Infiltrating Regulatory B Cells Control Neuroinflammation following Viral Brain Infection

Manohar B. Mutnal, Shuxian Hu, Scott J. Schachtele and James R. Lokensgard

*J Immunol* published online 10 November 2014
[http://www.jimmunol.org/content/early/2014/11/08/jimmunol.1400654](http://www.jimmunol.org/content/early/2014/11/08/jimmunol.1400654)
Infiltrating Regulatory B Cells Control Neuroinflammation following Viral Brain Infection

Manohar B. Mutnal, Shuxian Hu, Scott J. Schachtele, and James R. Lokensgard

Previous studies have demonstrated the existence of a subset of B lymphocytes, regulatory B cells (Bregs), which modulate immune function. In this study, in vivo and in vitro experiments were undertaken to elucidate the role of these Bregs in controlling neuroinflammation following viral brain infection. We used multicolor flow cytometry to phenotype lymphocyte subpopulations infiltrating the brain, along with in vitro cocultures to assess their anti-inflammatory and immunoregulatory roles. This distinctive subset of CD19⁺CD1dhiCD5⁺ B cells was found to infiltrate the brains of chronically infected animals, reaching highest levels at the latest time point tested (30 d postinfection). B cell–deficient Jh⁻/⁻ mice were found to develop exacerbated neuroimmune responses as measured by enhanced accumulation and/or retention of CD8⁺ T cells within the brain, as well as increased levels of microglial activation (MHC class II). Conversely, levels of Foxp3⁺ regulatory T cells were found to be significantly lower in Jh⁻/⁻ mice when compared with wild-type (Wt) animals. Further experiments showed that in vitro–generated IL-10–secreting Bregs (B10) were able to inhibit cytokine responses from microglia following stimulation with viral Ags. These in vitro–generated B10 cells were also found to promote proliferation of regulatory T cells in coculture studies. Finally, gain-of-function experiments demonstrated that reconstitution of Wt B cells into Jh⁻/⁻ mice restored neuroimmune responses to levels exhibited by infected Wt mice. Taken together, these results demonstrate that Bregs modulate T lymphocyte as well as microglial cell responses within the infected brain and promote CD4⁺Foxp3⁺ T cell proliferation in vitro. The Journal of Immunology, 2014, 193: 000–000.

The CNS is a target for acute viral infection, as well as a reservoir of latent and persisting viruses (1, 2). The prevailing paradigm has been that the inert immunological status of the CNS parenchyma is maintained through exclusion of key components of the immune system. However, it is now known that systemic T lymphocytes (3) normally transit into the CNS to participate in immune surveillance (4). Proinflammatory cytokines, chemokines, and other mediators produced by resident glial cells, both astrocytes and microglia, induce activation and recruitment of immune cells in response to viral infection (5–8). However, a balance is required between the neuroimmune responses that are necessary to deal with invading pathogens and those that prevent these responses from harming surrounding brain tissue. Long-term persistence of immune cells that infiltrate the CNS in several viral infections demonstrates that inflammation needs to be controlled to prevent immune-driven pathology through either native or external anti-inflammatory mechanisms. Various regulatory cell types and anti-inflammatory mediators have been shown to play critical roles in countering these overzealous neuroinflammatory responses and preventing immune-mediated neuropathology (9–11).

A number of anti-inflammatory mechanisms have been demonstrated to control excess neuroinflammation including the infiltration of inflammation-dampening regulatory T cells (Tregs) (12). IL-10 is a prototypical anti-inflammatory cytokine that suppresses cellular immunity and inhibits synthesis and release of proinflammatory mediators from activated microglia (13, 14). Tregs have been identified as a predominant source of IL-10, but studies have shown that this cytokine can also be produced by many other types of immune cells including B cells (15–17).

B lymphocytes have been traditionally considered APCs and Ab-producing cells. However, a number of studies in recent years have demonstrated the existence of a regulatory subset of B cells (Bregs) that exhibit immunosuppressive functions (reviewed in Ref. 18). An early study by Fillatreau et al. (19), using a chimeric model in which B cells were specifically deleted of IL-10 production, demonstrated that these cells inhibited immune-mediated pathology in murine experimental autoimmune encephalomyelitis. Further studies went on to reveal that Bregs play important roles in control of immunopathology during autoimmune diseases (16), cancer (20), and organ transplantation (21). Bregs have also been shown to modulate immune responses and immunopathology in infectious diseases (22, 23). Different phenotypes of Bregs have been described. These cells have been detected within splenic marginal zone populations (24). IL-10–producing B cells are found in the spleens of naive mice at low frequencies (1–5%), where they represent a subset of the CD19⁺CD1dhiCD5⁺ B cell subpopulation (15, 16, 25). These CD1dhiCD5⁺ B cells were found to be more enriched for IL-10–producing cells (9–15%) than other B cell subsets. Similar IL-10–producing B cell subsets have been identified in healthy and autoimmune individuals. The capacity of human and mouse Bregs to express IL-10 is central to their negative regulation of inflammation, autoimmunity, and adaptive as well as innate immune responses (26–29). B cell expression of cytoplasmic IL-10 protein parallels both their expression of IL-10...
transcripts and secretion of IL-10, as measured by ELISA (26–29). In this manuscript, we use the term Breg to identify cells expressing the CD19+CD1dhiCD5+ surface markers, whereas the term B10 cells is used to describe B lineage cells known to produce IL-10.

Our previous studies have demonstrated that viral brain infection with murine CMV (MCMV) triggers accumulation and long-term persistence of both T lymphocyte and B lymphocyte lineage cells within the brain (30, 31). Although CD8+ T cells were found to be required for viral clearance and control of brain infection (32), persisting B lineage cells were found to produce MCMV-specific Abs and played a significant role in controlling spread of reactivated virus (31). In the current study, we assessed the presence of CD19+CD1dhiCD5+ Bregs within the infected brain and investigated their anti-inflammatory and immunoregulatory roles.

Materials and Methods

Ethical statement

This study was carried out in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (protocol number 1105A99534) of the University of Minnesota. All surgery was performed under ketamine anesthesia, and all efforts were made to minimize suffering.

Viruses and animals

RM461, an MCMV expressing Escherichia coli β-galactosidase under the control of the human iel1/e2 promoter/enhancer (33), was kindly provided by Edward S. McAlcari. The virus was maintained by passage in weaning female BALB/c mice. Salivary gland–passaged virus was then grown in NIH 3T3 cells for two passages, which minimized any carryover of salivary gland tissue. Infected 3T3 cultures were harvested at 80–100% cytopathic effect and subjected to three freeze/thaw cycles. Cellular debris was removed by centrifugation (1000 g, 20 min) and the virus was pelleted in RPMI 1640 (2 g/L d-glucose and 10 mM HEPES; Sigma-Aldrich) at 4˚C, and the virus was pelleted at 10,000 g at 4˚C. The virus was resuspended in RPMI 1640 (Sigma-Aldrich) and stored at 4˚C. T cells due to B cell deficiency (35). IL-10–GFP knockin mice (B6.129S6-Il10tm1Flv/J) (28) were kindly provided by Dr. Sing Sing Way (Cincinnati Children’s Hospital, Cincinnati, OH). Poststimulation, cells were subjected to FACS using FACS-Aria (BD Biosciences). GFP expression indicated that the cells were IL-10 competent.

B cell isolation and stimulation

We employed two different protocols to generate B10 cells in vitro. B10 cells generated with the first protocol were used in microglia and Breg coculture studies. In these experiments, B10 cells were prepared following published protocols with minor modifications. Briefly, CD19+ lymphocytes from IL-10–GFP knockin mice were enriched by negative selection using a CD19+ cell purification kit, as per the manufacturer’s instructions (R&D Systems, Minneapolis, MN). For preparation of B10 cells, 1 × 106 purified B cells were cultured with agonistic CD40 mAb (1 µg/ml; Miltenyi Biotec, Auburn CA) and IL-4 (10 ng/ml; eBioscience), with agonistic CD40 mAb (1 µg/ml; Miltenyi Biotec, Auburn CA) and BAFF (20 ng/ml; BioXcell, West Lebanon, NH), IL-2 (10 ng/ml), and TGF–β1 (5 ng/ml; eBioscience) for 4 d. Approximately 45% of the B10 cells thus produced were found to be positive for intracellular IL-10, as measured by flow cytometry. Positive control cells, designated induced Tregs (iTregs) in Fig. 6, were generated using previously published protocols with minor modifications (42). Briefly, CD4+ T cells were isolated from spleens of Foxp3GFP reporter mice by negative selection, according to the manufacturer’s instructions (R&D Systems). Cells were cultured at a density of 2 × 106 cells/ml in 24-well plates for 5 d in RPMI 1640 medium supplemented with plate-bound anti-CD28 (1 µg/ml), anti-CD3 (10 µg/ml) (BioXcell, West Lebanon, NH), IL-2 (10 ng/ml), and TGF–β1 (5 ng/ml; eBioscience) to induce differentiation of Foxp3+CD4+ regulatory T cells (Tregs).

Marine microglial cell cultures

Microglial cells were isolated from cerebral cortices of 1-d-old mice as previously described (43, 44). Briefly, after a 30-min trypsinization (0.25%), dispersed cortical cells were plated in Falcon 75-cm2 culture flasks in DMEM (Sigma-Aldrich) containing 10% heat-inactivated FBS (Hyclone, Logan UT), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Sigma-Aldrich). Medium was changed at days 1 and 4 after plating. On day 8 of culture, media were changed to FCS (15%) in RPMI 1640 medium to remove the FBS. On day 14 of culture, floating microglia were harvested by gentle shaking, seeded into 24- or 48-well culture plates (1 × 106 cells/well), and washed after 60 min. Adherent microglial cells were ≥95% pure, as determined by MAC-1 (microglial cell marker; Roche, Indianapolis, IN) and glial fibrillary acidic protein (GFAP marker for astrocytes; Sigma-Aldrich) Ab staining (45, 46). All in vitro experiments used primary cell types that were genetically matched.

Adoptive transfer

Spleens from MCMV-primed (1 × 107 TCID50/mouse, i.p. injection) donor animals were collected aseptically at 7 d postpriming. Single-cell suspensions of immunocytes were depleted of RBC by treatment with 0.87% ammonium chloride, washed twice, and cell viability was confirmed using trypan blue. CD19+ lymphocytes were enriched by negative selection using...
a CD19+ cell purification kit, as per the manufacturer’s instructions (R&D Systems). Immune cells were transferred (5 × 10⁶ cells/mouse) via tail vein 1 d prior to infection with MCMV into syngenic (Jh⁻/⁻) recipients.

Results
Bregs persist within chronically infected brains
MCMV brain infection models have provided tremendous insights into viral neuropathogenesis and the role of immune responses in controlling infection (32, 47). In previous studies using our MCMV brain infection model, we have identified brain-infiltrating B lymphocyte lineage cells, identified as CD19⁺ B cells and CD138⁺ plasma cells (31). In this study, we also detected a B cell subset expressing the CD19⁺CD1d⁺⁺CD5⁺ phenotype, which possesses negative regulatory functions within the brains of MCMV-infected mice. We used a multicolor flow cytometry approach to phenotype lymphocyte subpopulations infiltrating the brain at various time points postinfection (p.i.). Brain-infiltrating mononuclear cells were isolated on a Percoll gradient as described in the Materials and Methods. These isolated cells were subsequently immunostained for expression of the markers CD45, CD11b, CD3, CD19, CD1d, and CD5. Bregs expressing the CD19⁺CD1d⁺⁺CD5⁺ cell-surface markers were identified among the CD45⁺ population, which represents cells of lymphoid origin. At 7 d p.i., 2.25% of the cells among the CD19⁺ population were reactive to CD1d⁺⁺CD5⁺, and this subset was found to increase to 5.19 and 18.06% at 14 and 30 d p.i., respectively (Fig. 1A). The absolute number of CD19⁺CD1d⁺⁺CD5⁺ cells was also determined. Presence of Bregs was apparent within the infected brain starting at 7 d p.i. (3.3 ± 1.0 × 10⁶), and they were present at 14 d p.i. (1.5 ± 0.4 × 10⁶) and persisted at least until 30 d p.i. (1.7 ± 0.5 × 10⁶). There was a significant increase in the accumulation of Bregs at both 14 and 30 d p.i. when compared with 7 d p.i. (1.5 ± 0.4 × 10⁶ versus 3.3 ± 1.0 × 10⁶, respectively; p < 0.05, Student t test) (Fig. 1B). We determined the percentage of CD19⁺CD1d⁺⁺CD5⁺-expressing cells at the indicated times p.i. from three independent experiments. Pooled data presented show that at 30 d p.i., there was a significant increase in the percentage of these cells infiltrating the brain when compared with 7 d p.i. (16.94 ± 1.2 versus 5.9 ± 4.8%; p < 0.05, Student t test) (Fig. 1C). To investigate the infiltration kinetics of Bregs (CD1d⁺⁺CD5⁺) relative to total B cells, we determined the absolute number of CD19⁺⁺ cells among the total infiltrating CD45⁺ population (i.e., including CD3⁺ cells). The presence of CD19⁺⁺ cells was observed as early as 7 d p.i. and there was a significant increase in their numbers at 14 and 30 d p.i. when compared to 7 d p.i. (1.6 ± 0.16 × 10⁶ and 2.5 ± 1.1 × 10⁶ versus 6.0 ± 1.6 × 10⁵, respectively, p < 0.05 Student t test) (Fig. 1D). The absolute number of Bregs (CD1d⁺⁺CD5⁺) within the total infiltrating CD45⁺ population was then determined. We observed that there was a significant increase in the presence of Breg cells at 30 d p.i. when compared to 7 d p.i. (5.3 ± 2.7 × 10⁵ versus 5.1 ± 2.7 × 10⁵, respectively, p < 0.05 Student t test) (Fig. 1E).

B cell deficiency alters long-term T cell persistence within the infected brain
Previous studies from our laboratory have shown that CD8⁺ T cells are essential in resolving MCMV brain infection, through a perforin-mediated mechanism (32). In subsequent studies, we showed that memory CD8⁺ T cells persisting in the brain drive chronic microglial activation through an IFN-γ-dependent manner, despite the absence of active viral replication, suggesting that long-term persisting CD8⁺ T cells may contribute to immunopathology in the CNS (30). In the current study, we found a correlation between the absence of B cells and an increased presence of CD8⁺ T cells along with decreased CD4⁺ T cell numbers within infected brains. MCMV-infected Wt as well as B cell–deficient (Jh⁻/⁻)
mice were analyzed for a number of neuroimmune responses that included phenotyping of the brain-infiltrating cell populations. The representative contour plots shown in Fig. 2 were obtained from isolated brain-infiltrating cells, gated on CD45hi, and subsequently analyzed for the presence of CD4+ and CD8+ T cells (Fig. 2A). A significant increase in the absolute number of infiltrating CD8+ T cells was observed in the Jh−/− mice (1.6 ± 0.2 × 10^6 versus 1.1 ± 0.11 × 10^6; p < 0.05), when compared with Wt animals (Fig. 2B). On the contrary, a significant decrease in the absolute numbers of infiltrating CD4+ T cells was observed in these animals (3.6 ± 0.1 × 10^6 versus 1.1 ± 0.3 × 10^6; p < 0.05) (Fig. 2B). Analysis of pooled data from three independent experiments showed a significantly elevated percentage of CD8+ T cell subsets in the brains of Jh−/− mice when compared with Wt controls (80.9 ± 1.2 versus 69.1 ± 0.9%, respectively; p < 0.05, Student t test) (Fig. 2C). Additionally, the CD4+ T cell percentage was also found to be significantly lower in Jh−/− mice when compared with Wt mice (13.9 ± 0.4 versus 20.4 ± 0.9%; p < 0.05, Student t test) (Fig. 2C). We also performed flow cytometry assays on single-cell suspensions prepared from draining lymph nodes (dLN) of MCMV-infected Wt and Jh−/− animals at 30 d p.i. Interestingly, lower numbers of CD4+ T cells and higher numbers of CD8+ T cells were observed within the CNS during B cell deficiency. Altered T cell presence in the brain was observed despite elevated levels of these populations within the draining lymph nodes of Jh−/− mice. The flow cytometric analysis shown in Fig. 2D indicated that there were elevated levels of both CD4+ and CD8+ T cells in the dLN of Jh−/− mice at 30 d p.i. We determined absolute numbers and percentages of both CD4+ and CD8+ T cells in the periphery (dLN) of Wt and Jh−/− animals and observed that there was a significant increase in the total number and percentage of CD4+ T cells Jh−/− mice when compared with Wt (3.5 ± 0.1 × 10^6 versus 2.8 ± 0.3 × 10^6 and 68.6 ± 3.5 versus 47.3 ± 6.5%, respectively; p < 0.05). On the contrary, we observed there was no significant increase in the total number of CD8+ T cells in dLN of Jh−/− animals when compared with Wt mice at 30 d p.i.; however, statistical analysis of percentage data showed a significant increase in CD8+ T cells in the Jh−/− animals in the dLN at 30 d p.i., when compared Wt mice (18.0 ± 0.5 versus 24.6 ± 2.3%; p < 0.05) (Fig. 2E, 2F).

**Frequencies of CD4+Foxp3+ Tregs were reduced in the brains of Jh−/− mice**

Tregs are critical for normal functioning immune responses by providing a mechanism to maintain self-tolerance, while still allowing for protective responses against microbes. Several studies have investigated the role of Tregs, defined as CD4+Foxp3+, in regulation of immune responses to infectious diseases (48–50). Because we observed a significant decrease in the presence of CD4+ T cells within the brains of Jh−/− mice at 30 d p.i., despite higher levels in the dLN, we next examined whether other CD4+ T cell subsets, particularly anti-inflammatory cells such as Tregs, were altered. In these experiments, we assessed the presence of Tregs in the brains of chronically infected Wt and Jh−/− mice. At 30 d p.i., we collected brain tissues and processed them for intranuclear staining for Foxp3. Representative contour plots from Wt and Jh−/− mice for CD4+Foxp3+ T cells, from brain and dLN, that were gated from the CD45hi cell population are shown (Fig. 3A). Analysis of pooled data showed a significant decrease in the absolute numbers of CD4+Foxp3+ T cell recruitment within the infected brains in Jh−/− mice (5.1 ± 1.1 × 10^6 versus 3.0 ± 0.6 × 10^6; p < 0.05) (Fig. 3B). Pooled data from
also determined from the dLN of Wt and Jh-/- mice collected at 30 d p.i. Brain leukocytes were collected and labeled with PE-Cy5–conjugated Abs specific for CD45, AF700-labeled anti-CD86, eFluor 450–CD4, and FITC–Foxp3 and analyzed using flow cytometry. (A) Representative contour plots show the percentages of CD4+Foxp3+ Tregs within the infiltrating CD45hi population in the infected brains at 30 d p.i. Also, representative contour plots are shown from dLN of Wt and Jh-/- mice collected at 30 d p.i. (B) FITC-labeled anti-Foxp3 Abs were used to determine the total number of infiltrating Tregs within the infiltrating CD45hi population (n = 5). Data shown are mean (± SEM) absolute number of infiltrating cells pooled from three independent experiments. (C) The percentage of CD4+Foxp3+ Tregs within CD45hi population from three independent experiments is also shown (n = 5). (D) The absolute number of CD4+Foxp3+ T cells was also determined from the dLN of Wt and Jh-/- animals and presented as pooled data from three independent experiments (n = 5). (E) The percentage of CD4+Foxp3+ T cell subsets in dLN from three independent experiments are shown (n = 5). *p < 0.05 versus infected Wt.

FIGURE 3. Absence of B cells leads to decreased numbers of Tregs within the brain. Wt and Jh-/- mice were infected with MCMV, and brain tissue samples were obtained at 30 d p.i. Brain leukocytes were collected and labeled with PE-Cy5–conjugated Abs specific for CD45, AF700-labeled anti-CD11b, eFluor 450–CD4, and FITC–Foxp3 and analyzed using flow cytometry. (A) Representative contour plots show the percentages of CD4+Foxp3+ Tregs within the infiltrating CD45hi population in the infected brains at 30 d p.i. Also, representative contour plots are shown from dLN of Wt and Jh-/- mice collected at 30 d p.i. (B) FITC-labeled anti-Foxp3 Abs were used to determine the total number of Tregs within the infiltrating CD45hi population (n = 5). Data shown are mean (± SEM) absolute number of infiltrating cells pooled from three independent experiments. (C) The percentage of CD4+Foxp3+ Tregs within CD45hi population from three independent experiments is also shown (n = 5). (D) The absolute number of CD4+Foxp3+ T cells was also determined from the dLN of Wt and Jh-/- animals and presented as pooled data from three independent experiments (n = 5). (E) The percentage of CD4+Foxp3+ T cell subsets in dLN from three independent experiments are shown (n = 5). *p < 0.05 versus infected Wt.

three independent experiments also showed a significant decrease in the percentage of CD4+Foxp3+ T cells in Jh-/- mice when compared with MCMV-infected Wt animals (3.5 ± 0.5 versus 8.77 ± 0.5%; p < 0.05, Student t test) (Fig. 3C). We also determined the percentage and absolute numbers for Tregs in the dLN from Wt and Jh-/- animals at 30 d p.i., and the data presented show that there were no significant differences in the presence of Tregs in the dLN of Jh-/- mice when compared with Wt animals (Fig. 3D, 3E).

Increased CD8+ T cell presence and reduced Tregs promote enhanced chronic microglial activation with B cell deficiency

To compare chronic activation of brain-resident microglia between MCMV-infected Wt versus Jh-/- animals, these cells were assessed by first gating on the CD45hiCD11b+ population, isolated at 30 d p.i., followed by evaluation of MHC class II (MHC II), CD40, and CD86 expression using flow cytometry. Representative histogram overlays demonstrate a shift in the expression levels of MHC II on microglia from Jh-/- mice (dotted line) when compared with Wt animals (solid line) (Fig. 4A). This shift indicated an increased level of microglial activation within the brains of B cell–deficient animals. In addition, the mean fluorescence intensity (MFI) of MHC II expression was also measured between groups and was found to be significantly higher among microglia isolated from Jh-/- mice (1.0 ± 0.1 × 10^6 versus 1.9 ± 0.1 × 10^5; p < 0.01, Student t test) (Fig. 4B). CD40 and CD86 expression on microglial cells was also assessed, and the flow cytometry histogram data showed increased expression of these molecules on microglia derived from Jh-/- animals at 30 d p.i. (Fig 4 C). Statistical analysis of percent expression of CD40 and CD86 molecules showed a significant increase in their expression on cells isolated from Jh-/- mice when compared with Wt animals (10.2 ± 0.9 versus 5.8 ± 0.3% and 26.2 ± 1.0 versus 17.6 ± 1.0%, respectively; p < 0.05, Student t test) (Fig 4D). In addition, mean fluorescence intensity for CD40 and CD86 expression on microglial cells was measured and was found to be significantly higher among those isolated from Jh-/- mice (170 ± 30 versus 380 ± 19 for CD40 and 330 ± 23 versus 540 ± 13 for CD86, p < 0.05 Student t test) (Fig 4E).

In vitro–generated B10 cells modulate microglial cell responses through IL-10 production

Previous studies have shown that the anti-inflammatory cytokine IL-10 inhibits secretion of proinflammatory mediators from macrophages (51). In this study, we examined the effect of IL-10, produced by B10 cells, on microglial cells stimulated with MCMV. To evaluate the effects of Bregs on cytokine production by microglia, we focused on the subset of IL-10–producing Bregs, B10 cells, for which we can generate enriched populations in vitro. TNF-α levels were quantified from supernatants of purified microglial cell cultures following stimulation with MCMV in either the presence or absence of B10 cells. Fig. 5A shows the protocol used for in vitro generation of B10 cells from naive B cells of MHC-matched IL-10–GFP knockin donor mice. Representative contour plots of B cells that were treated with the above protocol and analyzed for GFP+ cells, indicative of IL-10 production, are shown in Fig. 5B. The GFP+ cells were then enriched by FACS, and these enriched cells were used in subsequent coculture experiments. In the first set of experiments, we detected IL-10 production in supernatants from the B10/microglial cell cocultures. Data demonstrate that IL-10 was produced in the coculture system that included CD19-GFP+ cells, but it was not
detected when CD19\(^+\)GFP\(^-\) cells were used to reconstitute the cocultures (Fig. 5C). Further studies using this coculture system revealed that B10 cells had a suppressive effect on microglial cells that were stimulated with MCMV for 24 h. Microglia that were cocultured with CD19\(^+\)GFP\(^+\) B10 cells produced significantly lower levels of TNF-\(\alpha\) in response to stimulation with MCMV when compared with microglial cells cocultured with CD19\(^+\) GFP\(^-\) cells (Fig. 5D). We went on to demonstrate that addition of anti–IL-10 neutralizing Ab to the coculture system resulted in an abrogation of the suppressive effect of B10 cells on microglia, demonstrating an inhibitory role for B10-produced IL-10 (Fig. 5D).

**B10 cells promote proliferation of CD4\(^+\)Foxp3\(^+\) Tregs**

Our data show that the absence of B cells resulted in reduced numbers of Tregs persisting within the brain following viral infection. To address whether B10 cells promote conversion of CD4\(^+\) T cells into Tregs or induce their proliferation, we used an in vitro–generated B10 and CD4\(^+\) T cell coculture system. B10 cells were generated following the protocol noted in the Materials and Methods section. To confirm the successful generation of B10 cells using the specified stimulation protocol, we first examined the cells for their ability to produce IL-10 by intracellular staining and flow cytometry. In these experiments, IL-10 production was detected in \(\sim 8\%\) of the B cells. Correspondingly, these in vitro–generated B10 cells were subsequently phenotyped for typical B cell markers such as IgM\(^+\) (95%), IgD\(^+\) (51%), IgG1\(^+\) (0.7%), B220\(^+\) (87%), CD21\(^+\) (88%), CD93\(^+\) (23%), CD23\(^+\) (82%), and CD19\(^+\) (84%) prior to their use in coculture studies. Representative histogram plots show IL-10–producing CD19\(^+\) cells as well as the expression of various surface markers following treatment (Fig. 6A, 6B, respectively). These B10 cells were subsequently cocultured with CD4\(^+\) T cells from Foxp3\(^{EGFP}\) mice for 72 h, followed by analysis of Foxp3\(^{EGFP}\) expression using flow cytometry. Representative contour plots shown are from: CD4\(^+\) T cells from non-GFP animals for gating purposes, CD4\(^+\) T cells alone, CD19\(^+\)/CD4\(^+\) T cell cocultures, B10/CD4\(^+\) T cell cocultures, and iTregs as a positive control (using a standard protocol to generate iTregs from naive CD4\(^+\) T cells) (Fig. 6C). The ability of B cells to induce Treg proliferation, both in vitro and in vivo, has been previously reported (52). To differentiate between conversion and proliferation of Tregs, we performed intranuclear staining for Ki67 expression in CD4\(^+\) T cells cocultured with B10 cells. These experiments showed that \(\sim 8\%\) of the Foxp3\(^+\) cells also expressed Ki67, suggesting that the Tregs proliferated in presence of B10 cells. Representative contour plots, gated on CD4\(^+\) cells, are shown from CD4\(^+\) only, B10/CD4\(^+\), and iTregs (Fig. 6D). The amount of CD4\(^+\)Foxp3\(^+\) T cells present was found to be significantly higher in B10/CD4\(^+\) T cell cocultures when compared with the other treatment groups (11 ± 1.3 [CD4\(^+\) only] and 9 ± 1.3 [CD19\(^+\)/CD4\(^+\)] versus 27 ± 0.9% [B10/CD4\(^+\)]; \(p < 0.01\), Student \(t\) test). Data shown in the bar graph are from three independent coculture experiments (Fig. 6E).

**Transfer of B cells into MCMV-infected Jh\(^{-/-}\) mice restores T cell levels within chronically infected brains**

To evaluate the contributions of B cells in regulating neuroimmune responses during the chronic phase of disease, we went on to

**FIGURE 4.** B cell–deficient mice display increased levels of chronic microglial cell activation. Brain mononuclear cells isolated from animals at 30 d p.i. were stained using anti–CD45, anti–CD11b, anti–MHC-II, anti–CD40, and anti–CD86 surface marker Abs. (A) Upregulation of MHC II on the CD45\(^{int}\) CD11b\(^+\) resident microglial cells from Wt and Jh\(^{-/-}\) mice in response to viral brain infection was compared. An overlay of histograms from isotype (gray shaded), Wt (red solid line) mice, and Jh\(^{-/-}\) (blue dotted line) animals is shown. (B) Data presented show MFI of MHC II binding to microglia from Wt versus Jh\(^{-/-}\) mice (\(n = 5\)). (C) Modest upregulation of CD40 and CD86 on CD45\(^{int}\)CD11b\(^+\) resident microglial cells from Wt and Jh\(^{-/-}\) mice in response to viral brain infection was seen at 30 d p.i., although expression of these markers wanes at these later time points. Representative histogram overlays of microglial cells isolated from Wt (red, solid line) and Jh\(^{-/-}\) (blue, dotted line) animals stained for CD40 and CD86 expression, as well as isotype controls (grey, shaded) are shown. Isotype control staining is shown for Wt microglia, which was indistinguishable from Jh\(^{-/-}\) cells (data not shown). (D) Data presented in the bar graph show percentage of CD40 and CD86 expression on microglia from Wt versus Jh\(^{-/-}\) mice (\(n = 5\)). (E) Mean fluorescent intensity (MFI) of CD40 and CD86 expression on microglia obtained from Wt versus Jh\(^{-/-}\) mice. *\(p < 0.01\) versus infected Wt for (B), *\(p < 0.05\) versus infected Wt for (D) and (E).
FIGURE 5. Induced B10 cells modulate microglial cell activation. (A) Splenic CD19+ B cells from IL-10–GFP knockin mice were cultured with agonistic CD40 mAb (1 μg/ml; eBioscience) for 48 h. For the last 5 h, cells were treated with LPS (10 μg/ml; Sigma-Aldrich), PMA (50 ng/ml; eBioscience), ionomycin (100 ng/ml; eBioscience), and monensin (PIM; eBioscience) for 5 h to induce IL-10–producing B10 cells. (B) Representative flow cytometry contour plots showing IL-10 induction in CD19+ cells that were stimulated with the above protocol. (C) The induced B10 cells were then added to purified cultures of primary murine microglia (1:1 ratio), and IL-10 production in these cocultures was assessed using ELISA. (D) CD19+IL-10–GFP+ B10, as well as CD19+IL-10–GFP− cells, were enriched using FACS and then added to primary microglial cell cultures. Cocultures were incubated for 5 h, followed by stimulation with MCMV (multiplicity of infection of 5) and assessment of TNF-α production (at 24 h) using ELISA. *p < 0.05, CD19+GFP+ versus microglia alone. 

Discussion

Neuroimmune responses elicited during CNS infections are critical in controlling viral replication and promoting successful resolution; however, certain aspects of both innate and adaptive responses, if not appropriately regulated, may induce neuropathology. It is clear that excessive production of inflammatory mediators induced during various stages is a primary mechanism responsible for pathogenesis during many viral infections. Therefore, adequate function of the immune regulation arm, mediated by immunosuppressive cytokines and regulatory immune cells, plays a critical role in controlling excess inflammatory responses and their associated damage (53).

In addition to Ab production, specific subsets of B cells are also known to regulate T cell– and macrophage-driven immune responses and over the last decade the concept of anti-inflammatory Bregs has emerged (18). In the current study, our first experiments analyzed the presence of these Bregs during chronic viral brain infection with MCMV. Results obtained showed that there was a sustained presence of Bregs within the brains of mice following viral infection. While assessing function, we found that B cell–deficient animals developed exacerbated neuroimmune responses, as measured by enhanced accumulation of CD8+ T cells and increased levels of microglial activation. Additional experiments showed that the absence of B cells led to a significant reduction in accumulation of Tregs within the brains of infected Jh−/− mice when compared with Wt animals. Furthermore, in vitro–generated B10 cells were able to inhibit cytokine responses from MCMV-stimulated microglial cells. We went on to show that these in vitro–generated B10 cells also promoted proliferation of Foxp3-expressing Tregs. In the final experiments, adoptive transfer of B cells into MCMV-infected Jh−/− mice was
found to restore T cell levels to those of Wt animals within chronically infected brains.

A number of cell-surface molecules have been described as markers for Bregs, although complete elucidation of these regulatory cells remains to be achieved. It has been proposed that B cells with immunoregulatory function may represent a transient stage of activation and maturation (54). In this study, we phenotyped Bregs based on the expression of specific cell-surface markers that constituted reactivity to CD19+CD1dhiCD5+. B cells, Bregs, and B10 cells are known to regulate adaptive immune responses through multiple mechanisms that include: secretion of IL-10 (55) and promoting proliferation of CD4+Foxp3+ Tregs (52, 56), as well as through inhibitory B7 family molecules that promote Treg and IL-10 production (56). Previous studies have shown that IL-10–competent B cells (B10 cells) are predominantly found within this Breg subset, particularly in the spleen (41).

It has been shown that responses of Bregs can be induced by stimulation with TLR4 and TLR9 ligands, as well as signaling through the BCR (15, 57). Krug et al. (58) reported that MCMV engages with TLR9 to induce dendritic and IFN-producing cell activation and enhance immune responses to infection. It is also possible that the Breg response observed in this study results from direct stimulation of B cells by MCMV or its Ags. Although a number of studies involving various infectious agents have demonstrated the expansion of Bregs in peripheral immune appendages, to our knowledge, this is the first demonstration of the chronic presence of Bregs within the brain following MCMV infection.

We previously demonstrated that CD8+ T cells control replication and persist within the brain even after active viral replication ceases (32, 59). In addition, our studies and others have shown that IFN-γ produced from infiltrating T cells drives microglial activation (30, 60). Persistence of CD8+ T cells is particularly important because they constitute the major brain-infiltrating immune cell type during the chronic phase of infection; they are retained at a ratio of 3:1 to CD4+ T cells. In the current study, we observed that there were significantly higher numbers of CD8+ T cells present in the chronically infected brain (i.e., 30 d p.i.) in the absence of B cells. Conversely, we observed a lower frequency of CD4+ and CD4+Foxp3+ T cells than in Wt animals. Similar findings have been reported using an experimental autoimmune encephalomyelitis model in which the authors noted that depletion of B cells during disease development results in significant reduction in total CD4+ T cell, myelin oligodendrocyte glycoprotein–specific effector T, and Treg numbers within the CNS. However, these authors also state that before disease

**FIGURE 6.** Induced B10 cells promote proliferation of Foxp3+ Tregs. Regulatory B10 cells were prepared from CD19+ cells as described, and CD4+ T cells were isolated from MHC-matched donors, Foxp3EGFP+, using a negative selection kit. Induced B10 cells were added to the CD4+ T cell cultures at a 1:1 ratio. The cocultures were then incubated for 72 h, and Foxp3EGFP+ expression within the CD4+ T cell population was assessed by flow cytometry for GFP expression to assess Treg phenotype. (A) Flow cytometry histogram overlays show staining for isotype (gray shaded) and intracellular IL-10 (solid line) levels in the induced B10 cells. (B) B10 cells generated through this protocol were subjected to phenotyping to determine expression of various B cell surface markers. Representative histogram overlays, gated from the CD19(IL-10) population, which include isotype (gray shaded) and specific markers (solid line), are shown. (C) Representative flow cytometry contour plots of Foxp3EGFP+ within the CD4+ T cell population are shown: non-GFP, CD4+ T cells only (CD4), total B cells with CD4+ T cells (CD19+/CD4+), and induced B10 cells with CD4+ T cells (B10/CD4+), along with a standard protocol used to generate in vitro Tregs as a positive control (iTregs). (D) Representative flow cytometry analysis of Foxp3*Ki67* expression within CD4+ T cells cocultured with CD4 only, CD4/B10, and positive control iTreg is shown. (E) The percentages of CD4*Foxp3* in the various treatment groups from three independent experiments are shown. *p < 0.01 versus CD4+ T cells alone and total B cells + CD4+ T cells.
induction, there was significant increase in the accumulation of all
the above three cell types, suggesting that depletion of B cells at
specific stages of the disease may have had different outcomes on
encephalitogenic T cells (16).

Bregs have been shown to control macrophage-driven proin-
flammatory responses. Previous studies have revealed that B cell
depletion causes enhanced macrophage activation and cytokine
production during *Listeria* infection (61). Similarly, in this study,
we reported increased activation of brain resident microglia as
assessed by MHC II expression perhaps in the context of reduced
Treg presence and in turn insufficient control of CD8+ T cells
influx. This increased activation of microglia could be due to
enhanced production of IFN-\(\gamma\). IFN-\(\gamma\) has been shown to drive
microglial activation (60). There was also a small increase in the
expression levels of CD40 and CD86 molecules on the microglial
cells at 30 d p.i., which confirmed increased activation of these
cells in B cell–deficient animals. Our in vitro studies provide
a mechanistic explanation for the inhibition of microglial cell
activation through B10 cell–produced IL-10. The data presented in
this study are in agreement with findings that have demonstrated
that B10 cells downregulate macrophage activation following LPS
stimulation (61).

Bregs have also been shown to promote conversion of CD4+ T cells into a Treg phenotype. A recent study, using a collagen-
induced arthritis model, demonstrated that B10 cells promoted
Treg conversion when cocultured with naive CD4+ T cells (62).
T cell plasticity has been well recognized, and many factors are
known to influence plasticity including: cellular conditions, tran-
scriptional circuitry, and chrom atin modifications (63). Our in
vitro studies using B10/CD4+ T cell cocultures found that these
cells increased Treg proliferation that is demonstrated by Ki67
expression by Foxp3+ cells. Using both in vivo and in vitro
approaches, a recent elegant study by Ray et al. (52) has shown that
Treg proliferation in the presence of B cells is attributed to the

**FIGURE 7.** B cell replenishment restores T cell levels within chronically infected brains. MCMV-primed splenocytes and lymph node cells from donor
BALB/c mice were enriched for CD19+ cells using negative selection. These B cells were then adoptively transferred via tail vein injection into MHC-
matched Jh\(^{-/-}\) recipients 1 d prior to the infection with MCMV. Wt and Jh\(^{-/-}\) mice served as appropriate controls, and brain tissue samples were obtained
from each group at 30 d p.i. Brain leukocytes were collected and labeled with PE-Cy5–conjugated Abs specific for PE-Cy5–labeled anti-CD45, AF700-
labeled anti-CD11b, eFluor 450–labeled anti-CD4, PE-Cy7–labeled anti-CD8, FITC-labeled anti-Foxp3, and allophycocyanin-Cy7–labeled anti–MHC II
and analyzed using flow cytometry. (A) Representative contour plots showing the percentages of CD4+, CD8+ (top panel), and CD4+Foxp3+ T cells (bottom
panel) in the infiltrating CD45\(^{hi}\) population within the infected brains from each group. (B) The absolute numbers of CD4+, CD8+ and CD4+Foxp3+ T cells
were also determined among the brain-infiltrating CD45\(^{hi}\) cells from Wt, Jh\(^{-/-}\), and Jh\(^{-/-}\) with B cell AT animals. Pooled data obtained from three in-
dependent experiments are presented (\(n = 5\) / group). *p < 0.05 Jh\(^{-/-}\) + AT versus Jh\(^{-/-}\). (C) Histogram overlays from isotype (gray shaded), Wt (red solid),
Jh\(^{-/-}\) (blue dashed), and Jh\(^{-/-}\) mice that received CD19+ cells (green dotted) are shown for MHC II upregulation on CD45\(^{hi}\)CD11b\(^{+}\) resident microglia.
(D) Data presented show MFI of MHC II binding from Wt, Jh\(^{-/-}\), and Jh\(^{-/-}\) + AT mice (\(n = 5\) / group). *p < 0.01, Jh\(^{-/-}\) + AT versus Jh\(^{-/-}\).
interaction of glucocorticoid-induced TNFR family-related protein with its ligand, and the study also demonstrated that Treg proliferation was IL-10 independent. Although Tregs are not the main focus of this study, their role in controlling neuroinflammation is well established. Likewise, the presence of B10 cells during chronic phase of the disease could be beneficial, as they are capable of driving proliferation of Tregs and in turn may aid in controlling excessive neuroinflammation.

In animal models of allergic disease, Anu et al. (64) suggested that Bregs may have a more systemic effect on Treg expansion. Other studies demonstrated that IL-10–producing B10 cells function to downregulate autoimmunity and allergic disorders by inducing Tregs (15, 64, 65). These findings can be extended to our chronic viral brain infection model. In particular, adoptive transfer of B cells in this animal model resulted in enhanced amounts of Tregs, to a level exhibited by B cell–deficient animals. Thus, our data demonstrate that transfer of B cells inhibits the exacerbated neuroimmune responses that are observed in B cell–deficient mice.

Results generated from these studies demonstrate that the absence of B cells leads to exacerbated microglial cell– and T cell–mediated neuroinflammation following viral infection, suggesting the importance of these cells in controlling chronic and persistent immune activation in the brain. This knowledge may be useful in the development of therapeutic applications to modulate chronic neuroimmune activation and its associated neuropathology.

Disclosures
The authors have no financial conflicts of interest.

References


