Altered immune responses in apolipoprotein E-deficient mice

D. T. Laskowitz,[1,*] D. M. Lee,[*] D. Schmechel,[*] and H. F. Staats,[*,†]

Department of Medicine (Neurology)* and Department of Immunology,† Duke University Medical Center, Durham, NC 27710

Abstract Apolipoprotein E (apoE) is a 34 kDa glycosylated protein with multiple biological properties. In addition to its role in cholesterol transport, apoE has in vitro immunomodulatory properties. Recent data suggest that these immunomodulatory effects of apoE may be biologically relevant, and apoE-deficient mice have altered immune responses after bacterial inoculation and increased susceptibility to endotoxemia induced by lipopolysaccharide (LPS). To better understand the mechanism by which apoE-modulates immune responses, we tested the role of human apoE isoforms in assays of human T cell proliferation, and analyzed the immune responses of apoE-deficient mice. Both the E3 and E4 isoforms of apoE induced similar suppression of human lymphocyte function in assays of T cell proliferation, including mitogenic responses to phytohaemagglutin (PHA), stimulation of the T cell receptor with αCD3, and antigen-specific response to tetanus toxoid. ApoE-deficient mice showed no quantitative differences in thymic, splenic, or bone marrow lymphocyte populations, nor were there in vitro abnormalities in splenocyte proliferation after stimulation with αCD3 to suggest an inherent T cell defect in apoE-deficient mice. ApoE deficient animals, however, had significantly higher levels of antigen-specific IgM after immunization with tetanus toxoid, and impaired delayed type hypersensitivity responses as compared to control C57-BL/6 mice. These results support a growing body of evidence demonstrating an interplay between lipid metabolism and immune responses, and suggest that apoE plays a biologically relevant role in regulating humoral and cell-mediated immunity.—Laskowitz, D. T., D. M. Lee, D. Schmechel, and H. F. Staats. **Altered immune responses in apolipoprotein E-deficient mice.** ***J. Lipid Res.*** **2000.** 41: **613–620.**

Supplementary key words apolipoprotein E • transgenic mice • immunity

Apolipoprotein E (apoE) is a 34 kDa glycosylated protein with multiple biological properties. Although originally described in the context of cholesterol metabolism, apoE affects innate and acquired immune responses in vitro, as evidenced by its ability to suppress lymphocyte proliferation, generation of cytolytic T-cells, and stimulation of cultured neutrophils (1–4). Recent data suggest that the immunomodulatory properties of apoE are biologically relevant, as apoE-deficient animals have impaired immunity after bacterial challenge with *Listeria monocytogenes*, and increased susceptibility to endotoxemia after intravenous lipopolysaccharide (LPS) injection and inoculation with *Klebsiella pneumonia* (5,6). Although the mechanisms by which apoE down-regulates the immune response remain to be elucidated, this adds to the growing body of evidence suggesting an interplay between lipid metabolism and immunity.

There are three common human isoforms of apoE, designated E2, E3, and E4 which differ by single amino acid interchanges at residues 112 and 158: E3 ($Cys_{112}Arg_{158}$); E4($Arg_{112}Arg_{158}$); E2 ($Cys_{112}Cys_{158}$)(7). At present, it is unknown whether there is a differential immunomodulatory effect between the E3 and E4 isoforms. The E4 allele is a major determinant of risk for sporadic and late-onset familial Alzheimer's disease (8–10), a chronic progressive neurodegenerative disease pathologically characterized by neurofibrillary tangles and amyloid plaques. The findings of reactive microglia, complement fixation, and local expression of inflammatory cytokines surrounding amyloid plaques suggest an inflammatory component to these lesions (11–13).

In this study, we address the possibility of isoform-specific immunomodulatory properties of apoE, and demonstrate that apoE suppresses human lymphocyte proliferation in physiologically relevant concentrations in vitro by utilizing different models of lymphocyte stimulation, including direct stimulation of the T cell receptor, non-specific stimulation with phytohemagglutinin, and antigen-specific T-lymphocyte stimulation. We also address the in vivo functional relevance of apoE by characterizing the immune system and testing humoral and cell-mediated immune responses in apoE-deficient mice. We demonstrate that apoE-deficient mice have impaired delayed-type hypersensitivity responses (DTH), and generate higher levels of

Abbreviations: ap, apolipoprotein; DTH, delayed-type hypersensitivity; LPS, lipopolysaccharide; PHA, phytohaemagglutin; PBMC, peripheral blood mononuclear cells; TT, tetanus toxoid; AD, Alzheimer's disease.

[1] To whom correspondence should be addressed.

antigen-specific IgM relative to control mice. These results suggest that apoE plays a biologically relevant role in modulating immune responses that is not limited to direct interaction and neutralization of lipopolysaccharide (LPS).

MATERIALS AND METHODS

Human lymphocyte proliferation assays

Peripheral blood mononuclear cells (PBMC) were purified over lymphocyte separation medium (LSM, Organon Teknika, Durham, NC) as previously described (14) from whole blood obtained from normal volunteers through a Duke Institutional Review Board approved protocol. PBMC were cultured in 96-well culture dishes at a density of 2×10^6 cells/ml. For tetanus toxoid and PHA assays, cells were cultured in RPMI 1640 (Gibco, Grand Island, NY) containing heat-inactivated 15% human A (Worldwide Biologicals, Nashville TN); for monoclonal antibody stimulation, cells were cultured in RPMI 1640 containing 5% fetal calf serum (Gibco). Cells were preincubated with control media, human recombinant apoE3, apoE4 (Panvera Corp, Madison WI), or control apolipoprotein A-I (Sigma, St. Louis, MO). Cells were stimulated with phytohemagglutinin (PHA; Wellcome Diagnostics, Research Triangle Park, NC) at a concentration of 1 μg/ml, αCD3 (OKT3) at a dilution of 1:800, and tetanus toxoid at a dilution of 1:1000 (Wyeth-Ayerst, Marietta, PA). Cells were harvested 3 days after PHA stimulation, 4 days after stimulation with αCD3, and 7 days after incubation with tetanus toxoid. Four hours prior to harvest, the cells were pulsed with tritiated thymidine (0.4 μCi/well). DNA was collected on glass fiber filters using a PHD cell harvester (Cambridge Technologies, Cambridge MA), and radioactivity was measured by scintillation counting.

Animals

The apoE-deficient mice used in this study were 10–12-week-old male mice obtained commercially from Jackson Laboratories (Bar Harbor, ME) and had been previously backcrossed for 10 generations to a C57BL/6J background (15). Control animals were age- and sex-matched C57BL/6J mice (Jackson Laboratories). This study was approved by the Duke University Animal Care and Use Committee.

All blood samples were collected from the retroorbital plexus using a heparinized Natelson capillary tube (Baxter Healthcare Corporation, McGraw Park, IL) while mice were under isoflurane anesthesia. Plasma was stored at −20°C until assayed.

Preparation of cell suspensions and other samples

For all flow cytometry and mitogenic experiments, freshly obtained cell suspensions were used. Femurs were dissected and flushed with 2 ml ice-cold RPMI 1640 to obtain bone marrow. Splenocytes and thymocytes were teased into RPMI media using tuberculin syringe plungers. Splenocytes were subsequently isolated by Hypaque-Ficoll density centrifugation. Peripheral blood was collected from the retroorbital plexus using a heparinized pasteur pipette while mice were under isoflurane anesthesia and peripheral blood erythrocytes were removed with Immunolyse™ (Coulter, Miami, FL) per manufacturer's protocol. Cell suspensions were counted on a Coulter™ Counter (Coulter, Miami, FL).

Immunofluorescence assays and flow cytometry

Analysis of cell surface phenotype was performed in both direct and indirect immunofluorescence assays. Briefly, 50 μl of cells suspended in 2×10^7 cells/ml in PBS wash (phosphate-buffered saline containing 2% bovine serum albumin and 0.1% sodium azide) were mixed with 50 μl antibody solution at saturating titer for 30 minutes at 4°C. These cells were then washed twice with 1 ml of PBS wash and resuspended in fluorochrome-conjugated secondary reagent at saturating titer diluted in PBS wash for 30 min at 4°C. Cells were subsequently washed twice with 1 ml of PBS wash, resuspended in 1 ml PBS wash containing 4% paraformaldehyde, and stored at 4°C in foil wrap until flow cytometric analysis was performed. Directly conjugated primary reagents were performed as above, except fixation occurred after the first wash series. Cell suspensions were analyzed utilizing the FACStar^{Plus} fluorescence-activated cell sorter (Becton-Dickinson, San Jose, CA). Statistical significance between populations was determined with a two-tailed paired Student's *t*-test. The following monoclonal antibodies were used: α Thy1 (American Type Culture Collection, ATCC, Rockville, MD), αCD2 (gift from Dr. H. Yagita, Tokyo, Japan), αCD4(ATCC), αCD8(ATCC), PK-136 (ATCC), F4/80(ATCC), MAC-1 (ATCC), B220(ATCC), GR-1 (gift from Dr. R. Coffman, Palo Alto, CA), TER-119 (gift from Dr. I. Weissman, Stanford CA).

Mitogenic responses of control and apoE-deficient splenocytes

For mitogenic proliferation assays, single cell suspensions of splenocytes were created by harvesting and mechanically dissociating splenic tissue from apoE-deficient and wildtype control mice. Splenocytes were adjusted to 1×10^6 cells/ml in RPMI 1640, supplemented with 5% fetal calf serum and 10 mg/ml gentamycin. Cells (1×10^5) (100 ml) were incubated in the presence of either media, 1 μg/ml PHA (Murex Diagnostics Ltd., Dartford, England), or in media, αCD3 (ATCC), αCD28, or αCD3 plus αCD28 in round-bottom 96-well microtiter plates in triplicate, and incubated at 37°C in a 10% CO_2 air humidified environment for 3 (PHA) or 4 (αCD3/αCD28) days. Six hours before harvesting, 0.4 mCi [^{3}H]thymidine was added to each well. Cells were harvested onto glass filters using a PHD™ sample harvester. Incorporation was determined by placing the filters in CytoScint scintillation fluid (ICN, Costa Mesa, CA) and counting with a scintillation counter (Tri-Carb 4000, United Technologies Packard, Downers Grove, IL). Statistical significance was determined with the two-tailed paired Student's *t*-test.

Measurement of delayed-type hypersensitivity (DTH)

For the measurement of DTH responses, an ear swelling assay was used as previously described (16). DTH determination was performed 51 days after injection with 50 μg TT in complete Freund's adjuvant, and 16 days after mice were boosted with 50 μg TT in incomplete Freund's adjuvant. Briefly, 25 μg tetanus toxoid (TT) was injected into the right ear in 10 μl sterile PBS while 10 μl sterile PBS was injected into the left ear as a control. Ear swelling was measured 24 h after injection with a dial thickness gauge (Mitutoyo, Japan, code #7326). Antigen-specific ear swelling was calculated by subtracting the ear swelling of the PBS-injected ear from the swelling of the TT-injected ear. The two-tailed Student's *t*-test was used to determine the significance of differences between groups.

Determination of serum antibodies

For determination of total IgG and IgM levels in naive apoE-deficient and control animals, serum samples were tested by enzyme-linked immunoabsorbant assay (ELISA) as per manufacturer instructions (Southern Biotechnology Associates, Inc; Birmingham, AL). For determination of anti-tetanus toxoid IgG and IgM, animals were first immunized with 50 μg tetanus toxoid (TT) in complete Freund's adjuvant delivered subcutaneously. Mice were boosted on day 28 with 50 μg TT in incomplete Freund's adjuvant. Enzyme-linked immunosorbent assay (ELISA) was used to determine the presence of anti-tetanus toxoid antibodies in serum samples. TT was suspended in CBC buffer (15 mm Na_2CO_3

35 mm $NaHCO_3$, pH 9.6) at a concentration of 3 μg/ml and plated to 96-well microtiter plates (Costar #3590, Cambridge, MA) at 100 μl/well. After overnight incubation at 4°C, the contents of the well was discarded and blocking buffer (CBC with 3% non-fat dry milk) was added at 200 μl/well. After incubating at room temperature for 2 h, plates were stored at −20°C until used. ELISA plates were washed 4 times with ELISA wash buffer (PBS, 0.05% Tween-20, 0.1% sodium azide) before the addition of samples. Plasma samples were diluted in serum diluent (PBS, 2.5% bovine serum albumin, 2.5% non-fat dry milk, 5% normal goat serum. 0.1% sodium azide, 0.05% Tween-20) and added to ELISA plates at 100 μl/well. After overnight incubation at 4°C, plates were washed four times with ELISA wash buffer before the detection antibody was added. Alkaline phosphatase-conjugated, goat anti-mouse IgG, IgM, IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology Associates, Birmingham, AL) was diluted to 1:1000 (PBS, 0.05%, Tween-20, 0.5% bovine serum albumin) and used as the detection antibody (100 μl/well). After incubation at room temperature for 3 h, plates were washed 4 times with ELISA wash buffer and reacted with alkaline phosphatase substrate *p*-nitrophenyl phosphate. After a 10-min incubation, plates were read at 405 nm on a Titertek Multiscan Plus plate reader. For antigen-specific ELISAs, sample dilutions were considered positive when the optical density recorded for that dilution was at least 2-fold higher than the optical density recorded for a naive sample at the same dilution. For calculation of the average endpoint titer of anti-TT antibody responses, the mean log of the endpoint dilutions was determined and used to calculate the average endpoint titer.

RESULTS

Human T-cell proliferation assays

To determine whether the human apoE isoforms differ in their ability to modulate the immune response, we evaluated the role of apoE preincubation on peripheral blood mononuclear cell proliferation in response to stimulation with PHA, αCD3, and tetanus toxoid. ApoE suppressed lymphocyte stimulation in all three assays of T-cell proliferation in a dose-dependent and specific manner (**Fig. 1**).

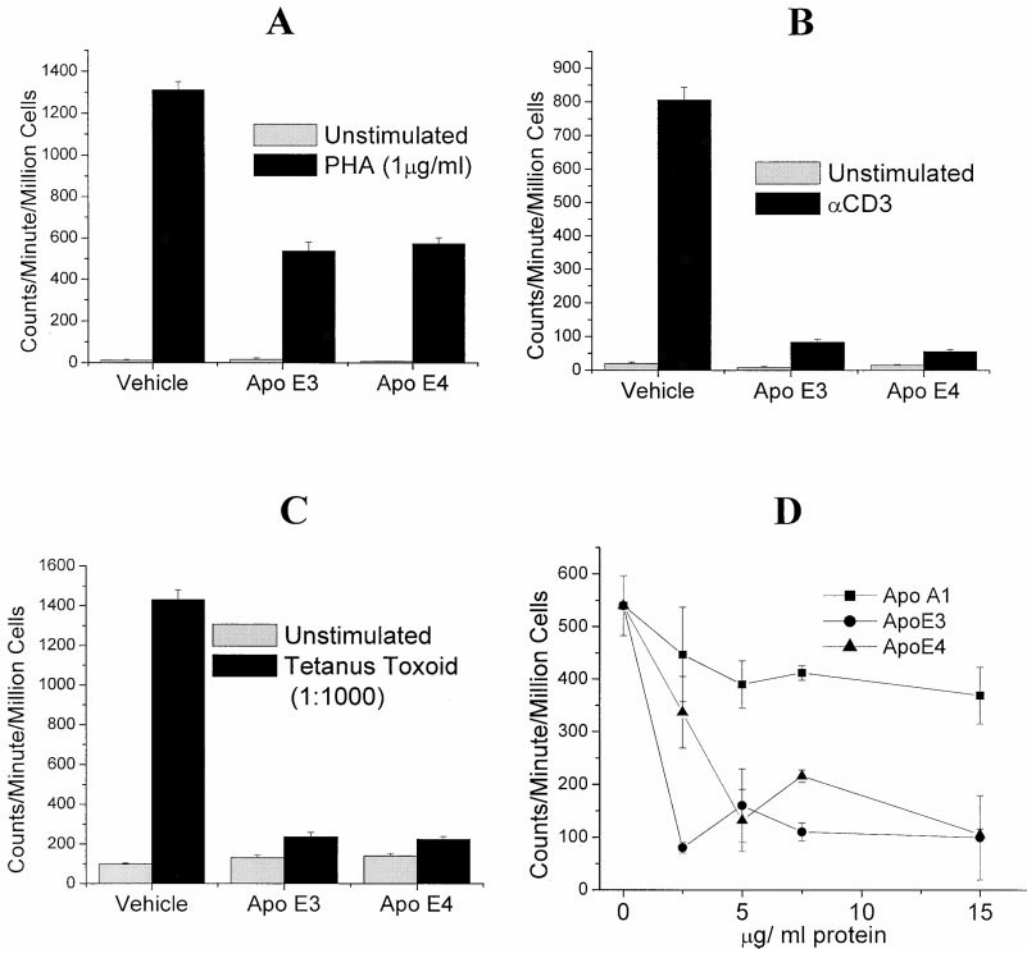

Fig. 1. Incubation of human recombinant apoE3, apoE4 (15 μg/ml) for 24 hours suppressed human mononuclear cell proliferation in three different paradigms: non-specific proliferation after 3 day exposure to 1 μg/ml PHA ($P < 0.01$ E3 or E4 vs. control, Fig. 1A); specific crosslinking of the T cell receptor after 4 days exposure to 1:800 αCD3 ($P < 0.01$ E3 or E4 vs. control, Fig. 1B); and antigen-specific responses after 7 days exposure to 1:1000 tetanus toxoid ($P < 0.01$ E3 or E4 vs. control. Fig. 1C). This effect was dose-dependent, and not present when apolipoprotein AI was preincubated in identical fashion prior to stimulation with αCD3 (Fig. 1D, $P < 0.05$ for A1 vs. E3 or E4 at 4, 8, 16, μL). Results are plotted as the average of three data points ± SEM.

ApoE had no direct effect on cell viability at any of the concentrations tested, as measured by trypan blue exclusion (data not shown). There were no statistically significant differences in the suppression induced by preincubation with human recombinant E3 versus E4.

To rule out the possibility that apoE suppressed lymphocyte proliferation by altering accessibility of the T-cell-receptor to mitogenic stimulation, we preincubated peripheral blood mononuclear cells with a concentration of apoE that had functional effects on suppressing proliferation (20 μg/ml). Preincubation of human recombinant apoE3 or E4 had no effect on the binding of αCD3 to peripheral blood mononuclear cells as demonstrated by flow cytometry. ApoB was used as a negative control, and had no effect on lymphocyte proliferation nor binding of αCD3 to the T-cell receptor (data not shown).

Immunologic evaluation of apoE-deficient mice

To assess whether apoE plays an immunomodulatory role in vivo, we analyzed the immune system of apoE-deficient mice that had been back-crossed for 10 generations onto a C57BL/6 background. Spleen weights of apoE-deficient animals (n = 18) were greater than age- and sex-matched C57-BL/6 controls (n = 24) when normalized to body weight (0.38% ± 0.012% vs. 0.29% ± 0.013%; $P = 0.0001$ ANOVA). There were no differences in thymus weight, nor were there any significant differences in thymocyte, splenocyte or PBMC numbers between apoE −/− mice and age- and sex-matched C57Bl/6 control mice. To determine whether there were differences in leukocyte subsets between apoE-deficient and matched wildtype control animals, a phenotypic analysis of thymus, spleen, and bone marrow was performed by incubating cells with a panel of monoclonal antibodies and quantifying binding by flow cytometry. Both wildtype and apoE-deficient animals had comparable percentage and total numbers of T-cell, B cell, monocyte, and granulocyte populations. Evaluation of erythrocyte precursors in bone marrow was also comparable between the two groups (**Table 1**).

We next tested for intrinsic functional differences in T-cells between the apoE-deficient animals and their age and sex matched controls by measuring splenocyte proliferation after stimulation with PHA, submitogenic concentrations of αCD3 with and without αCD28, and mitogenic

TABLE 1. Phenotypic analysis of spleen, bone marrow and thymus from apoE knockout mice compared to controls

Monoclonal Ab	Binding Specificity	Tissue	n (Matched Pairs)	% Labeled ± SEM +/+	% Labeled ± SEM −/−	*P*
Thy-1	T-cells	Thymus	3	100	100	NS
		Spleen	3	67 ± 2	68 ± 1	NS
		Bone marrow	1	60 ± 1	65 ± 1	NS
CD4	Helper/inducer	Thymus	3	90 ± 1	88 ± 3	NS
		Spleen	3	28 ± 1	32 ± 2	NS
		Bone marrow	3	4 ± 1	5 ± 1	NS
CD8	Suppressor/cytotoxic	Thymus	3	83 ± 1	82 ± 1	NS
		Spleen	3	12 ± 1	10 ± 2	NS
		Bone marrow	3	4 ± 1	4 ± 1	NS
B220	B cells	Thymus	3	2 ± 1	1 ± 1	NS
		Spleen	3	15 ± 1	17 ± 2	NS
		Bone marrow	3	10 ± 1	12 ± 1	NS
MAC-1	Macrophage	Thymus	3	0	1 ± 1	NS
		Spleen	3	6 ± 1	9 ± 1	NS
		Bone marrow	3	17 ± 3	15 ± 2	NS
F480	Macrophage	Thymus	3	1 ± 1	1 ± 1	NS
		Spleen	3	9 ± 1	12 + 1	NS
		Bone marrow	3	15 ± 2	19 ± 1	NS
GR-1	Granulocytes	Thymus	3	1 ± 1	3 ± 1	NS
		Spleen	3	5 ± 1	7 ± 1	NS
		Bone marrow	3	67 ± 2	61 ± 3	NS
PK-136	Killer T-cells	Thymus	3	0	0	NS
		Spleen	3	6 ± 1	7 ± 2	NS
		Bone marrow	3	3 ± 0	5 ± 1	NS
CD-2	T-cells	Thymus	3	96 ± 1	96 ± 1	NS
		Spleen	3	90 ± 1	90 ± 1	NS
		Bone marrow	3	13 ± 3	20 ± 8	NS
TER-119	Erythrocytes	Bone marrow	3	54 ± 9	50 ± 9	NS

To evaluate whether different populations of immunocompetent cells were present in apoE-deficient mice as compared to matched wildtype controls, tissue from thymus, bone marrow, and spleen was dissociated and the cells were incubated in a panel of monoclonal antibodies. Cells were subsequently evaluated by flow cytometry to determine the presence of specific cell surface markers. In both apoE-deficient and control mice, there were no significant differences between percentage and total numbers of T-cells (αThy-1, αCD2, αCD4, αCD8, PK-136), macrophage (F4/80, MAC-1), B cell (B220), granulocyte (GR-1), and erythrocyte populations (TER-119); matched Student's *t*-test; $P > 0.05$ in all groups.

TABLE 2. Splenocyte proliferation is similar in apoE-deficient and control mice

Group	Unstim	PHA (1 μg/ml)	αCD3 (1:3200)	αCD28 (1:200)	αCD3(1:3200) + αCD28(1:200)	CD3 (1:40)
Control (n = 4)	53 ± 6	2068 ± 828	621 ± 137	405 ± 159	3089 ± 686	2095 ± 329
ApoE-deficient (n = 4)	61 ± 3	2057 ± 384	475 ± 97	166 ± 61	2915 ± 561	2068 ± 166

Splenocytes were harvested and cultured and proliferation was measured by thymidine incorporation as detailed in Methods. There were no significant differences in splenocyte proliferation between apoE-deficient and control mice after exposure to PHA (1 μg/ml), αCD3 (1:40 and 1:3200), αCD28 (1:200), or αCD3 (1:3200) + αCD28 (1:200). Results are reported as counts/minute/million cells and are plotted as the average of three data points ± SEM.

concentrations of αCD3 (**Table 2**). No differences were observed between the two sets of animals. To determine whether there was an effect of apoE on Th1/Th2 responses, supernatant from the stimulated splenocyte cultures prepared from apoE-deficient and wildtype animals was collected and assayed for TNFα, γ-interferon, IL-4, and IL-10. No significant differences were noted between secreted cytokine levels in apoE-deficient or control splenocyte culture after stimulation (data not shown). Together, these results suggest that there were no ex vivo functional differences in splenocyte proliferative ability or cytokine secretion.

To evaluate the humoral arm of the immune response in apoE-deficient mice and paired controls in vivo, we first quantified total immunoglobulin levels. In naive apoE-deficient animals, there was no significant difference between total IgM (3902 ± 3363 μg/ml vs. 6502 ± 4059 μg/ml, $P = 0.55$) or total IgG (1614 ± 872 μg/ml vs. 1870 ± 1427 μg/ml, $P = 0.13$) as compared to controls. However, after inoculation with tetanus toxoid, the apoE deficient mice demonstrated a significant increase in antigen-specific IgM at 7, 14, and 51 days compared to control mice ($P = 0.026$, $P < 0.001$, $P < 0.001$, respectively). Despite these robust differences in antigen-specific IgM production, there were no significant differences in antigen-specific IgG at any time point tested (**Figs. 2A and 2B**).

To rule out the possibility of specific IgG subclass deficiencies, we tested for relative differences in antigen-specific IgG subclasses (IgG1, IgG2a, IgG2b, IgG3) at 7, 14, and 51 days after inoculation with tetanus toxoid. There was no IgG subclass deficiency in the apoE-deficient mice (**Table 3**).

Cell-mediated immunity was assessed in vivo by measuring the delayed-type hypersensitivity (DTH) response to tetanus toxoid 51 days after inoculation with tetanus toxoid plus adjuvant. There was a statistically significant decrease in the antigen-specific delayed-type hypersensitivity response in the apoE-deficient animals as compared to controls (68 ± 25.4 s vs. 106 ± 28 × 10^{-4} inches of ear swelling; $P < 0.001$).

DISCUSSION

ApoE has been demonstrated to exert in vitro immunomodulatory effects by a number of groups (1–4). We have confirmed that apoE inhibits human lymphocyte proliferation in non-specific (PHA-stimulated), TCR-specific (αCD3 crosslinking of the T-cell receptor), and antigen-specific (tetanus toxoid) assays of T-cell proliferation. This effect is dose-dependent in physiologically relevant concentrations, and is independent of cholesterol levels or any direct effect of apoE on cell viability. In our assays of lymphocyte proliferation, we observed no isoform-specific differences between apoE3 and apoE4. We also evaluated apoE-deficient mice and found no quantitative differences in leukocyte subsets of bone marrow, spleen, or thymus relative to controls. Furthermore, we demonstrated no intrinsic functional abnormality in the proliferation or

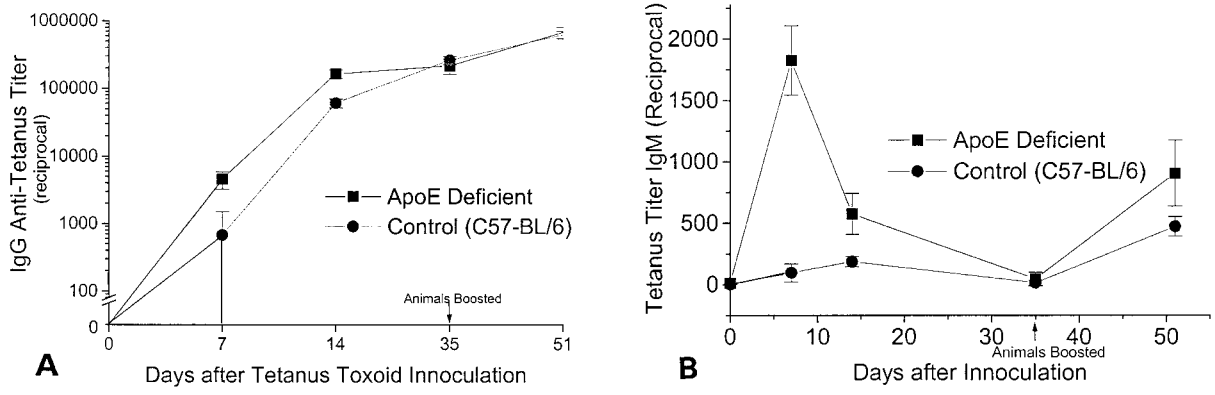

Fig. 2. After inoculation with tetanus toxoid, antigen specific IgM was increased in apoE-deficient mice at 7, 14, and 51 days (Fig. 2A; $P = 0.026$, $P < 0.001$, $P < 0.001$, respectively). There were no differences in antigen specific IgG between apoE-deficient mice and controls at any time point tested. (Fig. 2B). Twenty two mice were used in this experiment, which is representative of three trials performed.

TABLE 3. Antigen-specific IgG subclasses are different in apoE-deficient mice and controls after inoculation with tetanus toxin

	Day 7			Day 14			Day 51		
	KO	WT	P	KO	WT	P	KO	WT	P
IgG1	11.9 ± 0.4	8.6 ± 1.4	0.03	16.2 ± 0.2	16.2 ± 0.1	NS	17.8 ± 0.2	19.8 ± 0.2	<0.001
IgG2a	4.9 ± 1.3	0 ± 0	0.001	2.1 ± 1	0 ± 0	NS	11.8 ± 0.5	13 ± 0.4	NS
IgG2b	5.7 ± 1.5	1.3 ± 1.2	0.04	12 ± 0.3	11.1 ± 0.3	0.02	15.5 ± 0.2	16.3 ± 0.4	NS
IgG3	0.8 ± 0.8	0 ± 0	NS	1.4 ± 1	1 ± 1	NS	6.2 ± 1.5	3.6 ± 1.8	NS

IgG subclasses were measured after inoculation with tetanus toxoid. All titers represent serial dilutions, i.e., $1:2^n \pm$ SEM. Titers less than 2^6 are expressed as 0. *P* values were calculated using the two-tailed Student's *t*-test.

cytokine secretion of splenocytes prepared from apoE-deficient mice compared to control animals, as proliferation after stimulation with PHA and αCD3 ± CD28 was unaffected.

However, we found that apoE plays a biologically relevant role in modulating the immune response in vivo, as apoE-deficient mice had abnormalities of both humoral and cell-mediated immunity. Specifically, we observed significant elevations in antigen-specific IgM levels at 7 and 14 days after immunization with tetanus toxoid, although there was no difference at 35 days, and antigen-specific IgG levels were comparable at all time points tested. Therefore, it seems that some B cells on both strains of mice have switched to IgG, while more have remained as IgM secretors in the apoE −/− mice. ApoE-deficient mice also had evidence of impaired cell mediated immunity, as evidenced by their decrease in delayed-type hypersensitivity (DTH). There are two plausible explanations for this. Impaired DTH responses may be due to decreased numbers or functional impairment of antigen-specific T-cells. Alternatively, this abnormality may be due to a functional impairment of antigen processing or presentation by antigen presenting cells, resulting in poor stimulation of the antigen-specific T cells that are present. Impaired DTH responses have been associated with a variety of immune abnormalities, including a decreased functional capacity of CD4+ cells, and abnormalities of adhesion molecules such as L-selectin and ICAM-1 (17–19). This defect also suggests a possible abnormality of macrophage function, which is consistent with the prior observation that apoE-deficient mice are impaired in their ability to clear *Listeria monocytogenes* (6).

One potential difficulty with the interpretation of our in vivo data is that apoE-deficient animals have elevated serum cholesterol. Thus, the immune abnormalities that we observed may have been influenced by hypercholesterolemia. However, given our *in vitro* observations demonstrating a direct effect of biologically relevant concentrations of apoE on lymphocyte function independent of cholesterol, the most plausible explanation for our findings is that apoE directly modulates immune function.

The absence of any proliferative differences between apoE-deficient and wildtype splenocytes suggests the absence of any intrinsic functional defect in these cells. Moreover, no differences in representative cytokines involved in Th1 (TNFα, γ-interferon) and Th2 (IL-4, IL-10) responses were observed, when supernatant from these splenocyte cultures were examined before and after stimulation. This is consistent with a prior report demonstrating in vivo, but not ex vivo differences in cytokine production between apoE-deficient and wildtype mice (4).

It is possible that the immunomodulatory properties of apoE may also be relevant to the process of atherogenesis. ApoE-deficient mice are prone to premature atherosclerosis, which is usually attributed to their marked hypercholesterolemia and impaired cellular cholesterol efflux (20, 21). An alternative hypothesis is that local secretion of apoE by lipid-laden macrophage at the site of atheromatous vessels might modulate the inflammatory processes, which are believed to play a key role in atherosclerosis (22). In fact, macrophage-specific expression of human apoE reduced atherosclerosis in apoE null mice independent of systemic cholesterol levels (23). Conversely, after bone marrow transplantation, C57BL/6 mice that were reconstituted with macrophage null for the apoE gene developed significantly more atherosclerosis than those reconstituted with wildtype macrophage, in the absence of significant differences in serum cholesterol or lipoprotein profiles (24). These data suggest that apoE freshly secreted by macrophage at the vessel wall may have a local anti-atherogenic effect independent of systemic cholesterol level. Evidence to support a putative role for apoE in modulating the function of lesional T-cells is consistent with the observation that high levels of apoE are found in atheromatous plaques (25) and that early atheromatous plaques in apoE-deficient mice are associated with up-regulation of interleukin 6, vascular adhesion molecules, and increased numbers of $CD4^+$ lymphocytes (26–28). This is in contrast to a recent report that has suggested that T and B lymphocytes play only a minor role in the atherogenesis of apoE-deficient mice (29). Thus, it seems plausible that, in addition to its role in cholesterol transport, the immunomodulatory effects of apoE locally secreted by macrophage at the vessel wall may play an important role in the development of atherosclerosis.

An immunomodulatory role for apoE may also have particular relevance in the brain. ApoE is the primary apolipoprotein produced within the central nervous system, where its synthesis is up-regulated by astrocytes and oligodendrocytes after injury (30, 31). Microglia are the primary resident immunoeffector cells in the brain, and microglial activation is believed to mediate neuronal injury in both chronic neurodegenerative states such as Alzheimer's disease (AD), and after brain injury. It has been

recently demonstrated that physiologically relevant concentrations of apoE inhibit glial secretion of nitric oxide and TNFα after stimulation with LPS (32–34). A role for apoE in mediating glial activation and the endogenous CNS inflammatory response may be related to its association with AD and outcome after acute brain injuries such as stroke, intracranial hemorrhage, and brain trauma (35–42).

Although the mechanism(s) by which apoE down-regulates the immune response remain to be elucidated, it appears that this protein may be a biologically relevant mediator of immune responses. It has previously been reported that apoE-deficient mice have increased mortality after challenge with *Listeria monocytogenes*, which would be consistent with an abnormality of macrophage function (6). It has also been demonstrated that apoE-deficient mice have increased susceptibility to endotoxemia after administration of LPS or inoculation with *Klebsiella pneumonia* (5). This was attributed to a direct interaction between apoE and lipopolysaccharide (LPS), although no significant differences in LPS clearance were demonstrated in this study. Our results would suggest that the effects of apoE on the immune response are not limited to LPS neutralization or clearance. Our finding that preincubation of peripheral mononuclear cells with apoE suppresses proliferation without affecting binding of αCD3 to the T-cell receptor suggests that apoE does not bind non-specifically at the cell membrane to sterically hinder ligand–receptor interactions. Moreover, the fact that apoE suppresses proliferation in our three diverse assays of lymphocyte proliferation makes it unlikely that apoE acts by binding to and neutralizing mitogen. The most plausible mechanism to explain our results would be that apoE binds a specific cell surface receptor to modulate immune function. Further, our data indicate that both the E3 and E4 isoforms are capable of binding this putative apoE receptor (43).

In summary, these results support the hypothesis that apoE plays a functionally important role in modulating the immune response. These findings have relevance for autoimmune disease, atherosclerosis, and could be particularly relevant in the CNS, where apoE is the primary apolipoprotein produced, and is up-regulated after injury. Although in our in vitro model of human lymphocyte proliferation we did not detect statistically significant differences between suppression induced by exogenous recombinant E3 or E4, this does not preclude the possibility that there may be significant isoform-specific differences in secretion or metabolism that may be relevant in vivo. For example, E3 and E4 may differ in their susceptibility to being modified by the oxidative environment present in areas of tissue inflammation. These possibilities are currently being pursued in our laboratory.

We gratefully acknowledge the technical expertise of Richard Scearce. We thank Dr. Sal Pizzo for his advice and helpful discussions. This work was supported by NIH 1K08NS01949 and a pilot grant from the National Multiple Sclerosis Society PP-0450.

Manuscript received 11 August 1999 and in revised form 2 December 1999.

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