HHS Public Access

Author manuscript

J Leukoc Biol. Author manuscript; available in PMC 2024 July 31.

Published in final edited form as:

J Leukoc Biol. 2018 December ; 104(6): 1147–1157. doi:10.1002/JLB.3A0218-071RRR.

Production of IL-35 by Bregs is mediated through binding of BATF-IRF-4-IRF-8 complex to *il12a* and *ebi3* promoter elements

Cheng-Rong Yu,

Jin Kyeong Choi,

Anita N. Uche,

Charles E. Egwuagu

Molecular Immunology Section, Laboratory of Immunology, National Eye Institute, National Institutes of Health, Bethesda, Maryland, USA

Abstract

IL-10 and IL-35 suppress excessive immune responses and therapeutic strategies are being developed to increase their levels in autoimmune diseases. In this study, we sought to identify major cell types that produce both cytokines in-vivo and to characterize mechanisms that regulate their production. Experimental autoimmune uveitis (EAU) is a CNS autoimmune disease that serves as model of human uveitis. We induced EAU in C57BL/6J mice and investigated whether T cells, B lymphocytes, or myeloid cells are the major producers of IL-10 or IL-35 in blood, lymph nodes (LNs), spleen, and at the site of ocular inflammation, the neuroretina. Analysis of these tissues identified B cells as the major producers of IL-10 and IL-35 in-vivo. Compared to regulatory T cells (Tregs), IL-10- or IL-35-producing regulatory B cells (Bregs) are substantially expanded in blood, LNs, spleen, and retina of mice with EAU. We performed EMSA and chromatin immunoprecipitation (ChIP) assays on activated B cells stimulated with IL-35 or TLR agonists. We found that BATF, IFN regulatory factor (IRF)-4, and IRF-8 transcription factors were recruited and bound to AP1-IRF-composite elements (AICEs) of *il12a*, *ebi3*, and/or *il10* loci, suggesting their involvement in regulating IL-10 and IL-35 transcriptional programs of B cells. Showing that B cells are major source of IL-10 and IL-35 in-vivo and identifying transcription factors that contribute to IL-10 and IL-35 expression in the activated B-cell, suggest that the BATF/IRF-4/IRF-8 axis can be exploited therapeutically to regulate physiological levels of IL-10/IL-35-Bregs and that adoptive transfer of autologous Bregs might be an effective therapy for autoimmune and neurodegenerative diseases.

Correspondence: Charles E. Egwuagu, Molecular Immunology Section, National Eye Institute, National Institutes of Health, Building 10, Room 10N248G, 10 Center Drive, Bethesda, MD 20892–1857, USA. egwuaguc@nei.nih.gov.

AUTHORSHIP

C.-R.Y. conducted the EAU experiments, prepared the figures and edited the manuscript; J.K.C., assisted with EAU and other experiments; A.N.C., assisted with EAU experiments; C.E.E., conceived, designed and supervised the project and wrote the manuscript.

DISCLOSURES

The authors performed this study as part of their duties as United States Government employees and do not have any financial interest in relation to the submission.

Keywords

AP1-IRF complex; experimental autoimmune uveitis; IL-12p35; *ebi3*; retina; uveitis

1 | INTRODUCTION

The healthy immune system induces measured and appropriate responses to pathogens while exhibiting tolerance to innocuous or self-antigens. Central tolerance mechanism ensures the elimination of lymphocytes capable of inducing tissue pathology, whereas specialized regulatory immune cells contribute to peripheral tolerance mechanisms by secreting anti-inflammatory cytokines that curtail excessive immune responses and mediate rapid resolution of inflammatory diseases.[1] Specialized regulatory immune cells play important roles in limiting inflammation by secreting anti-inflammatory cytokines that curtail excessive immune responses and mediate rapid resolution of inflammatory diseases. Thus, reduced level of regulatory cells or immune-suppressive mediators is associated with persistent activation of proinflammatory cells and development of chronic inflammatory diseases. Developing biologics or immune-modulation strategies that target proinflammatory lymphocytes and/or cytokines is therefore a major objective in clinical management of autoimmune disease patients.

There is significant interest in adoptive immune-suppressive cell transfer as a potential treatment for autoimmune diseases.[2]Regulatory lymphocyte populations specialized in secreting large amounts of IL-10 and/or IL-35 are of particular interest because they have recently been shown to be critical regulators of immunity during autoimmune and infectious diseases.[3–6] T cells that suppress inflammation are collectively called T regulatory cells (Tregs). They include naturally occurring $CD4^+CD25^+Foxp3^+$ Tregs, induced $CD4^+CD25^+Foxp3^-$ Tregs, regulatory Th17 cells, and a variety of ill-defined Treg populations (Tr1, Tr3, $CD8^+CD28^-$, and Qa-1-restricted).[7] For the most part, Tregs suppress inflammation through the production of IL-10 and to a lesser extent, TGF-β.[8] On the other hand, B lymphocytes have historically been associated with inducing autoimmune pathology through the production of autoantibodies and by serving as Ag-presenting cells to auto-reactive T cells.[9] However, recent clinical trials using the B-cell-depleting CD20 Ab (Rituximab) in patients with arthritis or multiple sclerosis provided evidence that unique B-cell subsets possess immune suppressive activities.[10–13] These relatively rare immune-suppressive B cells in humans and mice are phenotypically diverse and collectively referred to as regulatory B cells (Bregs). The Bregs are phenotypically similar to B10 and B-1a cells and may derive from marginal zone or transitional 2-marginal zone precursor B cell subsets.[14] Although no unique marker or set of markers exclusively identifies the Bregs, they share the common attribute of suppressing immune responses through the production of IL-10 or TGF-β in response to activation by TLR, CD40, and/or B cell Ag receptors signaling pathways.[14] A Treg population that secretes IL-35 (iTr35) has been described in humans and mice and they mediate infectious tolerance and promote tumor progression through IL-35-dependent mechanisms independent of IL-10 or TGF-β.[15] This discovery was followed by description of B cells that produce IL-35.[3,4,15]

During embryonic development, the vertebrate retina and optic nerve originate as outgrowths of the diencephalon of the developing brain. There are 5 types of neurons in the retina: photoreceptors, bipolar cells, horizontal cells, ganglion cells, and amacrine cells. In humans, there are approximately 125 million Photoreceptors (rods and cones) that convert photons into sensory signals, which are transduced through bipolar and ganglion cells to the optic nerve and then to the brain. Consequently, the retina is widely considered a brain tissue and part of the CNS. Experimental autoimmune uveitis (EAU) is a well-characterized animal model of human uveitis and the most widely used mouse model of ocular inflammation.[25,26] EAU shares essential immunopathogenic features as experimental autoimmune encephalomyelitis, and both are considered as models of CNS autoimmune diseases.[27] In previous reports, we identified a unique B cell subpopulation that produced both IL-10 and IL-35 (i35-Bregs) and showed that i35-Bregs increase significantly during EAU and in their absence, mice develop exacerbated uveitis.[4] Furthermore, adoptive transfer of Bregs to mice with EAU suppressed uveitis and ameliorated ocular pathology by inhibiting the expansion of pathogenic Th17 cells.[4,16] Collectively, these studies helped establish the critical roles of IL-35 in regulating infectious and autoimmune diseases. In this. study, we have used the EAU mouse model to examine the major cell types that produce IL-35 during CNS inflammation and have characterized mechanisms that regulate production of IL-35 by B cells

2 | MATERIALS AND METHODS

2.1 Mice and human PBMCs

Six- to 8-week-old C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Both male and female mice were used and the mice were randomized for all the studies described. Mice were maintained and treated in accordance with National Institutes of Health (NIH) Animal Care and Use Committee guidelines and all animal studies were approved under the animal Study Protocol # NEI-597. Human PBMCs were obtained from the NIH Blood Bank administered by the NIH Department of Transfusion Medicine. Consent to donate blood was obtained from healthy human volunteers as required by NIH Institutional Review Board-approved patient protocols and adhered to the Declaration of Helsinki.

2.2 | Isolation of human B cells

PBMCs of normal human subjects were isolated from buffy coats by density gradient centrifugation by using a commercially available Lymphocyte Separation Medium (Mediatech. Inc., Manassas, VA). Human $CD19^+$ B cells were sorted using anti-CD19 Ab-conjugated magnetic beads (Miltenyl Biotec, Auburn, CA).

2.3 | Induction of EAU and histology

We induced EAU by active immunization with bovine interphotoreceptor retinoid-binding protein (IRBP; 150 μg) and human IRBP peptide (300 μg amino acid residues 1–20) (Bio Basic Canada, Canada) in a 0.2 ml emulsion (1:1 v/v with complete Freund's adjuvant [CFA]) containing mycobacterium tuberculosis strain H37RA (2.5 mg/ml) (Bifco, Morrice, MI). Mice also received Bordetella pertussis toxin (0.2 μg per mouse) (Sigma Aldrich, St.

Luis, MO) concurrent with immunization. For each study, 8 mice were used per group and they were matched by age and sex. Clinical disease was established and monitored by fundoscopy.[4,17] Eyes for histological evaluation were harvested 21 days postimmunization, fixed in 10% buffered formalin, and serially sectioned in the vertical pupillary-optic nerve plane. All sections were stained with hematoxylin and eosin.

2.4 | Imaging mouse fundus

Funduscopic examinations were performed at day 14 and 20 after EAU induction using a modified Karl Storz veterinary otoendoscope coupled with a Nikon D90 digital camera, as previously described.[18] Briefly, following systemic administration of systemic anesthesia (intraperitoneal injection of ketamine [1.4 mg/mouse] and xylazine [0.12 mg/mouse]) (NIH pharmacy, Bethesda, MD), the pupil was dilated by topical administration of 1% tropicamide ophthalmic solution (Alcon Inc., Fort Worth, TX). To avoid a subjective bias, evaluation of the fundus photographs was conducted without knowledge of the mouse identity by a masked observer. At least 6 images (2 posterior central retinal view and 4 peripheral retinal views) were taken from each eye by positioning the endoscope and viewing from superior, inferior, lateral, and medial fields and each individual lesion was identified, mapped, and recorded. The clinical grading system for retinal inflammation was as previously established.[19]

2.5 | Retinal cells isolation

To characterize inflammatory cells that cross the blood-retina barrier during EAU, mice were anesthetized and perfused with 1× PBS. Enucleated eyes were put in Petri dish containing culture medium (RPMI-1640) for immediate isolation of the retina under a dissecting microscope. The eye was cut along the limbus of the eye and the lens and cornea were carefully removed. Then, the retina was peeled off and the attached optic nerve was removed before digesting the freshly isolated retina with collagenase (1 mg/ml) in RPMI medium containing 10 μg/ml DNase (Sigma–Aldrich, Sigma Aldrich, St. Luis, MO) for 2 h at 37°C. During incubation, the cells were pipetted intermittently every 30 min and the digestion reactions was quenched with 5- to 10-folds volume 10% FBS in RPMI 1640 medium. The cells were washed twice in completed IRMI-1640 medium and counted using the Vi-Cell XR cell viability analyzer (Beckman Coulter).

2.6 | Characterization of Bregs and Tregs

Primary B cells isolated from the blood, spleen, or draining lymph node (LN) of unimmunized or EAU mice were sorted for $CD19^+$ or $B220^+$ cells and used for surface and intracellular FACS analysis. Some cells were reactivated with IRBP and anti-CD40 Ab as previously described.[4] For intracellular cytokine detection, cells were restimulated for 5 h with PMA (20 ng/ml) and ionomycin (1 μM). GolgiStop (BD PharMingen, San Diego, CA) was added in the last hour, and intracellular cytokine staining was performed using the BD Biosciences Cytofix/Cytoperm kit as recommended (BD Pharmingen, San Diego, CA). FACS analysis was performed on a MACSQuant analyzer (Mitenyi Biotec, San Diego, CA) using protein-specific monoclonal Abs and corresponding isotype control Abs (Pharmingen, San Diego, CA) as previously described by using protein-specific monoclonal Abs and corresponding isotype control Abs (PharMingen) as described.[4] Dead cells were

stained with dead cell exclusion dye (Fixable Viability Dye eFluor 450; eBioscience), and live cells were subjected to side-scatter and forward-scatter analyses. Bregs and Tregs were characterized by expression of CD4, CD19, CD5, CD27, CD38, CD138, B220, CD1d, IL-10 (BD Pharmingen, San Diego, CA), IL-12p35 (p35), and/or Ebi3 (R&D, Minneapolis, MN). FACS analysis was performed on cells stained with monoclonal Abs conjugated with fluorescent dyes; dead cells were excluded and each tube of cells was color compensated. Quadrant gates were set using isotype controls with less than 0.3% background.

2.7 | Western blotting analysis

Preparation of whole cell lysates and performance of Western blot analysis were as described.[20] Cell extracts (20–40 μg/lane) were fractionated on 4–12% gradient SDS-PAGE in reduced or non-educed condition and Western blot analysis was performed using Abs specific to IL-12p35, Ebi3, BATF, IRF-4, and IRF-8 or β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA or Cell Signaling Technology, Danvers, MA). Preimmune serum was used in parallel as controls and signals were detected with HRP-conjugated secondary $F(ab')_2$ Ab (Zymed Laboratories, San Francisco, CA) using the ECL-PLUS system (Amer-sham, Arlington Heights, IL). Each Western blotting analysis was repeated at least 3 times and band intensities were quantified using Image J and normalized to β-actin

2.8 | Detection of cytokine secretion by ELISA

$CD19^+$ B cells from EAU mice were reactivated in vitro with IRBP in the presence or absence of rIL-35 and culture supernatants were collected after a 48-h incubation. IL-10 secretion was quantified using ELISA Kit (R&D Systems, Minneapolis, MN).

2.9 | Chromatin immunoprecipitation analyses

Chromatin immunoprecipitation (ChIP) assays were performed using EZ Chip™ ChIP kits (Millipore Sigma, Darmstadt, Germany). Briefly, activated B cells were stimulated with recombinant mouse IL-35[4] or human recombinant IL-35 (rhIL-35 [Enzo Life Science, Farmingdale, NY], 20 ng/ml), and DNA-protein complexes were cross-linked for 10 min by addition of fresh formaldehyde (Sigma) to the culture medium at a final concentration of 1%, followed by quenching in 135 mM glycine. The cells were then washed in cold PBS (2×), lysed (EZ Chip™ lysis buffer), and sonicated (5×) in 15 s bursts (output 5 on Sonic Dismembrator Model 1000; Fisher Scientific). Lysates were then cleared with Protein G-agarose for 1 h, pelleted, and incubated overnight with control IgG or anti-BATF Ab (Cell signaling). Prior to Ab incubation, input samples were removed from the lysate and stored at −80°C until extraction. Immunoprecipitation was performed according to the manufacturer's instructions (EZ Chip™). The immunoprecipitated and input DNA were subjected to PCR using the following mouse *il12a* or *ebi3* primers: *il12a* region 1 primer (–1446 to –980), 5′-TCCTATAGCATTGACCTCTATCTC-3′ & 5′-CCC TCCTGAAGAAGTCCTGAAATG-3′; *il12a* promoter region 2 (−1933 to −1718), 5′-GTCGTCTCTGCTGGACATTATGA-3′ & 5′-TGTGGTGCTGAGGGCTGATTTC-3′; *ebi3* promoter region 1 (+49 to −288), 5′-TGATGATGGTGACGGGAACC-3′ & 5′-AGGTGGTAGTT GCTCCTTGTTGTC-3′; *ebi3* promoter region 2, 5′-CGCTCTC TTGCCTCTCTTGCCT-3′ & 5′-AT GTGGCTCTTTAGGGGAAG GC-3′ (–2429 to –

2188); *ebi3* promoter region 3 primer, 5′-ATCAG CAATGGCAG TTCAATCC-3′ & 5′-TGAGGCAAGCAGAAATC AGTGAC-3′ (−3679 to −3389).

2.10 | Electrophoretic mobility shift assay

EMSA was performed as described.[21] The double-stranded oligonucleotides containing motifs from the AICEs, 5′TGAnTCA/GAAA-3′,[22–24] were labeled by a fill-in reaction using Klenow polymerase (New England BioLabs, Beverly, MA) with [alpha-P^{32}]dATP or [alpha-32P]dGTP (3000 Ci/mmol; PerkinElmer Inc., Waltham, MA). Sorted $CD19^+$ B cells were stimulated with LPS (1 μg/ml) in the presence or absence rIL-35 (20 μg/ml) for 3 days and nuclear extracts were prepared in buffer containing the following pro-tease inhibitors: 2 μM leupeptin, 2 μM pepstatin, 0.1 μM aprotinin, 1 mM [4-(2-Aminoethyl)benzenesulfonyl fluoride, hydrochloride], 0.5 mM phenylmethyl-sulfonyl fluoride, and 1μM E-64 [*N*-(*N*-l-*trans*-carboxyoxiran-2-carbonyl)-l-leucyl]agmatine as described in previous study.[21] Protein levels were determined by the Bicinchoninic acid assay (BCA) method as recommended, and extracts were stored at −70°C until use. DNA-protein binding reaction was performed in a 20-μl mixture containing 5 μg nuclear protein and 1 μg double-stranded poly(d1:C) (Boehringer Mannheim, Pleasanton, CA), 12 mM HEPES (pH 7.9), 60 mM KCI, 0.5 mM DTT, 12% glycerol, and 2.5 mM MgCI. After 15-min incubation on ice, samples were further incubated with 1 μl P^{32}-labeled probe (15,000 cpm) at room temperature for 20 min and fractionated on 5% native polyacrylamide gel in 0.25xTris-borate-EDTA buffer. For super-shift analysis, before the addition of ^{32}P-labeled probes, extracts were preincubated with 1 μl Abs specific to BATF (Cell Signaling Technology, Danvers, MA), Jun B, Jun D, IRF-4, IRF-8, or IRF-1 (Santa Cruz Biotechnology).

2.11 | Statistical analysis

Statistical analyses were performed by independent 2-tailed Student's *t*-test. Probability values of ≤0.05 were considered statistically significant. The data are presented as mean + SEM. Asterisks denote *P*-value (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

3 | RESULTS

3.1 | Bregs are a major source of IL-10 and IL-35 during EAU

We used the EAU model to investigate the major sources of immune-suppressive cytokines that suppress CNS autoimmune diseases. EAU is induced in susceptible mouse strains by active immunization with the ocular auto-antigen IRBP in CFA supplemented with mycobacterium tuberculosis strain H37RA and pertussis toxin.[25,26] Similar to other organ-specific autoimmune diseases, while induction of EAU requires adjuvant,[28] the H37RA/pertussis toxin-supplemented CFA by itself cannot induce EAU.[25,26]

The development and progression of EAU were established and monitored by funduscopy, a procedure that uses an ophthalmoscope (funduscope) to view the inside of the fundus of the eye at various time points during the course of an intraocular inflammatory disease. Fundus images taken at day 14 and day 20 postimmunization established the development of characteristic clinical features of uveitis in the mice. The observed hallmark features of uveitis included blurred optic disc margins and enlarged juxtapapillary area, retinal vasculitis

with moderate cuffing and yellow–whitish retinal and choroidal infiltrates (Fig. 1A, top panels). Eyes were also enucleated 14 or 21 days after EAU induction, and histological analysis revealed the presence of substantial numbers of inflammatory cells in the vitreous, photoreceptor cell damage, choroiditis, and retinal in-folding (Fig. 1A, bottom panels). Clinical scores and assessments of disease severity were based on pathological changes at the optic nerve disc and retinal tissues (Fig. 1A; graph to the right). We next analyzed the inflammatory cells that mediate or regulate the disease and found significant increases in the absolute numbers of inflammatory cells present in the blood, draining LNs, and spleen of mice with EAU compared to unimmunized (control) mice (Fig. 1B). Consistent with established roles of Th1 and Th17 cells in EAU pathology,[4] intracellular cytokine staining analysis revealed marked increases in the frequency of IFN-γ-secreting and IL-17-producing T cells in the blood of mice with EAU (Fig. 1C). We also observed significant increases in frequency of IL-10 producing lymphocytes in the blood (Fig. 1C) and spleen (Fig. 1D) of EAU mice and this is in line with the critical roles of regulatory lymphocytes in mitigating CNS autoimmune diseases.[3,4,16,29,30] It is notable that there was a ~7-fold increase in the percentage of IL-10-producing B cells compared to IL-10-producing T cells (Fig. 1C and D). Further analysis revealed that IL-10-producing B cells in the blood during EAU derived from both the B-1 ($CD19^+CD11b^+$) and B-2 ($CD19^+CD11b^-$) B cell compartments, suggesting that Bregs that suppressed EAU can be of the adoptive B-2 (Fig. 1E) or the innate B-1a (Fig. 1F) lineage. Although absolute numbers of myeloid or T cells were much higher compared to B cells (Fig. 1G), we observed that the absolute numbers of IL-10-producing Bregs in the blood of mice with EAU was higher compared to IL-10-producing myeloid or T cells (Fig. 1H), suggesting that Bregs were preferentially activated. Similar to the blood, the frequency of IL-10-producing B cells in the spleen of mice with EAU was substantially higher in comparison to IL-10-producing T cells (Fig. 1H). Characterization of the IRBP-specific splenic B cells revealed that they were predominantly of the $CD1d^{Hi}CD138^{Hi}$ cells immunophenotype (Fig. 1I) and secretion of IL-10 was confirmed by ELISA (Fig. 1J).

Recent reports indicate that Bregs that produce IL-35 (i35-Bregs) play critical roles in regulating CNS autoimmune diseases.[3,4] Similar to our analysis of IL-10-producing Bregs, we investigated whether B cells are a major source of IL-35 production during EAU. Comparative analysis of the abundance of IL-35-producing cells in peripheral lymphoid tissues of mice with EAU showed that i35-Breg levels are highest in the blood (24.7%) compared to the spleen (8.6%). Furthermore, B cells are a major source of IL-35 as the level of i35-Bregs was comparable to all non-B cell types, albeit 2–3% and ~5% lower in the spleen or blood (Fig. 2A). It is notable that a substantial percentage of the IL-35-producing B cells also produced IL-10 (Fig. 2B) and were characterized by a $CD19^+B220^{Lo}CD38^+CD138^+$ immunophenotype (Fig. 2C and D). These results indicate that Bregs are a major source of IL-10 and IL-35 in secondary lymphoid tissues of mice with EAU.

3.2 | Bregs are recruited into the retina during EAU

We next investigated whether suppression and amelioration of uveitis required the recruitment of Bregs into the retina. We induced EAU in mice and 17 days postimmunization we harvested cells from the retina, spleen, or LN. The cells were

then analyzed for cell surface expressed proteins by FACS or cytokine expression by the intracellular cytokine-staining assay. Lymphocytes are not detectable in the normal retina. In the mice with EAU, we observed significant infiltration of T and B cells into the retina and compared to $CD19^+$ B cells, the level of $CD4^+$ T cells in the retinae was 3-folds higher (Fig. 3A). Consistent with the role of Th1 and Th17 cells in the etiology of EAU, we observed marked increase in the levels IFN-γ- and IL-17-producing T cells in the retina but surprisingly the level of IL-10-producing T cells was relatively low (Fig. 3B). Interestingly, a substantial proportion of the B cells was IL-10 and/or IL-35-producing Bregs, with i35-Bregs comprising more than 40% of B cells detected in the retina of mice with EAU (Fig. 3C). These observations are consistent with a previous study showing that IL-10-and IL-35-producing Bregs contribute to mechanisms that mitigate uveitis in the mouse.[4] Interestingly, compared to the B cells detected in the spleen and LN, the B cells in the retina of mice with EAU were disproportionately of the $CD19^+CD1d^{hi}CD21^{hi}CD138^{hi}$ phenotype and characterized by expression of CXCR4 and CCR6 (Fig. 3D–F).

3.3 | $CD1d^{hi}CD19^+$ B cells preferentially produce IL-35 during EAU

Previously, studies have suggested that IL-10-producing B cells that contribute to the suppression of CNS autoimmune diseases predominantly exhibit the $CD5^+CD1d^{Hi}Cd19^+$ phenotype.[3,4,31] Here, we isolated lymphocytes from spleens and draining LNs of EAU mice and examined whether the $CD5^+CD1d^{Hi}CD19^+$ B cell subset is also the main source of IL-35 during EAU. Similar to previous studies, IL-10 was predominantly produced by the $CD1d^{Hi}CD19^+$ B cell subset and secretion of IL-10 was enhanced following stimulation with IL-35 (Fig. 4A and B). Western blot analysis confirmed expression of both IL-12p35 and EbI3 subunit proteins indicating that $CD1d^{Hi}CD19^+$ B cells indeed produce IL-35 (Fig. 4C). Analysis of human peripheral blood CD19+ B cells also revealed that IL-35 also induces IL-12p35 and EbI3 expression (Fig. 4D), suggesting that i35-Bregs can increase IL-35 levels in vivo by a feed-forward auto-regulatory mechanism.

3.4 | Role of BATF-IRF complexes in the regulation of IL-35 expression by activated B cells

IRF-4 and IRF-8 are members of the IRF family of transcription factors. In contrast to other IRF members, IRF-4 and IRF-8 are constitutively expressed in B cells and play important roles in B cell development by regulating transcription of genes essential for B cell differentiation and germinal center events.[32–34] Both IRF members bind DNA through hetero-dimerization with ETS, PU-1 or BATF families of transcription factors, resulting in recruitment of the complex to ETS-IRF composite elements (EICEs) or AICEs.[22–24] AICEs are conserved enhancer elements that regulate transcription of BATF-IRF-dependent immune-regulatory genes that code for IL-17, IL-21, IL-10, and CTLA4.[22–24] In this study, we demonstrated that IL-35 can promote the expression of IRF-4, IRF-8, and BATF in activated $CD19^+$ B cells (Fig. 5A). It is of note that IRF-8 in the cytoplasm is largely phosphorylated while unphosphorylated IRF-8 predominates in the nucleus.[35,36] Interestingly, stimulation of activated B cell with IL-35 increased the unphosphorylated IRF-8 in nucleus (Fig. 5; lower band). We also identified putative AICEs in proximal, distal, or intronic regions of *il12a* or *ebi3*. We therefore used EMSA and ChIP assay to investigate whether expression of IL-35 in B cells might require physical interactions between BATF,

IRF-4, and IRF-8 at the *il12a* or *ebi3* locus. We activated CD19$^+$ B cells (purity >95%) with LPS in the absence or presence of rIL-35, prepared nuclear extracts from the cells as previously described[22] and we show here by EMSA, that BATF, IRF-4, and/or IRF-8 are indeed recruited to AICE motifs of activated mouse B cells (Fig. 5B; left panel). Super-shift analysis using Abs specific to BATF, JunB, JunD, IRF-1, IRF-4, or IRF-8 showed that Abs specific to BATF, JunD, IRF-4, or IRF-8 induced a mobility shift of the AICE complex (Fig. 5B; right panel). These results thus suggest that the binding and recruitment of BATF, IRF-4, and IRF-8 to AICEs of *il12a* or *ebi3* genes contributes to expression of IL-35 by Bregs. We also used the ChIP assay to verify these results. Pull-down assays using anti-BATF Ab revealed the presence of at least 2 IL-35-induced AICEs in the *il12a* promoter (Fig. 5C). Consistent with intracellular cytokine staining analysis showing that Ebi3 protein is constitutively expressed in activated B cells (Fig. 2A and B), the *ebi3*/anti-BATF CHIP assay confirmed constitutive binding of BATF to 3 regions within the *ebi3* locus (Fig. 5D). In view of our results showing that IRF-4, IRF-8, and BATF are recruited to AICE complexes of *il12a*, and/or *ebi3* loci of activated B cells, we investigated whether IRF-4 and IRF-8 might also be involved in expression of IL-10 by Bregs. We therefore analyzed IL-10 secretion by IRF-4$^-$IRF-8$^-$ IRF-4$^-$IRF-8$^+$ IRF-4$^+$IRF-8$^-$ IRF-4$^+$IRF-8$^+$ B cells in response to IL-35-stimulation. Intracellular cytokine-staining assay revealed increased expression of IL-10 by B cells expressing both IRF-4 and IRF-8 proteins (IRF-4^{hi}IRF-8^{hi}) but not IRF-4$^-$IRF-8$^-$ B cells (Fig. 5E), suggesting that corecruitment of IRF-4 and IRF-8 to the *il10* locus may be required for maximal production of IL-10 by Bregs.

4 | DISCUSSION

Uveitis is a CNS autoimmune disease characterized by aberrant infiltration of massive numbers of leukocytes into the uvea, vitreous, retina, or sclera.[25,37] EAU in the mouse shares essential immunopathologic features of uveoretinitis and serves as a model of human uveitis. Consistent with previous studies,[4] we show here that the suppression of pathogenic Th17/Th1 responses and amelioration of EAU required the expansion and recruitment of IL-10 and/or IL-35-producing cells into the retina. A goal of this study was to identify the major inflammatory cell types that produce these immune-suppressive cytokines during EAU, as they can be exploited therapeutically to suppress autoimmune diseases. While CD4$^+$ T cells and myeloid cells were the dominant cells recruited into the retina during ocular inflammation,[38,39] analysis of the blood, draining LNs, and spleen of mice with EAU revealed ~7-fold increase of IL-10-producing Bregs compared to Tregs or IL-10-secreting myeloid cells. The level of IL-35-expression Bregs was even higher and a significant proportion of the Bregs produced both IL-10 and IL-35. The Bregs in the retina were mainly of the CD1d^{hi}CD21^{hi} phenotype and expressed CXCR4 and CCR6, suggesting that Breg recruitment into the retina during intraocular inflammation might be facilitated by up-regulated expression of these chemokine receptors. Although previous studies established the critical roles played by Bregs in uveitis and encephalomyelitis,[3,4] it was assumed that the spleen was the major source of Bregs. Surprisingly, Bregs were most abundant in the blood of mice with EAU, indicating that Bregs in the peripheral blood are major source of IL-10 and IL-35 cytokines that mitigate CNS autoimmune diseases, such as uveitis.

No unique marker or set of markers exclusively identifies the Breg and designation of a B cell subtype as a Breg is mainly based on production of TGF-*β*, IL-10, and/or IL-35 as well as the capacity to suppress inflammation either in vivo or in vitro.[2,14] Although the capacity to produce immune-suppressive cytokines by Bregs is well established, the mechanisms that induce transcription of genes that code for these cytokines remains unknown. In T cells, the basic leucine zipper transcription factor ATF-like (BATF) is thought to regulate transcription of immune-suppressive genes.[23] BATF dimerizes with other transcription factors resulting in the recruitment of the complex to palindromic TPA response elements in target genes.[23] In fact, the immune cells-specific IRFs, IRF-4 and IRF-8, do not bind DNA strongly but depend on their interactions with BATF or ETS transcription to recruit them to AICEs or EICEs and activate their cognate genes.[23,40] In T cells, BATF and IRF-4 cooperate to induce transcription of *il10* through the recruitment of the BATF-IRF-4 complex to AICE in the *il10* locus.[22,23,41,42] In this study, we used the EMSA and ChIP assays to demonstrate that the recruitment of IRF-4, IRF-8, and BATF may be necessary for transcription of *il12a* and *ebi3* loci. Furthermore, while B cells coexpressing IRF-4 and IRF-8 (IRF-4^{hi}IRF-8^{hi}) secrete IL-10, activated B cells that do not express high levels of both factors (IRF-4^{-}IRF-8^{-}) do not efficiently express IL-10, indicating that corecruitment of IRF-4 and IRF-8 to immune suppressive genetic loci of B cells may be required for maximal production of IL-10 and/or IL-35 by Bregs.

In conclusion, we have shown that plasmablasts and plasma cells produce IL-10 and IL-35 cytokines and these IL-10- and IL-35-producing Bregs might contribute to the suppression of uveitis in the mouse. We have also established that the predominant phenotype of the Bregs detected in the retina during EAU exhibit a CD1d^{hi}CD21^{hi} phenotype and that their recruitment into the retina during intraocular inflammation might be facilitated by the expression of high levels of CXCR4 and CCR6. Our study might lead to better understanding of the role of Bregs in autoimmune diseases and provide impetus for exploring the use of adoptive transfer of autologous Bregs as new therapeutic strategies for autoimmune and neurodegenerative diseases.

ACKNOWLEDGMENTS

The Intramural Research Program of the National Eye Institute (NEI) and National Institutes of Health (NIH) supported the research (Projects # EY000350–15 and EY000280–23). We thank Rafael Villasmil (Head, NEI/NIH FLOW Cytometry facility) for cell sorting/FACS analysis and NEI Histology Facility for processing the eyes for histological analysis.

Abbreviations:

AICE	AP1-IRF-composite element
CFA	complete Freund’s adjuvant
ChIP	chromatin immunoprecipitation
EAU	experimental autoimmune uveitis
EICE	ETS-IRF composite element

IRBP	interphotoreceptor retinoid-binding protein
IRF	IFN regulatory factor
LN	lymph node
Treg	regulatory T cell

REFERENCES

1. Gery I, Egwuagu CE. Central tolerance mechanisms in control of susceptibility to autoimmune uveitic disease. Int Rev Immunol. 2002;21:89–100. [PubMed: 12424838]
2. Rosser EC, Mauri C. Regulatory B cells: origin, phenotype, and function. Immunity. 2015;42:607–612. [PubMed: 25902480]
3. Shen P, Roch T, Lampropoulou V, et al. IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. Nature. 2014;507:366–370. [PubMed: 24572363]
4. Wang RX, Yu CR, Dambuza IM, et al. Interleukin-35 induces regulatory B cells that suppress autoimmune disease. Nat Med. 2014;20:633–641. [PubMed: 24743305]
5. Carter NA, Rosser EC, Mauri C. Interleukin-10 produced by B cells is crucial for the suppression of Th17/Th1 responses, induction of T regulatory type 1 cells and reduction of collagen-induced arthritis. Arthritis Res Ther. 2012;14:R32. [PubMed: 22315945]
6. Carter NA, Vasconcellos R, Rosser EC, et al. Mice lacking endogenous IL-10-producing regulatory B cells develop exacerbated disease and present with an increased frequency of Th1/Th17 but a decrease in regulatory T cells. J Immunol. 2011;186:5569–5579. [PubMed: 21464089]
7. Ito T, Hanabuchi S, Wang YH, et al. Two functional subsets of FOXP3+ regulatory T cells in human thymus and periphery. Immunity. 2008;28:870–880. [PubMed: 18513999]
8. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. Nat Rev Immunol. 2008;8:523–532. [PubMed: 18566595]
9. Yanaba K, Bouaziz JD, Matsushita T, Magro CM, St Clair EW, Tedder TF. B-lymphocyte contributions to human autoimmune disease. Immunol Rev. 2008;223:284–299. [PubMed: 18613843]
10. Stasi R Rituximab in autoimmune hematologic diseases: not just a matter of B cells. Semin Hematol. 2010;47:170–179. [PubMed: 20350664]
11. Krumbholz M, Meinl E. B cells in MS and NMO: pathogenesis and therapy. Semin Immunopathol. 2014;36:339–350. [PubMed: 24832354]
12. Moreno Torres I, Garcia-Merino A. Anti-CD20 monoclonal antibodies in multiple sclerosis. Expert Rev Neurother. 2017;17:359–371. [PubMed: 27718747]
13. Yanaba K, Bouaziz JD, Haas KM, Poe JC, Fujimoto M, Tedder TF. A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. Immunity. 2008;28:639–650. [PubMed: 18482568]
14. Mauri C, Bosma A. Immune regulatory function of B cells. Annu Rev Immunol. 2012;30:221–241. [PubMed: 22224776]
15. Collison LW, Chaturvedi V, Henderson AL, et al. IL-35-mediated induction of a potent regulatory T cell population. Nat Immunol. 2010;11:1093–1101. [PubMed: 20953201]
16. Choi JK, Dambuza IM, He C, et al. IL-12p35 Inhibits Neuroinflammation and Ameliorates Autoimmune Encephalomyelitis. Front Immunol. 2017;8:1258. [PubMed: 29051763]
17. Oh HM, Yu CR, Lee Y, Chan CC, Maminishkis A, Egwuagu CE. Autore-active memory CD4+ T lymphocytes that mediate chronic uveitis reside in the bone marrow through STAT3-dependent mechanisms. J Immunol. 2011;187:3338–3346. [PubMed: 21832158]
18. Paques M, Guyomard JL, Simonutti M, et al. Panretinal, high-resolution color photography of the mouse fundus. Invest Ophthalmol Vis Sci. 2007;48:2769–2774. [PubMed: 17525211]

19. Xu H, Koch P, Chen M, Lau A, Reid DM, Forrester JV. A clinical grading system for retinal inflammation in the chronic model of experimental autoimmune uveoretinitis using digital fundus images. Exp Eye Res. 2008;87:319–326. [PubMed: 18634784]
20. Egwuagu CE, Yu CR, Zhang M, Mahdi RM, Kim SJ, Gery I. Suppressors of cytokine signaling proteins are differentially expressed in Th1 and Th2 cells: implications for Th cell lineage commitment and maintenance. J Immunol. 2002;168:3181–3187. [PubMed: 11907070]
21. Yu CR, Lin JX, Fink DW, Akira S, Bloom ET, Yamauchi A. Differential utilization of Janus kinase-signal transducer activator of transcription signaling pathways in the stimulation of human natural killer cells by IL-2, IL-12, and IFN-alpha. J Immunol. 1996;157:126–137. [PubMed: 8683106]
22. Li P, Spolski R, Liao W, et al. BATF-JUN is critical for IRF4-mediated transcription in T cells. Nature. 2012;490:543–546. [PubMed: 22992523]
23. Murphy TL, Tussiwand R, Murphy KM. Specificity through cooperation: bATF-IRF interactions control immune-regulatory networks. Nat Rev Immunol. 2013;13:499–509. [PubMed: 23787991]
24. Tussiwand R, Lee WL, Murphy TL, et al. Compensatory dendritic cell development mediated by BATF-IRF interactions. Nature. 2012;490:502–507. [PubMed: 22992524]
25. Caspi RR, Roberge FG, Chan CC, et al. A new model of autoimmune disease. Experimental autoimmune uveoretinitis induced in mice with two different retinal antigens. J Immunol. 1988;140:1490–1495. [PubMed: 3346541]
26. Caspi RR, Chan CC, Leake WC, Higuchi M, Wiggert B, Chader GJ. Experimental autoimmune uveoretinitis in mice. Induction by a single eliciting event and dependence on quantitative parameters of immunization. J Autoimmun. 1990;3:237–246. [PubMed: 2397017]
27. Calder VL, Lightman SL. Experimental autoimmune uveoretinitis (EAU) versus experimental allergic encephalomyelitis (EAE): a comparison of T cell-mediated mechanisms. Clin Exp Immunol. 1992;89: 165–169. [PubMed: 1638760]
28. Janeway. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol. 1989;54:1–13.
29. Stumhofer JS, Silver JS, Laurence A, et al. Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. Nat Immunol. 2007;8:1363–1371. [PubMed: 17994025]
30. Dambuza IM, He C, Choi JK, et al. IL-12p35 induces expansion of IL-10 and IL-35-expressing regulatory B cells and ameliorates autoimmune disease. Nat Commun. 2017;8:719. [PubMed: 28959012]
31. Matsushita T, Horikawa M, Iwata Y, Tedder TF. Regulatory B cells (B10 cells) and regulatory T cells have independent roles in controlling experimental autoimmune encephalomyelitis initiation and late-phase immunopathogenesis. J Immunol. 2010;185:2240–2252. [PubMed: 20624940]
32. Singh H, Glasmacher E, Chang AB, Vander Lugt B. The molecular choreography of IRF4 and IRF8 with immune system partners. Cold Spring Harb Symp Quant Biol. 2013;78:101–104. [PubMed: 24752218]
33. Pillai S, Cariappa A. The follicular versus marginal zone B lymphocyte cell fate decision. Nat Rev Immunol. 2009;9:767–777. [PubMed: 19855403]
34. Xu H, Chaudhri VK, Wu Z, et al. Regulation of bifurcating B cell trajectories by mutual antagonism between transcription factors IRF4 and IRF8. Nat Immunol. 2015;16:1274–1281. [PubMed: 26437243]
35. Unlu S, Kumar A, Waterman WR, et al. Phosphorylation of IRF8 in a pre-associated complex with Spi-1/PU.1 and non-phosphorylated Stat1 is critical for LPS induction of the IL1B gene. Mol Immunol. 2007;44:3364–3379. [PubMed: 17386941]
36. Li W, Nagineni CN, Ge H, Efiok B, Chepelinsky AB, Egwuagu CE. Interferon consensus sequence-binding protein is constitutively expressed and differentially regulated in the ocular lens. J Biol Chem. 1999;274:9686–9691. [PubMed: 10092656]
37. Caspi RR, Roberge FG. Glial cells as suppressor cells: characterization of the inhibitory function. J Autoimmun. 1989;2:709–722. [PubMed: 2529864]
38. Nussenblatt RB. The natural history of uveitis. Int Ophthalmol. 1990;14:303–308. [PubMed: 2249907]

39. Chan CC, Caspi RR, Ni M, et al. Pathology of experimental autoimmune uveoretinitis in mice. J Autoimmun. 1990;3:247–255. [PubMed: 2397018]

40. Glasmacher E, Agrawal S, Chang AB, et al. A genomic regulatory element that directs assembly and function of immune-specific AP-1-IRF complexes. Science. 2012;338:975–980. [PubMed: 22983707]

41. Iwata A, Durai V, Tussiwand R, et al. Quality of TCR signaling determined by differential affinities of enhancers for the composite BATF-IRF4 transcription factor complex. Nat Immunol. 2017;18:563–572. [PubMed: 28346410]

42. Li P, Spolski R, Liao W, Leonard WJ. Complex interactions of transcription factors in mediating cytokine biology in T cells. Immunol Rev. 2014;261:141–156. [PubMed: 25123282]

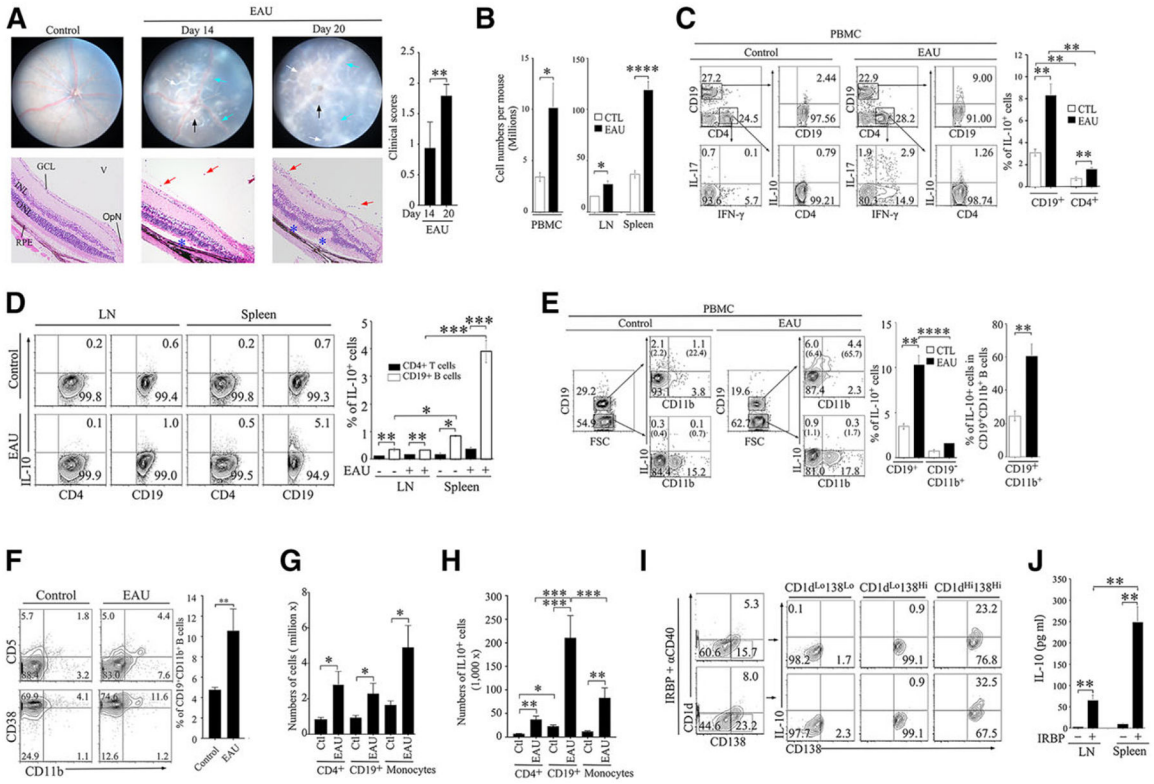

FIGURE 1. IL-10-producing Bregs are dominant regulatory population during EAU.

(A) EAU was induced in C57BL/6J mice by active immunization with the immunopathogenic ocular auto-antigen, IRBP, in CFA. Top panel shows Fundus images and bottom panels show histology images of eyes harvested 14 or 20 days after induction of EAU. Fundus images of the retina on day 21 after EAU induction show severe inflammation with blurred optic disc margins and enlarged juxtapapillary area (black arrows), retinal vasculitis with moderate cuffing (blue arrows), yellow–whitish retinal and choroidal infiltrates (white arrows). Histological analysis reveals substantial numbers of inflammatory cells in the vitreous (red arrows), photoreceptor cell damage, choroiditis, and retinal in-folding (blue asterisks). (B–E) Freshly isolated PBMCs, draining LN, and spleen cells were counted (B) and analyzed for cell surface protein expression by flow cytometry (FACS) and for cytokine expression by the intracellular cytokine-staining assay (C-I). Numbers in quadrants indicate percent cells in each throughout. (F and G) Cells were isolated from the draining LNs or spleen 21 days after induction of EAU and analyzed by the intracellular cytokine-staining assay and FACS analysis. (I and J) Cells isolated from the LN and spleen were reactivated ex-vivo with IRBP and anti-CD40 and analyzed by the intracellular cytokine-staining assay and FACS analysis (I) or ELISA (J). All plots were gated as described in the text and numbers in quadrants indicate percent of cells expressing the relevant proteins as indicated on Figures. The experiments were performed with at least 6 mice per group and results represent at least 3 independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$)

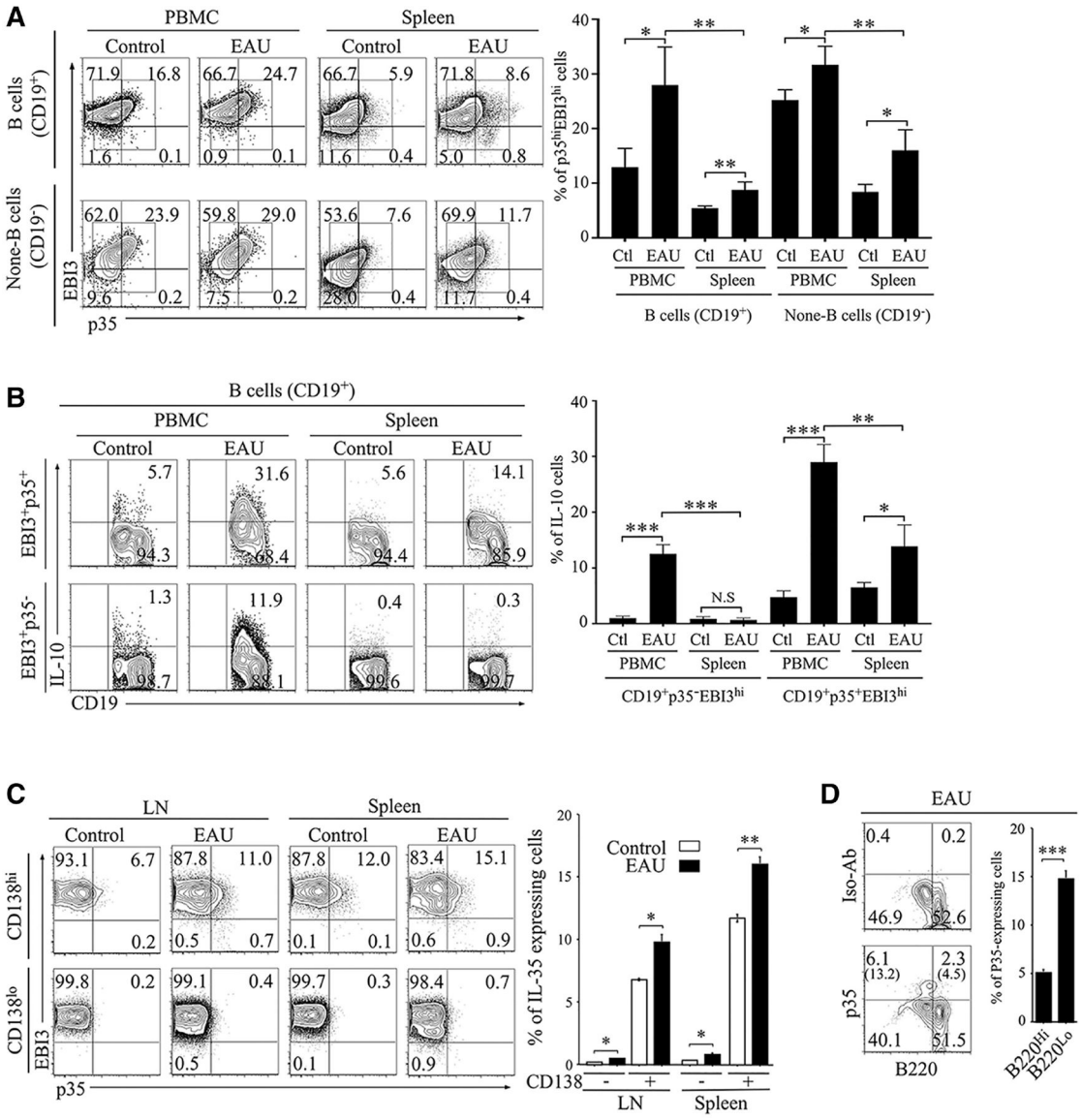

FIGURE 2. IL-35-producing Bregs are preferentially expanded in blood of mice with uveitis.

Cells were isolated from PBMC (A and B), LN, or spleen (C and D) of control mice (immunized with CFA) or EAU mice (IRBP/CFA immunized mice) on day 21 postimmunization and analyzed by the intracellular cytokine staining assay, FACS analysis of cell surface protein expression or Western blot analysis. (A) The frequency of B cells secreting IL-35 was determined by gating on $CD19^+$ (upper panels) and $CD19^-$ (lower panels) lymphocytes. (B) The frequency of B cells secreting both IL-10 and IL-35 was determined by gating on p35-expressing (upper panels) and nonexpressing (lower panels) $CD19^+Ebi3^+$ B cells. (C and D) Frequency of IL-35 producing B cells was also determined by gating on $CD138^+$ and $B220^{Lo}$ $CD19^+$ B cell pools. Numbers in quadrants indicate percent of cells expressing the relevant proteins as indicated on Figures. Results represent at least 3 independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

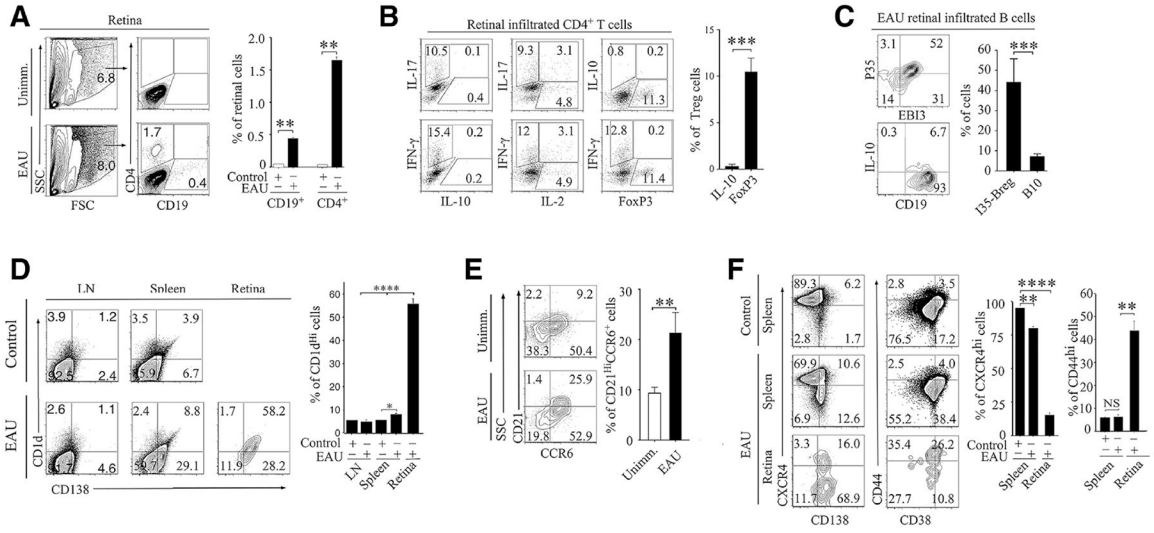

FIGURE 3. IL-35- and IL-10-producing B cells are expanded in the neuro-retina during EAU.

To characterize inflammatory cells that cross the blood–retina barrier during EAU, retinas from control and EAU mice were minced, digested with collagenase and the released cells were subjected to flow cytometry analysis. (A) We analyzed total retinal cell mixture for cell surface CD4 or CD19 expression by FACS. (B) CD4-gated cells were analyzed by intracellular cytokine staining assay and numbers in the quadrants are percentage of CD4 cells expressing IL-2, IL-10, IL-17, IFN-γ, or Foxp3. (C) $CD19^+$ B cells in the retina were analyzed by intracellular cytokine staining assay for expression of IL-10 or IL-35 (p35 & Ebi3). (D–F) We analyzed cells present in the retina, spleen, or LN during EAU for the expression of chemokine receptors and cell surface proteins that characterize the Breg phenotype by FACS. Numbers in quadrants indicate percent of cells expressing the relevant proteins as indicated on Figures. The experiments were performed with at least 6 mice per group and results represent at least 3 independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$)

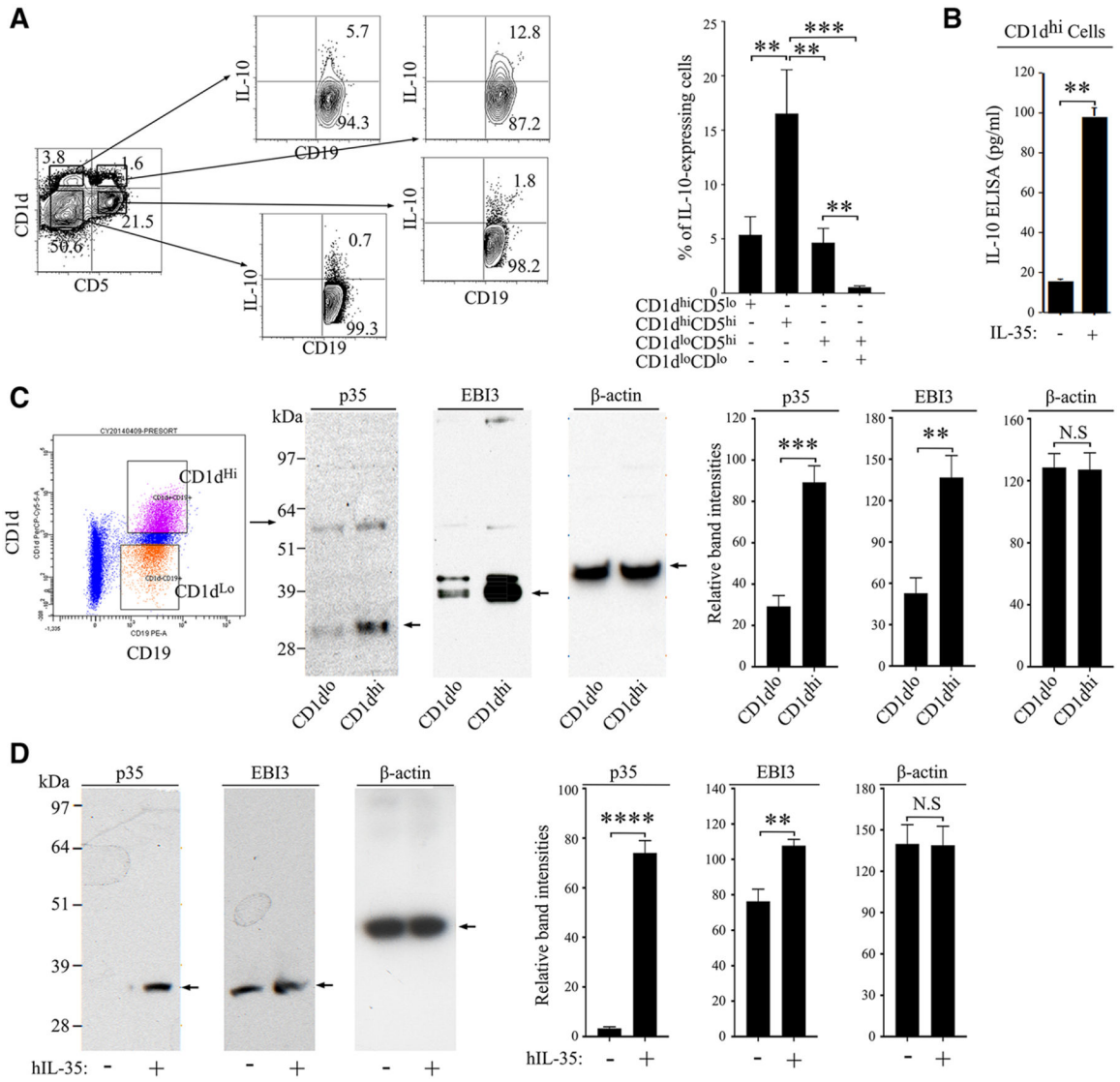

FIGURE 4. CD1d^{hi}CD19^{+} B cells express both IL-10 and IL-35 regulated by IL-35.

(A) CD19^{+} B cells from the spleen of EAU mice (20 days after induction of EAU) were sorted using magnetic beads and then subjected to FACS analysis as indicated by the gating strategy. (B) CD19^{+}CD1d^{hi} B cells are electronically sorted on a cell sorter, reactivated by stimulation with IRBP and anti-CD40 antibody in presence or absence of rIL-35 for 2 days and supernatants were analyzed for IL-10 secretion by ELISA. (C) CD19^{+}CD1d^{hi} and CD19^{+}CD1d^{lo} B cells were isolated by electronically sorting on a cell sorter and subjected to Western blot analysis. (D) Human CD19^{+} B cells were cultured with LPS (1ug/ml) for 3 days in the presence or absence of hrIL-35 (20 ng/ml). Total proteins were extracted for Western blotting. These results are representative of at least 3 independent experiments (*P < 0.05; **P < 0.01; ***P < 0.001)

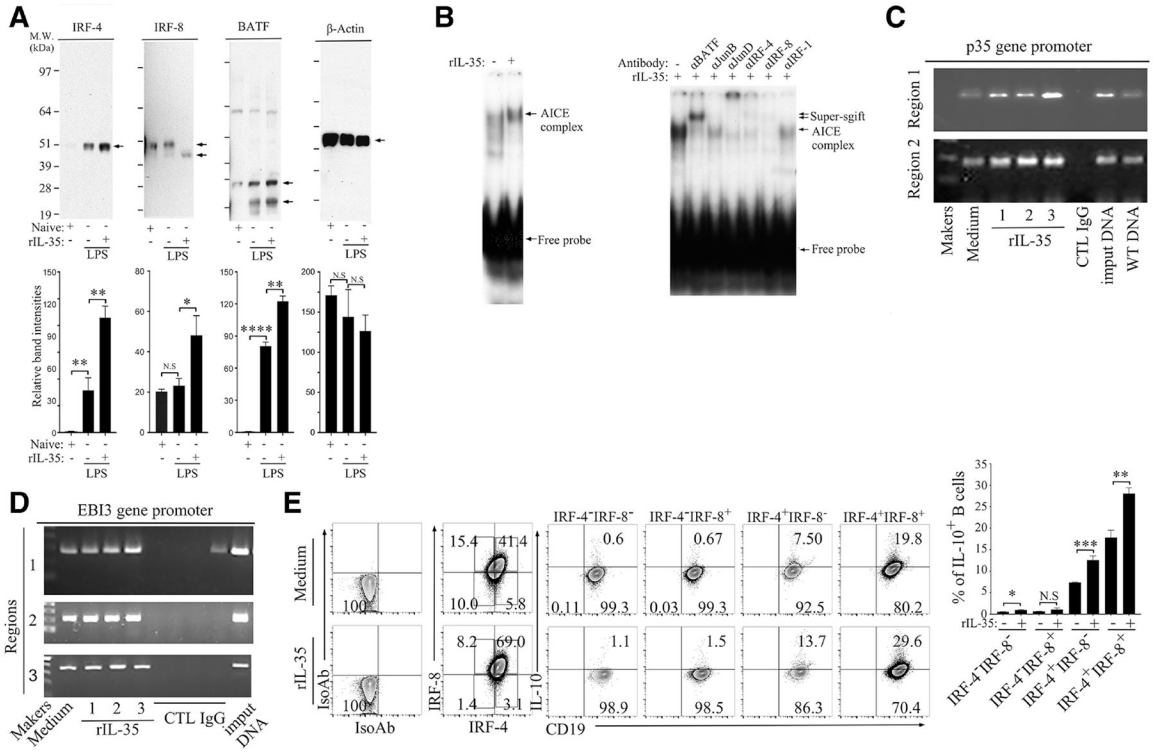

FIGURE 5. BATF, IRF-4, and IRF-8 recruitment to API-IRF composite elements (AICEs) in B cells.

Purified mouse CD19$^+$ B cells were activated with LPS in the absence or presence of rIL-35 for 48 h. (A) The total proteins are isolated and used to detect IRF-4, IRF-8, and BATF expression by Western blotting. Note: Densitometry analysis was performed on the lower band. (B) Nuclear proteins were isolated from the activated cells and subjected to EMSA to detect rIL-35-induced AICE complexes (left panel). Transcription factors recruited to the AICE were identified by super-shift assay (right panel). (C and D) BATF-ChIP assays were performed to detect potential AICE complexes in *il12a* (C) and *ebi3* (D) gene promoters. (E) Purified mouse CD19$^+$ B cells were activated with LPS in the absence or presence of rIL-35, and after 48 h, the cells were analyzed by the intracellular staining FACS assay. The numbers in the quadrants indicate the percentage of B cells expressing IL-10, IRF-4, and/or IRF-8