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Apolipoprotein E-Mediated Immune Regulation in Sepsis[1]

Omar M. Kattan, F. Behzad Kasravi, Erica L. Elford, Michael T. Schell, and Hobart W. Harris[2]

Lipids and lipoproteins have emerged as key constituents of the immune response to microbial infection. We, therefore, sought to understand the complex interaction between lipoprotein metabolism and sepsis. Apolipoprotein E (apoE), a component of plasma lipoproteins, has been suggested to bind and traffic Ags for NKT cell activation. However, apoE's role in sepsis has not been demonstrated. In this study, we examined the effect of exogenous apoE in a rat model of septic peritonitis, induced by cecal ligation and puncture. We demonstrate that 48 h after serial injections of apoE, septic mortality increased in a dose-dependent manner. While sepsis resulted in increased splenic and decreased hepatic and circulating NKT cell populations, serial injections of apoE for 24 h after cecal ligation and puncture increased the frequency, cell number, and BrdU uptake in splenic and hepatic NKT cell populations, while concomitantly depleting these populations in the circulation. These changes were correlated with elevated alanine amino transferase levels, an indicator of liver injury. Interestingly, while sepsis increased hepatic T cell apoptosis and necrosis, apoE reversed these changes. apoE also promoted increases in predominantly Th1 cytokine levels in sera and a decrease in IL-4, the main NKT cell-derived Th2 cytokine. Consequently, apoE treatment is associated with increased sepsis-induced mortality, and increased NKT cell frequency and proliferation in the liver and spleen, with concomitant decreases in these NKT cell parameters in the peripheral circulation. apoE treatment also promoted a Th1 cytokine response, increased the degree of liver injury, and decreased apoptosis in hepatic lymphocytes. *The Journal of Immunology,* **2008, 181: 1399–1408.**

C omponents of lipid metabolism have been co-opted to participate in microbial immunity. During the onset of sepsis, a fatal disease characterized by an acute systemic immune response to bacterial infections, the immune system becomes hyperactive and the distribution of circulating lipoproteins changes significantly, a phenomenon clinically termed the "lipemia of sepsis" (1). Although this phenomenon was originally thought to represent the fueling of the host response to infection (2), increasing evidence suggests a more direct role for lipids and lipoproteins as agents in the immune system (3).

Apolipoprotein E (apoE)[3] is a multifunctional component of plasma lipoproteins that is found on very low density lipoprotein, low density lipoprotein (LDL), high density lipoprotein, and chylomicron remnant lipoprotein complexes. First recognized as a major determinant in lipoprotein metabolism and cardiovascular disease, apoE, a ligand for the LDLR, has emerged as an important molecular mediator in immunoregulation (4). However, the evidence regarding apoE's role in the immune response is contradictory. Lipoproteins containing apoE have been shown to inhibit or stimulate Ag-induced T lymphocyte activation and proliferation (5). Additionally, mice deficient in apoE appear to have an impaired response to bacterial infection (6, 7). Studies have also shown that apoE can bind LPS, attenuate the host inflammatory response, and, thus, protect against LPS-induced mortality (8). Distinctively, apoE has also recently been implicated in the activation of NKT cells by acting as a molecular chaperone for bacterial Ags, delivering them to APCs via LDLR to activate NKT cells by CD1d, a nonclassical class-I-like Ag-presenting molecule (9). NKT cells are characterized by the NK marker (NK1.1) and a semi-invariant TCR expressing the $V\alpha14/J\alpha281$ gene segments. Upon stimulation, NKT cells can promptly secrete large amounts of Th1 and Th2 cytokines, IFN-γ and IL-4, respectively (10, 11). NKT cells appear to act as a functional bridge between innate and acquired immune systems, highlighting their importance as a target for infectious diseases (12).

In light of this contradictory evidence, we sought to characterize apoE's specific immunomodulatory role in a clinically relevant rat model of bacterial sepsis-cecal ligation and puncture (CLP). We found that apoE increased the response to septic shock through increased NKT cell frequency, number, and proliferation in the peripheral organs, and downstream responses, thereby contributing to increased mortality. Our results provide evidence for a role of apoE, a LDLR ligand, in increasing the immune response to polymicrobial sepsis, particularly by NKT cells.

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[3] Abbreviations used in this paper: apoE, apolipoprotein E; LDL, low density lipoprotein; CLP, cecal ligation and puncture; EJV, external jugular vein; 7-AAD, 7-aminoactinomycin D; ALT, alanine amino transferase.

Materials and Methods
Animals

Male Sprague-Dawley rats (Charles Rivers) weighing 250–300 g were maintained under standard conditions. All procedures were performed in full accordance with the policies of the Institutional Animal Care and Use Committee at University of California, San Francisco.

Apolipoprotein E

Recombinant apoE3 was produced in bacteria by using a vector expressing a thioredoxin fusion protein, as previously described (13). To

FIGURE 1. Effect of immediate administration of apoE on survival of wild-type rats subjected to CLP. After CLP or a sham operation, apoE (114 μg/kg or 1.6 mg/kg) or vehicle (saline) was immediately injected via the EJV catheter. Rats then received the same dose concentration every 4 h for 48 h and survival was monitored for 7 days. Differences between survival curves were determined by log-rank tests. Results are expressed as percentage of survival. Data represent three independent experiments. $p < 0.05$ [CLP/saline ($n = 20$) vs CLP/apoE 114 μg/kg ($n = 28$) and sham/apoE ($n = 7$) vs CLP/saline]; $p < 0.01$ [CLP/apoE 114 μg/kg vs CLP/apoE 1.6 mg/kg ($n = 8$)]; $p < 0.001$ (sham/apoE vs CLP/apoE 114 μg/kg, sham/apoE vs CLP/apoE 1.6 mg/kg, and CLP/saline vs CLP/apoE 1.6 mg/kg).
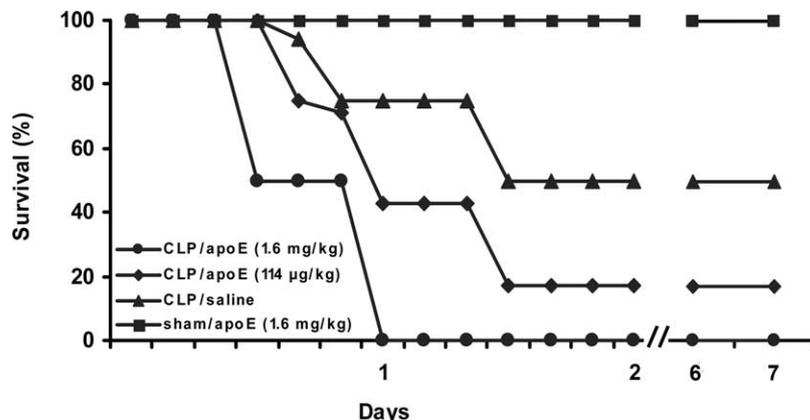
exclude toxicity, apoE was cultured for presence of bacterial contamination and a *Limulus* assay (Cape Cod) was performed to detect endotoxin contamination. Maximum potential endotoxin contamination is 5 pg/mg apoE.

Induction of sepsis by CLP

After general anesthesia was induced, a catheter was inserted in the rat's external jugular vein (EJV), as described by Thrivikraman et al. (14). In brief, after a 1.0-cm incision was made at the base of the neck, a catheter was placed into the EJV, tunneled behind the shoulder, and out through the skin on the rat's back. All incisions were closed using 4–0 silk sutures. After 24 h, rats either underwent CLP or a sham operation. CLP was performed as described previously (15, 16). In brief, rats were anesthetized and a 1.5-cm midline incision was made. The cecum was exposed and 40% was ligated with a 3–0 silk suture. The cecum was punctured through and through using an 18-gauge needle, and a small amount of fecal matter was expressed from each puncture hole. The cecum was returned to the abdominal cavity and the abdominal wall was closed in two layers using a 3–0 silk suture. Rats that underwent sham operations were treated identically except the cecum was not ligated or punctured.

Beginning immediately at the time of CLP, recombinant apoE3 (1.6 mg/kg or 114 μg/kg), or an equivalent volume of saline, was injected into the rats by using the EJV catheter every 4 h for a total of 48 h. Mortality was measured at day 7 in four groups: sham/saline, sham/apoE, CLP/saline, and CLP/apoE. For in vivo characterization, rats were given 114 μg/kg of apoE or an equivalent volume of saline exactly as above for 24 h, at which point liver and spleen were harvested for FACS analysis and blood samples were obtained to measure cytokine and transaminase levels.

Preparation of lymphocytes

Intrahepatic lymphocytes were isolated as described by Dao et al. (17). In brief, rats were perfused via the portal vein with digestion medium [RPMI 1640 medium containing 0.2 mg/ml collagenase and 0.02 mg/ml Dnase I (Roche) and 5% FCS]. The liver was minced and passed through a 70 μm nylon cell strainer (BD Falcon) and washed. The suspension was differentially centrifuged over a Percoll gradient [40% Percoll (EMB Biosciences), 150 × g, 15 min, 4°C]. Cells were washed in RPMI 1640 (300 × g, 5 min, 4°C) and resuspended in a red cell lysing buffer (0.15M NH$_4$Cl, 1.0 mM KHCO$_3$, 0.1 mM Na$_2$ EDTA) for 3 min. Cells were washed with PBS (300 × g, 5 min, 25°C) and resuspended in FACS staining buffer (0.1% BSA, 0.02% Na$_2$N$_3$ in PBS) for FACS analysis. Cell viability was determined by trypan blue exclusion.

The spleen was minced with two sterile glass slides, passed through a 40 μm nylon cell strainer (BD Falcon) and washed. Cells were resuspended in a red cell lysing buffer for 3 min. Splenocytes were washed with PBS and resuspended in FACS staining buffer for FACS analysis. Cell viability was determined by trypan blue exclusion.

Whole blood was incubated with red cell lysing buffer in a 1:14 ml ratio for 5 min on a rotator. Cells were washed (2000 rpm, 5 min) and resuspended in FACS staining buffer for FACS analysis. Cell viability was determined by trypan blue exclusion.

Flow cytometry analysis

Abs used included CD45-PECy5, CD161a-PE (NKR-P1A), $\alpha\beta$TCR-FITC, CD8a-PE, anti-CD23, Annexin V-APC, and 7-aminoactinomycin D (7-

AAD) (BD Pharmingen). For T cell counts, 1.0×10^6 cells from the liver and spleen were preincubated with anti-CD23 to block FC receptors and decrease nonspecific binding. Samples were then incubated with Abs for 30 min at 4°C in the dark, washed (1200 rpm, 5 min), and resuspended in FACS staining buffer. To determine positive double-staining, single-stain controls for each surface marker and IgG controls for each color were used. For apoptotic staining, 1.0×10^5 cells from the liver were incubated with Annexin V and/or 7-AAD and incubated for 15 min at 25°C. Three-color analysis was performed on a FACSCalibur (BD Biosciences) with a 100,000–200,000 event count. Data was analyzed using FlowJo software (TreeStar).

BrdU administration and detection

Rats received an i.p. injection of BrdU (50 mg/kg) 3 h after CLP. To detect BrdU incorporation in lymphocytes harvested 24 h after CLP, we used the Allophycocyanin BrdU Flow kit (BD Pharmingen). After surface staining, cells were fixed and permeabilized with Cytofix/Cytoperm buffer for 15–30 min on ice and then treated with DNase to expose incorporated BrdU. Subsequently, cells were stained with allophycocyanin-conjugated anti-BrdU Ab.

Multiplex analysis of serum cytokine concentration

Concentrations of IFN γ, IL-2, IL-4, IL-10, IL-1β, and TNF-α were simultaneously quantified in serum samples using an ELISA-based bead multiplex assay (Linco Research) according to the manufacturer's instructions. Samples were analyzed with a Luminex100 plate reader (Linco Research) to determine cytokine concentration. Concentrations for each cytokine in the multiplex assay were calculated from calibration curves using individual recombinant proteins as standards, according to the manufacturer's instructions. Serum samples were diluted (1/5). StatLIA software (Brendan Scientific) with a 5-parameter logistic curve-fitting method was used for data reduction. All specimens were tested in duplicate wells to assess interassay variability. The sensitivity of the cytokine assay was <5 pg/ml for all cytokines measured.

Serum transaminase

A total of 50 μl of rat serum was used to obtain alanine amino transferase (ALT) values by using ALT L$_3$K reagent (Diagnostic Chemicals) run by a standardized, automated analyzer (Cobas Mira Plus; Roche) at room temperature.

Statistical analysis

Flow cytometry and ALT values were compared using two-tailed t tests. Survival analysis was analyzed by the log-rank test. Multiplex serum cytokine levels were compared using Kruskal-Wallis and non-parametric Mann-Whitney U tests. A value of $p < 0.05$ was regarded as statistically significant.

Results

apoE increases mortality in sepsis

We first investigated whether apoE injected serially at the induction of and for the duration of septic insult affected the host defense against endotoxin-induced mortality. Mortality was determined in four groups of rats treated serially every 4 h for 48 h and followed for 7 days: sham/apoE-1.6 mg/kg, CLP/saline, CLP/apoE-114 μg/kg, and CLP/apoE-1.6 mg/kg. We found that apoE (1.6 mg/kg) had
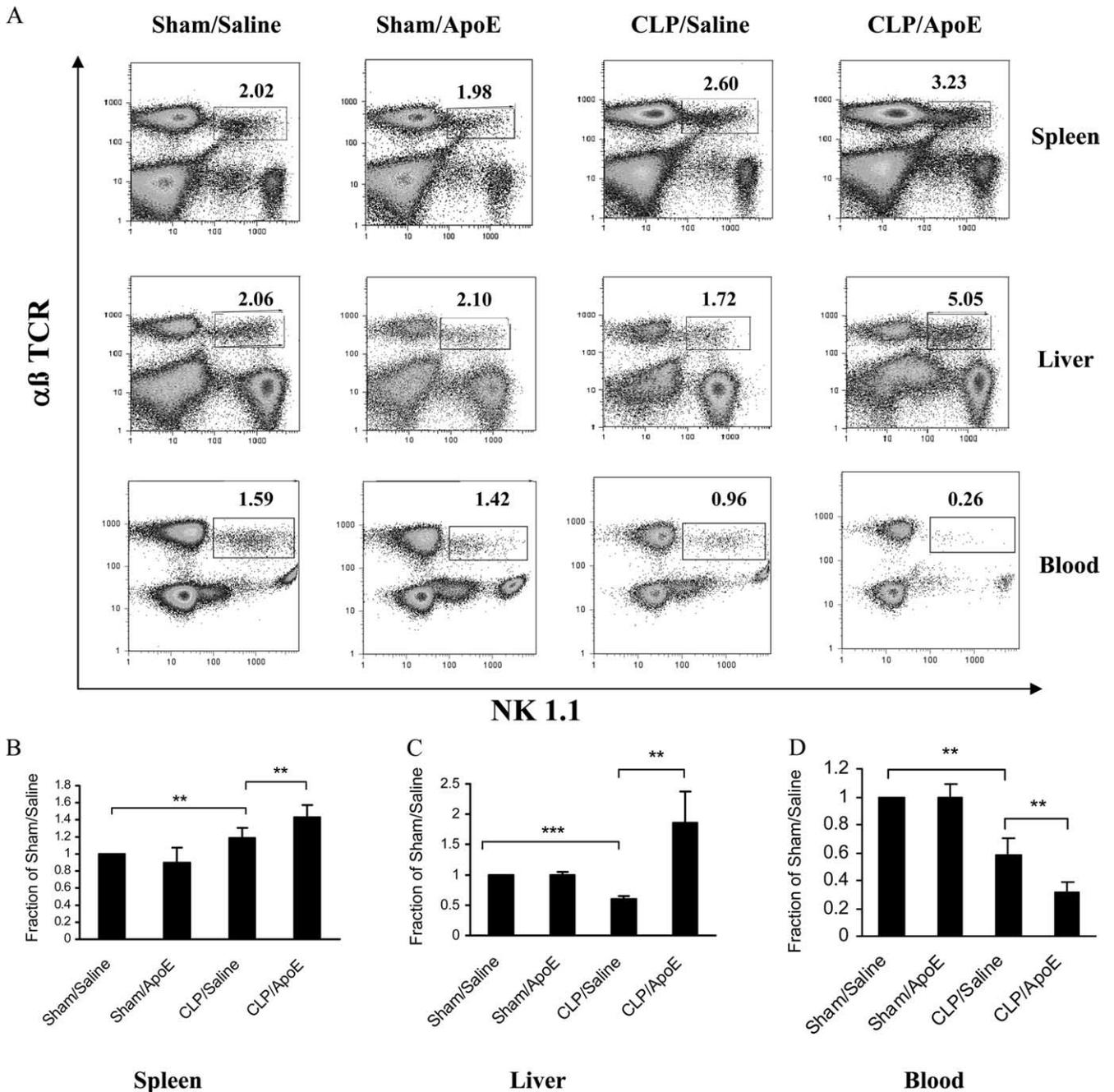
FIGURE 2. In vivo stimulation of CD1d-restricted NKT cell frequencies with apoE treatment during poly-microbial Gram-negative infection. Lymphocytes were isolated from spleens, livers, and whole blood of septic or sham rats, treated with apoE or vehicle. *A*, Numbers in flow cytometry panels indicate the percentage of gated lymphocytes. $\alpha\beta$TCR: $\alpha\beta$ TCR; NK1.1: NK cell-associated marker (CD161). *B–D*, Rats that underwent sham surgery with apoE or vehicle treatment had no significant differences in NKT cell frequencies in the spleen (*B*), liver (*C*), and circulation (*D*). Splenic lymphocytes from septic rats had 18% more NKT cell frequency than did lymphocytes from sham/saline rats ($p < 0.01$). NKT cell frequencies were reduced by 40% in the liver and by 42% in the circulation of septic rats as compared with sham/saline rats ($p < 0.001$; $p < 0.01$, respectively). Septic rats treated with apoE (114 μg/kg) immediately at the time of CLP and every 4 h for 24 h had increased ($p < 0.01$) NKT cell frequencies in their spleen and liver as compared with septic rats treated with vehicle, (17 and 300% more, respectively), whereas this decreased ($p < 0.01$) 47% further in the circulation. Percentages for individual cell types are shown. Data represent five to nine independent experiments and are reported as a fraction of sham/saline. **, $p < 0.01$; ***, $p < 0.001$. Error bars indicate SDs.

no effect on the mortality of sham-operated rats (Fig. 1). The mortality of CLP/saline rats increased significantly when compared with sham rats, and mortality after CLP was directly related to apoE concentration. Seven days after CLP, rats that received 114 μg/kg apoE had a mortality rate of 82.14%, whereas all of the rats that received 1.6 mg/kg died. These findings indicate that apoE, when administered at the time of and for the duration of the septic

insult, increases mortality in a dose-dependent manner in an in vivo model of sepsis.

apoE increases splenic and hepatic NKT cell frequencies in sepsis while depleting circulating NKT cells

In patients with sepsis, the subpopulation of lymphocytes changes considerably (18). NKT cells, a subset of T lymphocytes, have a
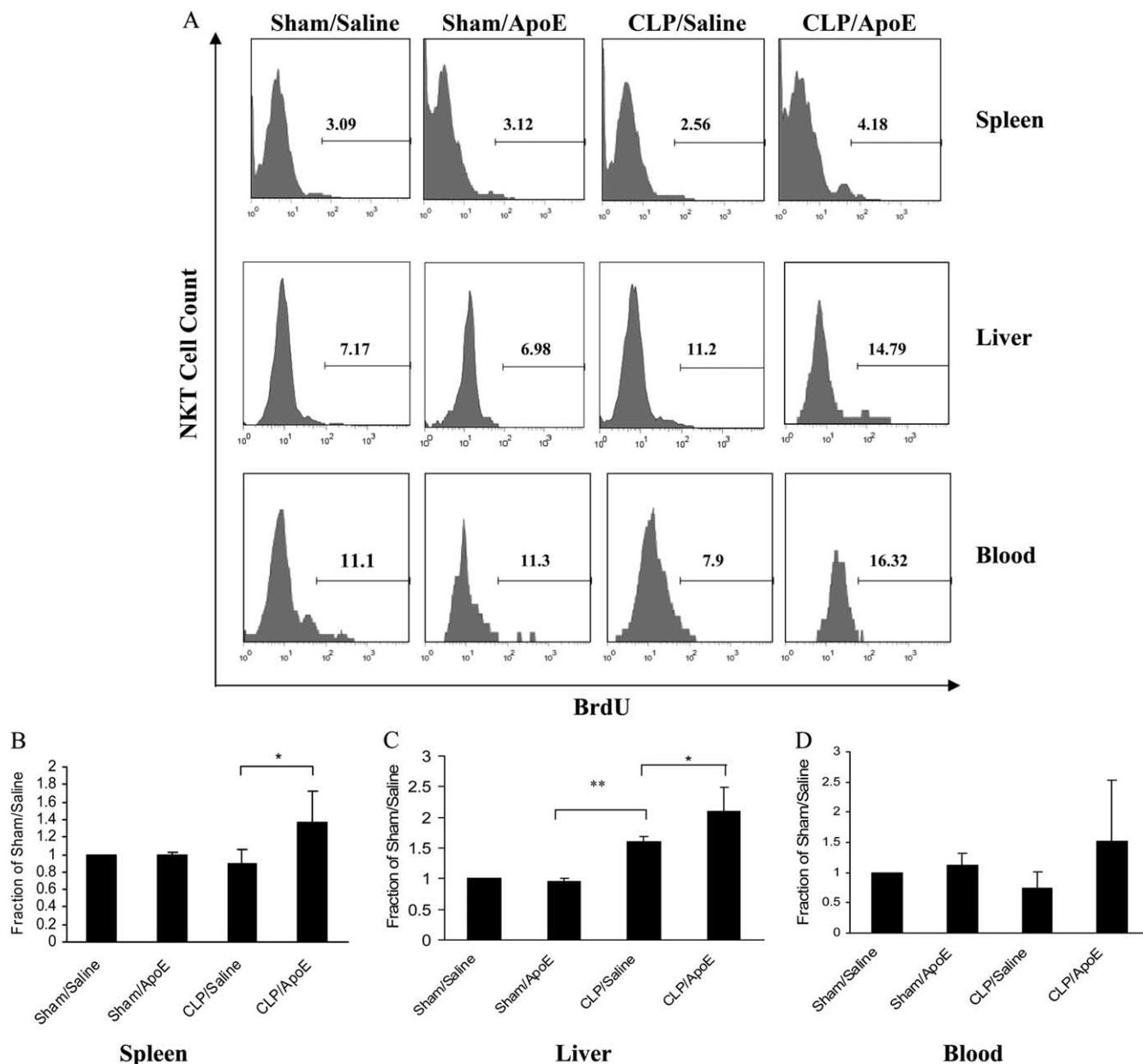
FIGURE 3. apoE-mediated increases in NKT cell proliferation in the spleen, liver, and circulation of septic rats. Rats were injected with BrdU (50 mg/kg) i.p. 3 h after CLP and lymphocytes were harvested 21 h later and were measured for BrdU incorporation in NKT cells. *A*, Percentages for BrdU$^+$ cells shown. *B–D*, Hepatic lymphocytes from septic rats had 60% more BrdU$^+$ NKT cells compared with sham rats treated with saline. Splenic and hepatic lymphocytes from septic rats treated with apoE had 54 and 31% more BrdU$^+$ NKT cells than septic rats that did not receive apoE, respectively. Data represent four independent experiments and are reported as a fraction of sham/saline. *, $p < 0.05$, **, $p < 0.001$. Error bars indicate SD.

relevant but poorly characterized role in the immunopathogenesis of septic shock induced by CLP (19). Therefore, we next investigated whether, in our model, apoE changed NKT cell frequency in the liver, spleen, and circulation, thereby contributing to immune activation and mortality. Lymphocytes from liver and spleen were harvested 24 h after CLP and treatment with saline or apoE.

Surface marker staining with the $\alpha\beta$TCR and NK1.1 Abs revealed that while there was no statistically significant difference in NKT cell frequencies in the liver, spleen, and circulation between the sham control groups, CLP-induced sepsis resulted in an increase in the splenic and a decrease in the hepatic and circulating NKT cells (Fig. 2*B*, *C*, and *D*). Septic rats receiving 114 μg/kg of apoE had significantly elevated splenic and hepatic NKT cell frequencies, whereas those in the circu-

lation were further depleted compared with septic rats that did not receive apoE (Fig. 2*B*, *C*, and *D*). This finding suggests that when apoE is added to an in vivo model of sepsis, NKT cell frequency increases in the liver and spleen, whereas it is depleted in the circulation.

apoE increases NKT cell proliferation and trafficking after CLP

Since the percentage of NKT cells was changing after CLP and treatment with apoE, we sought to determine the role of proliferation in these changes by BrdU labeling in NKT cells. Rats received one i.p. injection of BrdU 3 h after induction of CLP, and lymphocytes harvested at 24 h after CLP were measured for BrdU incorporation.

Flow cytometric analysis showed no significant changes between sham control groups in all three cell populations, as well as

no significant change in BrdU$^+$ NKT cells after CLP in the spleen and circulation (Fig. 3*B*, *C*, and *D*). However, CLP-induced sepsis did result in a significant increase in hepatic NKT cell proliferation (Fig. 3*C*). Septic rats that received apoE had significant increases in splenic and hepatic BrdU$^+$ NKT cells compared with those that did not receive apoE (Fig. 3, *B* and *C*). These results suggest that apoE causes increases in splenic and hepatic NKT cell proliferation in sepsis, whereas no significant changes occur in the circulation.

Despite clear differences in NKT and BrdU$^+$ NKT cell percentages, we sought to determine whether changes in an NKT cell population after apoE treatment were due to trafficking or local expansion by determining the absolute lymphocyte and NKT cell numbers. After CLP was induced, the total number of NKT and BrdU$^+$ NKT cells decreased in the liver by 62 and 40%, respectively, and in the circulation by 71 and 78%, respectively, when compared with sham controls (Table I). However, in septic rats that received apoE, the total number of NKT and BrdU$^+$ NKT cells increased significantly in the spleen (by 35 and 102%, respectively), and in the liver (by 275 and 437%, respectively), when compared with septic rats that received saline, but decreased in the circulation (by 78 and 64%) (Table I). Additionally, in septic rats treated with apoE, the total number of lymphocytes did not change in the spleen, increased by 35% in the liver, and decreased by 62% in the circulation when compared with septic rats that received saline. This suggests that apoE causes the absolute number of NKT and BrdU$^+$ NKT cells in the circulation to decrease while concomitantly increasing them in the spleen and liver during CLP-induced sepsis.

apoE selectively mediates stimulation in NKT cells

To investigate whether apoE selectively acts upon NKT cell populations, rather than all T lymphocytes, we next measured the levels of CD8$^+$ T cells in the spleen and liver of septic rats treated with apoE for 24 h. Flow cytometry analysis showed that after CLP, CD8$^+$ cytotoxic T cell populations increased by 12% in the spleen, 21% in the liver, and decreased by 48% in the circulation when compared with sham controls. After apoE treatment for 24 h, septic rats had 28, 101, and 68% more CD8$^+$ T cells in the spleen, liver, and circulation, respectively, than septic rats that received saline; however, these differences were not statistically significant (Fig. 4*B*, *C*, and *D*). Therefore, these results suggest that apoE selectively acts upon NKT cells, rather than producing a generalized stimulatory effect on all T lymphocytes within the spleen, liver, and circulation.

Increases in T cell populations predominantly correlate with increases in Th1 cytokines

Because activated NKT cells produce a variety of cytokines, we sought to test the systemic effect and contribution of apoE on cytokine secretion. Release of IL-10, IL-2, IL-4, IL-1β, TNF-α, and IFN-γ cytokine levels was measured in sera from septic rats 24 h after they received apoE. Multiplex cytokine analysis demonstrated that septic rats had higher levels of the Th1 cytokines IL-1β and TNF-α (Fig. 5, *A* and *B*), as well as the Th2 cytokines IL-10 and IL-2 (Fig. 5, *D* and *E*), than did sham/saline rats. Septic rats treated with apoE had higher levels of IL-1β and IFN-γ (Fig. 5, *A* and *C*), and IL-10 and IL-2 (Fig. 5, *D* and *E*), present in the sera than did septic rats that did not receive apoE.

An unexpected finding was that apoE treatment in sham rats resulted in a higher level of IFN-γ, a prototypical NKT cell cytokine, than was found in sham/saline conditions (Fig. 5*C*).

Table I. *apoE stimulates changes in absolute lymphocyte, NKT, and BrdU$^+$ NKT cell numbers in sepsis[a]*

	Spleen			Liver			Circulating		
	Lymphocyte	NKT	BrdU$^+$ NKT	Lymphocyte	NKT	BrdU$^+$ NKT	Lymphocyte	NKT	BrdU$^+$ NKT
Sham/Saline	$2.36 \pm 0.3 \times 10^8$	$5.16 \pm 1.0 \times 10^6$	$1.59 \pm 0.4 \times 10^5$	$1.59 \pm 0.1 \times 10^7$	$4.13 \pm 1.5 \times 10^5$	$2.97 \pm 1.0 \times 10^4$	$3.9 \pm 0.1 \times 10^7$	$5.18 \pm 1.0 \times 10^5$	$2.26 \pm 0.8 \times 10^4$
Sham/ApoE	$2.63 \pm 0.4 \times 10^8$	$5.13 \pm 0.2 \times 10^6$	$1.63 \pm 0.2 \times 10^5$	$1.68 \pm 0.2 \times 10^7$	$4.45 \pm 1.8 \times 10^5$	$3.02 \pm 1.1 \times 10^4$	$3.65 \pm 0.2 \times 10^7$	$4.78 \pm 0.9 \times 10^5$	$2.21 \pm 0.6 \times 10^4$
CLP/Saline	$1.94 \pm 0.1 \times 10^8$†	$5.24 \pm 0.7 \times 10^6$	$1.41 \pm 0.2 \times 10^5$	$9.84 \pm 0.1 \times 10^6$†††	$1.56 \pm 0.5 \times 10^5$††	$1.78 \pm 0.5 \times 10^4$†	$1.94 \pm 0.1 \times 10^7$†††	$1.46 \pm 0.3 \times 10^5$†††	$5.02 \pm 2.5 \times 10^3$†††
CLP/ApoE	$2.08 \pm 0.2 \times 10^8$	$7.07 \pm 1.6 \times 10^6$*	$2.85 \pm 0.5 \times 10^5$*	$1.33 \pm 0.1 \times 10^7$**	$5.86 \pm 1.1 \times 10^5$**	$9.55 \pm 1.9 \times 10^4$**	$7.4 \pm 0.3 \times 10^6$**	$3.14 \pm 0.4 \times 10^4$**	$1.83 \pm 0.6 \times 10^3$*

[a] Comparison of absolute lymphocyte, NKT, and BrdU$^+$ NKT cell numbers in the spleen, liver, and circulation after CLP and apoE treatment. Numbers are averages from five separate experiments. †, $p < 0.05$; ††, $p < 0.01$; †††, $p < 0.001$ for sham/saline v. clp/saline comparison; *, $p < 0.05$; **, $p < 0.001$ for CLP/saline vs CLP/apoE comparison.
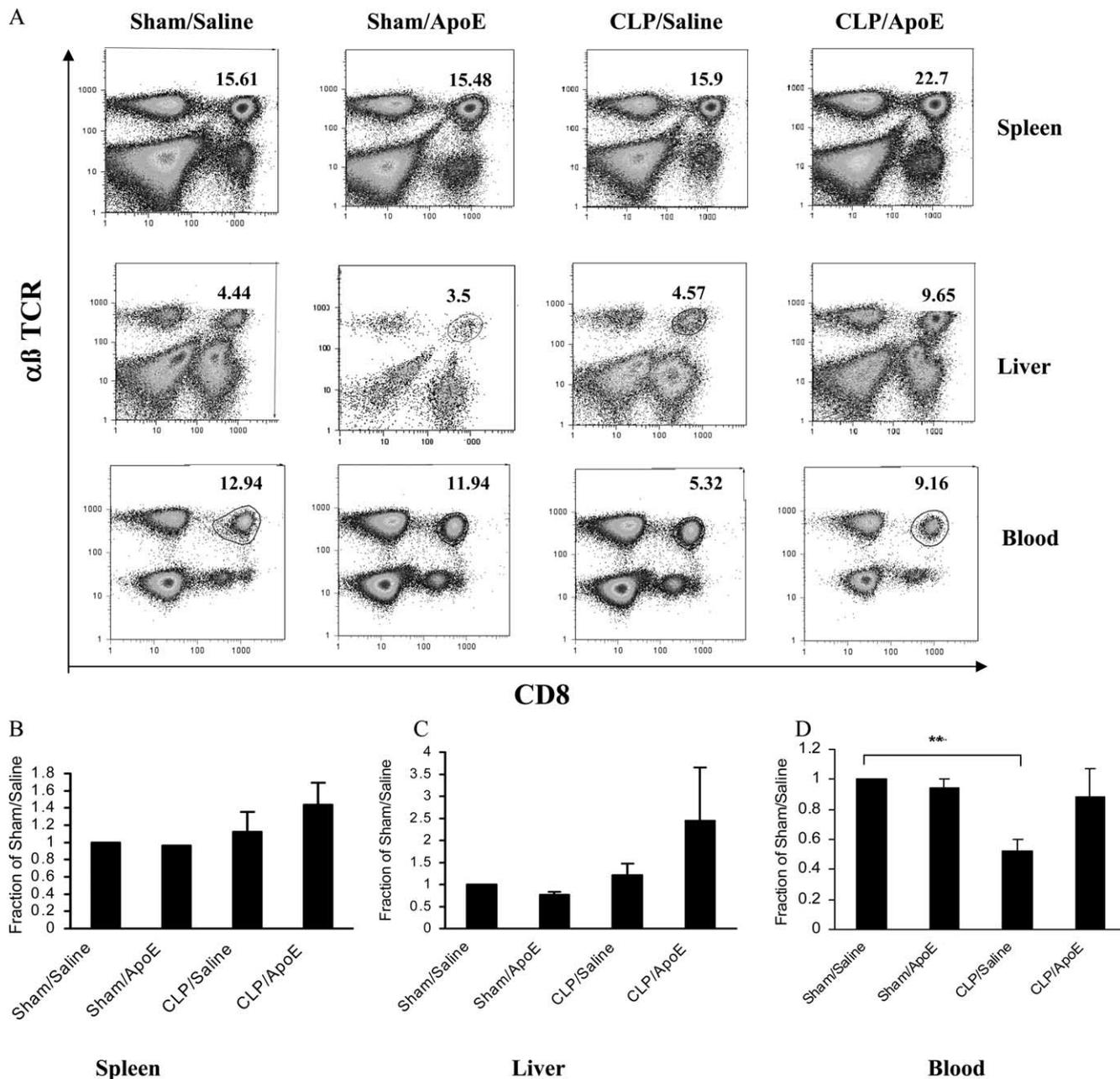
FIGURE 4. apoE-mediated stimulation is specific to NKT cell levels. *A*, Percentages of CD8$^+$ T cell types from the spleens, livers, and whole blood of rats from each treatment group are shown. *B–D*, Splenic lymphocytes (*B*) and hepatic lymphocytes (*C*) from rats subjected to CLP had more CD8$^+$ T cell populations than did sham/saline controls, whereas CD8$^+$ T cells in the circulation were reduced (*D*). The frequency of CD8$^+$ T cells in the spleens, livers, and circulation of septic rats treated with apoE (114 μg/kg) for 24 h were increased. Data represent three independent experiments and are reported as a fraction of sham/saline. Error bars indicate SDs.

Moreover, apoE treatment in septic rats decreased the elevated IL-4 levels of septic rats that received saline by 4-fold, to those of the sham/saline rats (Fig. 5*F*). These findings, therefore, suggest that whereas apoE increases Th1 and Th2 cytokines in the sera of septic rats, the predominant increase is in Th1 cytokines.

apoE increases immune cell activity by decreasing hepatic T cell apoptosis and necrosis in septic rats

Because apoptosis is known to result in immune dysfunction through the induction of Th2 responses in sepsis (20–22), we investigated whether our cytokine findings correlated with decreased apoptosis. To test apoE's effect on apoptosis-induced mortality in sepsis, T cell apoptosis and necrosis were measured

in the liver. Flow cytometry staining for Annexin V and 7-AAD demonstrated that 24 h after CLP, septic rats had 167% more hepatic T cells undergoing apoptosis than did sham controls. apoE in septic rats induced a 58% reduction in hepatic T cell apoptosis when compared with septic rats that did not receive apoE (Fig. 6*A*). Septic rats also had 37% more hepatic T cell necrosis 24 h after CLP than did sham controls. Similarly to its effect on apoptosis, apoE reduced T cell necrosis levels to those of sham control rats that did not receive apoE, although only by 29% (Fig. 6*B*). These results indicate that apoE prevented liver T cell apoptosis and necrosis, both of which are characteristic of sepsis, and thereby contributed to increased survival and activity of liver immune cells.
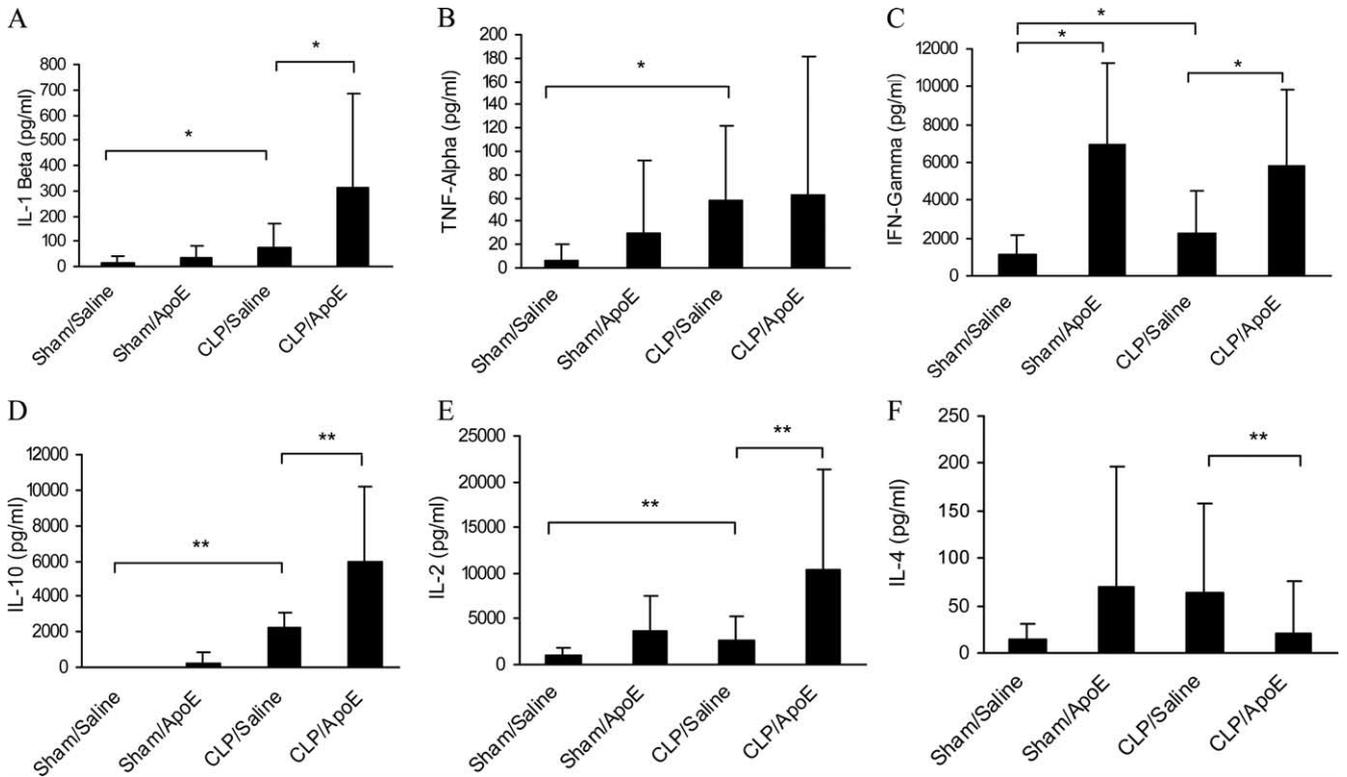
FIGURE 5. Predominant Th1 cytokine production in serum with apoE-mediated CD1d-restricted NKT cell proliferation during septic insult. Cytokine concentrations 24 h after CLP were determined by multiplex ELISA in sera of septic rats treated with apoE. *A*, IL-1β concentrations in rats treated with apoE increased by 2-fold ($p < 0.05$) over those in sham/saline rats. After treatment with apoE (114 μg/kg) at the time of CLP and every 4 h for 24 h, IL-1β concentrations increased by 4-fold ($p < 0.05$). *B*, TNF-α serum concentrations increased by at least 2-fold ($p < 0.05$) over those in sham controls, but did not change after apoE treatment. *C*, IFN-γ concentrations decreased by 3-fold ($p < 0.05$) over those in sham/apoE rats and were 2-fold higher in septic rats treated with apoE than in CLP/saline rats ($p < 0.05$). *D*, IL-10 serum concentrations in septic rats were 10-fold higher ($p < 0.01$) than those in sham/saline rats. After apoE treatment, IL-10 concentrations increased by 2-fold ($p < 0.01$) over those in rats subjected to CLP only. *E*, IL-2 concentrations in septic rats treated with apoE were 5-fold higher than those in CLP/saline rats ($p < 0.01$). *F*, apoE treatment reversed IL-4 concentrations in rats treated with CLP back to those of sham/saline controls ($p < 0.01$). Data represent means ± SDs of duplicate samples in each of at least four independent experiments. ∗, $p < 0.05$; ∗∗, $p < 0.01$.

apoE accentuates liver injury in septic rats

Hepatic dysfunction frequently accompanies a variety of bacterial infections (23). To examine the effect of apoE-induced NKT cell proliferation on hepatotoxicity, ALT serum levels were measured 24 h after CLP and apoE treatment. We found that serum ALT levels were increased in septic rats that had undergone CLP and were further increased in septic rats that received apoE. These findings indicate that bacterial sepsis induced by CLP resulted in liver injury, which was further exacerbated by apoE treatment (Fig. 7).
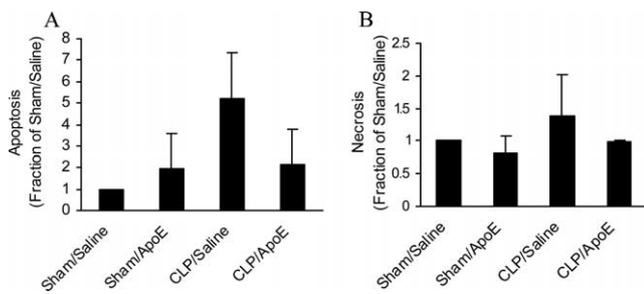

FIGURE 6. Reduction of apoptotic and necrotic T cells in the liver by apoE treatment in rats subjected to CLP. *A* and *B*, T cell apoptosis (*A*) and necrosis (*B*) levels were measured via Annexin V and 7-AAD cytometry analysis 24 h after CLP and apoE treatment. Levels in the liver were higher in CLP/saline rats than in rats that underwent sham surgery followed by either saline or apoE treatment. Treatment with apoE (114 μg/kg) in rats subjected to CLP serially every 4 h for 24 h reduced levels in the liver to those of sham controls. Data represent three independent experiments and are reported as a fraction of sham/saline. Error bars indicate SDs.
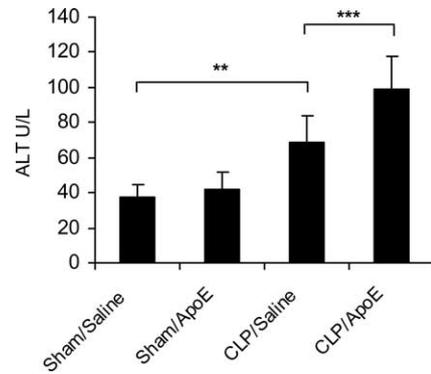

FIGURE 7. apoE increases liver injury in rats subjected to CLP. Serum concentrations of ALT were measured 24 h after rats were subjected to CLP and apoE treatment. Twenty-four hours after CLP, serum ALT levels were higher than in sham/saline rats (69.0 ± 14.7 vs 37.25 ± 6.9; $p < 0.01$). Treatment with apoE (114 mg/kg) every 4 h for 24 h after CLP further increased ALT serum levels (98.5 ± 19.4 vs 69.0 ± 14.7; $p < 0.001$) for rats subjected to CLP and saline treatment. ∗∗∗, $p < 0.001$. Error bars indicate SDs.

Discussion

Among previous characterizations of its immunomodulatory effects, apoE has recently been implicated in lipid Ag presentation to NKT cells, via CD1d (24). However, apoE's specific immunomodulatory role in sepsis is currently unknown. This study provides evidence that apoE, a LDLR ligand, has a role in increasing the immune response to polymicrobial sepsis, particularly through increased NKT cell number, proliferation, trafficking, and downstream responses, and thereby contributes to increased mortality. In our in vivo model of sepsis induced by CLP, apoE treatment increased sepsis-induced mortality, and increased NKT cell frequency and proliferation in the liver and spleen, with concomitant decreases in these NKT cell parameters in the peripheral circulation. apoE treatment also promoted a Th1 cytokine response, increased the degree of liver injury, and decreased apoptosis in hepatic lymphocytes.

Although apoE had no toxic effects on mortality in sham rats, the effect of apoE concentration on mortality during sepsis was dose-dependent; both concentrations tested resulted in significantly higher mortality in septic rats than did saline. The effect of sepsis on NKT cell frequency and proliferation differed in the spleen, liver, and circulation. The frequency of NKT cells increased in the spleen but significantly decreased in the liver and circulation. One possible explanation for this difference is the finding that hepatic apoE production decreases significantly after sepsis (25). Additionally, it has been shown that the percentages of circulating NKT cells are significantly lower in individuals with liver and infectious diseases (26–28). When compared with sham controls, septic rats had fewer NKT cells in the liver, but a greater proportion of these NKT cells were proliferating. apoE given during sepsis resulted in a significant increase in NKT cell frequency in the spleen and liver, and a decrease in the circulation, which probably contributed to the mortality we observed. Not only were the frequencies of splenic and hepatic NKT cells increased, a greater proportion of NKT cells were proliferating. This increase is consistent with studies demonstrating that mice deficient in apoE exhibited lower splenic and hepatic NKT cell activation than wild-type mice (29).

The increased percentage and proliferation of NKT cells in the spleen and liver, and the concomitant decrease in the frequency of NKT cells in the circulation after the addition of apoE, indicate that both trafficking from the circulation to the spleen and liver and local expansion of NKT cells may be occurring. The absolute number of lymphocytes, NKT, and BrdU$^+$ NKT cells confirm this. The addition of apoE in sepsis caused the total lymphocyte, NKT, and proliferating NKT cell populations to increase the most in the liver, while causing them to decrease in the circulation, suggesting a role for both pathways in the liver. However, there was no significant increase in the number of lymphocytes in the spleen, suggesting that the increase in the absolute number of splenic NKT cells may be due to local expansion, consistent with the increased number of proliferating NKT cells observed in the spleen. Furthermore, the circulation's small lymphocyte population may not significantly contribute in absolute cell number to the spleen as much as it can in the liver, which may also explain the greater hepatic response to apoE in sepsis. Evidence for preferential homing of NKT cells to the liver also supports this conclusion (30); however, apoptosis or down-regulation of T cell receptors cannot be ruled out as a cause for the depletion of circulating NKT cells (27). Studies on the mechanics of NKT cell trafficking, including the expression of chemokine and adhesion molecules, and contributions from the thymus, lymph nodes, and other organs may further characterize the dynamic redistribution of NKT cells. The lack of transgenic

models and a functional CD1d tetramer for detecting NKT cells in rats (31) limited this study. Future studies using murine transgenic models and appropriate blocking experiments could further characterize apoE's causative role in activation and proliferation of NKT cells in sepsis.

To test whether apoE affects activity of other T lymphocytes, we measured CD8$^+$ T cell populations. Despite a trend toward increased proliferation of CD8$^+$ T cells in septic rats treated with apoE as compared with septic rats that did not receive apoE, the difference was not statistically significant, indicating that apoE may selectively activate NKT cells rather than producing a generalized stimulatory effect on all T lymphocytes. As CD8$^+$ T cells are known to be influenced by NKT cell activation (32, 33), this trend could be attributed to the downstream effects of NKT cells. NKT cell activation reportedly plays a critical role in the intrahepatic immunity to several infections and liver injury (9, 34). We found ALT levels to be significantly increased after apoE treatment, which suggests that NKT cell activation contributes to sepsis-induced mortality. NK cells, which are key to the innate immune response, have been demonstrated to increase in sepsis (35). Although NK cells were significantly increased in the spleen and liver of rats 24 h after CLP, apoE treatment did not result in significant changes (data not shown), which suggests that the observed apoE-mediated Th1 response was due primarily to proliferation of NKT cells.

Endotoxic shock is frequently caused by a huge systemic cytokine response to Gram-negative bacteria and their characteristic cell-wall component, LPS. Clinically, bacterial sepsis is characterized by an imbalance between the pro- and anti-inflammatory responses of the immune system. Previous research suggests that both proinflammatory cytokines such as TNF-α, IL-1β, and IFN-γ, as well as anti-inflammatory cytokines such as IL-4, and IL-10, have relevant roles in sepsis (19). We found that sera from septic rats that received apoE had higher levels of IL-10, IL-1β, TNF-α, and IFN-γ than septic rats that received saline. Interestingly, IL-4, the main Th2 cytokine produced by NKT cells, was not elevated by apoE treatment after septic challenge. Instead, it was significantly reduced to that of control levels. Although both Th1 and Th2 cytokines were elevated, the observation that IL-4 was not suggests that apoE activation of NKT cells may predominantly elicit a Th1 cytokine response. This possibility is supported by recent data that suggest NKT cells exclusively produce IFN-γ after stimulation with LPS (36). A role for Th2 cytokines, such as IL-4, in counteracting LPS-induced shock, has been recently reported (37) and provides support for our findings. In addition, injection of a synthetic NKT cell ligand has been shown to protect against a systemic Shwartzmann reaction by increasing Th2 cytokines, depending on the timing of injection (37). Clearly, timing of NKT cell activation is a very important component of the host response to infection.

Clinical studies have shown sepsis to be a combination of a hyperinflammatory and immunosuppressive states (38). Lymphocytes (B and T cells) are central to the adaptive immune system. The profound decrease in their numbers documented in sepsis results in induction of Th2 responses by surviving immune cells (20–22). As dysregulated apoptotic immune cell death is proposed to contribute to this loss in lymphocytes, and, thus, increase in the immunosuppressive phase in sepsis (39), we sought to examine whether apoE's effect on inducing a Th1 response correlated with changes in apoptosis and necrosis. We found that whereas hepatic T cell apoptosis and necrosis were increased in septic rats 24 h after CLP, Annexin V and 7-AAD staining showed that apoE treatment equalized levels of hepatic T cell apoptosis and necrosis to those of sham animals. Although lymphocyte apoptosis is known

to contribute to septic mortality (39), as we have shown, apoE's activation of the splenic and hepatic lymphocytes mediated by NKT cells seems to have excited these cell populations into a hyperactivated state. The profound decrease in the number of T and B cells in sepsis impairs both the adaptive and innate immune response because of the important cross-talk between the systems (22, 40). Similarly, apoE's effect on preventing lymphocyte death increases the immune response, enhancing the proinflammatory response to polymicrobial infections, and, thus, increased mortality. Our findings support reports which demonstrate that inhibition of apoptosis by lipoproteins depends on APOE genotype (41), and that apoptotic cells and fragments accumulate markedly in a range of tissues in apoE-deficient mice (42). The predominant Th1 response observed in serum from septic rats treated with apoE correlates with our observations of decreased apoptosis and Th2 induction.

Among the evidence linking apoE and host immune responses to infection is the observation that apoE can bind LPS, attenuate the host inflammatory response, and protect against LPS-induced mortality (8). In contrast with these earlier findings, our results argue against a protective role for apoE. However, whereas we used serial injections of apoE in our CLP model of sepsis, the earlier study used injection of LPS as a model for sepsis and demonstrated protectability by preincubating LPS with apoE before injection, a possible reason for the different findings. Contradictory reports also exist regarding the role of NKT cells in bacterial sepsis. Some investigators have shown that α-GalCer treatment, which activates NKT cells, can induce septic shock, and propose it as a model for bacterial sepsis (43), while others have shown that activation of NKT cells can help reduce septic mortality (37). Confirming our finding that NKT cell activation contributes to CLP-induced mortality is the evidence that anti-CD1d reduces CLP-induced mortality in mice (19). Moreover, NKT cells have been shown to amplify the innate immune response to LPS (36). Although NKT cells are known to recognize glycolipid Ags, the identity of foreign and endogenous Ags remains a major question. The prototypic NKT cell Ag α-GalCer is derived from a marine sponge and is not recognized as a product of mammalian cells or pathogens. However, NKT cells have recently been found to react with a pathogen-derived lipid Ag from *Borrelia burgdorferi*, which causes Lyme disease (44, 45). We found that when apoE was given to sham rats, the level of IFN-γ, a prototypical cytokine produced by NKT cells, was higher than in sham rats treated with saline, perhaps suggesting that apoE was presenting an endogenous Ag to NKT cells. Other "indirect" mechanisms that have been suggested to amplify NKT cell activation are the dendritic cell-derived IL-12, a response to TLR activation by LPS (12, 46), and other APC-derived cytokines (36).

In conclusion, our study provides evidence that apoE hyperactivates the immune system, leading to increased morbidity and mortality in a rat model of sepsis. apoE, one possible vehicle for increased bacterial Ag presentation, increases CD1d-restricted NKT cell activity, Th1 cytokine release, and liver injury, and decreases lymphocyte apoptosis in polymicrobial infection. These findings emphasize that lipids do indeed play a significant role in the immune system and suggest that apoE may regulate the processing of foreign lipid Ags during bacterial sepsis. The *APOE* gene codes for three main isoforms of the protein: apoE2, 3, and 4 (4), which range in their binding affinities to the LDL receptor (E4 $\approx$ E3 $\gg$ E2) (47). Two intriguing questions are whether endogenous apoE could regulate host defense during sepsis, and whether deletion interferes with innate immune responses. Protection from bacterial sepsis may occur by modulating apoE's effect with anti-apoE Abs, or by taking advantage of other apoE isoforms

that may occupy LDL-receptor binding sites, to either block or delay apoE-mediated internalization of foreign Ags. Our findings raise new prospects for the role of apoE in regulating infection and immunity.

Disclosures

The authors have no financial conflict of interest.

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