plasma corticosterone concentration in the CLP-7 d SR-BI^{-/-} mice was significantly lower than that in the CLP-7 d SR-BI^{+/+} mice, indicating the presence of adrenal insufficiency in the CLP-7 d SR-BI^{-/-} mice. In addition, the CLP-7 d SR-BI^{-/-} mice presented a significantly lower survival rate than the CLP-7 d SR-BI^{+/+} mice (15 % vs. 92 %), indicating that the lack of iGC is associated with impairs survival in secondary infection in sepsis. Finally, we used the CLP-7 d SR-BI^{-/-} mice to study whether or not GC supplementation can benefit the mice with adrenal insufficiency in the secondary infection. Administration of a half-dose GC cocktail to the SR-BI^{-/-} mice after the secondary infection increased the survival rate from 8 % to 23 %, indicating that GC supplementation trends to improve the survival of adrenal insufficient mice in the secondary infection in sepsis.

Chapter 5 Discussion and future directions

The present dissertation demonstrates the important role of iGC in protecting against septic death and the beneficial effects of GC supplementation on survival in sepsis. First, by analyzing the difference between the adrenal-specific SR-BI-deficient mice and the control mice in CLP, we elucidate that iGC protects against septic death. The iGC in sepsis has several the functions, which includes: (1) preventing the kidney injury, (2) inhibiting the production of pro-inflammatory cytokines and inflammatory mediators, (3) increasing the phagocytosis and (4) enhancing the activation of lymphocytes in sepsis. Then, by administrating GC to SR-BI^{+/+} mice and SR-BI^{-/-} mice, we demonstrate that GC supplementation improves survival of mice with adrenal insufficiency during sepsis but is unhelpful for survival of mice without adrenal insufficiency. In addition, by investigating adrenal insufficiency in the “two-hit” model, we reveal that the adrenal insufficiency is associated with a reduced survival rate in secondary infection and GC supplementation has a trend to increase the survival rate of SR-BI^{-/-} mice in secondary infection in sepsis.

5.1 Adrenal-specific SR-BI-deficient mice as a model of iGC deficiency

To investigate the role of iGC in sepsis, we generated adrenal-specific SR-BI^{-/-} mice by transplanting SR-BI^{-/-} adrenal to adrenalectomized SR-BI^{+/+} mice. Some phenotypes of the adrenal-specific SR-BI^{-/-} mice have been previously studied. Hoekstra *et al.* measured the fasting plasma corticosterone level in the adrenal-specific SR-BI^{-/-} mice and showed that these mice have GC insufficiency.[198] Consistent with this study, using CLP, a surgery inducing

septic stress, we observed an impaired production of inducible corticosterone in adrenal-specific SR-BI-deficient mice.

Hoekstra *et al.* also revealed a decrease in the plasma very-low-density and low-density lipoprotein levels in atherogenic diet-fed adrenal-specific SR-BI-deficient mice, suggesting that adrenal SR-BI may play a role in modulating the lipoprotein profile.[198] However, we found that the adrenal-specific SR-BI^{-/-} mice under normal diet show no difference in the plasma lipoprotein profile compared to the control mice, indicating that adrenal SR-BI deficiency does not affect lipoproteins under normal conditions. Actually, unlike normal diet, the atherogenic diet itself can stimulate inflammation in the liver [228] and significantly increase the plasma corticosterone in mice[229]. Therefore, the changes of the lipoprotein profile in the atherogenic diet-fed adrenal-specific SR-BI-deficient mice could be a result of iGC insufficiency in the diet-induced inflammation. In addition, in their report, the authors concluded that general immune status is not affected in mice lacking adrenal SR-BI. In our study, we showed that although the basal immune status of the adrenal-specific SR-BI^{-/-} mice is generally normal, these mice are very susceptible to septic death due to the lack of iGC.

It has been reported that adrenalectomized mice have a significant increase in the number of mononuclear cells in the blood [219]. Our data show that the adrenal-specific SR-BI^{-/-} mice display normal counts of the circulating lymphocytes, neutrophils, macrophages and monocytes. Therefore, this result clearly demonstrates that the adrenal-specific SR-BI^{-/-} mice are different from the

adrenalectomized mice and implies the importance of basal GC in maintaining the homeostasis of immunity.

One limitation of the adrenal transplantation model is the cutoff of the communication between the preganglionic sympathetic neuron and the chromaffin cell in the adrenal medulla, which are responsible for the secretion of catecholamine vasopressors.[230] The lack of hemodynamic effects of catecholamine may make the mice more susceptible to sepsis. However, the control group and the experimental group were subjected to the same treatment (adrenal transplantation); therefore, the neuronal communication is similarly disrupted in adrenal-specific SR-BI^{-/-} mice and the control mice, and hence should not contribute to the differences between the adrenal-specific SR-BI^{-/-} mice and the control group in our endpoints.

It is worth noting that the response to sepsis in the adrenal specific SR-BI-deficient mice is different from that in the whole-body SR-BI-deficient (SR-BI^{-/-}) mice. Earlier work by our group showed that SR-BI^{-/-} mice display a delayed inflammatory response after CLP, as indicated by a delayed generation of pro-inflammatory cytokines and NOx, partly due to the role of SR-BI in LPS recruitment from inflammation site to circulation.[110] In the current study, the adrenal-specific SR-BI-deficient mice had a quick inflammatory response upon CLP, which implies that the delayed inflammatory response in SR-BI^{-/-} mice is not attributed to the lack of iGC. This feature of the adrenal-specific SR-BI-deficient mouse also makes it a better model to study adrenal insufficiency in sepsis than the whole-body SR-BI-deficient mouse.

5.2 GC and acute kidney injury in sepsis

Sepsis causes multi-organ dysfunction, and kidney is one of the organs frequently afflicted.[231] Acute kidney injury (AKI) occurs in about 19 % of patients with moderate sepsis, 23 % with severe sepsis and 50 % with septic shock when blood cultures are positive.[231] Patients with both sepsis and AKI have an mortality rate of 70 %, as compared with a 45 % mortality rate among patients with acute renal failure alone.[232] In clinic, AKI is diagnosed by a sudden decrease in GFR, the primary measure of kidney function, which is currently detected clinically as a rise in serum creatinine and BUN.

In animal studies, the CLP model does not develop reproducible AKI. AKI has been detected by elevation of BUN or creatinine in some [233-236] but not other studies [237]. In this study, we used BUN as the marker of AKI and showed that the adrenal-specific SR-BI^{-/-} mice, which have iGC deficiency, display an abnormally high BUN level at CLP 18 h, whereas the control mice with normal iGC production do not develop AKI in sepsis. Our data indicate that iGC protects the kidney function in sepsis and the occurrence of AKI may be associated with iGC deficiency in sepsis.

The detailed mechanism of sepsis-associated AKI is unknown. Several pathophysiological mechanisms have been proposed: vasodilation-induced glomerular hypoperfusion, dysregulated circulation within the peritubular capillary network, inflammatory reactions by systemic cytokine storm or local cytokine production, tubular dysfunction induced by oxidative stress and disseminated intravascular coagulation associated glomerular microthrombi.[238] GC has been

known to enhance the suppressor effects of catecholamines that improve the perfusion of the kidney and may also alleviate the pathological changes in renal tubules in sepsis by reducing mitochondrial damage and apoptosis [239] and prevent hypoxic injuries by suppressing iNOS activity after endotoxemia [240]. (Figure 4.2) These links between iGC and renal functions may lead future studies to understand how iGC protects from kidney injury in sepsis.

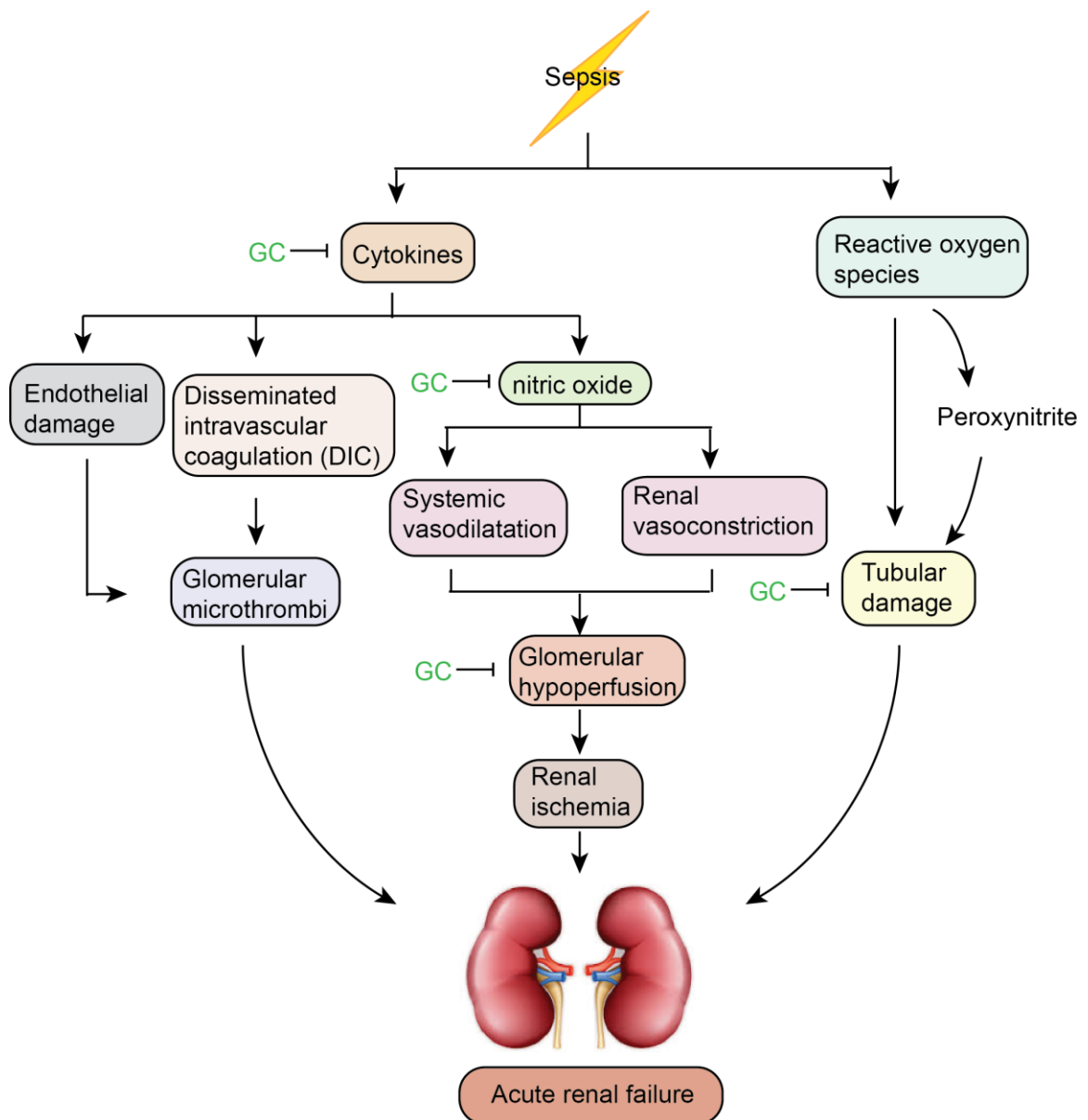


Figure 5.1 Acute kidney injury (AKI) in sepsis

Acute renal injury occurs in sepsis due to multiple factors. Sepsis induces the cytokine storm and an overwhelming production of reactive oxygen species. Cytokine storm is responsible for the endothelial damage, disseminated intravascular coagulation (DIC) and excess production of nitric oxide. Endothelial damage and DIC contribute to the formation of glomerular microthrombi. Nitric oxide leads to systemic vasodilation and simultaneously renal vasoconstriction, which together results in the glomerular hypoperfusion and renal ischemia. Reactive oxygen species can directly or indirectly damage the tubules. The glomerular microthrombi, renal ischemia and the tubular damage overall lead to the acute renal injury in sepsis.[238] GC can enhance the effect of catecholamine and therefore increase the perfusion of kidneys. GC may also inhibit cytokines and nitric oxide [239] and prevent mitochondria damage-induced apoptosis of tubules [240].

5.3 GC and cytokine production in sepsis.

The suppressive influences of GC on the synthesis of inflammatory cytokines and mediators are well documented.[101] Earlier studies showed that adrenalectomy leads to an exaggerated production of TNF- α in LPS or bacteria-induced sepsis [241, 242] and chronic replacement with a 5 mg corticosterone pellet inhibits TNF- α and IL-6 in adrenalectomized mice upon the stimulation with poly I:C, a ligand of TLR3 [243]. The mechanism by which GC inhibits inflammatory cytokine production involves multiple pathways. First, GC inhibits the activity of crucial transcriptional regulator of pro-inflammatory genes such as NF- κ B. Second, GC inhibits inflammatory gene expression at a post-transcriptional level via destabilization of mRNA or inhibition of translation. Third, the GC-induced expression of dual specificity phosphatase 1 (DUSP1) and glucocorticoid inducible leucine zipper (GILZ) can interfere with signaling pathways that are activated by pro-inflammatory stimuli and hence block pro-inflammatory gene expression.[244]

Intriguingly, our results of cytokines in the adrenal-specific SR-BI^{-/-} mouse model is different from previous studies using adrenalectomized mice or GC supplementation approaches. We provide *in vivo* evidence that iGC specifically regulates the production of IL-6 in sepsis. We show that there is no difference between the adrenal-specific SR-BI^{-/-} mice and the control mice in the plasma TNF- α level at CLP 4 h, although these mice have a significant difference in the plasma corticosterone concentrations at that time. In contrast to TNF- α , IL-6 is suppressed in the control mice but not in the adrenal-specific SR-BI^{-/-} mice in

CLP. IL-6 has a wider variety of cellular source than TNF- α which is mainly produced from macrophages. Many cells including fibroblasts, cells of the monocyte-macrophage lineage, endothelial cells and adipocytes can secrete IL-6 in inflammation.[245-247] Therefore, iGC may also exert the inhibitory effect on IL-6 production from other cell types, given the ubiquitous expression of GR. Actually, a normal down-regulation of TNF- α has been reported in mice with a dimerization-deficient glucocorticoid receptor (GR^{dim}) in LPS-induced septic shock, and the authors concluded that a classical transrepression of TNF- α by GC is fully functional in the GR^{dim} mice.[248] Based on our data, the normal regulation of TNF- α in the GR^{dim} mice could be explained by the cellular specificity of iGC action.

In addition to the different cellular sources, IL-6 presents different properties from TNF- α . TNF- α is characterized as a pro-inflammatory cytokine.[249] Nevertheless, IL-6 is a pleiotropic cytokine that plays disparate roles in inflammatory conditions depending on the infection model.[250] For example, it protects the host from death following infection since IL-6^{-/-} mice have higher mortality when infected with *Escherichia coli*, *Klebsiella pneumoniae*, or *Streptococcus pneumoniae*. [251-254] Treating mice with IL-6-blocking Abs may improve survival from CLP by reducing C5a receptor expression [255] or have no benefits [256]. A complete lack of IL-6 does not alter mortality in sepsis as indicated by no differences in the survival between IL-6 knockout mice and the wild types.[257, 258]

IL-6 is a candidate marker of sepsis mortality. As early as in 1989, it has been found that the plasma IL-6 levels in patients with sepsis are markedly increased, particularly in patients who develop a fatal septic shock.[259] Later investigations on clinical patients revealed that IL-6 appears to be a good candidate marker of severity during bacterial infection.[260, 261] In CLP models, a high IL-6 level after CLP also suggests a high mortality.[257] We show that a very significant correlation exists between the plasma corticosterone concentration and the plasma IL-6 level in CLP-induced sepsis, indicating an association between the plasma corticosterone and survival in sepsis. The point that plasma GC level is associated with survival in sepsis is also supported by our data from the SR-BI^{+/+} → SR-BI^{-/-} mice.

IL-10 is known as an important modulator in inflammation. It selectively blocks the expression of pro-inflammatory genes encoding cytokines and chemokine in myeloid cells activated by PRR ligands and simultaneously enhances the expression and production of anti-inflammatory molecules. [262, 263] It is known that GC can induce the gene expression of IL-10 as one of its anti-inflammatory actions[244]. The adrenal-specific SR-BI^{-/-} mice had the same IL-10 production as the control mice at CLP 4 h, indicating that the early production of IL-10 is not induced by iGC. However, iGC may be involved in promoting the IL-10 production in a later stage of sepsis, since we also observed higher IL-10 in the control mice than the adrenal-specific SR-BI^{-/-} mice at CLP 18 h. Because IL-10 can act at the adrenal gland and negatively regulate the corticosterone synthesis [264], the iGC-induced increase in IL-10 in a later stage

of sepsis could be a homeostatic mechanism to terminate HPA-axis activation once inflammation is resolving.

5.4 GC is not necessarily immunosuppressive.

GC has been considered as an immune-suppressive agent.[101] However, this concept has been challenged by several recent *in vitro* studies which demonstrated that GC has opposing effects on macrophage function dependent on the concentration [106, 107] and can significantly increase the phagocytic function of cultured human monocytes through regulating genes involved in the phagocytosis [108]. Our study revealed that iGC in sepsis significantly enhances the phagocytic activity of monocytes and neutrophils in blood and to some extent increases the phagocytic macrophages, neutrophils in the spleen in CLP-induced sepsis. Thus, we for the first time provide *in vivo* evidence that iGC is supportive to the functions of phagocytes in sepsis.

Synthetic GC can induce the apoptosis of lymphocytes [104] and inhibit the function of T lymphocytes [105]. Experimental and human studies suggest that the apoptosis of immune cells, in particular lymphocytes, may contribute to the immunosuppressive status in sepsis.[67, 265] We show that compared to the control mice, the adrenal-specific SR-BI^{-/-} mice display reduced lymphocyte activation in the spleen during CLP, indicating that endogenous iGC is supportive for the activation of adaptive immunity. In addition, the number of lymphocytes and apoptosis of them were not affected by iGC during sepsis. Overall, our data in adrenal-specific SR-BI^{-/-} mice show that iGC in sepsis support the function of both innate immunity and adaptive immunity.

5.5 GC therapy in sepsis

Septic patients represent an inhomogeneous group since the individual's immune response can be modulated by a variety of factors, such as the nature of the infectious stimuli, the genetic background of the host, the comorbidities, exogenous factors, etc. This heterogeneity makes it difficult for physicians and scientists to design effective therapies.[74]

In the last few decades, increasing evidence of relative adrenal insufficiency in septic shock evoked a reassessment of hydrocortisone therapy. Rather than high-dose GC supplementation used in old studies, low-dose (or stress-dose) hydrocortisone infusion has been evaluated for benefits on septic patients and is preferred as GC therapy.[169, 266] Actually, in line with our finding that iGC modulates the inflammatory responses and prevents kidney injury without suppressing the immune response in sepsis, some small clinical studies in the early 2000s provided evidence that low-dose hydrocortisone therapy may also benefit the septic patients in the same way.

First, it has been shown that stress-dose hydrocortisone infusion in septic patients decreases IL-6 levels but not TNF- α in septic shock patients [267], which indicates that low-dose GC may act in the same way as endogenous iGC to inhibit pro-inflammatory cytokines in sepsis. Second, clinical studies show that low-dose hydrocortisone improves the permeability of the glomerular endothelium and normalized free water clearance and renal sodium excretion in septic patients [268, 269], which is in consistent with the protective effect of iGC on kidney function in sepsis. Third, Keh D *et al.* showed that the infusion of low-

dose hydrocortisone does not induce severe monocyte or granulocyte dysfunction as indicated by the expression of HLA-DR on monocytes, the enhancement of monocyte phagocytosis, and only slightly depressed (2%) granulocyte phagocytosis [270], which indicate that low-dose GC is not suppressive to the innate immunity in sepsis. Finally, Hotchkiss and colleagues observed that low-dose GC treatment had no effect on the rates of lymphocyte apoptosis in severe sepsis [66] and Weber *et al.* showed that low-dose GC treatment may not influence the expression of apoptotic genes such as Bim, Bid, or Bak in severe sepsis.[271] These studies indicate that low-dose GC can exert modulating effects on immune responses that are similar to iGC in sepsis.

Although the low-dose GC treatment in sepsis showed some benefits on organ function [268, 269], the results of mortality in clinical studies remain controversial. Current guidelines from the Survival Sepsis Campaign do not recommend the diagnosis of adrenal insufficiency before the administration of GC in septic patients.[190] Our study provides evidence that GC supplementation should be given to the septic patients with adrenal insufficiency. Importantly, our data also indicate that for septic individuals without adrenal insufficiency, an additional GC supplementation maybe redundant and is completely unhelpful. Therefore, we recommend the diagnosis of adrenal insufficiency before GC supplementation in septic patients. This finding may also explain why the Surviving Sepsis Campaign showed no benefit of GC on survival of septic shock patients. In addition, the GC dose currently recommended for septic patients may be too high. Considering a factor of 1/12.3 that should be

applied when converting a drug dose from mouse to human according to the guidance from FDA, the dose of 100 µg hydrocortisone / mouse (~20g) in our study is converted to a human dose of ~61.5mg/kg, which is lower than the 200mg/kg hydrocortisone used in clinic as the “low-dose”. [272]

Older application of high-dose GC to septic patients led to an increased occurrence of secondary infection. In contrast, the recent randomized controlled HYPOLYTE study revealed that in trauma patients, the use of an intravenous stress-dose of hydrocortisone can decrease the risk of hospital-acquired pneumonia. [273] Our data in the secondary infection model show that iGC is also important to prevent secondary infection-induced death in sepsis. We did not use CLP-3 d SR-BI^{-/-} mice to investigate this issue, because CLP-3 d mice suffer from an unrecovered immune system, which makes it hard to distinguish the effect of adrenal insufficiency and that of the weak immunity. We show that after surviving the first insult of CLP-induced sepsis with the first injection of GC cocktail, the SR-BI^{-/-} mice still suffer from adrenal insufficiency in secondary infection and are very susceptible to the secondary infection-induced death. The second injection of GC cocktail in a half dose increased the survival time of SR-BI^{-/-} mice in secondary infection. The improvement in survival by the second GC injection in secondary infection is not as remarkable as that by the first injection in CLP, which may due to the lack of SR-BI in other organs and cells, such as the liver and macrophages, in the SR-BI^{-/-} mice. Therefore, it will be better to use adrenal-specific SR-BI^{-/-} mice to demonstrate the benefits of GC supplementation in the secondary infection.

In conclusion, a model of GC function and supplement stratagem in sepsis is proposed (Figure 5.2). Sepsis induced both pro-inflammatory and anti-inflammatory responses. iGC modulates the balance between anti-inflammatory and anti-inflammatory responses and thus helps to survive sepsis. For individuals without adrenal insufficiency, iGC regulates the balance and leads to survival in sepsis. Additional GC supplementation to these individuals may be unhelpful. For individuals with adrenal insufficiency, the lack of iGC leads to the loss of this balance, which may result in SIRS when the pro-inflammatory response overwhelms and CARS when the anti-inflammatory response dominants. The overwhelming cytokine storm in SIRS contributes to the septic death, while the immunosuppression in CARS can also lead to death. A proper GC supplementation can rescue the mice from systemic inflammation and survive CLP-induced sepsis. For the septic individuals that are in a status of CARS, a lower amount of GC supplementation may be helpful as well. However, further study is needed to investigate the optimal GC supplementation in CARS.

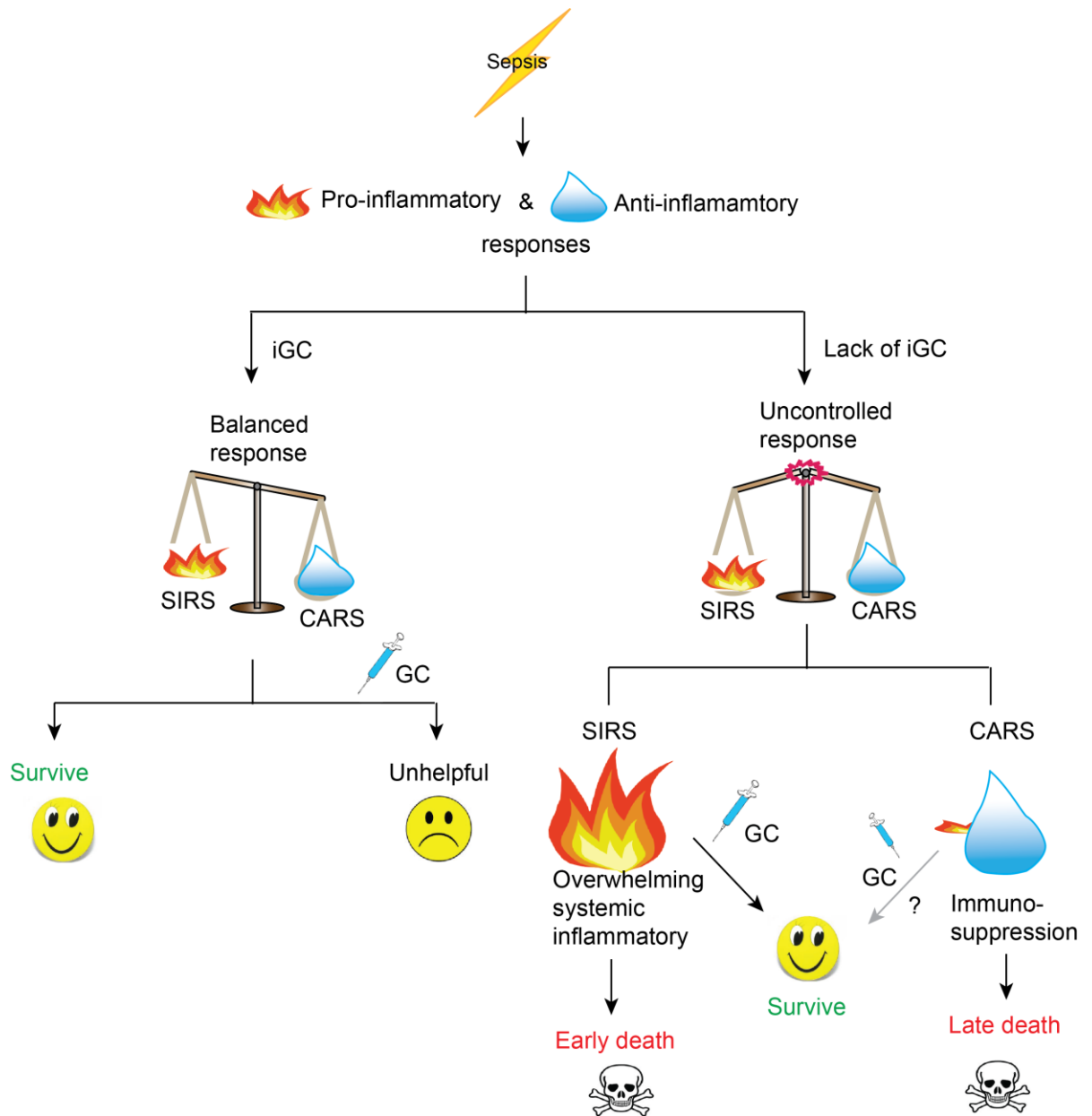


Figure 5.2 GC therapy in sepsis.

Variability in the production of GC upon sepsis influences the outcome of sepsis. Sepsis induced both pro-inflammatory and anti-inflammatory responses. iGC plays an important role in modulating the balance between pro-inflammatory and anti-inflammatory responses, like the balance of fire and water, and thus helps to survive sepsis. For individuals without adrenal insufficiency, iGC regulates the balance and leads to survive. Additional GC supplementation to these individuals may be unhelpful. For individuals with adrenal insufficiency, the lack of iGC leads to the loss of this balance, which may result in SIRS when the pro-inflammatory response overwhelms and CARS when the anti-inflammatory response dominates. The overwhelming cytokine storm contributes to the septic death in SIRS, and the immunosuppression in CARS can also lead to death. Optimal GC supplementation can help to control this balance. Our data have demonstrated that a proper GC supplementation can rescue the mice from systemic inflammation and survive CLP-induced sepsis.

Appendix

ACK lysis buffer

0.15 M NH_4Cl

10 mM KHCO_3

0.1 mM EDTA-2Na

PH=7.4

Proteinase K solution

1 M Tris-HCl PH 7.5 0.5 ml

0.1 M CaCl_2 10 ml

Glycerol 25 ml

H_2O 14.5 ml

Proteinase K 1 mg

Lysis buffer for tails for genotyping

50 mM Tris-HCl (PH 8.0)

0.45 % NP-40

RPMI 1640-5

RPMI 1640 medium 500 ml

FBS 25 ml

FACS staining buffer

DPBS 100 ml

BSA 1 g

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Publications

1. Guo L, Zheng Z, **Ai J**, Huang B, Li XA. Hepatic scavenger receptor BI protects against polymicrobial-induced sepsis through promoting LPS clearance in mice. *J Biol Chem*. 2014 Apr 9.

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Journal Articles Submitted or in Preparation

1. **Ai J**, Zheng Z, Guo L, Song Z, Howatt DA, Li Z, Daugherty A, Li XA. "Macrophage SR-BI deficiency and hypercholesterolemia cooperatively impair erythropoiesis". (Submitted to *Arterioscler Thromb Vasc Biol*., under revision)
2. **Ai J**, Zheng Z, Guo L, Li XA. "Low-dose glucocorticoid supplementation prevents septic death specifically in adrenal insufficient mice: a role of endogenous inducible glucocorticoids in sepsis". (In preparation)

Abstracts

1. **Ai J**, Zheng Z, Guo L, Song Z, Howatt DA, Li Z, Daugherty A, Li XA.

“Macrophage SR-BI deficiency and hypercholesterolemia cooperatively impair erythropoiesis”. The 16th Annual Gill Heart Institute Research Day, October 11, 2013; Lexington, KY

2. **Ai J**, Zheng Z, Guo L, Song Z, Howatt DA, Li Z, Daugherty A, Li XA.

“Hypercholesterolemia impairs erythropoiesis in an intrinsic scavenger receptor BI deficiency-dependent manner”. The Arteriosclerosis, Thrombosis and Vascular Biology 2013 Scientific Sessions, Lake Buena Vista, Florida

3. **Ai J**, Zheng Z, Feng H, Guo L, Li Z, Li XA. SR-BI deficiency and

hypercholesterolemia synergistically lead to impaired erythropoiesis. The 14th Annual Gill Heart Institute Research Day, October 21, 2011; Lexington, KY

4. **Ai J**, Zheng Z, Feng H, Guo L, Li Z, Li XA. SR-BI deficiency and

hypercholesterolemia synergistically lead to impaired erythropoiesis. The 2nd Barnstable Brown Obesity and Diabetes Research Day, May 14, 2012; Lexington, KY