IL-27–producing B-1a cells suppress neuroinflammation and CNS autoimmune diseases

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Regulatory B cells (Breg cells) that secrete IL-10 or IL-35 (i35-Breg) play key roles in regulating immunity in tumor microenvironment or during autoimmune and infectious diseases. Thus, loss of Breg function is implicated in development of autoimmune diseases while aberrant elevation of Breg prevents sterilizing immunity, exacerbates infectious diseases, and promotes cancer metastasis. Breg cells identified thus far are largely antigen-specific and derive mainly from B2-lymphocyte lineage. Here, we describe an innate-like IL-27–producing natural regulatory B-1a cell (i27-Breg) in peritoneal cavity and human umbilical cord blood. i27-Bregs accumulate in CNS and lymphoid tissues during neuroinflammation and confers protection against CNS autoimmune disease. i27-Breg immunotherapy ameliorated encephalomyelitis and uveitis through up-regulation of inhibitory receptors (Lag3, PD-1), suppression of Th17/Th1 responses, and propagating inhibitory signals that convert conventional B cells to regulatory lymphocytes that secrete IL-10 and/or IL-35 in eye, brain, or spinal cord. Furthermore, i27-Breg proliferates in vivo and sustains IL-27 secretion in CNS and lymphoid tissues, a therapeutic advantage over administering biologics (IL-10, IL-35) that are rapidly cleared in vivo. Mutant mice lacking irf4 in B cells exhibit exaggerated increase of i27-Bregs with few i35-Bregs, while mice with loss of irf8 in B cells have abundance of i35-Bregs but defective in generating i27-Bregs, identifying IRF8/BATF and IRF4/BATF axis in skewing B cell differentiation toward i27-Breg and i35-Breg developmental programs, respectively. Consistent with its developmental origin, disease suppression by innate i27-Bregs is neither antigen-specific nor disease-specific, suggesting that i27-Breg would be effective immunotherapy for a wide spectrum of autoimmune diseases.

B-1a cells | i27-Breg cells | CNS autoimmune diseases | neuroinflammation | uveitis and encephalitis

Regulatory B cells (Breg cells) play a critical role in maintaining immune tolerance and regulating immunity during autoimmune and infectious diseases via inhibitory cytokines including interleukin 10 (B10), TGF-β, or IL-35 (i35-Breg). However, it is not known whether Bregs are a dedicated B cell lineage, and developmental origins of Breg cells remain an active area of research. It is also not known if any B cell can become a Breg cell. Moreover, Breg cells identified thus far are largely antigen-specific and derive mainly from B2-lymphocyte lineage. Thus, our interest in this study was to determine whether the nonspecific innate-like B-1 cells can also function as classical Breg cells that suppress autoimmune diseases.

The two B cell lineages in mammals are B-1 and B-2 cells. B-1 cells derive from fetal hematopoietic stem cells in yolk sac or fetal liver, reside primarily in peritoneal cavity (PeC) or umbilical cord blood (CB), and are the earliest B cells to arise during development (1, 2). B-1 cells produce natural IgM antibodies, express polyreactive germline B cell receptor (BCR) with limited *N*-region diversity and are considered as innate lymphocytes (3). They express cell-surface CD5 (on B-1a but not B-1b cells), which serves to increase BCR signaling threshold, thereby restricting activation of B-1a cells to very strong signals induced by innate stimuli (4). B-1a cells have the dual role of maintaining tolerance to normal tissue proteins and regulating endogenous pathogens that induce high intensity toll-like receptor (TLR) signals in tissues or microbiota (2, 4). In contrast, B-2 cells appear much later during embryonic development from the bone marrow and reside mainly in lymphoid follicles (follicular B cells) or marginal zone of the spleen (MZ B cells). Follicular B cells are the largest Ag-specific B cell population and are activated by BCR signals, undergo germinal center reaction, and differentiate into long-lived plasma or memory B cells (5, 6). On the other hand, MZ B cells are self-renewing, long-lived, noncirculating mature B cells that respond mainly to T-independent Ag or innate stimuli. Although the best characterized Bregs are of B-2 lineage, it is not known whether B-1 cells exhibit suppressive functions attributed to B10 and i35-Bregs (7, 8).

Unlike B10 cells that derive from immature MZ B cells or plasmablasts, the CD138$^+$ IL-35–producing Bregs are terminally differentiated plasma cells (8, 9). The i35-Breg cells have recently been shown to suppress experimental autoimmune encephalomyelitis (EAE) and experimental autoimmune uveitis (EAU) that

Significance

Importance of regulatory B cells (Breg cells) is underscored by scores of autoimmune, infectious, or neoplastic diseases that result from loss of Breg functions in vivo. We identified an innate B-1a Breg population in peritoneal cavity and human umbilical cord that suppresses encephalomyelitis or uveitis by propagating inhibitory signals that convert conventional lymphocytes to IL-35–secreting regulatory cells and inhibiting Lag3$^+$PD-1$^+$ T cells that mediate CNS autoimmune diseases. Furthermore, IRF8 signaling is required for i27-Breg development and loss of IL-27Rα or IL-27 function in B cells abrogates capacity of i27-Bregs to suppress diseases or induce B-1a differentiation into i27-Breg. i27-Breg requires activation by innate stimuli and is not antigen-specific, suggesting that i27-Breg would be effective immunotherapy for diverse autoimmune diseases.

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serve as animal models of multiple sclerosis (MS) and uveitis, respectively (8, 9). The IL-35 Breg cell signature cytokine, IL-35, is structurally and functionally similar to IL-27, and both belong to the IL-12 family of cytokines that regulate lymphocyte developmental decisions (10). Like IL-35, IL-27 inhibits neuroinflammation (11, 12), leading us to posit the potential existence of IL-27–producing B cell (i27-Breg) subset and to investigate role of the putative i27-Breg cell in regulating immunity during autoimmune disease.

We describe a B-1 cell population that constitutively secretes IL-27 (i27-Breg) and suppresses encephalomyelitis and uveitis in mice. Analysis of the transcriptome and i27-Breg developmental program revealed that i27-Breg cell is developmentally and functionally distinct from B10 or i35-Breg and IRF8/IRF4 transcription factors play critical roles in skewing B cell differentiation toward i27-Breg or i35-Breg developmental pathway, respectively. Remarkably, we found that immunotherapy with as few as 500,000 i27-Breg cells conferred protection against central nervous system (CNS) autoimmune diseases and adoptive transfer of B-1a cells that do not produce IL-27 or respond to IL-27 (IL-27RαKO) could not suppress disease.

Results

B-1 Cells in PeC Constitutively Secrete IL-27 and Increase in Peripheral Tissues During Inflammation. To investigate whether B cells can produce IL-27, we analyzed B-1 cells in the PeC and B-2 cells in the spleen by the intracellular cytokine assay. The B-1 or B-2 cells constitutively secreted IL-27, and BCR or lipopolysaccharides (LPS) induced their expansion (Fig. 1 *A* and *B*). Regardless of the activating stimulus, B-1a cells are the major producers of IL-27. Analysis of the IL-27–producing B cells by immunohistochemical/confocal microscopy colocalized the p28 and Ebi3 subunits (white arrows) on the B cells (Fig. 1*C*). Proximity ligation assay demonstrated physical interaction between p28 and Ebi3 on the activated B cells (Fig. 1*D*), providing direct evidence that B cells do indeed secrete the heterodimeric IL-27 (p28/Ebi3) cytokine. Reciprocal immunoprecipitation and Western blot analysis of supernatant or whole cell extracts of activated B-1a cells also detected coexpression of p28 and Ebi3 (*SI Appendix*, Fig. S1*A*), further confirming that B cells secrete the heterodimeric IL-27 cytokine. We also show that B-1a cells express significantly higher IL-27 receptor (IL-27Rα) mRNA compared to B-2 cells (Fig. 1*E*, *Top*) and *Il27ra*$^{-/-}$ mice exhibited a defect in generating i27-Breg cells in response to BCR activation (Fig. 1*E*, *Bottom*), suggesting that IL-27 signaling is required for production of IL-27 by B-1 cells. We also generated *p28*$^{-/-}$ mice, and these mice, which do not produce IL-27, have significantly low levels of B-1a and i27-Bregs (Fig. 1*F*, *Top*) compared to wild-type (WT) mice while having normal levels of B-2 and IL-35–producing B cells (i35-Bregs) (Fig. 1*F*, *Bottom*), suggesting that IL-27 and/or IL-27 signals are required for the development of B-1a and/or i27-Breg cells but not B-2 or i35-Breg cells. Thus, the addition of IL-27 to activated B cell significantly increased IL-27 expression (*SI Appendix*, Fig. S1 *B* and *C*) and induced expansion of IL-27–producing B cells (*SI Appendix*, Fig. S1*D*), suggesting that autocrine and paracrine stimulation by the IL-27 produced by activated B-1a cells might mediate expansion of i27-Bregs in vivo. Chromatin immunoprecipitation (ChIP) assay revealed binding of activated STAT1 and STAT3 to *il27a* promoter (*SI Appendix*, Fig. S1 *E* and *F*), providing mechanistic insight into signals downstream of the IL-27 receptors of i27-Bregs. Henceforth, we refer to IL-27–producing B cells as i27-Breg.

Next, we used a mouse model of LPS-induced sepsis to examine whether i27-Bregs are induced in vivo. C57BL/6J mice were injected (intraperitoneally [i.p.]) with LPS, and 24 h post-immunization, percentage of i27-Bregs in the PeC increased from 20 to ∼55.6%, followed by a slow decline to basal level by day 4 of inflammation (Fig. 1*G*, *Left*). In contrast, percentage of i27-Bregs in spleen was very low at the 24 h time-point (∼2.5%) and progressively increased, reaching a peak of 11% by day 3 (Fig. 1*G*, *Right*), strongly suggesting that the rapid mobilization of i27-Bregs in response to inflammation was followed by their migration from the PeC into the spleen. These results are consistent with the report that activated B-1a cells respond to innate signals by exiting the PeC and migrating to secondary lymphoid tissues and sites of infection to participate in the ensuing inflammatory response (13). IL-27 signals regulate chemotactic responses of lymphocytes during infection (14) and Nano-String analysis of RNA from B-1a cells stimulated with IL-27 showed that IL-27 down-regulated *Cxcr4* and up-regulated *Cxcr3* transcription (*SI Appendix*, Fig. S1*I*). We therefore investigated whether IL-27 signals regulated the trafficking of i27-Bregs from the PeC to the spleen during inflammation in our sepsis mouse model. We observed an initial increase of CXCR4-expressing B-1a cells in the PeC that peaked at 24 h, followed by time-dependent decrease of CXCR4-expressing B-1a cells in PeC (Fig. 1*H*, *Right*) which preceded a rapid increase of CXCR3-expressing B-1a cells in the spleen (Fig. 1*H*, *Left*). In line with reports that down-regulation of CXCR4 is required for egress of B cells from bone marrow (15) while CXCR3 mediates recruitment of activated B cells into secondary lymphoid organs (16), our results thus suggest that the decrease of CXCR4-expressing B-1a cells in PeC during LPS-induced sepsis coincided with exit of i27-Bregs from PeC and their subsequent recruitment to the spleen. These in vitro and in vivo studies provide suggestive evidence that i27-Bregs are physiologically relevant and may regulate immunity during infectious and possibly autoimmune diseases.

i27-Breg Cells Are Abundant in B-1 Compartment of Human Umbilical CB and PBMC. Human CD20^{+}CD27^{+}CD43^{+} B cells are orthologs of murine B1 cells that are generally considered as human B1 cells and CD20^{+}CD27^{+}CD43^{+}CD11b^{+} B1 cells represent a B-1a subset that rapidly migrates from PeC to the spleen or inflammatory sites in response to innate stimuli (13, 17). We isolated and activated this unique CD11^{+} B-1a subpopulation in human peripheral blood mononuclear cell (PBMC) or CB and show that as much as 35% of the activated cells were i27-Bregs (Fig. 2*A*). We also show that ∼18.1% resting B-1a cells in human umbilical CB constitutively secrete IL-27 and activation of these B-1a cells in cultures containing IL-27 dramatically increased percentage of the CB i27-Bregs to 73.9% (Fig. 2*B*). These results established that i27-Bregs exist in humans and do respond to inflammatory stimuli. We next used the t-Distributed Stochastic Neighbor Embedding (t-SNE) clustering analysis approach to identify and group the other Breg subpopulations that might also reside in CB and detected three distinct subsets—B10, i27-Breg, and i35-Breg—that are spatially segregated in this tissue (Fig. 2*C*). To further determine the relative abundance of i27-Bregs vis-à-vis i35-Bregs or B10-Breg, we cultured unfractionated human CB cells for 6 d, and majority of the cells in day-3 cultures were i27-Bregs, which comprised 87.1% of the B1 cells (Fig. 2*D*). It is notable that the addition of IL-27 to the cultures had no effect on the relative abundance of i27-Bregs, suggesting that autocrine signaling induced by i27-Bregs was to sustain i27-Breg expansion. Although IL-10-Bregs and i35-Bregs were only 2.6 and 10.2% of B1 cells in day-3 cultures, respectively, they increased >2 fold (2.4 and 2.6-fold) in day-6 cultures, and this was accompanied by diminution of i27-Bregs from 87.1 to 67.5%. Similar analysis of B-2 cells revealed dramatic fluctuations in relative abundance of these Breg subsets (*SI Appendix*, Fig. S2), suggesting that steady-state levels of i27-Bregs, IL-10-Bregs, or

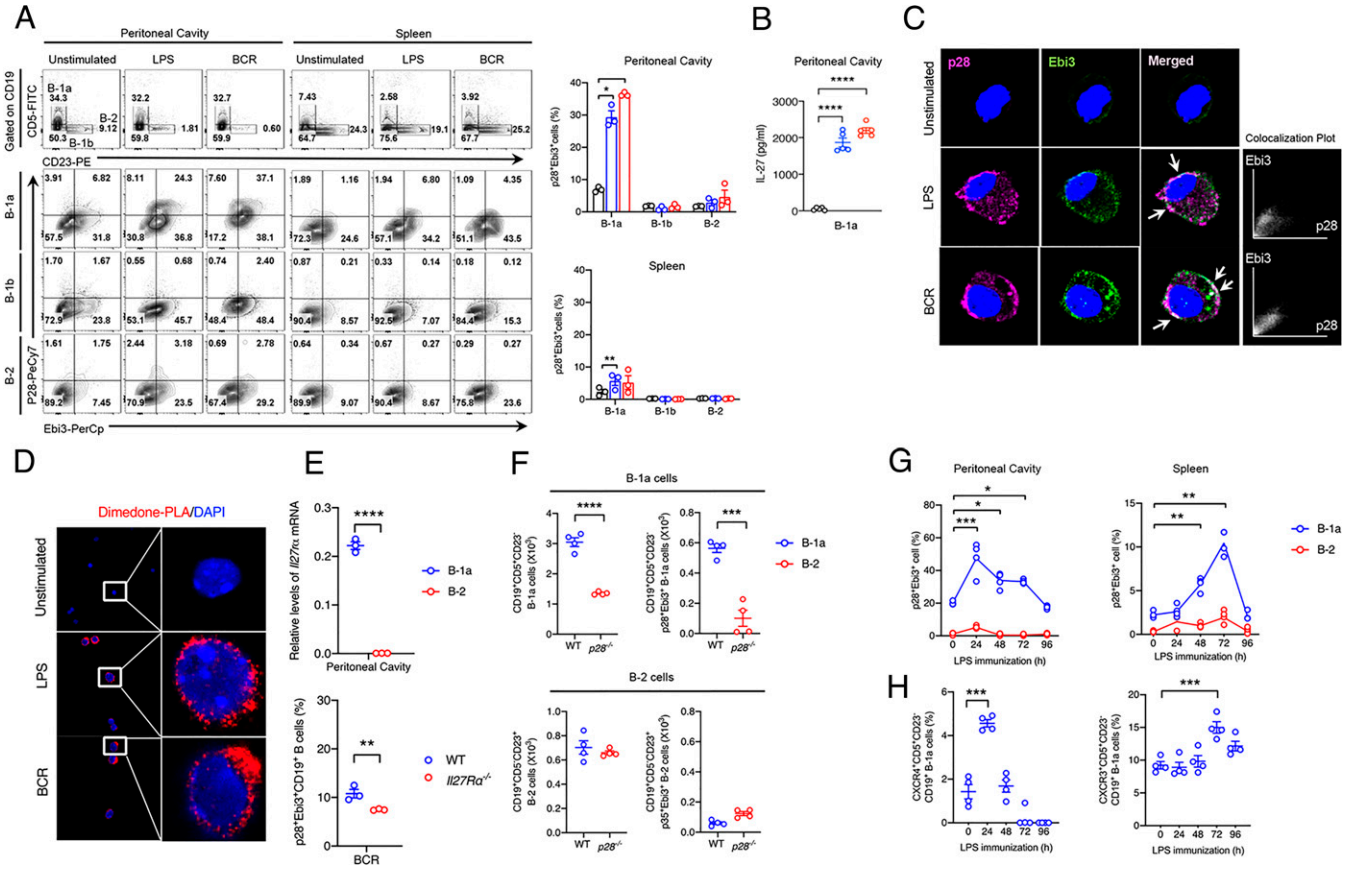

Fig. 1. Peritoneal i27-Breg cells traffic to peripheral tissues in response to inflammation. (*A*) Sorted CD19$^+$ B cells isolated from the PeC or spleen of C57BL/6J mice were activated in vitro for 72 h with LPS (5 μg/mL) or anti-CD40 (10 μg/mL)/anti-IgM antibodies (5 μg/mL, BCR). Representative flow cytometry plots and bar charts show percentage of B-1a and B2 cells expressing IL-27 (p28$^+$Ebi3$^+$). (*B*) Supernatants were analyzed by ELISA. (*C*) Cells coexpressing p28 and Ebi3 (IL-27) were detected by confocal microscopy (white arrows) and colocalization plot. (*D*) Proximity ligation assay was used to demonstrate physical interaction between p28 and Ebi3 subunits and colocalization of p28:Ebi3 heterodimers on the B cells. (*E*) Sorted CD19$^+$CD5$^+$CD23$^-$ B-1a cells or CD19$^+$CD5$^-$CD23$^+$ B-2 cells from the PeC of mice were activated in vitro for 72 h with BCR and relative abundance IL-27Rα expressed by B-1a or B-2 was assessed by qPCR analysis (*Top*). Cells expressing IL-27 (p28$^+$Ebi3$^+$) were detected by intracellular cytokine assay (*Bottom*). (*F*) The numbers of B-1a (CD19$^+$CD5$^+$CD23$^-$) or B-2 (CD19$^+$CD5$^-$CD23$^+$) cells in the PeC of WT or *p28*$^{-/-}$ mouse was determined, and the B-1a or B-2 cells expressing IL-27 (p28$^+$Ebi3$^+$) or IL-35 (p35$^+$Ebi3$^+$) was quantified by intracellular cytokine assay. (*G*) Mice were injected (i.p.) with LPS (50 μg/mouse), and frequency of B-1a (CD19$^+$CD5$^+$CD23$^-$) or B-2 (CD19$^+$CD5$^-$CD23$^+$) in the PeC or spleen expressing IL-27 was assessed at various time points by intracellular cytokine assay ($n = 4$) or (*H*) B-1a cells expressing cell-surface chemokine receptors. Data represent three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$, Student's *t* test.

i35-Bregs are temporally regulated during the course of an inflammatory response. Principal component and RNA-seq analyses also confirmed that human i27-Bregs, i35-Bregs, and CD19$^+$ B cells are distinct Breg subsets (Fig. 2*E*) and that i27-Breg and i35-Breg have distinct transcriptomic profiles (Fig. 2*F*). Of the 3,744 differentially expressed genes, 1,575 were elevated in i27-Bregs while 2,169 were down-regulated (Fig. 2*G*, *Top*). Similar comparison between unchallenged CD19$^+$ B cells and i27-Bregs found that of the 6,159 genes differentially expressed, 3,207 were up-regulated in i27-Breg (Fig. 2*G*, *Lower*).

i27-Breg Cells Confer Protection from Uveitis. EAU is a predominantly T cell–mediated CNS autoimmune disease, and we used this mouse model of human uveitis to investigate whether i27-Bregs are induced during EAU and if adoptive transfer of ex vivo generated i27-Bregs can be used to treat uveitis. EAU was induced by active immunization of C57BL/6J mice with $IRBP_{651–670}$-peptide (300 μg/mouse) in complete Freund's adjuvant (CFA) containing *Mycobacterium tuberculosis* strain H37RA. Mice were treated by i.p. injection of IL-27 (100 ng/mouse) or phosphate-buffered saline (PBS) on day 1 of immunization and every other day until day 10 postimmunization. Experimental detail on method of EAU induction is included in *SI Appendix*. Disease development was assessed by fundoscopy, histology, optical coherence tomography (OCT), and electroretinography (ERG). EAU clinical scores and assessment of disease severity were based on changes at the optic nerve disk or retinal vessels and detection of retinal and choroidal infiltrates in the eye. Fundus images (Fig. 3*A*), histopathologic examination of the retina (Fig. 3*B*), and OCT images (Fig. 3*C*) show characteristic features of uveitis in untreated mice that include papillitis, large numbers of inflammatory cells in the vitreous and optic nerve head nerve, destruction of retinal cells, and extensive retinal folding, a hallmark of uveitis in C57BL/6J mice. In contrast, IL-27–treated mice were protected and exhibited mild EAU with lower disease scores (Fig. 3*A*). Significant decreases in dark-adapted and light-adapted ERG *a* and *b* waves indicated development of visual impairment in the untreated mice, while ERG recordings of IL-27–treated mouse were comparable to normal unimmunized mice (Fig. 3*D*). Protection of IL-27–treated mice from severe uveitis was derived in part from reduced IL-17 cytokine and showed elevated levels of immune-suppressive cytokines,

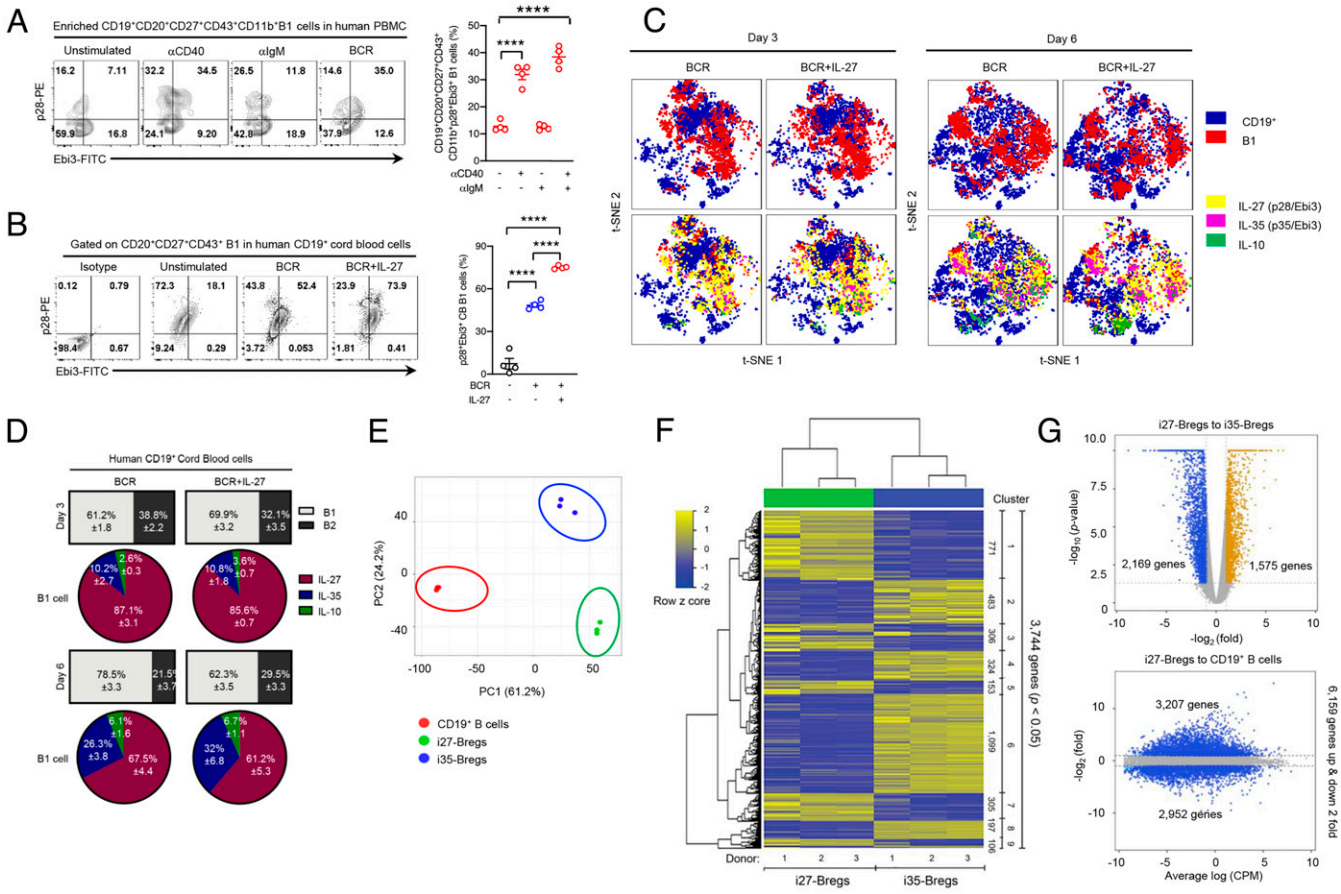

Fig. 2. Identification and characterization of i27-Bregs in human PBMC and umbilical CB. (*A* and *B*) B-1 cells in human PBMC or human umbilical CB were activated with anti-CD40 (5 μg/mL), anti-IgM (10 μg/mL), CPG (10 μg/mL), or BCR in the presence or absence of IL-27 (100 ng/mL). Representative flow cytometry plots and bar charts of showing expansion IL-27–producing $CD19^+CD20^+CD27^+CD43^+CD11b^+$ or $CD19^+CD20^+CD27^+CD43^+$ B-1 cells. (*C*) Representative t-SNE clustering plots (*D*) or flow cytometry pie charts showing the relative abundance of IL-27 (i27-Breg), IL-35 (i35-Breg) and IL-10–secreting Bregs in the B-1 ($CD19^+CD20^+CD27^+CD43^+$) compartment of activated human umbilical CB. (*E*–*G*) RNA-seq analysis using RNA from the conventional $CD19^+$ B-2, i27-Breg (BCR-stimulated $CD19^+CD20^+CD27^+CD43^+$ B cells from umbilical CB) or i35-Breg cells (BCR-stimulated $CD19^+CD38^+$ B cells from PBMC). (*E*) Principal component analysis and (*F*) differentially expressed genes heatmap analysis of i27-Breg and i35-Breg. (*G*) A volcano plot (*Top*) and smear plot (*Bottom*) showing differential gene expression between i27-Breg and i35-Breg cells or conventional $CD19^+$ B-2 and i27-Breg cells. Data represent three independent experiments. $^{****}P < 0.0001$, Student's *t* test.

including IL-27, IL-10, and IL-35 (Fig. 3*E*). We show that i27-Breg cells are induced during EAU (~8.2%) and increased significantly in mice treated with IL-27, with >25 of the IL-27–producing $CD19^+$ B cells deriving from the B-1a compartment (Fig. 3*F*). These results suggest that amelioration of EAU correlates with the expansion of i27-Bregs. To investigate whether i27-Breg cells can be used as therapy for uveitis, we sorted IL-27–producing B-1a cells from PeC of C57BL/6J mice (>80% i27-Bregs), transferred the ex vivo generated i27-Breg cells (5×10^5 cells/mouse) to C57BL/6J or *Il27rα*$^{-/-}$ mice immunized with IRBP/CFA, and evaluated severity of uveitis 17 d postimmunization. Fundus images show severe uveitis in *Il27rα*$^{-/-}$ and WT control mice, while mice administered i27-Bregs were protected from developing severe uveitis (Fig. 3*G*). However, i27-Breg therapy could not ameliorate uveitis in *Il27rα*$^{-/-}$ mice, suggesting that capacity to respond to IL-27 signal is required for EAU suppression. The suppression of EAU correlated with marked reduction of Th1 and Th17 responses and concomitant increase of regulatory T cells (Treg cells) and IL-27–producing B-1a cells in the eyes of i27-Breg–treated mice (Fig. 3 *H* and *I* and *SI Appendix*, Fig. S3*A*). Interestingly, B-2 cells producing IL-35 were also elevated in the eyes of i27-Breg–treated mice (Fig. 3*J*), suggesting that i27-Breg therapy also induced expansion of i35-Bregs during EAU.

Microglia are specialized macrophage-like cells that constitutively express low levels of IL-27 in CNS tissues, and increase in IL-27 has been implicated in the suppression of uveitis or encephalomyelitis (18–20). However, mechanisms that up-regulate IL-27 or its cognate receptor in the CNS during inflammation are not fully understood. To investigate whether IL-27 produced by i27-Breg cells recruited into the retina during EAU contributes to enhanced secretion of IL-27 by microglial cells, we sorted i27-Bregs and microglial cells from the retina of EAU mice and cocultured the cells in a two-chamber cell culture flask separated by 0.4 μm pores. Analysis of the cells after 3 d in culture shows increased expression of IL-27 in cocultures of the two cell types, suggesting that paracrine effects of i27-Bregs and microglial cells synergistically enhanced production of IL-27 (Fig. 3*K* and *SI Appendix*, Fig. S3*B*). On the other hand, addition of guide RNAs (sgp28 and sgpEbi3) specific to p28 or Ebi3 abrogated the synergistic increase of IL-27 (Fig. 3*L*), suggesting that production and binding of IL-27 to cognate receptors on i27-Breg and microglial cells might contribute to elevated levels of IL-27 in the retina during ocular inflammation. In a similar study, we coculture

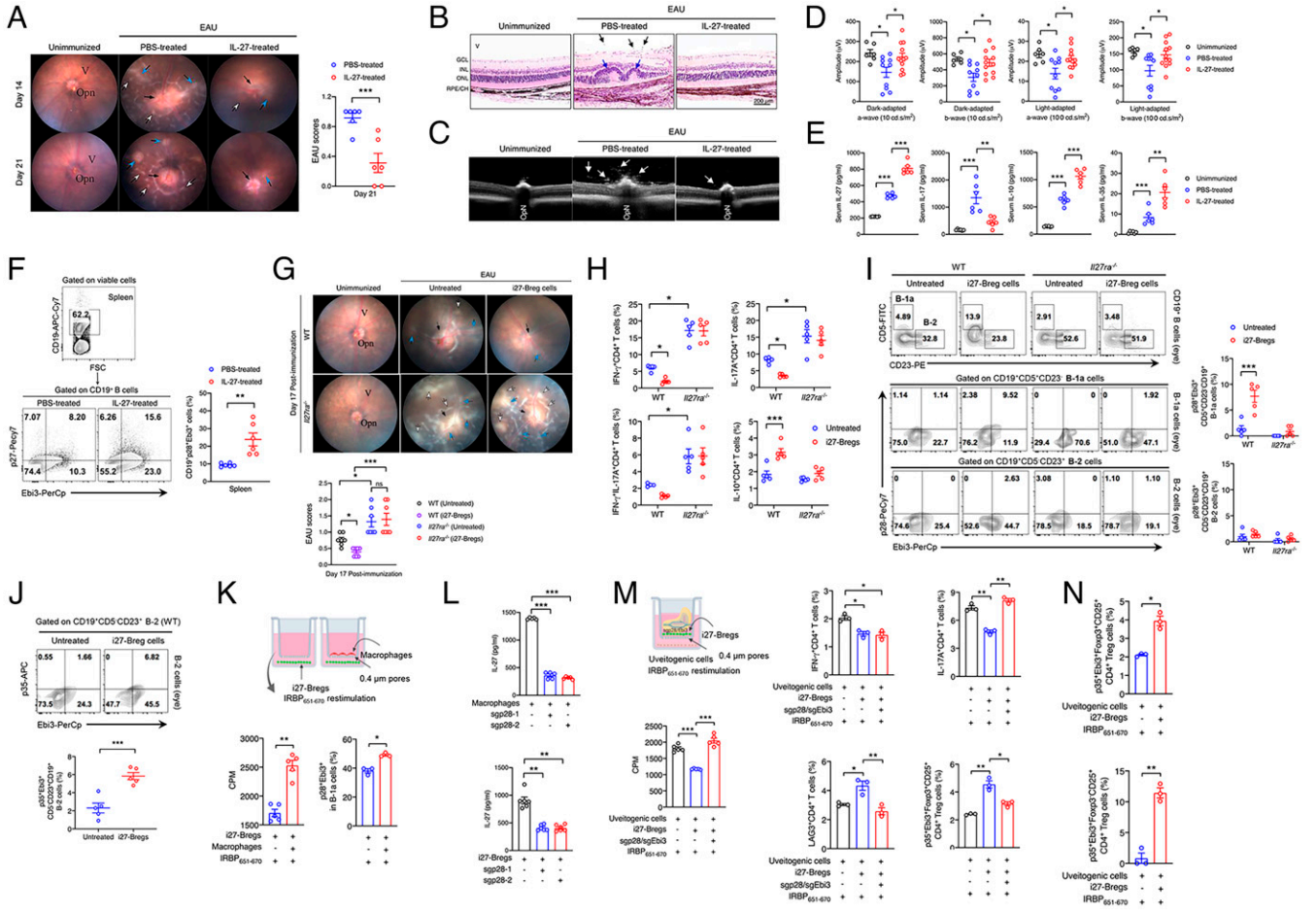

Fig. 3. i27-Bregs produce IL-27 and regulatory cells during autoimmune uveitis. (*A*) EAU clinical scores were based on changes at the optic nerve disk or retinal vessels, extent of retinal-choroidal infiltrates, and fundus images of the retina. Black arrow, inflammation with blurred optic-disk margins (papilledema); blue arrows, retinal vasculitis; white arrows, yellow-whitish retinal and choroidal infiltrates. (*B*) Hematoxylin and eosin histology sections: GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE/CH, retinal pigment epithelium and choroid; V, vitreous. Black arrows, lymphocytes; blue arrows, retinal folds (×200). (*C*) Representative OCT images showing inflammatory cells (white arrows) in the vitreous or optic nerve. (*D*) EAU day-20 light-adapted or dark-adapted ERG analysis showing a-wave or b-wave amplitudes plotted as a function of flash luminance. (*E*) Analysis of serum ELISA. (*F*) Representative flow cytometry plots showing percentage of IL-27–expressing B cells. The percentage of $CD19^+$ B cells expressing IL-27. (*G*) Clinical assessment of disease by fundoscopy show trace EAU in mice that received i27-Bregs (5×10^5 cells/mouse), while normal or *Il27ra*$^{-/-}$ mice that received control B1a cells developed severe EAU ($n = 7$). (*H*) Representative flow cytometry graphs/plot of IL-17–expressing, IL-10–expressing, or IFN-γ–expressing $CD4^+$ T cells, (*I*) i27-Breg cells, or (*J*) i35-Breg in the eye. (*K* and *L*) Coculture of uveitogenic i27-Bregs and $F4/80^+$ macrophages (microglia) from the retina of EAU mice (1:1) for 3 d. Representative flow cytometry percentage graphs showing percentage of IL-27–producing cells (*K*). The suppression of IL-27 production in cultures containing p28-specific (spg28) guide RNA (*L*). (*M* and *N*) T cells from the LN of EAU mice were cocultured for 3 d with i27-Bregs from WT EAU mice or EAU B-1a cells infected with lentivirus guide RNA that targets suppression of IL-27 (sgp28/Ebi3). (*M*) Thymidine incorporation analyzed and data presented as CPM and representative flow cytometry percentage bar graphs show uveitogenic $CD4^+$ T cells expressing IFN-γ, IL-17, LAG3, and/or $CD4^+CD25^+Foxp3^+$ producing IL-35. (*N*) $CD4^+CD25^+Foxp3^+$ or $CD4^+CD25^+Foxp3^-$ secreting IL-35. Data represent two independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, Student's *t* test.

interphotoreceptor retinoid-binding protein (IRBP)–specific uveitogenic $CD4^+$ T cells and i27-Bregs from mice with EAU and show that inhibition of Th17 responses correlated with the up-regulation of LAG3, an inhibitory receptor that induces T cell exhaustion or suppresses T cell expansion (Fig. 3*M* and *SI Appendix*, Fig. S3 *B–E*). Interestingly, we also observed expansion of IL-35–producing Treg cells (iTR35) characterized by expressing in the inhibitory IL-35 cytokine (Fig. 3*N* and *SI Appendix*, Fig. S3*F*).

i27-Bregs Suppress Neuroinflammation and EAE. EAE and EAU share essential immunopathogenic features (21–23). We therefore induced EAE in C57BL/6J mice and investigated whether i27-Breg cells also regulate immunity during EAE. EAE was induced by subcutaneous immunization with myelin oligodendrocyte glycoprotein peptide 35–55 ($MOG_{35–55}$; 200 μg/mouse) in CFA emulsion, containing heat-killed *M. tuberculosis* strain H37RA. Some mice received IL-27 (100 ng/mouse) concurrently with immunization with myelin oligodendrocyte glycoprotein (MOG) and every other day until day 12 postimmunization. Experimental detail on method of EAE induction is included in *SI Appendix*. Control mice treated with PBS developed pathognomonic features of EAE including infiltration of inflammatory cells into the brain and spinal cord, development of flaccid tail or front/hind limb paralysis, and moribund state, while these hallmark characteristics of EAE were attenuated in IL-27–treated mice (Fig. 4*A*). Furthermore, the amelioration of encephalomyelitis correlated with expansion of i27-Breg cells in the spinal cord (Fig. 4*B*) and spleen (*SI Appendix*, Fig. S4*A*). Like the EAU model, increase of Treg cells coincided with reduction of pathogenic Th17 cells (Fig. 4*C* and *SI Appendix*, Fig. S4*B*), and majority of the i27-Bregs that suppressed EAE

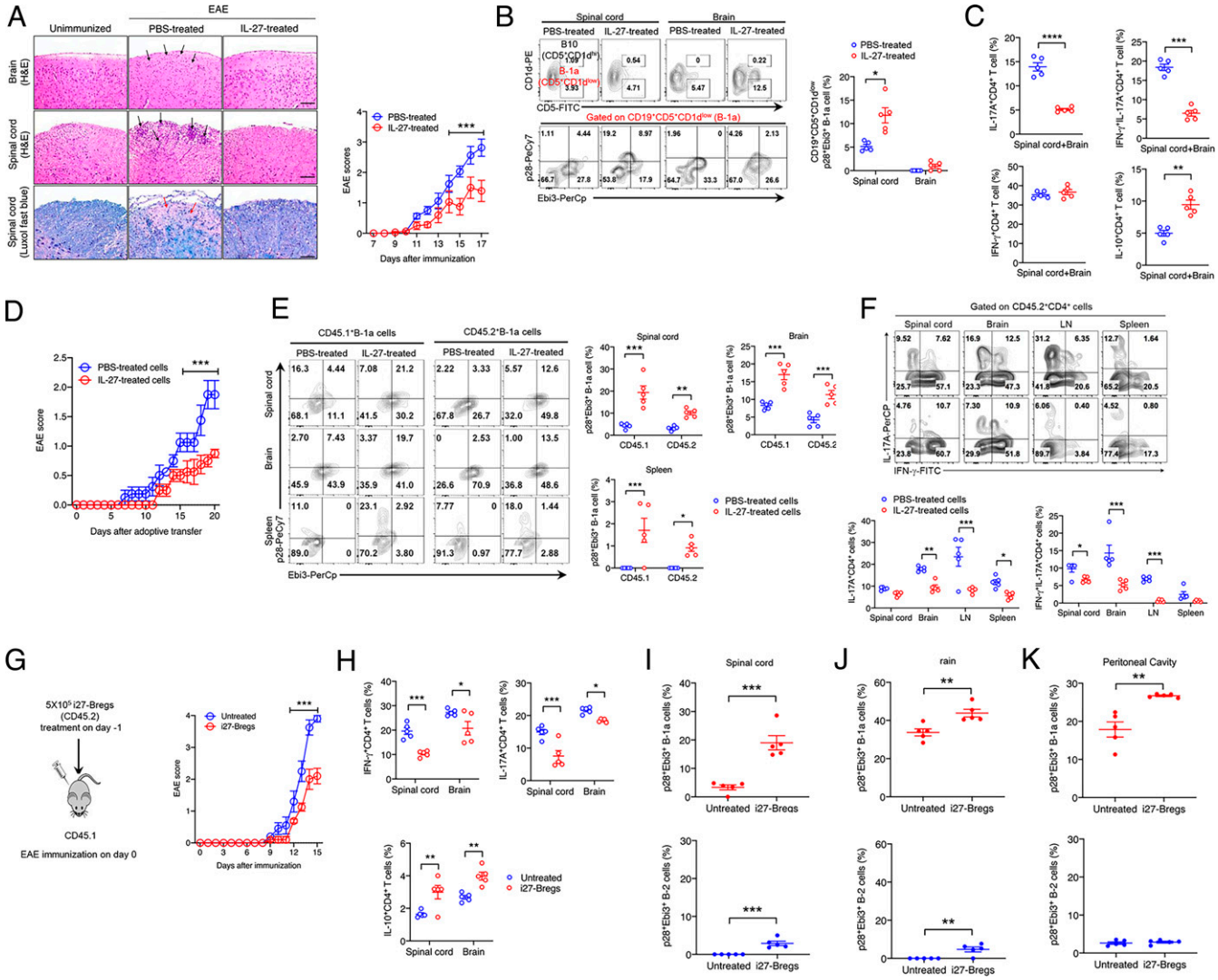

Fig. 4. i27-Bregs suppress EAE and suppress disease in antigen-independent manner. EAE was induced by immunizing C57BL/6J mice with $MOG_{35–55}$-peptide/CFA ($n = 12$). Mice were treated with IL-27 (100 ng/mouse) or PBS on day 0 of immunization and every other day until day-12 postimmunization. Disease severity was assessed by histology and clinical assessment of hallmark features of EAE. (*A*) Hematoxylin and eosin–stained sections on day-17 postimmunization and EAE scores. Black arrows, inflammatory cells in brain or spinal cord; demyelination was assessed by Luxol fast blue staining with red arrows denoting areas of demyelination (×200). (*B*) Brain and spinal cord of PBS-treated or IL-27–treated (100 ng/mL) mice were isolated on day 17 postimmunization, and the flow cytometry plots indicate percentage of i27-expressing B-1a cells or (*C*) IL-17–expressing, IL-10–expressing, or IFN-γ–expressing $CD4^+$ T cells in the spinal cord or brain. (*D–F*) $CD45.2^+$ splenocytes from control or IL-27–treated mice with EAE were restimulated with MOG35-55-peptide/anti-CD40 and adoptively transferred to unimmunized $CD45.1^+$ mice as described for EAU. (*D*) EAE scores. (*E* and *F*) Flow cytometry plots/graphs showing percent IL-27–expressing B-1a cells in spinal cord, brain, or spleen (*E*) or $CD4^+$ T cells expressing IL-17 or IFN-γ (*F*). (*G–K*) Sorted IL-27–secreting B-1a cells (>80% i27-Bregs) in PeC of WT donor $CD45.2^+$ mice were transferred (5×10^5 cells/mouse) to naïve syngeneic $CD45.1^+$ mice and 24 h later EAE was induced in the recipient mice by immunization with $MOG_{35–55}$ ($n = 5$). (*G*) Disease scores assessed by masked investigators indicate reduced EAE symptoms in mice treated with i27-Bregs. (*H*) Fifteen days after adoptive transfer, spinal cord and brain were isolated, digested with collagenase, and analyzed by intracellular cytokine staining assay. Representative flow cytometry graphs show percentage of $CD4^+$ T cells expressing IL-10, IL-17, or IFN-γ. The percentage of $CD19^+CD5^+CD23^-$ B-1a or $CD19^+CD5^-CD23^+$ B2 cells secreting IL-27 in the spinal cord (*I*), brain (*J*), or PeC (*K*). Data represent two independent experiments ($n = 5$). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$, Student's *t* test.

were $CD5^+$ B-1 cells (*SI Appendix*, Fig. S4*C*). We next performed adoptive transfer studies using the CD45.1 congenic mouse strains to examine therapeutic potential of i27-Breg cells. We induced EAE in $CD45.2^+$ mice by immunization with $MOG_{35–55}$, harvested cells from the spleen and lymph node (LN) of mice treated with PBS (control) or IL-27–treated mice, and restimulated and transferred the cells (5×10^5 cells/mouse) to unimmunized $CD45.1^+$ mice. The $CD45.1^+$ mice that received $CD45.2^+$ cells from the control mice developed severe EAE, while the IL-27–treated mice developed mild EAE with delayed onset (Fig. 4*D*). Analysis of spinal cord, brain, or spleen of the recipient mice show a strong correlation between EAE attenuation, expansion of $CD45.2^+$ i27-Bregs and $CD45.1^+$ i27-Bregs (Fig. 4*E*), and concomitant reduction of Th17 responses (Fig. 4*F*).

B-1 cells are highly responsive to pathogens and innate stimuli such as TLR agonists (1), suggesting that immune-suppressive activities of i27-Bregs might not be dependent on BCR-induced signals or BCR specificity. We investigated whether i27-Breg–mediated suppression of an autoimmune disease such as EAE requires prior activation by $MOG_{35–55}$-peptide. We injected (i.p.) $CD45.2^+$ mice with LPS, sorted IL-27–producing B-1a cells

(>83.5% i27-Bregs) from the PeC, and adoptively transferred the i27-Breg–enriched cells (5×10^5/mouse) into naive CD45.1$^+$ congenic mice. Twenty-four hours after transfer, the mice were immunized with $MOG_{35–55}$-peptide/CFA to induce EAE. Compared to B-1a cells from PBS-treated mice (<7.0% i27-Bregs), EAE was significantly suppressed in mice that received i27-Breg cells (Fig. 4*G*). The suppression of EAE correlated with reduction of Th1/Th17 cells and concomitant expansion of Treg cells in brain and spinal cord (Fig. 4*H* and *SI Appendix*, Fig. S4*D*), i27-Breg cells in spinal cord (Fig. 4*I* and *SI Appendix*, Fig. S4*E*), brain (Fig. 4*J* and *SI Appendix*, Fig. S4*F*), or PeC (Fig. 4*K* and *SI Appendix*, Fig. S4*G*). Thus, in line with its developmental origin, the innate-like i27-Bregs can suppress autoimmune diseases in antigen-independent manner.

IL-27/IRF8 Axis Regulates the i27-Breg Transcriptome and Developmental Program. Previous studies have identified and characterized IL-10–producing (B10) and IL-35–producing (i35-Breg) Breg sets that derive from the B-2 lymphocyte lineage (8, 9, 24, 25). It was therefore of interest to compare the transcriptome of i27-Breg, B10, and i35-Breg cells. First, we sorted B-1a cells in PeC, stimulated the B-1a cells with BCR and IL-27, obtained highly enriched IL-27–producing B-1a cells (>83%), and characterized the phenotypes of naive unstimulated B-1a cells and the i27-Bregs. Flow cytometry and enzyme-linked immunosorbent assay (ELISA) analyses revealed that conventional unstimulated B-1a cells secrete natural IgM while the i27-Breg cells do not, indicating an important distinction between i27-Breg and resting B-1a cell in the PeC (Fig. 5*A*). We then performed RNA-seq analysis of unstimulated CD19$^+$ B-1a or B-2 cells and i27-Bregs or i35-Bregs (>57% i35-Bregs) derived from the PeC of C57BL/6J mice. Principal component analysis segregated into B cells distinct gene-expression programs (Fig. 5*B*). Heatmap analysis identified 1,998 genes up-regulated in i27-Breg (*SI Appendix*, Fig. S5), and genes differentially induced in i27-Breg (>2-fold higher expression) include those that encode cytokines, cytokine receptors and chemokine receptors (*Il27*, *Ebi3*, *Il10*, *Il7r*, *Il21r*, *Cxcr3*, *Cxcr5*), inhibitory receptors (*Pdcd1*, *Lag3*), signaling molecules (*Notch4*, *Stat1*, *Stat3*, *Stat5, Akt1*, *Akt2*), and transcription factors (*Irf8*, *Irf1*, *Batf*, *Bhlhe40*, *Xbp1*, *Arid3a*, *Ikzf1*, *Ikzf2*, *Ikzf4*) (Fig. 5*C*). Repressed genes include *Il12a*, *Notch2*, *Cxcr4*, *Ccr2*, *Ccr7*), genes that encode inhibitory receptors (*Pdcd2*, *Cd1d1*, *Ctla4*), and transcription factors (*Irf4*, *Ikzf3*, *Bach2*, *Pax5*, *Ebf1*, *Runx1*, *Foxo1*, *Ets1*) (Fig. 5*C*). Genes required for B-1a development (*Bhlhe40*, *Arid3a*, and *Cd5*) are also increased (26), underscoring the developmental origin of i27-Breg from innate B-1 cells. However, the i27-Breg cell also exhibits a transcription signature characteristic of differentiating germinal center B cells (*Irf8*↑, *Batf*↑, *Pax5*↑, *Bach2*↑, *Ebf1*↑) (5) but not of terminally differentiated plasma cells (*Prdm1*↑, *Bach2*↓, *Pax5*↓, *Ebf1*↓) (6), demonstrating that i27-Breg has a unique transcriptome. Because of the essential role of IL-27 for the development of B-1a cells and expansion of i27-Bregs (Fig. 1*F*), we injected WT and $p28^{-/-}$ mice with LPS (intravenously), sorted B-1a cells in the PeC, and, consistent with our transcriptomic analysis, we observed a defect in the transcription of genes that characterize the i27-Breg phenotype, such as inhibitory-receptor genes (*Lag3*, *Pd1*, *Pd-l1*, *Pd-l2*) in $p28^{-/-}$ B-1a cells (Fig. 5*D*).

IRF-4 and IRF-8 transcription factors are expressed in developing B cells, and because concentration-dependent competition between these factors has been shown to control cell-fate decisions of differentiating B cells (27, 28), we examined whether IRF8/IL-27 axis might also regulate development and expansion i27-Breg subset. Consistent with our RNA-seq analysis showing that IRF8 is up-regulated in i27-Bregs while IRF4 is down-regulated (Fig. 5*C*), we also found 3 d after injection of mice with LPS, B-1a cells in the PeC preferentially express IRF8 but not IRF4 (Fig. 5*E*). We also show that activated $p28^{-/-}$ PeC B-1a cells do not express IRF8 (Fig. 5*F*), suggesting that IL-27/IRF8 axis might play critical role in skewing differentiation of B-1a cells toward i27-Breg developmental pathway. Because IRF4 and IRF8 regulate gene transcription by forming complexes with BATF family transcription factors and binding AP-1/IRF composite elements (AICEs) on target genes (29, 30), we performed gel-shift analyses to determine whether these IRF4 and/or IRF8 are downstream targets of IL-27 signaling in B cells. We injected C57BL/6J mice with LPS or LPS+IL-27 for 3 d, and electrophoretic mobility shift assay (EMSA)/supershift analysis of nuclear extracts derived from CD19$^+$ B cells show that both IRF4 and IRF8 were recruited to AICE of *Ctla4* (Fig. 5*G*, *Middle*) while IRF8 but not IRF4 was specifically recruited to AICE of *il27a* (Fig. 5*G*, *Right*). Taken together with the finding that IL-27 is required for B-1a cell development and i27-Breg expansion (Fig. 1*H*), these results lead us to posit a sequential feed-forward mechanism whereby IL-27 increases IRF8 expression and recruitment of IRF8-BATF complex to the p28 promoter, resulting in enhanced secretion of IL-27 and expansion of B cells expressing IL-27 receptor. We generated mice with targeted deletion of *irf8* ($Irf8^{fl/fl}Cd19^{+/cre}$) or IRF4 ($Irf4^{fl/fl}Cd19^{+/cre}$) in B cells (*SI Appendix*, *Methods*) and tested our hypothesis that IRF8 is required for B-1a and i27-Breg development. The $Irf4^{fl/fl}Cd19^{+/cre}$ or $Irf8^{fl/fl}Cd19^{+/cre}$ mice were immunized with anti-CD40/anti-IgM (BCR), and 3 d later, we performed t-SNE clustering analysis of the B cells in the PeC. The t-SNE plots of $Irf4^{fl/fl}Cd19^{+/cre}$ mouse PeC show significant increase of B-1a (Fig. 5*H*, *Top*) and i27-Breg (Fig. 5*H*, *Bottom*) populations. In contrast, t-SNE plots of $Irf8^{fl/fl}Cd19^{+/cre}$ mouse PeC show significant decrease of B-1a (Fig. 5*H*, *Top*) and i27-Breg cells (Fig. 5*H*, *Bottom*). On the other hand, B2 and i35-Bregs were markedly reduced in $Irf4^{fl/fl}Cd19^{+/cre}$ and significantly increased in $Irf8^{fl/fl}Cd19^{+/cre}$ mouse PeC (Fig. 5*H*). Western blot analysis also shows exaggerated increase in expression of IL-27 by the $Irf4^{fl/fl}Cd19^{+/cre}$ B cells (Fig. 5*I*), suggesting that the loss of *irf4* induced compensatory increase in IRF8 and skewing of B cells toward the i27-Breg phenotype. Taken together, these results suggest that IRF8 and IRF4 might play critical roles in skewing B cell differentiation toward i27-Breg or i35-Breg developmental pathway, respectively.

Discussion

This study demonstrates the existence of a unique IL-27-producing innate B-1a population in human umbilical CB and mouse PeC and that the i27-Breg cells are characterized by CD5$^+$CD11b$^+$CXCR4$^+$LAG3$^+$PD-1$^+$PD-L1$^+$ immunophenotype. Upon sensing innate stimuli or BCR activation, the resting B-1a cells undergo rapid expansion, exit the PeC, and migrate to peripheral lymphoid tissues or sites of ongoing inflammation in CNS tissues. In contrast to resting B-1 cell, which maintains immunological tolerance and suppresses endogenous pathogens through production of natural antibodies, the activated IL-27–secreting B-1a cell does not produce natural IgM antibodies but suppresses neuroinflammation through the regulatory effects of IL-27. The i27-Breg subset is also distinct from the newly described LAG3$^+$ regulatory plasma B cell population that suppresses inflammation by producing IL-10 and natural IgM antibodies (31).

Uveitis, a diverse group of potentially sight-threatening intraocular inflammatory disease and MS, a potentially paralyzing neurological disease, are classic chronic CNS autoimmune diseases characterized by cycles of remitting and recurrent inflammation. Antiinflammatory drugs, including steroids, are effective and the standard of care, but they can be toxic and ineffective with prolonged use, and there is an impetus to develop alternative therapies. Cell-based immunotherapy is an emerging therapeutic approach for CNS inflammatory and

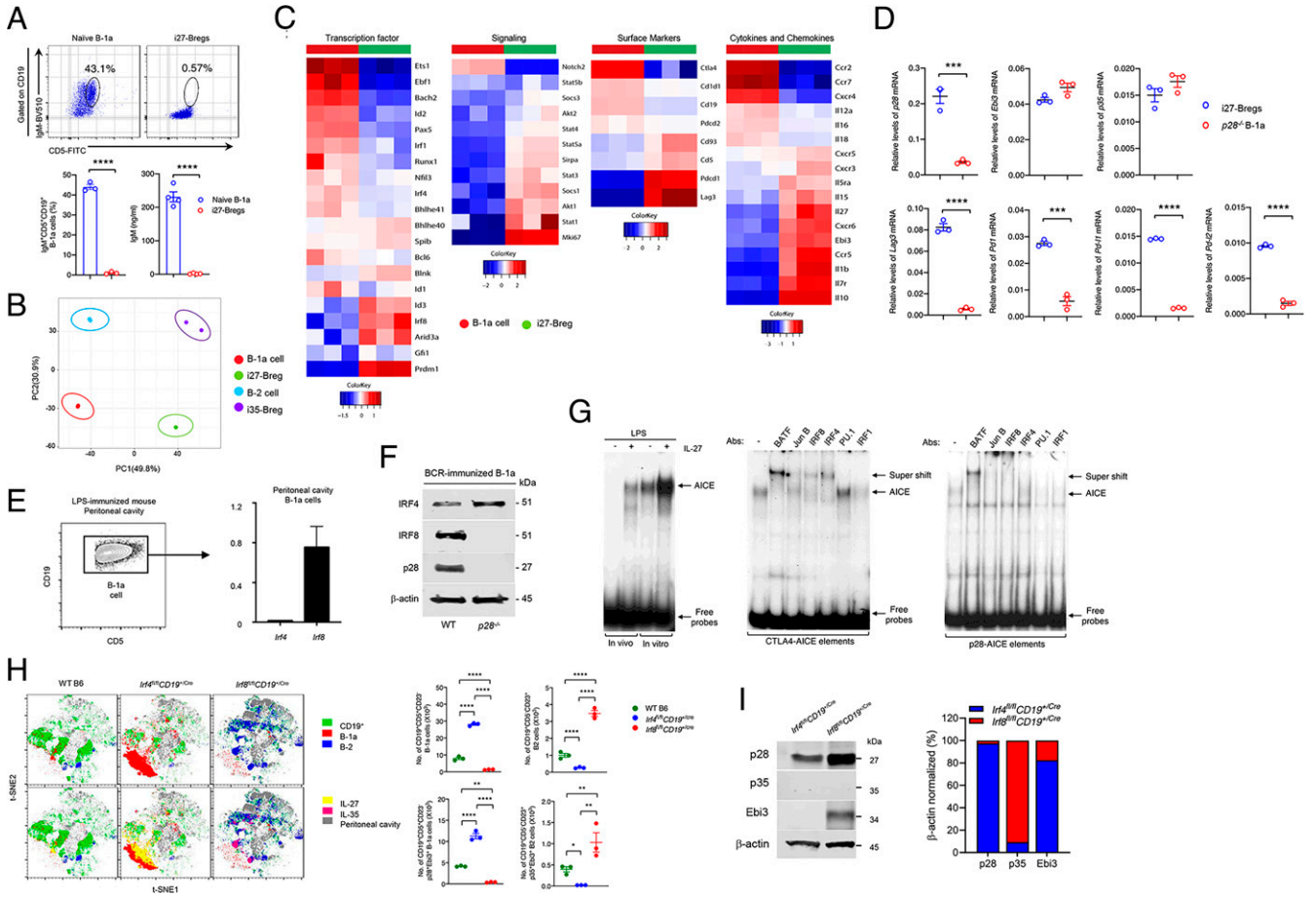

Fig. 5. Characterized i27-Bregs are IRF8 differentially skew naïve B cells toward their developmental pathway. (*A*) Analysis of IgM production by PeC B-1a or i27-Breg cells. Representative flow plot/graphs of intracellular cytokine analysis and ELISA (*Bottom*). (*B* and *C*) RNA-seq analysis of gene-expression program of i27-Breg (BCR-stimulated $CD19^+CD5^+$), i35-Breg (LPS-stimulated $B220^+CD138^+$), conventional B-1a ($CD19^+CD5^+$), or B-2 cells ($B220^+$). (*B*) Principal component analysis. (*C*) Heatmap showing genes by i27-Bregs compared to B-1a cells. (*D*) C57BL/6J (WT) and $p28^{-/-}$ mice were i.p. injected with LPS (50 μg/mouse), and after 24 h, RNA of purified B-1a cells from PeC was analyzed by qPCR for expression of inhibitory receptors. (*E*) C57BL/6J mice were injected (i.p.) with LPS. After 24 h, B-1a cells in the PeC were sorted and expression of *Irf4* or *Irf8* quantified by qPCR. (*F*) WT or $p28^{-/-}$ mice were injected (i.p.) with BCR. After 3 d, B-1a cells were isolated from PeC cell and analyzed by Western blotting. (*G*) Nuclear extracts from B cells in spleen of C57BL/6J mice injected (i.p.) with LPS or LPS plus IL-27 was analyzed by EMSA. EMSA/supershift analysis detected IL-27–induced AICE complexes (*Left*) and show recruitment of IRF4 and IRF8 to AICEs of *Ctla4* (*Middle*), while IRF8 but not IRF4 was recruited to AICE of *il27a* (*Right*). (*H*) Mice with B cell–specific deletion of IRF8 ($Irf8^{fl/fl}Cd19^{+/cre}$) or IRF4 ($Irf4^{fl/fl}Cd19^{+/cre}$) were injected with BCR and 3 d postinjection, IL-27–secreting or IL-35–secreting B-1a or B-2 cells in PeC were analyzed by t-SNE clustering analysis. Cytometry plots/graphs of percent i27-Bregs in PeC of $Irf4^{fl/fl}Cd19^{+/cre}$ or i35-Bregs in $Irf8^{fl/fl}Cd19^{+/cre}$ PeC. (*I*) Western blot data analysis of B cells in PeC of $Irf4^{fl/fl}Cd19^{+/cre}$ or $Irf8^{fl/fl}Cd19^{+/cre}$ mice. Data represent >3 independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$, one-way ANOVA with post hoc Tukey's test.

autoimmune diseases such as MS, uveitis, and a variety of neurodegenerative diseases. Increased expression of IL-27 in cerebrospinal fluid of MS patients and active MS plaques by astrocytes and up-regulation of IL-27 in the retina by microglia and ganglion cells during uveitis suggest that IL-27 might play critical role in regulating and suppressing neuroinflammation (18–20, 32). In this study, we have used EAE and EAU, mouse models of MS and uveitis, respectively, to investigate the therapeutic potential of i27-Breg immunotherapy for MS and uveitis. We show that i27-Breg cells are induced and expanded in response to neuroinflammation in mice, and mice that lack i27-Bregs developed severe encephalomyelitis or uveitis. The i27-Breg cells mitigated EAE or EAU by proliferating and sustaining IL-27 secretion locally in the brain, spinal cord, or retina, which is a therapeutic advantage over administering biologics such as IL-27 or IL-35 cytokine that are rapidly cleared in vivo. Mechanistically, i27-Bregs immunotherapy suppressed uveitis or encephalomyelitis by inducing conventional effector lymphocytes to up-regulate inhibitory receptors that promote T cell exhaustion and inhibition of pathogenic Th17 cells that mediate EAE and EAU.

Most Breg subsets described thus far are of the B2 lymphocyte lineage, suppress inflammation through secretion of IL-10, and derive from B10, MZP, or MZ cells (33). However, while the recently described natural IL-10–producing Bregs or the IL-35–producing Bregs (i35-Bregs) are also B2 cells, they derive from $CD138^+$ plasma cells (8, 9, 31). On the other hand, the i27-Breg is of the B1 lineage and exhibits features of innate lymphocytes. Despite their distinct developmental origins, the i27-Breg and i35-Breg cells suppress EAE or EAU in the mouse through production of their signature cytokines IL-27 and IL-35, respectively (11, 12, 34, 35), and induce expression of LAG3 and PD1 on encephalitogenic and uveitogenic lymphocytes, suggesting that both Breg subsets might suppress neuroinflammation by similar mechanisms that might involve T cell exhaustion (36). The structural and functional similarities of IL-27 and IL-35 thus beg the question of why the immune system requires the innate-like i27-Bregs and i35-Breg cells

with apparently redundant functions. Could it be that the IL-27–producing B-1a cells are the evolutionary antecedents of B2 Breg cells? Although at first glance i27-Breg appears to be functional akin to i35-Breg cells, data presented in this study reveal important differences between these Breg subtypes and suggest that i27-Bregs play critical roles in initiating and orchestrating immune response that mitigate pathogenic neuroinflammation. First, i27-Bregs suppressed EAE and EAU in part through propagating infectious-tolerance signals that induced the expansion of B10, i35-Bregs, and iTR35 in the brain, spinal cord, retina, or spleen. We also show that i27-Breg and i35-Breg cells encode distinct transcriptomes, and their differentiation is differentially regulated by BATF-IRF transcription factors, with BATF-IRF8 skewing naıve B-1a cells toward the i27-Breg developmental pathway while BATF-IRF4–promoting B2 cells develop into i35-Bregs (30). Thus, we observed significant increase of i27-Breg cells in mutant mice with targeted deletion of *irf4* in B cells that corresponded with marked reduction of i35-Bregs, while deletion of *irf8* in B cells correlated with expansion of i35-Bregs and absence of i27-Bregs in $irf8^{fl/fl}Cd19^{+/Cre}$ mice. These data suggest that net increase of IRF8 in B cells may serve as a developmental checkpoint event that promotes B-1a and i27-Breg development. Data presented here are consistent with reports that B cell gene-expression program is sensitive to fluctuations in steady-state concentrations of IRF4 or IRF8 and that mutual antagonism between IRF4 and IRF8 might regulate B cell developmental fate decisions (27, 28).

Therapeutic administration of IL-27 effectively suppressed uveitis and encephalomyelitis in mice (9, 35, 37), underscoring potential of IL-27 as biologics for CNS autoimmune disease, and begs the question whether i27-Breg immunotherapy provides significant therapeutic advantage over use of IL-27. Despite technical challenges associated with ex vivo generation of large amounts of functional i27-Breg, this study has revealed important advantages over use of the heterodimeric IL-27 cytokine with unpredictable pharmacokinetic profile pertaining to therapeutic dosing. Like IL-35, the association of the IL-27 subunit proteins, p28 and Ebi3, is not strong (noncovalent) and readily dissociates, making it difficult to ascertain the effective dose of bioactive IL-27 (p28/Ebi3) in tissues required to ameliorate disease. Interestingly, recent reports have identified Treg and Breg cells that secrete IL-35–containing exosomes (i35-exosomes), and the i35-exosomes were used to suppress neuroinflammation and uveitis in mice (38, 39). In addition, Treg cells were shown to display both p35 and Ebi3 in a membrane-associated form or as components of extracellular vesicles (38) but not as a water-soluble heterodimer, providing further evidence that IL-35 is an exosome-associated cytokine. Given that IL-27 is structurally and functionally similar to IL-35 and is a noncovalently linked heterodimeric cytokine that shares the Ebi3 subunit with IL-35, it is likely that IL-27 might also be an exosome-associated cytokine that can be exploited therapeutically. Nonetheless, i27-Breg immunotherapy obviates these constraints related to bioavailability of IL-27 as the adoptively transferred i27-Bregs proliferate in vivo, ensuring sustained provision of therapeutic effective concentration of IL-27 in target tissues. In contrast to i27-Bregs, i35-Bregs are terminally differentiated $CD138^{+}$ plasma cells that do not proliferate, suggesting that i27-Breg immunotherapy may be more effective, and this is reflected by the relatively low numbers of i27-Bregs (<500,000) required to suppress EAU or EAE. An important distinction between B1 and B2 Breg populations is that the i27-Breg is a natural Breg population primed to produce its signature immune-suppressing IL-27 cytokine within hours of sensing innate or BCR signal. In contrast, B2-derived B10 or i35-Breg cells require interaction with dendritic cells that license them to acquire their immune-suppressive functions (33, 40). In addition, we have shown that the innate-like i27-Breg cells induced expansion of B2 Breg cells (B10, i35-Bregs), and this is consistent with the well-recognized immunological principles that the innate system initiates immunological responses and activates adaptive immune cells that orchestrate the adaptive response.

In summary, data presented here suggest that i27-Bregs has the dual role of an innate Breg cell that senses TLR or BCR signals and orchestrates and adaptive regulatory loop that prevents exuberant autoimmune responses by inducing expansion of B2 regulatory Breg cells. Consistent with its developmental origin, the innate-like i27-Bregs suppresses inflammation through sustained production of the immunosuppressive IL-27 cytokine and does not require prior activation by a particular autoantigen to suppress an autoimmune disease. Because i27-Breg cell has the unique capacity of regulating innate and adaptive immune responses, we propose that i27-Breg immunotherapy can be used to treat diverse autoimmune diseases.

Materials and Methods

Mice. C57BL/6J, B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1), and B6N.129P2-*Il27ra*^{tm1Mak}/J (*Il27rα*$^{-/-}$) mice were purchased from Jackson Laboratory. *p28*$^{-/-}$, $Irf8^{fl/fl}Cd19^{+/cre}$, and $Irf4^{fl/fl}Cd19^{+/cre}$ mice were generated in this study and maintained and treated in accordance with NIH animal care guidelines (41), and studies were approved under Study Protocol # NEI-597. All mice analyzed were 6 to 12 wk old, and mice were randomized for all the studies described. Details on the protocols for in vivo experiments are provided in the *SI Appendix*.

Data Availability. All data discussed in this paper are included in this article and *SI Appendix*. Details on cell culture, i27-Breg activation, antibodies and reagents, cell sorting and flow cytometry, proliferation assays, RNA-seq, quantitative PCR, ELISA, Western blotting, CRISPR/Cas9-mediated gene deletion, proximity ligation assay, immunofluorescence staining and confocal imaging analysis, ChIP assay, EMSA, and statistical analysis are also provided in the *SI Appendix*.

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