Mycobacterium bovis Bacille Calmette-Guérin Infection in the CNS Suppresses Experimental Autoimmune Encephalomyelitis and Th17 Responses in an IFN-γ-Independent Manner

JangEun Lee, Emily K. Reinke, Alla L. Zozulya, Matyas Sandor and Zsuzsanna Fabry

*J Immunol* 2008; 181:6201-6212;
doi: 10.4049/jimmunol.181.9.6201
http://www.jimmunol.org/content/181/9/6201

**References** This article cites 62 articles, 33 of which you can access for free at: http://www.jimmunol.org/content/181/9/6201.full#ref-list-1

**Subscription** Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
**Mycobacterium bovis** Bacille Calmette-Guérin Infection in the CNS Suppresses Experimental Autoimmune Encephalomyelitis and Th17 Responses in an IFN-γ-Independent Manner

JangEun Lee,*† Emily K. Reinke,2* Alla L. Zozulya,3* Matyas Sandor,*† and Zsuzsanna Fabry4*†

Multiple sclerosis and an animal model resembling multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), are inflammatory demyelinating diseases of the CNS that are suppressed by systemic mycobacterial infection in mice and BCG vaccination in humans. Host defense responses against *Mycobacterium* in mice are influenced by T lymphocytes and their cytokine products, particularly IFN-γ, which plays a protective regulatory role in EAE. To analyze the counter-regulatory role of mycobacterial infection-induced IFN-γ in the CNS on the function of the pathological Th17 cells and the clinical outcome of EAE, we induced EAE in mice that were intracerebrally infected with *Mycobacterium bovis* bacille Calmette-Guerin (BCG). In this study, we demonstrate that intracerebral (i.c.) BCG infection prevented inflammatory cell recruitment to the spinal cord and suppressed the development of EAE. Comitantly, there was a significant decrease in the frequency of myelin oligodendrocyte glycoprotein-specific IFN-γ-producing CD4+ T cells in the CNS. IL-17+ CD4+ T cell responses were significantly suppressed in i.c. BCG-infected mice following EAE induction regardless of T cell specificity. The frequency of Foxp3+ CD4+ T cells in these mice was equivalent to that of control mice. Intracerebral BCG infection-induced protection of EAE and suppression of myelin oligodendrocyte glycoprotein-specific IL-17+ CD4+ T cell responses were similar in both wild-type and IFN-γ-deficient mice. These data show that live BCG infection in the brain suppresses CNS autoimmunity. These findings also reveal that the regulation of Th17-mediated autoimmunity in the CNS can be independent of IFN-γ-mediated mechanisms.


S
ystemic infection with *Mycobacterium bovis* bacille Calmette-Guerin (BCG)† or immunization with either heat-killed *Mycobacterium tuberculosis* (Mtb) or its purified protein derivative can suppress autoimmune responses in various autoimmune disease models, such as diabetes, arthritis, and experimental autoimmune encephalomyelitis (EAE), an animal model of human multiple sclerosis (MS) (1–7). A clinical trial using BCG vaccine as an adjuvant therapy for MS patients also showed a reduction in magnetic resonance imaging activity (8, 9). Multiple mechanisms have been put forth to explain the immunoregulatory effects of *Mycobacterium* in autoimmune diseases. For example, it was shown that mycobacteria-induced suppression of diabetes in NOD mice is mediated by T lymphocytes and MAC-1+ cells via Fas-ligand- and TNFR55-dependent apoptosis (10, 11). It was also demonstrated in EAE model that i.p. infection with BCG induces apoptosis, which deletes bystander CD4+ T cells in the periphery in an IFN-γ-dependent manner and reduces autoreactive T cells in the CNS (3, 4).

Studies performed with heat-killed BCG injection into the brain showed that rapid neutrophil influx into the brain was followed by the accumulation of MHC-II+ mononuclear cells (12, 13). However, the different subcellular localization of heat-killed vs live mycobacteria changes phagosomal maturation and protective immunity (14–16) and limits generalization of these results with heat-killed BCG immunization to live BCG-induced immune responses in the CNS. To understand the effects of live mycobacterial infection in the brain on immune function, particularly on autoimmunity in the CNS, we studied the clinical manifestation of EAE in animals harboring intracerebral (i.c.) live BCG.

We previously proposed a novel mechanism that contributes to the BCG-induced protection in EAE (3). We demonstrated that peripheral BCG-induced granulomas divert the traffic of neuronal Ag-specific T cells from the CNS to the liver granulomas and suppress the progression of EAE. The dynamic traffic of BCG-nonspecific T cells into well-established liver granulomas has been well-demonstrated (17–19). However, neither the functional consequence of BCG-nonspecific T cell traffic into granulomas formed in the CNS nor the effect of i.c. BCG infection on the development of CNS autoimmune disease has been studied.

To address these questions, we microinjected C57BL/6 mice intracerebrally with live BCG 3 wk before induction of EAE with myelin oligodendrocyte glycoprotein (MOG)35–55. We demonstrate here that i.c. BCG infection prevents the development of EAE. IFN-γ has been shown to be the key cytokine in host defense...
against intracellular mycobacterial infection (20–22). IFN-γ has also been shown to play a protective regulatory role in EAE, as evidenced by exacerbated clinical scores in studies with IFN-γ-deficient mice (23) and IFN-γ neutralization experiments (24). Furthermore, IFN-γ has been shown to induce apoptosis of autoimmune T cells (25) and inhibit the function of IL-17-producing T cells (Th17) (26). Th17 cells have been proposed to be critical in the pathogenesis of EAE (27, 28) and shown to reciprocally develop with Foxp3⁺ CD4⁺ T cells (29–31). Given the importance of IFN-γ in mediating cellular responses in EAE and mycobacterial infection and the potential for IL-17 regulation by IFN-γ, we examined the functional alteration, spatial distribution, and the frequency of IFN-γ- and IL-17-producing CD4⁺ T cells in i.c. BCG-infected mice following EAE induction. In parallel, we analyzed the frequency and distribution of Foxp3⁺ CD4⁺ T cells as well. Despite the fact that i.c. BCG infection itself induced IFN-γ and IL-17 cytokine-producing cell accumulation in the CNS, this infection suppressed mononuclear cell infiltration into the spinal cord and MOG-specific IFN-γ⁺ and IL-17⁺ CD4⁺ T cell responses in subsequently induced EAE. Furthermore, i.c. BCG-infected mice had a lower frequency of CD4⁺ IL-17-producing cells following EAE induction regardless of T cell Ag specificity, showing that IL-17⁺ CD4⁺ T cell responses were significantly suppressed. However, the frequency and distribution of Foxp3⁺ CD4⁺ T cells in BCG-infected mice was unaltered and comparable to control mice following EAE induction. Intracerebral BCG infection was also protective against the development of EAE and suppressed MOG-specific IL-17⁺ CD4⁺ T cell responses in IFN-γ-deficient mice, suggesting that suppressed IL-17 responses and inhibits the development of EAE and that these effects do not depend on the presence of IFN-γ.

Materials and Methods

Mice and EAE induction

Female C57BL/6 and IFN-γ-deficient (B6.12957-fng/m1ts1Jfs) mice were purchased from The Jackson Laboratory and maintained at the Animal Care Facility at the University of Wisconsin, Madison, WI. EAE was induced by s.c. injections of 100 μg of MOG35-55 peptide (synthesized by the University of Wisconsin Biotechnology Center, Madison, WI) in CFA (Difco) supplemented to 5 mg/ml Mtb H37Ra. A total of 200 ng of pertussis toxin (List Biological Laboratories) was injected i.p. at the time of EAE induction and 2 days following. To obtain a clinical score, mice were scored daily according to a standard procedure as follows: 0, no clinical symptoms; 1, limp/flaccid tail; 2, hind limb weakness with incomplete paralysis; 3, complete paralysis of hind limbs; 4, paraplegia; and 5, moribund. Intermediate scores were assigned for intermediate symptoms. All experiments were conducted in accordance with guidelines of the National Institutes of Health and the University of Wisconsin Medical School Animal Care and Use Committee.

Intracerebral injection

i.c. injection was performed as previously described (32, 33). PBS alone or 1 × 10⁵ CFU of BCG (substrain Pasteur, Staten Serum Institute) in 30 μl of PBS was injected 1.5 mm deep from the surface of the skull into the ventral-posterior region of the right frontal lobe of mice through an insulin syringe (28 and½G) via a penetrating depth controller.

Mononuclear cell isolation and flow cytometry

Mononuclear cells from the CNS were isolated and stained for flow cytometry as previously described (33). Mice were anesthetized with a ketamine and xylazine mixture and perfused transcardially with cold PBS. Organs were removed, weighed, and put on ice in HBSS (Mediatech). Spleen and cervical lymph nodes (CLN) were homogenized between frosted glass slides, and the resultant cell suspension was pelleted by centrifugation. Spleen RBC were lysed in ACK lysis buffer, and the remaining cells were washed three times with HBSS and counted. To isolate mononuclear cells from the CNS, brains and spinal cords were removed from perfused animals, weighed, minced, transferred to Medicon inserts, and ground in a MediMachine (BD Biosciences) for 20–30 s. The cell suspension was washed with HBSS, and cells were resuspended in 50% Percoll (Pharmacia) and overlaid with 30% Percoll. The gradient was centrifuged at 2250g for 30 min at 4°C. The interface was removed and washed once for further analysis. Absolute numbers were calculated based on the percentage of specific T cells from the total cell population acquired as determined using flow cytometry analysis and the weight of tissues.

Intracerebral cytokine staining, single-cell suspensions from various tissues were cultured at 37°C in complete RPMI 1640 supplemented with GolgiStop (BD Biosciences) in the presence of either 20 μg/ml MOG35-55 peptide or 5 μg/ml anti-CD3 (145–2C11) Ab for 5 h. After surface staining with anti-LFA-1 (2D7) and anti-CD4 (RM4–5) Abs, cell suspensions were fixed and permeabilized by Cytofix/Cytoperm solution (BD Biosciences) followed by staining with anti-IFN-γ (XMG1.2) and anti-IL-17 (TC11–18H10) Abs. Fluorochrome-labeled Abs against CD4, LFA-1, IFN-γ, and IL-17 were purchased from BD Biosciences. Anti-CD3 Ab was produced from hybridomas. Stained cells were collected using with a dual-laser FACScalibur (BD Biosciences) and analyzed with FlowJo software (TreeStar).

For Foxp3⁺ staining, anti-Foxp3 (FJK-16s) Ab (eBioscience) was used according to the manufacturer’s protocol.

Confocal microscopy and immunohistochemistry

Frozen sections were prepared for confocal microscopy as previously described (33). Mice were perfused with 50 ml of PBS, followed by perfusion with 50 ml of 3% paraformaldehyde in PBS. In these fixations of frozen sections, spinal cord was removed and postfixed in 3% paraformaldehyde/25% sucrose solution in PBS before freezing in Tissue-Tek OCT compound (Sakura Finetek) on dry ice. Sections (5 μm) were stored at −80°C until further use.

Frozen sections were thawed for 10 min at room temperature and blocked with 1% BSA solution in PBS for 15 min. Some sections were stained with anti-CD4-allophycocyanin or with anti-CD11b-FITC conjugated Abs for 1 h at room temperature (frozen sections), followed by extensive washes with PBS. The sections were examined on a Diaphot 200 microscope (Nikon) equipped with an MRC 1000 laser scanning confocal head (Bio-Rad), and the acquired digital images were processed and analyzed using Photoshop CS software (Adobe Systems).

Tissue section preparation

Spinal cord and optic nerve were removed from experimental and control mice, fixed in 10% formalin, and embedded in paraffin. Ten micrometer-thick sections were cut and stained with H&E or luxol fast blue (LFB) to detect infiltrating cells or demyelination, respectively.

Statistical analysis

All data are presented as mean ± SEM. The two-tailed Student’s t test was used for comparison of the two groups using customized routines written in the statistical programming language R (version 2.2.0). In all cases, p < 0.05 was considered statistically significant.

Results

Intracerebral BCG infection induces the accumulation of IFN-γ- and IL-17-producing CD4⁺ T cells in the CNS

It has been demonstrated that systemic infection with BCG induces a strong Th1 response characterized by IFN-γ production in the periphery (20, 34); however, the immune response to i.c. BCG infection has not been investigated. To model the effect of i.c. BCG infection on immune responses in the CNS, we infected mice intracerebrally with 1 × 10⁵ live BCG. Live BCG infection induced the formation of inflammatory lesions and cellular infiltration in the brain and spinal cord (data not shown). To characterize i.c. BCG infection-induced CD4⁺ T cell responses within the CNS, the cytokine responses of CD4⁺ T cells were analyzed by...
flow cytometry. Twenty-one days or 35 days post infection, single-cell suspensions were prepared from the brain and spinal cord for intracellular cytokine staining. Following i.c. BCG infection, activated CD4+ T cells infiltrated into the CNS, and 21% of the infiltrating activated CD4+ T cells produced IFN-γ in the brain (Fig. 1A). IL-17+ CD4+ T cells were also detected, but there were only one fifth as many of these as there were IFN-γ+ CD4+ T cells (Fig. 1B), suggesting that i.c. BCG infection induces a Th1-dominant immune response in the CNS. PBS-injected control mice had very few CD4+ T cells in the CNS (data not shown). Absence of response to MOG35–55 peptide above that of media indicates that the BCG-induced IFN-γ- and IL-17-producing CD4+ T cells were not MOG-specific. This shows that i.c. infection of live BCG resulted in the accumulation of more IFN-γ+ and many fewer IL-

**FIGURE 1.** Frequency of IFN-γ+ CD4+ T cells and IL-17+ CD4+ T cells in the CNS at 21 or 35 days post i.c. BCG infection. C57BL/6 mice were injected intracerebrally with 1 × 10^5 CFU of BCG. At 21 or 35 days, mice were perfused and their organs were removed. Single-cell suspensions were prepared from the brain and spinal cord and were restimulated with anti-CD3 Ab in the presence of GolgiStop. The frequency of IFN-γ+ or IL-17+ producing cells was measured by intracellular cytokine staining. A, Representative dot plots show IFN-γ+ cells on CD4+ T cell gate from brain and spinal cord. B, Representative dot plots show IL-17+ cells on CD4+ T cell gate from brain and spinal cord. Numbers represent the fraction of the gated population in boxed areas; n = 3 i.c. BCG-infected mice.
17^CD4^ T cells in the CNS without the induction of MOG-specific CD4^ T cells in the CNS or periphery.

**Intracerebral BCG infection suppresses EAE clinical symptoms**

It was previously shown that mice pre-exposed to systemic heat-killed *Mtib* or BCG were resistant to EAE induction, and the suppression was correlated with a reduction of CNS-derived Ag-specific T cells (3, 4). In this paper, we studied whether the mycobacterial-induced suppression persists when mice are intracerebrally infected with BCG. To investigate the role of i.c. BCG infection on the development of autoimmune T cell responses and disease induction in the CNS, C57BL/6 mice were intracerebrally injected with either PBS alone or 1 x 10^5 CFU of BCG and 3 wk later were induced for EAE by injection of MOG35-55 peptide in CFA with pertussis toxin (Fig. 2A). Control mice experienced disease onset at approximately day 13, disease peak between days 17 and 18, and a mean maximum disease score of 2.58 ± 0.51. In contrast, i.c. BCG-infected mice were completely resistant to EAE induction (Fig. 2B), even when EAE was induced 42 days post i.c. BCG infection (data not shown). These data show that a persistent protective mechanism was induced by i.c. BCG infection against EAE.
FIGURE 4. The frequency of IFN-γ⁺CD4⁺ and IL-17⁺CD4⁺ T cells in the CNS. EAE was induced in C57BL/6 mice 21 days after i.c. injection with PBS or 1 x 10⁵ CFU of BCG. Fourteen days post EAE induction, single cells were prepared from the brain and spinal cord, and the isolated cells were restimulated with either MOG₃₅–₅₅ peptide or anti-CD3 Ab in the presence of GolgiStop. The frequency of IFN-γ⁺ or IL-17⁺-producing cells was measured by intracellular cytokine staining. A and C. Representative dot plots show IFN-γ⁺LFA-1⁺ (A) or IL-17⁺LFA-1⁺ (C) cells on CD4⁺ T cell gate in control mice and i.c. BCG-infected mice following EAE induction. Numbers represent the fraction of the gated population in boxed areas. B and D. Mean data are presented for frequency of IFN-γ⁺ (B) or IL-17⁺ (D) producing LFA-1⁺ T cells on CD4⁺ T cell gate in each group from brain and spinal cord. Values shown were determined by subtraction of values obtained in medium control. Error bars represent the SEM. E and F. MFI of IFN-γ⁺LFA-1⁺ (E) and IL-17⁺LFA-1⁺ (F) cells on CD4⁺ T cell gate; n = 5 control mice, six i.c. BCG-infected mice combined from two independent experiments (#, p < 0.1, *, p < 0.05, and **, p < 0.005 i.c. BCG-infected mice vs control mice; p < 0.05 were considered statistically significant).
Intracerebral BCG infection suppresses EAE pathology, and it is primarily associated with reduced cellular infiltration into the spinal cord

To further analyze the protective effect of i.c. BCG infection on EAE, we examined cellular infiltration and demyelination in the spinal cord of i.c. BCG-infected and control mice by histopathology on day 21 of EAE. Optic nerves were also analyzed at day 14 of EAE as an early and sensitive indicator of the pathology. Consistent with the lack of EAE clinical scores, optic nerves and spinal cords from i.c. BCG-infected mice showed no cellular infiltrates or visible signs of demyelinating lesions by H&E or LFB staining, respectively. In contrast, control mice showed extensive demyelination closely associated with heavy cell infiltration into the spinal cord and optic nerve parenchyma (Fig. 2, C and D). These results suggest that in i.c. BCG-infected mice, there is a significant reduction in the frequency of infiltrating mononuclear cells and an absence of demyelination in the spinal cord and optic nerve during EAE.

Mononuclear cells recovered from the brain, spinal cord, CLN, and spleen of i.c. BCG-infected mice and control mice on day 14 of EAE were quantified by flow cytometry. More mononuclear cells were recovered from the brains of i.c. BCG-infected mice

![Graph showing percentage of cells](image)

**FIGURE 5.** The frequency of IFN-γ+/CD4+ and IL-17+/CD4+ T cells in the periphery. EAE was induced in C57BL/6 mice 21 days after i.c. injection with PBS or 1 × 10^6 CFU of BCG. Fourteen days post EAE induction, single cells were prepared from the CLN and spleen, and the isolated cells were restimulated with either MOG_35-55_ peptide or anti-CD3 Ab in the presence of GolgiStop. The frequency of IFN-γ- or IL-17-producing cells was measured by intracellular cytokine staining. A and C, Representative dot plots show IFN-γ+/LFA-1⁺ (A) or IL-17⁺/LFA-1⁺ (C) cells on CD4⁺ T cell gate in control mice and i.c. BCG-infected mice following EAE induction. Numbers represent the fraction of the gated population in boxed areas. B and D, Mean data are presented for frequency of IFN-γ- (B) or IL-17- (D) producing LFA-1⁺ T cells on CD4⁺ T cell gate in each group from CLN and spleen. Values shown were determined by subtraction of values obtained in medium control; n = 5 control mice, six i.c. BCG-infected mice combined from two independent experiments (*, p < 0.1, *, p < 0.05, and **, p < 0.005 i.c. BCG-infected mice vs control mice; p < 0.05 were considered statistically significant).
than those of control mice (Fig. 3, A and B), indicating that there was an ongoing i.c. BCG-induced inflammatory response in the brain. However, 2.1 times fewer total mononuclear cells and 5.7 times fewer CD4+ T cells were recovered from the spinal cords of i.c. BCG-infected mice compared with control mice during the expected time of EAE symptoms (Fig. 3B). In addition, fluorescent immunohistochemical staining was performed on frozen spinal cord sections to detect infiltrating CD4+ T cells and CD11b+ cells in the spinal cords of i.c. BCG-infected mice and control mice following EAE induction (Fig. 3C). In control mice, infiltrating CD4+ T cells and CD11b+ cells were abundantly found in the spinal cord parenchyma, in contrast to i.c. BCG-infected mice that had only a few infiltrating CD4+ T cells and CD11b+ cells (Fig. 3C), confirming that cellular infiltration into the spinal cord was indeed suppressed in i.c. BCG-infected mice following EAE induction.

Intracerebral BCG infection suppresses MOG-specific IFN-γ+CD4+ and IL-17+CD4+ T cell responses following EAE induction in the CNS

The critical counter-regulatory role of IFN-γ and IL-17 in the pathogenesis of EAE has been studied (23, 28, 35, 36). To define whether i.c. BCG infection alters the distribution and frequency of MOG-specific IFN-γ- and IL-17-producing CD4+ T cells following EAE induction, thus suppressing EAE clinical scores and pathology, we analyzed cytokine responses for IFN-γ and IL-17 after MOG35–55 restimulation. We show in Fig. 4 that in the brain, 0.7% were MOG-specific IFN-γ+CD4+ or IL-17+CD4+ T cells among isolated CD4+ T cells in i.c. BCG-infected mice following EAE induction compared with 15 and 4.7% of the CD4+ T cells in control mice. In the spinal cord, 0.4 and 3.9% were MOG-specific IFN-γ+CD4+ or IL-17+CD4+ T cells, respectively, in i.c. BCG-infected mice compared with 11.6 and 4.5% of the CD4+ T cells in control mice (Fig. 4, B and D) (the percentages above represent an average of 5–6 mice and include subtraction of background from medium control). These data demonstrate that i.c. BCG-infected mice had proportionally fewer MOG-specific CD4+ T cells compared with control mice following EAE induction. Therefore, the MOG-recall responses provide clear evidence of suppressed MOG-specific IFN-γ+ and IL-17+CD4+ T cell responses in i.c. BCG-infected mice following EAE induction.

Intracerebral BCG infection suppresses to a greater extent IL-17+CD4+ T cell responses compared with IFN-γ+CD4+ T cell responses in the CNS following EAE induction

To further characterize the CD4+ T cell cytokine response in i.c. BCG-induced protection against EAE, we evaluated IFN-γ and IL-17 cytokine responses in CD4+ T cells after stimulation with anti-CD3 Ab. The presence of IFN-γ- and IL-17-producing CD4+ T cells after anti-CD3 Ab activation in the CNS demonstrated intact T cell effector functions in i.c. BCG-infected mice following EAE induction (Fig. 4, A and C). Fig. 4 demonstrates that a significantly smaller proportion of IL-17+ cells among the total population of CD4+ T cells was detected in the CNS of i.c. BCG-infected mice after anti-CD3 Ab stimulation. In addition, there was a lower production of IL-17 per cell in the CNS of i.c. BCG-infected mice, as determined by the lower mean fluorescent intensity (MFI) of intracellular staining (Fig. 4, E and F). The frequency of IFN-γ+ cells among total CD4+ T cells in the CNS of i.c.
BCG-infected mice was also less than that of control mice, but the difference was not as pronounced as that observed for the IL-17 response, showing that i.c. BCG infection reduced the proportion of IL-17-producing CD4⁺ T cells following EAE induction to a greater extent than the proportion of IFN-γ-producing CD4⁺ T cells within the CNS.

### Intracerebral BCG infection suppresses IL-17⁺CD4⁺ T cell responses in the CNS

To understand whether the suppression of MOG-specific IFN-γ⁺ and IL-17⁺CD4⁺ T cell responses is localized in the CNS or can also be found in the periphery, we evaluated IFN-γ⁺CD4⁺ and IL-17⁺CD4⁺ T cell responses in the CLN and spleen. The frequency of MOG-specific IFN-γ⁺ and IL-17⁺ cells among CD4⁺ T cells was equivalent in the CLN and the spleen of both groups (Fig. 5). In the spleen, a significantly higher frequency of IFN-γ⁺-producing CD4⁺ cells was observed in the i.c. BCG-infected mice following EAE induction compared with control mice, supporting that i.c. BCG influenced peripheral immune responses. Similar to the cytokine responses in the CNS, there were fewer IL-17⁺ cells among CD4⁺ T cells in the periphery in i.c. BCG-infected mice than in control mice following EAE induction. However, the suppression of IL-17⁺CD4⁺ T cell responses in the CLN and spleen of i.c. BCG-infected mice, while reduced, was not statistically different from control mice either by analyzing the frequency (Fig. 5) or MFI of IL-17⁺CD4⁺ T cells (data not shown). This suggests that suppression of IL-17 responses upon i.c. infection with BCG is primarily localized to the CNS.

### Intracerebral BCG infection suppresses EAE clinical symptoms and MOG-specific IL-17⁺CD4⁺ T cell responses in the CNS of IFN-γ-deficient mice

It has been demonstrated that IFN-γ antagonizes the differentiation of naive CD4⁺ T cells into IL-17⁺CD4⁺ T cells (29, 37). Additionally, evidence has shown a disease-limiting role of IFN-γ in EAE. In comparison, preferential enrichment of IL-17⁺CD4⁺ T cells over IFN-γ⁺CD4⁺ T cells in the CNS during the peak of EAE suggests a pathogenic role for IL-17⁺CD4⁺ T cells in EAE (38). Thus, the ratio of IFN-γ to IL-17 is an important reference for the pathogenesis of EAE (39). Our data show that in control mice with EAE clinical symptoms, there were 1.6 or 1.9 times as many IFN-γ⁺CD4⁺ T cells as IL-17⁺CD4⁺ T cells in the brain and spinal cord, respectively. However, there were 6.2 or 11.4 times as many IFN-γ⁺CD4⁺ T cells as IL-17⁺CD4⁺ T cells in the brain and spinal cord, respectively, of EAE-resistant i.c. BCG-infected mice (Fig. 6). This data suggests that IFN-γ responses are more dominant in i.c. BCG-infected mice compared with IL-17 responses following EAE induction.

Therefore, to investigate whether an IFN-γ-dominant environment facilitates disease-limiting mechanisms in i.c. BCG-infected mice, we used IFN-γ-deficient mice. IFN-γ-deficient and C57BL/6 mice were infected i.c. with 1 × 10⁵ CFU of BCG and 3 wk later were induced for EAE. As previously reported, IFN-γ-deficient mice develop more severe EAE clinical symptoms (Fig. 7A) compared with C56BL/6 WT mice. However, i.c. infection of BCG provided complete protection to day 30 in both C57BL/6 and IFN-γ-deficient mice. Consistent
with the lack of EAE clinical scores, spinal cords from i.c. BCG-infected mice showed no cellular infiltrates or visible signs of demyelinating lesions by H&E or LFB staining, respectively. In contrast, control mice showed extensive demyelination closely associated with heavy cell infiltration into the spinal cord parenchyma at 41 days post EAE induction (Fig. 7B).

Next, we analyzed the cytokine response in IFN-γ-deficient mice for IL-17 with either MOG_{35-55} or anti-CD3 Ab stimulation at 20 days post EAE induction. IFN-γ-deficient, i.c. BCG-infected mice barely showed a MOG-specific IL-17+ response in CD4+ T cells from the CNS above the medium control and also had a lower frequency of IL-17+ T cells among total CD4+ T cells following anti-CD3 Ab restimulation (Fig. 8A). These data strongly suggest that the suppression of EAE by i.c. BCG infection was a result of changes in IL-17 responses and was independent of IFN-γ-mediated mechanisms.

**Intracerebral BCG infection and EAE lead to comparable induction of Foxp3+CD4+ T cells in the CNS**

Mycobacteria-induced regulatory T cells suppress host protective immunity against infection (40) and also inhibit allergic reactions and autoimmune diseases (41, 42). The disease-limiting role of regulatory T cells has been demonstrated in the EAE model (43–45), and recent studies have suggested that regulatory T cells might be reciprocally developed with pathogenic effector Th17 cells (29–31). Therefore, to gain a mechanistic insight for the i.c. BCG-induced protection against EAE, we examined whether i.c. BCG infection leads to the induction of regulatory T cells as a result of

![Image](http://www.jimmunol.org/DownloadedFrom/image_url)

**FIGURE 9.** The frequency and distribution of Foxp3+CD4+ T cells. Mice were i.c. injected with PBS or infected with 1 × 10^7 CFU of BCG. Then, 21 days later, EAE was induced in a subset of animals from each group. After fourteen days, single cells were prepared from the brain, spinal cord, CLN, and spleen and stained with anti-CD4 and anti-Foxp3 Abs. The frequency and distribution of Foxp3+CD4+ T cells from mice i.c. injected with PBS or BCG 35 days before single cell isolation are shown for comparison. A. Dot plots show CD4+ T cells and Foxp3+ cells on CD4+ T cell gate from indicated organs. The numbers shown are the percentage of the boxed population in CD4+ T cells. B. Mean data are presented for frequency of Foxp3+CD4+ T cells on CD4+ T cell gate in each group; n = 3 i.c. PBS, three i.c. BCG, 5 EAE, and 5 BCG/EAE mice. For EAE and BCG/EAE groups, data were combined from two independent experiments (*, p < 0.05; n.s., statistically not significant; p < 0.05 were considered statistically significant).
suppressing pathogenic effector Th17 cells and, thus, prevents disease development. The frequency and distribution of Foxp3$^{+}$CD4$^{+}$ T cells from four different experimental groups, (i.e. PBS, i.e. BCG, EAE, and i.e. BCG/EAE) were determined by flow cytometry. As we demonstrate in Fig. 9, the frequency of Foxp3$^{+}$CD4$^{+}$ T cells significantly increases in the brain and spleen of i.e. BCG-infected, EAE, and i.e. BCG-infected/EAE mice compared with naive control. However, there was no significant difference in the frequency and distribution of Foxp3$^{+}$CD4$^{+}$ T cells between i.e. BCG-infected and control mice following EAE induction (Fig. 9A, last two columns). These data show that i.e. BCG infection with or without subsequent EAE induction results in equivalent levels of Foxp3$^{+}$CD4$^{+}$ T cell accumulation in the CNS. These results indicate that Foxp3$^{+}$CD4$^{+}$ T cell-mediated regulatory mechanisms might not be critical in BCG-mediated protection from EAE.

**Discussion**

In the present study, we investigated the effect of i.e. BCG-infection on the development of CNS autoimmunity using a murine model of MS, EAE. We show that i.e. BCG infection protected mice against EAE. These data demonstrate that the suppressive effect of BCG can be achieved not only by i.p. (3, 4) but also by i.e. infection. The complete protection against EAE clinical symptoms by i.e. BCG infection is even more profound than the partial protection conferred by i.p. BCG infection (3). This result, demonstrating that i.e. BCG infection suppresses the development of CNS autoimmune responses more efficiently than i.p. infection, suggests that i.e. BCG infection exerts its suppressive effect primarily in the CNS (Fig. 5). Resistance to EAE induction in i.e. BCG-infected mice appears to be primarily associated with reduced mononuclear cell infiltration in the spinal cord. So far, the proposed mechanisms of infection-induced protection for autoimmunity are largely associated with reduced self-Ag-specific T cells at effector sites (3, 4, 25, 46). Infection can prevent autoreactive T cells from reaching the effector site of autoimmune disease, thus suppressing the given autoimmune responses on the target organ. Christen et al. (46) showed that lymphocytic choriomeningitis virus infections lead to a recruitment of T lymphocytes from the islet infiltrate to the pancreatic draining lymph node where increased apoptosis occurred in a chemokine IP-10-dependent manner, thus preventing the onset of diabetes in NOD mice. Our previous demonstration of the sink effect regarding peripheral BCG-induced granulomas for MOG-specific T cells also suggests that EAE is inhibited by an altered T cell trafficking induced by BCG infection (3). The results presented here show that i.e. BCG-induced inflammatory lesions in the brain partly act as a sink to recruit cells into the brain, as demonstrated by an increased number of cells isolated in the i.e. BCG-infected mice following EAE induction compared with control mice, thus preventing inflammatory cells from reaching the spinal cord, which is the main effector site of EAE. However, the presence of fewer MOG-specific IFN-γ CD4$^{+}$ and IL-17 CD4$^{+}$ T cells in i.e. BCG-infected mice both in brain and spinal cord following EAE induction implies that there are mechanisms that suppress CNS Ag-specific T cell responses other than diverted trafficking of inflammatory cells.

The critical role of IL-17 in autoimmune disease has been the focus of many recent studies. IL-17 has been shown to be involved in tissue inflammation in various models of organ-specific autoimmunity in the brain, synovium, and in allergic disorders, which leads to sustained tissue pathology (27, 47). In EAE studies, IL-17-deficient mice showed significantly delayed onset and milder clinical symptoms compared with WT mice, suggesting that IL-17 is indispensable for the onset and progression of EAE (28). However, despite the importance of IL-17 in EAE pathogenesis, a direct study into the effect of BCG infection on IL-17 CD4$^{+}$ T cells in the context of CNS autoimmunity has yet to be investigated. Recent data using in vitro naive CD4$^{+}$ T cells cultured with heat-killed mycobacteria show that mycobacteria effectively induce the differentiation of IL-17 CD4$^{+}$ cells in a TGF-β-dependent manner (48). Heat-killed mycobacteria in CFA favor IL-17 CD4$^{+}$ responses in EAE mice in association with up-regulated IL-23 mRNA. However, our results demonstrate that i.e. BCG-infected mice have a lower percentage of IL-17$^{+}$ cells among CD4$^{+}$ cells in the CNS following EAE induction compared with control mice. This suggests that immune responses induced by i.e. BCG infection, before EAE induction with MOG in CFA, suppress differentiation of IL-17 CD4$^{+}$ T cells. Furthermore, it indicates that ongoing IL-17 CD4$^{+}$ T cell responses do not facilitate further development of IL-17 CD4$^{+}$ T cells, including MOG-specific IL-17 CD4$^{+}$ T cells following EAE induction in the CNS.

As a proinflammatory cytokine, IFN-γ orchestrates inflammatory responses by regulating immune cell trafficking and activation and thus might appear to promote EAE pathology. However, IFN-γ can also exert a disease-limiting effect in the EAE inflammatory response as well. EAE clinical symptoms and pathology are more severe in IFN-γ-deficient mice (23, 25, 35) and IFN-γR-deficient mice (35, 49) compared with WT mice. Additionally, recent studies demonstrate that IFN-γ antagonizes the development of the Th17 lineage both in vitro and in vivo (26, 28, 29). Intracerebral BCG-infected mice developed a more Th1-biased immune response due to a decrease in the total number of IL-17 CD4$^{+}$ T cells following EAE induction compared with control mice. Thus, it may be possible that the IFN-γ-dominant environment facilitates disease-limiting mechanisms in a way that restrains IL-17 CD4$^{+}$ T cell differentiation in i.e. BCG-infected mice. Cruz et al. (50) demonstrated IFN-γ-mediated antagonism of IL-17 CD4$^{+}$ T cell responses during mycobacterial infection; therefore, we hypothesized that i.e. BCG infection may result in a suppressed differentiation of IL-17 CD4$^{+}$ T cells following EAE induction due to pre-existing BCG-induced IFN-γ responses at the time of EAE induction. However, our result using IFN-γ-deficient mice demonstrates that EAE protection in i.e. BCG-infected mice is independent of the contribution of IFN-γ-mediated mechanisms. Although initial studies on the differentiation of IL-17 CD4$^{+}$ T cells suggest counter-regulation between IFN-γ and IL-17, the necessity of IL-17 for optimal Th1 responses and vice versa has been demonstrated in both infection (51) and autoimmune disease (28) models. Additionally, if the master transcription factor for IFN-γ-producing T cells is nullified, it negatively regulates Th17 responses in both experimental autoimmune myocarditis (52) and EAE (53). This suggests that the interaction between IFN-γ and IL-17 is more complex and depends on the experimental system. Therefore, the interaction should be understood in the context of the exact location of the cytokine responses and acuity or chronicity of the disease. Altogether, our data using IFN-γ-deficient mice demonstrates that the protective effect of i.e. BCG infection on EAE is mediated by IFN-γ-independent mechanisms, and the suppression of IL-17 CD4$^{+}$ T cell responses is not the result of IFN-γ responses induced by BCG infection. The use of IL-17-deficient mice would validate the contribution of IL-17 CD4$^{+}$ T cells in protection; however, minimal EAE symptoms in these transgenic mice make the experiment nonfeasible (28) because i.e. BCG infection-induced protection would be immeasurable.

Inducing/facilitating anti-inflammatory or Th2 cytokines and inhibiting/blocking inflammatory responses have been studied as potential methods of preventing autoimmune responses or reversing
the established autoimmune pathology. Studies have shown a regulative role of IL-10 in amelioration and in the recovery phase of EAE and B cells and regulatory T cells as major sources of IL-10 (45, 54–56). Lampropoulou et al. (56) have demonstrated that mycobacteria trigger the regulatory function of B cells via TLR. They showed that the IL-10-producing regulatory function of B cells in EAE is mediated by MyD88 signaling through TLR2/4 by Mtb components contained in the Freund’s adjuvant used to induce EAE. Regulatory T cells are critical in maintaining peripheral tolerance, limiting the extent of inflammatory responses and ultimately keeping the host immunity balanced (57, 58). Mycobacterial infection induces regulatory T cells that contribute to limiting inflammatory responses and tissue damage during infection (40, 59). As shown in experimental models of allergic asthma (41) and insulin dependent diabetes mellitus (42), regulatory T cells induced during mycobacterial infection can suppress concomitant inflammatory responses; thus, induction of regulatory T cells has been attributed to being one of the mechanisms by which mycobacteria suppress autoimmune diseases. Recent studies have demonstrated a dichotomy in the differentiation of pathogenic Th17 cells and regulatory T cells from naive CD4+ T cells. In the presence of TGF-β, naive T cells predominantly differentiate into Foxp3+ regulatory T cells, whereas in the presence of TGF-β along with IL-6, a proinflammatory cytokine, naive T cells differentiate into pathogenic Th17 cells (29–31).

Based on this knowledge and our observation of suppressed Th17 responses in i.c. BCG-infected mice following EAE induction, we hypothesized that BCG in the brain facilitates more regulatory T cell induction than pathogenic Th17 cell differentiation, thus providing protection against EAE. In this study, our data shows that i.c. BCG infection, MOG immunization, and concomitant immunization result in equivalent levels of Foxp3+CD4+ T cell accumulation in the CNS. It was apparent that Foxp3+CD4+ T cells did not proportionally increase in frequency upon two independent inflammatory challenges as shown in i.c. BCG-infected mice following EAE induction. As shown in many experimental models, an increasing T regulatory:T effector ratio is a good reference of regulatory T cell-mediated disease resolution (60, 61). In this sense, the equivalent frequency of Foxp3+ T cells among CD4+ T cells in both i.c. BCG-infected and control mice following EAE induction weakens the possibility of regulatory T cells mediating EAE protection. Furthermore, a recent study showing that Foxp3+CD4+ T cells isolated from the CNS of EAE mice are not able to inhibit in vitro proliferation of encephalitogenic T cells questions the ability of Foxp3+ CD4+ T cells that have accumulated in the CNS during EAE to suppress EAE (62).

In summary, we have demonstrated that an ongoing i.c. BCG infection suppressed the development of CNS autoimmunity using the EAE model. Intracerebral BCG-infected mice had not only fewer infiltrating mononuclear cells in the spinal cord but also fewer MOG-specific IFN-γ+CD4+ and IL-17+CD4+ T cells following EAE induction. Although i.c. BCG-infected mice had a smaller proportion of both IFN-γ+ and IL-17+ cells among CD4+ T cells following EAE induction in the CNS, this suppressive effect was not IFN-γ-mediated and appears to be correlated with a decrease in IL-17+CD4+ T cells. Additionally, the equivalent frequency of Foxp3+CD4+ T cells in i.c. BCG-infected EAE and control EAE mice suggests that Foxp3+CD4+ T cells might not be responsible for protection from EAE. Altogether the data presented here support that manipulating local Th17 responses in the CNS must be considered in the treatment of autoimmune disease such as MS.


