Materials and Methods

Scavenger receptor BI and HDL regulate thymocyte apoptosis in sepsis

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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-*Scarb1*^{tm1Kri}/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 al[2]. 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-GR$^{fl/fl}$) were obtained from Dr Ashwell [3], and the GR$^{fl/fl}$ 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 CO_2 incubator at 37 ^oC 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 i*n vitro* apoptosis usually undergoes "secondary necrosis" due to lack of phagocytosis of the apoptotic cells[8, 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-dipalmitory 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

60min 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 [13]. Cells were cultured in 10 cm dishes in a CO_2 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[14].

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.

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