Scavenger Receptor BI and High-Density Lipoprotein Regulate Thymocyte Apoptosis in Sepsis

Ling Guo, Zhong Zheng, Junting Ai, Deborah A. Howatt, Paul R. Mittelstadt, Seth Thacker, Alan Daugherty, Jonathan D. Ashwell, Alan T. Remaley, Xiang-An Li

Objective—Thymocyte apoptosis is a major event in sepsis; however, how this process is regulated remains poorly understood.

Approach and Results—Septic stress induces glucocorticoids production which triggers thymocyte apoptosis. Here, we used scavenger receptor BI (SR-BI)–null mice, which are completely deficient in inducible glucocorticoids in sepsis, to investigate the regulation of thymocyte apoptosis in sepsis. Cecal ligation and puncture induced profound thymocyte apoptosis in SR-BI+/+ mice, but no thymocyte apoptosis in SR-BI−/− mice because of lack of inducible glucocorticoids. Unexpectedly, supplementation of glucocorticoids only partly restored thymocyte apoptosis in SR-BI−/− mice. We demonstrated that high-density lipoprotein (HDL) is a critical modulator for thymocyte apoptosis. SR-BI+/+ HDL significantly enhanced glucocorticoid-induced thymocyte apoptosis, but SR-BI−/− HDL had no such activity. Further study revealed that SR-BI+/+ HDL modulates glucocorticoid-induced thymocyte apoptosis via promoting glucocorticoid receptor translocation, but SR-BI−/− HDL loses such regulatory activity. To understand why SR-BI−/− HDL loses its regulatory activity, we analyzed HDL cholesterol contents. There was 3-fold enrichment of unesterified cholesterol in SR-BI−/− HDL compared with SR-BI+/+ HDL. Normalization of unesterified cholesterol in SR-BI−/− HDL by probucol administration or lecithin cholesteryl acyltransferase expression restored glucocorticoid-induced thymocyte apoptosis, and incorporating unesterified cholesterol into SR-BI+/+ HDL rendered SR-BI−/− HDL dysfunctional. Using lckCre-GRflox/flox mice in which thymocytes lack cecal ligation and puncture–induced thymocyte apoptosis, we showed that lckCre-GRflox/flox mice were significantly more susceptible to cecal ligation and puncture–induced septic death than GRflox/flox control mice, suggesting that glucocorticoid-induced thymocyte apoptosis is required for protection against sepsis.

Conclusions—The findings in this study reveal a novel regulatory mechanism of thymocyte apoptosis in sepsis by SR-BI and HDL. (Arterioscler Thromb Vasc Biol. 2014;34:966-975.)

Key Words: apolipoproteins □ apoptosis □ lipoproteins □ scavenger receptors, class B □ sepsis

Sepsis is a major health issue which claims >215,000 lives and costs $16.7 billion per year in America alone.1 The death rate of sepsis is high, exceeding 30%, because of the poor understanding of the disease. Sepsis induces profound lymphocyte apoptosis,2 which keeps adaptive immunity in check, but excessive lymphocyte apoptosis causes immunosuppression in the later stage of sepsis, resulting in susceptibility to secondary infections. Thymocyte apoptosis is a major mechanism of lymphocyte depletion in sepsis; however, how this process is regulated remains poorly understood.2-4 Scavenger receptor BI (SR-BI or Scarb1) is a well-characterized high-density lipoprotein (HDL) receptor mainly expressed in the liver and steroidogenic tissues.5-11 It regulates HDL cholesterol content through reverse cholesterol transport in which SR-BI selectively uptakes cholesteryl ester from HDL.9,10 Despite a 2-fold increase in plasma HDL cholesterol concentrations, SR-BI−/− mice are susceptible to atherosclerosis.12-16 Several earlier studies have shown that SR-BI deficiency leads to larger HDL with a profound increase in unesterified cholesterol concentrations on HDL,17-20 which may render HDL dysfunctional in SR-BI−/− mice.21-24 In support of this notion, an earlier study showed that HDL from SR-BI−/− mice loses activity of selective cholesteryl ester uptake25; several reports suggest that accumulation of unesterified cholesterol on HDL contributes to female infertility and disrupts red blood cell development26-24; we recently reported that HDL from SR-BI−/− mice also loses activity of suppressing lymphocyte proliferation.25 Although it has been recognized for years that HDL has immune regulatory activity, how
HDL regulates immunity and the role of SR-BI in the process remain largely unknown.

In this study, we report a novel function of HDL, namely, modulation of thymocyte apoptosis in sepsis. We demonstrate that SR-BI+/+ HDL enhances glucocorticoid-induced thymocyte apoptosis via promoting glucocorticoid receptor translocation, but SR-BI−/− HDL loses such regulatory activity. We further demonstrate that excessive accumulation of unesterified cholesterol on SR-BI−/− HDL likely renders it dysfunctional. Our findings reveal a novel regulatory mechanism of thymocyte apoptosis by SR-BI and HDL in sepsis.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>7-AAD</td>
<td>7-aminoactinomycin D</td>
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<tr>
<td>CLP</td>
<td>cecal ligation and puncture</td>
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<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
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<tr>
<td>iGC</td>
<td>inducible glucocorticoid</td>
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<td>LCAT</td>
<td>lecithin cholesterol acyltransferase</td>
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<td>LDL</td>
<td>low-density lipoprotein</td>
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<td>SR-BI</td>
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Results

SR-BI Is a Key Determinant of Thymocyte Apoptosis in Sepsis

We induced sepsis with cecal ligation and puncture (CLP) in SR-BI+/+ and SR-BI−/− mice and assessed thymocyte apoptosis 18 hours after CLP. Compared with sham, CLP caused significant thymic involution in SR-BI+/+ mice, as shown by a 80% reduction in thymus cell number, but only a slight decrease in thymus cell number in SR-BI−/− mice (Figure 1A). Trypan blue assay revealed a marked thymocyte death in CLP–SR-BI+/+ mice, but much less thymocyte death in CLP–SR-BI−/− mice (Figure 1B). Clear DNA ladders were observed in CLP–SR-BI+/+ mice, but not in CLP–SR-BI−/− mice (Figure 1C). Fluorescence-activated cell sorter analyses indicated profound thymocyte apoptosis in CLP–SR-BI+/+ mice as shown by 22% TUNEL+ thymocytes, whereas thymocyte apoptosis was almost completely absent in CLP–SR-BI−/− mice (Figure 1D and 1E). Hematoxylin and eosin staining showed significant apoptotic cell death in the thymic cortex of CLP–SR-BI+/+ mice, but no apoptotic cell death in CLP–SR-BI−/− mice (Figure 1F and Figure I in the online-only Data Supplement). Taken together, these findings demonstrate SR-BI as a key determinant of thymocyte apoptosis in sepsis.

Figure 1. Scavenger receptor BI (SR-BI) is a key determinant of thymocyte apoptosis in sepsis. SR-BI+/+ and SR-BI−/− mice were subjected to cecal ligation and puncture (CLP) or sham (22-gauge needle, full ligation) for 18 hours, and thymocyte apoptosis was analyzed. A, Changes in thymocyte number. B, Trypan blue assay. C, DNA ladder assay. D and E, TUNEL assay. n=7 to 14 per group. F, Hematoxylin and eosin staining of thymus cortex. Arrows indicate apoptotic cells with fragmented nuclei (representative data; also see Figure I in the online-only Data Supplement). **P<0.01.
SR-BI Controls Inducible Glucocorticoid Generation in Response to Septic Stress, Which Is a Determinant of Thymocyte Apoptosis in Sepsis

In the early stage of sepsis, adrenals produce high levels of glucocorticoids that trigger thymocyte apoptosis.2–4 SR-BI is highly expressed in the adrenals. As an HDL receptor, SR-BI mediates the uptake of cholesteryl ester from HDL for glucocorticoid synthesis, and several studies have shown that SR-BI+/+ mice have impaired glucocorticoid production in response to stress.26–28 To understand how SR-BI regulates thymocyte apoptosis, we measured plasma corticosterone concentrations in the early stages of sepsis. As early as 2 hours after CLP challenge, SR-BI+/+ mice produced high concentrations of corticosterone, but SR-BI−/− mice failed to upregulate glucocorticoids in response to septic stress (Figure 2A), which is consistent with our earlier report.26

Given the essential role of glucocorticoids in triggering thymocyte apoptosis,2–4 we reasoned that the lack of SR-BI–mediated inducible glucocorticoid (iGC) generation accounts for the impaired thymocyte apoptosis in sepsis. To test this, we administered dexamethasone to SR-BI+/+ and SR-BI−/− mice, expecting that supplementation of glucocorticoids would restore glucocorticoid-induced apoptosis in SR-BI−/− mice. Dexamethasone administration induced profound thymocyte apoptosis in SR-BI+/+ mice as shown by DNA ladders and a marked increase in TUNEL+ cell percentage (Figure 2B and 2C). Unexpectedly, dexamethasone administration only partly restored thymocyte apoptosis in SR-BI+/+ mice as shown by a lack of clear DNA ladders and 2-fold fewer TUNEL+ cell percentages in SR-BI+/+ mice (Figure 2B and 2C). Taken together, these findings not only indicate that SR-BI controls iGC generation in sepsis, but also imply that SR-BI modulates thymocyte apoptosis via an unidentified mechanism in addition to simply controlling iGC generation.

SR-BI Regulates Thymocyte Apoptosis Independent of Its Expression on Thymocytes

SR-BI is moderately expressed in the thymus (Figure IIA in the online-only Data Supplement). Our earlier study demonstrated that SR-BI induces apoptosis on serum depletion in vitro.29 This raised the possibility that SR-BI may regulate thymocyte apoptosis intrinsically. To test this, we isolated thymocytes from SR-BI+/+ and SR-BI−/− mice, incubated the cells with corticosterone, and monitored cell death. SR-BI+/+ and SR-BI−/− thymocytes displayed similar cell death in response to corticosterone or dexamethasone (Figure IIB and IIC in the online-only Data Supplement). To exclude an intrinsic effect in vivo, we conducted bone marrow transplantation by transferring SR-BI+/+ or SR-BI−/− bone marrow–derived cells into Rag-1−/− mice. Rag-1−/− mice are deficient in CD4+CD8+ (DP), CD4+CD8−, and CD4−CD8+ (SP) thymocytes (Figure IIIA in the online-only Data Supplement). Six weeks after bone marrow transplantation, the Rag-1−/− mice were populated with thymocytes derived from donor mice as shown by the presence of DP and SP thymocytes (Figure IIIA in the online-only Data Supplement). We then conducted CLP on these mice. As shown in Figure IIB and IIC in the online-only Data Supplement, CLP induced similar thymocyte apoptosis in mice receiving either SR-BI+/+ or SR-BI−/− bone marrow–derived cells and depleted DP thymocytes equally well in these mice. These findings indicate that SR-BI does not have an intrinsic effect on glucocorticoid-induced thymocyte apoptosis.

HDL Enhances Glucocorticoid-Induced Thymocyte Apoptosis, but HDL From SR-BI−/− Mice Loses Such Regulatory Activity

SR-BI plays an essential role in HDL metabolism. SR-BI−/− mice display abnormal HDL as shown by larger HDL particles associated with a 2-fold increase in HDL cholesterol content.17–20 We hypothesized that HDL modulates glucocorticoid-induced thymocyte apoptosis and SR-BI modulates glucocorticoid-induced thymocyte apoptosis via its effect on HDL. To test this hypothesis, we isolated HDL from SR-BI+/+ and SR-BI−/− mice and assessed its effect on glucocorticoid-induced thymocyte apoptosis. We incubated thymocytes isolated from SR-BI+/+ mice with corticosterone in the presence of SR-BI+/+ or SR-BI−/− HDL and quantified thymocyte apoptosis with 7-aminoactinomycin D (7-AAD) staining. As shown in Figure 3A and 3B, corticosterone induced significantly thymocyte apoptosis compared with noncorticosterone treatment in the absence of HDL. Interestingly, HDL from SR-BI−/− mice significantly enhanced
corticosterone-induced thymocyte apoptosis, but HDL from SR-BI−/− mice suppressed corticosterone-induced thymocyte apoptosis (Figure 3A and 3B). We further tested the dose effect of HDL and found that SR-BI+/+ HDL promotes corticosterone-induced thymocyte apoptosis, but SR-BI−/− HDL suppressed corticosterone-induced thymocyte apoptosis in a dose-dependent manner (Figure IV in the online-only Data Supplement). In vitro cell apoptosis usually undergoes secondary necrosis because of lack of phagocyte-mediated engulfment of the apoptotic cells. Indeed, we detected a significant increase in necrotic cell death (Figure 3C). We also used Trypan blue exclusion to test the effect of...
HDL on thymocyte cell death and obtained similar findings (Figure 3D). These data suggest that normal HDL promotes glucocorticoid-induced thymocyte apoptosis, but SR-BI−/− HDL loses this regulatory function. Similar results were obtained with dexamethasone incubations (data not shown).

**Human HDL Enhances Glucocorticoid-Induced Thymocyte Apoptosis**

We isolated HDL from human serum and tested its effect on corticosterone-induced thymocyte apoptosis. Similar to normal mouse HDL, human HDL significantly enhanced glucocorticoid-induced thymocyte apoptosis as shown by 7-AAD staining (Figure 3E–3G) and Trypan blue exclusion (Figure 3H) assays.

**HDL Enhances Glucocorticoid-Induced Thymocyte Apoptosis via Promoting GR Translocation, but HDL From SR-BI−/− Mice Loses Such Regulatory Activity**

On entering cells, glucocorticoid binds to GR, resulting in GR translocation from cytosol to the nucleus, where GR functions as transcriptional factor to activate apoptotic signaling. To further understand the molecular mechanisms by which HDL modulates glucocorticoid–GR apoptotic signaling, we tested the effects of HDL on GR translocation using L929 cells, a cell line widely used for glucocorticoid–GR signaling.32 As shown in Figure 3I, GR moved rapidly from cytosol to the nucleus in response to dexamethasone incubation. Importantly, HDL from SR-BI−/− mice markedly enhanced glucocorticoid-induced GR translocation, but HDL from SR-BI−/− mice suppressed glucocorticoid-induced GR translocation.

**Deficiency of HDL In Vivo Impairs Thymocyte Apoptosis in Sepsis**

Apoprotein A1 (apoAI−/−) mice are grossly deficient in HDL (Figure 4A)33 and thus have been used as an HDL-deficient model to determine the roles of HDL in vivo.34–36 We used apoAI−/− mice to elucidate the contribution of HDL to thymocyte apoptosis in vivo. On CLP, apoAI−/− mice displayed a 2-fold increase in the percentage of TUNEL+ thymocytes (Figure 4B) and less depletion in DP thymocytes compared with C57BL/6J mice (Figure 4C). An earlier study indicated that apoAI−/− mice have impaired glucocorticoid production in response to stress, which raised the possibility that apoAI−/− mice might produce less glucocorticoids in sepsis.37

To exclude such a possible effect, we induced thymocyte apoptosis by administering dexamethasone to apoAI−/− and C57BL/6J mice. As shown in Figure 4D, dexamethasone-treated apoAI−/− mice had significantly fewer TUNEL+ thymocytes than dexamethasone-treated C57BL/6J mice. Thus, these observations provided in vivo evidence that HDL modulates glucocorticoid-induced thymocyte apoptosis.

**Normalization of the Unesterified Cholesterol Content in SR-BI−/− HDL Restores Its Regulatory Activity of Modulating Glucocorticoid-Induced Thymocyte Apoptosis**

SR-BI deficiency leads to larger HDL with a profound increase in unesterified cholesterol ratio of HDL.18–20 We analyzed the cholesterol content of these HDL particles. Consistent with the earlier reports,18–20 we observed a 3-fold increase in unesterified cholesterol content in HDL, but only marginally increasing in the cholesteryl ester concentrations (Figure 5A, normal diet). The major form of cholesterol in SR-BI−/− HDL particles is cholesteryl ester, which is located in the inner core of HDL because of its hydrophobic nature. In contrast, unesterified cholesterol is more hydrophilic and polar and therefore is located on the surface of HDL.32 Given that earlier studies have shown that the accumulation of unesterified cholesterol in circulation causes female infertility and abnormal red blood cell develops in SR-BI−/− mice,21–24 it is likely that the massive accumulation of unesterified cholesterol in SR-BI−/− HDL disrupts the structure of HDL, leading to its dysfunction on regulating thymocyte apoptosis. To test this speculation in vivo, we administered SR-BI−/− and SR-BI−/− mice with probucol, a cholesterol-lowering drug that has been shown to normalize unesterified cholesterol levels in SR-BI−/− mice.21–24 As shown in Figure 5A (probulcol diet), probucol administration normalized the unesterified cholesterol content of SR-BI−/− HDL to almost normal levels. When we administered dexamethasone to the probucol-treated mice, we observed a 2-fold increase in the percentage of TUNEL+ thymocytes.
thymocytes in probucol-treated SR-BI−/− mice compared with untreated SR-BI−/− mice (Figure 5B).

We then tested the ability of HDL isolated from the probucol-treated mice to modulate corticosterone-induced thymocyte death. As shown in Figure 5C, HDL isolated from probucol-treated SR-BI−/− mice increased the corticosterone-induced thymocyte death by 3-fold compared with HDL isolated from non–probucol-treated SR-BI−/− mice. Thus, both in vivo and in vitro data indicated that correcting the unesterified cholesterol content of SR-BI−/− HDL restores its ability to modulate thymocyte apoptosis in sepsis.

Incorporation of Free Cholesterol to Normal HDL Renders the HDL Dysfunctional in Modulating Glucocorticoid-Induced Thymocyte Apoptosis

Probucol has functions other than lowering plasma cholesterol. To further support our hypothesis, we took an independent approach by incorporating free cholesterol into SR-BI−/− HDL following a method described by Cooper et al.38 Compared with control HDL that was treated with dipalmitoyl lecithin alone, the HDL treated with free cholesterol/dipalmitoyl lecithin had 3-fold enriched unesterified cholesterol content and a significant increase in the unesterified cholesterol to cholesteryl ester ratio (Figure 6A).

We tested the effect of this free cholesterol–enriched HDL on glucocorticoid-induced thymocyte apoptosis. The control HDL promoted glucocorticoid-induced thymocyte apoptosis by 2-fold as shown by 7-AAD stainings (Figure 6B and 6C). However, the free cholesterol–enriched HDL did not promote glucocorticoid-induced thymocyte apoptosis, as shown by only 37% positive 7-AAD stainings in free cholesterol–enriched HDL, versus 70% positive 7-AAD stainings in control HDL (Figure 6B and 6C). Similar findings were obtained with Trypan blue exclusion assay (Figure 6D). There was no significant difference in glucocorticoid-induced thymocyte apoptosis between SR-BI−/− HDL without dipalmitoyl lecithin treatment and control SR-BI−/− HDL treated with dipalmitoyl lecithin (Figures 3 and 6). Of note, we observed that the SR-BI−/− HDL strongly inhibited glucocorticoid-induced thymocyte apoptosis (Figure 3), but the free cholesterol–enriched HDL neither promoted nor inhibited glucocorticoid-induced thymocyte apoptosis (Figure 6). This difference was likely caused by a higher unesterified cholesterol concentration and higher unesterified cholesterol to cholesteryl ester ratio in SR-BI−/− HDL compared with the free cholesterol–enriched HDL.
Lecithin Cholesteryl Acyltransferase Corrects the Unesterified Cholesterol Content of SR-BI−/− HDL and Restores Its Regulatory Activity on Modulating Glucocorticoid-Induced Thymocyte Apoptosis

An earlier study showed that SR-BI−/− mice have a 90% decrease in endogenous lecithin cholesteryl acyltransferase (LCAT) activity on HDL, which may explain why SR-BI−/− HDL has accumulation of unesterified cholesterol. To further test whether accumulation of unesterified cholesterol on SR-BI−/− HDL renders it dysfunctional, we isolated HDL from LCAT transgenic mice in SR-BI+/+ or SR-BI−/− background (SR-BI−/−LCAT-tg). Overexpression of LCAT increased the total cholesterol levels in both SR-BI+/+LCAT-tg HDL and SR-BI−/−LCAT-tg HDL and markedly normalized the unesterified cholesterol ratio in SR-BI−/−LCAT-tg HDL compared with SR-BI−/− HDL (0.29, SR-BI−/−LCAT-tg HDL versus 0.59, SR-BI−/− HDL; see Table I in the online-only Data Supplement). Importantly, SR-BI−/−LCAT-tg HDL displayed much higher activity on glucocorticoid-induced thymocyte apoptosis compared with SR-BI−/− HDL (Figure 7A–7C).

Collectively, these in vivo and in vitro findings indicate that normal HDL enhances glucocorticoid-induced thymocyte apoptosis through promoting GR translocation, but SR-BI−/− HDL loses this regulatory activity, likely because of disruption of its structure as a result of the accumulation of unesterified cholesterol. To the best of our knowledge, this is the first evidence demonstrating that HDL regulates thymocyte apoptotic signaling in sepsis.

Mice Deficient in Glucocorticoid–GR Signaling in Thymocytes/T Cells Are Susceptible to CLP-Induced Septic Death

We asked whether a deficiency of SR-BI–mediated glucocorticoid–GR signaling in thymocyte/T cells contributes to septic death. Because deficiency of SR-BI–mediated glucocorticoid production will affect glucocorticoid–GR signaling in all types of cells, and glucocorticoid–GR signaling has been shown to play protective roles in macrophages and endothelial cells in sepsis, we used lckCre-GR fl/fl mice, a model in which glucocorticoid–GR signaling is deficient only in thymocytes/T cells. As expected, lckCre-GR fl/fl mice were completely resistant to CLP-induced thymocyte apoptosis (Figure 8A and 8B). Importantly, lckCre-GR fl/fl mice were significantly more susceptible to CLP-induced septic death than GR fl/fl control mice (Figure 8C), indicating that the glucocorticoid–GR signaling in thymocytes/T cells is critical for protection against sepsis.

Discussion

The findings in this study reveal a previously unrecognized regulatory mechanism of thymocyte apoptosis in sepsis. We
Hormone.43 Furthermore, heterozygous carriers of LDL receptor null displayed normal iGC production. However, patients with homozygous familial hypercholesterolemia (LDL receptor null) displayed normal iGC pathway. This raises a possibility that human mouse mainly has HDL, but human has both low-density lipoprotein (LDL) and HDL. This implies that SR-BI modulates thymocyte apoptosis and that SR-BI modulates glucocorticoid-induced thymocyte apoptosis through HDL. Our findings suggest that HDL profoundly enhances glucocorticoid-induced thymocyte apoptosis likely through promoting glucocorticoid-induced GR translocation. Interestingly, HDL from SR-BI−/− mice lost this regulatory function. Importantly, human HDL significantly enhanced glucocorticoid-induced thymocyte apoptosis. To determine the importance of HDL in modulating thymocyte apoptosis in vivo, we used apoAI−/− mice that are grossly deficient in HDL. As expected, mice deficient in HDL displayed impaired thymocyte apoptosis in sepsis, supporting our hypothesis.

**Accumulation of Unesterified Cholesterol on SR-BI−/− HDL Renders It Dysfunctional**

Nascent HDL acquires free cholesterol from peripheral tissues, and LCAT catalyzes the free cholesterol to form cholesteryl ester, which is a critical step for HDL metabolism. Interestingly, an earlier study showed that SR-BI−/− mice have a 90% decrease in endogenous LCAT activity in HDL.20 Thus, it is plausible that SR-BI−/− HDL would accumulate unesterified cholesterol because of the lack of LCAT activity. Indeed, in agreement with earlier reports,18–20 we observed a 3-fold increase in unesterified cholesterol contents of SR-BI−/− HDL compared with SR-BI+/+ HDL, but the cholesteryl ester levels of SR-BI−/− HDL were only minimally elevated compared with SR-BI+/+ HDL. Based on the difference in hydrophobic nature between unesterified cholesterol and cholesteryl ester,22 we proposed that SR-BI−/− HDL loses its regulatory activity on glucocorticoid-induced thymocyte apoptosis because of disruption of HDL structure as a result of the accumulation of unesterified cholesterol on the surface of HDL. To test this hypothesis, we took 3 independent approaches. First, we administered probucol to SR-BI−/− mice. Probucol normalized the unesterified cholesterol level of SR-BI−/− HDL; importantly, probucol effectively restored glucocorticoid-induced thymocyte apoptosis both in vivo and in vitro. Second, we incorporated free cholesterol to SR-BI−/− HDL. We found that this free cholesterol–enriched HDL loses its ability to promote glucocorticoid-induced thymocyte apoptosis. Finally, we isolated HDL from SR-BI−/−/LCAT-tg mice. The overexpression of LCAT in SR-BI−/− mice normalized the unesterified cholesterol contents in HDL and partly restored the activity of HDL on glucocorticoid-induced thymocyte apoptosis.

Although clinical studies have clearly established a negative correlation between plasma HDL cholesterol levels and cardiovascular diseases,16,47 there is growing appreciation that elevation in HDL Is a Critical Modulator of Glucocorticoid-Induced Thymocyte Apoptosis

When we administered glucocorticoid to SR-BI−/− mice, we unexpectedly found that supplementation of dexamethasone only partly restored thymocyte apoptosis in SR-BI−/− mice. This implies that SR-BI modulates thymocyte apoptosis via a secondary mechanism in addition to controlling iGC generation.

By excluding intrinsic effect of SR-BI, we tested our hypothesis that HDL is a modulator of glucocorticoid-induced thymocyte apoptosis and that SR-BI modulates glucocorticoid-induced thymocyte apoptosis through HDL. Our findings suggest that HDL profoundly enhances glucocorticoid-induced thymocyte apoptosis likely through promoting glucocorticoid-induced GR translocation. Interestingly, HDL from SR-BI−/− mice lost this regulatory function. Importantly, human HDL significantly enhanced glucocorticoid-induced thymocyte apoptosis. To determine the importance of HDL in modulating thymocyte apoptosis in vivo, we used apoAI−/− mice that are grossly deficient in HDL. As expected, mice deficient in HDL displayed impaired thymocyte apoptosis in sepsis, supporting our hypothesis.

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Although clinical studies have clearly established a negative correlation between plasma HDL cholesterol levels and cardiovascular diseases,16,47 there is growing appreciation that elevation in
HDL cholesterol levels may not always be beneficial and may even be deleterious.\textsuperscript{48-51} Indeed, SR-BI\textsuperscript{f/f} mice have a 2-fold increase in plasma HDL cholesterol concentrations, but are susceptible to atherosclerosis and other cardiovascular diseases,\textsuperscript{12-16} suggesting that a defect in SR-BI-mediated HDL metabolism may render HDL dysfunctional. Here, using 3 independent approaches, we demonstrated that the massive accumulation of unesterified cholesterol on SR-BI\textsuperscript{f/f} HDL likely accounts for loss of its regulatory activity on glucocorticoid-induced thymocyte apoptosis. Just as massive accumulation of unesterified cholesterol on HDL likely disrupts the structure of HDL, it may also impair the other functions of HDL, such as the selective cholesterol ester uptake. Further study is warranted to test this speculation.

Glucocorticoid–GR Signaling in Thymocytes/T Cells Is Required for Protection Against CLP-Induced Septic Death

As opposed to normal physiological conditions in which only \(\approx 1\%\) of thymocytes undergo apoptosis, in sepsis, thymocyte apoptosis is increased by 10- to 20-fold, which indicates a marked increase in thymocyte stress during sepsis. Whether this massive apoptotic cell death is simply a passive response to septic stress or a protective mechanism remains an open question. Using lckCre-GR\textsuperscript{fl/fl} mice in which thymocytes lack the GR, Mittelstadt et al\textsuperscript{52} demonstrated that glucocorticoid–GR signaling is required for proper selection of thymocytes and absence of glucocorticoid–GR signaling in thymocytes impairs thymocyte development, resulting in generation of functionally compromised T cells. Considering that thymocyte apoptosis is a major mechanism of thymocyte selection, we speculate that SR-BI and HDL-regulated glucocorticoid–GR signaling is required for the removal of the stressed thymocytes and a failure in this process impairs the development of thymocytes, resulting in functionally compromised T cells. We conducted CLP on lckCre-GR\textsuperscript{fl/fl} mice. The lckCre-GR\textsuperscript{fl/fl} mice were completely resistant to CLP-induced thymocyte apoptosis and significantly more susceptible to CLP-induced septic death compared with GR\textsuperscript{fl/fl} littermates, which supports apoptosis and significantly more susceptible to CLP-induced sepsis.

In summary, this study identifies SR-BI and HDL as key regulators of glucocorticoid–GR signaling in thymocytes in sepsis. As shown in Figure VI in the online-only Data Supplement, (1) in adrenals, SR-BI mediates the production of iGC which induces thymocyte apoptosis; (2) in thymus, HDL enhances glucocorticoid-induced thymocyte apoptosis likely via promoting GR nuclear relocation; (3) lack of SR-BI–mediated reverse cholesterol transport in liver causes accumulation of free cholesterol on HDL that renders it inactive on thymocyte apoptosis; and (4) the SR-BI and HDL-regulated glucocorticoid–GR signaling is likely required for removal of stressed thymocytes, proper selection of thymocyte, and protection against sepsis.

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Disclosures

None.

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**Significance**

Thymocyte apoptosis is an major event in sepsis; however, how this process is regulated remains poorly understood. In this study, we identify scavenger receptor BI (SR-BI) and high-density lipoprotein (HDL) as key regulators in this process. We found that mice deficient in SR-BI are completely resistant to sepsis-induced thymocyte apoptosis. We demonstrated that SR-BI determines thymocyte apoptosis through controlling inducible glucocorticoid production; we further demonstrated that HDL modulates glucocorticoid-induced thymocyte apoptosis through promoting glucocorticoid receptor translocation, but HDL from SR-BI–null mice loses such regulatory activity completely. We demonstrated that accumulation of unesterified cholesterol on HDL from SR-BI–null mice renders it dysfunctional. The SR-BI and HDL-regulated glucocorticoid–GR signaling is likely required for removal of stressed thymocytes, proper selection of thymocyte, and protection against sepsis.
Scavenger Receptor BI and High-Density Lipoprotein Regulate Thymocyte Apoptosis in Sepsis
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Materials and Methods

Scavenger receptor BI and HDL regulate thymocyte apoptosis in sepsis

Ling Guo1, Zhong Zheng1, 2, Junting Ai1, 2, Deborah A. Howatt3, Paul R. Mittelstadt4, Seth Thacker5, Alan Daugherty3, Jonathan D. Ashwell4, Alan T. Remaley5 and Xiang-An Li1, 2, 3, #

1Department of Pediatrics, 2Graduate Center for Nutritional Sciences and 3Saha Cardiovascular Research Center, University of Kentucky College of Medicine, Lexington, KY 40536. 4 Laboratory of Immune Cell Biology, NCI and 5Lipoprotein Metabolism Section, NHLBI, NIH, Bethesda, MD 20892.

Materials- Anti-GR (M-20) and anti-SP1 antibodies were from Santa Cruz; Water soluble dexamethasone (Dex), water soluble corticosterone, probucol, L-α-dipalmitory lecithin, cholesterol and Trypan Blue were from Sigma. TUNEL and apoptotic DNA ladder kits were from Roche. Antibodies for flow cytometry analysis were described previously 1.

Animals - SR-BI+/− on B6/129 mixed background (B6;129S2-Scarb1tm1Kri/J) were from the Jackson Laboratory. SR-BI−/− mice were generated by breeding SR-BI+/− mice, and SR-BI+/+ littermates were used as controls. ApoAI−/− and Rag-1−/− (in C57BL/6J background), and C57BL/6J mice were from the Jackson Laboratory, and bred as homozygous. LCAT transgenic mice overexpressing human LCAT were originally generated by Vaisman et al2. The mice were bred with SR-BI−/− mice to generate SR-BI+/−LCATtg and SR-BI−/−LCATtg mice (on mixed background). T cell specific GR knockout mice (lckCre-GRfl/fl) were obtained from Dr Ashwell 3, and the GRfl/fl littermates were used as control. Tail DNA was used for PCR genotyping. The animals were fed a normal rodent diet (0.015% wt/wt cholesterol, 5.7% wt/wt fat, Harlan Tekland 2018). For probucol administration, the animals were fed with 0.2% probucol supplemented to the normal rodent diet (probucol was dissolved in ethanol, sprayed on rodent diet pellets and dried thoroughly in a chemical hood). Animal care and experiments were approved by the Institutional Animal Care and Use Committee of the University of Kentucky. Both male and female mice were used.

CLP septic animal model - CLP was performed as we previously described 4.

Bone marrow transplantation - This procedure was performed as described previously 5. Briefly, Rag-1−/− mice were maintained on antibiotic water (sulfatrim, 4μg/mL) for 6-day and irradiated with a total of 900 Rads from a cesium source that was delivered in two doses within 4 hours. Bone marrow-derived cells from SR-BI−/− or SR-BI+/+ mice were obtained from the femurs of donor mice and were injected into the tail vein of 8-week-old irradiated recipient mice at 5×10^6 cells per mouse. Mice were maintained on antibiotic water for 4 weeks after irradiation and placed on regular water for 2 weeks before use.
Fluorescence-activated cell sorting (FACS) analysis- Single-cell suspensions from thymi or spleens were prepared and analyzed as previously described.1

Thymocyte apoptosis in sepsis – Sepsis-induced thymocyte apoptosis was assessed with 4 independent methods- DNA ladder assay, TUNEL staining, Trypan Blue exclusion and histological staining. Briefly, adult mice were subjected to CLP for 18 h and the thymi were harvested. For DNA ladder assay, the DNA ladder was isolated using an apoptotic DNA ladder assay kit and analyzed with 1% agarose gel electrophoresis as described previously.6 For TUNEL staining, single cell suspensions were prepared and the apoptotic cells were quantified with flow cytometry as described previously. 7 The Trypan Blue exclusion analysis and histological staining were done using standard techniques.

GC-induced thymocyte apoptosis – For in vivo assay, Dex was administered to mice at a dose of 8 mg/kg, i.p. After 18 h, thymocytes were isolated and subjected to apoptotic assays. For in vitro assay, thymocytes from mice were cultured in complete RPMI 1640 medium containing 10 µM corticosterone in a CO2 incubator at 37 °C for 18 h, and the cell apoptosis and total cell death were analyzed by 7-aminoactinomycin D (7-AAD) staining and Trypan Blue exclusion. Of note, we used different reagents for in vitro and in vivo assays. 1) We used 7-AAD staining to replace TUNEL assay because in vitro apoptosis usually undergoes "secondary necrosis" due to lack of phagocytosis of the apoptotic cells8,9, which cannot be detected by TUNEL assay; 2) we used Dex to induce thymocyte apoptosis in vivo but used corticosterone to induce thymocyte apoptosis in vitro. This was because the differential sensitivity of thymocyte to GC in vivo and in vitro. The primary thymocytes are very sensitive to GC in vitro; thus, we preferred to use corticosterone, an endogenous and less potent GC, for in vitro assay (similar data were obtained with Dex for in vitro assay). Thymocytes are less sensitive to GC-induced apoptosis in vivo than in vitro; thus, we used Dex, a much potent reagent than corticosterone, to induce thymocyte apoptosis in vivo.

Analysis of lipoprotein profiles by fast protein liquid chromatography (FPLC) - Plasma lipoprotein profiles were determined with FPLC as previously described.4

Isolation of mouse and human HDL - Mouse plasma (1.5 ml) pooled from 4-6 mice or human plasma was subjected to sequential gradient centrifugation using Optima MAX Ultracentrifuge (Beckman) as described previously.10,11 The HDL was obtained in 1.063 - 1.21 d fractions and dialyzed against PBS/EDTA. The HDL purity was confirmed by SDS-PAGE analysis. HDL fractions were adjusted to 1.5 ml with PBS/EDTA and added to complete RPMI 1640 medium at 20% for in vitro assay.

Incorporation of unesterified cholesterol to normal HDL – unesterified cholesterol was incorporated into normal mouse serum as described previously.12 Briefly, L-α-dipalmitylol lecithin (20mg) with free cholesterol (40mg) or without free cholesterol (control) were added to 5 ml of 0.15M NaCl and subjected to sonication at 30W for
60 min with a sonifier (Virsonic 100) in a water bath. After sonication, 2 ml of normal mouse serum was added and incubated on a rotor for 24 h at room temperature. Then, KBr was added to the solution to adjust density to 1.063 d and applied to sequential gradient centrifugation. The HDL was obtained in 1.063 - 1.21 d fractions.

Analysis of GR subcellular location – L929 cells express high levels of GR and thus have been used widely for studies of GR signaling. Cells were cultured in 10 cm dishes in a CO₂ incubator at 37°C to 90% confluence in RPMI 1640 medium supplemented with 10% FBS, 5 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin. Cells were harvested by scraping with a blade and suspended in complete medium, and incubated with/without 100 nM Dex in the presence/absence of SR-BI+/+ or SR-BI-/- HDL. Cells were incubated at 37°C for 15 and 30 min and subcellular fractions were isolated using a kit from Pierce following the manufacturer’s instructions. GR, tubulin (cytosol marker) and SP1 (nucleus marker) were detected with Western blot using chemiluminescence as we described previously.

Statistical Analysis - Data were represented as mean ± SD for in vivo analysis and as mean ± SEM for in vitro analysis. Comparison of two groups was by 2-tailed Student’s t-test. Significance in experiments comparing more than two groups was evaluated by One Way ANOVA, followed by post hoc analysis using Tukey’s test. Means were considered different at p < 0.05. P values for survival curves were determined from the Kaplan-Meier survival curves by use of the Log-Rank test using SPSS software.

References:
Supplemental Materials

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Supplemental figures and table:

**Figure sI. Deficiency of sepsis-induced thymocyte death in SR-BI+/+ mice.** SR-BI+/+ and SR-BI−/− mice were treated with/without cecal ligation and puncture (CLP, 22G needle, full ligation) for 18 h, the thymuses were fixed in 10% formaldehyde and the paraffin-embedded sections were stained with H&E. CLP induced profound cell death in SR-BI+/+ mice, predominately in the cortex (arrowhead). No significant cell death was observed in CLP-treated SR-BI−/− mice. n = 2 per group. Representative data is shown.

![Image of thymus sections showing cortical and medullary areas before and after CLP treatment with or without SR-BI expression](image.png)

**Figure sII. Intrinsic expression of SR-BI in thymus does not affect GC-induced thymocyte apoptosis.** A) SR-BI was moderately expressed in thymus. SR-BI expression was analyzed with Western blot using liver as positive control; B)
Corticosterone induced cell death equally in thymocytes isolated from SR-BI+/+ and SR-BI−/− mice. Thymocytes from SR-BI+/+ and SR-BI−/− mice were incubated with/without 10 µM corticosterone for 18 h, and cell death was analyzed by Trypan Blue assay. n = 6 per group with duplicate measurements. C) Dex induced cell death equally in thymocytes isolated from SR-BI+/+ and SR-BI−/− mice. Thymocytes from SR-BI+/+ and SR-BI−/− mice were incubated with/without 50 nM Dex for 18 h, and cell death was analyzed by Trypan Blue assay. n = 2 per group with duplicate measurements.

Figure sIII. Intrinsic expression of SR-BI in thymus does not affect CLP-induced thymocyte apoptosis. Bone marrow cells (5x10^6) from SR-BI+/+ or SR-BI−/− mice were transferred to γ-irradiated Rag-1−/− mice. After 6 weeks, the thymocytes were analyzed with flow cytometry. The Rag-1−/− mice were repopulated with thymocytes from SR-BI+/+ or SR-BI−/− mice as shown by the presence of CD4+CD8+ cells (A). n = 3 per group. Representative data is shown. The bone marrow-transplanted mice were subjected to CLP (22G needle, full ligation) for 18 h. Thymocyte apoptosis was determined by TUNEL assay (B) and CD4/CD8 profiling was analyzed (C). CLP induced similar thymocyte apoptosis in Rag-1−/− mice receiving bone marrow cells from SR-BI+/+ or SR-BI−/− mice, indicating that the intrinsic expression of SR-BI in thymus does not affect CLP-induced thymocyte apoptosis n = 4 - 5 per group. *p < 0.05 and **p < 0.01.
**Fig sIV.** Effect of HDL dose on GC-induced thymocyte apoptosis. Thymocytes harvested from wild-type mice were cultured in complete medium and incubated with indicated concentrations of SR-BI+/+ or SR-BI−/−HDL (vol/vol) in the presence of 10 µM corticosterone for 18 h, and the apoptotic (A) and apoptotic + necrotic thymocytes (B) were analyzed using FACS with 7-AAD staining. n = 3 with duplicate measurements. Significance between each group was evaluated by One Way ANOVA, followed by post hoc analysis using Tukey’s test. Data labeled with different letters are considered statistically different.

**Figure sV.** Effect of HDL on GC-induced SR-BI− thymocyte death. Thymocytes were harvested from 6-week old SR-BI−/− mice, cultured in complete medium and incubated with/without 10 µM of corticosterone in the presence/absence of SR-BI+/+ or SR-BI−/−HDL for 18 h, and cell death was analyzed with trypan blue assay. Number of trypan blue+ cells (A) and percentage of trypan blue+ cells (B). n = 3 with duplicate measurements. HDL had similar effect on GC-induced SR-BI− thymocyte death compared with SR-BI+/+ thymocytes.

**Figure sVI.** Schematic model of SR-BI and HDL regulating thymocyte apoptosis in sepsis. 1) In adrenals, SR-BI mediates the production of iGC which induces thymocyte apoptosis; 2) in thymus, HDL enhances GC-induced thymocyte apoptosis via promoting GR nuclear relocation; 3) lack of SR-BI-mediated reverse cholesterol transport in liver causes accumulation of free cholesterol on HDL that renders it inactive on thymocyte apoptosis; and 4) SR-BI and HDL regulated GC-GR signaling is likely required for
removal of stressed thymocytes, for proper selection of thymocyte and for protection against sepsis.

Table 1. Analysis of cholesterol content in isolated HDL

<table>
<thead>
<tr>
<th></th>
<th>SR-BI+/+</th>
<th>SR-BI-/-</th>
<th>SR-BI+/+ LCATtg</th>
<th>SR-BI-/- LCATtg</th>
</tr>
</thead>
<tbody>
<tr>
<td>UC:TC ratio</td>
<td>0.22±0.10</td>
<td>0.59±0.01</td>
<td>0.26±0.06</td>
<td>0.29±0.04</td>
</tr>
<tr>
<td>UC (unesterified cholesterol) mg/dL</td>
<td>11.22±5.26</td>
<td>29.5±2.68</td>
<td>21.34±5.31</td>
<td>30.63±4.85</td>
</tr>
<tr>
<td>TC (total cholesterol) mg/dL</td>
<td>49.77±0.46</td>
<td>49.73±3.91</td>
<td>81.77±0.02</td>
<td>104.02±2.35</td>
</tr>
<tr>
<td>CE (cholesterol ester) mg/dL</td>
<td>38.55±4.80</td>
<td>20.23±1.23</td>
<td>60.44±5.33</td>
<td>73.39±2.50</td>
</tr>
</tbody>
</table>

Table 1. Plasma was harvested from the mice indicated in the table and subjected to isolate HDL by sequential gradient centrifugation method. The total cholesterol (TC) and unesterified cholesterol (UC) were quantified.