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An Expanding Role for Apolipoprotein E in Sepsis and Inflammation

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Abstract

Background—Apolipoprotein E (apoE), a component of plasma lipoproteins, plays an important, but poorly defined role in sepsis. We have shown that injecting apoE increases septic mortality in a rat model of gram-negative bacterial sepsis, with concomitant hepatic natural killer T (NKT) cell proliferation and activation. The presumed mechanism for this apoE-mediated mortality is that apoE can bind and traffic antigens, presumed to include lipopolysaccharide (LPS), and promote activation of dendritic cells (DC) with subsequent NKT activation and cytokine release. Thus, we sought to prove that LPS was the antigen responsible for the increased NKT activation enhanced by the presence of apoE.

Methods—We isolated murine marrow-derived DCs, pulsed them with lipid antigen [LPS, and positive controls alpha-galactosylceramide (α -GalCer) and isogloboside 3 (iGb3)] with or without apoE, and then co-cultured the DCs with hybridoma NKTs. NKT activation was measured by interleukin-2 (IL-2) supernatant levels using ELISA.

Results—LPS at different concentrations was a weak stimulus for NKT activation regardless of apoE presence. When apoE was present, iGb3, an endogenous ligand analog, elicited more than a two-fold increase in IL-2 response when compared to iGb3 alone ($p < 0.05$).

Conclusions—These results indicate an endogenous ligand, not LPS, may be responsible for NKT activation. A molecular remnant similar to iGb3 could act as a damage-associated molecular pattern and play a prominent role in animal models of sepsis.

Keywords

Apolipoprotein E; Endogenous ligands; Lipopolysaccharide; Natural killer T cells; Sepsis

INTRODUCTION

Apolipoprotein E (apoE) is a 34 kDa glycoprotein that plays an integral role in triglyceride-rich lipoprotein catabolism. Principally synthesized in the liver, apoE can also be found in the

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brain, kidneys and spleen. It consists of an amino terminal receptor and heparin binding domain, and a carboxy terminal lipid binding site. The three isoforms of apoE, each of which differ by a single amino acid, confer differential binding affinities for the low density lipoprotein receptor (LDLR) (E4>E3>>>E2), which explains the hypertriglyceridemia found in E2 carriers [1].

ApoE was initially recognized for its role in lipoprotein metabolism, but has since been associated with cardiovascular disease and Alzheimer's disease. The E4 isoform has been linked to both, with increased plaque formation and decreased lipid clearance contributing to atherosclerosis, and enhanced neurofibrillary tangle formation promoting Alzheimer's progression in the brain. Neither mechanism is completely understood, but inflammation is believed to contribute significantly.

Only recently has the link between apoE and sepsis been investigated. Consistent with its ability to facilitate the clearance of triglyceride-rich lipoproteins by the liver, apoE has been shown both to redirect lipopolysaccharide (LPS) from Kupffer cells to hepatocytes and to protect against endotoxemia in rats [2]. ApoE-knockout mice are also more susceptible to LPS-induced lethality than are controls, despite elevated plasma lipid levels [3]. Similarly, when given intravenously, apoE has been shown to decrease LPS-induced morbidity and mortality in mice and has thus been identified to have anti-infective properties [4]. But, quite unexpectedly, our laboratory has recently discovered that infusion of apoE increased rather than decreased mortality after cecal ligation and puncture (CLP), an *in vivo* model of gram-negative bacterial sepsis [5] (Figure 1).

Interestingly, apoE has recently been implicated in the activation of Natural Killer T (NKT) cells by acting as a molecular chaperone for bacterial antigens, delivering them to antigen-presenting dendritic cells (DCs) via LDLR to activate NKT cells by CD1d, a nonclassical class-I-like antigen-presenting molecule (Figure 2) [6]. Predominantly located in the liver, activated NKT cells can secrete large amounts of T_H1 and T_H2 cytokines and appear to serve as a bridge between innate and acquired immunity [7]. When we investigated the NKT cell response in septic mice treated with apoE, we found that apoE infusion resulted in increased NKT number and proliferation, predominantly in the liver, with concomitant decreases in NKT cell number and proliferation in the periphery, implying hepatic trafficking [4]. ApoE treatment also increased T_H1 response and NKT-induced hepatocellular injury. Taken together, these observations support apoE as making a significant contribution to host defense against infection by regulating the processing of lipid antigens during sepsis, and by activating NKT cells. These observations, unlike those from previous studies [2-4], argue against a protective role for apoE, possibly because we used serial injections of apoE in our CLP model of sepsis, whereas earlier studies used an injection of LPS and demonstrated protection by pre-incubating LPS with apoE before injection.

To determine whether the observed hyperactive inflammatory response induced by apoE infusion in wild type mice was physiologically relevant, we recently performed a series of experiments showing that hypomorphic apoE transgenic mice expressing 2-5% of wild-type levels of apoE level are less susceptible to septic mortality than their induced, wild-type counterparts [unpublished data]. These findings appear to validate the pro-inflammatory contribution that apoE makes in sepsis. They also indicate that a subphysiologic level of apoE may be ideal in the face of sepsis. With all the findings to date, it appears that apoE may regulate a delicate counterbalance between sufficient systemic inflammation to clear the invading pathogen, yet insufficient to harm the host in the process of eradicating the infection.

In an effort to define apoE's role in lipid antigen presentation, we conducted *in vitro* studies looking at NKT cell activation in the presence of dendritic cells co-incubated with various lipid

antigens with apoE present or absent. We found that apoE plays no role in enhancing LPS presentation and immune activation in our *in vitro* system. Instead, we discovered that apoE enhances endogenous lipid antigen presentation and subsequent NKT cell activation. Our results have forced us to take a closer look at the murine model of sepsis and to redefine apoE's role as promoting damage-associated molecular pattern (DAMP) activation of host immunity rather than activation via pathogen-associated molecular patterns (PAMPs), like LPS.

MATERIALS AND METHODS

Isolation and preparation of dendritic cells derived from bone marrow

A protocol adapted from Lutz *et al.* was used to isolate murine bone marrow-derived dendritic cells (BMDCs) [8]. Bone marrow cells were harvested from femurs and tibias of 3-20 week-old female C57BL/6J mice (Charles River Laboratories, Wilmington, MA). Bone epiphyses were cut and the marrow was flushed out with a 25G needle and syringe. Cells were disaggregated with vigorous pipetting and filtered through a 70 μ m nylon cell strainer. Cells were washed and seeded in 100 mm bacteriological petri dishes. Cell culture medium (R10) was RPMI-1640 containing 100 U/ml penicillin and 100 μ g/ml streptomycin, 2 mM L-glutamine, and 10% heat-inactivated FCS (all from Sigma-Aldrich, St. Louis, MO). Complete R10 medium was supplemented with culture supernatant from NIH-3T3 cells transfected with the murine GM-CSF gene (a generous gift from S.-M. Kang, UCSF).

On day 0, bone marrow leukocytes were seeded at $2-3 \times 10^6$ cells per 100 mm dish in 10 ml R10 medium containing 20 ng/ml GM-CSF. On day 3, an additional 10ml of R10 containing 20 ng/ml GM-CSF was added to the plates. On days 6 and 8, half of the cell culture supernatant was collected and centrifuged; the cell pellet was resuspended in 10 ml R10 containing 20 ng/ml GM-CSF, and returned to the original plates. On day 9, non-adherent cells were collected by gentle pipetting, resuspended in fresh R10 containing 10 ng/ml GM-CSF, and plated into 100 mm tissue culture plastic dishes.

Hepatocyte preparation

Hepatocytes were isolated from 10-16 week-old C57BL/6J mice. Via the portal vein, the liver was perfused with 20 ml of liver perfusion medium (Life Technologies, Rockville, MD) followed by 5 ml of liver digestion medium (Life Technologies). The liver was removed and pressed through mesh. The liver cell suspension was collected and parenchymal cells were separated from the mononuclear cells by centrifugation at 50 X g for 5 minutes. Hepatocytes were washed twice in complete RPMI 1640 and resuspended in RPMI 1640 supplemented with 5% FCS (Life Technologies).

NKT-DC co-cultures

Bone-marrow-derived dendritic cells were then pulsed with antigens for 24 hours; 1, 10, and 100 μ g/ml LPS (Sigma-Aldrich), 100 ng/ml iGb3 (Axxora, San Diego, CA.), 100 ng/ml α -GalCer (Axxora), heat-killed aerobic and anaerobic bacteria (70° C for 1 hour, 1×10^8 bacteria/ml) or vehicle controls, with or without 1.63 μ g/ml recombinant apolipoprotein E (produced in bacteria expressing thioredoxin fusion protein as previously described [9]). Glycolipid antigens were dissolved in chloroform:methanol (2:1 ratio) and stored at -20°C. Aliquots from this stock were dried and iGb3 was reconstituted in methanol, whereas α -GalCer was reconstituted in DMSO (Sigma-Aldrich).

DN32 NKT hybridomas (a generous gift from A. Bendelac, University of Chicago) were maintained in 50% EHAA medium (Invitrogen, Carlsbad, CA.) and 50% RPMI-1640 medium supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin, 2 mM L-glutamine, 10% heat-inactivated FCS, 50 μ M β -ME, and 25 μ g/ml gentamycin (Sigma-Aldrich). Twenty-four

hours after antigenic stimulation, BMDCs were washed and co-cultured with DN32 NKT cell hybridomas at a 1:1 ratio in 96-well flat bottom plates for 24 hours. Supernatants were collected after 24 hours of culture for cytokine determination. Hepatocytes were stimulated in a similar manner as described in Trobonjaca *et al.* [10]

Cytokine detection by ELISA

Co-culture supernatants were assayed for IL-2 production by ELISA (R&D, Minneapolis, MN.).

Statistics

Differences between groups were analyzed using Student's *t*-test. Two-sided *P* values < 0.05 were considered statistically significant.

RESULTS

ApoE presumably contributes to host defense against infection by binding exogenous lipid antigens and targeting them for receptor-mediated uptake by DCs. DCs then present the antigen to NKT cells, resulting in a cytokine response [6]. In gram-negative bacterial sepsis, the lipid antigen in question is assumed to be LPS. Therefore, we sought to establish a dose-response relationship between LPS and NKT cytokine response. In our experiments, BMDCs were pulsed with varying doses of LPS (1, 10, 100 ug/mL) for 24 hours, after which the unbound LPS was washed away and the DCs were co-cultured with DN32 NKT hybridoma cells for an additional 24 hours. The IL-2 levels in the supernatant were measured using ELISA. IL-2 was chosen as our index of NKT hybridoma activation based on findings by Bendelac *et al.*, who noted robust CD1-dependent IL-2 production after thymocytes were stimulated [11]. We found that LPS alone elicited minimal cytokine response. Surprisingly, adding apoE failed to enhance the NKT response to LPS regardless of LPS dose (Figure 3). This suggests that apoE enhances inflammation through another unidentified lipid antigen, and not through LPS.

Since purified LPS did not appear to be the primary antigen that apoE presents, we wanted to confirm that LPS in the "reactive" state within the bacterial cell wall was not responsible for initiating apoE-mediated inflammation. For this experiment, we incubated DCs with heat-killed bacteria for several time periods (1, 3, 6, 24 hours), which resulted in lower IL-2 levels with longer incubation periods. When apoE was added to the heat-killed bacteria, IL-2 levels did not change significantly (data not shown). The results of this experiment indicate that although bacterial antigens caused a time-dependent decrease in cytokine levels, apoE was not involved in their enhanced presentation to NKTs.

Since LPS in the purified and natural states did not appear to be the apoE binding agents, we next sought to determine the role of endogenous ligands in apoE-mediated inflammation. Isoglobotrihexosylceramide (iGb3), a glycosphingolipid found in rat cancer cell lines and rat intestine, is a weak agonist for human and mouse NKT cells, as well as for NKT hybridomas [12]. As expected, when we used iGb3 as our endogenous ligand analog, adding it to DCs induced a moderate NKT cell response. Adding apoE to the media resulted in more than a 2-fold increase in IL-2 concentration (Figure 4). These results suggest that apoE may facilitate NKT cell activation by the endogenous antigen, iGb3. The potent NKT cell agonist alpha-galactosyl ceramide (α -GC) served as a positive control in our *in vitro* system. Its presence induced a robust NKT cell IL-2 response. The presence of apoE did not significantly alter this response (Figure 4).

Hepatocytes themselves, along with NKT cells and DCs in the liver, have been implicated in CD1d-based antigen presentation [10,13]. To determine whether the apoE-mediated antigen

presentation was unique to DCs, we isolated hepatocytes from B6 mice and exposed them for 2 hours to various antigens with or without apoE. As before, cells were washed and then co-cultured with DN32 NKT cells. We found that IL-2 release was moderate 24 hours after cells were stimulated with LPS, α -GC and iGb3. The presence of apoE did not stimulate further IL-2 release (Figure 5). Therefore, we did not demonstrate a role for hepatocytes as antigen presenting cells in the presence of apoE-chaperoned lipid antigens in our *in vitro* assay system.

Comments

Our previous work demonstrated that apoE enhances the immune response to gram-negative bacterial sepsis via increased NKT cell activation and T_H1 cytokine response. These initial findings were consistent with apoE's having been implicated in lipid antigen presentation to NKT cells via the class-I-like antigen-presenting molecule CD1d [6]. Our current study provides evidence that LPS is not the primary agent responsible for apoE-enhanced NKT cytokine elaboration during sepsis. Instead, apoE appears to enhance the presentation of an endogenous ligand, iGb3, to DCs for processing and re-presentation to NKT cells.

The cytokine-induced hyperlipoproteinemia following endotoxin exposure, the "lipemia of sepsis," is now understood to be the body's effort to decrease the bioavailability of LPS through direct neutralization and increased catabolism with subsequent hepatic clearance. With its ability to bind LPS via a large C-terminal lipid-binding domain, apoE is thought to play a critical role in endotoxin neutralization and transport. These properties support an anti-infective role for apoE that was corroborated by initial studies showing decreased LPS-induced mortality as a function of either endogenous or exogenous apoE levels [3,4]. But, recent *in vivo* studies linking increased inflammation and mortality in the more clinically relevant model of sepsis, CLP, together with *in vitro* studies implicating apoE as a lipid antigen-presenting agent, allude to its critical role in immunomodulation [5,6].

Our current findings contradict previous assumptions that LPS was the primary protagonist in the NKT-mediated pathway. Moreover, they suggest that apoE is not involved in enhancing the presentation of LPS to antigen-presenting cells and promoting inflammation. Instead, the classic TLR4 pathway may play a more prominent role in the direct inflammatory response to LPS in the *in vivo* setting. Likewise, heat-killed bacteria failed to elicit an impressive IL-2 response with or without apoE, ruling out non-LPS bacterial antigens as the NKT-inciting agent. Our search for this elusive agent led to our interest in the endogenous lipid antigen iGb3, a principal self-antigen of NKT cells and a weak agonist of NKT hybridomas and human and mouse NKT cells [14].

Natural Killer T cells have several indirect pathways of pathogen activation all mediated by DCs. In instances where exogenous antigen activation of DCs is insufficient to generate appropriate NKT cell activation, recognition of endogenous ligand by T-cell receptor is required [15]. Our findings suggest a novel mechanism by which apoE can bind iGb3, and perhaps other endogenous ligands, and deliver it by receptor-mediated uptake into endosomal compartments containing CD1, where it can be presented to NKT cells. Such a mechanism is in line with the theory that apoE hyperactivates the immune system.

The potential role of endogenous ligands in apoE-mediated septic mortality compelled us to take a second look at the classic murine model of sepsis, CLP, and the role that apoE plays in increasing septic mortality. Although the primary insult in CLP appears to be induction of fecal peritonitis and subsequent LPS-mediated inflammation (PAMPs), the traumatic ligation and puncture of the cecum could result in the release of endogenous ligands (DAMPs) and contribute significantly to inflammation as well. Our *in vitro* findings indicate that antigen presentation is more prominent with endogenous ligands than with LPS. The contribution of

DAMPs to DC and NKT cell activation is not unprecedented, having been studied in attempts to develop immunotherapies against malignancies [16].

Our study was not the first attempt to link iGb3 presentation and sepsis. Mattner *et al.* showed that Hexb (iGb3 synthesis enzyme) KO mice were unable to clear infectious bacteria, and that Hexb KO DCs exposed to LPS were deficient in stimulating NKT cells when compared to wild type DCs [17]. In addition, microbial stimulation of CD1+ APC's resulted in upregulation of glycolipid antigen synthesis [18]. How iGb3 is presented is poorly understood, but the LDLR is required for presentation of exogenous lipid antigens. Under physiological conditions, iGb3 can be internalized by DCs presumably via the LDLR, the same receptor that takes up apoE [12]. Thus, apoE, which binds LDLR with high affinity, presumably could enhance uptake of iGb3 (or similar ligands), which binds LDLR with presumably less affinity.

We believe our choice of iGb3 as our model of an endogenous ligand can be justified based on the existing literature. Biochemical evidence for iGb3 expression has been found in several mammalian species including rat intestine, the tissue that is damaged during CLP [19-23]. Although humans express the iGb3 synthase gene, it has not been purified from human tissue [24]. Despite the inability to isolate iGb3 from human cells, it is still recognized by human NKT cells and provokes robust IFN- γ secretion [14]. It appears that iGb3, or a close structural analog, is the principal self antigen of NKT cells.

In conclusion, our study provides evidence that apoE can increase NKT cell activation by enhancing endogenous ligand presentation. This finding builds upon recent evidence that apoE plays an immunomodulatory role in sepsis. It will be interesting to define the relative contribution of apoE presentation of endogenous ligands and pathogens to septic mortality, using limb ischemia reperfusion and intravenous LPS administration as model systems for each component. Protection from bacterial sepsis by means of apoE antagonism may hold more promise than previous anti-cytokine therapies because apoE can potentially act on pathogen-associated and damage-associated molecular patterns, two separate instigators of the host immune response during sepsis.

SUMMARY

Apolipoprotein E, a component of plasma lipoproteins, plays an important, but poorly defined role in sepsis. We pulsed dendritic cells with various lipid antigens in the presence or absence of apolipoprotein E, and then co-cultured these cells with NKT cells and determined NKT activation using ELISA. The results of this provide evidence that apoE can increase NKT cell activation by enhancing endogenous ligand presentation.

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Figure 1.

Effect of immediate administration of apoE on survival of wild-type rats subjected to cecal ligation and puncture (CLP). After CLP or a sham operation, apoE (114 µg/kg or 1.6 mg/kg) or vehicle (saline) was immediately injected via catheter. Rats then received the same dose concentration every 4 hours for 48 hours 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). Reproduced with permission from Kattan *et al.* 2008 [5], Copyright 2008 The American Association of Immunologists, Inc.

Figure 2.

Proposed model of apoE-mediated lipid antigen presentation and NKT cell activation. Pathogenic microbial lipids (e.g. lipopolysaccharide, LPS) are delivered to antigen-presenting cells as part of a triglyceride-rich lipoprotein (i.e. chylomicrons and very low density lipoproteins) or bound to apoE. The lipid complex binds to the LDL receptor (LDLr), is internalized, and while trafficked through the endosomal compartments, is processed and loaded onto CD1d for display on the cell surface, where it activates CD1d-restricted NKT cells. Adapted from van den Elzen *et al.* 2005 [6].

Figure 3.

ApoE does not play a role in LPS presentation to NKT cells. BMDCs were pulsed with LPS (1, 10, 100 µg/ml) for 24 hours, with or without apoE (1.63 µg/ml). Unbound antigen was washed away with PBS and BMDCs were then co-cultured with DN32 NKT hybridomas for 24 hours. Data are shown as mean IL-2 release $\pm$ SD.

Figure 4.

ApoE enhances the presentation of iGb3 to NKT cells. BMDCs were pulsed with iGb3 (100 ng/ml reconstituted in methanol), positive control α -GC (100 ng/ml reconstituted in DMSO), and the negative controls methanol and DMSO for 24 hours, with or without apoE (1.63 μ g/ml). Unbound antigen was washed away with PBS and BMDCs were then co-cultured with DN32 NKT hybridomas for 24 hours. Data are shown as mean IL-2 release $\pm$ SD.

Figure 5.

ApoE does not facilitate hepatocyte presentation of lipid antigens to NKT cells. Hepatocytes derived from B6 mice were incubated with either LPS (1 μ g/ml), α -GC (100 ng/ml), or iGb3 (100 ng/ml) with or without apoE (1.63 μ g/ml) for 2 hours. Hepatocytes were then co-cultured with DN32 NKT hybridomas for 24 hours.