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Regulatory T Cell Dysfunction Acquiesces to BTLA⁺ Regulatory B Cells Subsequent Oral Intervention of Experimental Autoimmune Encephalomyelitis

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Abstract

Regulatory T cells (Tregs) induced during autoimmunity often become quiescent and unable to resolve disease, suggesting inadequate activation. Resolution of established experimental autoimmune encephalomyelitis (EAE) can be achieved with myelin oligodendrocyte glycoprotein (MOG) fused to reovirus protein $\sigma 1$ (MOG- $\rho\sigma 1$) which activates Tregs, restoring protection, but requiring other regulatory cells to revitalize them. B cells have a dichotomous role in both the pathogenesis and recovery from EAE. While inflammatory B cells contribute to EAE's pathogenesis, treatment of EAE mice with MOG- $\rho\sigma 1$, but not OVA- $\rho\sigma 1$, resulted in an influx of IL-10-producing B220⁺CD5⁺ B regulatory cells (Bregs) enabling Tregs to recover their inhibitory activity, and in turn, leading to the rapid amelioration of EAE. These findings implicate direct interactions between Bregs and Tregs to facilitate this recovery. Adoptive transfer of B220⁺CD5⁻ B cells from MOG- $\rho\sigma 1$ -treated EAE or Bregs from PBS-treated EAE mice did not resolve disease while the adoptive transfer of MOG- $\rho\sigma 1$ -induced B220⁺CD5⁺ Bregs greatly ameliorated EAE. MOG- $\rho\sigma 1$ -, but not OVA- $\rho\sigma 1$ -induced IL-10-producing Bregs, expressed elevated levels of BTLA relative to CD5⁻ B cells, as opposed to Tregs or effector T (T_{eff}) cells, whose BTLA expression was not affected. These induced Bregs restored EAE Treg function in a BTLA-dependent manner. BTLA^{-/-} mice showed more pronounced EAE with fewer Tregs but, upon adoptive transfer of MOG- $\rho\sigma 1$ -induced BTLA⁺ Bregs, BTLA^{-/-} mice were protected against EAE. Hence, this evidence shows the importance of BTLA in activating Tregs to facilitate recovery from EAE.

Keywords

EAE/MS; Bregs; Tregs; BTLA; mucosal immunity; tolerance

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Disclosures

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Introduction

B cells have been classically considered a key player in the immune response against microbial infections, mainly through the production of Abs. B cells also have been shown to have a role in the pathogenesis of autoimmune disorders. New clinical interventions have targeted the elimination of B cells for the treatment of rheumatoid arthritis (¹), lupus (²), and MS (³). However, an increasing body of evidence supports the existence of a subpopulation of anti-inflammatory IL-10-producing B cells, that critically affect the outcome of autoimmune diseases both in animal models (⁴⁻⁸) and in human clinical disease (⁹⁻¹²).

EAE is a T cell-dependent inflammatory disease, principally mediated by IL-17 (¹³) and GM-CSF (¹⁴). EAE is highly reproducible in mice after immunization of susceptible strains with TCR-reactive peptides, and mimics many aspects of MS (¹⁵). Although EAE is predominantly a T cell-mediated disorder, B cells have been shown to play an important pathogenic role in this disease (reviewed by Gray *et al* in (¹⁶). On one hand, autoAb production may increase demyelination and inflammation, worsening the course of the disease (^{17, 18}). Additionally, B cells may function as APCs in early phases of EAE, contributing to the expansion of effector CD4⁺ T cells (^{19, 20}). On the other hand, B cell-deficient mice develop a more aggressive form of EAE (²¹⁻²⁴), and IL-10 production by B cells inhibits the lymphocyte response against auto-Ags, and thus ameliorates EAE (^{6, 7, 20, 25, 26}). As suggested by Matsushita *et al*, this contradiction may be explained if the existence of regulatory B cells (Bregs) is taken into account (⁶). While pathogenic B cells increase severity of the disease, Bregs can ameliorate symptoms. Therefore, a balance between pathogenic B cells and Bregs can affect the severity of EAE as well as other autoimmune diseases.

The induction of tolerance in humans is problematic, but some success has been achieved for treating allergies (^{27, 28}) and diabetes (²⁹). Oral tolerance in humans is plagued by the lack of effective means to absorb ingested tolerogens from the gastrointestinal tract. Hence, we postulated that targeting tolerogens or seeking alternative sites of treatment, e.g., nasal, offered a means to implement tolerance. We found that reovirus protein $\sigma 1$ ($\rho\sigma 1$) could facilitate uptake of genetically fused tolerogens, when applied mucosally, and stimulate the induction of tolerogen-specific regulatory T cells (Tregs; ³⁰⁻³³). $\rho\sigma 1$ effectively stimulated Ag-specific tolerance to defined Ags: OVA, proteolipid protein (PLP) peptide 139-151, and myelin oligodendrocyte glycoprotein (MOG₂₉₋₁₄₆) (³⁰⁻³³). PLP₁₃₉₋₁₅₁ fused to OVA- $\rho\sigma 1$ protected mice against PLP₁₃₉₋₁₅₁-induced EAE while OVA- $\rho\sigma 1$ did not (³³). MOG- $\rho\sigma 1$ protected against MOG₃₅₋₅₅-induced EAE, but not OVA- $\rho\sigma 1$, MOG, or MOG + $\rho\sigma 1$ (³²) further demonstrating that the tolerogens must be physically coupled to $\rho\sigma 1$ to elicit Ag-specific tolerance. Herein we show that treatment of EAE with MOG- $\rho\sigma 1$ increased the percentage of Bregs and their production of IL-10. This treatment provides the regulatory balance needed to resolve disease.

HVEM (Herpes virus entry mediator) belongs to the tumor necrosis factor receptor family. It regulates the immune response in various pathogen and autoimmune settings through interactions with multiple ligands such as Ig superfamily members, BTLA (B and T lymphocyte attenuator) and CD160, as well as, the TNF superfamily ligands, LIGHT and

LT α (34, 35). These receptors act bidirectionally having co-stimulatory or co-inhibitory properties. For instance, *trans* engagement of HVEM with BTLA or LIGHT is co-stimulatory for the HVEM⁺ cells and promotes cell survival via NF- κ B activation (36). Additionally, HVEM engagement of LIGHT potently activates T cells which can lead to T cell-mediated intestinal inflammation (37-39). In contrast, HVEM signaling through BTLA results in the inactivation of the BTLA⁺ cells mediated by SH2 domain-containing protein tyrosine phosphatase 1 and 2 (40, 41).

Although large numbers of Tregs are found at sites of chronic inflammation, these fail to curb disease (42-45). To address this paradox, we sought to determine the activation status of Bregs since these cells were induced subsequent to MOG-p σ 1 treatment. We found elevated numbers of BTLA⁺ Bregs following MOG-p σ 1 treatment. Neutralization of BTLA on Bregs inhibited their capacity to restore suppressive function to EAE Tregs. Thus, we hypothesize that restoration of Treg function is BTLA-dependent. Supporting this hypothesis, adoptive transfer of MOG-p σ 1-induced IL-10-producing B220⁺(CD19⁺)CD5⁺, but not B220⁺(CD19⁺)CD5^{neg} cells, significantly increased the percentage and activity of CD25⁺CD4⁺ Tregs which reduced EAE severity. Furthermore, these Bregs were protective against EAE in BTLA^{-/-} mice. Such findings underscore the relevance of Bregs and their potential benefit in treating human autoimmune diseases such as MS.

Material and Methods

Preparation of MOG-p σ 1 and OVA-p σ 1

MOG-p σ 1 and OVA-p σ 1 fusion proteins were prepared as previously described (31,32) consisting of MOG₂₉₋₁₄₆ or OVA genetically fused to the N-terminus of the whole reovirus p σ 1. These recombinant proteins were generated in *Pichia pastoris* featuring a his-tag to enable their purification (31, 32). Proteins were assessed for purity and quality by Coomassie-stained polyacrylamide gels and by Western blot analysis.

Mice

C57BL/6 females (6-8 wk old; Frederick Cancer Research Facility, National Cancer Institute, Frederick, MD) and breeding colonies of Foxp3-mRFP-transgenic, B cell deficient (μ MT), IL-10^{-/-}, and BTLA^{-/-} mice (Jackson Laboratory, Bar Harbor, ME) were maintained at Montana State University Animal Resources Center or the University of Florida Animal Center Services. Mice were kept in individual ventilated cages under high-efficiency particulate absorbing-filtered barrier conditions. All animal care and procedures are in compliance with institutional policies for animal health and well-being.

MOG-p σ 1-based therapies and EAE induction

Mice were given a single oral 50 μ g dose of MOG-p σ 1 or OVA-p σ 1 as previously described (30). For EAE induction, mice were challenged s.c. in the flank with 300 - 350 μ g of MOG₃₅₋₅₅ peptide (Global Peptide Services, LLC, Ft. Collins, CO or Bio-Synthesis, Inc., Lewisville, TX) in 100 μ l of IFA (Sigma-Aldrich, St. Louis, MO) containing 400 μ g killed *Mycobacterium tuberculosis* (Difco Laboratories, Detroit, MI) on day 0 followed by i.p. treatments with 200 ng of *Bordetella pertussis* toxin (List Biological Laboratories,

Campbell, CA) on days 0 and 2. Mice were monitored and scored daily for EAE progression (30, 33): 0, normal; 1, a limp tail; 2, hind limb paresis; 3, hind limb paralysis; 4, quadriplegia; 5, moribund state. In most instances, we observed that the PBS-treated EAE C57BL/6 mice made a slow recovery, and generally about days 40-45, exhibited a clinical score of 1, and these are referred to as “naturally recovered” EAE mice.

Isolation of mononuclear cells from CNS

Prior to dissection, mice were perfused with cold PBS through the left cardiac ventricle. Subsequently, the forebrain and cerebellum were dissected, and the spinal cords (SCs) flushed out with PBS by hydrostatic pressure, minced, and digested with 10 ml HBSS containing 500 U/ml of collagenase (Sigma-Aldrich) for 60-90 min at 37° C with shaking to release mononuclear cells. The tissues were then homogenized in cold HBSS buffer (Sigma-Aldrich) as previously described (30) to obtain a single cell suspension. Centrifuged cells collected from 4-5 mice were pooled, resuspended in 70% Percoll (Sigma-Aldrich), and underlaid with 30% Percoll. Cells were subjected to gradient density centrifugation, and mononuclear cells were isolated from the 30/70 interphase, then washed, and stained for flow cytometry analysis.

Flow cytometry

To block nonspecific Fc receptor binding, lymphocytes were preincubated for 15 min at room temperature with anti-CD16/32 FcR block, and stained 30 minutes in FACS buffer at 4°C with fluorochrome-labeled mAbs against B220, CD1d, CD4, CD5, CD19, CD25, and BTLA (clone 8F4, Biolegend). For intracellular expression of cytokines, lymphocytes were assessed stimulated for 5h with PMA (50 ng/ml) plus ionomycin (500 ng/ml) in the presence of brefeldin A (2 µg/ml), followed by permeabilization with saponin and subsequent staining with mAbs specific IL-10 (clone JES5-16E3) or IL-17 (clone eBio17B7). Nuclear Foxp3 expression by Tregs was measured by FACS analysis as per the manufacturer's instructions (Foxp3 Fix/Perm buffer set, Biolegend). Cells were analyzed using a LSRFortessa flow cytometer (Becton Dickinson, San Jose, CA) and analyzed with Flow Jo software (Tree Star Inc., Ashland, OR).

T cell proliferation and cytokine production

Single-cell splenic and LN suspensions were prepared as previously described (29,30). CD25⁺CD4⁺ Tregs and CD25⁻CD4⁺ T effector (T_{eff}) cells were purified using a FACSARIA (Becton Dickinson) and 50,000 T_{eff} cells were stimulated for 4 days on anti-CD3 mAb-coated wells (5 µg/ml) plus soluble anti-CD28 mAb (5 µg/ml) plus 5 U/ml of human IL-2 (28) Bregs were purified as described below. Splenic Tregs and Bregs were added when indicated at a 1:2 ratio. In some instances, Bregs or Tregs were pretreated (10 µg/ml) with anti-BTLA (clone 6F7; eBioscience) or isotype control mAb when indicated. One µCi per well of [³H]-thymidine was added during the last 12 h of culture. The cells were then harvested, and incorporated [³H]-thymidine was counted using a Beckman LS 6500 scintillation counter. For some experiments, purified CD4⁺ T cells were restimulated for 4 days with 5 µg/ml MOG₃₅₋₅₅ peptide in the presence of irradiated T cell-depleted splenic feeder cells (30-32).

Supernatants from cultured T cells were analyzed for the production of IFN- γ , TGF- β , IL-4, IL-6, IL-10, and IL-17 by ELISA as previously described (³²).

Adoptive transfers

Cell-sorting was accomplished using a FACSARIA as previously described (^{30, 32}). Briefly, splenic and LN (HNLN and MLN) cells from C57BL/6 and IL-10^{-/-} mice were sorted for Bregs or B cells based on the expression of B220 (or CD19) and CD5. Cell purity exceeded 95%. Sorted cells were kept at 4°C in PBS with 2% FBS until adoptive transfer. Cells were peri-orbitally transferred into anesthetized mice, and the amount transferred is indicated in each experiment.

Statistical analysis

The ANOVA followed by posthoc Tukey test was applied to show differences in clinical scores in treated vs. PBS mice. The Student's *t* test was used to evaluate the differences between two groups, and ANOVA was used to test differences between multiple groups. Values *p* < 0.05 were considered statistically significant.

Results

EAE-derived Tregs are dysfunctional

Previous studies have demonstrated the presence of Tregs during EAE and other inflammatory diseases, yet these remained dysfunctional or quiescent and did not reduce inflammation (⁴²⁻⁴⁵). Our previous work has shown that the inflammation in EAE mice is rapidly ameliorated with a single low nasal or oral dose of MOG-p σ 1 (^{30, 32}). Given this, purified splenic naïve Tregs, EAE-derived Tregs from mice at disease onset (d7 post-challenge [p.ch.], score 1), or Tregs from 15 days after EAE induction (score 3) were added to anti-CD3 plus anti-CD28 stimulated naïve T_{eff} cells to measure inhibition of T_{eff} cell proliferation. Naïve Tregs efficiently inhibited T_{eff} cell proliferation, while EAE Tregs did not (Fig. 1A). Tregs obtained from EAE mice 24 h after treatment with MOG-p σ 1 (day 16 p.ch.) were also quite effective in suppressing T_{eff} cell proliferation. Interestingly, Tregs obtained from naturally recovered mice (day 40 p.ch., score 0.5) were as effective as naïve Tregs in suppressing T_{eff} cell proliferation (Fig. 1A) suggesting these Tregs have regained their function.

MOG-p σ 1 treatment is only partially effective in μ MT mice

MOG-p σ 1's therapeutic effect has been shown to be IL-10-dependent (³²). To determine the source of IL-10-producing cells, splenic lymphocytes taken from EAE mice previously treated with PBS or MOG-p σ 1 were evaluated for their expression of TGF- β and IL-10. In addition to CD4⁺ T cells producing IL-10 (³²), a population of IL-10-producing B cells was also found in MOG-p σ 1-treated EAE mice (Fig. 1B). B lymphocytes isolated from EAE mice did not produce TGF- β regardless of the treatment (Fig. 1B). MOG-p σ 1 did significantly increase the percentage and total numbers of IL-10-producing B cells (Fig. 1B). Further studies set out to assess the impact of MOG-p σ 1 on B cells. C57BL/6 and B cell-deficient (μ MT) mice were induced with EAE, and 7 days later, orally treated with 50 μ g of MOG-p σ 1 or PBS (Fig. 1C). As expected (^{24, 46}), μ MT mice developed a more severe and

lingering disease, yet treatment of μ MT mice with MOG- ρ 1 reduced the disease severity (clinical score) by nearly 50%, showing that MOG- ρ 1's therapeutic effect is partially dependent on B cells.

MOG- ρ 1 treatment augments the IL-10-producing B220⁺ CD5⁺ regulatory B cells

Both CD5⁺CD1d^{Hi} (7, 47) and CD5⁺ (7) B cell subsets can serve as Bregs. An analysis was performed to determine which of these Bregs was induced following MOG- ρ 1 stimulation. When compared to naïve mice, a reduced presence of CD5⁺ B cells was observed in the spleen (Fig. 2A, B) and in the head and neck lymph nodes (HNLNs; Fig. 2C). Interestingly, treatment of EAE mice with MOG- ρ 1, but not OVA- ρ 1, restored the CD5⁺ B cells to levels similar to those of naïve mice (Fig. 2B). In contrast to CD5⁺ B cells that first decreased in diseased EAE mice and then increased either subsequent to MOG- ρ 1 treatment or following natural recovery (Fig. 2A, B), the B220⁺CD5⁺CD1d^{Hi} cells could be detected (data not shown), but did not increase as notably as did the CD5⁺ B cells following MOG- ρ 1 treatment (Fig. 2A-C). Thus, from this point forward, the described Bregs will be referred to as CD5⁺ (B220⁺ or CD19⁺) B cells. During EAE, the levels of CD5⁺ B cells diminished, and MOG- ρ 1 treatment essentially restored to basal levels in the spleens (Fig. 2B), and the total number of CD5⁺ B cells increased in the HNLNs (Fig. 2C). OVA- ρ 1 treatment failed to restore CD5⁺ B cells in the spleen (Fig. 2B), but did increase their numbers in the HNLNs (Fig. 2C). These findings support the importance of Bregs in protection conferred by MOG- ρ 1 in EAE.

The induced Bregs were further analyzed for their capacity to generate IL-10. EAE mice were evaluated 24h following MOG- ρ 1 or PBS treatment, and spleens, spinal cords (SCs), HNLNs, mesenteric LNs (MLNs), and peripheral LNs (PLNs) were analyzed for intracellular IL-10 production (Fig. 2D). Only SC-infiltrating Bregs were able to produce IL-10 by the PBS-treated group while MOG- ρ 1 treatment increased Breg IL-10 production in the SCs, PLNs, and MLNs (Fig. 2E). Of interest, the total number of IL-10-producing Bregs in the SCs was significantly greater in MOG- ρ 1-treated EAE than EAE mice (Fig. 2F). Thus, MOG- ρ 1 treatment augments these IL-10-producing CD19⁺ (B220⁺) CD5⁺ Bregs during EAE.

MOG- ρ 1-induced CD5⁺ Bregs are protective against EAE and require IL-10

To determine whether MOG- ρ 1-induced Bregs are protective against EAE, B cells were isolated from C57BL/6 mice induced with EAE, and treated 2 weeks later with PBS or MOG- ρ 1. Twenty-four hours after treatment, combined LN and splenic B cells were cell-sorted into B220⁺CD5⁻ (inflammatory) B cells and B220⁺CD5⁺ Bregs, and each was then adoptively transferred into different C57BL/6 mice that were induced with EAE 7 days earlier (Fig. 3). Neither CD5⁻ nor CD5⁺ B cells obtained from PBS-treated donors affected clinical manifestation of EAE (Fig. 3A). Donor B220⁺CD5⁻ B cells from MOG- ρ 1-treated mice also failed to ameliorate EAE; however, MOG- ρ 1-induced B220⁺CD5⁺ Bregs significantly reduced disease severity and accelerated recovery (Fig. 3B; $p < 0.01$). These findings underscore the importance of this IL-10-producing Bregs. The Breg subset, isolated from IL-10^{-/-} mice, failed to confer protection against EAE when adoptively transferred into C57BL/6 mice with EAE (Fig. 3C). This evidence further confirms the

critical role of IL-10⁺ Bregs induced by MOG-pσ1 in mediating protection against EAE. Notably, Bregs from diseased EAE mice failed to confer such protection.

Adoptive transfer of MOG-pσ1-treated CD5⁺ Bregs increases the number and activity of CD25⁺CD4⁺ Tregs in recipient mice

In an effort to understand how Bregs mediate their protection against EAE, both Bregs (B220⁺CD5⁺) and inflammatory B (B220⁺CD5⁻) cells were purified 48h after MOG-pσ1 treatment of EAE C57BL/6 mice. Subsequently, either of these B cell subsets was adoptively transferred into C57BL/6 mice induced with EAE 7 days earlier (Fig. 4A). At the peak of disease (day 14), the activity and quantity of Tregs were determined in recipients (Fig. 4B). Recipients given MOG-pσ1-induced Bregs showed an increase in the percentage of Tregs unlike those recipients given PBS-induced Bregs or MOG-pσ1-induced CD5⁻ B cells. Such evidence suggests that the MOG-pσ1-induced Bregs become activated and stimulate an influx of Tregs or in turn activate quiescent, disease-induced Tregs. The percentage of IL-17-producing T cells amongst the CD25^{Hi}CD4⁺ and CD25⁻CD4⁺ subsets⁽⁴⁸⁾ was measured. Mice adoptively transferred with MOG-pσ1-induced Bregs showed significantly fewer IL-17-producing cells than those found in EAE mice and recipients adoptively transferred with Bregs from PBS-treated EAE mice (Fig. 4C, $p = 0.01$).

To determine which cytokines correlated with protection induced upon Breg adoptive transfer, cytokine analyses were performed on purified splenic CD25⁺CD4⁺ T cells isolated 7 days following adoptive transfer. Tregs obtained from mice adoptively transferred with MOG-pσ1-induced Bregs showed significant elevations in IL-4 and IL-10 when compared to control groups or recipients given PBS-induced Bregs ($p < 0.01$) (Fig. 4D) with concomitant reductions in IFN-γ and IL-17 ($p < 0.01$). These results demonstrate that MOG-pσ1-induced Bregs are protective by increasing and activating Tregs to produce regulatory cytokines and to simultaneously inhibit proinflammatory cytokine production.

The influx of Bregs is BTLA⁺ and important for reactivating Tregs

Investigations of how Bregs become activated led to an examination of the role of the BTLA-HVEM-LIGHT pathway, since depending on the ligands' interactions to their HVEM receptor, this pathway can either be activating or suppressing^(34, 35). CD19⁺CD5⁺ B cells, from EAE mice treated with MOG-pσ1 (Fig. 5A), expressed BTLA (Fig. 5B, D, E), and these BTLA⁺ Bregs from MOG-pσ1-treated mice showed enhanced capacity for producing IL-10 unlike those BTLA⁺ CD5⁻ B cells (Fig. 5C, E). Although IL-10 production was mostly associated with Bregs in all treatment groups, MOG-pσ1 treatment of EAE mice resulted in a much higher percentage of IL-10-producing BTLA⁺ Bregs than was seen in either naïve mice or EAE mice treated with PBS or OVA-pσ1 (Fig. 5B, D). Thus, MOG-pσ1 enhances the frequency and the total number of IL-10-producing BTLA⁺ Bregs (Fig. 5B, D, E).

To determine if BTLA expression on Tregs was modulated as a consequence of EAE or MOG-pσ1 treatment, these Tregs were also examined (Table I). The percentage of BTLA⁺ Tregs did not change significantly as a consequence of treating EAE mice with MOG-pσ1 or OVA-pσ1 (Table I). Likewise, BTLA expression remained unchanged for CD25⁻ CD4⁺ T

cells (Table I). To determine whether Bregs enhanced the functionality of EAE-derived Tregs and contributed to EAE recovery, a T cell suppression assay was performed. To assess their function, splenic Tregs were either purified from naïve or EAE mice, and co-cultured with naïve CD25⁻CD4⁺ T_{eff} cells in the presence or absence of MOG-pσ1-induced Bregs. Interestingly, CD5⁺ Bregs, but not CD5⁻ B cells, were able to completely restore the inhibitory activity of EAE-derived Tregs (Fig. 6A). Of note, Bregs required the presence of Tregs since Bregs alone had no inhibitory effect upon T_{eff} cells, suggesting that Bregs act through Tregs to facilitate their inhibitory activity (Fig. 6A).

To confirm the relevance of BTLA expression by Bregs, studies were performed to assess whether blocking BTLA on MOG-pσ1-induced Bregs impacts the function of EAE-derived Tregs. MOG-pσ1-induced Bregs were pretreated with an anti-BTLA mAb or its isotype control. Isotype control-treated MOG-pσ1-induced Bregs were able to restore Treg function as evidenced by suppression of CD4⁺ T cell proliferation (Fig. 6B). In contrast, MOG-pσ1-induced Bregs treated with an anti-BTLA mAb blocked Bregs from interacting with EAE Tregs and the Tregs remained dysfunctional. BTLA^{-/-} Bregs were unable to activate EAE-derived Tregs, confirming the importance of BTLA expression on Bregs.

EAE exacerbation in BTLA^{-/-} mice is the result of the loss of IL-10 induction by Tregs and Bregs

BTLA^{-/-} mice induced with EAE exhibited significantly more severe clinical disease than did C57BL/6 mice ($p = 0.027$; Fig. 7A), similar to what others have shown (49). Moreover, these BTLA^{-/-} mice were refractive to MOG-pσ1 treatment (Fig. 7A). The observed EAE exacerbation was not due to an intrinsic defect by naïve BTLA^{-/-} Tregs since naïve BTLA^{-/-} Tregs retained the ability to inhibit CD4⁺ T cell proliferation and were able to proliferate after MOG₃₅₋₅₅ stimulation (Supplementary Fig. S1A-B). Further analysis of these BTLA^{-/-} (Foxp3⁺ CD25⁺) Tregs from EAE mice showed a failure to produce IL-10 relative to MOG-pσ1-treated BTLA-sufficient mice (Fig. 7B, C). Likewise, there were fewer IL-10-producing T_{eff} cells from BTLA^{-/-} mice when compared to C57BL/6 mice. Analysis of BTLA^{-/-} Bregs also showed their inability to upregulate IL-10 following MOG-pσ1 treatment (Fig. 7D, E). Thus, the absence of BTLA prevents the means to co-stimulate via BTLA, resulting in reduced IL-10 production by both Tregs and Bregs.

We next asked if MOG-pσ1-induced Bregs could restore Treg function in BTLA^{-/-} mice. To further address Tregs' dependency on BTLA⁺ Bregs, C57BL/6-derived BTLA⁺ Bregs were adoptively transferred into BTLA^{-/-} mice at the time of EAE induction, and recipients were monitored for development of disease. Consistent with the concept of requiring BTLA⁺ Bregs to complete Treg activation, BTLA^{-/-} recipients exhibited significantly less clinical disease (apparent by both delayed onset and reduced clinical score) than PBS-treated litter mates (** $p < 0.01$, Fig. 8A). In fact, very few Bregs were required since as few as 3×10^4 Bregs could effectively dampen EAE.

To determine which cytokines correlated with protection induced after Bregs' adoptive transfer, cytokine analyses were performed on purified splenic CD4⁺ T cells isolated at the peak of the disease (day 18 p.ch.). Cytokine production was significantly elevated in TGF-β and IL-6 as compared to PBS-treated mice with reduced IFN-γ and IL-17 production (* p

0.05, ** $p < 0.01$; Fig. 8B). No differences in IL-10 production were observed. These results show that MOG- $\rho 1$ -induced BTLA⁺ Bregs have a critical role in maintaining Treg function, and the adoptive transfer of BTLA⁺ Bregs was sufficient to suppress inflammation and disease as evidenced by reduced clinical scores, and less IFN- γ and IL-17 being produced.

Discussion

MS, the primary cause of paralysis in young adults, is a neurodegenerative disease of the CNS that involves infiltration by activated inflammatory cells, damaging both myelin and axons (13). EAE, a rodent model for MS, recapitulates and provides experimental support for the basic disease mechanism in MS (50). The role of B cells in MS and EAE has generated increasing interest (12, 46, 51-55). Distinct B cell subtypes have recently been associated with EAE disease progression and regulation (6, 7, 16, 53, 56-59), and are at least partially dependent on the choice of antigen (protein or peptide) used to induce the disease (18). A role for B cells has also been suggested in different models of recombinant MOG (rMOG)-induced EAE (18, 60-62). In general, when rMOG was used to induce EAE, pathogenic B cells were induced, and their depletion resulted in less EAE severity (18, 60-62). However, even these studies varied in the type of rMOG used, e.g., human (18) versus mouse (60-62). Whether a greater mobilization of Bregs can be achieved using MOG- $\rho 1$ in rMOG-induced EAE, remains to be determined.

Previous studies have shown that MOG₃₅₋₅₅-induced EAE does stimulate anti-MOG₃₅₋₅₅ Ab responses (23, 32, 63), but EAE can still be elicited in the absence of B cells (24, 50). Abs induced to different MOG peptides as a result of epitope spreading (64) or immunization with MOG protein do contribute to EAE pathogenicity (60-63, 65). Such evidence is consistent with the notion that Abs to the MOG protein are pathogenic to MS patients (66, 67). In contrast, a suppressive role for B cells has been suspected for many years (24, 68). The existence of a regulatory population of B cells has not yet been widely accepted, mainly because of the lack of a unique set of cell surface markers or a defined transcription factor. In mice, at least three different phenotypes have been associated with Bregs: O'Garra *et al* (68) first identified the CD5⁺ subpopulation among B cells responsible for IL-10 production; more recently, Bouaziz and colleagues have described CD1d^{Hi}CD5⁺ B cells, (generally referred as B10 cells) (69) while Evans *et al* attributed IL-10 production to CD21^{Hi}CD23⁺ cells (2B cells; 4). Recently, a role for GITR ligand-expressing (59), IL-35-producing (70), or PD-L1-expressing B cells (71) controlling autoimmunity independent of IL-10 has also been described, highlighting the complexity of the Breg-Treg interactions, where multiple mechanisms may be involved. Independent of the idea that Bregs constitute a true cell lineage or merely a state of activation by B cells, the production of IL-10 by a subset of B cells has proven critical for the resolution of EAE and other autoimmune disorders both in mice and humans (6, 10, 11, 21, 69). Bregs also have a role in the mechanism of action by Copaxone[®], a glatiramer acetate copolymer, approved for the treatment of relapsing-remitting MS (5, 12, 25, 52, 56). In a recent clinical trial, Ireland *et al* showed that treatment of relapsing-remitting MS patients with glatiramer acetate restored IL-10 production while reducing lymphotoxin- α production by peripheral B cells, thus contributing to the

therapeutic effects of glatiramer acetate (⁵³). This finding further supports our contention that IL-10-producing Bregs contribute to suppression of autoimmunity.

Upon encountering Ag, lymphocyte signaling via T or B cell receptor is modulated by a cognate signal from a co-stimulatory or co-inhibitory molecule (⁷²). One such B cell activation signal, CD40, has recently been found to be important for the generation of Bregs (⁷³). Moreover, the role of HVEM and its ligands BTLA, LIGHT, and CD160 has generated interest (^{30, 35, 44, 74-77}). HVEM is often referred to as a “molecular switch” of the immune system since it facilitates either a co-stimulatory or co-inhibitory signal. In addition, HVEM has bidirectional signaling capacity, transducing a co-inhibitory signal by the ligand (BTLA)-positive cells and simultaneously transducing a co-stimulatory signal for the HVEM (receptor)-positive cells (reviewed by Murphy et al., (³⁵)).

BTLA is highly expressed by anergic cells (⁷⁸), but for Tregs and T_{eff} cells remain unchanged despite EAE disease or MOG-pσ1 treatment (Table I). In EAE mice, we demonstrate that both Breg and Treg function is absent. Regarding the absence of Breg function in EAE mice, purified Bregs isolated from PBS-treated EAE mice adoptively transferred into EAE mice failed to reduce clinical disease (Fig. 3A). In contrast, purified Bregs from MOG-pσ1-treated EAE mice adoptively transferred into EAE mice reduced disease (Fig. 3B). Such reduction in clinical disease was the result of Breg-induced augmentation Tregs noted by the increased number of Tregs and their activation status to protect against EAE. This combination reduced the presence of IL-17-producing encephalitogenic T_{eff} cells (Fig. 4). Although BTLA levels on Bregs were not augmented as a consequence of MOG-pσ1 treatment, the percentage of and total BTLA⁺ Bregs producing IL-10 were enhanced, further showing that MOG-pσ1 activates these Bregs in an Ag-specific fashion. OVA-pσ1's inability to replicate MOG-pσ1's action was evidenced by the failure to elevate the percentage of or total number of activated IL-10-producing Bregs. Increases in IL-10 production by MOG-pσ1-treated mice were not significantly increased for CD5⁻ B cells. Aspects of Breg intervention were also recapitulated using a T suppressor assay in which MOG-pσ1-induced Bregs, but not CD5⁻ B cells, reversed the loss of T cell suppressor activity. Furthermore, neutralization of BTLA interfered with the ability of the MOG-pσ1-induced Bregs to restore EAE Treg suppressor activity (Fig. 6). In addition, IL-10 production by BTLA^{-/-} mice with EAE was substantially reduced by both Bregs and Tregs. These mice are refractive to MOG-pσ1 treatment of EAE, and clinical disease remained enhanced relative to BTLA-sufficient mice. Only upon adoptive transfer of BTLA-sufficient Bregs from MOG-pσ1-treated C57BL/6 mice did we observe reduction in EAE. Even though these donor cells were IL-10⁺, IL-10 production by BTLA^{-/-} mice remained undetectable; however, TGF-β was induced by this treatment suggesting alternative mechanisms may have been activated. The universality of BTLA⁺ Bregs' role to suppress EAE remains to be determined. It is important to bear in mind that EAE is a very heterogeneous disease in terms of its induction, clinical and pathological presentations, and mouse susceptibilities, which have raised some concerns about its relevance as a MS model (^{15, 79}).

The results presented here begin to address the paradox of having large numbers of Tregs at sites of chronic inflammation, but still having no apparent effect in subduing this

inflammation^(42,45). We know that EAE Tregs become activated subsequent MOG-pσ1 treatment since tetramer-specific Foxp3⁺ Tregs were previously found to express CD69⁽³⁰⁾. This stimulation reverses the Treg quiescent status, initiating a cascade of events that leads to the restoration of suppressor cell activity via the production of regulatory cytokines which abate clinical disease. Furthermore, the results from this present study show that Tregs purified from EAE mice remain inactive as evidenced by their inability to suppress polyclonally activated CD4⁺ T cell proliferation (Fig 1A). In contrast, Tregs from EAE mice treated with MOG-pσ1 showed restored suppressor activity prompting us to study their reactivation. Tregs purified from mice naturally recovering from the disease (5 or 6 weeks after EAE induction) also regained their capacity to inhibit CD4 T cell proliferation to a level similar to those Tregs derived from naïve mice. Future work will be needed to determine if the naturally recovered Tregs share similar suppressor mechanisms as those with the MOG-pσ1-induced Tregs.

Treg engagement of BTLA⁺ Bregs represents one mechanism for stimulating EAE Tregs. We are currently investigating whether this interaction is HVEM-dependent or whether other co-stimulatory molecules are involved. It has been shown that BTLA interacts in *cis* with HVEM on cells in which both BTLA and HVEM are expressed⁽³⁵⁾. The fact that BTLA acts *cis* with HVEM on Tregs offers one potential pathway by which BTLA⁺ Tregs are rendered quiescent resulting in the inability to control EAE. Tregs possibly become quiescent during EAE as a consequence of persistent co-inhibitory molecule signaling via BTLA on Tregs with concomitant down-regulation of activation or co-stimulatory molecules. The introduction of BTLA⁺ cells, much like the Bregs described here, could compete with Tregs' own BTLA, and be rendered active resulting in suppressor function restoration. Defining which specific ligands are induced on Tregs during EAE, and how these are regulated by MOG-pσ1 is currently being investigated.

The role of B cells in MS and EAE has generated significant interest, especially since the discovery of IL-10-producing B cells^(4, 21, 68, 69). Here we show that CD5⁺ Bregs dramatically restore Tregs' suppressive properties while B220⁺CD5⁻, BTLA⁻ B cells, or Bregs from diseased mice, do not. In fact, adoptive transfer of disease-induced Bregs into EAE mice failed to stimulate Treg production of regulatory cytokines. In contrast, the EAE recipients given MOG-pσ1-induced Bregs stimulated Tregs to generate regulatory cytokines. Furthermore, BTLA^{-/-} mice developed a severe form of EAE⁽⁴⁹⁾, but adoptive transfer of wild-type (BTLA⁺) Bregs restored protection against EAE (Fig. 8). These BTLA-expressing Bregs interact with Tregs, providing an alternative (or synergistic) pathway for Treg rescue. Of note, the derived Bregs, especially those induced with MOG-pσ1, cannot substitute for Tregs since they are unable to directly suppress T_{eff} cells. Rather, they are crucial modulators of Tregs' activity. In contrast, a recent publication⁽⁸⁰⁾ reported that innate B regulatory progenitors (c-kit^{low}Sca-1^{low} CD127⁺B220⁺CD19⁺IgM⁻CD1d^{int}CD43⁺) directly suppress CD4⁺ T cell proliferation. These data suggest a number of regulatory cell subsets, each dampening autoimmune disease.

In summary, these studies show a critical role for BTLA-dependent signaling in the activation of both Bregs and Tregs. The lack or failure of Treg activation has critical consequences for EAE induction, which in turn is important for amelioration of disease by

MOG-pσ1. Although previous studies (81, 82) have shown that Bregs are able to selectively induce Foxp3⁺ Treg proliferation, our work provides critical insights to explain how Treg function can be restored following their quiescent status during autoimmune disease. Moreover, our hypothesis proposes a method to restore Treg function, namely with the addition of therapeutically induced Bregs. Since both regulatory subsets are necessary to reduce EAE, utilizing the HVEM-BTLA pathway has promise in the development of effective therapies for inflammatory diseases such as MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this article

Breg	regulatory B cell
BTLA	B and T lymphocyte attenuator
HNLNs	head and neck lymph nodes
HVEM	Herpes virus entry mediator
IKDCs	interferon-producing killer dendritic cells
LIGHT	lymphotoxin-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T cells
MLNs	mesenteric lymph nodes
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
PLNs	peripheral LNs
p.ch.	post-challenge
pσ1	protein sigma one
SCs	spinal cords

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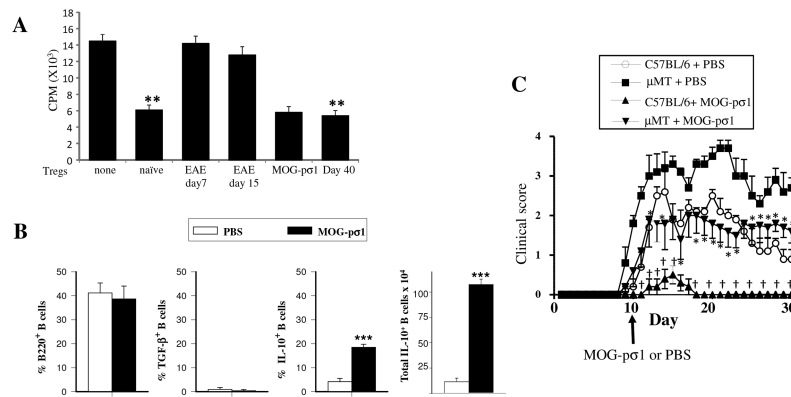
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**Figure 1.**

EAE derived Tregs are dysfunctional and B cells modulate EAE. (A) Tregs were purified from naïve or EAE mice (day 7 or 15 p.ch., scores 1 and 3 respectively), MOG-p01-treated mice (day 15 p.ch.), those naturally recovering from EAE (day 40, scores 0-1), and CD25⁻ CD4⁺ T_{eff} cells from naïve mice. Tregs were then co-cultured with T_{eff} cells (Tregs/effector ratio 1:2) in the presence of anti-CD3 plus anti-CD28 mAbs for 4 days before measuring extent of proliferation using a ³H-thymidine incorporation assay. A representative experiment of 5 is shown; ***p* = 0.01 versus naïve cell proliferation. (B) C57BL/6 mice (8/group) were induced with MOG₃₅₋₅₅ EAE, and 10 days later, treated with PBS or MOG-p01. The percentage and total combined LN and splenic B220⁺ B cells expressing TGF-β⁺ or IL-10 from individual mice was measured 20 days p.ch.; ****p* < 0.001 versus PBS-treated EAE mice. (C) C57BL/6 or B cell-deficient μMT mice were orally dosed with 50 μg MOG-p01 or PBS 10 days after EAE challenge and monitored daily for disease course. An average of 5 mice per group is represented; **p* < 0.05 versus PBS-dosed μMT, †*p* < 0.05 versus PBS-dosed C57BL/6 mice.

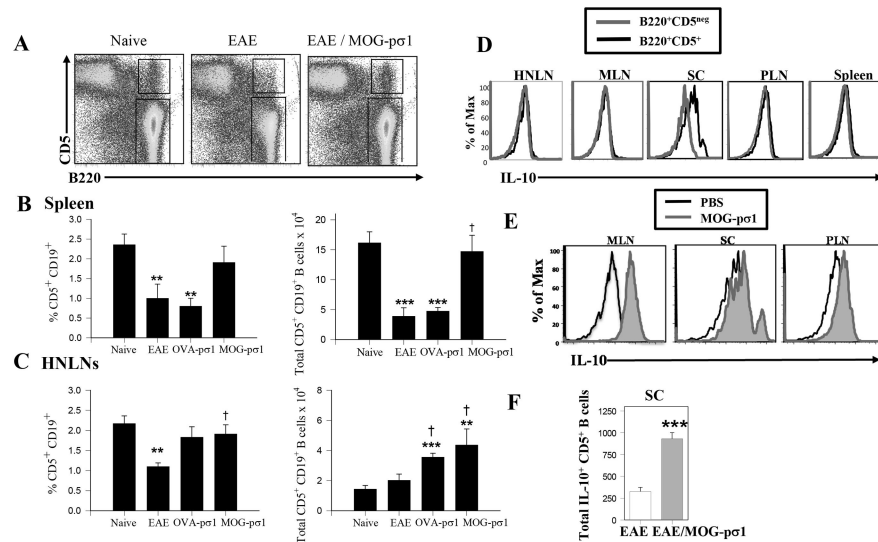
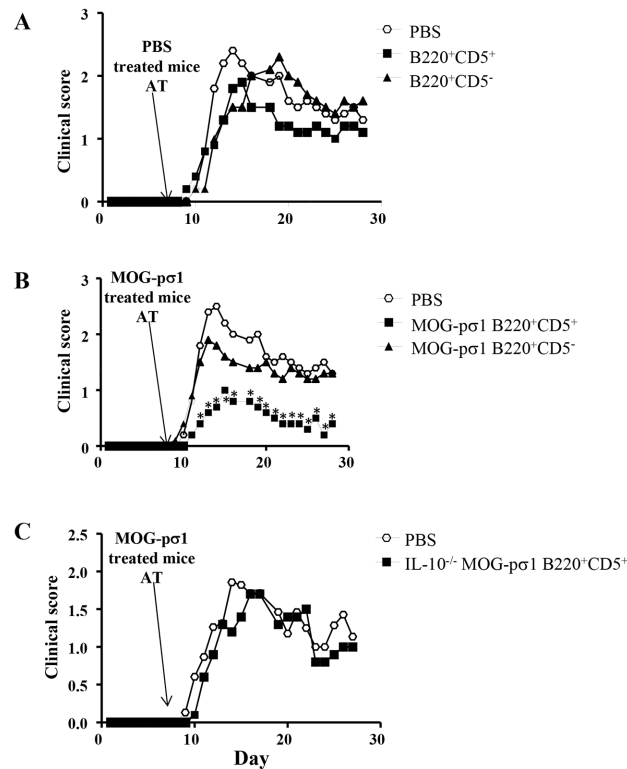


Figure 2.

MOG- $\rho\sigma 1$ intervention promotes the development of regulatory B cells (Bregs). EAE was induced on C57BL/6 mice, and 14 days later, mice were treated with PBS or MOG- $\rho\sigma 1$. (A) A representative dot-plot showing the percentage of B220⁺CD5⁺ Bregs is depicted. Bar graphs indicate mean (\pm SEM) percentages of and total (B) splenic and (C) HNLN CD19⁺CD5⁺ B cells (9 mice/group) in naïve mice, EAE mice, and EAE mice treated with OVA- $\rho\sigma 1$ or MOG- $\rho\sigma 1$; *** p < 0.001, ** p = 0.01, * p < 0.05 versus naïve B cells. (D) EAE mice were evaluated d14 p.ch. for IL-10 production by B220⁺CD5⁺ and B220⁺CD5^{neg} cells in HNLNs, MLNs, SCs, PLNs, and spleens. (E) EAE (day 14 p.ch.) mice (5/group) were analyzed 24h after PBS or MOG- $\rho\sigma 1$ treatment for intracellular IL-10 production for pooled SCs, MLNs, and PLNs. (F) The total number of IL-10-producing CD5⁺ B cells from the SCs of PBS- and MOG- $\rho\sigma 1$ -treated EAE mice is depicted (right panel); *** p = 0.001 vs. EAE mice. A representative experiment of three is shown.

**Figure 3.**

MOG-pσ1-, not PBS-induced Bregs, confers protection against EAE. C57BL/6 or IL-10^{-/-} mice (10/group) were induced with EAE and 2 weeks later treated with PBS or MOG-pσ1. Twenty four hours after MOG-pσ1 treatment, combined LNs and splenic B cells were sorted into B220⁺CD5⁻ B cells and B220⁺CD5⁺ B cell subsets, and 2.0x10⁶ B cells were adoptively transferred (AT) into C57BL/6 recipients (5/group) that were already induced with EAE 7 days earlier. (A) PBS-induced B220⁺CD5⁻ B cells or Bregs failed to ameliorate EAE. (B) MOG-pσ1-induced Bregs, not B220⁺CD5⁻ B cells were able to significantly reduce EAE severity. (C) In the same experiment, IL-10^{-/-} B220⁺CD5⁺ Bregs induced with MOG-pσ1 failed to significantly reduce EAE, indicating that their effect is IL-10-mediated. Panels A and B share the same control PBS group. **p* < 0.01 versus PBS-treated mice or recipients adoptively transferred with MOG-pσ1-induced B220⁺ CD5⁻ B cells.

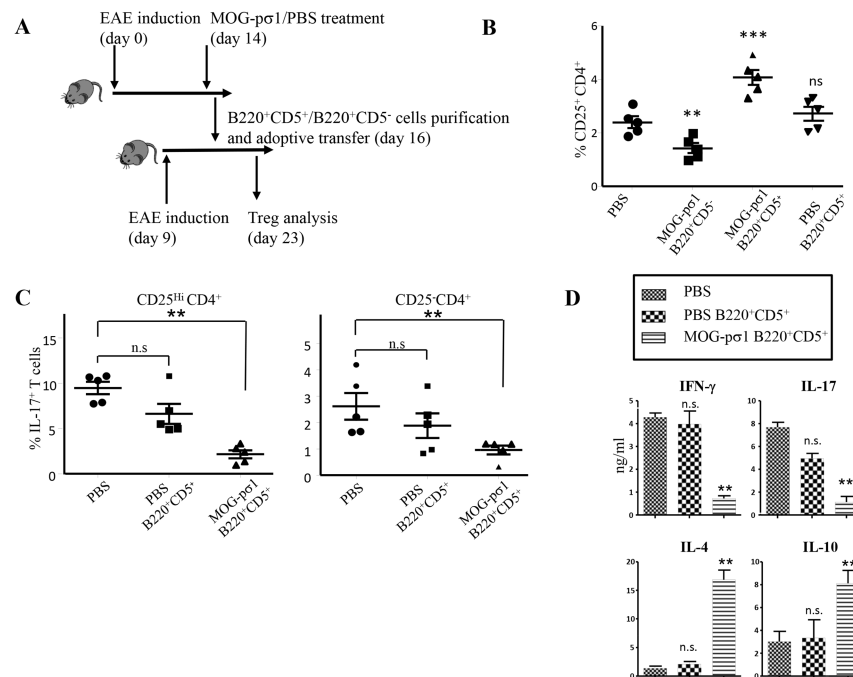


Figure 4. Adoptive transfer of MOG-pσ1-treated B220⁺CD5⁺ cells increases both the frequency and activity of CD25⁺CD4⁺Tregs in recipient mice. C57BL/6 mice (10/group) were treated with MOG-pσ1 or PBS 2 wks after EAE induction, and B220⁺CD5⁺ and B220⁺CD5⁻ cells were purified 48 h after treatment from combined LNs and spleens. B cells (10⁵ cells/mouse) were adoptively transferred into C57BL/6 mice (5/group) previously induced with EAE 7 days earlier. (A) A schematic depicting the experiment is provided. (B) The percentage of CD25⁺CD4⁺ T cells in each group was measured 1 wk later. Only recipients given MOG-pσ1-induced B220⁺CD5⁺ Bregs showed an increase in the percentage of CD25⁺CD4⁺ T cells; *** $p = 0.002$, ** $p < 0.01$ versus PBS-treated mice; ns, not significant. (C) Analysis of the percentage of IL-17-expressing cells between CD25^{Hi}CD4⁺ and CD25⁻CD4⁺ T cells. Recipients given MOG-pσ1-induced Bregs showed significantly fewer IL-17-producing cells; ** $p < 0.01$; ns, not significant. (D) Bregs stimulate Tregs to produce IL-4 and IL-10 and suppress IFN-γ and IL-17 production. CD25⁺CD4⁺ T cells were purified from PBS-treated mice and recipients adoptively transferred with PBS- or MOG-pσ1-induced B220⁺CD5⁺ Bregs, and stimulated in vitro with anti-CD3 + anti-CD28 mAbs. Cytokine levels (mean ± SEM from triplicate cultures) in culture supernatants were measured by ELISA 4 days after culture, and values corrected for spontaneous production from unstimulated cells; ** $p < 0.01$ versus PBS-treated mice; ns, not significant. Data are representative from two experiments.

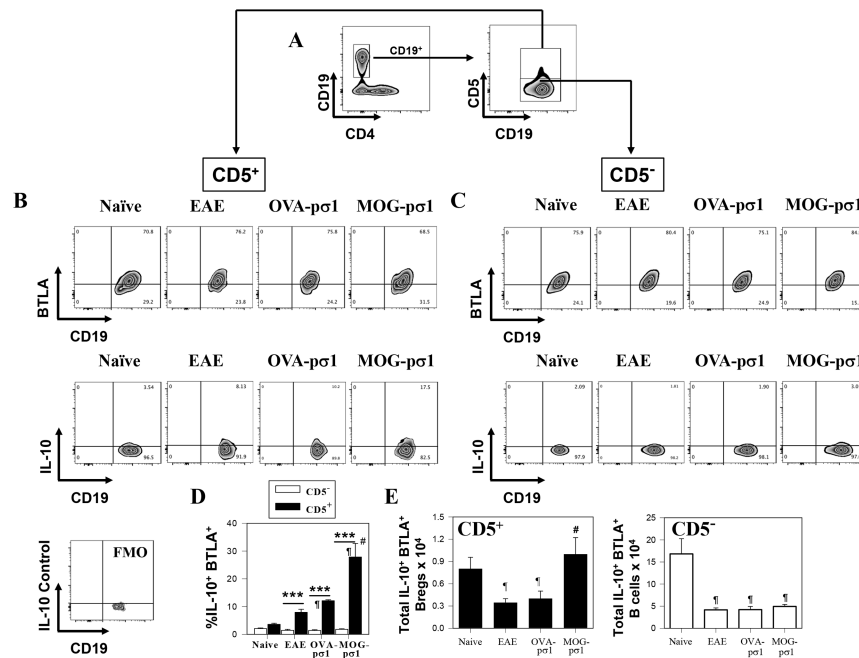


Figure 5. MOG-pσ1 intervention upon EAE promotes increased BTLA expression/activation of Bregs for enhanced IL-10 production. EAE was induced in C57BL/6 mice, and mice were treated with PBS, OVA-pσ1, or MOG-pσ1 at the peak of disease (day 14), and splenic B cells were subsequently evaluated for BTLA expression. (A) Gating strategy used to identify Bregs staining for CD19 vs. CD4, and CD19⁺ B cells further analyzed for CD5 expression. (B) CD5⁺ and (C) CD5⁻ CD19⁺ B cells from naïve, PBS-treated EAE, and MOG-pσ1-treated EAE mice were analyzed for BTLA and IL-10 expression; a fluorescence minus one (FMO) control histogram was performed for intracellular IL-10 staining. (D) The percent and (E) total IL-10⁺ BTLA⁺ CD5⁺ or CD5⁻ B cells ± SEM from individual mice from naïve (n=9), PBS- (n=11), OVA-pσ1- (n=9), and MOG-pσ1-treated (n=11) mice are shown; ****p* < 0.001, **p* < 0.05 versus the corresponding CD5⁻ B cell subset; #*p* < 0.005 versus EAE mice; and †*p* < 0.05 versus naïve mice.

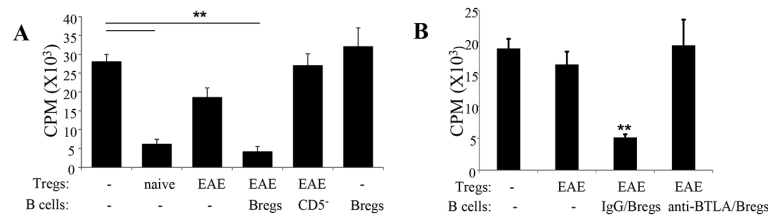


Figure 6.

Bregs activate EAE Tregs to restore Treg function. (A, B) To test if Bregs can reactivate Tregs *in vitro*, (A) splenic Tregs obtained from naïve or EAE mice were stimulated as described in Fig. 1A, co-cultured with naïve CD4⁺ T cells in the absence or presence of CD19⁺ CD5⁺ Bregs or CD19⁺ CD5⁻ B cells obtained from MOG-p01-treated EAE mice, and measured their ³H-thymidine incorporation 4 days later (Tregs/B cells/T_{eff} cells ratio 1:1:2). As a control, naïve CD4⁺ T cell proliferation in the presence of B220⁺ CD5⁺ cells (without Tregs) was also included. An average of 6 replicates/treatment group is depicted, and these data are representative of three experiments; ***p* < 0.01 versus naïve T cell proliferation. (B) Tregs obtained from naïve or EAE mice were co-cultured with naïve CD4⁺ T cells in the presence or absence of B220⁺ CD5⁺ Bregs (Tregs/Bregs/T_{eff} cells ratio 1:1:2), and the extent of CD4 T cell proliferation was measured 4 days later. Where indicated, B220⁺ CD5⁺ Bregs were pretreated with an anti-BTLA mAb. An average of 6 replicates/treatment group is depicted, and these data are representative of two experiments; ***p* < 0.01 versus naïve T cell proliferation.

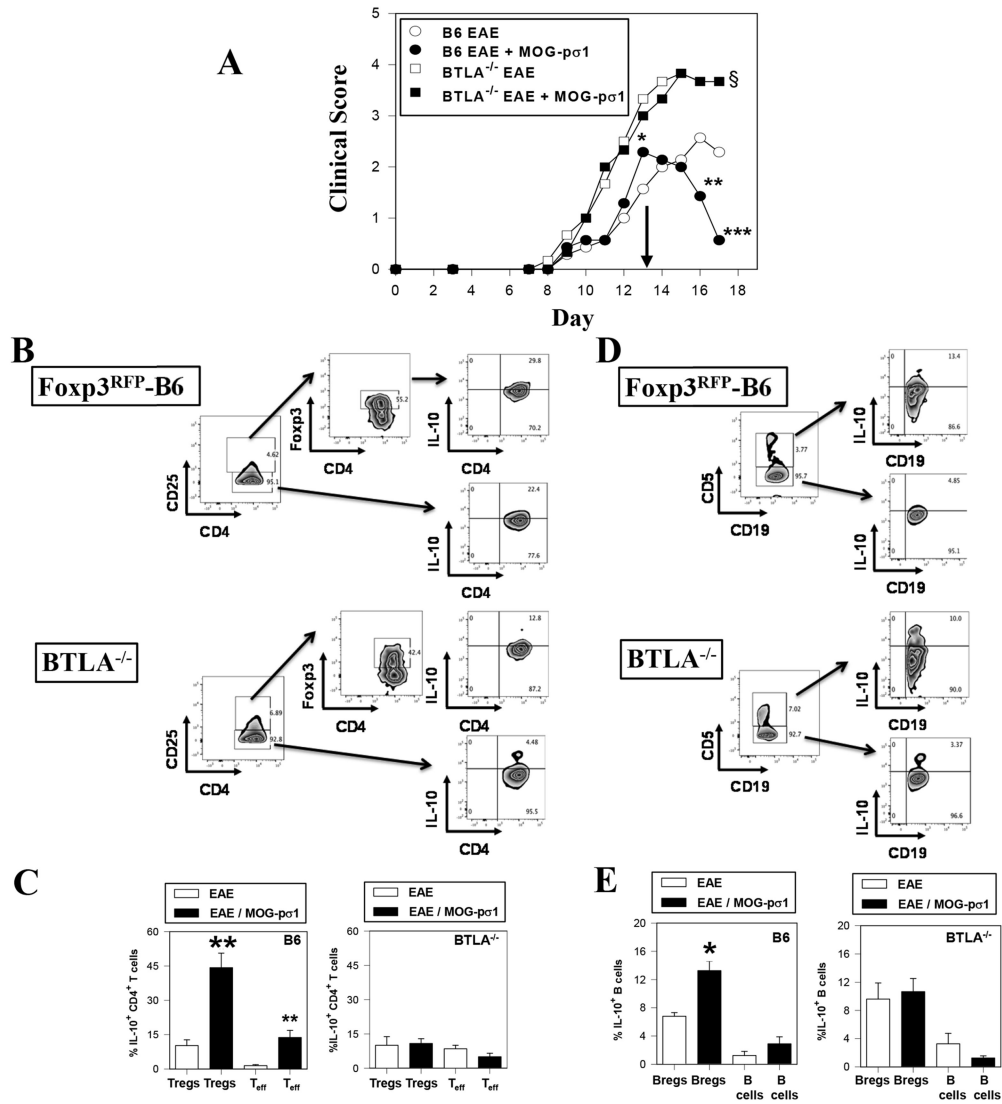


Figure 7. A lack of IL-10 activation in BTLA^{-/-} accounts for increased susceptibility to MOG-induced EAE. (A) Groups of C57BL/6 and BTLA^{-/-} mice were induced with EAE on day 0, and were subsequently treated with PBS (B6 mice, n=7; BTLA^{-/-} mice, n = 6) or MOG-pσ1 (B6 mice, n=7; BTLA^{-/-} mice, n = 6) on day 13 (arrow). Clinical scores were measured throughout the disease; ****p* < 0.001, ***p* < 0.005, **p* = 0.036 versus PBS-treated B6 mice; §*p* < 0.030 for PBS- or MOG-pσ1-treated BTLA^{-/-} mice versus PBS-treated B6 mice. (B) MOG-pσ1-treated BTLA^{-/-} mice failed to show an induction of IL-10. (B-E) Additional groups of PBS- and MOG-pσ1-treated Foxp3^{RFP}-B6 and BTLA^{-/-} mice were treated as described in (A), and four days after treatment, isolated HNLNs were evaluated for IL-10 expression by (B and C) Tregs and T_{eff} cells and (D and E) CD5⁺ Bregs and CD5⁻ B cells. In BTLA-sufficient mice, MOG-pσ1 was able to significantly induce IL-10 by both (C) Tregs and (E) Bregs while no changes in IL-10 production by these same cells from BTLA^{-/-} mice remained unchanged; ***p* = 0.003, **p* = 0.019 versus cells from EAE mice. Depicted data are representative of two experiments.

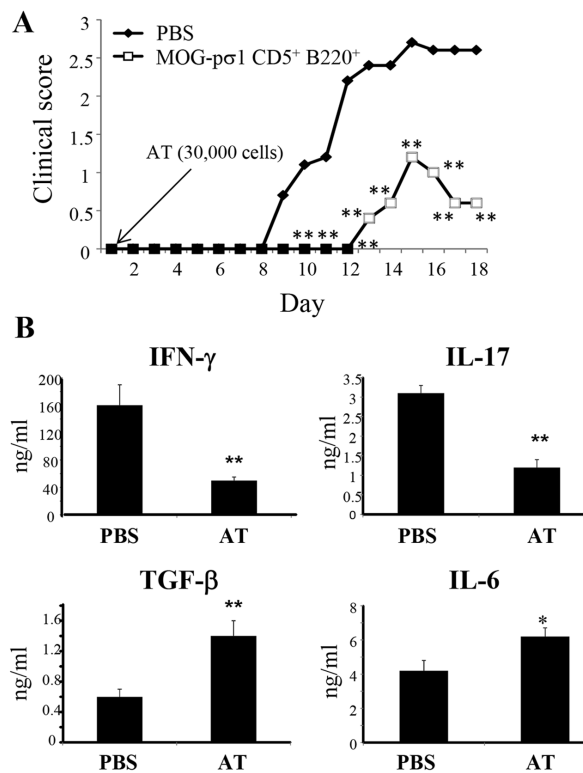


Figure 8.

Adoptive transfer of Bregs restores protection to EAE in $BTLA^{-/-}$ mice. (A) $BTLA^{+} CD19^{+} CD5^{+}$ Bregs were purified from MOG-pσ1-treated C57BL/6 mice and 3×10^4 Bregs were adoptively transferred (AT) into $BTLA^{-/-}$ mice at the time of EAE induction; clinical scores were measured throughout the disease (5/group, ** $p < 0.01$). (B) Cytokine analyses were performed on purified splenic $CD4^{+}$ T cells at the peak of the disease (day 18 p.ch.). Purified $CD4^{+}$ T cells were cultured for 4 days in the presence of MOG₃₅₋₅₅ (5 μg/ml) and irradiated APCs. Cytokine production showed significant elevations in TGF-β and IL-6 when compared to PBS mice with concomitant reductions in IFN-γ and IL-17 production (* $p < 0.05$, ** $p < 0.01$).

Table I

. Percent BTLA⁺ Tregs following treatment of EAE mice with PBS, OVA-pσ1, or MOG-pσ1

Treatment Group ^a	%BTLA ⁺ Foxp3 ⁺ CD25 ⁺ CD4 ⁺ T cells ^b		%BTLA ⁺ CD25 ⁻ CD4 ⁺ T cells ^b	
	Mean ± SEM	p-value ^c	Mean ± SEM	p-value ^c
Naive (no EAE)	17.52 ± 0.79	NS	9.07 ± 2.08	NS
PBS-treated EAE	26.40 ± 5.64	-	13.22 ± 3.29	-
OVA-pσ1-treated EAE	27.86 ± 8.61	NS	14.15 ± 5.90	NS
MOG-pσ1-treated EAE	30.72 ± 4.95	NS	18.40 ± 5.99	NS

^a C57BL/6 mice were induced with EAE, and treated with PBS or 50 μg of OVA-pσ1 or MOG-pσ1 orally at the peak of EAE.

^b FACS analysis was performed on BTLA⁺ Tregs and CD25⁺ CD4⁺ T cells isolated from the spleen. Mean ± SEM of 5-6 mice per group is presented.

^c Statistical significance was calculated by the one-way ANOVA to test differences among the treatment groups relative to PBS-treated EAE mice; NS = not significant.