Extrapituitary prolactin promotes generation of Eomes-positive helper T cells mediating neuroinflammation

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Induction of eomesodermin-positive CD4+ T cells (Eomes+ Th helper [Th] cells) has recently been correlated with the transition from an acute stage to a later stage of experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis. Moreover, these cells’ pathogenic role has been experimentally proven in EAE. While exploring how the pathogenic Eomes+ Th cells are generated during the course of EAE, we unexpectedly found that B cells and MHC class II+ myeloid cells isolated from the late EAE lesions strikingly up-regulated the expression of prolactin (PRL). We demonstrate that such PRL-producing cells have a unique potential to induce Eomes+ Th cells from naive T cells ex vivo, and that anti-MHC class II antibody could block this process. Furthermore, PRL levels in the cerebrospinal fluid were significantly increased in the late phase of EAE, and blocking the production of PRL by bromocriptine or Zbtb20-specific siRNA significantly reduced the numbers of Eomes+ Th cells in the central nervous system (CNS) and ameliorated clinical signs in the later phase of EAE.

The PRL dependency of Eomes+ Th cells was confirmed in a series of in vitro and ex vivo experiments. Collectively, these results indicate that extrapituitary PRL plays a crucial role in the CNS inflammation mediated by pathogenic Eomes+ Th cells. Cellular interactions involving PRL-producing immune cells could be considered as a therapeutic target for the prevention of chronic neuroinflammation.

Significance

We have previously demonstrated that induction of pathogenic eomesodermin-positive CD4+ T cells (Eomes+ Th helper [Th] cells) is associated with transition from an acute stage to a later stage of experimental autoimmune encephalomyelitis (EAE). In the late phase of EAE, B cells and non-B cell antigen-presenting cells (APCs) recruited to the central nervous system strikingly up-regulate prolactin (PRL). The PRL-producing APCs have the potential to promote generation of Eomes+ Th cells from naive T cells in an MHC class II-restricted manner, and therapies inhibitory for PRL production suppress the induction of Eomes+ Th cells and ameliorate clinical signs of EAE. Our study highlights the unexpected role of extrapituitary PRL in the development of persistent neuroinflammation.

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antigen(s) presented by the CNS-recruited APCs. To explore this possibility, we isolated CD19<sup>+</sup> B cells and CD19<sup>-</sup> MHC class II-positive cells from the CNS of mice with EAE (SI Appendix, Fig. S2B) and cultured these cells with splenic CD4<sup>+</sup> T cells isolated from naive congenic mice or CD226<sup>+</sup>CD4<sup>+</sup> T cells as responders to assess the potentials of inducing Eomes<sup>+</sup> Th cells (SI Appendix, Fig. S2 A and C). The proportions of CD19<sup>+</sup> B cells and CD19<sup>-</sup> MHC class II-positive cells in the CNS increased greatly during the subacute phase (or at a middle time point) of EAE, followed by some reductions in the late phase (SI Appendix,
understand the reason why APCs from late EAE lesions are su-

mune-related Gene Ontology terms (Analysis of gene sets that were high up-regulated in late EAE. Notably, anti-MHC class II antibody significantly inhibited were most significantly induced when the responder T cells were cultured with the B cells and non-B cell APCs derived from late EAE. Notably, anti-MHC class II antibody significantly inhibited the induction of CD107a on responder T cells, as well as induction of CD107a+ Eomes+ cells after culture with the B cells or non-B cells (SI Appendix, Fig. S3). By contrast, the addition of anti-CD3 antibody significantly promoted CD107a expression and induction of the CD107a+Eomes+ cells in the presence of B cells but not in the presence of non-B cells. In the absence of the B cells or non-B cell APCs, anti-CD3, anti-CD28, or a combination of the 2 antibodies did not show any significant effects. Collectively, these results indicate the possible involvement of MHC II-associated ligand in the induction of cytotoxic Eomes+ Th cells ex vivo.

PRL Is Produced by APCs Capable of Inducing Eomes+ Th Cells. To understand the reason why APCs from late EAE lesions are superior to others in their ability to induce CD107a+Eomes+ Th cells, we analyzed the gene expression profiles of the APCs obtained from early, middle, and late time points during EAE (Fig. 1C). Analysis of gene sets that were highly up-regulated in late EAE revealed the enrichment of biological processes associated with immune-related Gene Ontology terms (SI Appendix, Table S1), indicating a drastic change in the properties of APCs after they enter the CNS. Unexpectedly, we found that PRL and growth hormone (GH), the hormonal sibs produced predominantly by pituitary glands, were highly up-regulated in both B cells and non-B cell APCs from late EAE (Fig. 2A). Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis confirmed an outstanding up-regulation of PRL in the APCs recruited to the late lesions (Fig. 2B). GH expression was also up-regulated in those samples, although this was less significant in non-B cell APCs compared with B cells. We further observed that concentrations of PRL and GH in the CNS of EAE mice were significantly increased at the late time point during EAE compared with the early or middle time point (Fig. 2C). In contrast, PRL transcripts in the pituitary glands were reduced in mice showing signs of EAE, as reported previously (8). More conspicuously, serum PRL levels were greatly reduced in EAE mice, although GH levels were not changed significantly (SI Appendix, Fig. S4 A and B). These results indicate that the production of PRL by CNS APCs is not a trivial phenomenon but could play a pathogenic role in the late stage of EAE.

As immunostimulatory functions of PRL have been described previously (9–13), we next examined the ability of PRL and GH to induce Eomes+ Th cells in vitro. Eomes expression was significantly up-regulated in naive CD26+CD4+ T cells from the spleen after they were cultured with exogenous PRL for 4 h (Fig. 2 D and E). In contrast, GH showed only a marginal effect on the induction of Eomes (Fig. 2F). A very narrow range of PRL concentrations are effective for the induction of Eomes, as has been described for the effect of PRL on T-bet expression in T cells (14). Since the ability of CNS APCs to induce Eomes+ Th cells is correlated with the potential to produce PRL, we speculated that the ectopic secretion of PRL by the APCs might facilitate the generation of Eomes+ Th cells in the inflamed CNS tissue.

Zbtb20 Is Necessary for PRL Expression in CNS APCs. The transcription factor Zbtb20 is essential for the development of lactotrophs and is responsible for the production of PRL in the anterior pituitary gland (15, 16). Since Zbtb20, originally cloned from human dendritic cells (DCs) (17), is expressed in the B cell lineage (18, 19), we examined the expression of Zbtb20 in the CNS-recruited B cells and non-B cell APCs in the late phase of EAE. Flow cytometry assessments showed that the proportion of Zbtb20-expressing cells in these myeloid cells were increased in late EAE compared with early EAE (Fig. 3A). Moreover, the in vivo injection of Zbtb20-specific siRNA inhibited the expression of PRL in the APCs from late EAE (Fig. 3B), suggesting that Zbtb20 plays a critical role in the production of extrapituitary PRL.

Based on the flow cytometry results, we next analyzed the expression of PRL and Zbtb20 in subsets of B cells and non-B cells: CD5+ B cells, CD5− B cells, conventional DCs (cDCs), plasmacytoid DCs (pDCs), and CD11c+ PDCA-1− (CD11c+) cells (SI Appendix, Fig. S4 C and D). In all of these subsets, PRL expression was selectively up-regulated in the cells obtained from late EAE (Fig. 3 C and D). Expression of Zbtb20 in CD5+ B cells and pDCs was found in early stages of EAE, whereas late EAE-specific expression of Zbtb20 was confirmed in CD5− B cells, cDCs, and CD11c+ cells (Fig. 3 C and D). Consistent with a previous report (18), CD5+ B cells (Fr. 2 and Fr. 3 in SI Appendix, Fig. S4C) contained a higher proportion of Zbtb20-expressing cells compared with conventional CD5+ B cells (Fr. 1 and Fr. 4). Interestingly, in all subsets except pDCs, B cells and non-B cell APCs isolated from the spleens of naïve mice expressed only marginal levels of Zbtb20 or PRL (orange dashed lines in Fig. 3 C and D). These results imply that a wide variety of B cells and non-B cell APCs acquire an ability to express PRL after entering the CNS of EAE mice.

Inhibition of PRL and Zbtb20 Attenuates the Development of Late EAE. Although hyperprolactinemia can be associated with autoimmune diseases, such as systemic lupus erythematosus and MS (10, 20), the role of PRL in the pathogenesis of EAE/MS remains controversial. In fact, a previous report has suggested that the development of acute EAE might not depend on PRL (8). We evaluated the effect of the PRL inhibitor BRC in a model of EAE induced in NR4A2KO mice. Because NR4A2KO mice are deficient for Th17 cells but maintain Eomes+ Th cells, the chronic EAE induced in these mice is suitable for evaluating the pathogenicity of Eomes+ Th cells (2, 3). Treatment with BRC significantly reduced the clinical symptoms of EAE in this model (Fig. 4A), which is not attributable to the effect of BRC on Th17 cells. Moreover, we observed a reduced infiltration of Eomes+ CD4+ T cells in the CNS lesions of the mice treated with BRC (Fig. 4B). The CNS APCs isolated from BRC-treated mice showed a reduced capability to induce Eomes+ T cells from naive Th cells ex vivo (Fig. 4C and D). These results are consistent with our postulation that PRL produced by CNS APCs plays a critical role in the induction of Eomes+ Th cells.

Because BRC is a dopamine D2 receptor agonist, we further examined the effect of dopamine and its precursor L-DOPA in late EAE. We observed that in vitro treatment with dopamine markedly reduced the expression levels of PRL and Zbtb20 in APCs isolated from late EAE (SI Appendix, Fig. S5A). Clinical manifestations of late EAE were significantly ameliorated by the administration of L-DOPA in vivo (SI Appendix, Fig. S5B), whereas the numbers of Eomes+ Th cells and Zbtb20+ CNS APCs were reduced. PRL expression was significantly suppressed in the CNS APCs derived from L-DOPA-treated mice (SI Appendix, Fig. S5C). Furthermore, late/chronic EAE induced in NR4A2KO mice was significantly ameliorated by the systemic administration of Zbtb20-specific siRNA (Fig. 4F). In parallel, the proportions of Eomes+ Th cells and Zbtb20+ B cells in the CNS were significantly reduced in mice treated with this siRNA (Fig. 4G). These results indicate that the up-regulation of Zbtb20 in the CNS APCs is essential for the ectopic production of PRL, generation of Eomes+ Th cells, and progression of late EAE.

Inflammatory Cytokines Induce the Ectopic Expression of Zbtb20 and PRL in the CNS APCs. Since the persistent inflammatory milieu appears to be a prerequisite for the expression of Zbtb20/PRL in the
APCs recruited to the CNS lesions, we next evaluated the ability of inflammatory cytokines to induce the production of Zbtb20/PRL in splenic B cells. Among a panel of cytokines used, IL-1β, IL-6, IL-9, IL-12, IL-33, type I IFNs (IFN-α and -β), and IFN-γ were able to significantly increase the proportion of Zbtb20+ cells in splenic B cells (SI Appendix, Fig. S6A and B). Many of the cytokines and chemokines in the CSF were differentially expressed during the course of EAE (Fig. 5 and SI Appendix, Fig. S7). Although the kinetics suggest that IL-6 and IL-12 (increased from mid to late stages of EAE) might be involved in the induction of Zbtb20/PRL in the CNS APCs, other cytokines may also participate in the induction of Zbtb20/PRL. This is because cytokines produced in the CNS parenchyma might not always be identified by CSF analysis. We actually observed that type I IFNs are produced by CNS pDCs (SI Appendix, Fig. S8); therefore, further study is needed to identify cytokines that make the greatest contribution to ectopic PRL expression.

Amelioration of the Late Phase of EAE by B Cell Depletion Accompanies a Reduction of Eomes+ Th Cells. Efficacy of B cell depletion by the anti-CD20 mAb has been reported in the treatment of autoimmune diseases, such as MS (21, 22). We hypothesized that the efficacy of B cell depletion therapy might be achieved in part through the depletion of PRL-producing B cells and subsequent inhibition of the induction of Eomes+ Th cells. To explore this possibility, we
treated EAE mice with B cell-depleting anti-CD20 mAb and evaluated its effects on clinical EAE symptoms and Eomes<sup>+</sup> Th cell numbers in the CNS. The depletion of B cells before immunization with MOG<sub>35–55</sub> EAE (day −7) modestly increased disease severity, probably due to a depletion of B regulatory cells, as reported previously (23) (SI Appendix, Fig. S9A). In contrast, B cell depletion shortly after the onset of disease (day 13) reduced EAE severity at the peak phase and at later phases. The suppression of EAE symptoms was accompanied by reduced numbers of Zbtb20<sup>+</sup> B cells and Eomes<sup>+</sup> Th cells in the CNS (SI Appendix, Fig. S9 B–D). These results suggest that the efficacy of the anti-CD20 mAb in MS might be related to its effect on ectopic PRL production in the CNS.

Discussion

An increasing body of evidence suggests that bidirectional interactions between the neuroendocrine and immune systems play critical roles in the maintenance of homeostasis in the body. Consequently, disturbing the mutual communication between these systems might trigger or promote the development of a wide variety of diseases (24). Among the endocrine hormones known to influence the
Fig. 4. Inhibition of PRL and Zbtb20 attenuates the transition to late EAE. (A) NR4A2cKO mice were immunized with MOG35–55 peptide emulsified in CFA and were given i.p. injections of pertussis toxin on days 0 and 2. The mice were then given i.p. injections of BRC or DMSO/PBS every other day starting on day 4 postimmunization. Clinical EAE scores are shown, with error bars representing SEM values. On the right, solid lines represent cumulative disease scores and dashed lines indicate the 95% confidence intervals. ****P < 0.0001, linear regression analysis. (B) Freshly isolated CNS CD4+ T cells were stained intracellularly to detect the presence of Eomes. (Left) Flow cytometry plots showing representative data from 3 independent experiments. (Right) Collective data indicating the frequency and exact number of CNS Eomes+CD4+ T cells. Error bars represent mean ± SD values. P values were calculated using Student’s t test. (C and D) CNS CD19+B cells and CNS CD19–CD45hi MHC II+ non-B APCs were purified by FACS sorting, then cocultured with FACS sorted splenic CD4+ T cells derived from congenic naïve mice in the presence of FITC-conjugated anti-CD107a antibodies. After 8 h of culture, cells were then stained intracellularly for expression of Eomes. (C) FACS plots showing the expression of Eomes and CD107a surface trapping in CD4+ T cells cocultured with CNS B cells (Top) or CNS non-B APCs (Bottom) from control (Left) or BRC-treated (Right) mice. (D) Summary of expression of Eomes+, CD107a, and Eomes+CD107a+ in CD4+ T cells after coculturing with CNS B cells or CNS non-B APCs from control or BRC-treated mice. Error bars show mean ± SD. *P < 0.05, Student’s test. Data are representative of 3 independent experiments. (E) CNS APCs were isolated from late EAE mice. CNS CD19–CD45hi MHC II+ non-B cell APCs were purified from the pooled APCs by FACS sorting, then transfected with Zbtb20-specific or control scrambled siRNA and cultured in the presence of LPS for 24 h. The expression levels of Zbtb20 and PRL were determined by qRT-PCR. (F) Zbtb20-specific (green triangle) or control scrambled (blue triangle) siRNA was stabilized in the atelocollagen substrate and administered i.v. into WT B6 mice that had been sensitized for MOG35–55 peptide 7 d earlier. Clinical EAE scores are shown, with error bars representing SEM values. On the right, solid lines represent cumulative disease scores, and dashed lines indicate the 95% confidence intervals. P = 0.0012, linear regression analysis. (G) CNS CD4+ T cells and B cells were freshly isolated from untreated, control siRNA-treated, or Zbtb20-specific siRNA-treated EAE mice and then stained intracellularly for Eomes or Zbtb20. Flow cytometry plots show representative data from 2 independent experiments. Summarized data are shown in the bar graphs. Error bars represent mean ± SD values. P values were calculated using 1-way ANOVA test with Dunnett’s multiple comparisons test. NS, not significant; *P < 0.05; **P < 0.01.
immune system, those produced by the specialized endocrine organs have been widely studied (25). However, hormones of ectopic origin have not been a major area of research with regard to the pathogenesis of human disorders. Although PRL is a pituitary hormone known to be involved in lactation, lutal functioning, and reproduction (26), we here disclose the unexpected role of extrapituitary PRL in the pathogenesis of persistent neuroinflammation mediated by Eomes+ Th cells, and report that cytotoxic Eomes+ Th cells are under the control of extrapituitary PRL.

After noting the biased TCR use by Eomes+ Th cells in the CNS (SI Appendix, Fig. S1), we examined the properties of potential APCs that may interact with precursors of Eomes+ Th cells. Subsequently, we came across the up-regulation of PRL in B cells and non-B cells recruited to the chronic EAE lesions, implying that CNS-recruited myeloid cells acquire the ability to secrete PRL several days after CNS entry. Further analysis indicated that such PRL-producing APCs are able to induce potentially cytotoxic CD107a+ Eomes+ Th cells from the splenic naive T cells in vitro, which is blocked in the presence of anti-MHC class II antibody (SI Appendix, Fig. S3). The link between extrapituitary PRL and Eomes+ Th cells has been explored in a series of experiments using anti-PRL drugs and siRNA, which showed that blocking PRL production by BRC, Zbtb20-specific siRNA, or L-DOPA all led to reduced induction of Eomes+ Th cells in vivo.

Although the efficacy of BRC for EAE has been reported previously (20, 27), another study has shown that the development of EAE does not necessarily require PRL (8). In the present study, we have demonstrated a role of PRL in maintaining T cells of the late phase of EAE that is dependent on Eomes+ Th cells, by introducing NR4A2 cKO mice in which Th17 cells are dysfunc-
tional (3). These results from in vitro and ex vivo analyses allow us to propose a model in which B cells and non-B cells recruited to the CNS acquire the ability to secrete PRL and induce Eomes+ Th cells via the presentation of MHC class II-associated antigen in the presence of PRL (SI Appendix, Fig. S10). In support of this, costimulatory roles of PRL in the in vitro activation of T cells and B cells have been reported previously (28, 29). Analysis with MOG35–55-loaded class II tetramer revealed that MOG35–55 is not the epitope recognized by Eomes+ Th cells (SI Appendix, Fig. S1A). Although the TCR ligands remain to be identified, autoantigens from the CNS tissue, superantigens (30, 31), or gut-derived antigens can be considered candidates.

There is a critical incubation period for the CNS-recruited APCs to acquire an ability of promoting the induction of Eomes+ Th cells, although such APCs are most abundant at the middle phase of EAE. We have indicated that expression of Zbtb20 in the APCs is probably induced by inflammatory cytokines; however, we have not specified which cytokine is most critical. Our next goal is to determine this Zbtb20/PRL-inducing cytokine in vivo and identify its cellular source. We also need to learn more about the cellular interactions leading to the generation of Eomes+ Th cells on the molecular terms, as such efforts may provide a better understanding of the pathogenesis of CNS inflammatory diseases and lead to the design of novel therapeutic strategies for preventing persistent neuroinflammation.

Methods

Mice. All mice used were 6 to 8 wk old and maintained in specific pathogen-free conditions in accordance with institutional guidelines. C57BL/6 (WT) mice were purchased from CLEA Japan. CD4-Cre NR4A2 mice were generated in house as described previously (3). All animal experiments were approved by the National Center of Neurology and Psychiatry’s Committee for Small Animal Research and Animal Welfare. All efforts were made to minimize animal suffering in clinical disease experiments.

EAE Induction and Scoring. Mice were injected s.c. with 50 mg of MOG35–55 peptide (synthesized at the Toray Research Center) and 1 mg of heat-killed Mycobacterium tuberculosis H37RA emulsified in complete Freund’s adjuvant (CFA; Difco). Then 100 ng of pertussis toxin (List Biological Laboratories was injected i.p. on days 0 and 2 after immunization). Neurologic deficits were evaluated on a scale of 0 to 5 (0, no clinical signs; 0.5, tail weakness; 1, partial tail paralysis; 1.5, severe tail paralysis; 2, flaccid tail; 2.5, flaccid tail and hind limb weakness; 3, partial hind limb paralysis; 3.5, severe hind limb paralysis; 4, total hind limb paralysis; 4.5, hind and fore leg paralysis; 5, dead).

BRC Treatment. BRC (Wako) was dissolved in DMSO and diluted by PBS, and a dose of 2.5 mg/kg was administered i.p. starting on day 4 postimmunization and continuing every other day up to day 32.
l-DOPA Treatment. l-DOPA (Sigma-Aldrich) was dissolved in DMSO and diluted with PBS, and a dose of 50 mg/kg was administered i.p. starting on day 4 postimmunization and continuing every other day up to day 30.

Anti-CD20 Treatment. Here 250 μg of anti-mouse CD20 or isotype control (all from BioLegend) was administered i.v. on day -7 or day 13 related to MOG35-55 peptide immunization.

Systemic sRNA Treatment. Mice received i.v. 20 mM Zbtb20-specific sRNA (sequence 50-ggaacauacuaauauauu-30; synthesized by Koken) or negative control sRNA stabilized with an AteloGene collagen system kit (Koken) on day 7 after MOG35-55 peptide immunization.

Cell Isolation. Single-cell suspensions of splenocytes were generated by mechanical disruption of tissues. To obtain CNS cell suspensions, the spinal cord was flushed out with PBS, and the brain was removed from the skull. The brain and spinal cord were cut into small pieces, followed by digestion with 1.4 mg/mL collagenase H and 100 μg/mL DNase I (Roche) in RPMI medium for 40 min at 37 °C. The cell suspension was filtered through a 70-μm cell strainer and enriched by a discontinuous 37%/70% Percoll gradient centrifugation (GE Healthcare Life Sciences).

Flow Cytometry. Cells were treated with anti-mouse CD16/CD32 (BioLegend) to block Fc receptors before staining. Staining was performed in PBS solution containing 5% FCS. For all experiments, dead cells were excluded using an Aqua Live/Dead fixable staining reagent (Invitrogen). mAbs CD1d (1B1), CD4 (GK1.5), CD5 (30F11), CD107a (1D4B), CD138 (281-2), CD226 (TX42.1), CD317 (129C1), Eomes (Dan11Mag), IL-9 (RM9A), IL-10 (JES5-16E3), TCRβ (H57-597), MHCI class II + Aa-E (MS/141.15.2), and Zbtb20 (AA3) were obtained from BD Biosciences, BioLegend, or eBioscience. For surface staining, cells were incubated with mAbs for 30 min on ice. For intracellular cytokine staining, cells were incubated for 6 h at 37 °C in 10% FCS in RPMI medium with PMA (50 ng/mL; Sigma-Aldrich) and ionomycin (500 ng/mL; Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences; 1:1,000 dilution). For Eomes and Zbtb20 intracellular staining for CNS cells, fresh isolated cells were used without restimulation. After surface staining, a Foxp3 staining kit (eBioscience) was used according to the manufacturer's instructions, and then cells were incubated with the respective antibodies for 30 min at room temperature. Flow cytometry analysis was performed with a FACS Canto II flow cytometer (BD Biosciences) with FACS Diva software, and data were analyzed using FlowJo software (Free Star).

Cell Sorting. From splenocytes, T cells and B cells were isolated using a CD4-CD19 cell or CD19 cell MACS isolation kit, respectively, with an AutoMACS separator (Miltenyi Biotech) according to the manufacturer’s instructions. CD226+ CD4+ T cells were further purified using a FACS Aria IIu cell sorter (BD Biosciences). For CNS cells, CD45hiCD19- B cells, CD45hiCD19+ MHCI class II+ CD11c+CD177+CD11b- DCs, CD45hiCD19+CD11b+ microglia cells were sorted using a FACS Aria IIu cell sorter (BD Biosciences).

Mouse Vβ TCR Analysis. Groups of NR4A2 cKO and control mice were immunized with MOG35-55 to induce EAE. During the late phase of EAE (day 26), single-cell suspensions were prepared from spleen and CNS tissues. Cells were stained with antibodies against TCRβ+ and CD4 combined with the Mouse Vβ TCR screen panel kit (BD Biosciences), followed by fixation and permeabilization with an intracellular transcription factor staining reagent kit (eBioscience). Subsequently, intracellular staining with antibodies against Eomes was conducted for flow cytometry analysis.

Cell Culture. The culture medium was RPMI supplemented with 10% FCS, 100 μM penicillin-streptomycin, and 50 mM 2-mercaptoethanol (all from Invitrogen).

T Cell-APC Coculture. Flow cytometry-sorted CNS B cells or non-B APCs from EAE mice (5×2) were cultured with flow cytometry-sorted splenic CD226+ CD4+ T cells from naive congenic mice (5×1) in the presence of an anti-CD107a or fluorochrome-conjugated antibody with or without anti-CD3 (17A2; BioLegend) stimulation for 8 h at 37 °C. In the indicated experiments, anti-mouse class II Aa-E (MS/141.15.2) was added. Cells were subjected to FACS analysis as described above.

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Tetramer Staining. CNS-derived lymphocytes were treated with neuraminidase (Sigma-Aldrich), followed by staining with MOG35-55/MHC class II tetramer-BV421 (BioLegend). Core Factor was used at room temperature. Human CD1d+ cells or ex vivo tetramer-BV421 served as a staining control. Cells were stained with antibodies against surface markers, followed by fixation and intracellular staining for Eomes.

Induction of Zbtb20. AutoMACS-isolated splenic B cells from naive mice were cultured in the presence of IL-1β (5 ng/mL; BioLegend), IL-6 (40 ng/mL; BioLegend) IL-9 (12.5 ng/mL; BioLegend), IL-12 (5 ng/mL; R&D Systems), IL-33 (40 ng/mL; Peprotech), IFN-γ (1000 U/mL; BioLegend), IFN-α (500 U/mL; BioLegend), or IFN-β (200 ng/mL; BioLegend) with lipopolysaccharide (LPS; 10 ng/mL) for 24 h at 37 °C. Cells were subjected to Zbtb20 intracellular staining or lysed for qRT-PCR assays.

L-DOPA Treatment. L-DOPA (Sigma-Aldrich) was dissolved in DMSO and diluted with PBS, and a dose of 50 μmol/L dopamine (Sigma-Aldrich) with 10 ng/mL LPS for 24 h, 48 h, and 96 h at 37 °C. Cells were lysed for qRT-PCR assays.

Microarray Analysis. Expression microarrays were carried out on FACS-sorted CNS B cells and non-B APCs using GeneChip Mouse Gene 2.0 ST Arrays prepared using the First-Strand cDNA Kit (Takara) according to the manufacturer’s instructions. Gene expression was measured by real-time qPCR using a LightCycler instrument (Roche) with SYBR Green Master Mix (Roche). All primers were purchased from Qiagen. Expression levels were normalized to Rplpol or β2m.

Microarray Analysis. Expression microarrays were carried out on FACS-sorted CNS B cells and non-B APCs using GeneChip Mouse Gene 2.0 ST Arrays prepared using the RNeasy Mini Kit (Qiagen), then transcribed into cDNA using the First-Strand cDNA Kit (Takara) according to the manufacturer’s instructions. Arrays were washed in a GeneChip Fluidics Station 450 and scanned using a GeneChip Scanner 3000 7G (Qiagen). Array data were compiled using Affymetrix GCO5 software. Differential gene expression analysis was performed using MeV software (32), and Gene Ontology analysis was performed using PANTHER (33).

Block of PRL Signaling. Flow cytometry-sorted CNS B cells or non-B APCs from late EAE mice were cultured in the presence of 50 μmol/L dopamine (Sigma-Aldrich) with 10 ng/mL LPS for 24 h, 48 h, and 96 h at 37 °C. Cells were lysed for qRT-PCR assays.

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