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Deficiency of Fatty Acid-Binding Proteins in Mice Confers Protection from Development of Experimental Autoimmune Encephalomyelitis¹

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Fatty acid-binding proteins (FABPs) act as intracellular receptors for a variety of hydrophobic compounds, enabling their diffusion within the cytoplasmic compartment. Recent studies have demonstrated the ability of FABPs to simultaneously regulate metabolic and inflammatory pathways. We investigated the role of adipocyte FABP and epithelial FABP in the development of experimental autoimmune encephalomyelitis to test the hypothesis that these FABPs impact adaptive immune responses and contribute to the pathogenesis of autoimmune disease. FABP-deficient mice exhibited a lower incidence of disease, reduced clinical symptoms of experimental autoimmune encephalomyelitis and dramatically lower levels of proinflammatory cytokine mRNA expression in CNS tissue as compared with wild-type mice. In vitro Ag recall responses of myelin oligodendrocyte glycoprotein 35–55-immunized FABP^{−/−} mice showed reduced proliferation and impaired IFN- γ production. Dendritic cells deficient for FABPs were found to be poor producers of proinflammatory cytokines and Ag presentation by FABP^{−/−} dendritic cells did not promote proinflammatory T cell responses. This study reveals that metabolic-inflammatory pathway cross-regulation by FABPs contributes to adaptive immune responses and subsequent autoimmune inflammation. *The Journal of Immunology*, 2007, 179: 313–321.

The adipocyte fatty acid-binding protein (A-FABP³; also referred to as aP2) and the epidermal FABP (E-FABP; also referred to as mal1) are members of the FABP family of cytosolic proteins and are products of the genes designated *FABP4* and *FABP5*, respectively. The FABP family consists of nine highly homologous proteins expressed with distinct tissue specificity (1). FABPs bind long-chain fatty acids and are believed to function as transporters of fatty acids to intracellular targets. A-FABP-deficient mice show protection from development of diet-induced insulin resistance (2), as well as resistance to development of atherosclerosis when crossed onto an apoE-deficient background (3). Mice deficient for both A- and E-FABP display an even greater degree of protection from atherosclerosis, improved glucose and lipid metabolism, and an increased survival rate (4, 5).

A recent study has shown that a genetic polymorphism of the *FABP4* locus in humans resulting in reduced A-FABP expression produces a similar phenotype as in mice, suggesting that the biological roles of FABPs may be similar between species (6).

Ligands of A- and E-FABP include arachidonic acid and metabolites of the cyclooxygenase and lipoxygenase pathways, including compounds that also act as ligands for peroxisome proliferator-activating receptor γ (PPAR γ). PPAR γ , a lipid-regulated nuclear transcription factor, both induces expression of genes involved in cholesterol trafficking (7) and represses transcriptional activation of proinflammatory genes in macrophages (8, 9). We have shown that macrophage expression of A-FABP is antagonistic to PPAR γ activity suggesting that FABPs may act to restrict accessibility of PPAR γ ligands to the nucleus (10). A-FABP-deficient macrophages displayed elevated PPAR γ activity, which was accompanied by enhanced CD36 and ABCA1 expression and accelerated cholesterol trafficking. In contrast, macrophage A-FABP deficiency resulted in reduced activity of the I κ B kinase (IKK)-NF- κ B pathway, accompanied by reduced inflammatory potential (10). These findings indicate that expression of A-FABP directs macrophages toward inflammatory function and restricts PPAR-regulated lipid trafficking, thus providing a mechanistic basis for the atheroprotective effect of FABP deficiency.

Experimental autoimmune encephalomyelitis (EAE)/multiple sclerosis (MS) is an autoimmune disease of the CNS marked by demyelination, axonal injury, and loss of neurological function (11). The disease process is initiated by perivascular infiltration of mononuclear cells, primarily inflammatory T cells and macrophages into the CNS with subsequent retention and activation of self-reactive T cells (12). A role of both Th1 and Th17 T cell subsets in EAE has been demonstrated. The presence of Th1 cells in CNS of EAE-diseased mice and the ability of Th1 cells to transfer EAE are well-documented (13). However, the role of Th1 cells as the central mediators of EAE/MS was challenged by the observation that IFN- γ and IFN- γ receptor

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³ Abbreviations used in this paper: A-FABP, adipocyte fatty acid-binding protein; E-FABP, epidermal FABP; PPAR, peroxisome proliferator-activating receptor; IKK, I κ B kinase; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; MOG, myelin oligodendrocyte glycoprotein.

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deficiency resulted in exacerbated EAE (14, 15). It has also been shown that the administration of IL-12 suppressed EAE and that induction of IFN- γ was responsible for this effect (16). Recent studies have demonstrated that EAE pathogenesis is dependent on IL-23 and the maintenance of the Th17 subset of T cells (17, 18). An explanation for the exacerbated EAE seen in IFN- γ -deficient mice was found by the demonstration that IFN- γ negatively regulates Th17 development (16). Thus, published data indicate that both Th1 and Th17 participate in EAE, potentially at different stages of disease, and counterregulatory mechanisms may serve to limit disease progression.

Although the precise contributions of Th1 and Th17 subsets remain uncertain, it has been established that the retention of CNS-infiltrating macrophages initiates destruction of myelin and oligodendrocytes and is associated with onset of clinical symptoms (19). Studies have demonstrated that partial depletion of macrophages delayed the onset of clinical signs and reduced the severity of disease (20) and near complete depletion of macrophages strongly suppressed clinical symptoms of EAE (21). We have shown that many of the macrophage functions shown to contribute to EAE disease progression are regulated by FABPs. For example, MCP-1/CCL2 and IL-6 are well-documented as major contributors to development of EAE (22, 23) and the production of these proteins is reduced in A-FABP-deficient macrophages (10). In addition, it has been demonstrated that administration of PPAR γ ligands reduced symptoms of EAE (24–26) and that PPAR γ -deficient heterozygous mice showed exacerbated disease (27). Given the enhanced PPAR activity in FABP-deficient macrophages and the reduced production of cytokines/chemokines that mediate EAE, we hypothesized that FABPs may contribute to the pathogenesis of EAE/MS. In the present study, we evaluated the impact of FABP deficiency on the development of EAE via immunization of A-FABP $^{-/-}$, E-FABP $^{-/-}$, and A, E-FABP $^{-/-}$ (double deficient) mice with myelin oligodendrocyte glycoprotein peptide (MOG) 35–55. FABP $^{+/+}$ and FABP $^{-/-}$ mice were compared for clinical symptoms of EAE, Ag recall responses, and the ability of myeloid APC to induce proinflammatory T cell responses. Our results demonstrate a protective role of FABP deficiency in the development of EAE and reveal altered APC function of both macrophages and dendritic cell (DC) derived from FABP-deficient mice.

Materials and Methods

Mice

Mice deficient for A-FABP and E-FABP were generated as previously described (2, 5, 28). The FABP-deficient mice were backcrossed >10 generations onto a C57BL/6J background. OT-II breeding pairs were obtained with permission of Dr. W. R. Heath (Walter and Eliza Hall Institute, Victoria, Australia) (29). FABP-deficient and OT-II mice are bred and maintained in the University of Louisville Research Resources Facility and all animal care and experimental procedures used in this study were approved by the University of Louisville Institutional Animal Care and Use Committee.

Induction of EAE

MOG_{35–55} peptide corresponding to the sequence MEVGWYRSPFSRV VHLRYNGK was purchased from Bio-Synthesis. Mice were injected in the flank with a 100- μ l emulsion containing 150 μ g of MOG_{35–55} in CFA (Sigma-Aldrich) supplemented with 500 μ g of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories). Mice were injected i.p. with 500 ng of pertussis toxin (List Biological Laboratories) immediately following MOG_{35–55} injection and again 2 days postimmunization. The animals were weighed and scored daily for clinical symptoms of EAE. Clinical scores were designated numerically according to the following: 0, no detectable EAE symptoms; 1, tail paralysis/loss of tonic; 2, abnormal gait; 3, hind limb paralysis; 4, hind and forelimb paralysis; 5, moribund or dead; 0.5 gradations were assigned for intermediate scores.

Analysis of FABP and cytokine mRNA by real-time RT-PCR

Brain and spinal cord tissue was harvested, frozen in liquid nitrogen, and ground into a fine powder. mRNA was isolated from brain and spinal cord tissue and converted to cDNA using μ MACs One-step cDNA columns (Miltenyi Biotec). Real-time RT-PCR was performed using a DNA Opticon 2 monitor (MJ Research, currently Bio-Rad) using SYBR Green (New England Biolabs). Primers for A-FABP and E-FABP were prepared by MWG Biotech, based on sequences provided by Dr. D. A. Bernlohr (University of Minnesota, Minneapolis, MN): A-FABP (forward 5'-CAGAAG TGGGATGGAAAGTCG-3' and reverse 5'-CGACTGACTATTGTAGTG TTTGA-3') and E-FABP (forward 5'-AACCGAGAGCACAGTGAAG-3' and reverse 5'-ACACTCCACGATCATCTTCC-3'). TNF- α , MCP-1/CCL2, IL-6, IL-10, IL-17, IFN- γ expression was analyzed by Quantitect Primer Assays (Qiagen). β -actin mRNA was analyzed using primers purchased from Bio-Rad. Relative expression of mRNA transcripts was quantified using the relative expression software tool (30).

Immunohistochemical analysis

Spinal cord samples were frozen in isopentane cooled in liquid nitrogen and cryosectioned at 6 μ m. Slides were incubated in Dual Endogenous Enzyme Blocking reagent (DakoCytomation) for 10 min, followed by a 30-min incubation with rat anti-CD45 mAb (ab25386; Abcam) or diluent, for negative controls. Incubation in primary Ab was followed by 15-min incubations with rat probe, followed by rat HRP-polymer, components of a Rat-on-Mouse HRP-polymer kit (BioCare Medical). Nuclei were stained with hematoxylin (DakoCytomation) for 5 min and the slides were dehydrated and mounted in Permount (Fisher Scientific).

Generation of bone marrow-derived macrophages and DC

Femurs and tibias from 8- to 10-wk-old mice were flushed with DPBS (Mediatech), supplemented with 2% FBS (Atlanta Biologicals) to collect bone marrow. For generation of macrophages, the bone marrow was washed in DPBS and plated overnight in RPMI 1640 (HyClone) with 10 ng/ml M-CSF (R&D Systems) in 100-mm tissue culture dishes (BD Biosciences). After overnight incubation, nonadherent cells were plated in RPMI 1640 containing 25% filtered supernatant of L929 fibroblasts (American Type Culture Collection) with 10 ng/ml M-CSF in 6-well Ultra Low Cluster (low attach) plates (Corning). Cells were harvested on day 7 and purified by centrifugation on 35% Ficoll (Atlanta Biologicals). For generation of DCs, bone marrow mononuclear cells, prepared as described above, were plated at 2×10^6 cells/10 ml of RPMI 1640 with 20 ng/ml GM-CSF (R&D Systems) and incubated at 37°C/5% CO₂ for 7 days in 100-mm petri dishes (BD Biosciences). Both macrophages and DC were subjected to flow cytometric analysis for the expression of CD11c, CD11b, CD80, CD86, CD40, and I-A^b. Macrophage cultures were consistently 98–100% CD11b⁺ and DC cultures were consistently 98–100% CD11c⁺.

Analysis of T cell responsiveness and APC activity

For analysis of MOG_{35–55} recall responses, splenocytes were harvested from control and MOG-immunized animals and plated at 2.5×10^5 cells in 96-well plates (Nalge Nunc International). Cells were left untreated or stimulated with MOG_{35–55} (10, 50 μ g) for 48 h. Culture supernatants were harvested and assayed for IFN- γ content by ELISA (OptEIA; BD Biosciences). Analysis of macrophage and DC APC activity was performed with use of responder T cells isolated from OT-II mice. Bone marrow-derived macrophages or DC were plated at 10^5 cells/well in 96-well plates and pulsed for 4 h with 100 μ g/ml OVA (Sigma-Aldrich). OT-II T cells, enriched by nylon wool column purification, were added to the Ag-pulsed APC at 2.5×10^5 /well and incubated at 37°C/5% CO₂ for 48 h. For neutralization of IL-10 activity, cultures were treated with the indicated concentrations of neutralizing anti-IL-10 (clone JES5-16E3; BD Pharmingen) at the time of addition of OT-II T cells. Supernatants were harvested and assayed for cytokine content by ELISA (OptEIA). For analysis of T cell proliferation, cultures were incubated for 72 h, pulsed with 1 μ Ci/ml [³H]thymidine (Amersham Biosciences, currently GE Healthcare) for 12 h, then lysed using a Filtermate Harvester (Packard Instrument) onto glass fiber filters (Packard Instrument). Counts per million were read on a TopCount NXT (Packard Instrument) scintillation counter.

For analysis of cytokine production by purified T cell populations, splenocytes were harvested from healthy FABP $^{+/+}$ and A-, E-FABP $^{-/-}$ mice and T cells were enriched using a Pan T Cell Isolation kit (Miltenyi Biotec). Cells were then plated at 2.5×10^5 cells/well in 96-well plates (Nalge Nunc International) coated with 5, 10, or 20 μ g/ml anti-CD3 ϵ (BD Pharmingen) at 37°C/5% CO₂ for 48 h. Supernatants were harvested and assayed for IFN- γ (OptEIA; BD Biosciences). Purified T cell populations were consistently 97–100% CD3 ϵ ⁺.

Table I. *MOG₃₅₋₅₅ induced EAE in FABP-deficient mice^a*

Group	n	Day of Onset (Mean ± SD)	Peak Clinical Score (Mean ± SD)	Incidence of EAE
FABP ^{+/+}	52	15.35 ± 2.12	2.38 ± 1.13	49/52 (94%)
A-FABP ^{-/-}	18	16.20 ± 2.04	1.56 ± 1.16*	15/18 (83%)
E-FABP ^{-/-}	15	15.50 ± 1.31	1.10 ± 1.18**	8/15 (53%) [†]
A, E-FABP ^{-/-}	20	19.53 ± 4.60***	1.55 ± 1.21*	15/20 (75%) ^{††}

^aFABP^{+/+} and FABP^{-/-} mice were immunized with MOG₃₅₋₅₅ and clinical scores were assessed daily. Student's *t* tests were performed to compare FABP^{-/-} mice with FABP^{+/+} controls in day of onset and peak clinical score. *, *p* < 0.01; **, *p* = 0.0003; ***, *p* = 0.0002. Fisher's exact tests were performed to compare FABP^{-/-} mice with FABP^{+/+} controls in incidence of EAE. †, *p* = 0.0006 and ††, *p* = 0.03.

Analysis of DC cytokine production

Bone marrow-derived DC were isolated from healthy FABP^{+/+} and FABP^{-/-} animals and plated at 10⁵ cells/well in 96-well plates (Nalge Nunc International). Cells were left untreated or stimulated with the indicated concentrations of LPS (Sigma-Aldrich) at 37°C/5% CO₂ for 24 h. Supernatants were harvested and assayed by ELISA for IL-12p70 (eBioscience), IL-10, IL-1β, IL-6, and TNF-α (OptEIA; BD Biosciences). To evaluate the impact of IL-10 on DC inflammatory cytokine production, bone marrow-derived DC were isolated from healthy mice and plated as described above. Cells were left untreated or stimulated with LPS with or without the indicated concentrations of neutralizing anti-IL-10 (clone JES5-16E3; BD Pharmingen). Supernatants were harvested and assayed for IL-12p70 (eBioscience) and TNF-α (OptEIA; BD Biosciences).

Western blot analysis of FABP expression

Bone-marrow-derived macrophages and DCs were generated then lysed in buffer containing 25 mM Tris-HCl, 1% deoxycholate, 0.35 M NaCl, and 1% Triton X-100 (Fischer Scientific). Protein quantity was assayed by bicinchoninic acid (Pierce) and 8 μg of protein was loaded per well on a 15% Tris-HCl gel (Bio-Rad). The contents of the gel were transferred in a Trans-Blot SemiDry Transfer Cell (Bio-Rad) onto nitrocellulose membranes (Amersham Biosciences). The membranes were incubated with rabbit anti-A-FABP and anti-E-FABP (provided by Dr. D. A. Bernlohr, University of Minnesota, Minneapolis, MN), followed by donkey anti-rabbit-HRP (Jackson ImmunoResearch Laboratories). Ab-labeled proteins were detected using an ECL Western blot analysis system (Amersham Biosciences) and the bands were visualized on Kodak Biomax film. The blots were also

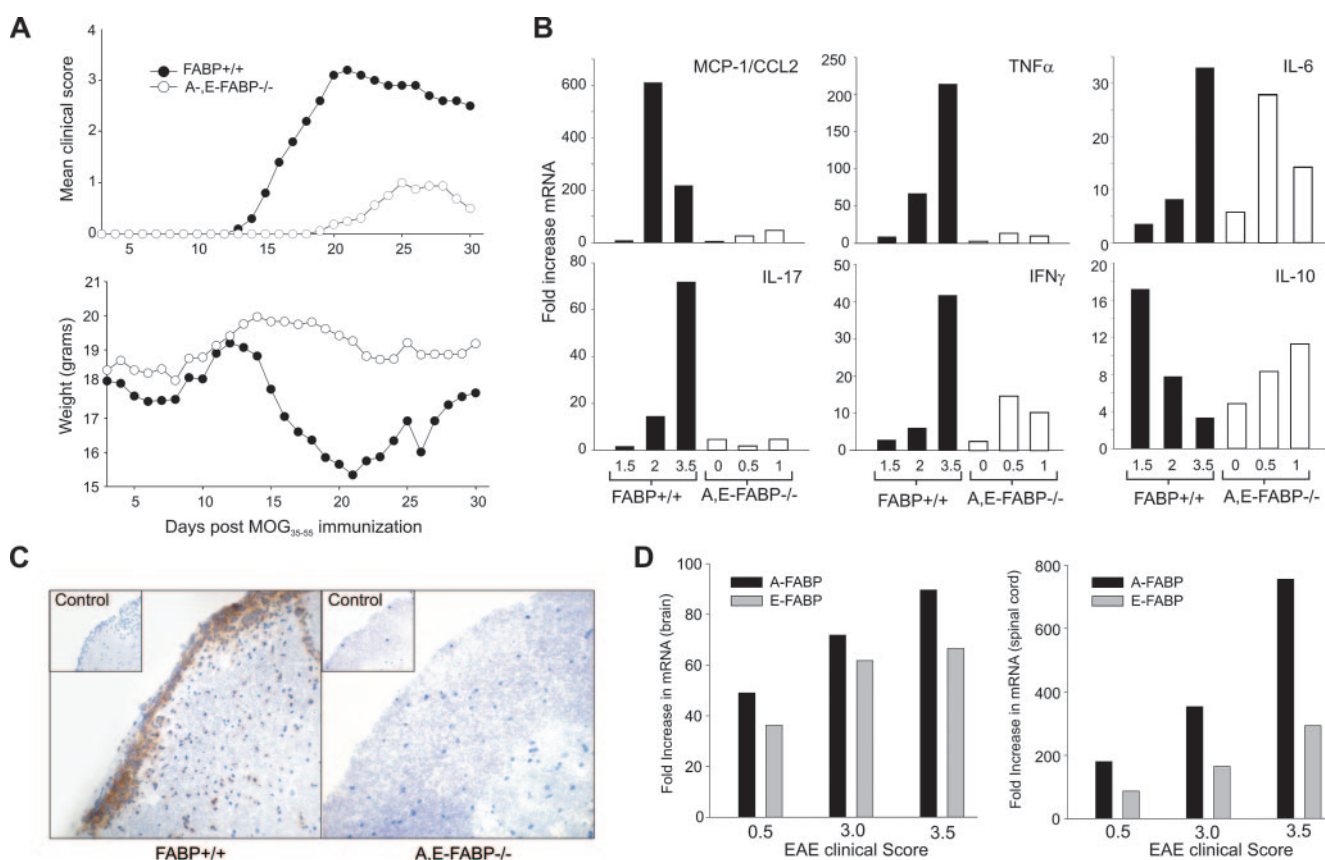


FIGURE 1. FABP deficiency results in reduced clinical symptoms of EAE and reduced CNS inflammation in MOG₃₅₋₅₅-immunized mice. **A**, FABP^{+/+} mice (*n* = 10) and A-, E-FABP^{-/-} mice (*n* = 8) were MOG-immunized and weighed and scored daily for 30 days postimmunization. **B**, Brain tissue from FABP^{+/+} and A-, E-FABP^{-/-} at various stages of EAE was analyzed for cytokine/chemokine mRNA expression by real-time RT-PCR. Mice were euthanized on day 30 post-MOG immunization. Clinical scores are indicated on the x-axis. Fold increase in mRNA was quantified using β-actin as a reference gene and a nonimmunized brain sample as the baseline for expression using the relative expression software tool. **C**, Immunohistochemical labeling of spinal cord tissue with anti-CD45 Abs revealed more extensive leukocyte infiltration in tissue from FABP^{+/+} mice than in tissue from FABP^{-/-} mice. Original magnification, ×20. **D**, Real-time RT-PCR analysis of A-FABP and E-FABP mRNA content of CNS tissue. Mice were euthanized on days 21 and 26 post-MOG immunization.

labeled with mouse anti- β -actin (Sigma-Aldrich) followed by goat-anti-mouse-HRP (Jackson ImmunoResearch Laboratories) as a loading control.

Statistical analysis

The Student *t* test was performed for comparisons of disease severity, levels of cytokine production in recall assays, and assays of macrophage and DC Ag presentation between FABP^{+/+} and FABP^{-/-} mice. A *p* value of <0.05 was considered significant.

Results

FABP-deficient mice show reduced clinical symptoms of EAE

EAE was induced via immunization of FABP^{+/+} and FABP^{-/-} mice with MOG₃₅₋₅₅ peptide and clinical scores were assessed daily. Table I summarizes data from pooled experiments. A minimum of two separate experiments comparing FABP^{+/+} and FABP^{-/-} mice were performed for each FABP^{-/-} genotype. Overall, FABP^{-/-} mice developed less severe disease than FABP^{+/+} animals, with a lower incidence of disease and significantly lower peak clinical scores. In addition, mice deficient for both A-FABP and E-FABP showed a significant delay in disease onset as compared with FABP^{+/+} mice (mean of 19.5 days, FABP^{-/-}, vs mean of 15.3 FABP^{+/+}). Clinical data obtained from a representative experiment comparing FABP^{+/+} with A, E-FABP^{-/-} mice are shown in Fig. 1A. Mean clinical scores assessed over a 30-day period postimmunization were dramatically reduced in the A, E-FABP^{-/-} as compared with FABP^{+/+} mice (*top panel*), as was the weight loss typically associated with disease onset (*bottom panel*). Real-time RT-PCR analysis of cytokine expression in brain tissue of representative animals is shown in Fig. 1B. Fold increases in MCP-1/CCL2, TNF- α , IL-17, and IFN- γ mRNA expression over baseline levels present in healthy, nonimmunized mice were substantially higher in brain tissue of FABP^{+/+} mice as compared with FABP^{-/-} mice and, with the exception of MCP-1/CCL2, the levels of these cytokines in FABP^{+/+} mice increased with increasing clinical score. Conversely, levels of IL-10 decreased with increasing clinical score in the FABP^{+/+} mice, yet, interestingly, increased with increasing clinical score in the FABP^{-/-} mice. Levels of IL-6 mRNA expression reached comparable levels in both FABP^{+/+} and FABP^{-/-} mice. TGF- β mRNA expression was low in brain tissue and was comparable between the two groups of mice (data not shown). Leukocyte infiltration into CNS tissue of MOG-immunized FABP^{+/+} and FABP^{-/-} mice was evaluated by immunohistochemistry using anti-CD45 Abs. Both spinal cord (Fig. 1C) and brain (data not shown) of FABP^{+/+} mice showed more extensive CD45 labeling than CNS tissues of FABP^{-/-} mice, indicative of a greater degree of leukocyte infiltration.

Levels of FABP expression in CNS tissue are associated with disease progression

Brain and spinal cord mRNA isolated from healthy wild-type mice was compared with mRNA isolated from mice at the early stages of EAE (clinical score of 0.5) or those at later stages of disease (clinical scores of 3 and 3.5). As shown in Fig. 1D, real-time RT-PCR analysis revealed that levels of both A- and E-FABP mRNA increased substantially in both brain (*left panel*) and spinal cord (*right panel*) following MOG immunization and disease progression, indicating an association of FABP mRNA expression in CNS tissue with clinical symptoms of EAE.

FABP deficiency alters T cell priming in MOG-immunized mice

Splenocytes from FABP^{+/+} and A, E-FABP^{-/-} mice were harvested at day 10 post-MOG₃₅₋₅₅ immunization, stimulated in vitro with MOG₃₅₋₅₅, and assayed for proliferative response via [³H]thymidine incorporation after a 72-h culture period (Fig. 2A,

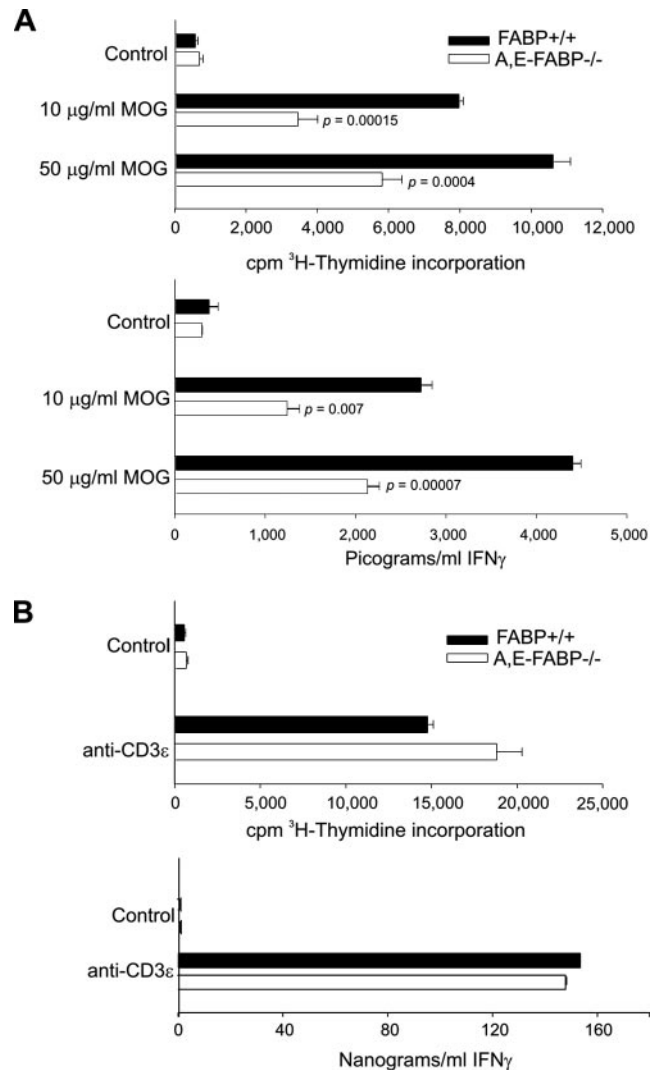


FIGURE 2. FABP deficiency alters T cell priming in MOG-immunized mice. *A, Top panel*, Proliferation of splenocytes from MOG₃₅₋₅₅-immunized mice was assessed by [³H]thymidine incorporation 72 h poststimulation. *A, Bottom panel*, The levels of IFN- γ production by MOG₃₅₋₅₅-stimulated splenocytes were assayed by ELISA at 48 h poststimulation. *B, Top panel*, Proliferation of anti-CD3 ϵ -stimulated FABP^{+/+} and FABP^{-/-} splenocytes was assessed by [³H]thymidine incorporation 72 h poststimulation. *B, Bottom panel*, Purified T cells derived from FABP^{+/+} and FABP^{-/-} mice were stimulated by culture on anti-CD3 ϵ -coated plates. IFN- γ production was assayed by ELISA 48 h poststimulation. Each panel displays a representative of three experiments yielding similar results. Data are presented as the means of triplicate determinations \pm SD.

top panel). Splenocytes from both FABP^{+/+} and FABP^{-/-} MOG-immunized mice proliferated in response to in vitro MOG stimulation, although the response of FABP^{-/-} splenocytes was reduced as compared with the response of FABP^{+/+} cells (57 and 45% reduction at 10 and 50 μ g/ml MOG, respectively). The reduced clinical scores of FABP^{-/-} mice combined with the reduced proliferative response of FABP^{-/-} splenocytes in MOG recall assays suggested that priming and/or maintenance of proinflammatory T cell responses is blunted in FABP^{-/-} mice. Indeed, supernatants harvested from MOG-stimulated splenocytes from FABP^{-/-} mice contained <50% of the levels of IFN- γ present in supernatants of MOG-stimulated splenocytes from FABP^{+/+} mice (Fig. 2A, *bottom panel*). No difference in the percentage of CD3⁺

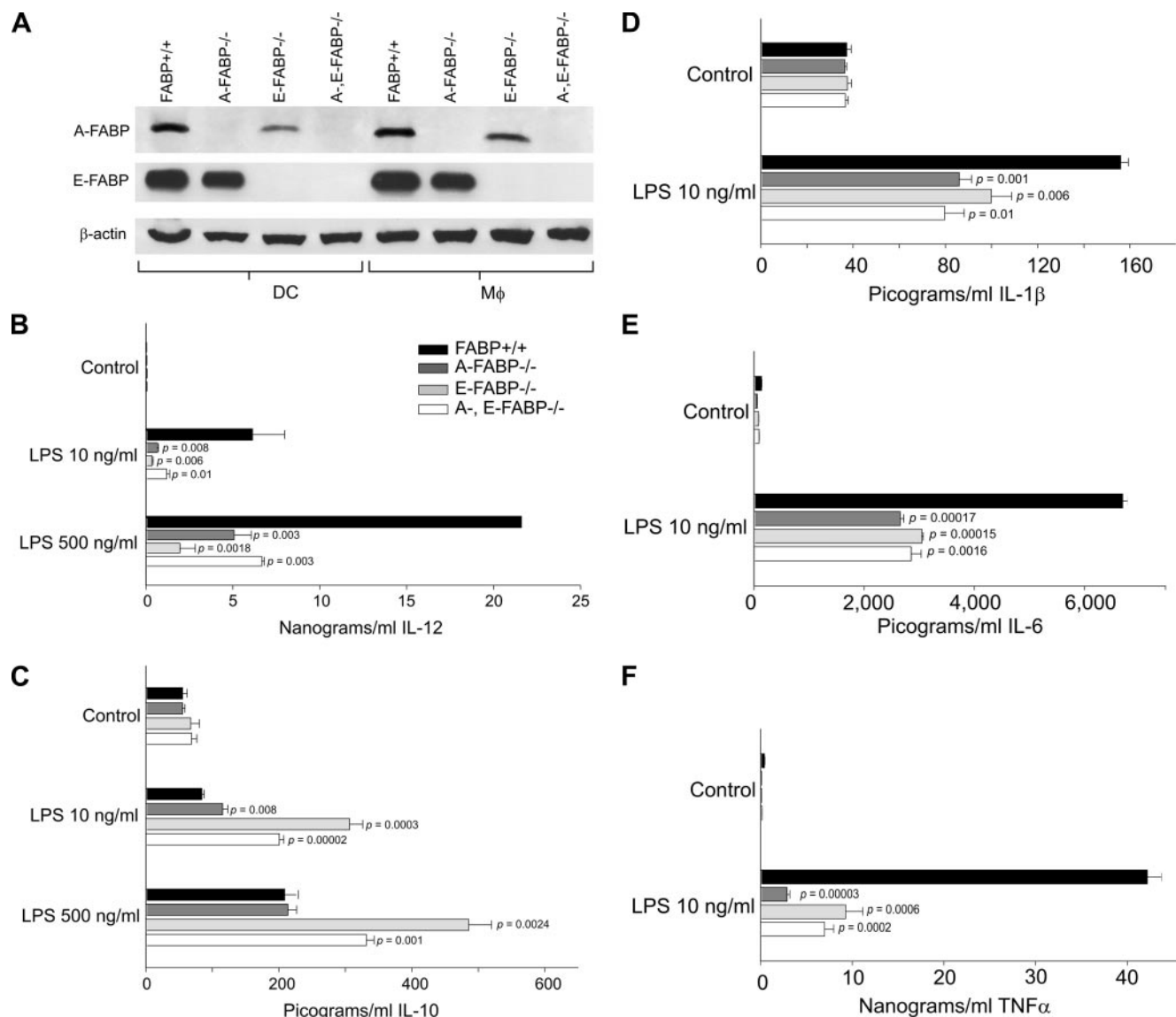


FIGURE 3. FABP expression by DC contributes to inflammatory responsiveness. *A*, FABP content of bone marrow-derived DC and macrophages was evaluated by Western blot. *B–F*, Bone marrow-derived DC from FABP^{+/+} and FABP^{-/-} mice were evaluated for production of (B) Th1-driving IL-12, (C) Th2-driving IL-10, and proinflammatory IL-1β (D), IL-6 (E), and TNF-α (F) by ELISA. Each panel displays a representative of two experiments yielding similar results. Data are presented as the means of triplicate determinations ± SD.

T cells present in spleens of FABP^{+/+} and FABP^{-/-} mice was detected by flow cytometric analysis (data not shown), indicating that the functional differences observed were not due to differences in numbers of T cells present in the splenic compartment. In addition, proliferation of T cells in response to anti-CD3ε stimulation was not significantly different between wild-type and FABP^{-/-} mice (Fig. 2*B*, top panel) and purified T cells isolated from wild-type and FABP^{-/-} splenocytes produced equivalent levels of IFN-γ in response to immobilized anti-CD3ε (Fig. 2*B*, bottom panel). Therefore, the reduced proliferation and IFN-γ production of splenocytes from FABP^{-/-} mice does not appear to be due to intrinsic defects in T cell responsiveness. Levels of IL-17 and IL-10 were low to undetectable in MOG recall cultures and no significant differences in production of these cytokines between the FABP^{+/+} and FABP^{-/-} cultures were observed (data not shown). Given the presence of both IL-17 and IL-10 mRNA in CNS tissue (Fig. 1*B*), these data suggest that, although Th2 and Th17 subsets appear to be present at the site of inflammation, at the time of

harvest, MOG-specific representatives of these subsets were not prominent in the splenic compartment.

FABP deficiency results in impaired proinflammatory cytokine production by DC

We had previously shown that macrophages from A-FABP^{-/-} deficient mice display impaired production of inflammatory cytokines (10) and macrophages deficient for E-FABP are likewise impaired in proinflammatory cytokine production (J. Suttles, unpublished data). Thus, we hypothesized that the altered cytokine environment of the FABP^{-/-} mice may not be favorable for the development or support of proinflammatory T cell responses. The cytokine profiles of CNS tissue (Fig. 1*B*) support this hypothesis. The impact of FABP deficiency on T cell priming revealed in Fig. 2*A* implicated DC as contributing to the protective effect of FABP deficiency in the EAE model. DC expression of both E-FABP and A-FABP has been reported previously (31, 32). Given that both A- and E-FABP are expressed in macrophages, we considered it likely that myeloid-lineage DC express comparable levels of both A- and

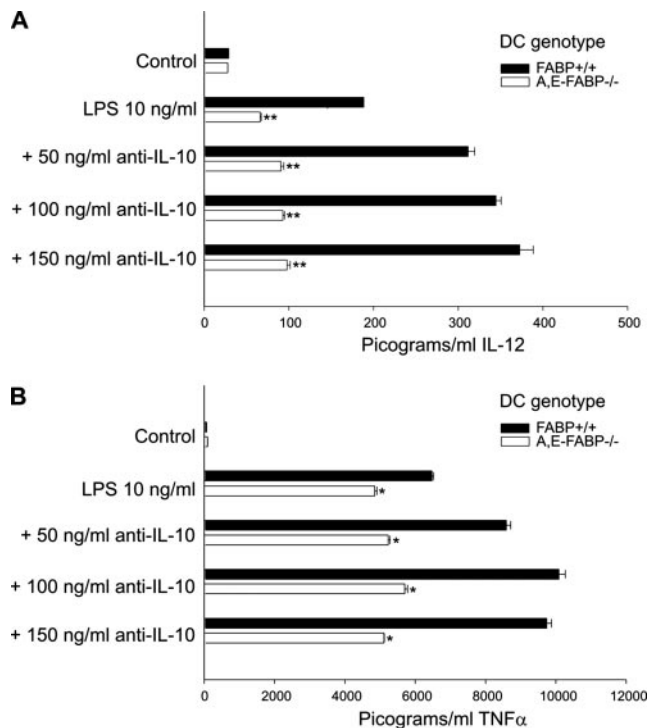


FIGURE 4. Proinflammatory cytokine production by FABP^{-/-} DC is not restored by neutralization of IL-10. Bone marrow-derived DC from FABP^{+/+} and A-, E-FABP^{-/-} mice were stimulated with LPS in the presence of neutralizing anti-IL-10 Ab. Supernatants were analyzed for IL-12p70 (A) and TNF-α (B) content by ELISA. Each panel displays a representative of two experiments yielding similar results. Data are presented as the means of triplicate determinations ± SD. **, $p < 0.00015$; *, $p < 0.003$.

E-FABPs and that these FABPs function similarly in this closely related cell type. Analysis of lysates of bone marrow-derived DC by Western blot revealed expression of A- and E-FABP by DC at levels comparable to those expressed by macrophages (Fig. 3A). It is noteworthy that in both cell types, deficiency of A-FABP resulted in reduced E-FABP expression, and, likewise, deficiency of E-FABP resulted in reduced A-FABP expression. FABP^{-/-} DC were found to be deficient in expression of IL-12 in response to LPS stimulation (Fig. 3B), whereas levels of IL-10 proved to be somewhat higher in FABP^{-/-} DC (Fig. 3C). Stimulation of DC did not result in a substantial increase in IL-23 levels above background, with 500 pg/ml produced in response to 500 ng/ml LPS vs a 300 pg/ml background level, and differences between the two groups did not reach statistical significance (data not shown). Analysis of IL-1β (Fig. 3D), IL-6 (Fig. 3E), and TNF-α (Fig. 3F) revealed a diminished level of these cytokines in FABP^{-/-} DC as compared with FABP^{+/+} DC. We considered the possibility that the reduced inflammatory cytokine production by FABP^{-/-} DC was due to an autocrine inhibitory effect of the elevated IL-10 production we observed (Fig. 3C). To address this possibility, DC were treated with neutralizing anti-IL-10 at the time of LPS stimulation. Analysis of culture supernatants revealed that treatment with neutralizing anti-IL-10 did not restore IL-12 (Fig. 4A) or TNF-α (Fig. 4B) production. The levels of these cytokines remained significantly lower than those produced by wild-type DC.

Ag presentation by FABP-deficient macrophages and DC does not promote proinflammatory T cell responses

The data presented above, as well as the reduced IL-12 production by FABP-deficient macrophages (10) and by DC (shown herein)

indicate that FABP-deficient APC do not support Th1 responses. This possibility was tested directly via culture of OVA-pulsed FABP-deficient macrophages and DC with OT-II cells. As shown in Fig. 5, cultures of both FABP^{-/-} macrophages (Fig. 5A) and FABP^{-/-} DC (Fig. 5B) with OT-II T cells produced significantly less IFN-γ than cultures of OT-II T cells incubated with FABP^{+/+} macrophages or DC, indicating a reduced ability of the FABP-deficient APC to stimulate classical Th1 responses. Similar results were seen when MOG was presented by FABP^{-/-} DC to T cells harvested from MOG-immunized FABP^{+/+} mice. All genotypes of FABP^{-/-} DC were very poor inducers of IFN-γ production by MOG-specific T cells (Fig. 5C). Importantly, as has been shown previously (32, 33), we observed via flow cytometric analysis that expression of the costimulatory molecules, CD80, CD86, and expression of CD40 by FABP^{+/+} and FABP^{-/-} DC were comparable, as was expression of I-A^b (data not shown). Thus, both populations display a maturing/mature DC phenotype and the altered T cell responses to Ag presentation by FABP^{-/-} APC are not due to deficiencies in costimulatory molecule or class II MHC expression. Identical results were obtained in our analysis of surface phenotypes of A-FABP^{-/-} and E-FABP^{-/-} macrophages (data not shown). Our data indicate that, although T cells responding to FABP^{-/-} APC are not efficient IFN-γ producers, effective Ag presentation by FABP^{-/-} APC does occur. As shown in Fig. 5D, equal levels of IL-2 are produced by FABP^{+/+} T cells in response to OVA presented by either FABP^{+/+} or FABP^{-/-} DC. To address the possibility that increased IL-10 production by FABP^{-/-} APCs (Fig. 3C) may inhibit Th1 responses by OT-II responder T cells, neutralizing anti-IL-10 Ab was added to the DC-OT-II cultures. Neutralization of IL-10 failed to reconstitute IFN-γ production to level of FABP^{+/+} cultures (Fig. 5E).

Discussion

In this study, we demonstrate that mice deficient for A-FABP and E-FABP show protection from the development of EAE, exhibiting reduced clinical symptoms of disease, reduced inflammatory cytokine mRNA expression, reduced leukocyte infiltration into CNS tissue, and impaired inflammatory T cell activity. This study was performed as a test of our hypothesis that the inflammatory outcome of autoimmune disease would be blunted in FABP-deficient mice. Although, the study was based on our previous findings demonstrating that FABP-deficient macrophages are impaired in inflammatory function, we acknowledged that the effect of A-FABP and E-FABP-deficiency likely extends beyond the role of FABPs in macrophages. Although A-FABP expression had been thought to be limited to adipocytes and myeloid cells, recent work has shown that expression of A-FABP by airway epithelial cells can be induced by stimulation with IL-4 or IL-13 and A-FABP expression in this cell type contributes to allergic airway inflammation in a murine model (34). This finding suggests that A-FABP may be expressed as a result of specific stimuli in a more diverse array of tissue types than originally supposed. In contrast, expression of E-FABP has been found in a wide variety of tissues, including brain (35), suggesting that the protection from EAE exhibited by E-FABP^{-/-} mice may include contributions of the target tissues (i.e., CNS). We have found that expression of both A-FABP and E-FABP in CNS correlated with increased disease severity (Fig. 1D). This is likely a result of infiltration of FABP-expressing leukocytes into the CNS, but may also be a result of local induction of FABP expression in target tissues in response to inflammation.

Although a potential role of nonhemopoietic cells has yet to be determined, our results demonstrate a substantial impact of FABP deficiency on the immune response to MOG_{35–55} immunization.

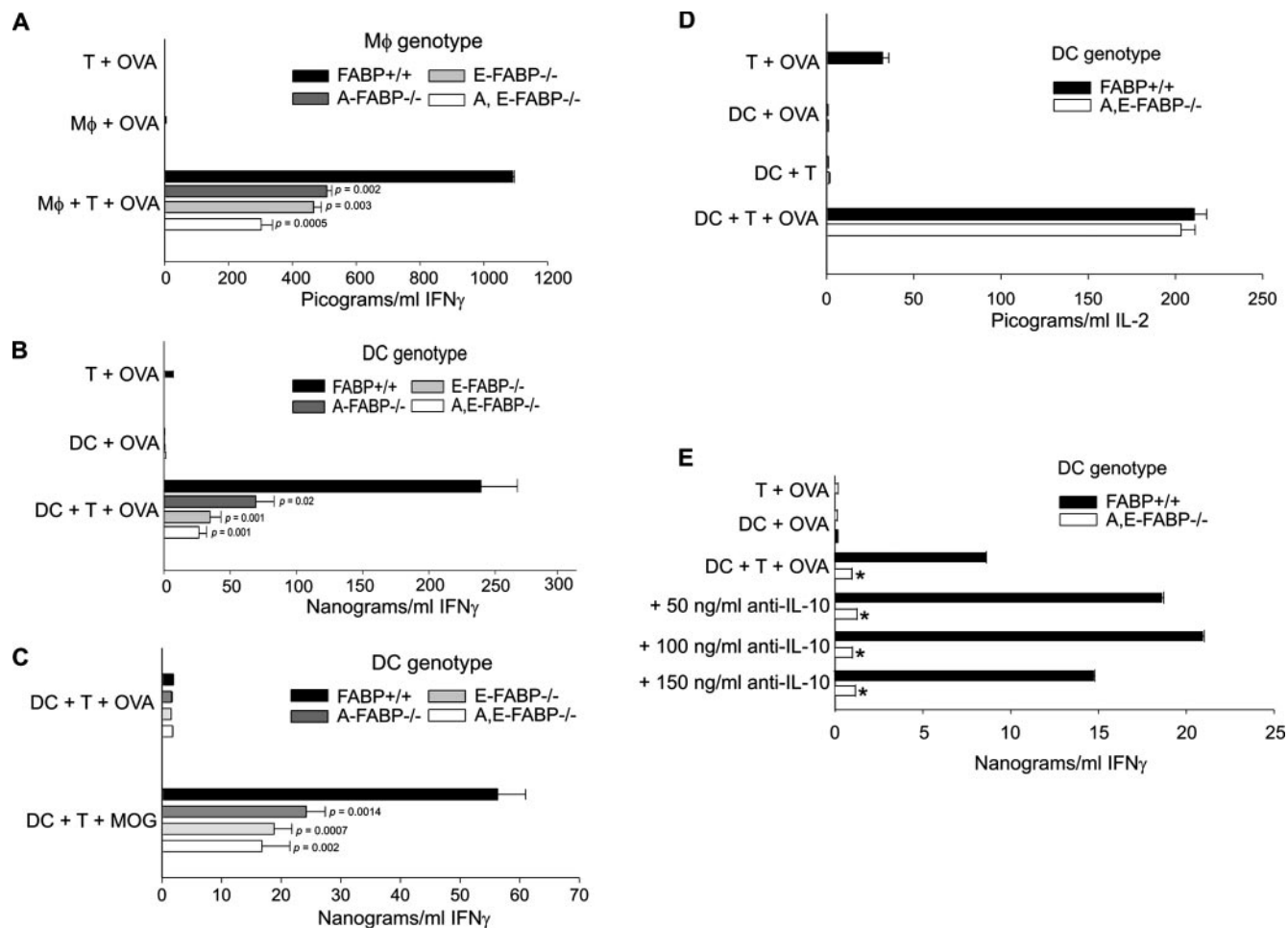


FIGURE 5. Ag presentation by FABP^{-/-} macrophages and DC does not promote proinflammatory T cell responses. *A*, Bone marrow-derived macrophages and *B*) DC were pulsed with OVA and cocultured with OT-II T cells. Analysis of supernatants by ELISA revealed a deficiency of FABP^{-/-} APC in the induction of OT-II IFN- γ production. *C*, DC were pulsed with MOG₃₅₋₅₅ before coculture with purified T cells from MOG₃₅₋₅₅-immunized wild-type animals. In this experiment, OVA-pulsed DC served as an Ag-specificity control. *D*, Production of IL-1 by OT-II cells in the presence of OVA-pulsed FABP^{+/+} and FABP^{-/-} DC was evaluated by ELISA. *E*, DC were pulsed with OVA and cocultured with OT-II T cells in the presence of neutralizing anti-IL-10 Ab. IFN- γ content of the supernatants was assessed by ELISA. Each panel displays a representative of two to four experiments yielding similar results. Data are presented as the means of triplicate determinations \pm SD. *, $p < 0.00004$.

CNS tissue showed reduced levels of both IFN- γ and IL-17, as well as TNF- α and MCP-1/CCL2 (Fig. 1*B*) and recall responses of splenocytes from MOG-immunized mice revealed a diminished MOG-reactive Th1 compartment in FABP^{-/-} mice (Fig. 2*A*). The reduced IL-12 production by APC derived from FABP^{-/-} mice most likely contributes to the suppressed IFN- γ production by T cells in response to Ag stimulation. Likewise, although our studies thus far have not revealed an impact of FABP deficiency on the Th17-supporting cytokines IL-23 and TGF- β , the production of the Th17-supporting cytokines IL-6 and IL-1 (35) by FABP^{-/-} APC was impaired, which may contribute to the reduced IL-17 synthesis observed in vivo. Although our finding that A-FABP^{-/-} DC were impaired in IL-12 production in response to LPS stimulation is in agreement with a recent report (32), our results showing a similar defect in E-FABP^{-/-} DC conflict with recent published work showing enhanced expression of IL-12 by E-FABP^{-/-} DC (33). These authors analyzed the function of DC derived from spleen, whereas our studies examined bone marrow-derived DC. Otherwise, technical aspects of the two studies are not dissimilar. Thus, the cause for this discrepancy in results is not clear.

Both the in vivo and in vitro data presented herein suggest that A-FABP and E-FABP have overlapping as well as unique func-

tions. For example, although deficiency of either A- or E-FABP, independently, resulted in protection from EAE, deficiency of E-FABP resulted in a more pronounced reduction in peak clinical scores and incidence than did deficiency of A-FABP, whereas only the combined deficiency resulted in a significant delay in onset of disease. In addition, in vitro experiments demonstrated that DC derived from double-deficient mice were more severely impaired in their ability to induce Th1 responses than DC derived from either A-FABP^{-/-} or E-FABP^{-/-} mice. Interestingly, as shown in Fig. 3*A*, we found that deficiency of A-FABP is accompanied by a reduction in expression of E-FABP, and likewise, deficiency of E-FABP is accompanied by a reduction in expression of A-FABP. In contrast, in adipocytes, A-FABP deficiency is accompanied by an up-regulation of E-FABP expression, suggesting a compensatory effect (2). Our results suggest that there may be mechanisms of cross-regulation of these two proteins in macrophages and DC and that this phenomenon may explain why, despite significant overlap in function, A-FABP^{-/-} and E-FABP^{-/-} mice display similar functional phenotypes. In other words, presence of an intact *A-FABP* gene cannot completely compensate for loss of E-FABP and vice versa.

Taken together, our data suggest that APC expression of FABPs affects the induction of EAE during Ag presentation by DC to naive T cells, as well as the ability of macrophages to exacerbate the disease state via reactivation of T cells at sites of inflammation and via the direct effect of macrophage production of inflammatory mediators (e.g., TNF- α , NO). In addition, defects in chemokine (e.g., MCP-1/CCL2) production may result in impaired leukocyte trafficking. T cells derived from FABP^{-/-} mice do not appear to have intrinsic defects in proliferation or Ag recognition, as shown by their equivalent ability to proliferate and produce IFN- γ in response to anti-CD3 ϵ stimulation (Fig. 2B) and equivalent IL-2 production (Fig. 5D) in response to Ag presentation. Therefore, it is likely that the altered T cell responses in FABP^{-/-} mice are primarily due to the altered cytokine profiles of FABP^{-/-} APC.

In previous work, we demonstrated that expression of FABPs is antagonistic to PPAR γ activity. Macrophages deficient for A-FABP possess enhanced PPAR γ activity and reduced IKK activity, resulting in elevated cholesterol trafficking activity and reduced production of proinflammatory mediators (10). The reduced inflammatory potential of FABP^{-/-} macrophages may be linked to the enhanced PPAR γ activity, due to the ability of PPAR γ to antagonize macrophage proinflammatory pathways (8). However, a number of the common ligands of both PPARs and FABPs are capable of direct interference with IKK activity, suggesting that FABP regulation of the availability of these ligands could play a role independent of PPARs (36). Although there is evidence for a role of FABPs in shuttling of PPAR ligands to the nucleus in nonhemopoietic cells (37), thus far we have found both A-FABP and E-FABP located exclusively in the cytosol in macrophages (J. Suttles, unpublished data). In addition, the finding that FABP deficiency enhanced, not reduced, PPAR γ activity, argues against a role of FABPs in delivery of ligands to the PPARs and favors the hypothesis that FABPs may restrict ligand access to the nucleus. The impact of FABP deficiency on DC function likely involves similar mechanisms. For example, PPAR γ agonists have been shown to inhibit TLR signaling in DC and to reduce the production of IL-12 (38, 39). It should also be noted that both A-FABP and E-FABP are capable of binding arachidonic acid, as well as many of its metabolites. Thus, both production and responsiveness to cyclooxygenase and lipoxygenase metabolites, which include PPAR agonists, may also be affected by FABP deficiency, which in turn could modulate macrophage and DC activities and/or disease progression. FABPs are emerging as pivotal to the interrelationship between metabolic and inflammatory pathways (40). This report extends the role of FABPs through the demonstration that the ability of FABPs to promote macrophage and DC inflammatory function, in turn, impacts the adaptive immune response by promoting proinflammatory T cell activity.

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Disclosures

The authors have no financial conflict of interest.

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