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***APOE 4* is associated with enhanced *in vivo* innate immune responses in humans**

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Abstract

Background—The genetic determinants of the human innate immune response are poorly understood. Apolipoprotein (apo)E, a lipid-trafficking protein that impacts inflammation, has well-described ‘wild type’ (ε3) and disease-associated (ε2, ε4) alleles, but its connection to human innate immunity is undefined.

Objective—To define the relationship of *APOE4* to the human innate immune response.

Methods—We evaluated *APOE4* in several functional models of the human innate immune response including intravenous lipopolysaccharide challenge in human subjects, and assessed *APOE4* association to organ injury in human severe sepsis, a disease driven by dysregulated innate immunity.

Results—Whole blood from healthy *APOE3/APOE4* volunteers induced higher cytokines upon *ex vivo* stimulation with Toll like Receptor (TLR)2, TLR4, or TLR5 ligands than blood from *APOE3/APOE3* subjects, whereas TLR7/8 responses were similar. This was associated with increased lipid rafts in *APOE3/APOE4* monocytes. By contrast, *APOE3/APOE3* and *APOE3/APOE4* serum neutralized lipopolysaccharide equivalently and supported similar lipopolysaccharide responses in

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ApoE-deficient macrophages, arguing against a differential role for secretory APOE4 protein. After intravenous lipopolysaccharide, *APOE3/APOE4* human subjects had higher hyperthermia and plasma TNF α and earlier plasma IL-6 than *APOE3/APOE3* subjects. APOE4-targeted replacement mice displayed enhanced hypothermia, plasma cytokines, and hepatic injury, and altered splenic lymphocyte apoptosis after systemic lipopolysaccharide compared with APOE3 counterparts. In a cohort of 828 severe sepsis patients, *APOE4* was associated with increased coagulation system failure among European American subjects.

Conclusions—*APOE4* is a determinant of the human innate immune response to multiple TLR ligands, and associates with altered patterns of organ injury in human sepsis.

Keywords

Apolipoprotein; Toll like Receptor; Lipopolysaccharide

INTRODUCTION

Apolipoprotein (apo)E is a 34-kDa lipid-binding protein expressed by macrophages and carried on serum lipoproteins (i.e., very low density lipoprotein [VLDL], high density lipoprotein [HDL]). Beyond its well-described role in lipoprotein clearance, apoE has also been shown to have immunomodulatory actions. *ApoE*^{-/-} mice display higher mortality than wild type counterparts after challenge with lipopolysaccharide (LPS) or live bacteria.¹⁻³ In addition to anti-inflammatory effects on macrophages,⁴ apoE binds LPS and promotes its clearance by the liver.⁵

In humans, there are three well-described major isoforms of apoE designated apoE2, apoE3, and apoE4, encoded by allelic variants of the *APOE* gene on chromosome 19 (frequencies ~5–10%, 65–70%, and 15–20%, respectively), allowing for inheritance of six possible genotypes.⁶ The isoforms differ by cysteine-arginine interchanges at two residues (i.e., apoE2 [Cys¹¹², Cys¹⁵⁸], apoE3 [Cys¹¹², Arg¹⁵⁸], apoE4 [Arg¹¹², Arg¹⁵⁸]). Compared with the common ('wild type') apoE3 variant, which is found mostly in HDL particles, apoE4 has increased N-C terminal interactions that favor its association with VLDL⁷ and decreased stability.⁸ In addition to associations with cardiovascular disease and Alzheimer's disease,⁶ apoE4 has more recently been linked to enhanced inflammation in transfected cell lines and rodent models.^{9, 10} Herein, we investigated whether *APOE4* modifies the innate immune response of primary human cells to Toll like Receptor (TLR) ligands, and translated our findings to human sepsis by evaluating the association of *APOE* genotype to *in vivo* LPS responses in humans, and to disease severity measures in a large cohort of human sepsis patients.

METHODS

Human *ex vivo* whole blood assay of TLR-induced cytokines

Normal, healthy subjects were identified by *APOE* genotype using the NIEHS Environmental Polymorphisms Registry,¹¹ and enrolled in accordance with an NIEHS IRB-approved protocol. Exclusion criteria included age <18 years, active smoking, use of anti-inflammatory (e.g., non-steroidal anti-inflammatory drugs) or immunosuppressive (e.g.,

corticosteroids) agents, confirmed/suspected immunodeficiency, and recent gastrointestinal or respiratory illness. Blood was drawn from fasting subjects per an NIEHS IRB-approved protocol before 10:00 A.M. and was anticoagulated with pyrogen-free citrate (0.1 M, pH 7.2), diluted 1:1 with RPMI 1640 medium, and then added to each well of a 96-well plate containing TLR ligands (1 µg/ml Pam3CKS4; 8×10^7 heat-killed *Listeria monocytogenes*; 1 ng/ml LPS; 1 µg/ml flagellin; 1 µg/ml CL075). After incubation (3h, 37°C, 5% CO₂), supernatants were harvested for cytokine analysis, and results were normalized to absolute monocyte number for each subject as previously reported.¹²

Human intravenous LPS exposure

All subjects provided written, informed consent under guidelines approved by the IRB of the UMDNJ-Robert Wood Johnson Medical School. Inclusion criteria were: adults aged 18–40 years and “normal general health” as demonstrated by medical history, physical examination, and laboratory testing. Women of childbearing potential were screened for pregnancy risk and included if utilizing reliable contraception. Subjects underwent an initial screening visit that included the comprehensive history, physical, and laboratory testing to establish suitability for inclusion; eligible subjects returned within 3 weeks for admission to the clinical research center (CRC). Volunteers were administered LPS 2 ng/kg (CC-RE, lot 2) as an intravenous (i.v.) bolus, as reported.¹³ Vital signs and blood samples were obtained and recorded at various times (–24, 0, 0.5, 1, 1.5, 2, 3, 4, 6, 24h) post-LPS.

Murine *in vivo* LPS exposure

APOE3- and APOE4-TR female mice on a C57BL/6 background (Taconic Farms, Hudson, NY), 8–12 weeks old and weighing 18–22g, were injected intraperitoneally (i.p.) with saline or 2 mg/kg *E. coli* 0111:B4 LPS. All experiments were performed in accordance with the Animal Welfare Act and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals after review by the NIEHS Animal Care and Use Committee. Plasma was analyzed for ALT and LDH concentrations using a AU400e clinical analyzer (Beckman Coulter, Irving, TX).

Severe sepsis patients in Consortium to Evaluate Lung Edema Genetics (CELEG)

The CELEG study¹⁴ enrolled patients with severe sepsis or septic shock. This study was approved by human subjects research committees at Johns Hopkins University and the University of Maryland, Emory University, the Medical College of Wisconsin (Milwaukee, WI), and the University of Colorado (Denver, CO).

Cytokine analysis

Cytokines were quantified by multiplex assay (Bio-Plex, Bio-Rad, Hercules, CA) or by ELISA (eBioscience).

Statistical analysis

Associations between *APOE* genotype and disease risk among septic patients in CELEG were tested by multivariate logistic regression analysis, including age, sex, and ancestry as covariates. The relationship of *APOE4*-positivity to injury in individual organ systems, as

quantified by the Brussels score,¹⁵ was performed using linear regression. Age was recorded as a categorical variable with three categories: 40 years, 41–59 years, and 60 years. CELEG analyses were performed with Stata 9.0 (Stata Corp., College Station, TX). Analysis of human and murine responses to TLR ligands was performed using GraphPad Prism (San Diego, CA) or SPSS (IBM, Armonk, NY) and Statistica Software v7 (StatSoft, Tulsa, OK). Data are represented as mean $\pm$ SEM. Student's *t* test was applied for comparisons of two groups, and repeated measures ANOVA for analysis of measurements over time. For all tests, *p* < 0.05 was considered significant.

Additional information on the methods is provided in an online supplement.

RESULTS

Blood from *APOε 4* human subjects displays enhanced responses in multiple TLR cascades

In order to first define whether *APOε4* is associated with an altered human innate immune response, we collected peripheral blood from 7 *APOε3/APOε3* and 7 *APOε3/APOε4* healthy adult volunteers, and stimulated it *ex vivo* with a panel of TLR ligands.¹² Whole blood was selected rather than isolated leukocyte populations as a model of the integrated human *in vivo* innate immune response given that apoE is both expressed in circulating monocytes and present in serum. Total leukocyte count, % monocytes, % neutrophils, and % lymphocytes were equivalent in blood from the two *APOε* genotypes (Figure E1).

APOε3/APOε4 whole blood induced significantly higher TNF α than *APOε3/APOε3* blood in response to ligation of TLR2 (as well as TLR1, which dimerizes with TLR2 in response to Pam3CSK4¹⁶), TLR4, and TLR5, but not TLR7/8 (Figure 1). *APOε3/APOε4* blood also induced higher levels of a much wider panel of pro-inflammatory cytokines (Figure 2A) and chemokines (Figure 2B–C) after exposure to TLR2 or TLR4 ligands. LPS transduces early signals through TLR4 via the adaptor myeloid differentiation primary response protein 88 (MyD88), and other responses through a MyD88-independent pathway.¹⁷ Figure 2C demonstrates that LPS-induced levels of CXCL10/IP-10, a MyD88-independent chemokine,¹⁷ were significantly higher in *APOε3/APOε4* blood than *APOε3/APOε3* blood, indicating that apoE4 is also associated with enhanced MyD88-independent TLR4 signaling. In contrast, *APOε3/APOε3* and *APOε3/APOε4* blood induced equivalent levels of IL-1 receptor antagonist, an anti-inflammatory mediator (Figure 2D). Neither LPS nor Pam3CSK4 induced significant levels of the Th2 cytokine IL-4 in blood from either genotype (data not shown). Taken together, these results indicate that apoE4 is associated with broad, yet selective enhancement of pro-inflammatory output from multiple human TLR cascades.

APOε 3/APOε 3 and *APOε 3/APOε 4* sera are equally permissive to LPS signaling

ApoE is expressed by monocytes/macrophages and, as a secreted protein, is also abundant in serum, where it binds LPS.⁵ *APOε3/APOε3* and *APOε3/APOε4* humans have very similar serum apoE levels.⁶ In order to address, however, whether serum apoE3 and apoE4 confer differential LPS responsiveness upon leukocytes, we next cultured RAW 264.7

macrophages (which are apoE-null⁴) in DMEM with 10% serum from *APOε3/APOε3* or *APOε3/APOε4* subjects, and exposed them to LPS. Macrophages cultured in the two sera induced equivalent TNFα (Figure 3A). Consistent with similar LPS neutralization, equivalent LPS was also detected by Limulus amoebocyte lysate assay in *APOε3/APOε4* and *APOε3/APOε3* serum after spiking of LPS (data not shown). These findings suggest that the heightened TLR responsiveness of *APOε4*⁺ human blood is unlikely to be conferred by differential effects of secretory apoE4.

Augmented lipid rafts in *APOε 3/APOε 4* human monocytes

Lipid rafts are cholesterol-enriched membrane microdomains that organize cellular signaling events.¹⁸ A cell surface-associated pool of apoE attenuates raft-dependent cell signaling by depleting lipid rafts through induction of cholesterol efflux.¹⁹ ApoE4, however, is less effective than apoE3 at inducing cholesterol efflux from macrophages.²⁰ Several TLR cascades are initiated in lipid rafts and are enhanced by raft cholesterol loading, such as occurs with deficient cholesterol efflux. Given this, we next quantified lipid rafts in human peripheral blood monocytes, using fluor-tagged cholera toxin B subunit, a widely used raft probe that recognizes the raft-specific marker GM1.¹⁹ Flow cytometric analysis revealed higher raft signal in CD14^{hi} monocytes from *APOε3/APOε4* subjects than in those from *APOε3/APOε3* counterparts (Figure 3B–D). A similar trend for higher raft signal with *APOε3/APOε4* genotype was observed within the CD14^{lo} monocyte subpopulation. By contrast, we found that total cell-surface expression of CD14, itself a co-receptor for TLR2, TLR4, and TLR7,^{21–23} was equivalent between *APOε3/APOε3* and *APOε3/APOε4* monocytes (Figure E2). Monocytes are major innate responder cells in blood. Taken together, our data suggest that the enhanced TLR responses of *APOε3/APOε4* blood may arise, at least in part, from augmented lipid raft assembly in monocytes.

APOε 3/APOε 4 human subjects display enhanced responses to *in vivo* LPS challenge

In order to further verify the functional relationship of *APOE* variants to the human innate immune response as it occurs *in vivo*, we next utilized the human endotoxemia model. Endotoxemia (i.e., LPS in the bloodstream) occurs frequently during human sepsis, originating from Gram-negative bacterial infection and/or LPS translocation from the intestine, and is predictive of sepsis severity.²⁴ Thirty-five healthy human volunteers (age 24.5 ± 1.0 years, M:F = 22:13; Figure E3, Table E1) were challenged with intravenous LPS.¹³ Of these, 18 subjects were *APOε3/APOε3*, and 7 were *APOε3/APOε4*.

Following LPS challenge, *APOε3/APOε4* subjects demonstrated greater, sustained body temperature increases compared to *APOε3/APOε3* subjects (Figure 4A), as well as significantly higher plasma TNFα (Figure 4B). Maximal plasma IL-6 was equivalent between genotypes, although IL-6 appeared to peak earlier in *APOε3/APOε4* subjects (Figure 4C). In order to determine whether a single copy of ε4, whether paired with ε3 or ε2, is sufficient to confer enhanced LPS responsiveness, we also compared ε4[−] (i.e., *APOε3/APOε3* or *APOε2/APOε3*) to ε4⁺ (i.e., *APOε3/APOε4* or *APOε2/APOε4*) subjects in the study cohort. ε4⁺ subjects indeed experienced significantly higher increases in temperature and plasma TNFα after LPS, as well as a trend to earlier induction of plasma IL-6 that approached significance ($P=0.064$; Figure E4).

APOE4-targeted replacement mice display augmented inflammation and organ injury after LPS challenge

We next further modeled endotoxemia by systemic injection of *E. coli* 0111:B4 LPS into mice with targeted replacement (TR) of the *ApoE* locus with either human *APOE3* or *APOE4*. In addition to allowing study of sepsis-induced organ injury, this model provides the advantage in comparison with the former two of avoiding possible confounding by unknown functional polymorphisms in the human genome outside of the *APOE* coding sequence that might be in linkage disequilibrium with *APOE4*. Similar to our findings in the human *in vivo* LPS model, endotoxemic APOE4-TR mice displayed greater thermal dysregulation than did APOE3-TR mice, in this case a more profound hypothermic response (Figure 5A). APOE4-TR mice also displayed a greater increase in plasma TNF α and G-CSF than APOE3-TR counterparts, whereas plasma IL-6 was equivalent between strains (Figure 5B–D).

Six hours post-LPS, APOE4-TR mice displayed higher plasma alanine aminotransferase, a marker of hepatic injury, than did APOE3-TR counterparts (Figure 5E), as well as a trend to higher plasma lactate dehydrogenase, a clinical marker of cell lysis (Figure 5F). Blood urea nitrogen, a marker of renal function, and creatinine kinase, a marker of muscle injury, were equivalent between the two strains 6 hours after LPS challenge (Figure 5G). Splenic lymphocyte apoptosis may contribute to the immunocompromised state seen in septic patients. Compared to APOE3-TR counterparts, APOE4-TR mice displayed equivalent apoptosis of CD8⁺ T cells, but lower apoptosis of CD4⁺ T cells and B cells after LPS (Figure 5G).

APOE 4 is associated with altered patterns of organ injury in severe sepsis patients

Given the enhanced immune responses observed in *APOE4*⁺ human subjects and the exacerbated endotoxemia phenotype in *APOE4*-TR mice, we hypothesized that $\epsilon 4$ would also associate with increased illness severity in human sepsis. Sepsis syndrome arguably represents the prototypical example of dysregulated innate immunity in humans. In order to address this, we analyzed data from the Consortium to Evaluate Lung Edema Genetics (CELEG), a study of European American (EA) and African American (AA) severe sepsis patients recruited upon ICU admission, a subset of whom also developed acute lung injury (ALI).¹⁴

Using PLINK (<http://pngu.mgh.harvard.edu/purcell/plink>), all *APOE* genotypes were found to be in Hardy Weinberg equilibrium in the CELEG study population. Genotype composition of the study group is depicted in Figure E6. Stratification of the EA and AA subjects by *APOE4* status indicated no significant differences between *APOE4*⁺ (i.e., *APOE2/APOE4*; *APOE3/APOE4*; *APOE4/APOE4*) and *APOE4*[−] (i.e., *APOE2/APOE2*; *APOE2/APOE3*; *APOE3/APOE3*) patients in age, site of infectious insult, or underlying comorbidities (Table E2). Just under half of all subjects had ALI or acute respiratory distress syndrome (ARDS), but there was no significant difference between *APOE* genotypes. Among EA subjects, *APOE4*⁺ patients had an increased Acute Physiology and Chronic Health Evaluation (APACHE) II score, a grading system that quantifies sepsis severity,²⁵ although this difference fell marginally short of statistical significance. There was no significant

difference between *APOε* genotypes in the percentage of subjects requiring vasopressor support, ICU duration, ventilator-free days, or 60-day survival.

By logistic regression, no association was found between *APOε4* genotype and development of ALI/ARDS in either EA or AA severe sepsis patients (data not shown). Nonetheless, given the borderline difference in APACHE II score between *APOε4*⁺ and *APOε4*⁻ subjects, we examined organ injury by *APOε4* status further with the use of the Brussels score, a scale that quantifies organ injury during critical illness across six organ systems.¹⁵ In a linear regression of organ injury severity by *APOε* genotype (Table I), among EAs, *APOε4* was associated with an increase in coagulation system dysfunction (i.e., thrombocytopenia) that was of borderline statistical significance. By contrast, among AAs, *APOε4*⁺ status was associated with reduced hepatic dysfunction (i.e., hyperbilirubinemia). Pursuing the coagulation finding further, we confirmed that, among EAs, a significantly higher proportion of *APOε4*⁺ than *APOε4*⁻ subjects had an abnormal Brussels coagulation score (43.6% vs. 30.5%; *P*=0.011)(Table II). Finally, in a multivariate logistic regression analysis adjusting for APACHE II score, we confirmed that *APOε4*⁺ patients were 1.67 times likelier than *APOε4*⁻ patients to have an abnormal coagulation status (*P*=0.018)(Table III).

DISCUSSION

APOε4 has been associated in many reports with increased risk for diseases that are thought to arise in part from chronic inflammation, most notably atherosclerotic cardiovascular disease and Alzheimer's disease. Here, we show for the first time, using primary *ex vivo* and *in vivo* human systems, that a single copy *APOε4* is associated with marked augmentation of the human innate immune response. This enhancement extends across several plasma membrane-localized TLR pathways (TLR1, TLR2, TLR4, TLR5), but, at least as judged by studies with one model ligand, does not extend to TLR7/8, an endosomal TLR.

Several roles have been identified for apoE in innate immunity; however, how the apoE4 variant impacts these functions remains unclear. While it is interesting to speculate that the preference of apoE4 for VLDL may impact LPS presentation and/or clearance, our finding of differential effects of apoE4 on TNFα vs. IL-6 in the *in vivo* LPS models argues against this. Moreover, we did not detect a differential effect of *APOε3/APOε4* serum on LPS neutralization. Soluble apoE is also reported to suppress inflammation through a mechanism involving ligation of LDL receptor (LDLR) family members.⁴ ApoE3 and apoE4 display equivalent LDLR binding,⁶ suggesting that differential receptor interaction is an unlikely mechanism. Our finding of increased GM1 signal, a widely used metric of raft abundance that tracks with raft cholesterol,¹⁹ may not provide comprehensive information on raft microenvironment/topography in *APOε3/APOε4* monocytes. Nonetheless, it does suggest that apoE4 may enhance TLR signaling via relative augmentation of lipid raft assembly. Slightly larger, but still modest increases in raft GM1 are reported in murine hematopoietic stem cells in the setting of apoE deletion, and correlate with enhanced raft-dependent cytokine signaling.¹⁹

In analysis of the CELEG study population, hard clinical outcomes, such as survival, vasopressor requirement, and ventilator-free days showed no relation to *APOE4* status. Important differences between the functional models and the clinical analysis should be acknowledged. In addition to the fact that clinical sepsis is fundamentally different from and far more complex than the functional systems used in this study, patients in CELEG were recruited after onset of organ injury. Thus, unlike the *ex vivo* or *in vivo* functional challenges, the innate immune response in CELEG subjects was likely well beyond its initial hours. These contextual differences notwithstanding, our data may suggest that, despite its clear regulation of human *in vivo* responses to i.v. LPS, *APOE4* does not meaningfully impact human severe sepsis. As *APOE4* has been shown to associate with several other diseases in which innate immunity plays a role, including atherosclerosis, Alzheimer's disease, and, more recently, viral infection,^{6, 26} our findings raise the interesting possibility that it may do so through regulation of the innate immune response. For example, our findings may serve to explain an earlier report that *APOE4* carriers have heightened systemic inflammation after cardiopulmonary bypass.²⁷

The finding of increased coagulation system failure in *APOE4*⁺ EA sepsis patients, while of uncertain overall clinical impact, raises interesting issues. Platelets play complex roles in inflammation and sepsis pathogenesis.^{28, 29} Several studies indicate that apoE protein modifies platelet function.^{7, 30–32} Thus, *ApoE*-deficient mice display enhanced platelet reactivity that contributes to vascular injury.^{30, 33} Conversely, apoE protein inhibits platelet aggregation through induction of platelet nitric oxide,³² a phenomenon that may be altered in *APO* deficient mice display *4*⁺ subjects.³¹ Increased cholesterol loading of platelets in mice causes platelet hyperreactivity as well as thrombocytopenia due to increased platelet clearance.^{34, 35} Our finding of increased lipid rafts in *APOE4*⁺ monocytes, together with past reports that apoE4 protein is defective in promoting cholesterol efflux from cells,²⁰ raises the interesting possibility that the *APOE4* genotype may confer abnormalities in platelet function and number through effects upon platelet cholesterol.

Future studies will be required to determine whether the EA-specific association of *APOE4* with coagulation system failure can be replicated. Prior studies have found race-specific associations of *APOE* genotypes with several phenotypes,^{36–40} and it has been proposed that this could arise from race-specific patterns of linkage disequilibrium with other causal variants, from gene-gene epistatic effects, or from gene $\times$ environment interactions. Indeed, race-specific differences in distribution of functional *APOE* promoter variants across *APOE2*, *APOE3*, and *APOE4* alleles have been reported.³⁹ Of note, our *ex vivo* whole blood cytokine induction studies used predominantly AA donors (N=5/7, *APOE3/APOE3*; N=6/7, *APOE3/APOE4*), and stratification of our analyses of monocyte rafts and human *in vivo* TNF α induction by race revealed similar increases in outcome measures with *APOE3/APOE4* in AAs and EAs (data not shown). Together, this suggests that any mechanism of *APOE4* on induction of the innate immune response may be dissociated from its relationship to sepsis-associated thrombocytopenia. Genetic determinants have been proposed for differences in platelet count between EAs and AAs,^{41, 42} and recently a miR-376c-PCTP-PAR4 axis was identified in platelets from AA subjects that underlies their increased reactivity to the PAR4 thrombin receptor.⁴³ Taken together with reports that cholesterol

efflux can directly regulate platelet number through impacting signaling in platelet progenitors,⁴⁴ this suggests the possibility that *APOE4* could directly impact platelet biology independently of its effect on systemic inflammation.

ApoE mimetic peptides that show potent anti-inflammatory efficacy in the lung, intestine, and other organs of rodents^{45, 46} are currently under preclinical development as human therapeutics. Perhaps these agents will prove specifically useful in ‘rescuing’ the deficient anti-inflammatory function of native apoE protein in *APOE4*⁺ humans. Intriguing in light of our coagulation findings, an apolipoprotein mimetic peptide was recently shown to inhibit platelet aggregation and prolong bleeding time in apoE-deficient, but not apoE-sufficient mice.⁴⁷ Investigation of apolipoprotein mimetics in human sepsis, along with their potential pharmacogenomic interactions with *APOE*, may thus be warranted.

In sum, we report for the first time that *APOE4* is associated with augmented human innate immunity, and we provide functional confirmation in several primary models, including human *in vivo* LPS challenge. Given the high prevalence of *APOE4* and the growing application of TLR-based therapeutics in a wide variety of immune-based diseases, our findings have important public health implications that mandate further investigation. In a broader sense, as innate immunity has been implicated in a large number of disorders, infectious and non-infectious, and lipid rafts have been implicated in multiple signaling pathways in many cell types, we propose that our findings may impact a broad spectrum of human disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AA	African American
ALI	acute lung injury
Apo	apolipoprotein
ARDS	acute respiratory distress syndrome
CELEG	Consortium to Evaluate Lung Edema Genetics
EA	European American
HDL	high density lipoprotein

LPS	lipopolysaccharide
MyD88	myeloid differentiation primary response gene 88
TLR	Toll like Receptor
VLDL	very low density lipoprotein

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Key Messages

- Intravenous injection of lipopolysaccharide induces higher hyperthermia and plasma TNF α in *APO ϵ 3/APO ϵ 4* human subjects than in *APO ϵ 3/APO ϵ 3* subjects.
- Whole blood from *APO ϵ 3/APO ϵ 4* human subjects produces higher cytokines in response to *ex vivo* stimulation with Toll like Receptor ligands than whole blood from *APO ϵ 3/APO ϵ 3* subjects.
- *APO ϵ 4* is associated with increased coagulation system failure in severe sepsis patients.

Figure 1. Enhanced TNFα production by *APOε3/APOε4* human whole blood in response to TLR ligands

Whole blood from *APOε3/APOε3* (N=7) and *APOε3/APOε4* (N=7) healthy volunteers was left nonstimulated (NS) or exposed to a panel of ligands for TLR2 (1 μg/ml Pam3CKS4 ['Pam'], 8×10^7 heat-killed *Listeria monocytogenes* [HKLM]), TLR4 (1 ng/ml LPS), TLR5 (1 μg/ml flagellin [FLA]), or TLR7/8 (1 μg/ml CL075). After 3h, TNFα protein release into supernatant was quantified by ELISA. ND, not detected. *, $P < 0.05$, **, $P < 0.01$.

Figure 2. Enhanced production of a wide panel of cytokines and chemokines by *APOε3/APOε4* human whole blood in response to TLR2 and TLR4 ligands

Whole blood from *APOε3/APOε3* (N=7) and *APOε3/APOε4* (N=7) healthy volunteers was nonstimulated (NS) or stimulated for 3h with LPS or Pam3CSK4, and then the indicated cytokines (A), chemokines (B–C), and anti-inflammatory mediators (D) quantified in the supernatant by multiplex assay. *, P<0.05, **, P<0.01.

Figure 3. *APOε3/APOε4* human monocytes display increased lipid rafts

(A) RAW 264.7 macrophages were plated in DMEM with 10% human serum (pooled by *APOε* genotype) and then left nonstimulated (NS) or exposed to LPS (1 ng/ml, 2h). TNF α was then quantified in the media. Results are representative of two independent experiments. ND, not detected. (B–D) Peripheral blood monocytes from *APOε3/APOε3* (N=9) and *APOε3/APOε4* (N=10) human subjects were gated by CD14 (B), and then left unstained or stained with Alexa Fluor 488-cholera toxin B (CtB). CtB was quantified by flow cytometry, as shown by a representative histogram (C). Mean fluorescence intensity (MFI) for CtB were pooled by *APOε* genotype for quantification (D). *, $P < 0.05$; **, $P < 0.01$; #, $P = 0.11$.

Figure 4. Association of APOE genotype with *in vivo* LPS responses in humans

(A) Temperature curve in *APOE*3/*APOE*3 (N=18) and *APOE*3/*APOE*4 (N=7) subjects at various time points following i.v. LPS injection. $P < 0.05$ for inter-genotype difference. (B) Plasma TNFα levels in subjects from A. $P < 0.000001$ for inter-genotype difference. (C) Plasma IL-6 levels in subjects from A. $P = 0.11$.

Figure 5. Response of *APOE3*- and *APOE4*-targeted replacement mice to systemic LPS
(A) Core body temperature of *APOE3*- and *APOE4*-targeted replacement (hereafter, E3 and E4) mice at timepoints following i.p. saline or LPS (2 mg/kg). **(B–D)** Plasma TNF α **(B)**, G-CSF **(C)**, and IL-6 **(D)** 1.5h following i.p. saline or LPS in E3 and E4 mice. **(E–F)** Plasma alanine aminotransferase (ALT)**(E)** and lactate dehydrogenase (LDH)**(F)** 6h after i.p. saline or LPS in E3 and E4 mice. **(G)** Apoptosis was quantified in splenic CD4⁺ T cells, CD8⁺ T cells, and B cells 6h after i.p. LPS with the poly-caspase activity reporter FLIVOTM. *, P<0.05 and **, P<0.01 for inter-genotype comparison. †, P<0.05 compared to saline control within genotype. N=5/genotype. ND, not detected.

Table I

Organ injury in CELEG severe sepsis and ALI population by *APOε4* status*

	European American				African American			
	Beta	(95% CI)	Z	P	Beta	(95% CI)	Z	P
Organ system								
Cardiovascular	-0.02	(-0.32, 0.28)	-0.13	0.897	-0.04	(-0.44, 0.37)	-0.18	0.861
Pulmonary	-0.07	(-0.28, 0.08)	-0.61	0.539	-0.13	(-0.44, 0.18)	-0.80	0.423
Central nervous system	0	(-0.23, 0.15)	0	1.0	0.25	(-0.12, 0.61)	1.34	0.183
Coagulation	0.20	(-0.01, 0.41)	1.89	0.059	-0.17	(-0.43, 0.09)	-1.27	0.206
Renal	0.11	(-0.14, 0.36)	0.83	0.405	0	(-0.37, 0.38)	0.02	0.981
Hepatic	0.02	(-0.20, 0.25)	0.21	0.836	-0.38	(-0.69, -0.08)	-2.51	0.013

* Linear regression of the relationship of *APOε4*-positivity to injury in individual organ systems, as quantified by the Brussels score, which scores 4 grades of increasing dysfunction in 6 systems (Cardiovascular: systolic blood pressure; Pulmonary: PaO₂/FiO₂ ratio; Central nervous system: Glasgow score; Coagulation: platelet number; Renal: plasma creatinine concentration; Hepatic: plasma bilirubin concentration)¹⁵. Adjustment was performed as follows: 1) European American population – APACHE II score; 2) African American population – anemia comorbidity, insult (lung disease as reference), and African ancestry.

Table II
CELEG severe sepsis and ALI patients with an abnormal Brussels coagulation system score by *APOε4* status*

	European American		African American	
	<i>APOε4</i> ⁺ (N=117)	<i>APOε4</i> ⁻ (N=427)	<i>P</i>	<i>P</i>
Abnormal coagulation, N (%)	51 (43.6)	130 (30.5)	0.011	0.081

* Subjects with an abnormal coagulation system per Brussels score (defined as platelets < 150,000/mm³)¹⁵.

Table III

Relationship between *APOε4* genotype and the number of European American CELEG severe sepsis and ALI patients with abnormal coagulation *

	OR	SE	χ^2	L95	U95	P value
<i>APOε4+</i>	1.67	0.217	5.61	1.09	2.56	0.018

* Multivariate logistic regression of relationship of *APOε4*-positivity to abnormal Brussels coagulation score (platelets 150,000/mm³), adjusting for APACHE II score.