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Interleukin-35 Induces Regulatory B Cells that Suppress CNS Autoimmune Disease

Ren-Xi Wang[1,4], **Cheng-Rong Yu**[1], **Ivy M. Dambuza**[1], **Rashid M. Mahdi**[1], **Monika Dolinska**[2], **Yuri V. Sergeey**[2], **Paul T. Wingfield**[3], **Sung-Hye Kim**[1], and **Charles E. Egwuagu**[1,*]

[1]Molecular Immunology Section, Laboratory of Immunology, National Eye Institute, Bethesda, Maryland, USA

[2]Ophthalmic Genetics & Visual Functions Branch, National Eye Institute, Bethesda, Maryland, USA

[3]Protein Expression Laboratory, National Institutes of Arthritis and Musculoskeletal & Skin Diseases; National Institutes of Health, Bethesda, Maryland, USA

Abstract

Interleukin 10-producing regulatory B-cells (Breg-cells) suppress autoimmune diseases while aberrant elevation of Breg-cells prevents sterilizing immunity, promotes carcinogenesis and cancer metastasis by converting resting $CD4^+$ T-cells to regulatory T-cells (Tregs). It is therefore of interest to discover factors that induce Breg-cells. Here we show that IL-35 induces Breg-cells *in-vivo* and promotes their conversion to a unique Breg subset that produces IL-35 (IL-35^+Breg). Treatment of mice with IL-35 conferred protection from uveitis and mice lacking IL-35 or defective in IL-35-signaling produced less Breg-cells and developed severe uveitis. Ex-vivo generated Breg-cells also suppressed uveitis by inhibiting pathogenic Th17/Th1 while promoting Tregs expansion. We further show that IL-35 induced the conversion of human B-cells into Breg-cells and suppressed uveitis by activating STAT1/STAT3 through IL-35-Receptor comprising IL-12Rβ2/IL-27Rα subunits. Discovery that IL-35 converts human B-cells into Breg-cells, allows *ex-vivo* production of autologous Breg-cells for immunotherapy and investigating Breg/IL-35^+Breg cells roles in autoimmune diseases and cancer.

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* Charles E. Egwuagu. (egwuaguc@nei.nih.gov).

[4]Present address: Laboratory of Immunology, Institute of Basic Medical Sciences, Beijing 100850, China

AUTHOR CONTRIBUTIONS

R-X.W conducted all experiments, prepared the figures and edited the manuscript. C-R.Y assisted with EAU experiments, FACS analysis, prepared the figures and editing of the manuscript. I.M.D assisted with EAU experiments, FACS analysis, protein purification, prepared the figures and editing of the manuscript. R.M.M performed Western blot analyses and assisted with rIL-35 preparation. M.D and Y.V.S assisted with FPLC analysis. P.T.W performed equilibrium ultracentrifugation analysis. S-H.K assisted with EAU experiments and fundoscopy. C.E.E conceived, designed, supervised the project and wrote the manuscript.

COMPETING INTEREST STATEMENT

The authors declare that they have no competing financial interests.

Supplementary Material

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INTRODUCTION

B-cell depletion is an effective therapy for a number of T-cell mediated autoimmune diseases, suggesting that B-cells may contribute to autoimmunity[1-4]. However, subsequent studies showed that the efficacy of anti-CD20 antibody rituximab in some autoimmune diseases derived in part from the expansion of a rare regulatory B-cell population with greater resistance to anti-CD20 antibodies[5,6]. The B-cell-mediated suppression of autoimmunity is independent of autoantibody production but due to secretion of the potent anti-inflammatory cytokine, interleukin 10 (IL-10) [7] The IL-10-producing regulatory B-cells (Breg-cells) are very rare, lack a specific marker and play pivotal role in maintaining immunological tolerance and restraining excessive inflammation during auto-inflammatory diseases[8]. However, aberrant elevation of Breg-cells levels can prevent sterilizing immunity to pathogens and inhibit immune responses to infectious agents by impairing optimal T-cell responses[8]. Tumor-induced Breg cells are recruited and expanded in tumors and constitute an important mechanism utilized by tumor cells to evade protective immunity and support metastatic growth[9-11].

There is significant interest in identifying factors that induce or regulate Breg cells *in vivo* and recent studies suggest that IL-21 and CD40-dependent cognate interactions with T cells induce Breg cells that suppressed experimental autoimmune encephalomyelitis (EAE)[12,13]. Similarly, a GM-CSF and IL-15 fusokine induced Breg cells that suppressed EAE, suggesting involvement of cytokines in the development or expansion of Breg-cells[14]. Recent studies have also uncovered the role of Interleukin 35 (IL-35) in inducing Tregs[15,16]. Given the close relationship between these lymphocyte populations we speculated that IL-35 might also play a role in inducing Breg cells *in vivo*. IL-35 is the newest member of the IL-12 family of heterodimeric cytokines [15,17] and is composed of Ebi3, a β chain subunit encoded by the Epstein-Barr virus (EBV)-induced gene 3 (*IL27b*), and the IL-12p35 α subunit encoded by *IL-12*α[15,18,19]. It is produced by nTregs and contributes to their suppressive activities[15,20]. However, it is not clear whether other lymphoid cell types also produce IL-35 and its *in vivo* functions are not known because the native IL-35 is not available.

In this study, we have genetically engineered a functional heterodimeric mouse IL-35 (rIL-35). We show here that rIL-35 induces Breg cells and a unique IL-35-producing Breg (IL-35$^+$Breg) subpopulation that conferred protection from experimental autoimmune uveitis (EAU), an animal model of human autoimmune uveitis[21]. Adoptive transfer of Breg cells induced by rIL-35 ameliorated EAU even when the disease was already established. Thus, *ex-vivo* production of functional Breg cells with the rIL-35 would undoubtedly facilitate investigations of the role of Breg and IL-35$^+$Breg cells in autoimmune diseases and cancer.

RESULTS

IL-35 mediates the induction of regulatory B-cells (Breg cells)

To study the potential regulatory role of IL-35 in autoimmune diseases and examine whether it can be used to treat uveitis, we genetically engineered and produced mouse IL-35 in insect

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cells **(Fig. 1a)**. Details of the production and purification of the mouse recombinant IL-35 (rIL-35) are presented **(Supplementary methods/Supplementary Fig.1)**. Single chain Ebi3 or p35 migrated as 33 kDa monomeric protein on denaturing SDS gels while rIL-35 migrated as ~67 kDa heterodimeric protein on native, non-denaturing gel **(Fig.1b)**. rIL-35 was further purified by two cycles of FPLC (**Supplementary Fig.1a,1b)** and characterized by SDS-PAGE **(Supplementary Fig.1c)**. Accurate mass determination was obtained by sedimentation equilibrium analysis **(Supplementary Fig.1d,1e)**. Western blotting and coimmunoprecipitation analyses using anti-Flag and anti-V5 Abs revealed specific association of Ebi3 with p35 as a stable p35:Ebi3 heterodimeric complex **(Fig.1c)**, consistent with a previous study[18]. As control for functional studies we used pMIB, an unfractionated heterogeneous collection of irrelevant secretome of the insect cells. Western blot analysis of the pMIB control established that pMIB does not exhibit immunoreactivity to p35, Ebi3, Flag or V5 epitope (**Fig.1c**). Identity of the heterodimer was derived from dual reactivity with anti-p35 and Ebi3 monoclonal antibodies **(Fig.1d)**. In line with a previous report[15], we demonstrated that the heterodimeric protein is biologically active by showing that rIL-35 suppressed T-cell proliferation **(Supplementary Fig.2a)**.

rIL-35 also inhibited proliferation of primary mouse B-cells **(Fig.1e)** and most surprising rIL-35 induced the generation or expansion of IL-10-producing B-cells[22-24] (Breg-cells) **(Fig.1f,1g)**. rIL-35 also inhibited proliferation of the mouse B-cell line, WEHI-279, in a dose-dependent manner **(Fig.1h)**, excluding the possibility that the suppressive effects might have derived from contaminating cells in our primary B-cell preparation. rIL-35 also increased IL-10 production by WEHI-279 **(Fig.1i)**, indicating that rIL-35 mediates their conversion into Breg cells. It is notable that p35 and Ebi3 inhibited lymphocyte proliferation but had marginal effect on IL-10 production by B-cells **(Fig.1f)** or T-cells **(Supplementary Fig.2b,2c)**, suggesting that the induction of Breg cells was mediated mainly by rIL-35. We co-cultured purified rIL-35-induced Breg cells with freshly isolated $CD19^+$ B-cells and suppression of B-cell proliferation **(Fig. 1j)** established that the rIL-35-induced Breg cells indeed possess suppressive activity. Addition of anti-IL-10 Ab abrogated the inhibitory effect of rIL-35 **(Supplementary Fig.2d)**, suggesting that rIL-35 inhibited proliferation, in part, by inducing conversion of B-cells into Breg cells. Similarly, rIL-35 suppressed T-cell proliferation in co-culture experiments by inducing Tregs **(Supplementary Fig.2e,2f)**. We confirmed the authenticity of our rIL-35 by use of commercially available Abs specific to p35 or Ebi3, which abrogated the growth inhibitory effect of rIL-35 or its capacity to induce conversion of B-cells into Breg cells **(Supplementary Fig.2g,2h,2i)**.

IL-35 induces an IL-35-producing Breg population (IL-35$^+$Bregs)

IL-35 induces an IL-35-producing regulatory T-cell population called iTR35 that suppresses inflammation[16]. We therefore examined whether rIL-35 could induce IL-35-producing B-cells. LPS-stimulated B-cells co-express Ebi3 and p35 (IL-35$^+$) and following stimulation with rIL-35 the frequency of the Ebi3/p35-expressing B-cell population increased tremendously from 7.8% to 35.3% (**Fig.2a;** left panels) and 17.8% of the IL-35-producing B-cells also expressed IL-10 (**Fig.2a;** right panels). Henceforth we refer to this B-cell subset co-expressing p35 and Ebi3 as IL-35$^+$Breg. Analysis of IL-10$^-$ and IL-10$^+$ B-cells revealed that approximately 20% of the Breg-cells produced IL-35 and frequency of the IL-35$^+$Bregs

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increased by >50% following stimulation with rIL-35 (**Fig.2b**). We further show that rIL-35 induced the binding of STAT1 to p35 or Ebi3 proximal promoter in B-cells (**Fig.2c**), up-regulated p35 and Ebi3 mRNA expression **(Fig.2d, Supplementary Fig.2i)** and secretion of IL-35 by Breg cells **(Supplementary Fig.3)**. Interestingly, substantial percentage rIL-35-induced Breg cells express cell-surface CD5 and/or Tim-1 but not Foxp3 (**Fig.2e**). We examined effects of rIL-35 *in vivo* by injecting mice with LPS and/or rIL-35 and found that IL-35 induced a substantial decrease of $B220^+$ B-cells (42.68% to 27.92%) and this was accompanied by appearance of a prominent population of $CD5^+B220^{lo}$ B-cells in mice treated with rIL-35 but not with LPS alone (see red arrow in **Fig.2f**). Analysis of $B220^{lo}CD5^+$ B-cell compartment further revealed that rIL-35 preferentially induces expansion of $CD19^+CD5^+B220^{lo}$ Breg cells (**Fig.2g,2h**). Together, these results suggest that IL-35 induces IL-35^+Breg and while rIL-35 suppressed the proliferation of conventional $B220^+$ B-cells, it selectively induced expansion of $CD19^+CD5^+B220^{lo}$ Breg cells *in-vivo*.

IL-35 suppressed autoimmune uveitis by inducing expansion of Breg and IL-35^+Breg cells

We next induced EAU, a rodent model of human uveitis[25,26] and investigated whether rIL-35 can be used to treat EAU. Mice were treated with pMIB or rIL-35 (100ng/mouse) and disease severity was assessed on day-21 post-immunization as described (Supplementary Methods). Histology and fundus images of control mice (no treatment) or mice that received pMIB revealed severe inflammation characterized by papilledema, retinal vasculitis, retinal folds, substantial infiltration of inflammatory cells into the vitreous and chorio-retinal infiltrates (**Fig.3a**). In contrast, rIL-35-treated mice had trace or very mild EAU with significantly lower EAU scores (**Fig.3a**). Amelioration of EAU was accompanied by significant reduction of Th17 and Th1 cells in the draining LN (**Fig.3b**) with concomitant increase of Breg cells (**Fig.3c**). Breg cells comprised ~5.18% of the B-cells in spleen and draining LNs of rIL-35-treated EAU mice compared to ~0.91% in mice treated with pMIB (**Fig.3d**) and the Breg population in rIL-35-treated mice contained IL-35^+Breg-cells (**Fig. 3e**), suggesting that IL-35 induces both Breg and IL-35^+Breg cells *in vivo*. Although there is no unique marker, or set of markers, that exclusively identifies Breg cells[8], we observed a >4-folds increase of $CD1d^{hi}CD5^+$ B-cells during EAU **(Fig.3f)**, a Breg cell phenotype previously shown to suppress inflammation in a contact hypersensitivity model[27]. Importantly, administration of Breg cells from rIL-35-treated mice suppressed EAU induced by adoptive transfer of IRBP-specific pathogenic T cells (**Fig. 3g**), consistent with recent reports showing that Breg cells restrain excessive inflammation during autoimmune diseases[8,28]. Collectively, these observations provide evidence that IL-35 induced Breg and IL-35^+Breg cells that suppress and ameliorate uveitis.

IL-35-induced Breg cells suppressed established uveitis

To examine whether *ex-vivo* generated Breg cells can be used to treat ongoing uveitis, we generated highly enriched Breg cells (>93% IL-10^+ B-cells) and IL-10^- B-cells (<1% IL-10^+ B-cells) by sorting (**Fig.4a**). We induced EAU by active immunization and on day-4 post-immunization intravenously injected the purified Breg-cells or IL-10^- B-cells (1×10^6/mouse). Fundoscopy and histological analysis of the eyes 21 days post-immunization show that the untreated mice or mice that received IL-10^- B-cells developed full-blown EAU

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(**Fig.4b,4c**). In contrast, mice treated with Breg cells had relatively low EAU scores with very few cells in the vitreous and no evidence of retinal folds (hallmark of severe EAU) (**Fig.4b,4c**). More than 31.1% of the Breg-cells generated during EAU were IL-35^{+}Bregs and as many as ~64.4% of the Breg-cells in spleen of rIL-35-treated mice were IL-35^{+}Bregs (**Fig.4d;** lower panels). The increase in Breg and IL-35^{+}Breg cells was accompanied by substantial increase in Foxp3^{+}CD4^{+} T-cells (8.36% versus 2.81%) and a corresponding decrease in Th1 and Th17 levels in the LN (**Fig.4e**), suggesting that Breg and Treg cells may synergize to suppress uveitis. Similar experiments using congenic mouse strains suggest that Breg cells might inhibit EAU by inducing expansion of endogenous Breg and Treg cells while inhibiting pathogenic Th17/Th1 in recipient mice (**Fig.4f,4g**). Of particular significance to therapy, human recombinant IL-35 induced human Breg cells (**Fig.4h**) and inhibited human B-cell proliferation (**Fig.4i**).

Generation of regulatory B-cells that mitigate uveitis require IL-35 signaling and IL-10

We induced EAU in p35KO, IL-12Rβ2KO or IL-10KO mice and examined whether loss of IL-35 or its signaling component[29] would compromise generation of Breg-cells and exacerbate uveitis. Compared to WT, IL-12Rβ2KO mice developed severe EAU **(Fig.5a)** with marked expansion of Th17 **(Fig.5b)**. Also, B-cells from IL-12Rβ2KO or IL-10KO could not induce Breg cells in response to rIL-35 **(Fig.5c)** or suppress proliferation of uveitogenic T-cells **(Fig.5d)**. Disease in p35KO mice was comparable to WT and it is not clear whether the modest effect of p35-deficiency stemmed from the fact that IL-12 (utilizes p35 subunit) is required for the induction and exacerbation of EAU[30]. Use of p35KO or IL-12Rβ2KO mice to examine requirement of IL-35 for *in-vivo* Breg generation or Breg/IL-35^{+}Breg-mediated EAU suppression is complicated because IL-12Rβ2KO and p35KO mice also have defective IL-12 signaling. We therefore sorted B-cells from WT, p35KO, IL-12Rβ2KO or IL-10KO EAU mice, stimulated them *ex-vivo* with rIL-35 and investigated whether they could suppress EAU induced by adoptive transfer of uveitogenic cells. Mice that received pMIB-treated B-cells from WT or rIL-35-treated B-cells from p35KO, IL-12Rβ2KO or IL-10KO mice developed EAU with relatively high EAU scores while mice that received rIL-35-treated B cells from WT mice were protected from EAU pathology **(Fig.5e)** and had marked reduction of Th17 cells **(Fig. 5f).** Although the Balb/c mouse strain is very resistant to EAU induction[31], Ebi3KO mice on a Balb/c background developed EAU characterized by optic neuritis, papilledema, retinal vasculitis and hemorrhage **(Fig.5g)** and had 3.6-folds less Breg cells **(Fig.5h).** Nonetheless, Ebi3KO mice produce Breg cells, albeit at very low frequency, suggesting existence of alternative pathways that induce Breg cells expansion[13,14]. Requirement of Breg/IL-35^{+}Breg-mediated suppression of uveitis is further underscored by development of severe EAU by B-cell deficient mice (*mu*MT) that have intact Treg compartment. While rIL-35 suppressed EAU in WT mice, rIL-35 treatment could not effectively ameliorate uveitis in *mu*MT mice **(Fig.5i)**. Although the data indicates that Breg/IL-35^{+}Breg cells possess intrinsic immunosuppressive activities, it also suggests that there is a meaningful non-B-cell IL-35 driven suppressor response in vivo.

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IL-35 induced Breg cells and suppressed B-cell proliferation by activating STAT1/STAT3 pathways through IL-35 receptor comprising of IL-12Rβ2 and IL-27Rα

We used siRNA to specifically silence receptor subunits utilized by IL-12 family cytokines (**Fig.6a**) and examined effect of silencing each receptor subunit on rIL-35-mediated suppression of B-cell proliferation or IL-10 induction. Silencing IL-12Rβ1 or gp130 subunit did not affect IL-35-mediated inhibition of B-cell proliferation (**Fig.6b**) or IL-10 induction (**Fig.6c**). Furthermore, anti-gp130-neutralizing Abs did not block IL-35-induced inhibition of B-cell proliferation (**Supplementary Fig.4a)** or suppress IL-35-induced production of IL-10 (**Supplementary Fig.4b**). In contrast, silencing of IL-12Rβ2 and IL-27Rα completely abrogated the effects of IL-35 on B-cell proliferation and IL-10 production (**Fig.6b,6c**). We confirmed requirement of these receptors in IL-12Rβ2- or IL-27Rα-deficient B-cells as rIL-35 could not inhibit proliferation of IL-12Rβ2- or IL-27Rα deficient B-cells (**Fig.6d**) or induce expansion of Breg cells (**Fig.6e**). Moreover, reciprocal coimmunoprecipitation analysis confirmed the expression and interaction of IL-12Rβ2 and IL-27Rα on IL-35-stimulated B-cells (**Fig.6f**). Furthermore, generation of IL-35^{+}Bregs required IL-35-induced signaling through IL-12Rβ2 or IL-27Rα chains (**Fig.6g**). Western blot analysis also revealed that rIL-35 preferentially activates STAT1, STAT3 and STAT4 in T-cells **(Fig.6h)** while in B-cells it signals primarily through STAT1 and STAT3 but not STAT4 **(Fig.6i; Supplementary Fig.4c,4d)**. Taken together, these results support the notion that IL-35 signals in B-cells through IL-12Rβ2 and IL-27Rα and suggest that IL-35 mediates its biological activities in T- and B-cells through differential activation of overlapping and distinct STAT pathways.

Discussion

Since its discovery in 2007, investigations into mechanisms that mediate biological functions of IL-35 have been stymied because the native cytokine is not available. We have genetically engineered and produced a highly purified mouse recombinant IL-35. We show for the first time that IL-35 induces the expansion of Breg cells and conversion of Breg into a Breg subpopulation that produces IL-35 (IL-35^{+}Breg). Our data suggest that Breg and IL-35^{+}Breg cells are at different stages of regulatory B-cell development and that exposure of Breg to IL-35 *in-vivo* may induce their terminal differentiation into IL-35^{+}Breg plasma cells. While our manuscript was under revision another group identified an IL-35-producing B cells[32], validating the existence and physiological relevance of IL-35^{+}Breg plasma cells. Discovery that IL-35 promotes Breg expansion and conversion of Breg-cells into IL-35^{+}Bregs now allows *ex-vivo* production of these regulatory cells for therapeutic use.

It is of note that while IL-35 induces the expansion of Breg and IL-35^{+}Breg cells, it also induces Tregs and a regulatory T-cell population, iTR35, with immune-suppressive activities[16]. This raises the intriguing possibility that Breg and IL-35^{+}Breg populations may be the B-cell counterparts of Treg and iTR35, respectively. However, there appears to be important differences between iTR35 and IL-35^{+}Bregs. For example, the suppression of inflammation by iTR35 is mediated by IL-35 and does not require IL-10 [16]. On the other hand, B-cells deficient in IL-10, IL-12Rβ2 or Ebi3 could not inhibit proliferation of

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pathogenic T cells **(Fig.5d)** or suppress inflammation **(Fig.5e,5g),** suggesting that both IL-10 and IL-35 signaling may be required for suppressive functions of IL-35^{+}Bregs.

In B-cells IL-35 signals through a receptor comprised of IL-12Rβ2 and IL-27Rα (**Fig.6d, 6e**) and mediates its biological effects by activating STAT1 and to a lesser extent, STAT3 (**Fig.6h,6i**). However, a recent report suggests that IL-35 signals in T cells through unconventional receptors comprising of IL-12Rβ2/IL-12Rβ2, IL-12Rβ2/gp130 or gp130/gp130 and preferentially activates STAT1 and STAT4[33,29]. Although we also found that IL-35 activates STAT1 and STAT4 in T cells, it also activated STAT3 **(Fig.6h).** It is therefore not clear whether IL-35 utilizes different receptors and signaling components in different cell types or if the different results derived in part from the IL-35 preparations and/or the levels of inhibitory p35:p35 and Ebi3:Ebi3 homodimers in the preparations **(Supplementary Fig.1a)**. In this regard, it is notable that rIL-35 activated STAT1 and STAT3 in B-cells while the single-chain p35 could not activate these STATs but instead inhibited IL-6-induced STAT3 activation while enhancing activation of STAT1 by IL-27 **(Supplementary Fig.5)**. This is reminiscent of recent findings that IL-27p28 and IL-12p40 could not activate STATs but suppressed T cell proliferation by inhibiting STAT1, STAT3 and STAT4 activation by IL-27, IL-6 and IL-12, respectively[34,35]. Thus, factors that regulate the stability of the non-covalently linked p35/Ebi3 heterodimer[33] and the ability of the individual subunits to inhibit STAT activation suggest an additional layer of complexity that may underlie the physiological regulation of IL-35. The finding that p35 and Ebi3 inhibit lymphocyte proliferation but could not induce Breg or Treg cells, suggests that p35 and Ebi3 have functions, independent of IL-35, and may constitute a new class of therapeutic “cytokines” that can be used to inhibit infectious agents and cancer cells without the danger of inducing Breg-cells that prevent sterilizing immunity.

Uveitis is a diverse group of potentially sight-threatening CNS intraocular inflammatory diseases of infectious or autoimmune aetiology and accounts for more than 10% of severe visual handicaps in the United States. Although steroids and other anti-inflammatory drugs are effective therapy, renal toxicity and other adverse effects preclude prolonged usage[36]. Thus, use of biologics and regulatory T-cell therapy to treat uveitis are alternative approaches under investigation. Here, we show that treatment of mice with IL-35 suppressed uveitis and conferred protection against ocular pathology by inhibiting Th17 and Th1 pathogenic T-cells while inducing the expansion of regulatory Breg/IL-35^{+}Breg-cells, as well as, Tregs. Our data is consistent with the recent report showing that IL-35-producing B cells play critical roles in host immunity during autoimmune diseases[32]. The expansion of IL-35^{+}Bregs during EAU raises the possibility that production of IL-35 by IL-35^{+}Breg in lymphoid tissues or the retina may orchestrate a positive feedback loop that further increases the levels of Breg and Treg cells, thereby contributing to the suppression of uveitis. Thus, effective suppression of EAU may require the combined actions of Breg-cells, IL-35^{+}Bregs and Tregs. Of significance to therapy are our findings that IL-35 also induces the expansion of human Breg cells and inhibited human B-cell proliferation **(Fig.4h,4i)**, suggesting possibility of using IL-35 or Breg/IL-35^{+}Breg cells for treatment of human uveitis and other CNS autoimmune diseases, including multiple sclerosis. The expansion of Breg-cells in human PBMC also suggests that the effect of IL-35 on lymphocytes is evolutionarily

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conserved between humans and mice. In view of recent reports implicating Breg cells in carcinogenesis[9-11] and promoting breast cancer metastasis by converting resting $CD4^+$ T cells to Tregs[37], our discovery that IL-35 can convert B-cells to Breg-cells or Breg-cells into IL-35^+Bregs has implications in context of designing therapeutic strategies to inhibit cancer metastasis, autoimmune diseases and prevent graft-versus-host disease (GVHD).

ONLINE METHODS

Normal Human subjects

Blood samples were obtained after IRB approval and consent from 10 normal human subjects at the NIH Department of Transfusion Medicine. PBMCs were isolated from heparin treated whole blood by density gradient centrifugation and $CD19^+$ B-cells were isolated using human CD43 MicroBeads (AutoMACS, Miltenyi Biotic). PBMC or $CD19^+$ B cells were stimulated for 4 days with LPS (10μg/ml)+IL-4 (10ng/ml) or PMA (5ng/ml) and or several concentrations of (10-100ng/ml human IL-35 single-chain fusion protein (Enzo Life Sciences, Farmingdale, NY).

Mice

Wild type C57BL/6j, C57BL/6 CD45.1, IL-12p35KO, Ebi3KO (Balb/c background), IL-12Rβ2KO, *mu*MT, and IL-10KO mice were purchased from Jackson Laboratory. STAT1KO and STAT4KO mice were purchased from Taconic animal facility. CD4-STAT3KO mice were generated as previously reported [38]. Drs. Aisling O'Hara and Christopher A. Hunter kindly provided IL-27RαKO B-cells (U Penn). Mice were maintained and treated in accordance with NIH Animal Care & Use Committee guidelines.

Production and characterization of the recombinant IL-35 (rIL-35)

Mouse recombinant IL-35 (rIL-35) construct was generated by recombinant PCR. The p35 cDNA was fused to an amino-terminal melittin (HBM) secretion signal sequence and the Ebi3 cDNA was at the carboxyl-terminus. The construct encoded V5-epitope, Flag and polyhistidine tags that facilitated isolation and characterization of the recombinant protein. The construct was cloned into a 3.6 kb bicistronic pMIB vector containing FLAG-IRES (internal ribosomal entry site) and V5-His sequences as described for pMIB/V5-His A, B, and C Vector Kit (Catalog # V8030-01; Invitrogen, Carlsbad, CA). As controls, Flag-tagged p35 and V5-tagged Ebi3 single chain recombinant proteins were also engineered. The expression construct was then transfected into insect High Five cells and stable transfectants were identified by drug selection (Blasticidin S; 100 μg/ml). To ensure that the recombinant clone expressed bona fide IL-35 (p35/Ebi3), we isolated the expression vector (HBM-p35-Flag-IRES-HMB-Ebi3-V5-His) and verified that no mutations were introduced during cloning or drug selection by DNA sequencing and the construct was in the correct orientation. The recombinant protein(s) secreted by the insect cells was sequentially purified by Ni-NTA Purification system (Invitrogen), size-exclusion centricon filtration and two consecutive cycles of FPLC gel filtration chromatography. The highly enriched p35/Ebi3 preparation was characterized by SDS-PAGE gels stained with coomassie blue, Western blot/immunoprecipitation assays using V5, Flag, p35 or Ebi3 mAbs or sedimentation equilibrium ultracentrifugation.

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Induction of EAU

EAU was induced by active immunization with 150μg bovine interphotoreceptor retinoid-binding protein (IRBP) and 300μg human IRBP peptide, amino acid residues 1–20 ($IRBP_{1-20}$) in 0.2 ml emulsion 1:1 v/v with Complete Freund's adjuvant (CFA) containing *Mycobacterium tuberculosis* strain H37Ra (2.5mg/ml). Mice also received *Bordetella pertussis* toxin (0.3μg/mouse) concurrent with immunization. Clinical disease was established and scored by fundoscopy as described previously[39,40]. For adoptive transfer studies, following co-culture of Breg cells and uveitogenic T-cells, sorted B-cells from control or EAU mice were re-activated, *ex-vivo* in $IRBP_{1-20}$-coated plates containing anti-CD40 Abs[41] with or without rIL-35 for 3 days. Cells were then washed to remove residual rIL-35 or pMIB and then co-cultured with freshly isolated EAU draining LN cells (1:5). Following co-culture for 4 days with freshly isolated draining LN cells from EAU mice in medium containing IRBP (20 μg/ml), the cells were adoptively transferred i.v to naïve syngeneic recipient mice at 1×10^7 cells/mouse. Ten days after cell transfer, disease was assessed by fundoscopy and histology. For some experiments IL-10-expressing B cells were isolated and purified by a three steps procedure using the Cytokine Secretion Assay technology developed Miltenyi Biotec (#130-095-873). Briefly, B cells from mice with EAU were isolated and cultured for 3 days in IRBP-coated plates containing anti-CD40 antibody and rIL-35. Cells were then washed and the pre-enriched IL-10-producing B cells were incubated with the patented IL-10 Catch Reagent. After attaching the Reagent to the cell surface, cells were cultured in medium containing IL-35 to enhance IL-10 secretion. The cells were subsequently labeled with the Breg Detection Antibody (IL-10-specific antibody) conjugated to PE. The IL-10-secreting cells were then magnetically labeled with Anti-PE MicroBeads and magnetically labeled Breg cells were then isolated over MACS columns placed in the magnetic field of a MACS Separator.

Imaging mouse fundus

Fundoscopic examinations were performed after EAU induction using a modified Karl Storz veterinary otoendoscope coupled with a Nikon D90 digital camera, as previously described[40,42]. Fundus photography was conducted without knowledge of the mouse identity by masked observers. At least six images (2 posterior central retinal view, 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[43,44].

Isolation of regulatory B cells

The isolation of regulatory B cells is performed in three steps using the Cytokine Secretion Assay technology (Regulatory B Cell Isolation Kit) developed by Miltenyi Biotec. (Miltenyi Biotec GmbH, Order no. 130-095-873). First, B cells were pre-enriched by depletion of non-B cells and cultured for 3 days in IRBP-coated plates containing anti-CD40 antibody and rIL-35. Second, mg/mL 50 ng/mL PMA, 1 culture for 5 hours to induce IL-10 secretion. Third, the viable ionomycin were added IL-10–producing cells were specifically to the isolated by using the cytokine secretion assay technology. Briefly, the pre-enriched B cells

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were incubated with the patented IL-10 Catch Reagent and after attaching the Reagent to the cell surface the cells were cultured in medium containing IL-35 to enhance IL-10 secretion. Cells were subsequently labeled with Breg Detection Antibody (IL-10-specific Abs) conjugated to PE. The IL-10-secreting cells were then magnetically labeled with Anti-PE MicroBeads and magnetically labeled Breg cells were then isolated over MACS columns placed in the magnetic field of a MACS Separator.

Lymphocyte proliferation assay

B cells were stimulated with LPS (1.5 μg/ml) while $CD4^+$ T cells were cultured in plate bound anti-CD3 Abs and medium containing anti-CD28 Ab. B-cells, T-cells or WEHI-279 B-cells were propagated in presence or absence of pMIB or rIL-35 (50 ng/ml). For some co-culture experiments, purified B cells were stimulated with LPS (1.5 μg/ml) or $IRBP_{1\text{-}20}$ (40 μg/ml)+anti-CD40 (5μg/ml) in presence of pMIB or rIL-35. Cells were then washed and co-cultured with LPS-stimulated B cells or IRBP-stimulated uveitogenic cells. After 72 hours, cultures were pulsed with ^{3}H-thymidine (0.5 μCi/10 μl/well) as described[40]. Presented data are mean c.p.m ± S.E. of responses of 5 replicate cultures.

Analysis of IL-10-producing B-cells (Breg) or $CD4^+$ T-helper cells

Primary B cells isolated from the spleen and lymph nodes (LN) (sorted for $CD19^+$ or $B220^+$) were stimulated with LPS. WEHI-279 B-cells were propagated without stimulation with LPS. $CD4^+$ T-cells (>98%) from the spleen and/or LN were activated in plate-bound anti-CD3 Abs (10μg/ml) and soluble anti-CD28 Abs (3 μg/ml) as described [38]. For intracellular cytokine detection, cells were re-stimulated for 5 hours with PMA (20 ng/ml) and ionomycin (1μM). Golgi-stop was added in the last hour and intracellular cytokine staining was performed using BD Biosciences Cytofix/Cytoperm kit as recommended (BD Pharmingen, San Diego, CA). FACS analysis was performed on a Becton-Dickinson FACSCalibur (BD Biosciences) using protein-specific monoclonal antibodies and corresponding isotype control Abs (PharMingen, San Diego, CA) as previously described [45]. FACS analysis was performed on samples stained with mAbs conjugated with fluorescent dyes and each experiment was color-compensated. Dead cells were stained with dead cell exclusion dye (Fixable Viability Dye eFluor® 450; eBioscience) and live cells were subjected to side-scatter (SSC) & forward scatter (FSC) analysis. Quadrant gates were set using isotype controls with less than 0.2% background. Supernatants were analyzed for IL-10 secretion by ELISA kit (R&D systems, Minneapolis, MN) as described[46].

Reverse transcription (RT) PCR and quantitative RT-PCR analysis

All RNA samples were DNA free. cDNA synthesis, RT-PCR and qPCR analyses were performed as described [45]. Each gene-specific primer pair used for RT-PCR analysis spans at least an intron. Primers and probes used for RT-PCR or qPCR were purchased from Applied Biosystems and mRNA expression was normalized to the levels of *β-Actin* and *GAPDH* genes. For siRNA silencing experiments, WEHI-279 B cells were cultured to 60–70% confluency and transfected with siRNA oligonucleotides (Santa Cruz). Cells were replated in six-well plates, cultured for 72 hours and then analyzed by PCR using gene-specific primers.

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Immunoprecipitation/Western analysis

Preparation of whole cell lysates was as described[47]. Cleared lysates or cellular supernatants were immunoprecipitated with antibody that was pre-coupled to protein G-sepharose beads as described [48]. Immunoprecipitates were resolved by SDS-PAGE and blots were probed. The following antibodies were used for Western blotting and/or immunoprecipitation: anti-Flag or anti-V5 (Invitrogen Life Technologies, Grand Island, NY); antibodies specific to STAT1, pSTAT1, STAT3, pSTAT3, STAT4, pSTAT4, STAT5, pSTAT5, STAT6, pSTAT6 (Cell signaling Technology,); antibodies specific to IL-12p35, Ebi3, IL-12Rβ1, IL-12Rβ2, IL-27Rα, gp130, and Danvers, β-actin MA(Santa Cruz Biotechnology, Santa Cruz, CA). Pre-immune serum was used in parallel as controls and signals were detected with HRP conjugated-secondary F(ab')2 (Zymed Labs, San Francisco, CA) using ECL system (Amersham, Arlington Heights, IL).

Chromatin Immunoprecipitation (CHIP) analysis—DNA-protein complexes in B cells stimulated with rIL-35 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 fixed cells were lysed with lysis buffer (EZ Chip™, Upstate Biotechnology) and sonicated five times for 15 seconds (output 5 on Sonic Dismembrator Model 1000, Fisher Scientific). Lysates were then cleared with Protein G-agarose for 1 hour, pelleted, and incubated overnight with control IgG or anti-STAT1 antibody (Cell signaling, CHIP grade). Prior to antibody incubation, input samples were removed from the lysate and stored at −80°C until extraction. Immunoprecipitation was performed according to the manufacture's instructions (EZ Chip™). The immunoprecipitated and input DNA were subjected to PCR and using mouse p35 Chip primers: 5'-AGAAAGGAGTTAAGTTCCAAGGAATC-3' and 5'-GATTTCAGCAGCAGTGTAGACGC –3'; mouse Ebi3 primers 5'- AGGTGGTAGTTGC TCCTTGTTGTC -3' and 5'- TGATGATGGTGACGGGAACC -3'.

Statistical analysis

Statistical analysis was performed by Student *t*- test (two-tailed). EAU scores were analyzed by nonparametric Mann-Whiney U test (two-tailed). Asterisks denote p value (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The Intramural Research Programs of the National Eye Institute and National Institutes of Health provided funding for this research. We thank Dr. Howard Young (NCI, NIH); Dr. Rachel R. Caspi and Dr. Chi-Chao Chan (NEI, NIH) for critical reading of the manuscript. We also thank Dr Chan for expert analyses of Fundus images and histology sections. Authors also thank Drs. Aisling O'Hara and Christopher A. Hunter (U Penn), for providing IL-27Rα KO B-cells and Rafael Villasmil (NEI/NIH FLOW Cytometry Core facility) for cell sorting and FACS analysis.

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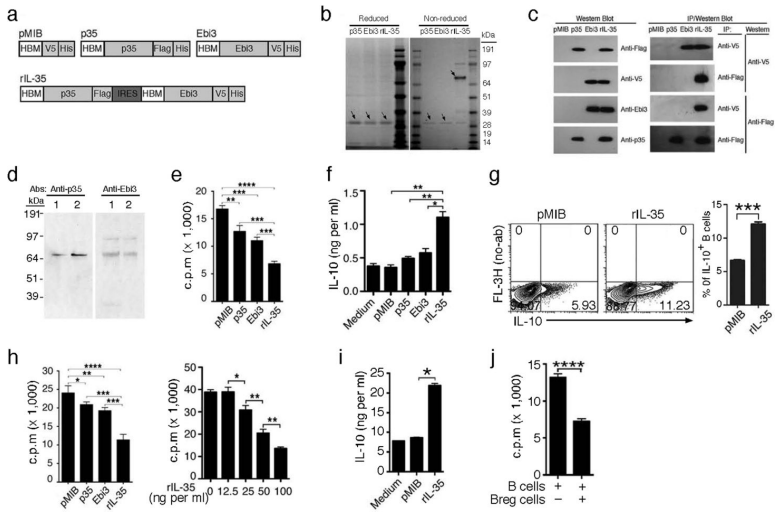

Figure 1. IL-35 induced regulatory B cells (Breg)

(a) Schematic of the cDNA constructs used to genetically engineer IL-12p35 (p35), Ebi3 and IL-35 (p35/Ebi3) recombinant proteins. (b) Coomassie blue gels of the recombinant proteins characterized on reducing SDS or non-reducing polyacrylamide gels. (c) Detection and characterization of p35, Ebi3 or IL-35 recombinant proteins by immunoprecipitation/ Western blot analysis under reducing condition. (d) Characterization of 2 independently generated batches of the purified rIL-35 preparations by Western blotting under non-reducing conditions with anti-p35 or anti Ebi3 Abs. (e, f, g) Purified $B220^{+}CD19^{+}$ B cells from C57BL/6 mouse spleen were stimulated with LPS (5μg/ml) in medium containing 50 ng/ml pMIB, p35, Ebi3 or rIL-35 for 72 h. Lymphocyte proliferation was analyzed by $[^{3}H]$-thymidine incorporation assay (e) and IL-10 production was analyzed by ELISA (f) or intracellular cytokine staining assay (g). (h, i) WEHI-279 B-cells were cultured in medium containing 50 ng/ml pMIB, p35, Ebi3 or rIL-35. Lymphocyte proliferation was analyzed by $[^{3}H]$-thymidine incorporation assay (h) and IL-10 production was analyzed by ELISA (i). (j) $CD19^{+}$ primary B cells were stimulated with rIL-35 (50ng/ml) and the purified Breg cells were co-cultured (1:5) for 3 days with freshly isolated $CD19^{+}$ B cells in medium containing LPS. Proliferation was analyzed by the $[^{3}H]$-thymidine incorporation assay. Results represent at least 3 independent experiments (**P*<0.05; ***P*<0.01; ****P* < 0.001; *****P*<0.0001).

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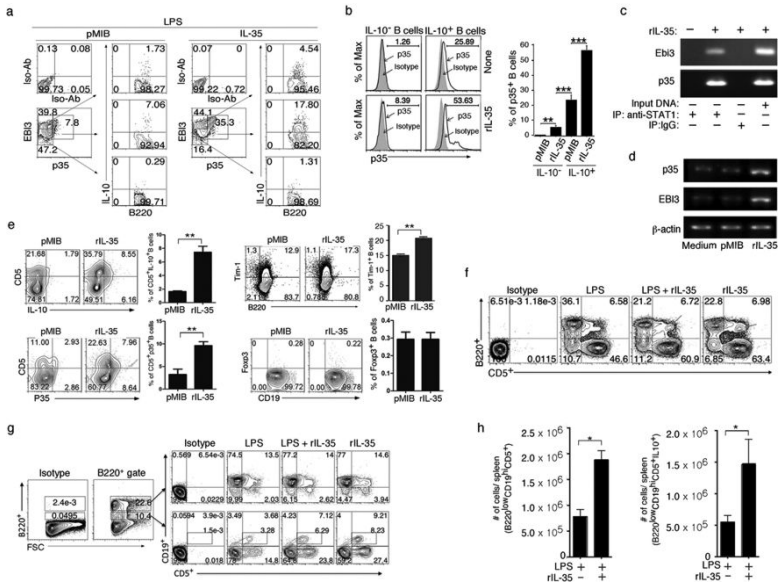

Figure 2. IL-35 preferentially induced CD5$^+$CD19$^+$B220^{lo} Breg cells and a unique IL-35-producing Breg subpopulation (IL-35$^+$Breg)

(a) Purified CD19$^+$ B cells were stimulated for 3 days in medium containing 50ng/ml pMIB or rIL-35 and numbers in quadrants indicate the percent of B220$^+$ B cells expressing Ebi3, p35 and/or IL-10. (b) IL-10$^-$ or IL-10$^+$ B cells enriched with a Breg Isolation Kit (see Methods section) were analyzed by intracellular cytokine staining assay. (c,d) Activated B-cells were stimulated with rIL-35 and subjected to chromatin immunoprecipitation (ChiP) assay (c) or RT-PCR (d). (e) Purified B cells were stimulated for 3 days in medium containing pMIB or IL-35 and expression of B220, CD19, CD5, Foxp3 or Tim-1 and/or IL-10 was analyzed by intracellular cytokine staining assay or cell surface FACS analysis. (f, g) C57BL/6 mice were injected with LPS (15 μg/mouse) and/or rIL-35 (1μg/mouse) and after 4 days splenic cells were analyzed by FACS. (h) Absolute numbers of B220^{lo}CD19$^+$CD5$^+$ or B220^{lo}CD19$^+$CD5$^+$IL-10$^+$ cells in the spleen. Results are representative of at least 3 independent experiments (****P<0.05; **P<0.01; ***P < 0.001).

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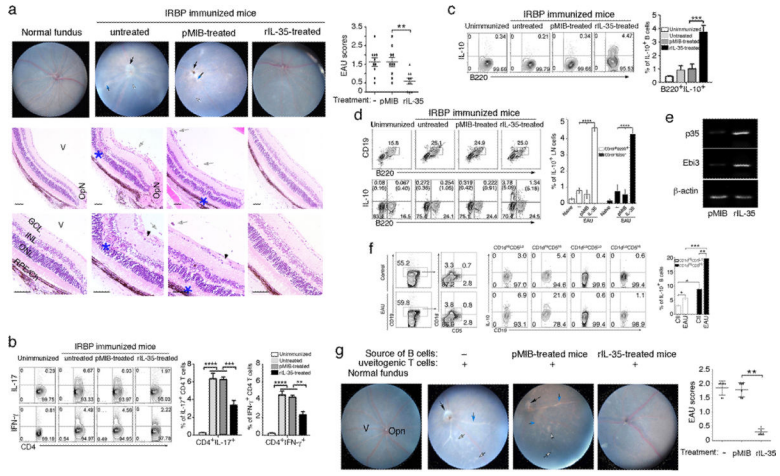

Figure 3. rIL-35 induced *in vivo* expansion of Breg cells and suppressed EAU development

EAU was induced in C57BL/6 mice and eyes were analyzed at day-21 post-immunization by funduscopy or histology. (a) Fundus images of the retina (top): Black-arrow, inflammation with blurred optic-disc margins (papilledema); Blue-arrows, retinal vasculitis; White-arrows, retinal/choroidal infiltrates. H&E histological sections: Scale bar, 500 μM. OPN, optic nerve; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE/CH retinal pigmented epithelial and choroid. Open white-arrow, lymphocytes; black-arrowhead, enlarged retinal blood vessels; Blue-asterisk, retinal-folds. EAU scores were based on changes at the retina, optic nerve disc and choroid (ONLINE METHODS). (b-d) Intracellular-cytokine analysis of IL-17- or IFN-γ-expressing T cells in draining LN (b) or IL-10-producing B cells in spleen (c, d) on day-21 post-immunization. Spleen cells isolated from control, pMIB, rIL-35-treated EAU mice were also analyzed for IL-35 (p35 and Ebi3) expression by RT-PCR (e) or intracellular cytokine staining assay (f). (g) B cells from untreated, pMIB-treated or rIL-35-treated EAU mice were re-activated *ex-vivo* with IRBP/anti-CD40 and $CD19^+$ B-cells were isolated on a cell sorter. The purified $CD19^+$ B cells were co-cultured with uveitogenic LN and spleen cells for 3 days in medium containing IRBP and the cells (1×10^7) were then transferred into naïve syngeneic mice. Fundus images of the eyes and EAU scores recorded 10 days after adoptive transfer. Data represents at least 3 independent experiments (***P*< 0.01; ****P* <0.001; *****P*< 0.0001).

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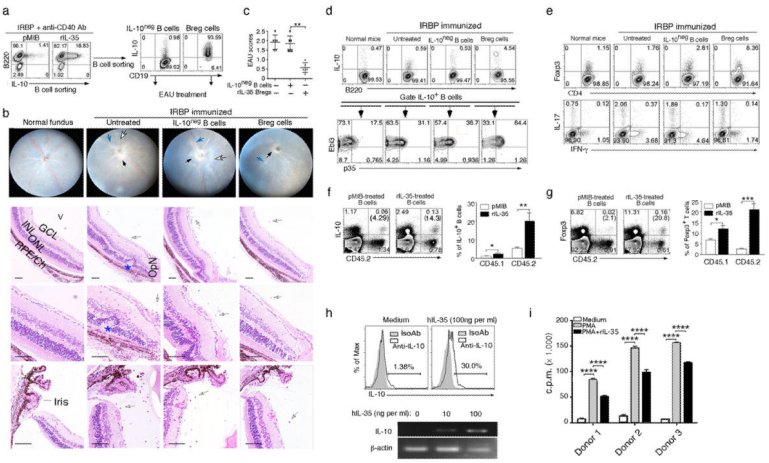

Figure 4. Breg cells suppressed EAU by inducing Breg/IL-35^{+}Breg and Treg cells while inhibiting Th17/Th1

(a) Purified rIL-35-induced Breg cells and IL-10^{negative} B cells were derived as described (Supplementary methods). WT mice with EAU were treated with 1×10^{6} Breg or IL-10^{-} B cells. (b) Eyes were analyzed by fundoscopy or histology 21 days after disease induction and characteristic features of EAU are as described in Fig. 3a. Scale bar, 500 μM. (c) EAU scores were determined as described in Fig. 3a, 3b. (d, e) Cells from spleen or draining LN were gated on CD19 or CD4, respectively and the percentage of IL-10-, p35- or Ebi3-expressing B cells (d) or Foxp3-, IFN-γ or IL-17-expressing CD4^{+} T cells (e) was determined by intracellular-cytokine staining assay. (f, g) B cells from the pMIB- or rIL-35-treated mice (described in a) were co-cultured with IRBP-stimulated uveitogenic LN cells (5:1) for 3 days and the cells (1×10^{7}) were transferred into naïve CD45.1 congenic mice. Ten days after adoptive transfer, recipient (CD45.1) and donor (CD45.2) cells were analyzed for IL-10-producing B cells (gated on CD19) in spleen (f) or Foxp3-expressing T cells (gated on CD4) in LN (g). (h, i) Purified human CD19^{+} B cells were cultured for 3 days with PMA in medium with or without rIL-35 and analyzed by FACS or RT-PCR (h) or [^{3}H]-thymidine-incorporation assay (i). Data is representative of analysis of PBMC from 10 donors. Other results represent at least 3 independent experiments (*P<0.05; **P<0.01; ***P < 0.001; ****P<0.0001).

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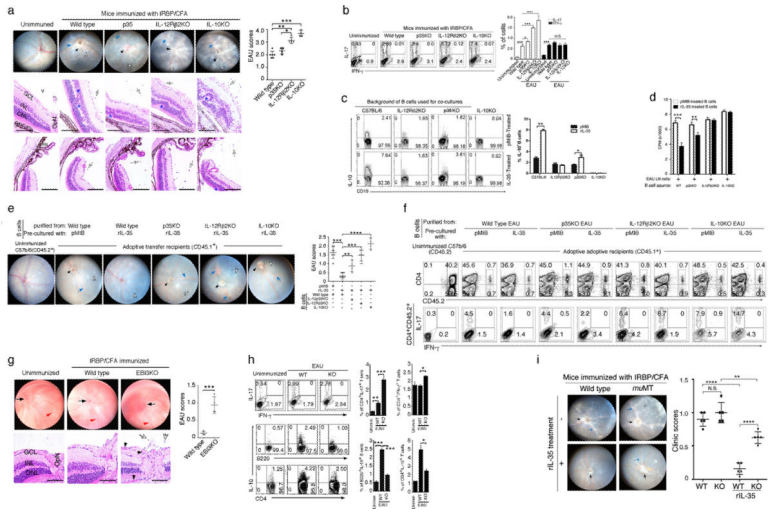

Figure 5. IL-35-signaling is required for suppressive functions of Breg and i35-Breg cells

(a) EAU was induced in WT, p35KO, IL-12Rβ2KO or IL-10KO mice. Fundus images (top panels) and H&E-stained sections (bottom panels) of eyes enucleated 21 days after disease induction. Scale bar, 500 μM. (b) Analysis of IL-17- or IFN-γ-expressing T cells in the draining LN. (c) B cells from indicated EAU mice were re-activated *ex-vivo* with IRBP/anti-CD40 in medium containing pMIB or rIL-35 and percentage of Breg cells was determined by intracellular cytokine assay. (d) $CD19^+$ B cells from (c) were co-cultured with draining LN cells from WT EAU mice (1:5) for 4 days in medium containing IRBP or pMIB and analyzed by $[^3H]$-thymidine incorporation assay. (e, f) Ex-vivo activated B-cells from (c) were co-cultured with uveitogenic LN T cells (1:5) for 3 days and cells were then transferred to naïve congenic $CD45.1^+$ mice. Fundus images and EAU scores were obtained 10 days after adoptive transfer (e) and the percentage of $CD4^+CD45.2^+$ T cells, IFN-γ- or IL-17-expressing $CD4^+CD45.2^+$ T cells in the draining LNs was determined by cell surface and intracellular cytokine staining (f). (g, h) Balb/c or Ebi3KO (Balb/c background) mice were immunized with IRBP/CFA and eyes enucleated on day-21 post-immunization were analyzed by fundoscopy or histology and red-arrowheads indicate blood vessels. Scale bar, 500 μM (g). (h) IL-17, IFN-γ or IL-10-expressing LN $CD4^+$ T cells or IL-10-producing splenic B cells were analyzed by intracellular cytokine staining assay. (i) EAU was induced in WT or muMT mice by active immunization with IRBP/CFA and the mice were treated with rIL-35 as described (Supplementary Methods) and eyes examined by fundoscopy on day 21 post-immunization. Results represent at least 3 independent experiments (*P<0.05; **P<0.01; ***P < 0.001).

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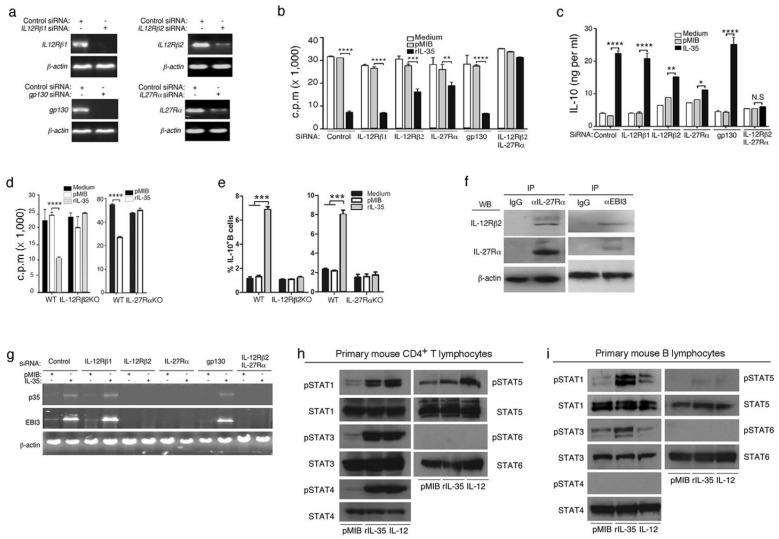

Figure 6. IL-35 mediates its biological effects on B-cells by activating STAT1 and STAT3 pathways through an IL-35 receptor comprising of IL-12Rβ2 and IL-27Rα

(a) WEHI-279 B-cell line were transduced with control siRNA or IL-12Rβ1-, IL-12Rβ2-, IL-27Rα- or gp130-specific siRNA and after 3 days total RNA was analyzed by RT-PCR. (b, c) siRNA-treated B-cells were cultured in medium containing pMIB or rIL-35 for 3 days and B-cell proliferation (b) or rIL-35-induced production of IL-10 (c) was assessed by [^{3}H]-thymidine incorporation assays or ELISA, respectively. (d, e) Primary B cells from WT, IL12Rβ2KO or IL27RαKO mice were stimulated by LPS in the presence of pMIB or rIL-35 and analyzed by [^{3}H]-thymidine incorporation assay (d) or intracellular cytokine IL-10 staining assay (e). (f) WEHI-279 cells were cultured overnight in medium containing rIL-35 and co-expression of IL-27Rα and IL-12Rβ2 was detected by Western blotting and immunoprecipitation using anti-IL-27Rα anti-Ebi3 or control isotype-specific IgG Abs. (g) Primary B cells were transduced with IL-12Rβ1-, IL-12Rβ2-, IL-27Rα-, gp130 or both IL-12Rβ2- and IL-27Rα-siRNA. The cells were then stimulated in presence of pMIB or rIL-35 and expression of *p35* and *Ebi3* was detected by RT-PCR analysis. (h, i) Primary T cells (h) or B cells (i) from WT C57BL/6 mice were stimulated with anti-CD3/CD28 or LPS, respectively, and cells were then washed and starved for 2 h in serum free medium (0.5% BSA). Cells were then stimulated for 30 minutes with pMIB, rIL-35 or IL-12 and analyzed for STAT activation by Western blotting. Data represent at least 3 independent experiments (**P*<0.05; ***P*<0.01; ****P* < 0.001; *****P*<0.0001).