Deficiency of Scavenger Receptor BI Leads to Impaired Lymphocyte Homeostasis and Autoimmune Disorders in Mice

Hong Feng, Ling Guo, Dan Wang, Haiqing Gao, Guihua Hou, Zhong Zheng, Junting Ai, Oded Foreman, Alan Daugherty, Xiang-An Li

Objective—Scavenger receptor BI (SR-BI) is a high-density lipoprotein (HDL) receptor. Recent studies revealed that SR-BI protects against sepsis via modulating innate immunity. However, its role in adaptive immunity is unclear.

Methods and Results—SR-BI-null mice exhibited impaired lymphocyte homeostasis as shown by splenomegaly and imbalanced expansion of T and B lymphocytes in the spleens. Importantly, the activated T and B lymphocytes were increased 3- to 4-fold, indicating a heightened active status of T and B lymphocytes. More importantly, in line with the accumulation of the activated T and B lymphocytes, SR-BI-null mice developed systemic autoimmune disorders characterized by the presence of autoantibodies in circulation, the deposition of immune complexes in glomeruli, and the leukocyte infiltration in kidney. Further analyses revealed that SR-BI deficiency enhanced lymphocyte proliferation, caused imbalanced interferon-γ and interleukin-4 production in lymphocytes, and caused elevated inflammatory cytokine production in macrophages. Furthermore, HDL from SR-BI-null mice exhibited less capability of suppressing lymphocyte proliferation.

Conclusion—SR-BI regulates lymphocyte homeostasis, likely through its roles in modulating the proliferation of lymphocytes, the cytokine production by lymphocytes and macrophages, and the function of HDL. Its deficiency leads to impaired lymphocyte homeostasis and autoimmune disorders. Our findings reveal a previously unrecognized role of SR-BI in adaptive immunity. (Arterioscler Thromb Vasc Biol. 2011;31:2543-2551.)

Key Words: lipoproteins ■ HDL ■ SR-BI ■ Scarb1 ■ adaptive immunity

Lymphocytes are essential regulators and effectors of the adaptive immune system. Unlike the innate immune cells, activated T and B lymphocytes form long-lived immunologic memory, which provides protective immune responses to a diversity of pathogens. However, improper activation of T and B lymphocytes breaks down the lymphocyte homeostasis that leads to a variety of diseases, such as autoimmune disorders.

Scavenger receptor BI (SR-BI, or Scarb1) is a well-established high-density lipoprotein (HDL) receptor.3,4 It mediates intracellular uptake of cholesterol ester from HDL, which plays a key role in regulating plasma HDL cholesterol levels.4-7 Mice lacking SR-BI exhibit a 2-fold increase in plasma cholesterol levels and are susceptible to atherosclerosis.4-12 Recent reports showed that mutations in human SR-BI cause elevated plasma HDL concentration associated with infertility and changes in cholesterol metabolism in macrophages and platelets, indicating a role of SR-BI in humans.13-15 In addition to regulating HDL metabolism, recent studies indicated that SR-BI is a multifunctional protein. It activates endothelial nitric oxide synthase in endothelial cells in the presence of HDL,16-19 induces apoptosis in the absence of HDL/endothelial nitric oxide synthase,20 and negatively regulates cytokine and IgM generation in B lymphocytes.21 Recent studies revealed that SR-BI protects against endotoxic and septic animal death via multiple mechanisms, including protection against nitric oxide (NO)-induced oxidative damage and suppression of inflammatory cytokine generation in macrophages.22-24 In spite of this established role in innate immunity, the function of SR-BI in adaptive immunity remains largely unknown. In this study, we report that mice deficient in SR-BI exhibited impaired lymphocyte homeostasis and developed autoimmunity. Additional studies revealed that SR-BI deficiency enhanced lymphocyte proliferation, caused imbalanced interferon-γ (IFN-γ) and interleukin-4 (IL-4) production in lymphocytes and elevated inflammatory cytokine production in macrophages, and reduced the inhibitory effect of HDL on lymphocyte proliferation. The current findings extend our understanding about SR-BI and its role in autoimmunity.

Materials and Methods

SR-BI-Null Mice

SR-BI+/− (B6;129S2-Scarb1m1Kr/J) mice were from the Jackson Laboratory. SR-BI−/− mice were generated by breeding SR-BI+/− mice.
mice, and SR-BI−/− littermates were used as controls. The animals were fed a standard laboratory diet (0.015% wt/wt cholesterol, 5.7% wt/wt fat, Harlan Tekland 2018). 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. For detailed Materials and Methods, see the supplemental materials, available online at http://atvb.ahajournals.org.

Results

Splenomegaly, Imbalanced T and B Lymphocyte Expansion, and Heightened Lymphocyte Activation in Mice Lacking SR-BI

To understand the significance of SR-BI in adaptive immunity, we examined the lymphatic organs from SR-BI-null and wild-type littermates. SR-BI-null mice exhibited splenomegaly (0.173 ± 0.051 g versus 0.083 ± 0.031 g for the wild-type) associated with a 2.1-fold increase in total splenocyte number (Figure 1a and 1b). Histological examination of the enlarged spleens revealed disrupted spleen structure, as shown by increased numbers of small lymphoid nodules that lack a defined marginal zone (Figure 1c). Immunofluorescent staining showed that these small lymphoid nodules contained densely packed T and B lymphocytes (Supplemental Figure Iia). Enlarged lymph nodes were more frequently observed in SR-BI-null mice than in wild-type controls (Supplemental Figure Iib). There was no significant difference in thymus weight between SR-BI-null and wild-type littermates, but the thymus cellularity in SR-BI-null mice was slightly decreased (Supplemental Figure Iic).

Fluorescence-activated cell sorting analysis revealed that, compared with wild-type littermates, SR-BI-null mice had a 2-fold increase in the number of B lymphocytes in the spleen (Figure 1e). Interestingly, compared with the marked increase in the number of B lymphocytes, the T lymphocytes were less expanded (1.4-fold) (Figure 1c). Actually, the percentages of CD3+, CD4+, and CD8+ T lymphocytes were moderately decreased (Figure 1d and 1f), and there was a 30% reduction in T- to B-cell ratio compared with that of wild-type controls (Figure 1g), indicating an imbalanced T- and B-cell expansion in mice lacking SR-BI. A 1.8-fold increase in B lymphocytes and a 30% reduction in T- to B-cell ratio were also observed in circulating blood (Supplemental Figure IId).

We next investigated the effect of SR-BI deficiency on lymphocyte activation. Both T and B cells exhibited an overall hyperreactive feature, as shown by significant increases in the percentages of activated CD3CD69high T and CD19 CD69high B cells (Figure 2a), and 3- to 4-fold increases in the number of activated CD3CD69high T and CD19 CD69high B cells (Figure 2b). We also looked at the effect of SR-BI deficiency on memory T cells using CD44 as marker. Mice lacking SR-BI had significant increases in both the percentage and the number of effector memory (CD4+CD44highCD62Llow) CD4+ T lymphocytes (Figure 2c and 2d). In contrast, there was a significant decrease in the ratio of naïve (CD4+CD44lowCD62Lhigh) CD4+ T lymphocytes (Figure 2c). The increase in memory T-cell ratio and decrease in naïve T-cell ratio indicate a heightened active status of T lymphocytes and accumulation of the memory T cells.

We also examined activated B lymphocytes using CD138 as a marker. Although there was no significant change in the percentage of plasmablasts (CD138+), the number of plasmablasts was increased 2.2-fold in SR-BI-null mice compared with wild-type mice (Figure 2e).

SR-BI Deficiency Did Not Affect T or B Lymphocyte Development

To understand how SR-BI deficiency leads to disrupted lymphocyte homeostasis, we investigated the effect of SR-BI on lymphocyte development in thymus, bone marrow, and spleen of adult mice. We analyzed the CD4/CD8 profile in the thymus for T lymphocyte development. No difference was observed between SR-BI-null and wild-type littermates (Supplemental Figure IVa), suggesting that SR-BI deficiency does not affect T lymphocyte development in the thymus. We then examined the development of B lymphocytes. Immature B lymphocytes are first produced in bone marrow; after reaching the IgM+ immature stage, these B cells migrate to the spleen, where they continue to develop and differentiate into mature B lymphocytes. As shown in Supplemental Figure IVb and IVc, there was no difference in the percentages of B lymphocyte subsets in bone marrow or spleen between SR-BI-null and wild-type littermates. To determine whether SR-BI deficiency affects T- or B-cell development in early age, we analyzed the T- and B-cell development in 5- to 6-week old mice. No difference was observed between SR-BI-null and wild-type littermates (data not shown).

Taken together, these data suggest that the disordered lymphocyte homeostasis in SR-BI-null mice may not be the consequence of lymphocyte development.

Increased In Vivo Lymphocyte Proliferation in Mice Lacking SR-BI

We then asked whether an increase in lymphocyte proliferation is responsible for the accumulation of lymphocytes in the spleen. We administered bromodeoxyuridine (BrdU) in drinking water and quantified in vivo lymphocyte proliferation by fluorescence-activated cell sorting analysis of the BrdU incorporated cells. Compared with wild-type littermates, SR-BI-null mice displayed a 2-fold increase in proliferating B-cell (B220+BrdU+) populations and a slight increase in the percentage of proliferating B cells (Figure 3a); there was a slight increase in the proliferating T-cell (CD3+BrdU+) populations and a moderate decrease in the percentage of proliferating CD3+ cells (Figure 3b). Interestingly, the number of proliferating CD4+CD44highBrdU+ T cells was significantly increased, but their percentage was slightly decreased in SR-BI-null mice compared with wild-type littermates (Figure 3b and 3c). This may not be surprising considering the longer life span of the memory T cells, which results in their accumulation.

SR-BI Deficiency Caused Enhanced In Vitro Lymphocyte Proliferation and HDL From SR-BI-Null Mice Lost Inhibitory Effect on Lymphocyte Proliferation in Response to T Cell Receptor/B Cell Receptor Stimuli

To understand the mechanisms of how SR-BI deficiency contributes to the impaired lymphocyte homeostasis, we
assessed the effect of SR-BI deficiency on lymphocyte proliferation and cytokine production. We isolated T and B cells from spleen and detected SR-BI expression in T and B cells. Moderate SR-BI expression was detected in T and B cells (Figure 4a and 4b), suggesting that SR-BI may have an intrinsic effect on lymphocyte. Indeed, as shown in Figure 4c and 4d, T and B cells from SR-BI-null mice had 2-fold and 1.5-fold increases in proliferating rate compared with wildtype controls, respectively, indicating enhanced proliferation in basal conditions in the absence of SR-BI.

We next looked at the effect of SR-BI deficiency on lymphocyte proliferation in stimulated conditions. Unexpectedly, T or B cells from SR-BI-null mice did not display a difference in proliferation in response to T cell receptor activation.

Figure 1. Splenomegaly, disrupted splenic architecture, and imbalanced T- and B-cell expansion in mice lacking scavenger receptor BI (SR-BI). a, Splenomegaly and increased spleen/body weight in SR-BI−/− mice. b, Increased splenic cellularity in SR-BI−/− mice. c, Disrupted splenic architecture in SR-BI−/− mice. Hematoxylin/eosin staining showed increased numbers of small lymphoid nodules (arrows) that lack a defined marginal zone in SR-BI−/− mice. d to g, Imbalanced T- and B-cell expansion in SR-BI−/− mice. Fluorescence-activated cell sorting analysis indicated a 2-fold increase B-cell populations and a 1.4 increase in T-cell populations (d and e); a 30% decrease in the percentages of CD3+, CD4+, and CD8+ T cells (d and f); and a 30% decrease in T/B-cell ratio (g) in SR-BI−/− spleen. Fluorescence was analyzed on gated cells with forward and side light scatter properties of lymphocytes. Spleens were harvested from 20- to 26-week-old SR-BI−/− and SR-BI+/+ littermates. n=30 each group. ***P<0.001 vs SR-BI+/+ mice.
(TCR) stimulus by anti-CD3 IgG or B cell receptor stimulus by lipopolysaccharide in the presence of fetal bovine serum (Figure 4e and 4f). A possible explanation for the difference between the basal and stimulated conditions is that the strong stimuli by TCR or B cell receptor ligands overshadowed the effect of SR-BI.

Early reports showed that HDL has inhibitory effect on T- and B-cell proliferation in stimulated status. SR-BI is an HDL receptor, and mice deficient in SR-BI have abnormal HDL (as shown by larger HDL particles, with a 2-fold increase in cholesterol contents) and changes in associated proteins. To test whether this HDL loses its inhibitory effect on lymphocyte proliferation, we stimulated splenocytes with TCR or B cell receptor ligands in the presence or absence of HDL. In agreement with the early reports, HDL from wild-type mice displayed 30% inhibition on CD4 T-cell and marked inhibition on B-cell proliferation rate (Figure 4e and 4f, SR-BI+/+ HDL), but HDL from SR-BI-null mice displayed no inhibition on CD4 T-cell and much less inhibition on B-cell proliferation rate (Figure 4e and 4f, SR-BI-/− HDL). Similar results were obtained with respect to CD8 T-cell proliferation (data not shown).

We also looked at the effect of SR-BI on cytokine production in response to TCR stimuli. Interestingly, despite no difference in IFN-γ production (Figure 4g), T cells from SR-BI-null mice produced markedly less IL-4 (Figure 4h) and displayed a 2- to 3-fold increase in IFN-γ/IL-4 ratio (Figure 4i) in response to anti-CD3 stimulus. Similar data were obtained when cells were stimulated by both anti-CD3 and anti-CD28, which is a costimulator of CD3 that stimulates T cells. We also examined the effect of SR-BI on cytokine production stimulated by concanavalin A or phorbol 12-myristate 13-acetate/ionomycin, which bypass the TCR to induce T-cell activation, and found no difference suggesting that the SR-BI likely functions via TCR signaling (Figure 4g and 4h). It is worth noting that IFN-γ belongs to proinflammatory cytokine (Th1 type) and IL-4 belongs to antiinflammatory cytokine (Th2 type). Thus, SR-BI deficiency causes imbalanced Th1/Th2 cytokine production, which may lead to an inflammatory status and contribute to impaired lymphocyte homeostasis.

**Increased Monocyte/Macrophage Populations and Elevated Proinflammatory Cytokine Production in Mice Lacking SR-BI**

Recent studies indicated that SR-BI suppresses inflammatory cytokine production by macrophages by regulating Toll-like receptor-4/nuclear factor-κB signaling pathway. Macrophage cytokines, such as IL-6, can act as growth factor for...
Thus, it is possible that SR-BI deficiency may affect lymphocyte homeostasis, partly through its role in modulating cytokine production in macrophages. To test such a possibility, we quantified CD11c<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells, a major type of monocyte/macrophage in the spleen. As shown in Figure 5a, there was a 1.9-fold increase in the number of CD11c<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells in SR-BI-null mice compared with wild-type littermates. Importantly, in line with the significant increase in monocytes/macrophages, SR-BI-null mice had 3- to 4-fold increases in tumor necrosis factor-α, IL-6, and inducible nitric oxide synthase expression and significant increases in tumor necrosis factor-α, IL-6, and nitrite/nitrate levels in circulation (Figure 5b to 5d).

**Development of Autoimmunity in Aged SR-BI-Null Mice**

Impaired lymphocyte homeostasis with overactivation of T and B lymphocytes, especially the accumulation of long-lived memory CD4<sup>+</sup>T cells, is characteristic of systemic autoimmune diseases, such as human systemic lupus erythematosus, and the systemic lupus erythematosus-prone mouse. To assess whether SR-BI deficiency leads to autoimmunity, we quantified circulating autoantibodies in adult and aged mice. The autoantibody levels in adult SR-BI-null mice (30 weeks) were slightly elevated compared with wild-type littermates. For aged SR-BI-null mice (>30 weeks), there were marked increases in circulating autoantibodies against double-stranded DNA, single-stranded DNA, and histone (Figure 6a), indicating age-dependent development of autoimmunity in mice lacking SR-BI.

Circulating autoantibodies can deposit as immune complexes in kidney glomeruli through entrapment of circulating immune complexes, as well as formation of immune complexes in situ following cross-reaction with components of glomerular basement membranes, which is a common feature of systemic autoimmune disorders. As shown in Figure 6b, using fluorescent-labeled anti-mouse IgG and C3 antibodies, we detected profound positive staining of IgG and C3.
C3 in the glomeruli of SR-BI-null mice (1 of 7 in wild-type versus 7 of 7 in SR-BI-null mice were positive for IgG staining; 2 of 7 in wild-type versus 7 of 7 in SR-BI-null mice were positive for C3 staining). Furthermore, most of the deposition of IgG was colocalized with C3, which suggests the formation of IgG/C3 immune complexes. We also evaluated leukocyte infiltration in the kidney, another common feature of systemic autoimmune disorders.32,33 We found that there was a large perivascular accumulation of leukocytes in SR-BI-null mice that was seldom observed in control mice (Figure 6c). All of these observations were evidence of age-dependent development of autoimmunity in SR-BI-deficient mice.

**Discussion**

In this study, we report that SR-BI deficiency leads to impaired lymphocyte homeostasis characterized by splenomegaly and imbalanced expansion of T and B lymphocytes. Importantly, T and B lymphocytes in SR-BI-null mice exhibited a heightened active status as shown by 3- to 4-fold increases in activated T and B lymphocytes. More importantly, in line with the accumulation of the activated T and B
lymphocytes, SR-BI-null mice developed systemic autoimmune disorders characterized by the presence of autoantibodies in circulation, the deposition of immune complexes in glomeruli, and the leukocyte infiltration in kidney. To understand the mechanisms of how SR-BI deficiency contributes to the impaired lymphocyte homeostasis, we assessed the effect of SR-BI on lymphocyte proliferation and cytokine production. We found that SR-BI is moderately expressed in both T and B cells, and its deficiency enhances lymphocyte proliferation in basal status and causes imbalanced IFN-γ/IL-4 production in stimulated status. Furthermore, HDL from SR-BI-null mice exhibited less capability of suppressing lymphocyte proliferation. An early report by Zhu et al demonstrated that SR-BI negatively regulates Toll-like receptor-9–dependent B-cell activation induced by CpG.21 Combined with the current finding, these studies suggest that SR-BI has both intrinsic and extrinsic effects on lymphocyte activation and function.

As SR-BI has been shown to suppress inflammatory cytokine production by macrophages via regulating Toll-like receptor-4/nuclear factor-κB signaling pathway,24 and the nuclear factor-κB signaling in macrophages plays an important role in lymphocyte activation,30 we elucidated the effect of SR-BI on macrophage populations and proinflammatory cytokine production. We found a significant increase in the number of splenic monocytes/macrophages; marked increases in tumor necrosis factor-α, IL-6, and inducible nitric oxide synthase expression; and significant increases in serum tumor necrosis factor-α, IL-6, and nitrite/nitrate levels in mice lacking SR-BI.

Taken together, these findings suggest that SR-BI regulates lymphocyte homeostasis likely through multiple ways, by modulating the proliferation of lymphocytes, the cytokine production by lymphocytes and macrophages, and the function of HDL.

Glucocorticoid is an immunosuppressive hormone. As an HDL receptor, SR-BI plays an essential role in providing cholesterol for glucocorticoid synthesis in stressed conditions such as endotoxemia, sepsis, and long-term fasting.23–24,34 However, SR-BI deficiency does affect glucocorticoid production in physiological condition,23–24,34 which suggests that the impaired lymphocyte homeostasis may not be caused by a change in glucocorticoid levels.

Recent studies revealed that excess accumulation of cellular cholesterol due to a defect in HDL-mediated cholesterol...
efflux disrupts lymphocyte homeostasis in mice lacking liver X receptor-β, ABCA1/ABCG1, or apolipoprotein A-1/low-density lipoprotein receptor.31,35–38 SR-BI is an HDL receptor that mediates intracellular uptake of cholesterol from HDL. This raises a possibility that SR-BI may modulate lymphocyte homeostasis via regulating cellular cholesterol levels. To address this speculation, we isolated T and B cells from the spleen and analyzed their cholesterol contents. No difference in free or esterified cholesterol levels was found between SR-BI-null and wild-type littermates (Supplemental Figure Va). We also assessed the plasma membrane cholesterol level of T and B cells by filipin staining, and no difference was observed between SR-BI-null and wild-type control mice (Supplemental Figure Vb). Our finding is consistent with a recent report by Ji et al demonstrating that SR-BI expression enhances both cell cholesterol efflux and cholesterol influx from HDL but does not lead to altered cellular cholesterol mass.39 These data suggest that the impaired lymphocyte homeostasis may not be caused by a change in cellular cholesterol contents.

Regulatory T cells and regulatory B cells play important roles in lymphocyte proliferation and activation. To examine whether SR-BI deficiency affects regulatory T cells or regulatory B cells, we quantified regulatory T cells and regulatory B cells using CD4\(^+\)CD25\(^+\)Foxp3\(^+\) and CD19\(^+\)CD1 day-

highCD5\(^+\) as markers, respectively. As shown in Supplemental Figure VI, SR-BI-null mice displayed no change in the percentage and a moderate increase in the number of regulatory T cells; the percentage of regulatory B cells was significantly decreased, but their number remained unchanged. Further investigation is required to determine a role of SR-BI in regulatory T cells and regulatory B cells and their contribution to lymphocyte homeostasis.

Early studies showed that mice lacking SR-BI exhibit extramedullary erythropoiesis because of defects in erythrocyte maturation.40–41 To assess the contribution of the erythrocytes to the observed splenic hypercellularity, we quantified erythrocytes (Ter119\(^+\)) with fluorescence-activated cell sorting in the splenocyte suspension after ACK treatment and found that \(\approx 14\%\) of the splenocytes in SR-BI-null mice were Ter119\(^+\) cells, whereas only 3\% of the splenocytes in wild-type littermates were Ter119\(^+\) cells (Supplemental Figure VIIa). Further analysis showed that most of the Ter119\(^+\) cells were premature erythrocytes (Supplemental Figure VIIb). Thus, the accumulation of erythrocytes accounted for about a 10\% increase in splenocyte populations after ACK treatment. We also prepared single-splenocyte suspension without ACK lysis to determine erythrocyte populations. As shown in Supplemental Figure VIIc and VIIId, SR-BI-null mice had a 20\% increase in Ter119\(^+\) cells compared with wild-type littermates (48.2\% \pm 9.6\% in SR-BI\(^-/-\) versus 28.5\% \pm 3.2\% in SR-BI\(^+/+\)), and a great portion of the erythrocytes were premature, as illustrated by a significant increase in the ratio of early (Ter119\(^{high}\)CD71\(^{high}\), region II) to-late (Ter119\(^{high}\)CD71\(^{low}\), region IV) phase erythrocytes in SR-BI-null mice. Thus, accumulation of erythrocytes accounted for about a 20\% increase in total splenic cellularity observed in SR-BI-null mice. These data confirmed the presence of extramedullary erythropoiesis in the spleens of SR-BI-null mice, but it only partly contributed to the observed splenic hypercellularity. It is of interest to determine whether and how extramedullary erythropoiesis contributes to the impaired lymphocyte homeostasis in SR-BI-null mice.

In summary, SR-BI plays critical roles in modulating lymphocyte activation, proliferation, and cytokine production, and its deficiency leads to impaired lymphocyte homeostasis and extramedullary erythropoiesis, which warrants further investigation.

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Disclosures

None.

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Supplement Materials

Detailed Materials and Methods

Materials

Fetal bovine serum (FBS, heat inactivated, SH30070.03HI) was obtained from Thermo scientific. Bovine serum albumin (BSA), total calf thymus histones, poly-L-lysine, bromodeoxyuridine (BrdU), concanavalin A (Con A) phorbol myristate acetate (PMA) and ionomycin were from Sigma-Aldrich. Anti-CD3 (145-2C11), anti-CD28 mAb (37.51), Tregs kit, and enzyme-linked immunosorbent assay (ELISA) kits for quantifying IFN-γ, IL-4, IL-6 and TNF-α were from eBioscience. LPS (E. coli serotype K12) was from InvivoGen. AffiniPure F(ab′)² anti-IgM and Cy3-conjugated goat anti-mouse IgG was from Jackson ImmunoResearch. FITC-conjugated goat anti-mouse complement C3 was from MP Biomedicals. Bregs kit was from Biolegend. Fixation/Permeabilization solution kit and 100μm cell strainer were from BD Biosciences. Salmon sperm DNA was from GE healthcare. Alkaline phosphatase-conjugated goat anti-mouse IgG was from Southern Biotechnology.

SR-BI null mice

SR-BI\textsuperscript{+/−} (B6;129S2-Scarb1\textsuperscript{tm1Kri/J}) mice were from the Jackson Laboratory. SR-BI\textsuperscript{−/−} mice were generated by breeding SR-BI\textsuperscript{+/−} mice, and SR-BI\textsuperscript{+/+} littermates were used as controls. The animals were fed a standard laboratory diet (0.015% wt/wt cholesterol, 5.7% wt/wt fat, Harlan Tekland 2018). 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.
Fluorescence-activated cell sorting (FACS) analysis

Single-cell suspensions from the thymus, bone marrow and peripheral lymph nodes were obtained by disrupting tissues through 100µm cell strainers. Splenocytes were prepared using Stomacher 80 (Seward) and incubated with 5 mL of ACK lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA-2Na, pH 7.4) for 5 minutes at RT to deplete erythrocytes. 1x10⁶ cells were incubated immediately with a panel of fluorochrome-conjugated antibodies, including FITC-, PE-, APC-, PerCP-Cy5.5, or PE-Cy5-conjugated anti-CD3 (17A2), CD4 (GK1.5), CD8 (53-6.7), CD19 (ID3), B220 (RA3-6B2), CD23 (B3B4), CD43 (S7), CD44 (IM7), CD69 (H1.2F3), IgM (R6-60.2), CD138 (281-2), CD62L (MEL-14), Ter119 (TER-119), CD71 (C2), CD117 (2B8), CD93 (AA4.1) (BD Biosciences). Cells were sorted using FACSCalibur flow cytometer (BD Biosciences) and data were analyzed with FlowJo software (Treestar). For FACS analysis of lymphocyte subtypes, fluorescence was analyzed on gated cells with forward and side light scatter properties of lymphocytes; for other cell types, fluorescence was analyzed on gated cells with forward and side light scatter properties of viable cells (Supplement Fig. 1).

In vivo BrdU proliferation assay

Mice were administered 0.8 mg/mL BrdU in drinking water for 6 days. Splenocytes were prepared as described above and first stained with surface antibodies to CD3, B220, CD4 and CD44, followed by BrdU incorporation detection using the APC BrdU flow kit following the manufacturer’s protocol (BD Biosciences). Splenocytes were sorted by FACS. A wild type mouse receiving water alone was included as a negative control to show background levels.

In vitro BrdU proliferation assay
Splenocytes isolated from 8-10-week old mice, were plated at a concentration of 4 x 10^6 cells/mL in 96-well plate and cultured in RPMI 1640 medium supplemented with 10% FBS, 5 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, 50 µM 2-mercaptoethanol (2-ME) and 10 µM BrdU. For lymphocyte proliferation in stimulated conditions, the splenocytes were stimulated with anti-CD3 (10 µg/mL) or LPS (10 µg/mL) in the presence/absence of 40 µg/mL HDL isolated from SR-BI null or wild type littermates. Cells were incubated with 10 µM BrdU at 37°C. After 96 hours, cells were stained with surface antibodies against T and B cells first, and then the BrdU incorporation was detected using the APC BrdU flow kit and analyzed by FACS. Cells without BrdU incubation were included as a negative control to show background levels.

**Serum TNF-α, IL-4, IL-6 and nitrite/nitrate (NOx)**

Serum was diluted for 2 to 10 times and TNF-α, IL-4, IL-6 and NOx were quantified with corresponding kit following the manufacturer’s instructions.

**In vitro cytokine production**

Splenocytes isolated from 8-10 week old mice were cultured in RPMI 1640 medium supplemented with 10% FBS, 5 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, and 50 µM 2-ME. Cells were plated at a concentration of 4 x 10^6 cells/mL in 96-well plates and stimulated for 48 hours at 37°C with 1µg/mL Con A, 6.5 ng/mL PMA plus 500 ng/mL ionomycin, platebound anti-CD3 alone (10 µg/mL) or together with soluble anti-CD28 mAb (10 µg/mL) in the presence of 10% of FBS. The concentrations of IFN-γ and IL-4 in the
supernatants were determined with corresponding ELISA kit according to the manufacturer’s instructions.

**Cellular and membrane cholesterol**

To quantify cellular cholesterol levels in T and B cells, splenic T or B cells were sorted with FACS. After three times wash with PBS, $1 \times 10^7$ cells were lysed in lysis buffer and the cholesterol levels were measured with total and free cholesterol kits (Wako) as we previously described.\(^1\) To assess membrane cholesterol levels, splenocyte were stained with anti-CD3 and B220, fixed with 3% Paraformaldehyde, stained with filipin (Sigma) at 100µg/ml for 2 hours and analyzed by FACS.\(^2\)

**Histochemistry and immunofluorescence**

For hematoxylin and eosin (H&E) staining, tissues were fixed in 3% paraformaldehyde for 24 hours. Specimens were then embedded in paraffin, sectioned (5 µm), and stained using standard techniques. For immunofluorescence, tissues were embedded in optimal cutting temperature embedding media (OCT), snap frozen, sectioned (7 µm) and fixed in chilled acetone. To identify immune complexes in the kidney, the sections were blocked with 5% goat serum and incubated with Cy3-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-mouse complement C3 at 4°C overnight. Images were taken with a confocal microscope (Olympus FluoView).

**RT-PCR and qRT-PCR**

To detect SR-BI expression in T and B cells, T or B cells were sorted with FACS, total RNA was isolated and used for RT-PCR using SR-BI specific primers: forward 5’- CTG CTG TTT GCT
GCG CT and reverse 5’- TCC CGG ACT ACT GGC TTC T. The same primers were used for quantitative RT-PCR using LightCycler® 480 Real-Time PCR System (Roche Applied Science) with SYBR-green method. Liver total RNA from SR-BI null and wild type mice was used as control. To quantify TNF-α and IL-6 expression in spleen, total spleen RNA was isolated and the TNF-α and IL-6 expression were quantified using LightCycler® 480 Real-Time PCR System (Roche Applied Science) with probe method. The following primers were used: TNF-α primers, forward 5’- TGC CTA TGT CTC AGC CT CTC TTC and reverse 5’- GAG GCC ATT TGG GAA CTT CT; IL-6 primers, forward 5’- GCT ACC AAA CTG GAT ATA ATC AGG A and reverse 5’- CCA GGT AGC TAT GGT ACT CCA GAA; 18s rRNA, forward 5’- CGATTGGATGGTTTAGTGAGG and reverse 5’- AGT-TCG-ACC-GTC-TTC-TCA-GC. The data were normalized to 18s rRNA expression.

Quantification of serum autoantibodies by ELISA

Autoantibodies against dsDNA, ssDNA and histone were detected using ELISA as described previously. Briefly, sonicated and phenol extracted salmon sperm DNA was employed as dsDNA. ssDNA was prepared by boiling the dsDNA for 10 min and cooling on ice/water for 15 minutes. Total calf thymus histones were dissolved in a 6 M urea solution and then dialyzed against PBS. Polyvinyl microtiter plates were coated with optimal concentrations of either dsDNA (5µg/mL), ssDNA (5µg/mL) or histone (20µg/mL). For the anti-dsDNA and anti-ssDNA ELISA, the plates were first coated with poly-L-lysine (5µg/mL) before incubation with autoantigens. Antigens were added to plates and the plates were incubated overnight at 4°C. The plates were then washed and blocked with BBT (BBS, 0.4% Tween 20, and 0.5% BSA) for 2 hours at 4°C. Serum samples, diluted 1:200 in BBT, were added and the plates were incubated
overnight at 4°C. Alkaline phosphatase-conjugated goat anti-mouse IgG was added as secondary antibody. The plates were washed, incubated with p-nitrophenyl phosphate and read at A_405 nm.

**Statistical Analyses**

Data were presented as mean ± SD. Significance in experiments was determined by a 2-tailed Student's t test. Data from multiple groups were analyzed with one-way ANOVA and Student-Newman-Keuls multiple comparison test. Means were considered significantly different at \( p < 0.05 \).

**Supplemental figure legends:**

**Fig. I Gating of lymphocytes and viable cells.**

For FACS analysis of lymphocyte subtypes, fluorescence was analyzed on gated cells with forward and side light scatter properties of lymphocytes (a); For other cell types, fluorescence was analyzed on gated cells with forward and side light scatter properties of viable cells (b).

**Fig. II Effect of SR-BI on T and B cell distribution in lymphoid nodules of spleen, lymph node and thymus**

(a) Immunofluorescent staining of T and B cells in lymphoid nodule. Frozen spleen sections from SR-BI/- and SR-BI+/+ littermates were stained for T (green) and B (red) lymphocytes. 1st antibodies: hamster monoclonal IgG against CD3 (Santa Cruz. Biotechnology, SC-1174) and rat anti mouse CD45-R (B220) (BD Biosciences, 550286); 2nd antibody: Cy5-conjugated AffiniPure goat anti American hamster IgG (H+L) (Jackson Immuno research, 127-175-160) and B220-Cy3-conjugated goat anti-rat IgG (H+L) (Invitrogen, A10522). The nuclei were stained
with DAPI (blue). Small lymphoid nodule from SR-BI-/ contains densely packed T and B lymphocytes. n = 6 per group. Representative lymphoid nodule stainings from SR-BI-/ and SR-BI+/+ littermates are shown.

(b) Effect of SR-BI on lymph node. The weight and the cellularity of lymph nodes were increased in SR-BI-/ mice, but not statistically significant. n = 12 each group.

(c) The thymus weight was unaffected by SR-BI deficiency and the cellularity of thymus were decreased in SR-BI-/ mice, but not statistically significant. n = 12 each group.

(d) FACS analysis of T and B lymphocytes in circulation. Blood was isolated from SR-BI-/ and SR-BI+/+ littermates. After lysis of red blood cells, T and B lymphocytes were analyzed by FACS on gated CD45+ cells. A 1.8-fold increase in B lymphocytes and a 30% reduction in T to B cell ratio were observed. p > 0.05, n = 3 each group.

Fig. III Isotype control staining for CD69 and CD138

Splenocytes from SR-BI-/ and SR-BI+/+ littermates were stained with fluorescent labeled CD69 or CD138 isotype antibodies. Representative plots are shown.

Fig. IV SR-BI deficiency did not affect T or B lymphocyte development

FACS analysis of lymphocytes from thymi, bone marrow and spleens of 20-26-week-old SR-BI-/ and SR-BI+/+ littermates. n = 10 each group.

(a) CD4/CD8 profile analysis indicate that T cell development in thymus was unaffected by SR-BI deficiency.

(b) B lymphocyte development in bone marrow was unaffected by SR-BI deficiency. The first row divides B cells into mature recirculating (B220^{high}IgM^+), immature (B220^{low}IgM^+), and B
cell progenitors ($B220^{\text{low}}\text{IgM}^-$). The $B220^{\text{low}}\text{IgM}^-$ gate is further resolved in the second row into CD43$^+$ pro-B cells and CD43$^-$ pre-B cells.

(c) B lymphocyte development in spleen was unaffected by SR-BI deficiency. The first row divides B cells into immature ($B220^+\text{AA4.1}^+$) and mature ($B220^+\text{AA4.1}^-$) B cell subsets. The immature B cell gate is further resolved in the second row into T1 B ($B220^+\text{AA4.1}^+\text{IgM}^{\text{high}}\text{CD23}^-$), T2 B ($B220^+\text{AA4.1}^+\text{IgM}^{\text{high}}\text{CD23}^+$) and T3 B ($B220^+\text{AA4.1}^+\text{IgM}^{\text{low}}\text{CD23}^+$) cells. The mature B cell gate is resolved in the third row into marginal zone B ($B220^+\text{AA4.1}^+\text{IgM}^{\text{high}}\text{CD23}^-$) and follicular B ($B220^+\text{AA4.1}^+\text{IgM}^{\text{low}}\text{CD23}^+$) cells.

**Fig. V** SR-BI deficiency did not affect cellular cholesterol levels.

(a) Quantification of cellular cholesterol levels in T and B cells. Splenic CD3$^+$ and B220$^+$ cells were sorted by FACS and the total and free cholesterol levels were quantified with corresponding kit. n=4 each group.

(b) Filipin staining of T and B cells. Splenocytes were stained with filipin and analyzed by FACS on gated CD3$^+$ and B220$^+$ cells. n=4 each group.

**Fig. VI** Effect of SR-BI deficiency on splenic regulatory T and B cells.

(a) Effect of SR-BI on regulatory T cells (Tregs, CD4$^+$CD25$^+$Foxp3$^+$). Compared to wild type littermates, SR-BI null mice displayed no change in the percentage of Tregs and a moderate increase in the number of Tregs.
(b) Effect of SR-BI on regulatory B cells (Bregs, CD19<sup>+</sup>CD1d<sup>high</sup>CD5<sup>+</sup>). Compared to wild type littermates, SR-BI null mice displayed a significant decrease in the percentage of Bregs but the number of Bregs remained unchanged.

Spleens were harvested from 20-26-week-old SR-BI+/+ and SR-BI-/ littermates. n = 5 each group. *p < 0.05 vs. SR-BI+/+ mice.

Fig. VII Extramedullary erythropoiesis partly contributed to the splenic hypercellularity in SR-BI null mice.

Spleens were harvested from 20-26-week-old SR-BI/- and SR-BI+/+ littermates. Single-cell suspensions were prepared with/without ACK lysis. Cells were then stained with indicated antibodies for FACS analysis. n = 8 each group. *p < 0.05, **p < 0.01 and ***p < 0.001 versus SR-BI+/+ mice.

(a) Increased proportion of erythrocytes (Ter119<sup>+</sup>) in the splenocytes after ACK lysis in SR-BI/- mice.

(b) Most of the Ter119<sup>+</sup> cells in the splenocytes after ACK lysis in SR-BI null mice were early stage erythrocytes. Regions I to IV were gated as indicated following the order of erythroid precursor differentiation stages, with region I representing proerythroblasts (Ter119<sup>med</sup>CD71<sup>high</sup>), region II basophilic erythroblasts (Ter119<sup>high</sup>CD71<sup>high</sup>), region III late basophilic erythroblasts and polychromatic erythroblasts (Ter119<sup>high</sup>CD71<sup>med</sup>), and region IV late orthochromatic erythroblasts (Ter119<sup>high</sup>CD71<sup>low</sup>).
(c) More erythrocytes (Ter119⁺) accumulated in SR-BI-/ mouse spleen than that of wild type controls. The proportion of erythrocyte lineage (Ter119⁺) in splenocytes without lysis was assessed by FACS.

(d) The ratio of early-to-late erythroblasts was dramatically increased in SR-BI-/ mouse splenocytes without ACK lysis.

References:


Supplemental figures:

Fig I
Fig II

a

b

SBR-I+/+

SR-BI-/−

LN/body weight (mg/g)

Total number of LN cells (×10⁶)

Total number of thymocytes (×10⁶)

SR-BI+/+

SR-BI-/−

c

d

% CD3 B220

Cell number (×10⁶/mL)

CD3 B220

CD3 B220

CD3 B220
Fig III

(a) SR-BI+/+ vs SR-BI-/

(b) SR-BI+/+ vs SR-BI-/

(c) SR-BI+/+ vs SR-BI-/
Fig IV

(a) Thymus

(b) BM

(c) Spleen

Legend:

SR-BI+/+

SR-BI-/ 

Pro: B220^hi^IgM^CD43+

Pre: B220^hi^IgM^CD43+

Imm: B220^hi^IgM'

Mat: B220^hi^IgM'

T1: B220^AA4.1'l^IgM^CD23+

T2: B220^AA4.1'l^IgM^CD23+

T3: B220^AA4.1'l^IgM^CD23+

MZ: B220^AA4.1'l^IgM^CD23+

Fo: B220^AA4.1'l^IgM^CD23+
Fig. V

**a**

Cholesterol (mg/g protein) displayed for CD3+ and B220+ cells in different conditions: Total, Free, and Esterified. The graphs show the concentration levels with error bars indicating variability.

**b**

Flow cytometry data illustrating the cell number distribution in CD3+ and B220+ cells treated with Filipin and stained with SR-BI+ or SR-BI- markers. The histograms are grouped according to SR-BI genotypes.
Fig. VI

[Diagram showing bar graphs for Treg and Breg percentages and cell numbers for SR-BI+/+ and SR-BI-/- in panels a and b.]