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IFN-γ Production by Amyloid β–Specific Th1 Cells Promotes Microglial Activation and Increases Plaque Burden in a Mouse Model of Alzheimer's Disease

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Alzheimer's disease (AD) is characterized by the presence of amyloid-β (Aβ)–containing plaques, neurofibrillary tangles, and neuronal loss in the brain. Inflammatory changes, typified by activated microglia, particularly adjacent to Aβ plaques, are also a characteristic of the disease, but it is unclear whether these contribute to the pathogenesis of AD or are a consequence of the progressive neurodegenerative processes. Furthermore, the factors that drive the inflammation and neurodegeneration remain poorly understood. CNS-infiltrating T cells play a pivotal role in the pathogenesis of multiple sclerosis, but their role in the progression of AD is still unclear. In this study, we examined the role of Aβ-specific T cells on Aβ accumulation in transgenic mice that overexpress amyloid precursor protein and presenilin 1 (APP/PS1). We found significant infiltration of T cells in the brains of APP/PS1 mice, and a proportion of these cells secreted IFN-γ or IL-17. Aβ-specific CD4 T cells generated by immunization with Aβ and a TLR agonist and polarized in vitro to Th1-, Th2-, or IL-17–producing CD4$^+$ T cells, were adoptively transferred to APP/PS1 mice at 6 to 7 mo of age. Assessment of animals 5 wk later revealed that Th1 cells, but not Th2 or IL-17–producing CD4$^+$ T cells, increased microglial activation and Aβ deposition, and that these changes were associated with impaired cognitive function. The effects of Th1 cells were attenuated by treatment of the APP/PS1 mice with an anti–IFN-γ Ab. Our study suggests that release of IFN-γ from infiltrating Th1 cells significantly accelerates markers of diseases in an animal model of AD. ***The Journal of Immunology*, 2013, 190: 2241–2251.**

A role for inflammation in the pathogenesis of Alzheimer's disease (AD) is suggested by epidemiological studies that have reported a decreased incidence of AD in patients treated with nonsteroidal anti-inflammatory drugs (1, 2); these findings are supported by evidence of preventative effects of these drugs in animal models of AD (3). Whereas the classical characteristics of AD are the presence of amyloid-β (Aβ) plaques and neurofibrillary tangles, together with selective neuronal loss, there is also evidence of innate immune activation in AD, with activation of microglia, the primary resident immune cell of the CNS. Activated microglia are found in the brain of AD patients with mild to moderate dementia (4) and in a significant proportion of cases with mild cognitive impairment (5). Microglia secrete inflammatory cytokines like IL-1β and TNF-α, which increase activity and expression of secretases (6, 7), contributing to Aβ deposition and the early pathogenic changes in AD (8). Inflammatory cytokines released from activated microglia are known to be potentially cytotoxic, but there is evidence indicating a positive effect of an inflammatory environment on Aβ clearance (9–11). Microglia demonstrate significant plasticity and also adopt other phenotypes that are associated with tissue repair (12). Furthermore, immune cells in the AD brain can have an alternative activated state as well as the classical proinflammatory phenotype (13). Cell-surface expression of MHC class II and costimulatory molecules is enhanced on activated microglia (14, 15), enabling them to act as APC. However, circulating cells, including T cells, are infrequently observed in the normal CNS, although there is a population of perivascular macrophages, distinct from microglia (16–18), and these cells may play an important anti-inflammatory function, perhaps mediated by a change in hypothalamic–pituitary–adrenal axis function (19).

The blood–brain barrier plays a key role in protecting the brain, restricting the entry of pathogens and macromolecules. An intact blood–brain barrier is also important in restricting entry of circulating cells, and increased blood–brain barrier permeability, which is a characteristic of several neurodegenerative conditions including multiple sclerosis, AD, and Parkinson's disease (20–22), is associated with infiltration of circulating immune cells. Studies in multiple sclerosis and experimental allergic encephalomyelitis (EAE), a mouse model of multiple sclerosis, have shown that T cells, particularly IL-17–producing CD4$^+$ T cells (Th17) cells, infiltrate the brain and spinal cord and are central to the pathogenesis of the disease (23). The role of Th1 cells in CNS inflammation associated with EAE is more controversial, with some studies suggesting that Th1 cells contribute to pathology and

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Abbreviations used in this article: Aβ, amyloid-β; AD, Alzheimer's disease; APP, amyloid precursor protein; dH_2O, distilled H_2O; EAE, experimental autoimmune encephalomyelitis; HBSS/FBS, HBSS containing 3% FBS; PS1, presenilin 1; RT, room temperature; Th17 cells, IL-17–producing CD4$^+$ T cells; WT, wild-type.

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others suggesting a protective role for IFN-γ through inhibition of Th17 cells. As well as their role in demyelination, the interaction of T cells with microglia contributes to the inflammatory changes observed in EAE (24).

T cells are also present in the brain of patients with AD (25–28), and infiltration may result from increased expression of CXCR2 and MIP-1α on the T cells (29). Although T cells, in particular Th2 or regulatory T cells, can have a protective role in the brain (30, 31), the entry of activated effector T cells, particularly Th1 or Th17 cells, into the brain in which inflammatory changes are ongoing, is likely to escalate the inflammatory cascade. Consistent with this is the finding that Aβ-induced release of inflammatory cytokines from glia was exacerbated by Th1 and Th17 cells (32), and this effect was attenuated by Th2 cells. Immunization with Aβ peptides, formulated with various adjuvants, is being evaluated both in preclinical models and in the clinic as a potential therapy for AD-based on Ab-mediated reduction of Aβ plaque burden (33). However, a proportion of AD patients who received a vaccine containing Aβ peptide formulated with the adjuvant QS21 (AN1792) developed meningoencephalitis (34). It is possible that the generation of certain subtypes of Aβ-specific T cells may contribute to inflammatory pathology in AD.

In this study, we used a transgenic mouse model of AD that overexpresses amyloid precursor protein (APP) with the Swedish mutation and exon-9–deleted presenilin 1 (PS1; APP/PS1 mice) to determine whether Aβ-specific T cell subsets can modulate Aβ burden and affect microglial activation. Aβ-specific effector T cells were generated by immunization with Aβ and CpG, polarized in vitro to Th1, Th2, and Th17 cells, and adoptively transferred to 6- to 7-mo-old APP/PS1 mice. We found that Aβ-specific Th1 cells increased Aβ deposition and microglial activation in APP/PS1 mice and negatively impacted on spatial

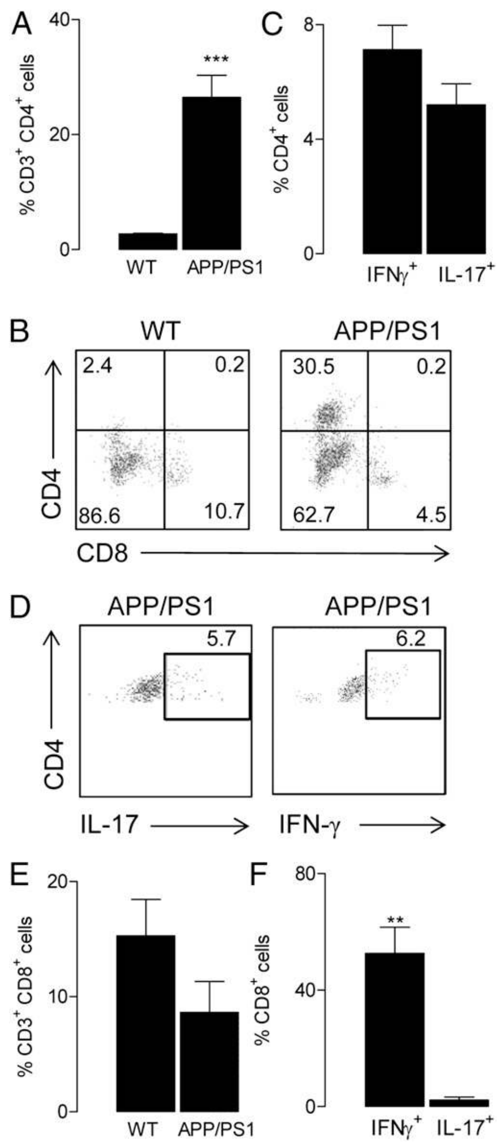

FIGURE 1. Th1 and Th17 infiltrate the brain of APP/PS1 mice. Mononuclear cells were prepared from the brain of APP/PS1 and WT mice, and cells were surface stained with Abs specific for CD3, CD4, CD8, intracellular IL-17, and IFN-γ, and flow cytometric analysis was performed. Mean frequency (**A**) and representative dot plots (**B**) of $CD4^+$ and $CD8^+$ cells in brain of WT and APP/PS1 mice. Mean frequency (**C**) and representative dot plots (**D**) of $CD4^+$ cells stained positively for IFN-γ and IL-17 in brain of APP/PS1 mice. (**E**) Mean frequency of $CD8^+$ in brain of tissue prepared from WT and APP/PS1 mice. (**F**) Mean frequency of $CD8^+$ cells stained positively for IFN-γ and IL-17 in brain tissue prepared from APP/PS1 mice. $^{**}p < 0.01$, Student *t* test for independent means ($n \geq 4$), $^{***}p < 0.001$, Student *t* test for independent means. Representative of three experiments.

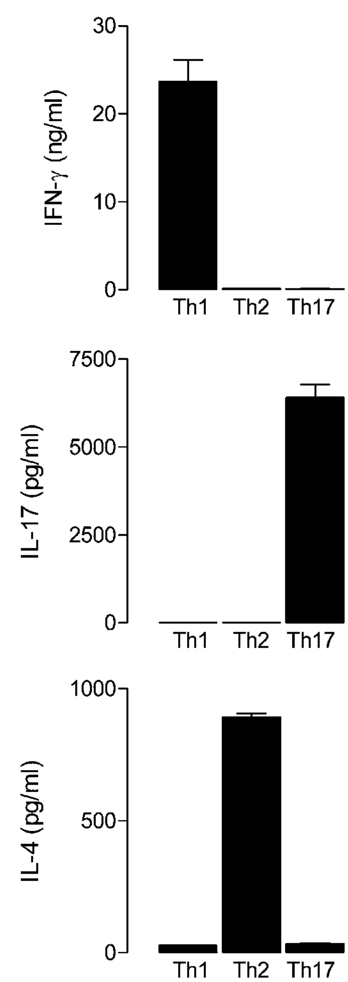

FIGURE 2. Cytokine production by in vitro polarized Aβ-specific T cells. Popliteal lymph nodes harvested from mice immunized with Aβ and CpG were cultured with $A\beta_{1-42}$ in the presence of IL-12 to generate Th1 cells, dexamethasone, IL-4, and anti–IFN-γ to generate Th2 cells, or IL-23 and anti–IFN-γ to generate Th17 cells. IFN-γ, IL-4, and IL-17 concentrations were determined by ELISA on supernatants removed 3 d after stimulation with Ag and APC. Values are expressed as means ± SEM (n = 4); representative of four experiments.

learning. Treatment of mice with anti–IFN-γ Ab ameliorated these changes, suggesting that release of IFN-γ from infiltrating Th1 cells accelerates the pathology in these animals.

Materials and Methods

Animals

APP/PS1 mice and wild-type (WT) littermates (6 to 7 mo old) were obtained from The Jackson Laboratory and subsequently bred in a specific pathogen-free unit in the Bioresources Unit in Trinity College Dublin. GFP mice were a gift from Matthew Campbell, School of Genetics and Microbiology, Trinity College Dublin. Mice used were transgenic animals on a C57/Bl6J background expressing eGFP cDNA under the control of a chicken β-actin promoter and CMV enhancer. All mice were maintained in controlled conditions (temperature 22 to 23˚C, 12-h light-dark cycle, and food and water ad libitum) under veterinary supervision, and experimentation was carried out under a license granted by the Minister for Health and Children (Ireland) and with the appropriate ethical approval.

Isolation and FACS analysis on mononuclear cell isolation from CNS tissue

APP/PS1 mice and nontransgenic littermates were anesthetized with sodium pentobarbital (40 μl) and perfused intracardially with sterile ice-cold PBS (20 ml). The brain was removed and placed in HBSS (2 ml) containing 3% FBS (HBSS/FBS; Sigma-Aldrich). Tissue was dissociated through a sterile 70-μm nylon mesh filter, washed with HBSS/FBS, and centrifuged at 170 × *g* for 10 min at room temperature (RT). The supernatant was removed and the remaining pellet resuspended in HBSS/FBS (2 ml) containing collagenase D (1 mg/ml; Roche) and DNAse I (10 μg/ml; Sigma-Aldrich) and incubated for 1 h at 37˚C. Cells were washed in HBSS/FBS and centrifuged at 1200 rpm for 5 min. Supernatants were removed, and cells were resuspended in 1.088 g/ml Percoll (9 ml; Sigma-Aldrich). This was underlaid with 1.122 g/ml Percoll (5 ml) and overlaid with 1.072 g/ml Percoll (9 ml) followed by 1.030 g/ml Percoll (9 ml) and finally PBS (9 ml). Percoll gradients were centrifuged at 1250 × *g* for 45 min at 18˚C. Mononuclear cells were removed from between the 1.088/1.072 and 1.072/1.030 g/ml interfaces, washed twice in HBSS/FBS, and counted.

Mononuclear cells prepared from CNS tissue were prepared for intracellular staining using a cell permeabilization kit (DakoCytomation). Cells were centrifuged at 1200 rpm for 5 min before stimulation with X-Vivo media (200 μl) containing PMA (10 ng/ml; Sigma-Aldrich), ionomycin (1 μg/ml; Sigma-Aldrich), and brefeldin A (5 μg/ml; Sigma-Aldrich) for 5 h. Following stimulation, cells were centrifuged at 1200 rpm for 5 min and resuspended. Low-affinity IgG receptors (FcγRIII) were blocked by incubating cells in FACS buffer (50 μl/sample) containing CD16/CD32 FcγRIII (1:100) for 10 min at RT. Cells were incubated in 50 μl/sample FACS buffer containing the appropriate FACS Abs for 15 min at RT and fixed in IntraStain Reagent A (50 μl/sample; DakoCytomation) for 15 min at RT. Cells were washed twice with FACS buffer and centrifuged at 1200 rpm for 5 min, permeabilized with IntraStain Reagent B (50 μl/sample; DakoCytomation) plus intracellular Abs for 15 min at RT in the dark, washed twice in FACS buffer, and centrifuged at 1200 rpm for 5 min. Immunofluorescence analysis was performed on a DakoCytomation Cyan,

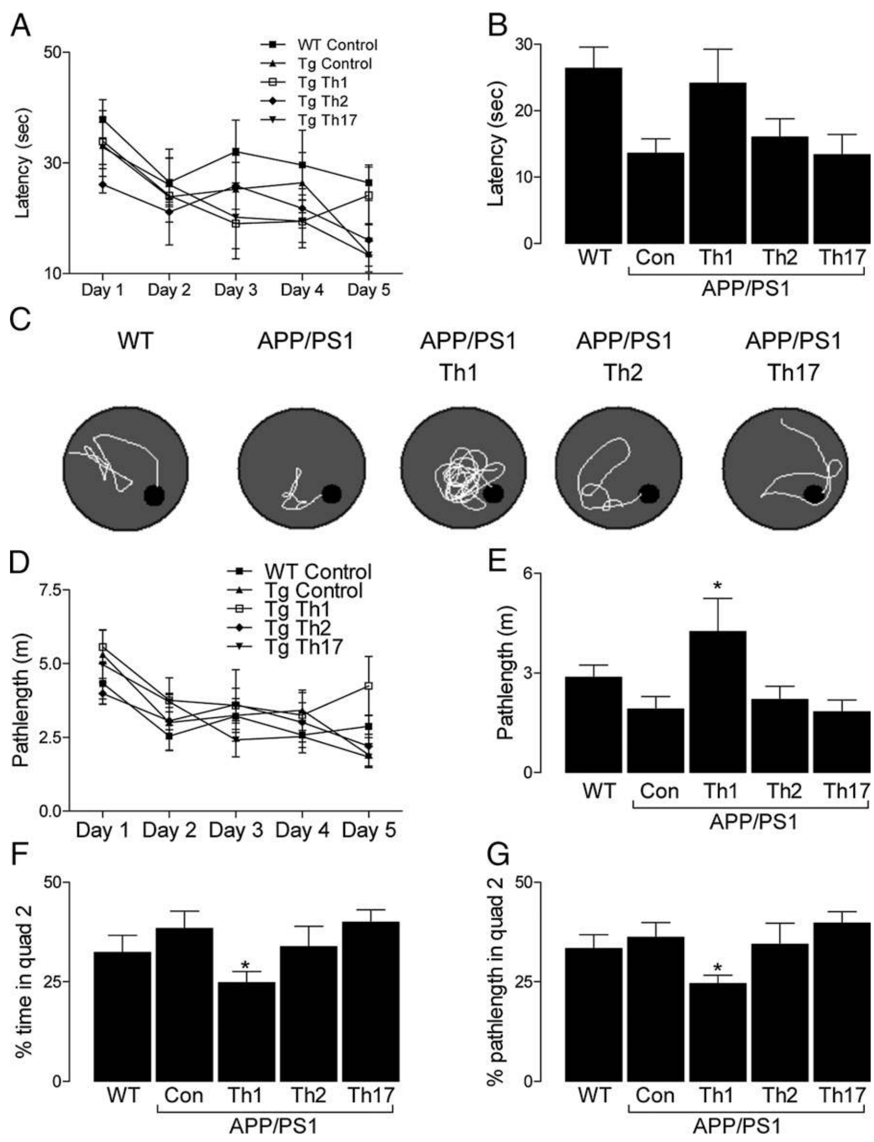

FIGURE 3. Spatial learning is impaired in APP/PS1 mice that received Aβ-specific Th1 cells. Aβ-specific Th1, Th2, and Th17 cells, generated as described in Fig. 2, were injected i.v. into APP/PS1 mice at 6 to 7 mo of age. Two weeks after injection, cognitive function was analyzed in the Morris Water Maze test. Training commenced after 1 d of habitation and continued for 5 consecutive days on which the mice underwent four 1-min trials with an intertrial interval of 5 min. The day after the final day of training, the platform was removed, and mice were given a single 60-s probe trial. The percentage of time each animal spent swimming in the quadrant previously containing the platform was measured. Path length was also assessed. (**A**) The latency to reach the platform of all groups. (**B**) Mean latency on day 5 of training. (**C**) Sample paths for individual mice in each treatment group. (**D** and **E**) The path length taken to reach the platform. (**F** and **G**) In the probe test, the percentage of the total time and distance (i.e., path length) each animal spent swimming in the quadrant was measured. *$p < 0.05$, ANOVA; $n \geq 5$; representative of two experiments. Con, Control; Tg, transgenic.

data acquired using Summit software (DakoCytomation), and the results analyzed using FlowJo software (Tree Star).

Generation of Aβ-specific T cell lines and in vivo transfer

WT mice were immunized in the footpad with $A\beta_{1-42}$ (75 μg/mouse) and CpG (25 μg/mouse) and boosted after 21 d. Mice were sacrificed 7 d later; the spleens and popliteal lymph nodes were harvested and restimulated with $A\beta_{1-42}$ (25 μg/ml) in the presence of IL-12 (10 ng/ml) to generate Th1 cells, dexamethasone (1×10^{-8} M), IL-4 (10 ng/ml), and anti–IFN-γ (5 μg/ml) to generate Th2 cells, or IL-23 (10 ng/ml) and anti–IFN-γ (5 μg/ml) to generate Th17 cells. After 4 d, IL-2 (5 ng/ml) was added to the Th1 and Th2 cell preparations, RPMI-1640 culture medium only was added to the Th17 cell cultures, and incubation continued for a further 7 d. Cells were washed and injected i.v. (15×10^6 cells/mouse in 300 μl serum-free medium) into 6- to 7-mo-old APP/PS1 mice. Control animals received in 300 μl serum-free medium alone. Behavior analysis was assessed 2 wk after T cell transfer. Samples of supernatant were assessed by ELISA (see below) for IFN-γ, IL-4, IL-10, IL-17, and IL-5 production.

In a separate series of experiments, 6- to 7-mo-old APP/PS1 and WT control mice were injected i.p. with anti–IFN-γ Ab (600 μg) or a control Ab (anti–β-galactosidase: 600 μg; R&D Systems) and after 24 h were injected i.v. with Th1 cells (15×10^6 cells/mouse) as described above. Anti–IFN-γ or anti–β-galactosidase Ab injections were repeated 3, 7, 10, 14, 17, 21, 24, 28, and 31 d after T cell transfer. Behavioral analysis was assessed 21 d after T cell transfer.

Tracking of Aβ-specific Th1 cells into the brain

Aβ-specific Th1 were generated from GFP mice immunized with $A\beta_{1-42}$ and CpG, restimulated in vitro with $A\beta_{1-42}$ and IL-12, and expanded with IL-2 as described above. Cells were washed and injected i.v. (15×10^6 cells/mouse) into 6- to 7-mo-old APP/PS1 mice or WT mice. Mice were sacrificed 14 d later and mononuclear cells prepared from CNS tissue. Cells were stimulated with PMA and ionomycin and stained for surface CD3, CD4, CD8, and intracellular IFN-γ. Immunofluorescence analysis was performed on a DakoCytomation Cyan as described above.

Behavioral analysis

Gait was analyzed in WT and APP/PS1 mice using the footprint test to assess stride length and hind and front limb base widths. Muscular strength and coordination were assessed using the inverted screen and wire-hang tests. Two days later, 2 wk after administration of Aβ-specific T cells, mice were tested for spatial memory in the Morris water maze. The pool (1.2 m diameter; 0.6 m high; 0.24 m water depth; 0.15 m platform diameter placed in the northwest quadrant of the pool; 0.13 m from the edge of the pool) was sited in a well-lit room (22 to 23°C), and distinct visual cues were placed on the curtains that encircled the pool. Training commenced after 1 d of habitation and continued for 5 consecutive days on which the mice underwent four 1-min trials with an intertrial interval of 5 min. Each trial ended when mice located the platform or after 60 s when mice that failed to locate the platform were led to it; animals remained on the platform for 20 s. The day after the final day of training, the platform was removed, and mice were given a single 60-s probe trial. The percentage of time each animal spent swimming in the quadrant previously containing the platform was measured. Path length was also assessed.

Preparation of tissue

In the first study, in which the effect of transfer of Th1, Th2, and Th17 cells was assessed, mice were killed 24 h after the last behavioral analysis. In the second study, in which the effect of anti–IFN-γ Ab was assessed, mice were killed 34 d after the first injection of Ab. They were anesthetized with sodium pentobarbital (40 μl; Euthatal; Merial Animal Health) and perfused intracardially with ice-cold PBS (20 ml). The brains were rapidly removed, and one half of the brain was stored for later extraction and analysis of Aβ. The second half of the brain, which was used for immunohistochemical analysis, was placed onto cork discs, coated with optimum cooling temperature compound (Sakura Tissue-Tek), snap-frozen in prechilled isopropanol, and stored at −80°C. Before sectioning, the tissue was allowed to equilibrate to −20°C for 2 h. Sagittal sections (10-μm thick) were prepared using a cryostat (Leica, Meyer, U.K.), mounted on gelatin-coated (Flukaerland) glass slides, allowed to dry for 20 min, and stored at −20°C for later immunohistochemical analysis.

Detection of Aβ

Snap-frozen cortical tissue was homogenized in five volumes (w/v) of homogenizing buffer (SDS/NaCl in distilled H_2O [dH_2O] with proteases) and centrifuged (15,000 rpm, 40 min, 4°C). The supernatant samples were removed to extract SDS-soluble Aβ, and the pellets were kept for extraction of insoluble Aβ. Supernatants were equalized (3 mg/ml) with homogenizing buffer using a BCA protein assay, and samples were neutralized by the addition of 10% (w/v) 0.5 M Tris-HCl (pH 6.8). Samples were stored at −20°C for later detection of soluble Aβ. Pellets were incubated in guanidine buffer (50 μl; 5 M guanidine-HCl/50 mM Tris-HCl, pH 8; Sigma-Aldrich) for 4 h on ice. Samples were centrifuged (15,000 rpm, 30 min, 4°C), and the supernatant samples were equalized (0.3 mg/ml) with guanidine buffer and stored at −20°C for later detection of insoluble Aβ using MSD 96-well multi-spot 4G8 Aβ triple ultra-sensitive assay kits according to the manufacturer's instructions (Meso Scale Discovery). Standards ($A\beta_{1-38}$, 0–3,000 pg/ml; $A\beta_{1-40}$, 0–10,000 pg/ml; $A\beta_{1-42}$, 0–3,000 pg/ml) and samples were added to the 96-well plates, incubated

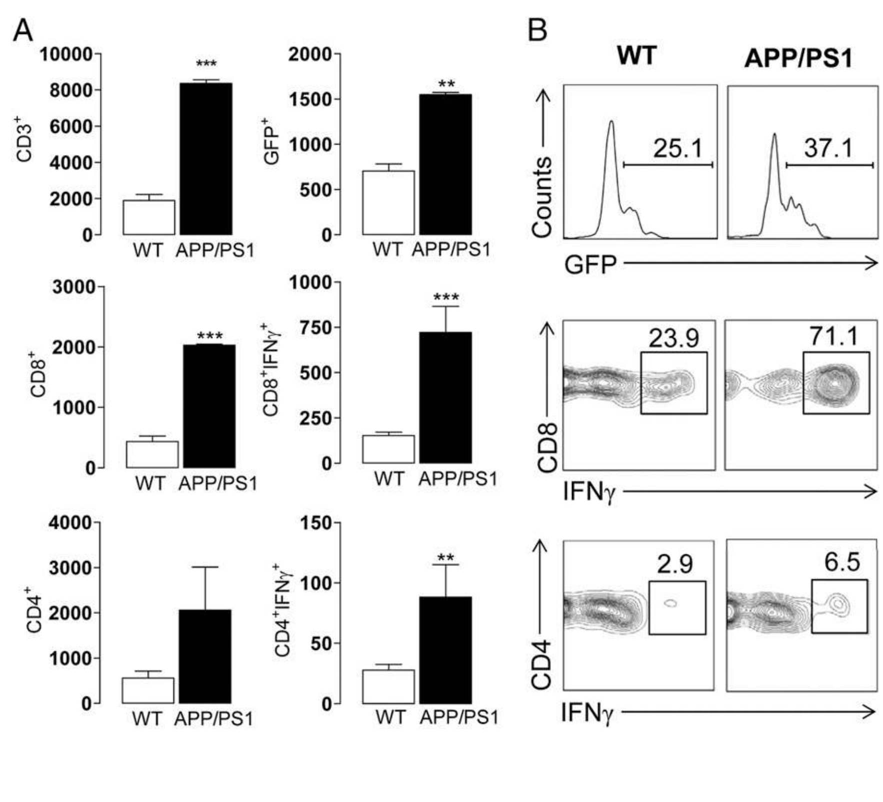

FIGURE 4. Transferred Aβ-specific Th1 cells migrate into the brain of APP/PS1 mice. Aβ-specific Th1 cells, generated from GFP mice were injected i.v. into 6- to 7-mo-old WT or APP/PS1 mice. Two weeks after injection, mice were sacrificed, and mononuclear cells were prepared from the brain. Cells were surface-stained with Abs specific for CD3, CD4, and CD8 and intracellularly stained for IFN-γ, and flow cytometric analysis was performed to quantify GFP-expressing and IFN-γ–secreting T cells in the brain. (**A**) Results are mean absolute number of the indicated cells in the brain. (**B**) Sample FACS plots of GFP^+ T cells (gated on CD3), $IFN\text{-}\gamma^+CD8^+$, and $IFN\text{-}\gamma^+$ $CD4^+$ cells; number represent percentage positive. Data in (A) represent mean ± SEM from four animals per experimental group from one experiment; data in (B) representative of four mice. $^{**}p < 0.01$, $^{***}p < 0.001$, Student *t* test for independent means.

(2 h, RT), washed, and read in a Sector Imager plate reader (Meso Scale Discovery) immediately after addition of the MSD read buffer. Aβ concentrations were calculated with reference to the standard curves and expressed as picograms per milliliter.

Immunohistochemistry

Cryostat sections were assessed for Aβ plaque deposition by staining with Congo red. Sections, equilibrated to RT, were fixed in ice-cold methanol for 5 min, washed in PBS, and incubated at room temperature for 20 min in an alkaline solution prepared by adding NaOH (2 ml; 1 M) to saturated NaCl (200 ml; 80% ethanol in dH_2O). Thereafter, sections were incubated in filtered Congo red solution (0.2% Congo red dye in the same alkaline solution) for 30 min, rinsed in dH_2O, incubated in methyl green solution (1% in dH_2O) for 30 s, washed, and dehydrated by dipping in 80, 95, and then 100% ethanol. Sections were dried, incubated in 100% xylene (3 × 5 min), mounted onto slides using dibutyl phthalate in xylene (RA Lamb), and allowed to dry overnight.

To assess CD11b, sections were fixed in an acetone/ethanol mixture (1:1) for 5–10 min, and endogenous peroxidase activity was blocked by incubating in 0.3% H_2O_2 in PBS for 5 min. Sections were washed, blocked in 10% rabbit serum (Vector Laboratories), incubated overnight at 25°C in rat anti-CD11b Ab (1:100 in 5% rabbit serum in PBS; clone 5C6; Serotec), washed, and incubated for 2 h at RT in biotinylated rabbit anti-rat IgG (1:200 in 5% rabbit serum in PBS; Vector Laboratories). Sections were washed, incubated in Vectastain Elite ABC reagent (two drops of A/B in 5 ml PBS; Vector Laboratories) for 1 h at RT, washed, and developed using the substrate 3,3 diaminobenzidine–enhanced liquid substrate system tetrahydrochloride (one drop solution B in 1 ml solution A) for ∼10 min until the color developed and counterstained with 1% methyl green for 10 min. Samples were dehydrated by dipping in graded ethanol (70, 80, 95, and 100%) and incubating in xylene (VWR International). Sections were mounted with dibutyl phthalate in xylene, dried overnight, and stored at RT. The sections were examined using an Olympus lx51 light microscope (Olympus, Tokyo, Japan), and micrographs were taken using an Olympus UCMAD3 (Olympus) at ×40 magnification. Data were quantified using the Immunoratio plugin (http:\\imtmicroscope.uta.fi/immunoratio/) available for the ImageJ software package (National Institutes of Health) (35). Colocalization of Aβ and CD11b was examined by confocal microscopy. Frozen brain sections brought to RT, fixed in ice-cold methanol, washed, permeabilized in 0.1% Trition (Sigma-Aldrich) in PHEM buffer, and washed. Nonspecific binding was blocked by incubating sections in 10% normal goat serum (2 h, RT), and sections were incubated overnight with pan-Aβ Ab (1:1000; Calbiochem) and rat anti-CD11b Ab (1:100, clone 5C6, AbD; Serotec) in 5% normal goat serum in PHEM buffer. Sections were washed, incubated in secondary Ab ALEXA 488 (1:4000; Invitrogen) and Alexa 546 (1:1000; Invitrogen; 90 min, RT), washed, mounted, and analyzed using confocal microscopy (Axioplan 2; Zeiss).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad). Data were analyzed using Student *t* test, two-way ANOVA, or one-way ANOVA followed by Newman–Keuls post hoc test. Data are expressed as means with SEM and deemed statistically significant when $p < 0.05$.

Results

Th1 and Th17 cell are present in the periphery and infiltrate the brains of APP/PSI mice

We used flow cytometry to assess the presence of T cells in the brain of WT and APP/PS1 mice. We found that there were very few $CD3^+CD4^+$ cells in the brain of WT mice but a significantly

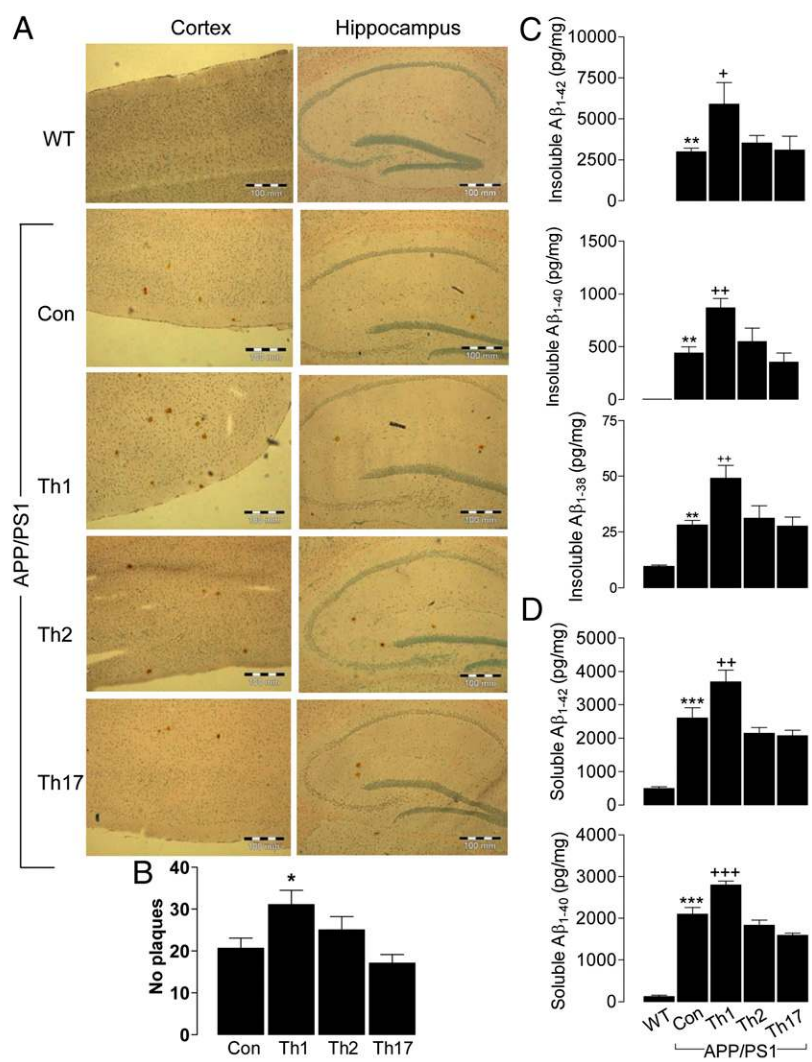

FIGURE 5. Transfer of Aβ-specific Th1 cells enhanced Aβ deposition in brains of APP/PS1 mice. APP/PS1 mice mice were injected with Aβ-specific Th1, Th2, or Th17 cells as described in Fig. 3. (**A**) Cryostat sections were stained with Congo red to assess Aβ-containing plaques in hippocampus and cortex; the mean number of plaques was recorded (**B**). (**C**) The concentrations of insoluble $Aβ_{1–42}$, $Aβ_{1–40}$, and $Aβ_{1–38}$ in the cortical tissue were quantified by ELISA. (**D**) The concentrations of soluble $Aβ_{1–42}$ and $Aβ_{1–40}$ in cortical tissue were quantified by ELISA. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ANOVA, APP/PS1 versus WT. $^+p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, ANOVA versus control untreated APP/PS1 mice (n = 5 to 6); representative of two experiments. Con, Control.

greater number in brain tissue prepared from APP/PS1 mice (***$p <$ 0.001; Student t test for independent means; Fig. 1A). Intracellular staining revealed that a proportion of $CD4^+$ cells stained positively for IFN-γ and also for IL-17 (Fig. 1B–D). There was no genotype-related difference in the number of $CD3^+CD8^+$ cells in the brain (Fig. 1E), although intracellular staining indicated that a greater proportion of these cells stained positively for IFN-γ compared with IL-17 (**$p < 0.01$; Student t test for independent means; Fig. 1F).

Aβ-specific Th1 cells impair cognitive function in APP/PS1 mice

Having demonstrated the presence of Th1 and Th17 cells in the brain of APP/PS1 mice, we set out to evaluate the effect of administration of Aβ-specific T cells on cognitive function, Aβ accumulation, and microglial activation in 6- to 7-mo-old APP/PS1 mice in which early pathological changes have been reported (36). To amplify Aβ-specific T cells, WT mice were immunized twice (0, 21 d) with Aβ and CpG, an adjuvant known to promote Th1 and Th17 responses. Short-term Aβ-specific Th1, Th2, and Th17 cell lines were generated by restimulation with Ag and APC in the presence of polarizing mixture described in the *Materials and Methods* section. This protocol resulted in the generation of highly polarized populations of Th1, Th2, and Th17 cells; Th1 cells produced high levels of IFN-γ and low IL-4 and IL-17, Th2 cells secreted high levels of IL-4 and low IL-17 and IFN-γ, and Th17 cells produced high levels of IL-17 and no IL-4 or IFN-γ (Fig. 2). After one round of Ag-stimulation, surviving T cells were washed and injected i.v. (15×10^6 cells/mouse) into 6- to 7-mo-old APP/PS1 or WT mice. Mice were tested for spatial memory in the Morris water maze 2 wk after administration of Aβ-specific T cells. The latency to reach the platform decreased over the 5-d training period but changes were similar in WT mice and control-treated APP/PS1 mice or APP/PS1 mice that received T cells (Fig. 3A), and no treatment effect was observed on day 5 of training (Fig. 3B). The path length taken to reach the platform decreased with training, except in APP/PS1 mice, which received Th1 cells (Fig. 3D) as shown by the representative traces obtained on day 5 (Fig. 3C). The mean path length on day 5 was significantly increased in these mice compared with untreated APP/PS1 mice (*$p < 0.05$; ANOVA; Fig. 3E). In contrast, transfer of Th1 cells into WT mice had no significant effect on path length taken to reach the platform or mean path length on day 5 (Supplemental Fig. 1). The day after the final day of training, the platform was removed, and mice underwent a single 60-s probe trial. The percentage of the total time and distance (i.e., path length) each animal spent swimming in the quadrant that previously contained the platform was significantly decreased in APP/PS1 mice that received Th1 cells compared with untreated APP/PS1 mice (*$p <$ 0.05; ANOVA; $n \geq 5$; Fig. 3F, 3G). Therefore, Th1 cell transfer induces a deficit in spatial learning in APP/PS1 mice at an age at which such deficits are not generally observed. Importantly, no motor deficits were observed in these animals; stride length, hind limb base width, and front limb base width were similar in all groups of mice, and, on the hangwire task, there were no differences in the latency to fall between groups (data not shown). These findings suggest that transfer of Th1, but not Th2 or Th17 cells, around the time of onset of Aβ plaque formation impairs cognitive function in APP/PS1mice.

We tracked the migration of transferred T cells into the CNS using Aβ-specific Th1 cells generated from GFP mice immunized with Aβ and CpG and polarized with IL-12. We found a higher proportion of $CD3^+$ T cells in the brain of APP/PS1, compared with WT, mice after transfer of Aβ-specific Th1 cells (Fig. 4). Furthermore, we detected GFP^+ cells in the brain 14 d following transfer of Th1 cells, and this was significantly greater in APP/PS1 mice. Finally, we found that $CD8^+$ as well as $CD4^+$ cells infiltrated

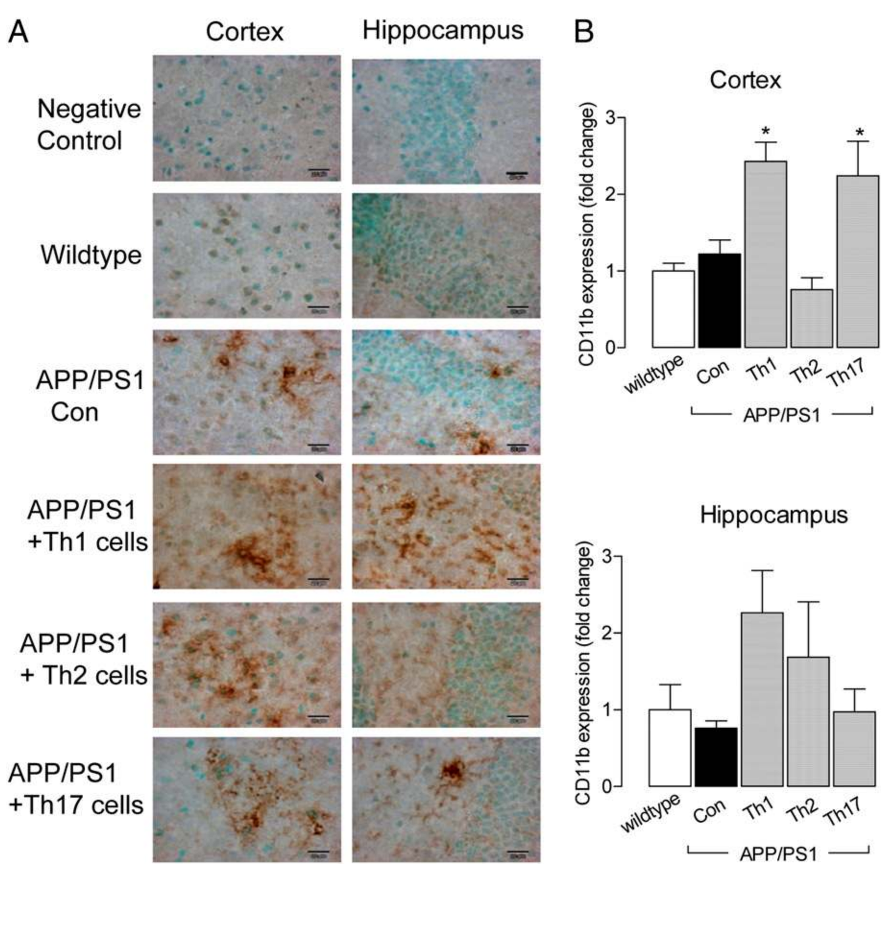

FIGURE 6. Transfer of Aβ-specific Th1 cells increase CD11b immunoreactivity in hippocampus and cortex of APP/PS1 mice. APP/PS1 mice were injected with Aβ-specific Th1, Th2, or Th17 cells as described in Fig. 3. (**A**) Microglial activation was assessed by CD11b immunoreactivity. (**B**) Data are means ± SEM. *$p < 0.05$, ANOVA versus control (n = 3–5) representative of two experiments. Con, Control.

the brain, and a significant number of these secreted IFN-γ (Fig. 4). These findings suggested that at least a proportion of Aβ-specific Th1 cells migrate into the brain following systemic delivery, and this is more pronounced in APP/PS1 when compared with WT mice. In addition, IFN-γ–secreting CD8 T cells are detected in higher numbers in the brains of APP/PS1 compared with WT mice.

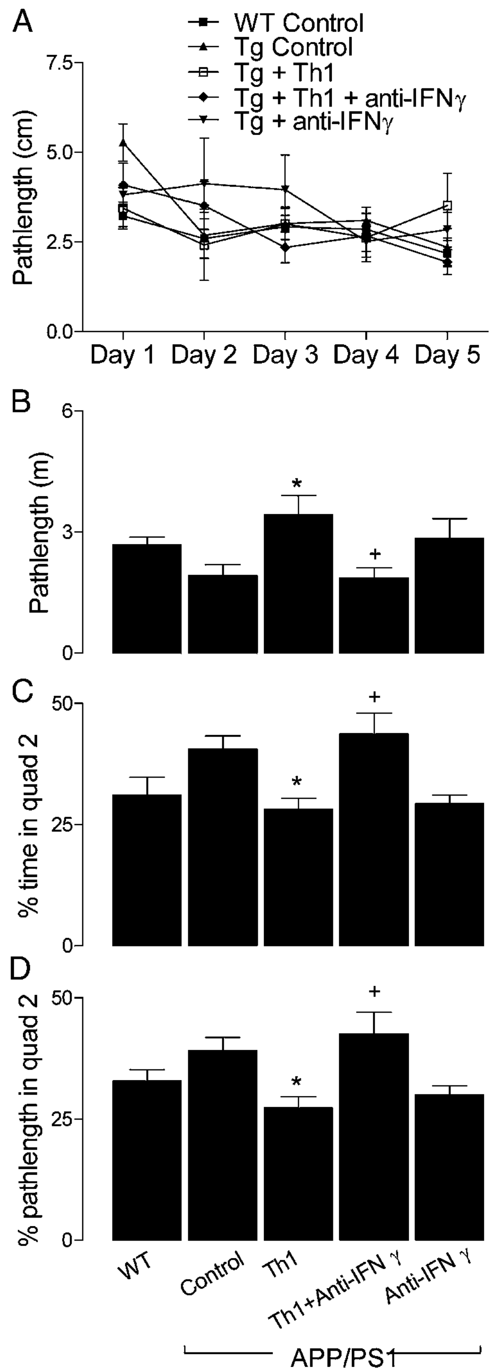

FIGURE 7. Anti–IFN-γ Ab attenuated the effect of Th1 cells on behavioral deficits. APP/PS1 mice were injected with Aβ-specific Th1 cells as described in Fig. 3, and mice were treated with anti–IFN-γ Ab or anti–β-galactosidase as a control Ab before and after injection of the cells. Three weeks after injection, cognitive function was analyzed in the Morris Water Maze test as described in Fig. 3. (**A**) The path length taken to reach the platform. (**B**) Mean path length on day 5 of training. (**C** and **D**) In the probe test, the time and path length in the quadrant that previously contained the platform (expressed as a percentage of the total) was measured. Data represent mean ± SEM from four to five animals per experimental group from two experiments. $*p < 0.05$, ANOVA, APP/PS1+Th1 cells versus control APP/PS1 mice, $^{+}p < 0.05$, ANOVA, APP/PS1 + Th1 cells versus APP/PS1 plus Th1 cells plus anti–IFN-γ Ab. Tg, Transgenic.

Aβ-specific Th1 cells enhance Aβ plaque burden and enhance microglial activation in APP/PS1 mice

Aβ deposition has been reported in the brain of APP/PS1 mice as early as 6 mo of age (37). Cryostat sections prepared from the 6- to 7-mo-old APP/PS1 mice used in this study confirm the presence of Aβ-containing plaques in cortex and hippocampus. Adoptive transfer of Aβ-specific Th1 cells markedly increased Aβ load, particularly in cortex, whereas transfer of Th2 or Th17 cells had little effect (Fig. 5A). Mean plaque number was significantly increased in sections prepared from mice that received Th1 cells ($*p < 0.05$; ANOVA; Fig. 5B). Insoluble $A\beta_{1-38}$, $A\beta_{1-40}$, and $A\beta_{1-42}$ were all significantly increased in tissue prepared from APP/PS1, compared with WT, mice ($*p < 0.05$; $**p < 0.01$; ANOVA; Fig. 5C). Injection of Th1 cells induced a further increase in the concentration of the three Aβ species ($^{+}p < 0.05$; $^{++}p < 0.01$, ANOVA, versus control APP/PS1 mice). Furthermore, soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ were also significantly increased in tissue prepared from APP/PS1 following transfer of Th1 cells (Fig. 5D), although soluble $A\beta_{1-38}$ was unchanged between treatment groups (data not shown). Neither Th2 nor Th17 cells exerted any significant effect on soluble or insoluble Aβ.

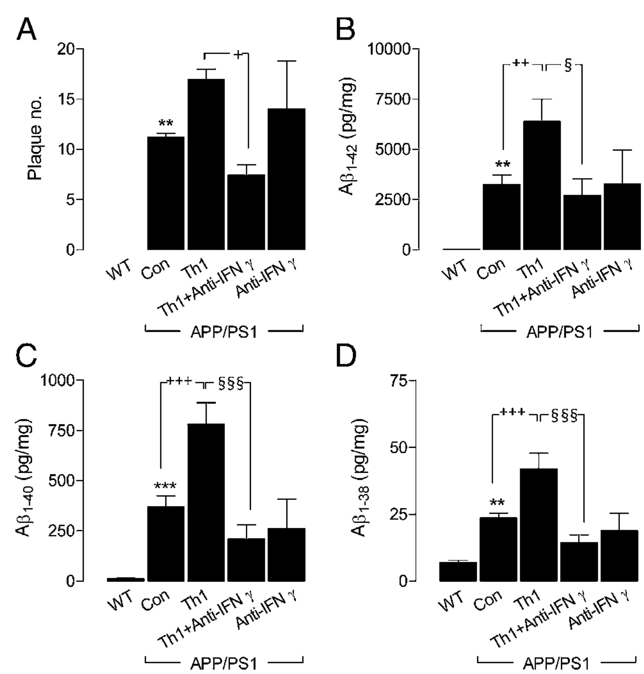

FIGURE 8. Anti–IFN-γ Ab attenuated the effect of Th1 cells on Aβ accumulation. APP/PS1 mice mice were injected with Aβ-specific Th1 cells and treated with anti–IFN-γ Ab or a control Ab as described in Fig. 6. Mice were sacrificed 5 wk after cell transfer. Cryostat sections were stained with Congo red to assess Aβ-containing plaques in hippocampus and cortex; the mean number of plaques was recorded (**A**), and the concentrations of insoluble $A\beta_{1-42}$ (**B**), $A\beta_{1-40}$ (**C**), and $A\beta_{1-38}$ (**D**) were quantified by ELISA in brain tissue prepared from APP/PS1 and WT mice. Data represent mean ± SEM from four to five animals per experimental group from two experiments. $**p < 0.01$, $***p < 0.001$, ANOVA; (n = 4–6); $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, ANOVA; control APP/PS1 mice versus APP/PS1 mice that received Th1 cells; $^{\S}p < 0.05$; $^{\S\S\S}p < 0.001$, ANOVA, APP/PS1 + Th1 cells versus APP/PS1 + Th1 cells + anti–IFN-γ Ab. Con, Control.

Sections prepared from WT and APP/PS1 mice were assessed for CD11b immunoreactivity as a measure of microglial activation. Immunoreactivity was negligible in sections of hippocampus and cortex prepared from WT mice (Fig. 6), whereas CD11b staining was observed in both areas in some but not all APP/PS1 mice. Quantification of the data indicated that CD11b expression was markedly increased in APP/PS1 mice that received Th1 cells, and the increase was significant in the case of the cortex ($*p < 0.05$; ANOVA), where Th17 cells exerted a similar effect.

Neutralization of IFN-γ attenuates the effect of Th1 cells on behavioral deficits

Having shown a specific effect of Th1 cells on spatial memory and Aβ accumulation, we assessed the role of the key Th1 cytokine, IFN-γ, by treating APP/PS1 mice with a neutralizing anti–IFN-γ Ab prior to, and following, Th1 cell transfer. There was no significant effect of treatment on latency to reach the platform (data not shown), confirming the data shown in Fig. 3. However, we found that the path length taken to reach the platform decreased with training in all groups except in APP/PS1 mice, which received Th1 cells (Fig. 7A), and analysis of the mean data indicates that path length was significantly increased in this group compared with APP/PS1 mice that did not receive Th1 cells ($*p < 0.05$; ANOVA; Fig. 7B). Administration of anti–IFN-γ Ab significantly attenuated the Th1 cell–induced effect ($^{+}p < 0.05$, ANOVA, versus APP/PS1 mice that received Th1 cells). In the probe test, treatment with Th1 cells decreased the percentage of the total time and distance each animal spent swimming in the quadrant that previously contained the platform ($*p < 0.05$; ANOVA; Fig. 7C, 7D), confirming the findings presented in Fig. 3. Treatment with anti–IFN-γ significantly reversed the effect of Th1 cells ($^{+}p < 0.05$, ANOVA, versus APP/PS1 mice that received Th1 cells).

Neutralization of IFN-γ attenuates the effect of Th1 cells on Aβ plaque burden

Anti–IFN-γ Ab attenuated the effects of Th1 cells on plaque number and concentration of insoluble $A\beta_{1-38}$, $A\beta_{1-40}$, and $A\beta_{1-42}$ in tissue prepared from APP/PS1 mice (Fig. 8). These measures were increased in tissue prepared from APP/PS1 mice compared with WT mice ($**p < 0.01$, $***p < 0.001$, ANOVA; Fig. 8), and these were significantly increased by administration of Th1 cells ($^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, ANOVA, control APP/PS1 mice versus APP/PS1 mice that received Th1 cells). The increase in $A\beta_{1-38}$, $A\beta_{1-40}$, and $A\beta_{1-42}$ induced by Th1 cells was attenuated when mice were treated with anti–IFN-γ Ab ($^{\S}p < 0.05$, $^{\S\S\S}p < 0.001$, ANOVA, APP/PS1 mice that received Th1 cells versus Ab-treated APP/PS1 mice that received Th1 cells).

CD11b immunoreactivity was negligible in sections prepared from hippocampus and cortex of WT mice (Fig. 9), whereas some staining was observed in both areas in APP/PS1 mice. This was greater in APP/PS1 mice that received Th1 cells, but this effect was ameliorated to some degree in sections prepared from APP/PS1 mice that received Th1 cells and anti–IFN-γ Ab. Immunoreactivity was similar in sections prepared from control APP/PS1 mice and APP/PS1 mice, which received anti–IFN-γ Ab. Analysis of staining using confocal microscopy indicated that CD11b immunoreactivity (green staining; Fig. 10) was colocalized with Aβ deposition (red staining) in hippocampus and cortex. As shown in Figs. 6 and 9, Aβ accumulation was increased in sections prepared from APP/PS1 mice, which received Th1 cells, and this effect was attenuated by anti–IFN-γ Ab treatment (Fig. 10). These findings demonstrate that the impact of Th1 cells on Aβ plaque burden and microglial activation was mediated through IFN-γ.

Discussion

The significant finding of this study is that adoptive transfer of Th1 cells increases Aβ accumulation and microglial activation in

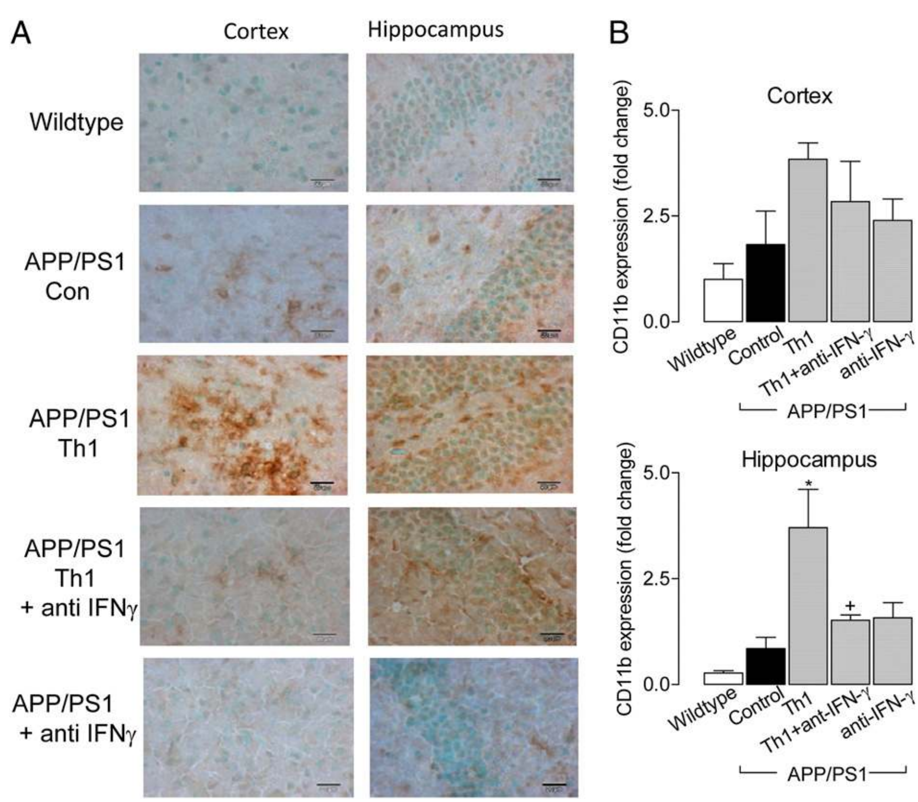

FIGURE 9. Anti–IFN-γ Ab attenuated the effect of Th1 cells on CD11b immunoreactivity. APP/PS1 mice mice were injected with Aβ-specific Th1 cells and treated with anti–IFN-γ Ab or a control Ab as described in Fig. 6. Mice were sacrificed 5 wk after T cell transfer. (**A**) Microglial activation was assessed by CD11b immunoreactivity in the cortex and hippocampus. (**B**) Data are means ± SEM. Data represent mean ± SEM from four to seven animals per experimental group from two experiments. $*p < 0.05$, ANOVA, versus control, $^{+}p < 0.05$, ANOVA, versus Th1. Con, Control.

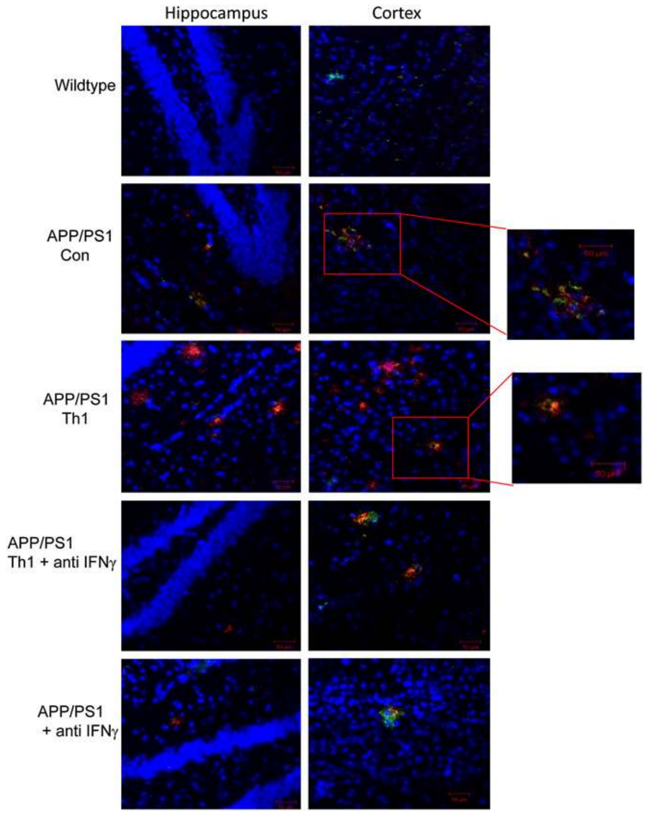

FIGURE 10. Anti–IFN-γ Ab attenuated the effect of Th1 cells on CD11b immunoreactivity. APP/PS1 mice were injected with Aβ-specific Th1 cells and treated with anti–IFN-γ Ab or a control Ab as described in Fig. 6. Mice were sacrificed 5 wk after T cell transfer. Microglial activation and Aβ deposition was assessed by confocal microscopy. Cells were stained with DAPI (nuclei; blue), amyloid-β (red), and CD11b (green). Original magnification ×40 and enlarged panels ×60. Data are representative of five mice per experimental group from two experiments. Con, Control.

the brain of 6- to 7-mo-old APP/PS1 mice and impairs performance in a Morris water maze; these effects are attenuated by treatment of mice with anti–IFN-γ Ab.

It has been recognized for some time that T cells can infiltrate the brain (38, 39). T cell infiltration is significantly enhanced under pathological conditions (for example, in multiple sclerosis and EAE), and this is due, at least to some extent, to an increase in blood–brain barrier permeability (23, 40). In this study, we report that there is a significant increase in the number of $CD3^+CD4^+$ cells in the brain of APP/PS1 mice compared with WT mice and that a proportion of these are Th1 and Th17 cells. Consistent with this is the observation that significant numbers of peripheral T cells are present in the postmortem brain of AD patients compared with the relatively low numbers of cells in other degenerative dementia cases and, importantly, that these cells are clustered in areas of the brain in which pathology is more marked, such as the hippocampus and limbic regions (25). However, the role of T cells in the pathogenesis of AD is not clear, with circumstantial evidence of both host protective and damaging roles for Aβ-specific T cells. Peripheral T cells specific for $A\beta_{1-40}$ have been detected in healthy individuals, but were absent in patients with AD (41), possibly suggesting that $A\beta_{1-40}$-specific T cells may prevent the development of Aβ plaques. It has also been reported that Th1 cells directed against $A\beta_{1-42}$ are present in young individuals but decline with age and are lost in patients with AD, in whom IL-10–producing regulatory T cells predominate (42).

Vaccine studies in mouse models have shown that immunization with $A\beta_{42}$ in CFA prevented the development of Aβ plaques and reduced the development of AD-like neuropathology (33, 43). The protection was associated with Ab and could be mimicked by passive transfer of Aβ-specific Abs (44). There is also evidence from a clinical trial that active immunization with $A\beta_{42}$, formulated with the adjuvant QS21 (AN 1792), can reduce plaque burden in AD patients (45), though a number of patients developed meningoencephalitis, and the trial was halted. Although the cause of the meningoencephalitis is not clear, it has been suggested that it could result from the induction of inflammatory T cell responses (46). Interestingly, QS-21, the adjuvant used in AD vaccine, has been shown to promote Th1 responses to coadministered foreign Ag in mice (47). Our findings are consistent with a pathogenic role for Th1 cells, at least in a mouse model of AD.

To evaluate the impact of different T cell subtypes on plaque burden in the brain, we adoptively transferred Aβ-specific Th1, Th2, and Th17 cells into 6- to 7-mo-old APP/PS1 mice. Consistent with previous findings (36), we found that there was some Aβ accumulation in the brain of the 6-mo-old APP/PS1 mice. This was accompanied by increased concentrations of $A\beta_{1-42}$, $A\beta_{1-40}$, and $A\beta_{1-38}$ in cortical tissue. However, transfer of Th1 cells increased deposition of Aβ (determined by Congo red staining) and markedly increased cortical Aβ concentration. This suggests that Aβ-specific Th1 cells may play a role in the development of Aβ plaques in the brain. This was confirmed by treatment of mice with a neutralizing anti–IFN-γ Ab, which attenuated the effect of Th1 cells on Aβ accumulation. In contrast with the effect of Th1 cells, transfer of Th17 cells, which have been associated with pathology in EAE and other autoimmune/inflammatory diseases, and Th2 cells, which have a more anti-inflammatory function in other diseases, did not enhance Aβ accumulation in the brain. These findings are consistent with our earlier report that Aβ-specific Th1 cells enhance proinflammatory cytokine production and MHC class II and costimulatory molecule expression by Aβ-stimulated microglia, whereas Aβ-specific Th2 cells suppress cytokine production by glial cells (32).

Under resting conditions, microglia are maintained in a quiescent state in the brain because of the presence of neuroimmune regulatory molecules that enable the interaction with other cells, low concentrations of stimulatory factors such as IFN-γ and other inflammatory cytokines, and the presence of minimal numbers of immune cells like T cells (13). However, microglial activation occurs following any insult, and an activated state is a characteristic of most, if not all, neurodegenerative diseases in which these cells can assume the role of APC. Modest microglial activation was observed in the hippocampus and cortex of 6- to 7-mo-old APP/PS1 mice but transfer of Th1 cells markedly increased activation. This is consistent with our previous findings that showed that Aβ-specific Th1 cells increased microglial activation in vitro (32). In parallel with its effect on Aβ accumulation, treatment of mice with anti–IFN-γ Ab attenuated the effect of Th1 cells on microglial activation. It is well established that IFN-γ is among the most potent activators of microglia (15, 48) and synergizes with Aβ to increase expression of cell-surface markers of activation and production of inflammatory cytokines (15, 49). Chakrabarty et al. (50) reported that viral delivery of IFN-γ gene promotes microglial activation and clearance of Aβ. We observed that Th1 cells also promoted microglial activation but that this was associated with an increase in Aβ plaques. We do not have a definitive explanation for the discrepancy in these studies other than the differences in the experimental approaches: virally-delivered IFN-γ, which had effects, such as basal ganglia calcification, in

WT as well as Tg mice, compared with i.v. injected Aβ-specific Th1 cells, in which the effects were largely confined to Tg mice. One interpretation of the data, as suggested in this study, is that anti–IFN-γ prevents Th1 cell–induced activation of microglia, but it is possible that the Ab treatment affects infiltration of cells, perhaps by altering chemotaxis or exerting an effect on blood–brain barrier permeability.

Our studies with Th1 cells expressing GFP demonstrated that at least a proportion of the transferred Th1 cells did migrate from the periphery into the CNS. Interestingly, IFN-γ–secreting CD8 as well as CD4 T cells were detected in the brain following i.v. injection of Aβ-specific Th1 cells. This is consistent with studies in the EAE model that have demonstrated that Th1 cells preferentially infiltrate the CNS and facilitate recruitment of other inflammatory T cells (51). Interestingly, the migration of T cells into the brain and subsequent behavioral deficits was significantly more pronounced following transfer of Aβ-specific Th1 cells into APP/PS1 when compared with WT mice. This may reflect the higher Aβ burden in the APP/PS1 mice and might suggest local Ag stimulation of IFN-γ–secreting T cells, which were at a significantly higher frequency in the brains of APP/PS1 compared with WT mice.

Previous studies from this laboratory have shown that Aβ-specific Th1 cells enhanced Aβ-induced activation of microglia (32). Furthermore, the increase in microglial activation in APP/PS1 mice was accompanied by increased expression of inflammatory cytokines, including TNF-α and IL-1β (52). Interestingly, TNF-α and IL-1β have been shown to increase activity and/or expression of γ- and β-secretases (6, 7), which leads to Aβ deposition. Although activated microglia may phagocytose and remove Aβ aggregates (50), IL-1β–expressing microglia are associated with Aβ plaques and neurofibrillary tangles in the brain of AD patients, where they correlate with progressive neuronal damage (53). Furthermore, IL-1β can promote synthesis of APP in endothelial cells (54). It has also been reported that IFN-γ–induced activation of microglia enhanced processing of APP and suppressed Aβ clearance (55). We found that Th1 cells, which increase IL-1β expression by microglia (32), enhanced soluble and insoluble Aβ concentrations in the brains of APP/PSI mice. However, it must be acknowledged that Aβ potently activates microglia in vitro and in vivo (56, 57), and therefore it is possible that there may be a feedback loop, leading to persistent microglial activation and Aβ accumulation with the subsequent pathogenic consequences.

Although there was significant Aβ accumulation in the brain of 6- to 7-mo-old APP/PS1 mice, we found no evidence of genotype-related changes during the training phase in the Morris water maze or during the probe test, contrasting with previous reports that indicated a deficit in slightly older (8-mo-old) APP/PS1 mice (58, 59). It has been suggested that cognitive deficits correlate with insoluble Aβ in Tg2576 mice (60) and APP/PS1 mice (61), but this view is not supported by the present findings. However, we report that transfer of Th1 cells doubled the concentration of insoluble Aβ in brain tissue, and this was associated with deterioration in cognitive function in the probe test; this raises the possibility that a threshold concentration of Aβ must be reached before an impact on spatial learning is exerted. In contrast to the effect of Th1 cells on APP/PS1 mice, transfer of Th2 cells or Th17 cells exerted no effect in the spatial learning task or on either plaque number or Aβ accumulation. The present findings are at variance with an earlier report that indicated that adoptive transfer of a mixed T cell preparation improved performance of 10-mo-old APP/PS1 mice in a radial arm maze task (62). Although no effect on insoluble Aβ or Aβ plaque numbers was observed, the authors suggested that microglia or monocytes were stimulated to clear Aβ because the distribution of Aβ-immunoreactive cells in hippocampus of mice that received T cells was similar to the distribution of MHC class II–positive cells. More recent data from this group suggested that the beneficial effects on behavior may be Th2 cell–mediated because the effect was evident when T cells had been incubated in vitro in the presence of IL-2 and IL-4 (31). We have recently reported that the Aβ-induced microglial activation in vitro is attenuated by Th2 cells (32), and the current study found that Th2 cells, unlike Th1 cells, did not enhance plaque burden in vivo.

Although beneficial effects of T cells in the brain have also been observed (63), the evidence presented in this study indicates that Th1 cells, but not Th2 or Th17 cells, contribute to Aβ accumulation and development of a functional deficit in APP/PS1 mice during the early stages of development of pathology. In this model, the effects appear to be mediated by IFN-γ and are associated with enhanced microglial activation, which may trigger inflammatory changes that propagate a damaging cascade of events and further development of pathology.

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Disclosures

K.H.G.M. is a cofounder and shareholder in Opsona Therapeutics and TriMod Therapeutics Ltd., startup companies involved in the development of immunotherapeutics. The other authors have no financial conflicts of interest.

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