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The Alzheimer's Disease–Associated Protein BACE1 Modulates T Cell Activation and Th17 Function

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β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) is best known for its role in Alzheimer's disease amyloid plaque formation but also contributes to neurodegenerative processes triggered by CNS injury. In this article, we report that BACE1 is expressed in murine CD4$^+$ T cells and regulates signaling through the TCR. BACE1-deficient T cells have reduced IL-17A expression under Th17 conditions and reduced CD73 expression in Th17 and inducible T regulatory cells. However, induction of the Th17 and T regulatory transcription factors RORγt and Foxp3 was unaffected. BACE1-deficient T cells showed impaired pathogenic function in experimental autoimmune encephalomyelitis. These data identify BACE1 as a novel regulator of T cell signaling pathways that impact autoimmune inflammatory T cell function. ***The Journal of Immunology*****, 2019, 203: 000–000.**

Multiple sclerosis (MS) is an autoimmune disease of the CNS initiated by myelin-reactive T cells that produce cytokines that cause direct damage to CNS tissue as well as trigger recruitment and activation of macrophages and myelin-reactive autoantibody-producing B cells. Th17 cells were initially defined as a distinct proinflammatory CD4$^+$ Th cell subset in the mouse model of MS, experimental autoimmune encephalomyelitis (EAE) (1). Although previously considered a Th1-mediated disease, numerous studies have consistently demonstrated the vital contribution of Th17 cells to EAE development (2), including the finding that the majority of IFN-γ$^+$ Th1 cells found in the CNS of mice with EAE are in fact derived from the Th17 lineage (3). Clinical trials testing blockade of IL-17A in relapsing-remitting MS are showing promise, supporting the importance of this pathway in MS (4). IL-17A acts on multiple CNS-resident cells to potentiate inflammation. Astrocytes respond to IL-17A by producing chemokines to facilitate recruitment of inflammatory cells, such as macrophages and neutrophils (5–9). Likewise, oligodendrocytes contribute to the Th17 inflammatory response (10, 11) and are also induced to undergo apoptosis in response to IL-17A signaling through Act1 (10). IL-17A can also be directly neurotoxic by activating Ca$^+$ flux in neurons (12). Hence, accumulated damage, not only to neurons but also to the cells that support them, impairs future CNS function, leading to permanent disability.

BACE1 is a transmembrane aspartyl protease that was initially identified for its role in cleavage of amyloid precursor protein (APP) to generate amyloidß peptides that form plaques in Alzheimer's disease (AD) (13). Blockade of BACE1, either by genetic deletion or chemical inhibitors, greatly reduces amyloid plaque formation in mouse models of AD, and BACE1 inhibitors are now being tested in clinical trials for AD (14). Inflammatory signals, including hypoxia and cytokines such as IL-1 and TNF, contribute to upregulation of BACE1 in AD (15, 16), whereas nonsteroidal anti-inflammatory drugs reduce BACE1 expression and associated plaque burden (17, 18). Furthermore, BACE1 expression increases following damage to the CNS due to ischemia (stroke) (19–22) and traumatic brain injury (23, 24), and BACE1-deficient mice show reduced lesion volume and better recovery following traumatic brain injury (24), although not all studies find the same result (25). Thus it appears that BACE1 may have additional functions in neuroinflammatory or neurodegenerative responses beyond AD, although this has not been investigated in MS.

Intriguingly, the IL-23–IL-17A axis has also been found to promote neurodegeneration and impair recovery following brain ischemia (26–28), but any connection between BACE1 and Th17 cells has not been probed. We therefore queried a potential role for BACE1 in the immune system and report in this article that BACE1 is expressed in CD4$^+$ T cells and modulates T cell response to TCR ligation as well as some effector molecules in Th17 and T regulatory cell (Treg) differentiation. Ultimately, BACE1-deficient T cells were found to have reduced inflammatory capacity in the EAE model, indicating an important functional role for this neurodegenerative protein in the immune system.

Materials and Methods

Mice

BACE1$^{-/-}$ (29), C57BL/6, CD45.1$^+$, 2D2, and RAG1$^{-/-}$ mice were purchased from The Jackson Laboratory. Animals were housed and bred under specific pathogen–free conditions in an Association for Assessment and Accreditation of Laboratory Animal Care–approved facility, and all

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Abbreviations used in this article: AD, Alzheimer's disease; dLN, draining lymph node; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; Treg, T regulatory cell; WT, wild-type.

animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

In vitro CD4$^+$ T cell differentiation

CD4$^+$ T cells from spleens and lymph nodes of naive mice were purified by magnetic separation (Miltenyi Biotec). T cells were activated by plate-bound anti-CD3 (clone 145-TC11, 5 μg/ml; Bio X Cell) in RPMI medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME, HEPES, and Na pyruvate. For Th17 differentiation, cells were cultured in the presence of recombinant mouse IL-1β (40 ng/ml), IL-23 (20 ng/ml), IL-6 (100 ng/ml), and TGF-β1 (10 ng/ml); all cytokines were from R&D Systems, Minneapolis, MN. In all Th0 cell cultures, anti–IFN-γ neutralizing Abs (10 μg/ml; Bio X Cell) were added. For Th1 cultures, IL-12 (PeproTech) was added at 10 ng/ml. For Treg differentiation, T cells were cultured in the presence of recombinant mouse TGF-β1 (20 ng/ml), recombinant human IL-2 (100 U/ml), and anti–IFN-γ neutralizing Abs (10 μg/ml).

To assess cAMP production, 4 million CD4$^+$ T cells isolated from wild-type (WT) or BACE1$^{-/-}$ mice were stimulated for 30 min with 10 μg/ml forskolin (EMD Millipore). cAMP levels in cell lysates were then analyzed using the cAMP Assay Kit from Abcam.

EAE induction

For active immunization, mice were immunized s.c. with 100 μg of MOG (35–55) (Bio-Synthesis, Lewisville, TX) emulsified in 200 μl of CFA (Difco Laboratories, Detroit, MI) containing 100 μg of heat-killed *Mycobacterium tuberculosis* H37Ra (Difco Laboratories) distributed in four sites on the flank. A total of 200 ng of Pertussis Toxin (List Biological Laboratories) was given i.p. on day 0 and 2. For RAG$^{-/-}$ transfer experiments, all lymph nodes and spleen were harvested from donor C57BL/6 mice or BACE1$^{-/-}$ mice, and CD4$^+$ cells were isolated by magnetic separation using CD4 microbeads (Miltenyi Biotec). Ten to 14 million CD4$^+$ cells were transferred i.p. to naive RAG1$^{-/-}$ recipients, which were immunized the following day, as described above.

For the passive transfer of EAE, LN and spleen were harvested from WT or BACE1$^{-/-}$ 2D2 mice and stimulated in vitro, according to protocol described by Jäger et al. (30). Briefly, cells were activated with MOG (35-55) (20 μg/ml) in the presence of TGF-β and IL-6 (5 and 50 ng/ml, respectively) for 4 d. Cells were washed, split, and resuspended in complete RPMI 1640 containing IL-2 (10 U/ml). After 3 d of resting, cells were reactivated in 24-well plates with plate-bound αCD3 (1 μg/ml) and soluble IL-23 (20 ng/ml) for 2 d before transferring to recipient mice. IL-17A expression was determined by flow cytometry at the end of the first activation stage (day 4).

For clinical scoring, EAE was assessed according to the following clinical grades: 1) flaccid tail, 2) impaired righting reflex and hindlimb weakness, 3) partial hindlimb paralysis, 4) complete hindlimb paralysis, 5) hindlimb paralysis with partial forelimb paralysis, and 6) moribund/dead. Atypical EAE was noted when mice demonstrated advanced ataxia, circling, or head tilt with or without classical signs of EAE, and these mice were scored as grade 2 if other classical signs of EAE were not present.

Flow cytometry and ImageStream

The following FACS Abs were purchased from BD Biosciences: CD4 (RM4-5), IL-17A (TC11-18H10), and CD25 (7D4). The following were purchased from eBioscience (Invitrogen): RORγt (AKFJS9), Foxp3 (FJK-16s), CD73 (eBIOTY/11.8), IFN-γ (XMG1.2), and GM-CSF (MP1-22E9). For cytokine analysis, cells were cultured in complete medium (RPMI media containing 10% FCS, supplemented with penicillin-streptomycin, L-glutamine, HEPES, sodium pyruvate, and 2-ME) with 50 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of Golgiplug (BD Biosciences) for 3 to 4 h, followed by FACS staining and analysis. For intracellular cytokines, staining was performed using Cytofix-Cytoperm kit from BD Biosciences; RORγt and Foxp3 intracellular stains were performed using eBioscience Foxp3 Staining Kit, according to manufacturer's instructions. Samples were acquired on a BD Fortessa and analyzed using FlowJo (TreeStar).

For ImageStream analysis, cells were stained with anti-CD4, then fixed overnight before staining with anti-BACE1 (D10E5 rabbit mAb; Cell Signaling) in permeabilization buffer containing 2% BSA, followed by secondary Ab labeling using FITC-conjugated or PE-conjugated donkey-anti-rabbit. Samples were acquired on an ImageStream^X MK II imaging cytometer, at ×60 magnification, with low flow rate/high sensitivity, using INSPIRE software. Data were analyzed using IDEAS 6.2 software, according to the manufacture guidelines.

ELISA

IL-17A and IFN-γ production was analyzed using Ready-Set-Go kits (eBioscience) in culture supernatants from in vitro T cell differentiation (as described above) or supernatants from ex vivo–stimulated cultures, as follows: draining lymph nodes (dLN) were isolated from mice with EAE and single-cell suspensions were obtained, and cells were cultured for indicated times with 50 μg/ml MOG (35–55) and 10 ng/ml IL-12 (Peprotech) to promote IFN-γ or 20 ng/ml IL-23 (R&D Systems) to promote IL-17 production.

Quantitative real-time PCR

RNA was isolated using an RNeasy Mini Kit (QIAGEN) and converted to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosciences). Gene expression was quantified using Excella SYBR MasterMix Rox (WorldWide Life Sciences) with RT^2 quantitative PCR primers (Qiagen: Gapdh-PPM02946E, Il17a-PPM03023A, Rorc-PPM25095A, Il23r-PPM33761A, Tbx21-PPM03727A, Foxp3-PPM05497F) in a 7300 Real-Time PCR System (Applied Biosystems).

Western blotting

Western blots were performed using Abs from Cell Signaling Technology: phospho-473 Akt (C67E7), PTEN (D4.3), and β-actin (D6A8). T cells for Western blot analysis were stimulated with soluble anti-CD3 and anti-CD28 cross-linked by streptavidin, in the presence of Th17-inducing cytokines.

Statistics

One-way ANOVA (for multiple groups) or Student *t* test was performed for experiments with parametric values (such as FACS percentage); Mann–Whitney *U* test was performed for EAE experiments, analyzing scores for each day separately. The *p* values are shown as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, in which statistical significance was found, and all data are represented as mean ± SD.

Results

RNA sequencing analysis of different immune populations by the ImmGen consortium (31) indicated widespread expression of BACE1 throughout the immune system (Supplemental Fig. 1). Of the T cell populations tested, double-negative thymocytes showed the highest expression of BACE1, but all mature T cells appear to maintain BACE1 (SF1). We confirmed expression of BACE1 protein in mature CD4$^+$ T cells by ImageStream, using cells from BACE1$^{-/-}$ mice (29) as controls (Fig. 1A). BACE1$^{-/-}$ had normal numbers and proportions of thymic CD4$^+$, CD8$^+$, double-positive, and double-negative thymocytes (Fig. 1B). Likewise, ratios of the four stages of double-negative thymocyte development (defined by expression of CD44 and CD25) were unaffected by BACE1 deficiency (Fig. 1C). Numbers of cells in peripheral LN and spleen (Fig. 1D) and frequencies of mature CD4$^+$ T cells, CD8 T cells, and B220$^+$ B cells were similar in WT and BACE1$^{-/-}$ mice (Fig. 1E). Finally, BACE1$^{-/-}$ mice had normal CD25 expression on peripheral Foxp3$^+$ Tregs (Fig. 1F) as well as normal frequencies of Tregs in peripheral LN (Fig. 1G). Although we have not analyzed TCR repertoire in these mice, we can conclude that BACE1 does not alter absolute numbers in thymic generation or maintenance of mature T cells.

BACE1-deficient T cells have altered cAMP and PTEN regulation

Separate from its proteolytic functions on APP, BACE1 has been reported to regulate adenylyl cyclase to decrease cAMP production in neurons (32). Accordingly, we tested the cAMP response to forskolin-mediated adenylyl cyclase activation. Indeed, BACE1$^{-/-}$ T cells showed a significant increase in cAMP production compared with WT T cells (Fig. 2A). In T cells, cAMP acts as a second

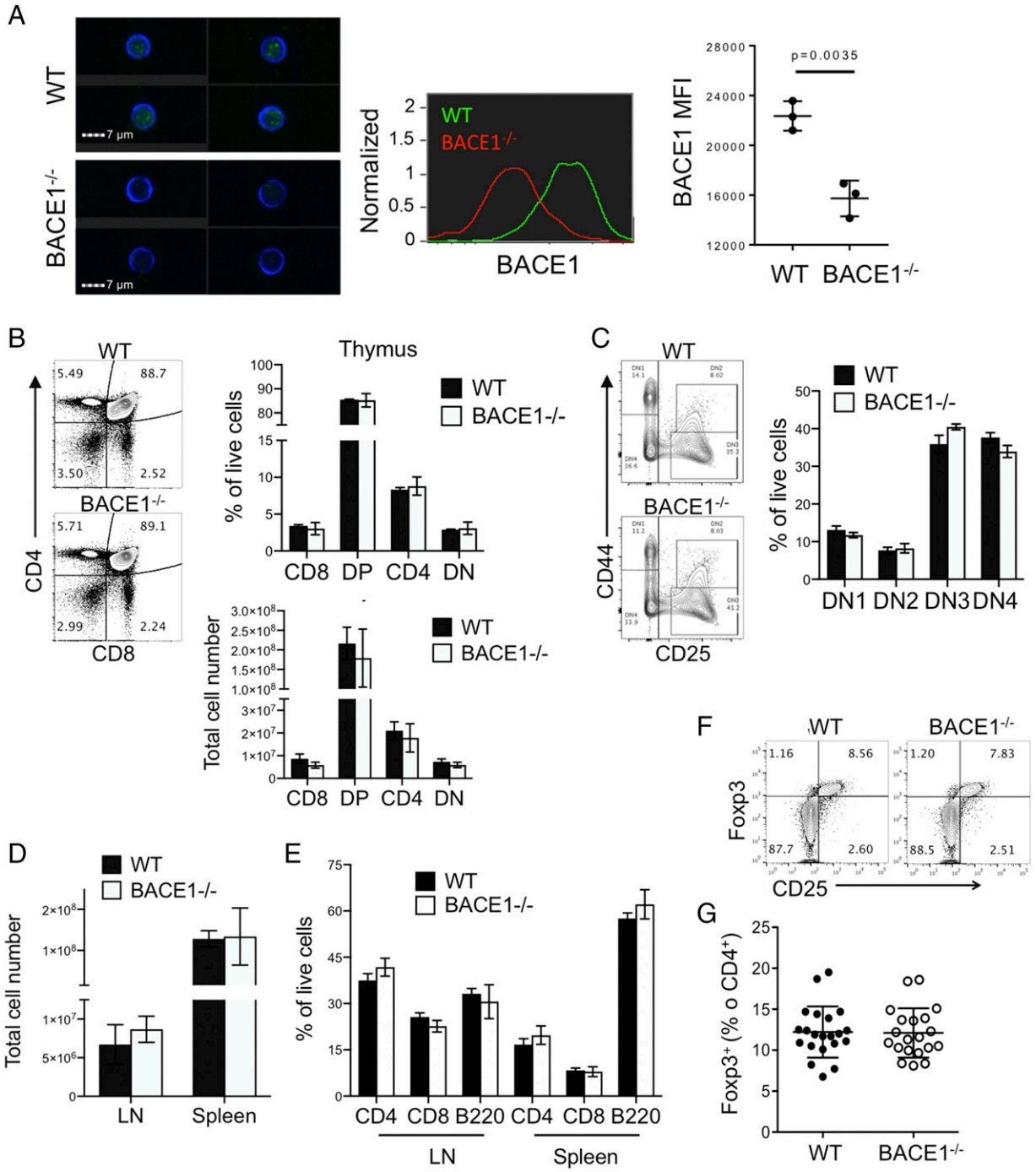

FIGURE 1. BACE1$^{-/-}$ T cells develop at normal frequencies. (**A**) Expression of BACE1 in mature WT and BACE1$^{-/-}$ T cells analyzed by ImageStream; data representative of three independent experiments with three mice per group. Blue, CD4; green, BACE1. (**B**) Numbers and proportions of CD8^{+}, CD4^{+}, CD8^{+}CD4^{+}double-positive (DP) and CD8^{-}CD4^{-}double-negative (DN) thymocyte populations in 6-wk-old WT and BACE1$^{-/-}$ thymus analyzed according to representative FACS plot. (**C**) Frequencies of thymus DN populations gated as shown in representative FACS plot. (**D**) Numbers of cells in cutaneous LN and spleen. (**E**) Proportions of live CD4^{+} T cells, CD8^{+} T cells, and B220^{+} B cells in those LN and spleen. (**F**) Representative FACS plots of Foxp3 and CD25 expression in live CD4^{+} T cells from peripheral LN. (**G**) Frequencies of Foxp3^{+} cells in CD4^{+} T cells from WT and BACE1$^{-/-}$ mice. Graphs show mean ± SD of three to four mice per group representative of three to four independent experiments, except (F) is pooled from three experiments.

messenger to activate multiple pathways that can impact TCR signaling (33). One of the first events in TCR signaling is activation of store-operated calcium entry leading to calcium flux. Following TCR/CD28 stimulation, WT and BACE1$^{-/-}$ T cells showed a similar rapid increase of intracellular calcium; however, BACE1$^{-/-}$ T cells consistently showed a slower loss of intracellular calcium, leading to a more sustained calcium flux (Fig. 2B). Similarly, BACE1$^{-/-}$ T cells showed enhanced and sustained Akt activation responses compared with WT T cells, following stimulation under Th17 conditions (Fig. 2C, 2D). This led us to examine PTEN, the key lipid phosphatase regulator of PI3K. Corresponding to exaggerated Akt activation, PTEN expression was reduced in BACE1$^{-/-}$ T cells compared with WT, even in unstimulated T cells (Fig. 2C, 2E). These results suggest

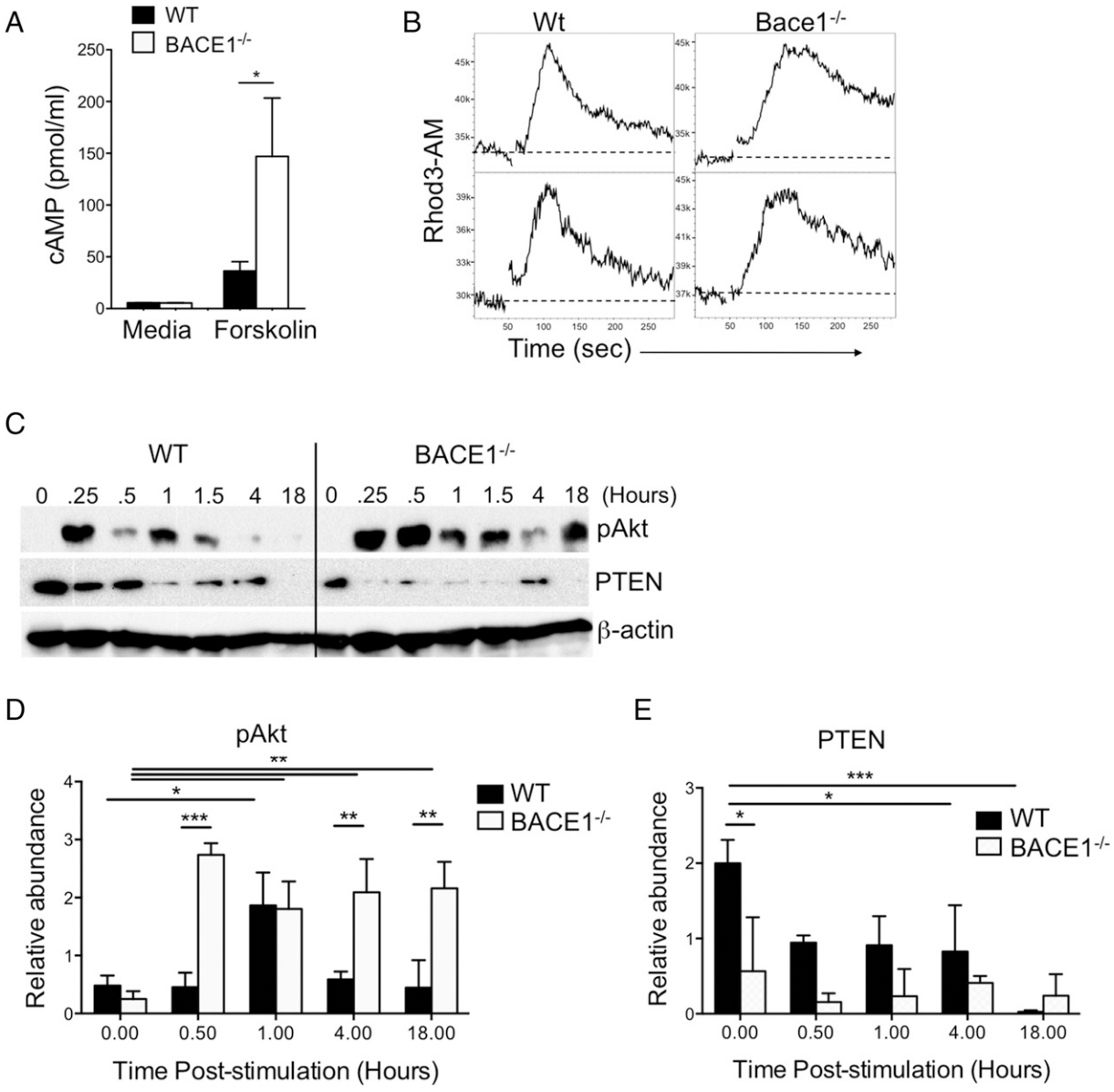

FIGURE 2. Altered cAMP and TCR signaling in BACE1$^{-/-}$ T cells. (**A**) Intracellular cAMP concentration in WT and BACE1$^{-/-}$ T cells after 30 min stimulation with forskolin or media-only control; mean ± SD of pooled data from three experiments with two to three replicates. (**B**) Calcium flux assessed in Rhod3AM-labeled WT and BACE1$^{-/-}$ T cells, activated with anti-CD3/CD28 at 50 s; T cell responses from two individual mice per group shown, representative of three independent experiments. (**C**) Immunoblots of p-Akt(Ser473), PTEN, and β-actin, following stimulation of WT and BACE1$^{-/-}$ T cells for indicated time periods. (**D**) Densitometry of p-Akt relative to β-actin; mean ± SD data pooled from three experiments. (**E**) Densitometry of PTEN relative to β-actin; mean ± SD data pooled from three experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

that BACE1 negatively regulates T cell signaling, including during Th17 differentiation.

BACE1-deficient Th17 cells have impaired IL-17 production

PTEN deletion was recently shown to impair Th17 differentiation (34). We determined whether low PTEN expression could affect Th17 development by stimulating CD4-Cre/PTEN$^{fl/+}$ cells, which have reduced but not complete knockdown of PTEN expression. Indeed, PTEN$^{fl/+}$ Th17 cells had significantly fewer IL-17–producing Th17 cells compared with PTEN$^{+/+}$ controls (Fig. 3A). Given that BACE1$^{-/-}$ T cells had reduced PTEN expression, we tested their capacity to differentiate into Th17 cells. BACE1$^{-/-}$ Th17 cells had significantly reduced frequencies of IL-17A–producing cells compared with WT cells, and although IL-17^{+} cell frequencies varied between experiments, typically BACE1$^{-/-}$ T cells had one-third to half the frequency of IL-17^{+} cells compared with WT Th17 cells (Fig. 3B). It was clear that BACE1$^{-/-}$ Th17 cells also produced less IL-17A on a per-cell basis, as demonstrated by the geometric mean fluorescence intensity of IL-17A^{+} cells in these cultures (Fig. 3C). *Il17a* gene expression and secreted IL-17A protein were also significantly decreased in BACE1$^{-/-}$ Th17 cultures versus WT controls (Fig. 3D, 3E). Presence or absence of IL-23 or IL-1 had no additional effects on the IL-17A defect in BACE1$^{-/-}$ cells (data not shown). Although IL-17 was decreased, BACE1$^{-/-}$ T cells cultured under Th17-differentiating conditions expressed normal levels of the Th17 master transcription factor RORγt (Fig. 3F, 3G).

Although PTEN has been reported to enhance Th17 differentiation by limiting IL-2 and IFN-γ, we did not see a consistent change in IL-2 or IFN-γ production by flow cytometry or ELISA (data not shown). Similarly, proliferation of BACE1$^{-/-}$ cells was

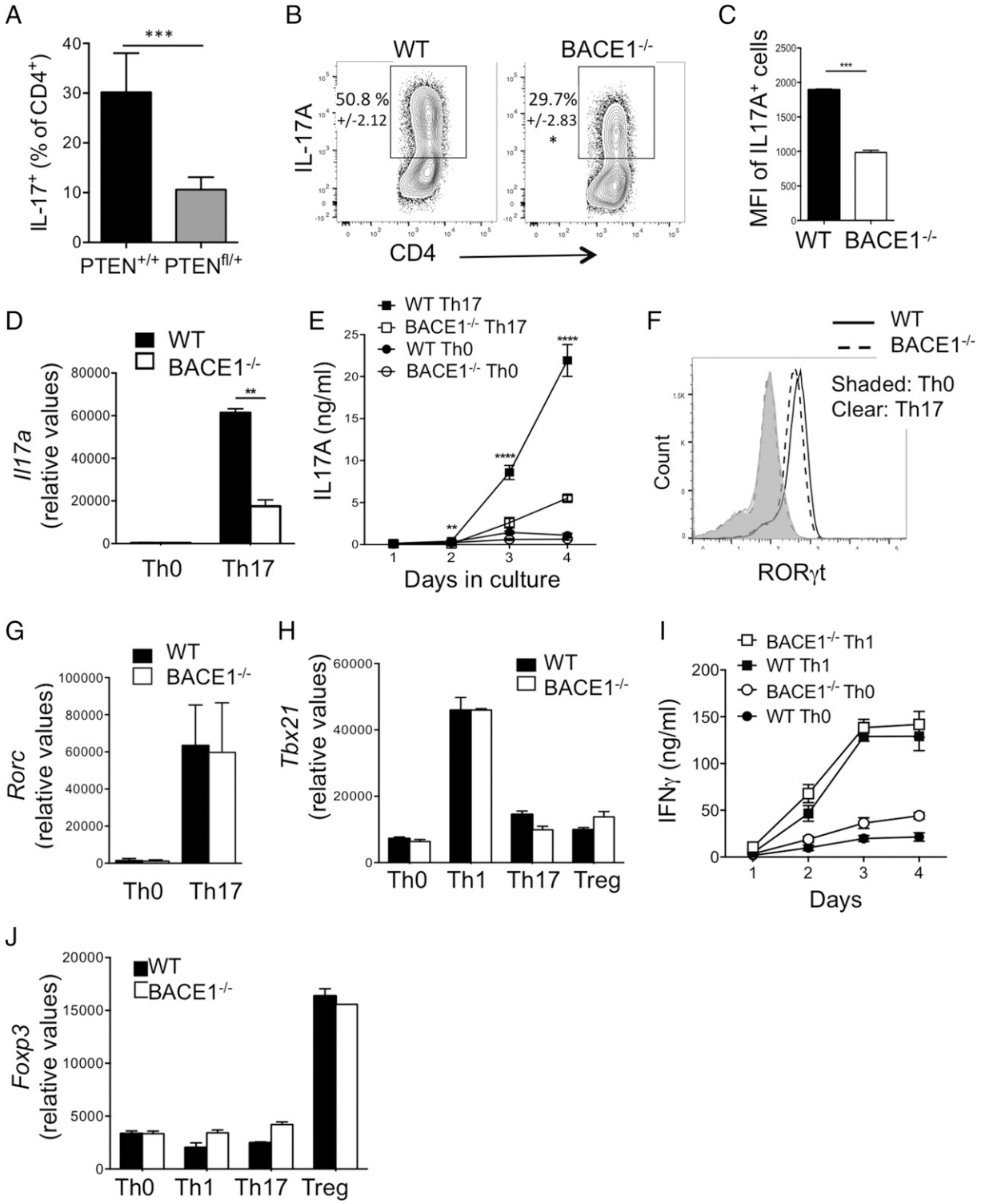

FIGURE 3. T helper subset differentiation in BACE1$^{-/-}$ T cells. (**A**) CD4-cre/PTEN$^{+/+}$ (WT control) and CD4-cre/PTEN$^{fl/+}$ T cells were cultured under Th17 conditions for 3 d, and IL-17A^{+} cells were assessed by flow cytometry. (**B**) WT or BACE1$^{-/-}$ CD4^{+} T cells were differentiated under Th17 conditions, and intracellular IL-17A was analyzed by flow cytometry on indicated days of culture; mean ± SD indicated. (**C**) Mean fluorescence intensity of IL-17A, gated on IL-17A^{+} cells, analyzed by flow cytometry on day 3 of culture under Th17 differentiating conditions. (**D**) Gene expression of *Il17a* in Th0 and Th17 cells on day 3 of culture, normalized to *Gapdh*. (**E**) IL-17A in culture supernatants analyzed by ELISA at indicated times; IL-17A levels reflect accumulated cytokine from start of culture. (**F**) RORγt protein expression analyzed by flow cytometry on day 3 of indicated cultures. (**G**) *Rorc* gene expression in T cells cultured under indicated differentiation conditions for 2 d, normalized to *Gapdh*. (**H**) *Tbx21* (Tbet) gene expression in T cells cultured under indicated differentiation conditions for 2 d, normalized to *Gapdh*. (**I**) IFN-γ production in Th0 and Th1 cells, analyzed by ELISA at indicated times; IFN-γ levels reflect accumulated cytokine from start of culture (**J**) *Foxp3* gene expression in T cells cultured under indicated differentiation conditions for 2 d, normalized to *Gapdh*. Data indicate mean ± SD of two to three replicates representative of at least four experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

similar to WT T cells (data not shown). Similarly, BACE1 deficiency had no effect on *Tbx21* gene expression (Fig. 3H) or IFN-γ production (Fig. 3I) in Th1 cells. Similar to in vivo Tregs, induction of *Foxp3* gene or Foxp3 protein expression by TGF-β was unaffected by BACE1 deficiency (Fig. 3J and data not shown). Compared with other T cell subsets, Th17 cells seem particularly prone to conversion to other Th cell phenotypes, particularly Th1 or Tregs (35). However, these data demonstrate that BACE1$^{-/-}$ cells cultured under Th17 conditions did not upregulate either *Tbx21* (Fig. 3H) or *Foxp3* expression (Fig. 3J). Hence, we concluded that BACE1 affects Th17 differentiation, leading to reduced production of IL-17A, but does not alter regulation of T helper subset master transcription factors or conversion to Th1 or Treg phenotypes.

CD73 expression is regulated by BACE1 in Th17 and Tregs

Preliminary gene expression profiling of WT and BACE1$^{-/-}$ Th17 cells confirmed that IL-17A was reduced in the absence of BACE1 and surprisingly few genes were changed in BACE1$^{-/-}$ Th17 cells (data not shown). One clearly Th17-assocated gene other than IL-17A found to be significantly downregulated in BACE1$^{-/-}$ Th17 cells was *Nt5e*, encoding ecto-5′-nucleotidase or CD73 (36, 37). CD73 expression is regulated by several pathways, including purinergic signaling through the second messenger intracellular cAMP (38). We confirmed that both the frequency of CD73-expressing cells and the amount of CD73 protein expressed per cell were increased on WT Th17 cells compared with Th0 and Th1 cells, and CD73 expression was significantly reduced on BACE1$^{-/-}$ Th17 cells (Fig. 4A, 4B). CD73 acts on the purinergic pathway, converting extracellular AMP to adenosine, which is thought to play an immunoregulatory role in cancer (39). As expected, in vitro–generated Tregs expressed high levels of CD73, which were partially dependent on BACE1 (Fig. 4A, 4B). In vivo, BACE1$^{-/-}$ Tregs showed a partial but significant decrease in CD73 expression by Tregs as well as reduced CD73 expression by Foxp3^{-} CD4^{+} T cells (Fig. 4C, 4D), supporting a role for BACE1 in promoting T cell expression of the immunoregulatory enzyme CD73.

BACE1 deficiency in the CNS versus T cells differentially affects susceptibility to EAE

The data so far indicate that BACE1$^{-/-}$ T cells have limited but potentially important defects in the expression of effector molecules associated with inflammation (IL-17) and immunoregulation (CD73). We therefore investigated the contribution of BACE1 to CNS inflammation in the animal model of MS, EAE.

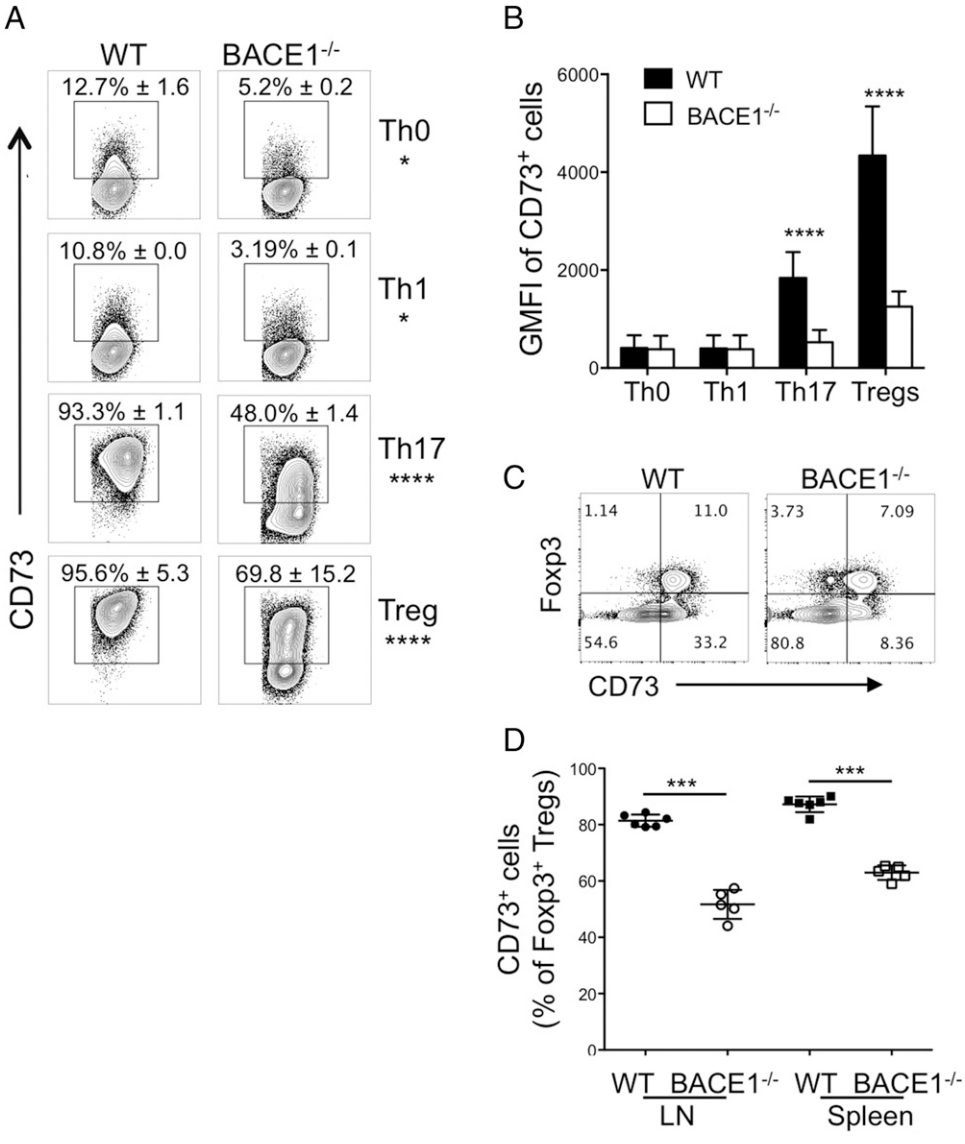

FIGURE 4. CD73 expression is reduced on BACE1$^{-/-}$ Th17 and Tregs. (**A**) WT and BACE1$^{-/-}$ T cells were cultured under indicated conditions for 4 d, and CD73 expression was assessed by flow cytometry on live CD4^{+} cells; representative FACS plots show mean ± SD of four experiments with two replicates each. (**B**) Geometric mean fluorescence intensity of CD73 expression on CD73^{+} cells gated as in (A); data show mean ± SD of four experiments with two replicates per experiment. (**C**) Representative FACS plots showing Foxp3 and CD73 expression in peripheral LN CD4^{+} T cells from naive WT and BACE1$^{-/-}$ mice. (**D**) Frequencies of CD73^{+} cells out of Foxp3^{+}CD4^{+} cells from naive WT and BACE1$^{-/-}$ mice, mean ± SD of three experiments. $*p < 0.05$, $***p < 0.001$, $****p < 0.0001$.

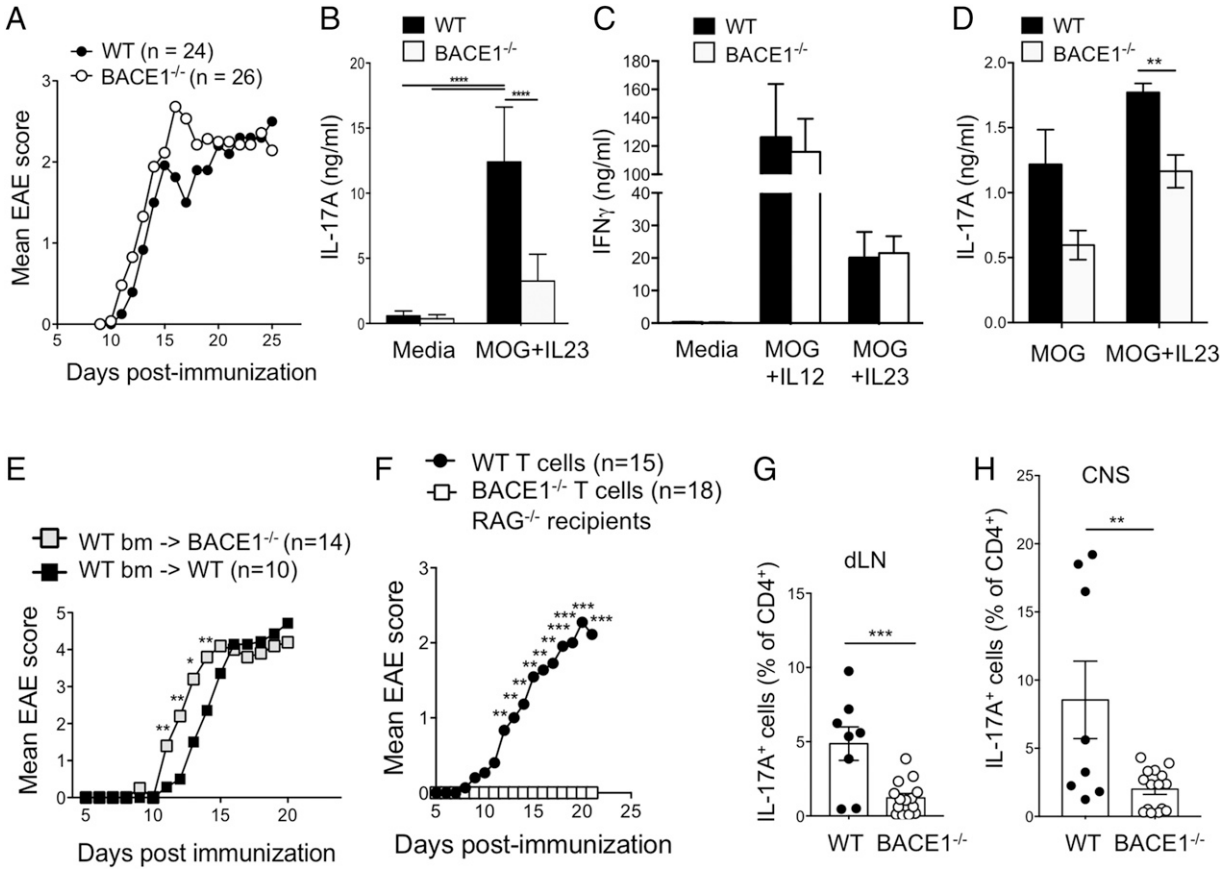

FIGURE 5. Differential roles for BACE1 in CNS and Th17 cells during EAE. (**A**) WT and BACE1$^{-/-}$ mice were immunized to induce EAE, and clinical scores were monitored; data pooled from five experiments. (**B**) Day 8 postimmunization, dLN cells were stimulated with MOG (35–55) + IL-23 for 3 d, then secreted IL-17A was analyzed by ELISA. (**C**) Day 8 postimmunization, dLN cells were stimulated with MOG (35–55) + IL-12 or MOG (35–55) + IL-23 for 3 d, then secreted IFN-γ was analyzed by ELISA. (**D**) Day 12 postimmunization, dLN cells were stimulated for 18 h with MOG (35–55) ± IL-23, then secreted IL-17A was analyzed by ELISA. (B–D) Data pooled from three experiments. (**E**) WT bone marrow was transferred into irradiated WT or BACE1$^{-/-}$ recipients, and EAE was induced following 8 wk reconstitution; data pooled from two experiments. (**F**) WT or BACE1$^{-/-}$ CD4$^+$ T cells were transferred into RAG1$^{-/-}$ recipients. EAE was induced the following day, and clinical signs were monitored. Data pooled from three experiments. (**G**) IL-17A$^+$ cells analyzed by flow cytometry in dLN on day 12 post-EAE induction following PMA/ionomycin stimulation. (**H**) IL-17A$^+$ cells analyzed by flow cytometry in CNS on day 12 post-EAE induction, following PMA/ionomycin stimulation. Data in (G) and (H) pooled from two experiments, each point represents an individual mouse. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

BACE1$^{-/-}$ and WT controls were immunized with MOG (35–55) in CFA. Incidence and clinical severity of EAE were similar between WT and BACE1$^{-/-}$ mice (Fig. 5A). However, ex vivo stimulation of dLN Th17 cells with MOG (35–55) and IL-23 for 3 d demonstrated reduced IL-17 response on day 8 postimmunization (Fig. 5B), similar to the in vitro differentiation results. In contrast, IFN-γ production under Th1 or Th17 conditions was unaffected in BACE1$^{-/-}$ dLN (Fig. 5C). IL-17 production after 18-hr culture with MOG (35–55) and IL-23 still showed a significant defect in BACE1$^{-/-}$ cells (Fig. 5D), suggesting that these affects were unlikely to be due to differences in the in vitro proliferation or survival of BACE1$^{-/-}$ T cells responding to Ag.

These data suggested the possibility of different roles for BACE1 in CNS-resident cells versus immune cells for determining EAE susceptibility. To test the role of BACE1 in CNS-resident cells in EAE susceptibility, we generated bone marrow chimeras in which irradiated WT or BACE1$^{-/-}$ recipients were reconstituted with WT bone marrow, thus restricting BACE1 deficiency to the nonimmune compartment. BACE$^{-/-}$ recipients of WT bone marrow had significantly earlier onset of EAE disease signs compared with controls (Fig. 5E), suggesting that BACE1 deficiency in nonimmune cells in the CNS causes increased susceptibility to CNS damage. To directly test the requirement for BACE1 in T cell function in vivo, RAG1$^{-/-}$ recipients (lacking T cells) were reconstituted with WT or BACE1$^{-/-}$ T cells before induction of EAE. Mice with T cells that lacked BACE1 (but all other cells including CNS were BACE1 sufficient) were resistant to EAE induction (Fig. 5F). Accordingly, IL-17 production was reduced in transferred BACE1$^{-/-}$ T cells in dLN and CNS (Fig. 5G, 5H).

To confirm the Th17 cell–intrinsic requirement for BACE1 in vivo, BACE1$^{-/-}$ 2D2 cells were transferred into CD45.1$^+$ WT recipients and tracked by expression of CD45.2 following immunization with MOG (35–55) in CFA. BACE1$^{-/-}$ 2D2 cells again showed a clear reduction in frequencies of IL-17A producers (Fig. 6A), whereas Th17 responses were not different in recipients of WT or BACE1$^{-/-}$ 2D2 cells (Fig. 6A). Of the remaining BACE1$^{-/-}$ cells that did produce IL-17A, the mean level of IL-17A protein per cell was reduced (Fig. 6B). To further test the role of BACE1 in autoimmune Th17 cells, WT and BACE1$^{-/-}$ 2D2 cells, bearing transgenic TCRs reactive to MOG (35–55), were differentiated under Th17 conditions in vitro, and IL-17A production was confirmed (Fig. 6C). Upon transfer to RAG$^{-/-}$ naive hosts, BACE1$^{-/-}$ 2D2 Th17 cells were significantly impaired in their ability to induce EAE (Fig. 6D, Table I). Similar results were observed after transfer of 2D2 Th17 cells into WT recipients (Fig. 6E, Table II). It was particularly striking that

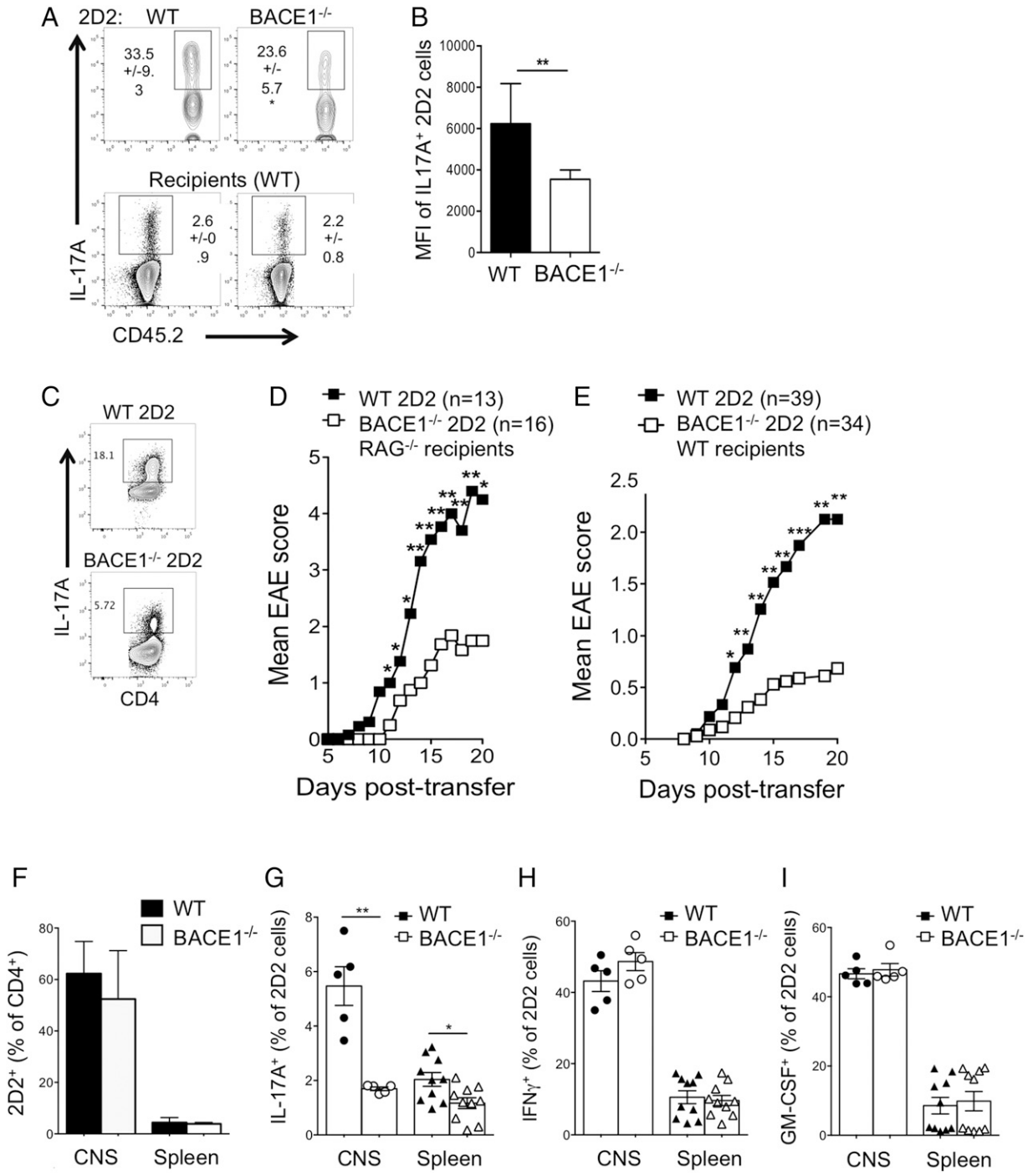

FIGURE 6. BACE1$^{-/-}$ Th17 cells have reduced pathogenicity in vivo. (**A**) CD45.2$^+$ WT or BACE1$^{-/-}$ 2D2 cells were transferred into CD45.1$^+$ recipients, which were then immunized with MOG (35–55) in CFA. IL-17 production in CD45.2$^+$ 2D2 cells was analyzed following PMA/ionomycin stimulation in dLN on day 8; mean ± SD shown. (**B**) Mean fluorescence intensity of IL-17$^+$ cells in 2D2 cells analyzed in (A). Data in (A) and (B) pooled from two independent experiments with three to four mice per group. (**C**) WT and BACE1$^{-/-}$ 2D2 Th17 cells were activated in vitro for passive transfer of EAE; IL-17A expression assessed by flow cytometry on day 4 of culture. (**D**) EAE clinical scores following transfer of WT or BACE1$^{-/-}$ 2D2 Th17 cells into RAG$^{-/-}$ recipients (see also Table I). (**E**) EAE clinical scores following transfer of WT or BACE1$^{-/-}$ 2D2 Th17 cells into WT recipients (see also Table II). Data in (D) and (E) pooled from five experiments. (**F–I**) WT and BACE1$^{-/-}$ Th17 cells were generated and transferred into WT recipients as described in (C) and (E). 2D2 cells and cytokine production analyzed by flow cytometry following PMA/ionomycin stimulation in live CD4$^+$ Vβ11$^+$ cells in CNS and spleen after EAE onset (day 12–14); data pooled from independent experiments ($n = 5$ for CNS, $n = 10$ for spleen). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

many recipients of WT 2D2 cells developed signs of atypical EAE, including severe ataxia and circling behavior, with higher rates of mortality, but these atypical signs were not apparent in recipients of BACE1$^{-/-}$ 2D2 cells (Table II). This form of atypical EAE has previously been associated with a strong Th17 response (7). Frequencies of WT and BACE1$^{-/-}$ 2D2 cells were similar after transfer (Fig. 6F). We did not analyze frequencies of 2D2 cells at late timepoints of EAE, so we cannot rule out long-term survival or proliferation defects in BACE1$^{-/-}$ T cells. After EAE onset, IL-17 production as a proportion of 2D2 cells was

Table I. Th17 2D2 cell passive transfer of EAE into RAG$^{-/-}$ recipients is reduced in absence of BACE1

	WT 2D2, *n* (%)	BACE1$^{-/-}$ 2D2, *n* (%)	*p* Value (Fisher)
Number of recipients	13	16	
Incidence	13 (100)	9 (56)	0.0084
Death	3 (23)	0 (0)	0.0783

Data pooled from four independent experiments.

Table II. Th17 2D2 cell passive transfer of EAE into WT recipients is reduced in absence of BACE1

C57BL/6 Recipients	WT 2D2, *n* (%)	BACE1$^{-/-}$ 2D2, *n* (%)	*p* Value (Fisher)
Number of recipients	39	34	
Incidence	26 (67)	11 (32)	0.0049
Death	8 (21)	1 (3)	0.0316
Atypical EAE	19 (49)	2 (6)	<0.0001

Data pooled from six experiments.

significantly reduced in BACE1$^{-/-}$ 2D2 compared with WT 2D2 in CNS and spleen, whereas IFN-γ and GM-CSF were unaffected (Fig. 6G–I). Taken together, these results demonstrate that BACE1-deficient T cells have impaired proinflammatory functions, resulting in reduced pathogenicity.

Discussion

Our data indicate a role for the CNS-associated protein BACE1 in CD4$^+$ T cells. BACE1 regulates baseline cAMP responses, calcium signaling, and PTEN levels with resultant effects on Th17 and Treg differentiation. Although there was not a complete block in Th17 development, the defects in BACE1$^{-/-}$ T cells were sufficient to strongly impair their capacity to induce disease in the EAE model. Typically, defects in IL-17A production that are observed during early stages of Th17 development are also accompanied by broader defects in the Th17 program, including reduced expression of RORγt. However, there is precedent for modulation of IL-17A independently of other Th17 factors downstream of RORγt. Serum amyloid A produced by inflamed epithelium strongly promotes production of IL-17A by RORγt$^+$ effector Th17 cells in epithelial tissues (40). Closer to the initial activation events for Th17 differentiation, PKCα acts as a signaling intermediary to promote TGF-β–mediated SMAD activation, and deficiency results in impaired IL-17A but not IL-17F production by Th17 cells (41). Similarly, TGF-β–regulated inhibition of the transcriptional repressor Gfi1 is required for Th17 development, and Gfi1 deficiency or overexpression has a marked effect on IL-17A but comparatively minor effects on RORγt and IL-17F (36, 42). Strikingly, Gfi1 repression is also required for CD73 expression (36), which we found to be coregulated with IL-17A in BACE1$^{-/-}$, making Gfi1 a good candidate to mediate the effects of BACE1. However, we did not observe any difference in Gfi1 expression between BACE1$^{-/-}$ and WT cells (data not shown), suggesting that Gfi1 is not responsible for defective IL-17A and CD73 expression in BACE1$^{-/-}$ cells. It is interesting to note that Akt activation by TCR engagement was also found to inhibit Gfi1 (43), as BACE1$^{-/-}$ cells demonstrated heightened Akt activity.

Aside from activation of Akt, TCR ligation results in Lck-mediated ZAP70 phosphorylation that in turn activates the LAT signaling complex including the TEC kinase Itk that then activates PLCγ1 (44). Itk$^{-/-}$ Th17 cells show strikingly similar defects to BACE1$^{-/-}$ cells, with reduced IL-17A but not RORγt or IL-17F upon in vitro differentiation (45). However, Itk$^{-/-}$ cells have reduced PI3K activation, reduced calcium signaling, and retain high PTEN expression following activation (46), whereas BACE1$^{-/-}$ T cells have increased Akt activation concomitant with reduced PTEN expression. Therefore, it seems that altering TCR signaling either above or below the optimal threshold contributes to defects in Th17 cells that, although subtle, may impact their function. Itk$^{-/-}$ cells are also more prone to converting to Tregs under Th17 differentiating conditions (46), as high PTEN promotes Tregs (47, 48), in contrast to BACE1$^{-/-}$ Th17 cells, which showed no increase in Foxp3 expression. In this regard, it is interesting to note that BACE1$^{-/-}$ Tregs showed reduced CD73 expression because Treg instability has previously been associated with reduced PTEN localization to TCR that resulted in reduced CD73 expression and Treg function in tumor settings (49). Hence, reduced CD73 corresponds well with reduced PTEN expression. We did query whether CD73 expression contributes to the Th17 phenotype of BACE1$^{-/-}$ cells but found that CD73 is not required for IL-17 production by Th17 cells and is dispensable for EAE induction (50). Furthermore, addition of adenosine to Th17 cultures did not restore IL-17A production in BACE1$^{-/-}$ Th17 cells (data not shown). Thus, we conclude that CD73 is dysregulated concomitantly with IL-17 in BACE1-deficient T cells, rather than being an upstream regulator of IL-17A responsible for the effects of BACE1.

A recent report demonstrated that deletion of PTEN in developing Th17 cells inhibited Th17 differentiation (34), and our data confirmed that reduced PTEN inhibits IL-17 production. Regulation of PTEN is complex, with a plethora of mechanisms targeting mRNA transcription and translation as well as degradation of mature protein, for example, through ubiquitination (51). The relationship between cAMP and PTEN expression has not been well studied in T cells, but there is evidence for direct downregulation of PTEN protein by cAMP in glial cells and thyroid cells (52, 53). cAMP also activates PKA, which can feed-forward to activate PI3K (54, 55), and because activation of PI3K signaling pathways negatively regulates PTEN levels (56), this provides an indirect mechanism by which increased cAMP could lead to dysregulation of PTEN. It is also feasible that cAMP activation of PKA leads to inhibition of the downstream mediators of Ca^{2+} activation, such as NFAT. However, we could not find evidence to support defective NFAT nuclear localization in BACE1$^{-/-}$ T cells (data not shown). Furthermore, cAMP induced following TCR engagement has been shown to negatively regulate TCR signaling through Lck inhibition (57) or PKA-Csk activation (58, 59), which would ultimately reduce Ca^{2+} flux if unchecked. Because PTEN is reduced at baseline in BACE1$^{-/-}$ T cells, we hypothesize that in naive CD4$^+$ T cells, cAMP and p-Akt induced by survival signals through growth factor receptors, chemokines, and TCR "tickling" by MHC all contribute to alterations in PTEN that are negatively regulated by BACE1, hence setting the threshold for outcome of eventual effector cell differentiation.

It was intriguing that the global BACE1 knockout mice did not reveal the defect in Th17 pathogenicity that was demonstrated when BACE1 deficiency was restricted to T cells in either active or passive EAE. It is possible that other cytokines, such as IFN-γ or GM-CSF, are sufficient to induce disease when presented with a CNS that is already suffering some physiological defects or when occurring in sufficient numbers to override the IL-17A defect. This premise is supported by the finding that WT recipients of BACE1$^{-/-}$ 2D2 cell transfers (arguably at higher cell numbers than would occur physiologically following immunization) were

not completely protected from EAE induction. However, the clinical characteristics of EAE were different, and IL-17A–producing capability corresponded to increased incidence of atypical EAE, as has previously been reported (7–9, 60). We did not further verify ratios of neutrophils versus macrophages, which are indicators of the Th17 to Th1 ratios (7–9); nevertheless, the body of evidence from the active and passive EAE models supports the loss of pathogenicity when BACE1 is specifically lacking in T cells.

One outstanding question is whether the proteolytic activities of BACE1 are required for its IL-17A–promoting effects because these are the current target of AD therapy. In neuronal cells, BACE1 regulates cAMP through nonproteolytic interactions with adenylyl cyclase (32), making it likely that at least some of the effects in T cells could be through this noncanonical BACE1 function. Similarly, the precise mechanisms by which BACE1 contributes to neuroinflammation and degeneration following injury are still not fully clear. Many studies have used BACE1-deficient animals to address the role of BACE1 in determining outcomes of CNS injury. Intriguingly, IL-17A has also been reported to increase following CNS injury (26–28), often produced by γδ T cells, which rapidly enter the site of damage. IL-17A–producing γδ T cells also accompany myelin-reactive Th17 cells during the early phases of EAE and contribute to inflammation (61). IL-17A has been shown to promote neuronal cell death poststroke, and blocking IL-17A reduces lesion size and enhances functional recovery in rodent models (26–28). Recently, IL-17A–producing γδ T cells that exacerbate damage following CNS ischemia were found to be programmed by gut microbiota (26), corresponding to a rapidly growing body of evidence that the microbiome sets the rheostat for immune responses, and particularly Th17 and Treg responses, throughout the body (62, 63). Our findings that BACE1 regulates IL-17A production therefore cast a new light on previous findings on outcomes of CNS injury in BACE1$^{-/-}$ animals because effects on both CNS cells and immune cells, particularly IL-17A–producing cells, may have contributed to these observed outcomes. The finding that BACE1-deficient T cells demonstrate enhanced AC-stimulated production of cAMP corresponds to findings in neurons. It would therefore be interesting to determine whether CNS neurons in BACE1-deficient mice also have defects in the PTEN, Akt, and PLCγ pathways, or indeed CD73 expression, and whether these contribute to BACE1-mediated effects in healthy and diseased brain.

From a therapeutic point of view, these data suggest that blocking BACE1 has the potential to target both inflammation and neurodegeneration, prompting further investigation of the role of BACE1 in neuroinflammation provoked by CNS injury, be it autoimmune, traumatic, or ischemic. The finding that the same molecule can have very different effects and outcomes on disease depending on the cell type targeted is a recurring theme in immunology: for example, STAT3 deletion in all CD4$^+$ T cells renders mice resistant to Th17 induction and associated inflammation (64), whereas STAT3 deletion in Foxp3$^+$ Tregs results in spontaneous development of Th17-associated autoimmune disease (65). Another example is CD47: blockade of CD47 has completely opposite effects on EAE development depending on the cell type targeted (immune cells versus CNS) and timing of blockade (66). It will be interesting in future studies to conditionally delete BACE1 in specific T cell populations (e.g., Tregs versus Th17 cells) and also in other immune cells to determine additional roles in immune function, including inflammatory disease, infection control, and tumor eradication.

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Disclosures

The authors have no financial conflicts of interest.

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