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Apolipoprotein E Mediation of Neuro-inflammation in a Murine Model of Multiple Sclerosis

Soomin Shin^{1,*}, Katharine A. Walz^{1,*}, Angela S. Archambault¹, Julia Sim², Bryan P. Bollman¹, Jessica Koenigsnecht-Talboo¹, Anne H. Cross^{1,3}, David M. Holtzman^{1,2,3}, and Gregory F. Wu^{1,3,4,**}

¹Department of Neurology, Washington University in St. Louis School of Medicine; Box 8111, 660 S. Euclid Avenue, St. Louis, MO 63110

²Department of Developmental Biology, Washington University in St. Louis School of Medicine; Box 8111, 660 S. Euclid Avenue, St. Louis, MO 63110

³Hope Center for Neurological Disorders, Washington University in St. Louis School of Medicine; Box 8111, 660 S. Euclid Avenue, St. Louis, MO 63110

⁴Department of Pathology and Immunology, Washington University in St. Louis School of Medicine; Box 8111, 660 S. Euclid Avenue, St. Louis, MO 63110

Abstract

Apolipoprotein E (ApoE) functions as a ligand in receptor-mediated endocytosis of lipoprotein particles and has been demonstrated to play a role in antigen presentation. To explore the contribution of ApoE during autoimmune central nervous system (CNS) demyelination, we examined the clinical, cellular immune function, and pathologic consequences of experimental autoimmune encephalomyelitis (EAE) induction in ApoE knockout (ApoE^{-/-}) mice. We observed reduced clinical severity of EAE in ApoE ^{-/-} mice in comparison to WT mice that was concomitant with an early reduction of dendritic cells (DCs) followed by a reduction of additional innate cells in the spinal cord at the peak of disease without any differences in axonal damage. While T cell priming was enhanced in ApoE ^{-/-} mice, reduced severity of EAE was also observed in ApoE ^{-/-} recipients of encephalitogenic wild type T cells. Expression of ApoE during EAE was elevated within the CNS of wild type mice, particularly by innate cells such as DCs. Overall, ApoE promotes clinical EAE, likely by mediation of inflammation localized within the CNS.

Keywords

EAE; Antigen Presentation; Apolipoprotein E; Dendritic Cells

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^{**}Corresponding Author, wug@neuro.wustl.edu, phone: 314-362-3293, fax: 314-747-1345.

^{*}These authors contributed equally to this work

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1. Introduction

Antigen-specific responses by autoimmune T cells targeting the central nervous system (CNS) are critical for the initiation and propagation of experimental autoimmune encephalomyelitis (EAE), an established animal model for multiple sclerosis (MS) (Fletcher et al., 2010, Kuchroo et al., 2002). Presentation of encephalitogenic epitopes by antigen presenting cells (APCs) within the CNS is necessary for T cell-mediated immune responses that induce myelin damage in EAE (Becher et al., 2006, Slavin et al., 2001, Tompkins et al., 2002, Wilson et al., 2010). However, the crucial interactions between APCs and T cells that promote neuro-inflammation during EAE have yet to be fully established. Of the numerous APCs that are capable of driving adaptive immune responses during EAE, dendritic cells (DCs) have been shown to perform critical APC functions during every phase of EAE (Chastain et al., 2011, Deshpande et al., 2007, Ji et al., 2013, Wu and Laufer, 2007).

Apolipoprotein E (ApoE) is a glycoprotein that functions as a ligand in receptor-mediated endocytosis of lipoprotein particles (Kim et al., 2009a). ApoE is expressed in both the liver and CNS as well as produced by APCs such as DCs and macrophages (Kim, Basak, 2009a, van den Elzen et al., 2005). The $\epsilon 4$ isoform of ApoE is a major genetic risk factor for sporadic Alzheimer's disease (AD) (Kim, Basak, 2009a). Polymorphisms in ApoE may also contribute to the risk of developing MS and cognitive dysfunction in patients with MS (Shi et al., 2011, Yin et al., 2012), although this remains controversial (Lill et al., 2012, Yin, Zhang, 2012). Recent work has shown that ApoE regulates blood-brain barrier integrity and function (Bell et al., 2012). Therefore, the pleiotropic functions of ApoE are highly relevant to neurologic disease.

ApoE, secreted by APCs, associates with lipids and facilitates their uptake and presentation as antigens (van den Elzen, Garg, 2005). As myelin is over 70% lipid (Quarles et al., 2006), ApoE may serve as an important intermediate during the process of antigen presentation to induce autoimmune T cell responses during diseases such as MS. In addition to differing results reported recently on the role of ApoE in EAE (Dayger et al., 2012, Wei et al., 2013), limited data exist to substantiate the role of APC-derived ApoE in EAE. To assess the importance of ApoE in neuroinflammation, we subjected mice that had undergone targeted genetic deletion of ApoE (ApoE $-/-$) to EAE. We found that ApoE $-/-$ mice prime auto-reactive CD4 T cells more efficiently than WT mice, but exhibit less severe clinical disease. In addition, ApoE is expressed at increased levels within the CNS of WT mice affected by EAE, with infiltrating DCs expressing especially high levels of ApoE compared to the periphery. There was a reduction in DC infiltration at the onset of disease in ApoE $-/-$ mice with EAE compared with WT mice. These results indicate that ApoE contributes to the disease state of EAE and suggest a mechanism by which APCs, including DCs, express ApoE to promote neuro-inflammation within the CNS compartment.

2. Materials and Methods

Mice

C57BL/6 (B6), ApoE $-/-$ and CX3CR1^{GFP} (Jung et al., 2000) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). ApoE $-/-$ mice were confirmed to be on the B6

background, with 99% identity verified by congenic analysis (accounting for the ApoE mutation on chromosome 7; data not shown). Mice were housed in specific pathogenfree conditions and all animal experiments were performed in strict compliance with Animal Studies Committee regulations of Washington University in St. Louis.

Flow Cytometry and sorting

CNS mononuclear cells were isolated as described (Archambault et al., 2006) separately from the spinal cord and brains of mice. Flow Cytometry was performed on a BD FACSCalibur or Beckman Coulter Gallios flow cytometers using CD3, NK1.1, CD45, and CD11b antibodies from BD Biosciences (San Jose, CA) as well as CD3, CD11c, CD4, CD8a, MHCII and CD11b antibodies from eBioscience (San Diego, CA). Data were analyzed using FlowJo software (Treestar; Ashland, OR). Fluorescence-activated cell sorting (FACS) of CX3CR1^{GFP+} or CD11c⁺ cells was performed with a FACSARIA cell sorter (BD Biosciences) at the Washington University in St. Louis School of Medicine Department of Pathology and Immunology Flow Cytometry and Fluorescence Activated Cell Sorting core. FACS-sorted populations were typically of >95% purity and were sorted after gating out cells labeled with 7-AAD (BD Biosciences).

Induction and clinical assessment of EAE

To induce active EAE, mice were injected subcutaneously with 100–200µg of a peptide consisting of amino acids 35–55 of myelin oligodendrocyte glycoprotein (MOG_{35–55}) (CS Bio, Menlo Park, USA) or recombinant rodent MOG protein (rMOG; (Oliver et al., 2003, Wu et al., 2011) emulsified in complete Freund's adjuvant (CFA) containing 2.5 mg/ml heat inactivated Mycobacterium Tuberculosis strain H37Ra (Difco; Detroit, MI). Immediately following immunization, 200ng of pertussis toxin (Enzo; Farmingdale, NY) was administered intraperitoneally. Clinical evaluation was performed using following criteria: 0: no disease; 1: tail paralysis; 2: mild hind limb paresis; 3: severe hind limb paresis; 4: hind limb paralysis; 5: moribund or dead. Passive EAE was induced by intravenous transfer of encephalitogenic CD4 T cells as previously described (Archambault, Sim, 2006). Initial onset of disease was defined by the development of EAE in WT mice (days 11–12); peak of disease was defined by establishment of disease by days 16–18, and chronic disease was on or after day 30 post-immunization.

ELISPOT Assay

Millipore multiscreen filter plates were coated with IFN-γ or IL-17 capture antibodies (BD Bioscience; San Jose, CA) overnight. Lymphocytes from draining lymph nodes were plated with media, 25 µg/ml MOG_{35–55} or 100 µg/ml rMOG, and incubated at 36 °C for 24 hours. The plates were washed, followed by incubation with detection antibodies and AKP streptavidin (BD Biosciences; San Jose, CA) for two hours. Subsequently, the plates were washed and then developed using SigmaFast BCIP/NBT (Sigma; St. Louis, MO). An ImmunoSpot® S5 FluoroSpot (CTL; Shaker Heights, OH) was used to score the plate, with spot separation set to 0 and size set for lymphocytes at 0.1mm–001 mm. The same analysis criteria were applied across separate individual experiments.

Antigen presentation assays

Mouse bone marrow was harvested by flushing femur and tibial shafts from B6 or ApoE^{-/-} mice with PBS to generate bone marrow-derived DCs (BMDCs). Cells isolated after red blood cell lysis were then plated at $2-10 \times 10^6/3$ ml with media containing 10ng/mL GM-CSF (R&D Systems; Minneapolis, MN). To establish ApoE-free conditions, cultures were diluted to serum-free media containing identical concentrations of GM-CSF over the course of nine days. T cell hybridomas, generated as described (Carrero et al., 2012), were screened for specific reactivity to MOG₃₅₋₅₅. The MOG₃₅₋₅₅-specific T cell hybridoma, MOG.15, was added along with media, 25 µg/ml MOG₃₅₋₅₅, or 100 µg/ml rMOG to B6 or ApoE^{-/-} BMDCs for 24 hours. The culture supernatant was freeze/thawed twice and assayed for the concentration of IL-2 by CTLL cell line ³H Thymidine uptake (Carrero, Vivanco-Cid, 2012).

Histology and quantification of spinal cord damage

Following perfusion with ice-cold PBS, spinal cords were isolated and placed in 4% paraformaldehyde for 24 hours or longer. Tissue was then dehydrated and embedded in paraffin. Myelin was stained using 8 µm sections with solochrome cyanine as previously described (Kiernan, 1984). Briefly, sections were stained for 45 minutes with Eriochrome Cyanine R (Sigma; St. Louis, MO). Sections were washed, and then differentiated for 30 seconds in 10% Iron (III) Chloride (Sigma). Next, sections were counterstained with Van Gieson's stain for two minutes, washed, dehydrated in sequential concentrations of ethanol, cleared in xylene, and covered slipped. 8 µm sections were autoclaved in Trilogy solution (Cell Marque; Rocklin, CA) using a Cuisinart pressure cooker (model CPC-600; East Windsor, NJ) for 15 minutes. After rinsing, sections were blocked with 10% horse serum (Biowest; Nuaille, France) and then incubated overnight at 4° C with primary antibodies. These included Goat anti-CD3 antibody (Santa Cruz Biotechnology; Santa Cruz, CA), mouse anti-phosphorylated neurofilament H (SMI-31) and mouse anti-non-phosphorylated neurofilament H (SMI-32, Covance; Princeton, NJ) diluted in 3% fetal calf serum in PBS. For fluorescent imaging, sections were then washed with PBS and subsequently incubated with secondary antibodies (goat antimouse IgG1-Alexa 488 and goat anti-rabbit IgG-Texas Red; Invitrogen) for one hour at room temperature. Slides were washed three times in PBS and coverslipped using Vectashield mounting medium with DAPI (Vector Labs; Burlingame, CA). For nonfluorescent imaging, sections were washed with PBS and incubated in 3% hydrogen peroxide solution for 20 minutes at room temperature to block the endogenous peroxidase activity. Sections were subsequently incubated with secondary antibodies (donkey biotinylated IgG anti-goat, Rockland; Boyertown, PA) for one hour at room temperature. Subsequently, slides were washed in PBS and then incubated with Streptavidin HRP (Invitrogen; Carlsbad, CA) for 30 minutes at room temperature. Slides were then developed using DAB substrate kit (Cell Marque; Rocklin, CA) for 10 minutes and then counterstained in hematoxylin (Cell Marque; Rocklin, CA) for 60 seconds. Slides were then dehydrated using ethanol and xylene and cover-slipped using permount (Fisher; Hampton, New Hampshire). Blinding was achieved by covering the slide label and assigning a unique, random number to each blinded slides (performed by a member of the lab not involved in the project). Images were obtained with a Nikon 90i digital microscope using Metamorph software (Molecular Devices; Sunnyvale, CA). Quantification of axonal

injury was performed using ImageJ (NIH; <http://rsb.info.nih.gov/ij/>). Regions of white matter were manually traced after thresholds were set for isolated detection of antibody labeling above background in 8-bit images. SMI-32 index was calculated according to the following formula: (% SMI-32+ pixels in ROI of white matter)/(% SMI-32+ pixels in ROI of white matter + % SMI-31+ pixels in ROI of white matter). All image analysis was performed in a blinded manner by SS or BPB. Quantification of myelin loss and CD3 staining was based on reported methods (Piccio et al., 2013). Specifically, myelin loss was assessed in a blinded fashion in at least three axial sections from the rostral to caudal spinal cord on a four point scale: 0 = no myelin loss; 1 = partial loss of myelin in the dorsal or ventral edge of the spinal cord; 2 = loss of myelin in the peripheral edge of the dorsal and ventral portion of the spinal cord; 3 = extensive loss of myelin extending from the peripheral edge to the gray matter in the dorsal and/or ventral spinal cord. Quantification of CD3 staining was also assessed using samples from at least three axial sections from the rostral to caudal spinal cord. Grading was performed in a blinded manner using a four point scale: 0 = no CD3+ cells; 1 = CD3+ cells in the sub-pial region only; 2 = CD3+ cells in the sub-pial and perivascular space; 3 = CD3+ cells throughout the parenchyma of the spinal cord.

Quantitative RT-PCR

RNA from whole CNS and spleen was homogenized in trizol (Invitrogen; Carlsbad, CA) and isolated following the manufacturer's instructions. Precipitated RNA was resuspended in THE RNA Storage solution (Invitrogen) and treated with rDNase I (Invitrogen). Complementary DNA was generated with the high capacity cDNA reverse transcription kit using the random hexamer primer protocol (Invitrogen). Quantitative real-time PCR was performed on an ABI PRISM 7000 System (Applied Biosystems; Foster City, CA) using SYBR® Green detection (Invitrogen). Primers for APOE (5' CGCAGG TAATCCCAGAAGC 3') and (5' CTGACAGGATGCCTAGCCG 3') along with 18s rRNA (5' TTCGGAAGTGGCCATGATT 3') and (5' TTTCGCTCTGGTCCGTCTTG 3') were obtained from IDT (Coralville, IA). The relative quantitation (RQ) value was calculated using the $\Delta\Delta C_t$ method with 18s as the internal control.

Statistical Analysis

Two-tailed Student t-tests (unpaired) were performed for comparisons between different groups of mice as well as comparisons between cellular populations. Additionally, two-way ANOVA was employed to analyze differences between group means.

3. Results

ApoE deficiency is associated with reduced severity of EAE

To determine whether ApoE contributes to inflammatory autoimmune responses targeting the CNS, we induced EAE in WT and ApoE $-/-$ mice by active immunization with MOG protein and with MOG peptide. A typical course of disease, manifesting as chronic persistent hindlimb weakness, was observed in WT mice after immunization with rMOG in CFA. In comparison, ApoE $-/-$ mice exhibited a delay in onset and reduction of maximal severity of clinical disease ($p < 0.05$ for days 10–15, 20, 22, 23, 27, and 29 by unpaired t-test; $p < 0.0001$ by two-way ANOVA; Fig. 1A). To determine if reduced disease in ApoE $-/-$

– mice was due to incomplete antigen processing of the immunodominant fragment from MOG protein, we immunized both WT and ApoE $-/-$ mice with MOG_{35–55} peptide in CFA. Again, ApoE $-/-$ mice immunized with MOG peptide had less severe clinical disease compared to WT mice ($p = 0.0002$, two-way ANOVA; Fig. 1B). While variability was observed between experiments, a prominent reduction in clinical severity was seen in ApoE $-/-$ mice whether the immunogen was rMOG, which requires extensive processing by APCs, or MOG_{35–55}, which is a short peptide requiring little to no processing (Table 1). These results demonstrate the essential role for ApoE in generating maximal disease severity in standard murine models of MS.

Priming to myelin antigens elicits robust CD4 T cell responses in ApoE $-/-$ mice

Murine EAE is primarily a T cell-driven model of CNS disease (Fletcher, Lalor, 2010, Kuchroo, Anderson, 2002). ApoE has been shown to participate during development of T-cell immune responses, including presentation of antigen to CD4 T cells (Bell, Winkler, 2012, Tenger and Zhou, 2003, van den Elzen, Garg, 2005). That EAE severity was reduced – and to a similar degree – in ApoE $-/-$ compared with WT mice whether using rMOG or MOG_{35–55} as the inducing antigen indicated that ameliorated disease in absence of ApoE was not due to impaired antigen processing. To determine if the reduction of EAE disease severity in ApoE $-/-$ mice was a result of altered Th1 priming, antigen-specific production of IFN- γ was measured by ELISPOT. Fifteen days after SQ immunization with MOG_{35–55}, draining LN (dLN) cells were isolated and incubated *ex vivo* with MOG_{35–55} or rMOG. More cells producing IFN- γ in response to either MOG_{35–55} or rMOG were detected in ApoE $-/-$ compared with WT mice (Fig. 2A). A balance between Th1 and Th17 CD4 T cells is thought to be critical for the development of EAE (Miossec et al., 2009, Steinman, 2007), and thus we also measured IL-17-producing dLN cells in response to MOG_{35–55}. Similar to IFN- γ , MOG_{35–55}-specific IL-17-producing cells were greater in number in ApoE $-/-$ mice compared with WT mice (Fig. 2B)

DCs are a specialized APC capable of eliciting potent responses from CD4 T cells (Lewis and Reizis, 2012, Reis e Sousa, 2006). ApoE is expressed by DCs (van den Elzen, Garg, 2005) and could influence CD4 T cell responses essential for EAE. Thus, we generated BMDC from WT and ApoE $-/-$ mice for use as APCs *in vitro*, in the absence of serum to prevent exogenous ApoE contamination during culture. When BMDC from WT mice were combined with the MOG-specific CD4 T cell hybridoma, MOG.15 along with MOG_{35–55} *in vitro*, IL-2 was produced in an antigen dose-dependent manner (Fig. 2C). ApoE $-/-$ BMDCs elicited the same antigen-specific response (Fig. 2C). Similar results were obtained using rMOG as antigen (data not shown). Despite several reports of a role of ApoE in antigen presentation (Tenger and Zhou, 2003, van den Elzen, Garg, 2005), these data indicate that the reduced clinical manifestations of EAE in ApoE $-/-$ mice were not due to limitations in antigen processing or presentation during T-cell priming. Indeed, priming of CD4 T cells toward MOG was exaggerated after active EAE induction in ApoE $-/-$ mice, consistent with prior reports of elevated priming efficiency by ApoE-deficient APCs (Tenger and Zhou, 2003).

ApoE promotes an early accumulation of CNS DCs and innate cells during EAE

ApoE is known to participate in regulation of the blood-brain barrier (BBB) integrity (Bell, Winkler, 2012, Methia et al., 2001). Given that peripheral MOG-specific CD4 T cell responses after active immunization did not explain clinical differences between WT and ApoE $-/-$ mice, we examined the CNS compartment at three key time points after the induction of active EAE: onset, peak and late stages of disease. Mononuclear cells were isolated from the spinal cord, the major site of inflammatory demyelination during EAE (Ransohoff, 2006), of EAE-affected mice and examined using flow cytometry. Because DCs are critical to the initiation and propagation of EAE (Greter et al., 2005, Ji, Castelli, 2013, McMahon et al., 2005, Wu, Shindler, 2011), we compared the frequency of total CD11c⁺ cells during EAE in both WT and ApoE $-/-$ mice. At onset, there was a statistically significant reduction in the percentage and absolute number of CD11c⁺ cells in the spinal cord of ApoE $-/-$ mice compared with WT mice (Fig. 3A). Frequencies and quantities of other innate cells or CD4 T cells within the spinal cord did not differ at this time when comparing ApoE $-/-$ and WT mice (Fig. 3B). At the peak of disease, CD45^{hi}CD11b^{hi} invading myeloid cells and activated microglia (Ponomarev et al., 2005, Vom Berg et al., 2012) were observed to be reduced in frequency and number within the spinal cords of ApoE $-/-$ mice compared with WT mice (Fig. 3C & D). The frequency and number of spinal cord CD4⁺ T cell infiltrates were not different in ApoE $-/-$ mice in comparison to WT mice (Fig. 3D). Furthermore, when we examined the infiltration of T cells using immunohistochemical detection of CD3, a trend toward fewer T cells in the spinal cord of ApoE $-/-$ mice compared with WT mice was observed, but this did not reach statistical significance (WT = 1.5 ± 0.6 , ApoE $-/-$ = 0.8 ± 0.7 , $p = 0.5$; Fig. 4). At chronic timepoints after disease was well-established, the amount of immune cell infiltrates, measured by flow cytometry or CD3⁺ infiltration assessed by immunohistochemistry, did not differ between ApoE $-/-$ and WT mice (Figs. 3E&4).

Finally, due to the role of ApoE in antigen presentation to NKT cells (van den Elzen, Garg, 2005), we examined the frequency of NKT in the CNS and the spleen after immunization, again without any significant difference observed between WT and ApoE $-/-$ mice (Fig. 3F). Overall, differences in CNS accumulation of APCs and T cells were observed between WT and ApoE $-/-$ mice in the spinal cords were observed early during the course of disease, but normalized during chronic phases of EAE.

ApoE is required during effector phases of EAE for maximal disease

EAE can be divided into stages consisting of the initial T cell priming against myelin antigens in secondary lymphoid organs (initiation stage), followed by migration of auto-reactive T cells and other immune system cells across the blood-CNS barrier, and subsequent disease pathology within the CNS (effector stage). Our data does not suggest that decreased EAE severity is related to the initiation of disease. Thus, we next used an adoptive transfer system for the induction of EAE (Stromnes and Goverman, 2006b), which limits ApoE deficiency to the effector stage. Encephalitogenic T cells were generated from WT B6 mice as described (Archambault, Sim, 2006). Equal numbers of cells were transferred to either WT or ApoE $-/-$ recipients, and the resulting disease was compared. In these experiments we observed more variability in clinical disease than with active EAE.

Due to excessive mortality the dose of donor encephalitogenic cells was scaled down by a third in some experiments. WT mice exhibited ascending weakness following receipt of encephalitogenic T cells with maximal disease severity peaking around two weeks. In contrast, fewer ApoE $-/-$ recipients of donor cells demonstrated clinical signs of EAE, and those that did showed less hindlimb weakness (Table 1), indicating that the expression of ApoE in the recipient was an important factor in the reduced severity of disease. Of note, ApoE $-/-$ donor cells, cultured in the absence of serum to eliminate ApoE, were highly potent in disease induction, and could not be reliably used in comparison with WT lines (data not shown). Taken together, these results, along with the T cell priming results and the similar numbers of infiltrating inflammatory cells in CNS, indicate that ApoE is critical during the effector phase of EAE. Moreover, the critical location for ApoE modulation of disease appears to be within the CNS.

The degree of axonal injury is similar between ApoE $-/-$ and WT mice with EAE

Axonal injury and loss subsequent to CNS inflammation are central pathologic features of MS and are considered the key factors in permanent disability (De Stefano et al., 1998, Dutta and Trapp, 2007). B6 mice with EAE exhibit similar pathology, including accumulation of SMI-32 labeled injured axons (Soulika et al., 2009). We examined the degree of axonal injury in the spinal cord of ApoE $-/-$ and WT mice using SMI-32 and SMI-31 immunohistochemistry at early, peak and chronic times during disease. We found a trend toward a decrease in the degree of axonal injury at the onset of disease in ApoE $-/-$ mice; however, this did not reach statistical significance (Fig. 5). Of note, no difference in myelin loss at any timepoint was observed (data not shown). Overall, there was no statistically significant difference between axonal injury in the spinal cords of ApoE $-/-$ and WT mice during any phase of disease (Fig. 5).

ApoE expression is elevated within CNS sites affected by EAE and is driven in part by DCs

Our results suggest that ApoE may mediate neuro-inflammation within the CNS compartment during the latter phases of EAE. For EAE to develop, antigen-specific CD4⁺ T cells must be reactivated by cognate interactions with APCs in the CNS (Bartholomaeus et al., 2009, Becher, Bechmann, 2006, Slavin, Soos, 2001). ApoE is known to be expressed in the CNS predominantly by astrocytes and microglia (Kim, Basak, 2009a). Therefore, we examined the CNS before and after the induction of active EAE for expression of ApoE by quantitative RT-PCR. At the peak of active EAE disease in WT, expression of ApoE was increased almost two-fold in the spinal cord, the primary site of inflammatory demyelination in EAE, relative to the spleen (Fig. 6A). After the induction of passive EAE, a similar elevation in ApoE gene expression was observed within the spinal cord relative to the spleen (Fig. 6B). APOE expression was only slightly increased in brain, which is not a major site of pathology in this EAE model (Goverman, 2009, Stromnes and Goverman, 2006a) (Fig. 6B). Macrophages and DCs, innate cells that accumulate within the CNS during EAE, are known to utilize ApoE for antigen presentation (Tenger and Zhou, 2003, van den Elzen, Garg, 2005). We isolated innate cells from the CNS based on expression of CX3CR1, which identifies microglia along with subsets of monocytes, macrophages and DCs (Saederup et al., 2010) and compared gene expression levels of ApoE to these same cells in the spleen. At the peak of disease, we found that expression of ApoE was over 20-fold greater in

CX3CR1⁺ cells isolated from the CNS as compared to the spleen (Fig. 6C). DCs and their progenitors can express CX3CR1 (Anandasabapathy et al., 2011, Lyszkiewicz et al., 2011) and are known to regulate APC function via ApoE. Therefore, we sorted CD11c⁺ cells from the CNS as well as the spleens of mice with EAE to determine the relative level of ApoE expression. At the peak of EAE clinical disease, gene CNS CD11c⁺ DC expression of ApoE was over 17 times that compared with CD11c⁺ DCs within the spleen (Fig. 6C).

4. Discussion

To explore the molecular basis for cell-mediated inflammatory CNS diseases such as MS, we examined the effect of ApoE deficiency in EAE. In the present study, mice genetically deficient in ApoE were less susceptible to EAE. Priming of T cells to myelin antigens was not impaired in ApoE ^{-/-} mice. Rather, peripheral antigen-specific T cell responses were enhanced compared to WT. Although less severe than WT, ApoE ^{-/-} mice did develop EAE which was characterized by an early reduction in DC accumulation within the spinal cord. Moreover, transfer of encephalitogenic WT T cells into ApoE ^{-/-} mice resulted in less severe disease compared with WT recipients of the same cells. Together these data suggest that the clinical differences in EAE in mice lacking ApoE localize to the CNS compartment. Indeed, we found that ApoE expression is highly elevated within the spinal cord, the primary site of inflammatory demyelination in this model system. Further, we observed that within the CNS, ApoE is expressed by DCs to a far greater extent than by DCs in the periphery. Based on these data, we propose that the major function of ApoE during EAE is to propagate T cell autoreactivity within the CNS.

Our data stand in direct contrast to earlier work that reported increased severity of EAE in ApoE ^{-/-} mice (Karussis et al., 2003). Use of spinal cord homogenate as the immunogen in the prior work is a major difference between our work and that of Karussis et al, who detected minimal disease in WT mice (Karussis, Michaelson, 2003). Induction of active EAE using MOG₃₅₋₅₅ as immunogen in the present study represents a more standard and commonly used system (Racke, 2001, Stromnes and Goverman, 2006a). Our attempts to induce active EAE using spinal cord homogenate based on the Karussis et al report resulted in no disease in either WT or ApoE ^{-/-} mice (data not shown).

Targeting of lipid antigens is thought to occur during MS and EAE (Cudaback et al., 2011). Immune responses to lipids may exacerbate disease, as EAE induced by immunization with lipid-bound protein exhibited much greater demyelination than that induced by protein devoid of lipid (Jordan et al., 1999, Raine et al., 1981, Zehetbauer et al., 1991). As ApoE is critical for lipid metabolism as well as shuttling lipid antigens to APCs, the reduction in severity in ApoE ^{-/-} mice in our work suggests that ApoE may participate in pro-inflammatory responses that occur upon myelin breakdown.

ApoE-dependent antigen presentation is most clearly described in relation to NKT cells (Brigl and Brenner, 2004, van den Elzen, Garg, 2005). DCs actively participate in lipid presentation to NKT cells (van den Elzen, Garg, 2005), a potentially critical step in the destruction of lipid-rich myelin during EAE and MS. NKT cells are a unique subset of T cells that recognize glycolipid antigens presented by the major histocompatibility complex

(MHC) class I-like molecule CD1 (Brigl and Brenner, 2004). Following recognition of the CD1d/glycolipid complex via their T cell receptor (TCR), NKT cells produce cytokines such as IFN- γ and IL-4 and participate in immune regulation and effector processes (Rossjohn et al., 2012). The regulatory qualities of NKT cells in EAE (Furlan et al., 2003, Mars et al., 2008, Singh et al., 1999) are not likely to be responsible for early differences in EAE in our model, as we did not use a glycolipid antigen to induce EAE. As well, numbers of NKT cells did not differ between WT and ApoE $-/-$ mice in our studies. The potential suppressive qualities of NKT cells responding to lipid targets in spinal cord homogenate is a possible explanation for the greater EAE severity in ApoE $-/-$ than WT mice induced by spinal cord homogenate but not peptide or protein.

ApoE has been shown to participate not only in priming and antigen presentation, but also in blood-brain barrier (BBB) integrity (Bell, Winkler, 2012, Tenger and Zhou, 2003, van den Elzen, Garg, 2005) which is critical for regulation of immune cell entry into the CNS. The reduced clinical manifestation of EAE in ApoE $-/-$ mice was not a result of limitations in antigen processing and presentation during the initiation of disease, as priming of CD4 T cells toward myelin antigens is exaggerated after active immunization, consistent with prior reports of elevated priming efficiency by ApoE-deficient APCs (Tenger and Zhou, 2003). Furthermore, BMDCs from ApoE $-/-$ and WT mice are equivalent in eliciting MOG₃₅₋₅₅-specific CD4 T cell responses from rMOG protein (data not shown). Similar reduction in EAE in ApoE $-/-$ mice compared with WT mice following immunization with either MOG₃₅₋₅₅ or rMOG; thus, reduced EAE early in the course of disease is likely to be independent of APC processing and presentation of protein targets during priming given the minimal requirements for antigen processing in peptide immunization with MOG₃₅₋₅₅. However, it is important to note the potential contribution of ApoE in the peripheral compartment, particularly in light of the ameliorating effects on EAE resulting from peripheral administration of A β peptides (Grant et al., 2012). ApoE, known to complex with A β peptides and modulate aggregation and toxicity within the CNS (Kim et al., 2009b), may participate in the modulation of EAE after engagement with A β peptides outside of the brain and spinal cord.

Recent reports of EAE in ApoE $-/-$ mice are in conflict (Dayger, Rosenberg, 2012, Wei, Zheng, 2013). Our results are in agreement with Dayger et al who reported less severe EAE in ApoE $-/-$ mice using the same model system as in our studies (Dayger, Rosenberg, 2012). This group also observed later disease onset and peak of disease with less severity, similar to our findings. However, in some of our studies ApoE $-/-$ mice remained less severely affected throughout the disease course out beyond day 30 post-immunization, whereas in Dayger et al the clinical and pathologic EAE was no different in the presence or absence of ApoE at later time points. In contrast, our results are similar to those reported by Wei et al (Wei, Zheng, 2013). While our detailed immunologic analysis revealed no difference in CD4 T cell frequency within the spinal cord of ApoE $-/-$ and WT mice, ApoE $-/-$ mice were reported to accumulate greater numbers of Th17 CD4 T cells in ApoE $-/-$ brains (Wei, Zheng, 2013). When we quantified immune cell subsets from the entire CNS, including CD4 $^{+}$ T cells, DCs, and NKT cells using flow cytometry only differences in innate cell fractions were observed between ApoE $-/-$ and WT mice with EAE, with reductions early during disease in ApoE $-/-$ mice. In contrast, Dayger et al. measured T

cells using immunohistochemistry and found lower fractions of CD4⁺ T cells in lesioned areas and less demyelination at day 17, which normalized to WT levels by day 30. Although more quantitative than immunohistochemistry, a limitation of flow cytometry of bulk CNS tissue is that immune cell frequency differences may be diluted and *in situ* localization of immune cells is not preserved. Nonetheless, the different techniques used in the two studies offer complementary information. In our experiments we measured several different immune cell subsets, immune responses to antigen by MOG_{35–55}-reactive CD4⁺ T cells, and we pinpointed the main difference due to lack of ApoE to the effector phase of EAE, likely within the CNS. Unfortunately, technical issues have limited our ability to test the hypothesis that T cell expression of ApoE also mediates disease. Namely, in order to generate and maintain encephalitogenic T cells from ApoE ^{−/−} mice, passage of donor T cells in serum-free conditions was performed to exclude ApoE prior to transfer. In these experiments, we have consistently observed an increase in encephalitogenicity of T cells deprived of serum. Thus, our ability to compare the effect of passive EAE resulting from ApoE ^{−/−} and WT cells is currently not feasible.

ApoE was greatly upregulated within the CNS of EAE-affected WT mice in our studies, with expression most abundant in the spinal cord during EAE. Although intrinsic CNS cells may express ApoE, that the most pronounced upregulation was in the main site of pathology in this model suggests that invading cells may be responsible for part or all of the enhanced ApoE. Certainly, ApoE deficiency may result in alterations of inflammatory responses unique to the CNS, as microglial migration is affected by ApoE (Cudaback, Li, 2011). Additionally, ApoE may contribute in some fashion to the process of axonal injury that is a central pathologic feature of EAE and MS and is considered a key factor in the consequent disability in each. As no difference in axonal injury was seen in our work, we favor the idea that ApoE deficiency promotes repair mechanisms or axonal function of remaining nerves rather than inhibiting axonal injury.

Our finding that ApoE contributes to disease severity in EAE carries clinical implications for patients with MS. Risk of MS development has not been convincingly linked to ApoE polymorphisms (Lill, Liu, 2012, Sadeghi et al., 2011, Yin, Zhang, 2012), which is consistent with the present findings that EAE could be induced in ApoE ^{−/−} mice. However, ApoE polymorphisms have been linked with the severity of impairments in MS patients, particularly cognitive functional impairment (Shi, Tu, 2011). This would be in accord with our and others' results of altered manifestations of EAE with ApoE deficiency. A future avenue of research might be to explore the effects of ApoE isoforms in EAE outcomes.

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Highlights

- Demonstrates deficiency of ApoE is beneficial in EAE
- Clarifies controversy between reports on the role of ApoE in EAE
- Narrows the role for ApoE in EAE to the secondary phases of disease
- Identifies a major source of ApoE within the CNS during EAE as dendritic cells

Figure 1. ApoE is required for maximal disease in MOG-induced EAE

Mean clinical scores $\pm$ SEM of rMOG-immunized (A) and MOG₃₅₋₅₅-immunized (B) mice.

Two separate experiments are depicted in B. WT mice ($n = 12-14$) are shown in black squares and ApoE^{-/-} mice ($n = 14-15$) are shown in open circles. Data shown is representative of two independent experiments for A and four independent experiments for B with three to 15 mice per group. * = $p < 0.05$ by Student's *t*-test. Two-way ANOVA testing also distinguishes ApoE^{-/-} and WT mice (A, $p < 0.0001$; B, $p = 0.0002$ (left), $p < 0.0001$ (right)).

Figure 2. Activation of CD4 T cells is not inhibited by ApoE deficiency

(A and B) WT and ApoE $^{-/-}$ mice were immunized with MOG₃₅₋₅₅. (A) Fifteen days after immunization, single cell suspensions of dLN cells were incubated *ex vivo* with media alone, MOG₃₅₋₅₅ or rMOG. Production of IFN- γ was determined by ELISPOT. Error bars represent SEM. * = $p < 0.01$, ** = $p < 0.001$ by Student's t test. (B) IL-17 and IFN- γ production by single cell suspensions of dLN cells were incubated *ex vivo* with media alone or MOG₃₅₋₅₅. (C) BMDC were isolated from WT (black squares) and ApoE $^{-/-}$ (open circles) mice and cultured in serum-free conditions. BMDCs were then combined with the

MOG-specific hybridoma, MOG.15 in the presence of increasing concentrations of MOG_{35–55}. Proliferation of the IL-2-dependent T cell line, CTLL (Carrero, Vivanco-Cid, 2012) was measured after supernatants from these cultures were added. For each panel, data are representative of two separate experiments with three mice per group.

Figure 3. Early innate cell accumulation in the CNS during EAE is ApoE dependent

(A) Representative flow plots of spinal cord mononuclear cells stained with CD11c and MHCII from WT (left flow plot) and ApoE ^{-/-} (right flow plot) mice 11 days after immunization with MOG₃₅₋₅₅. Quantification of frequencies and absolute numbers of CD11c⁺ cells are shown to the right. (B) Frequencies of CD45^{int}CD11b^{int} ("gate 1"), CD45^{hi}CD11b^{hi} ("gate 2"), and CD4 T cells from the spinal cord 11 days post-immunization. (C) Representative flow plots demonstrating gate 1 and gate 2 frequencies and (D) Quantification of individual mononuclear cell subsets identified by flow cytometry

at peak of disease. (E) Quantification of individual mononuclear cell subsets identified by flow cytometry during chronic EAE. (F) Splenocytes (left) and CNS mononuclear cells (right) were isolated from WT and ApoE $-/-$ mice 15 days after the induction of active EAE and stained for NK1.1 and CD3. Data are representative of three separate experiments with three mice per group at each time point.

Figure 4. Spinal cord T cell infiltration during EAE is independent of ApoE
Representative images of immunohistochemical staining for CD3 in axial spinal cord sections from WT (left) and ApoE ^{-/-} (right) mice at onset, peak and chronic phases of EAE. Arrows indicate CD3⁺ labeling of T cells. Scale bar = 100 μ M.

A.

B.

Figure 5. Quantities of axonal injury are similar in ApoE^{-/-} and WT mice with EAE

(A) Representative photomicrographs of lumbar spinal cords from WT (left) and ApoE^{-/-} (right) mice at day 20 post-immunization. Immunohistochemical labeling of non-phosphorylated neurofilaments (green) along with DAPI staining (blue) of lumbar spinal cord sections reveals accumulation of axonal injury within white matter tracts (arrows).

Scale bar = 100 μ m. Data shown represents images obtained from three mice per group in two separate experiments. (B) Quantification of axonal injury was performed as described in the materials and methods. Data presented represent mean $\pm$ SEM and are from spinal cords from at least three separate mice per group.

A.

B.

C.

Figure 6. ApoE expression is elevated in the CNS by DCs and other innate cells during EAE
 (A) RQ value for spleen and spinal cord tissue from WT mice at day 16 post-immunization in comparison to naïve tissue. The ratio of RQ values from spinal cord and spleen samples from each individual mouse is shown to the right. (B) EAE was induced in WT mice by transfer of encephalitogenic T cells. RQ value for spleen, brain and spinal cord tissue at day 13 post-transfer in comparison to naïve tissue. (C) Gene expression of ApoE by FACS-sorted CNS GFP⁺ mononuclear cells from CX3CR1^{GFP} mice (day 22) or CD11c⁺ cells

from WT mice (day 17) compared with splenocytes expressing the same marker following active EAE induction. Data shown are averages of at least three samples per group.

Table 1Summary of EAE clinical features in ApoE $-/-$ and WT mice

Mouse group	Model	Incidence	Day of Onset (mean $\pm$ SEM)	Maximum Disease* (mean $\pm$ SEM)
WT (n=26)	Active MOG ₃₅₋₅₅	92%	12.8 $\pm$ 0.5 ^a	3.3 $\pm$ 0.2 ^b (n=16)
ApoE $-/-$ (n=29)	Active MOG ₃₅₋₅₅	69%	15.1 $\pm$ 0.7	2.5 $\pm$ 0.3 (n=19)
WT (n=8)	Active rMOG	100%	9.6 $\pm$ 0.6 ^c	3.8 $\pm$ 0.2 (n=3)
ApoE $-/-$ (n=13)	Active rMOG	92%	15.9 $\pm$ 1.8	3.6 $\pm$ 0.6 (n=7)
WT (n=24)	Passive WT cells	96%	6.9 $\pm$ 0.4	3.5 $\pm$ 0.3 ^d
ApoE $-/-$ (n=21)	Passive WT cells	71%	7.2 $\pm$ 0.4	2.2 $\pm$ 0.4
WT (n=12)	Passive ApoE $-/-$ cells	92%	5.7 $\pm$ 0.3 ^e	3.5 $\pm$ 0.5
ApoE $-/-$ (n=11)	Passive ApoE $-/-$ cells	100%	6.7 $\pm$ 0.4	4.5 $\pm$ 0.3

* Only mice available at day 30 post-immunization were used to calculate maximum disease scores

^a $p < 0.005$ by Student's t test vs. ApoE $-/-$ active EAE with MOG₃₅₋₅₅

^b $p < 0.05$ by Student's t test vs. ApoE $-/-$ active EAE with MOG₃₅₋₅₅

^c $p < 0.01$ by Student's t test vs. ApoE $-/-$ active EAE with rMOG

^d $p < 0.005$ by Student's t test vs. ApoE $-/-$ passive EAE receiving WT cells

^e $p < 0.05$ by Student's t test vs. ApoE $-/-$ passive EAE receiving ApoE $-/-$ cells