Interleukin-10-Producing Plasmablasts Exert Regulatory Function in Autoimmune Inflammation

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SUMMARY

B cells can suppress autoimmunity by secreting interleukin-10 (IL-10). Although subpopulations of splenic B lineage cells are reported to express IL-10 in vitro, the identity of IL-10-producing B cells with regulatory function in vivo remains unknown. By using IL-10 reporter mice, we found that plasmablasts in the draining lymph nodes (dLNs), but not splenic B lineage cells, predominantly expressed IL-10 during experimental autoimmune encephalomyelitis (EAE). These plasmablasts were generated only during EAE inflammation. Mice lacking plasmablasts by genetic ablation of the transcription factors Blimp1 or IRF4 in B lineage cells developed an exacerbated EAE. Furthermore, IRF4 positively regulated IL-10 production that can inhibit dendritic cell functions to generate pathogenic T cells. Our data demonstrate that plasmablasts in the dLNs serve as IL-10 producers to limit autoimmune inflammation and emphasize the importance of plasmablasts as IL-10-producing regulatory B cells.

INTRODUCTION

In the context of autoimmune disorders, B cells can be pathogenic effectors through their production of autoantibodies. However, evidence is accumulating that B cells can also be immunosuppressive in T-cell-mediated autoimmune and inflammatory diseases. Examples are collagen-induced arthritis (CIA) (Mauri et al., 2003), systemic lupus erythematosus (SLE) (Watanabe et al., 2010), and experimental autoimmune encephalomyelitis (EAE), an animal model of human multiple sclerosis (MS) (Fillatreau et al., 2002; Matsushita et al., 2008). The regulatory function of B cells is considered to be mainly determined by the secretion of interleukin-10 (IL-10), which is controlled by signals from Toll-like receptors (TLRs) (Lampropoulou et al., 2008), CD40 (Mauri et al., 2003), and B cell antigen receptors (BCR) (Fillatreau et al., 2002). To date, several unique populations of splenic IL-10-competent B cells (regulatory B cells) have been described. They include $CD21^{hi}CD23^{hi}IgM^{hi}$ transitional 2-marginal zone precursor (T2-MZP) B cells (Evans et al., 2007) and $CD1d^{hi}CD5^{+}$ B cells (Matsushita et al., 2008) that have been reported to inhibit autoimmunity. In addition, splenic $CD138^{+}$ plasma cells were also reported to express IL-10 (Shen et al., 2014). However, these populations produce detectable IL-10 only when stimulated ex vivo. Thus, despite progress made in understanding the importance of B-cell-derived IL-10, there has been no definitive identification of in vivo IL-10-producing B cells with regulatory function during autoimmunity.

In humans, a role for B-cell-derived IL-10 in downregulation of inflammatory reactions has been suggested in autoimmune diseases such as MS or SLE (Blair et al., 2010; Duddy et al., 2007; Mauri and Bosma, 2012). Treatment with rituximab for B cell depletion efficiently ameliorated the disease progression in some autoimmune diseases, presumably because of elimination of pathogenic B cells (Gürcan et al., 2009). However, this might work in part because of selective survival and repopulation of regulatory B cell subsets (Duddy et al., 2007; Todd et al., 2014). The functional and clinical importance of human IL-10-competent B cells has begun to be elucidated but more must be learned about their characteristics.

Here we have exploited IL-10 reporter mice to identify in vivo IL-10-producing B cells and demonstrate that $CD138^{+}$ plasmablasts, proliferating immature plasma cells, are the predominant source of IL-10 during EAE development. IL-10-producing plasmablasts were generated specifically in the draining lymph nodes (dLNs) but not in the spleen after EAE induction. By genetic

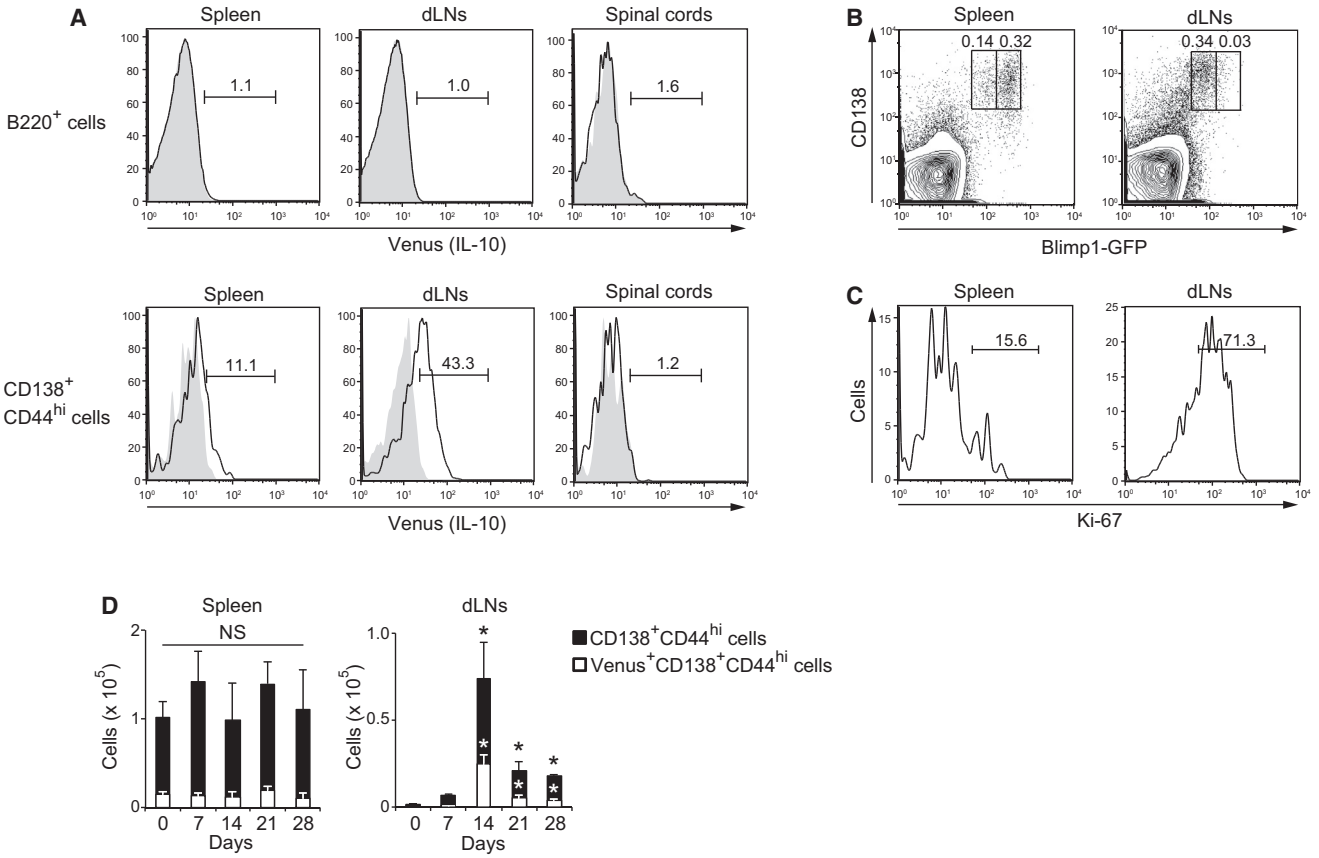

Figure 1. Plasmablasts Are the Dominant IL-10-Producing B Lineage Cells during EAE

(A) Flow cytometry of Venus expression in B220$^+$ and CD138$^+$CD44^{hi} cells harvested from spleen, dLNs, and spinal cords of wild-type (shaded histogram) and *Il10*$^{Venus/+}$ (open histogram) mice 14 days after MOG_{35-55} immunization. Percentages of Venus$^+$ B cells are shown.

(B) Flow cytometry of cells from spleen and dLNs of *Prdm1*$^{gfp/+}$ mice 14 days after MOG_{35-55} immunization. GFP^{int} and GFP^{hi} populations of CD138$^+$ cells are gated and their percentages are shown.

(C) Flow cytometry of CD138$^+$CD44^{hi} cells from spleen and dLNs of wild-type mice 14 days after MOG_{35-55} immunization. Percentages of Ki-67$^+$ cells are shown. Data are representative of at least three independent experiments in (A)–(C).

(D) Absolute number of CD138$^+$CD44^{hi} or their Venus$^+$ cells from spleen and dLNs of *Il10*$^{Venus/+}$ mice before and 7, 14, 21, and 28 days after MOG_{35-55} immunization. Data are representative of two independent experiments. Data are presented as mean ± SEM for four mice. NS, not significant. *$p < 0.05$ versus day 0 (Welch's t test).

See also Figure S1.

approaches, we show that plasmablasts in the dLNs were critical for limiting EAE progression. In addition, IL-10 production by plasmablasts requires IRF4 and can prevent dendritic cells from generating pathogenic T cells. Furthermore, human plasmablasts also preferentially secrete IL-10, further highlighting plasmablasts as the IL-10-producing regulatory B cells.

RESULTS

Plasmablasts Are the Main IL-10-Producing B Cells during EAE

To identify in vivo IL-10-producing B cells and their distribution during autoimmune disease, we elicited EAE in mice carrying a transgene of *Venus*, a variant of yellow fluorescent protein (*Il10*$^{Venus/+}$), which allows tracking of IL-10$^+$ cells (Atarashi et al., 2011). Although previous reports suggested that several splenic B cell subsets can produce IL-10 (Mauri and Bosma, 2012; Yanaba et al., 2008), we observed little Venus expression in B220$^+$ B cells before and 14 days after immunization with myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}) (Figure 1A and Figures S1A–S1D available online). In contrast, CD138$^+$CD44^{hi} cells expressed Venus markedly in the dLNs, only modestly in spleen, and not at all in the spinal cords (Figures 1A, S1C, and S1D). ELISA assays also demonstrated that the CD138$^+$CD44^{hi} population expressing Venus had a potential to produce IL-10 (Figure S1E). CD138$^+$ cells are composed of highly proliferative plasmablasts and nondividing plasma cells that express intermediate and high amounts of Blimp1 (encoded by the *Prdm1*), respectively (Kallies et al., 2004). By utilizing EAE-induced heterozygous *Prdm1*^{gfp} knockin mice (*Prdm1*$^{gfp/+}$), we confirmed that CD138$^+$ cells in spleen and dLNs were largely GFP^{hi} plasma cells and GFP^{int} plasmablasts, respectively (Figure 1B). CD138$^+$CD44^{hi} cells in dLNs, but not spleen, were proliferating, as demonstrated by Ki-67 staining

(Figure 1C). Whereas the absolute number of CD138$^+$CD44^{hi} cells and their Venus$^+$ cells was essentially constant in spleen, those in dLNs expanded to a peak on day 14 after MOG_{35-55} immunization (Figure 1D). Thus, these results indicate that CD138$^+$ plasmablasts in dLNs are the principal IL-10-producing B lineage cells during EAE.

Plasmablasts in the dLNs Negatively Regulate EAE Inflammation

A key question is whether IL-10$^+$CD138$^+$ cells are functionally competent to inhibit EAE. To directly address this issue, we elicited EAE in mice conditionally lacking Blimp1 in B lineage cells by crossing of *Prdm1*$^{f/f}$ with *Mb1*$^{Cre/+}$ mice (called *Prdm1*$^{f/f}$*Mb1*$^{Cre/+}$ here). Plasma cell differentiation and antibody responses were impaired in these mice (Figures S2A and S2B). EAE development in *Prdm1*$^{f/f}$*Mb1*$^{Cre/+}$ mice was greatly exacerbated as compared to *Mb1*$^{Cre/+}$ control mice (Figure 2A). Consistent with the exacerbated EAE, CD4$^+$ T cells, particularly those producing interferon-γ (IFN-γ) (Th1 cells) and IL-17 (Th17 cells), increased in the spinal cords of *Prdm1*$^{f/f}$*Mb1*$^{Cre/+}$ mice (Figure 2B). When stimulated with MOG_{35-55}, *Prdm1*$^{f/f}$*Mb1*$^{Cre/+}$ LN cells produced more IFN-γ and IL-17 than *Mb1*$^{Cre/+}$ cells (Figure 2C). Thus, we conclude that CD138$^+$ plasmablasts/plasma cells limit EAE inflammation.

Given that IL-10-producing CD138$^+$ cells are detected in both spleen and dLNs during EAE, it remained important to test which secondary lymphoid organ is critical for EAE attenuation. L-selectin (CD62L), also known as Sell, is an essential homing receptor that governs migration into the peripheral LNs. To explore the involvement of LN B cells in EAE suppression, we generated mixed bone marrow (BM) chimeras by transferring a mixture of BM cells from μMT (80%) and *Sell*$^{-/-}$ (20%) mice into lethally irradiated wild-type mice. The Sell deficiency was restricted to B cells in the resultant BM chimera (B-*Sell*$^{-/-}$) mice. These mice lacked B lineage cells in LNs, but not spleen, and exhibited increased disease severity compared with control mice (Figure 2D). In striking contrast, mice that had splenectomy developed EAE normally (Figure 2E). B cell population and plasmablast differentiation in the dLNs was not affected by splenectomy. Collectively, these data suggest that plasmablasts in the dLNs negatively regulate EAE, but that splenic B lineage cells are dispensable for its suppression.

Nevertheless, published studies have claimed a functionally important role of splenic B cells to reduce EAE in adaptive transfer experiments (Fillatreau et al., 2002; Matsushita et al., 2008). Based on our above findings, we reasoned that adoptively transferred splenic B cells might give rise to plasmablasts in the dLNs that then regulate EAE. We therefore examined EAE in μMT mice with adoptively transferred splenic B cells isolated from *Prdm1*$^{f/f}$*Mb1*$^{Cre/+}$ or *Sell*$^{-/-}$ mice and control mice. Although the mice that received B cells from control mice resolved EAE symptoms, these suppressive effects were not observed when *Prdm1*$^{f/f}$*Mb1*$^{Cre/+}$ and *Sell*$^{-/-}$ B cells were transferred (Figures 2F and 2H). As expected, plasmablast differentiation from control B cells, but not *Prdm1*$^{f/f}$*Mb1*$^{Cre/+}$ and *Sell*$^{-/-}$ B cells, occurred in LNs (Figures 2G and 2I). These results suggest that splenic B cells can suppress EAE in an adoptive transfer setting but that their plasmablast differentiation in the dLNs might be required.

EAE Induces Generation of GC-Independent Plasmablasts that Produce IL-10 Preferentially

To gain insight into cellular aspects of IL-10-producing plasmablasts in the dLNs, we first investigated the cell surface phenotype. Most LN plasmablasts in EAE mice expressed high amounts of CD43, CXCR4, and major histocompatibility complex II (MHCII) and low amounts of B220, CD38, and CXCR5 (Figure 3A). Many of them also had undergone immunoglobulin (Ig) class-switch recombination (Figure 3B), which commonly occurs in both extrafollicular and germinal center (GC) responses (Klein and Dalla-Favera, 2008). Because an extensive expansion of GC B cells in the dLNs was detected during EAE (Figure 3C), we investigated the involvement of GCs in regulatory plasmablast generation. We elicited EAE in mice in which the transcription factor Bcl6 was functionally disrupted by inserting a YFP gene in both of the *Bcl6* alleles (*Bcl6*$^{yfp/yfp}$) (Kitano et al., 2011) and found that *Bcl6*$^{yfp/yfp}$ mice exhibited normal EAE despite of their lack of GC B cells (Figures 3D and 3E). The plasmablast generation was not significantly influenced by loss of Bcl6 (Figure 3E). Thus, EAE attenuation does not necessarily require GC responses.

We next assessed the potential contribution of anti-inflammatory cytokines besides IL-10 in plasmablasts. Because published studies have suggested that splenic B cells or plasma cells secrete IL-4, IL-13, IL-35, and transforming growth factor-β (TGF-β) (Mauri, 2010; Shen et al., 2014), we examined their expression in plasmablasts by quantitative RT-PCR analysis. In agreement with our data obtained with IL-10 reporter mice, CD138$^+$CD44^{hi} plasmablasts, but not CD19$^+$CD138$^-$ B cells, highly expressed IL-10 (Figure 3F). By contrast, the amount of *Il4*, *Il13*, *Il27* (*Il27b/p28*), *Il35* (*Il12a/Il27b*), and *Tgfb1* mRNA in CD138$^+$CD44^{hi} cells was decreased or comparable to that in CD19$^+$ cells. Consistent with that, ELISA and Bio-Plex suspension assay demonstrated preferential IL-10 secretion by CD138$^+$CD44^{hi} cells (Figure 3G). Although IL-6 and IFN-γ produced by B cells have been reported to contribute to EAE pathogenesis (Barr et al., 2012; Matsushita et al., 2006), CD138$^+$CD44^{hi} cells had little expression of their mRNA and proteins. Collectively, EAE-induced plasmablasts in the dLNs predominantly produce IL-10.

IRF4 Is Essential for Plasmablast IL-10 Production

We next investigated the mechanisms by which plasmablasts produce IL-10. In a previous study, we found that B cells could secrete IL-10 after BCR stimulation in a Ca^{2+} influx-dependent way (Matsumoto et al., 2011). However, this occurred only when B cells were preactivated with TLR agonists. Thus, we reasoned that TLR-dependent transcription factors would be required for plasmablast differentiation and/or IL-10 production. To this end, LPS-stimulated B cells from *Prdm1*$^{gfp/+}$ mice were sorted on the basis of GFP and CD138 expression followed by stimulation with anti-IgM (Figure 4A). IL-10 secretion was restricted to GFP$^+$ fractions and drastically enhanced by BCR ligation (Figure 4B). Because both CD138$^+$GFP$^+$ and CD138$^-$GFP$^+$ populations are known to have characteristics of antibody secretion and proliferative responses (Kallies et al., 2004), we concluded that plasmablasts are the principal IL-10 producers in vitro. Unexpectedly, however, B cells lacking Blimp1 proteins secreted IL-10 normally despite having impaired CD138$^+$

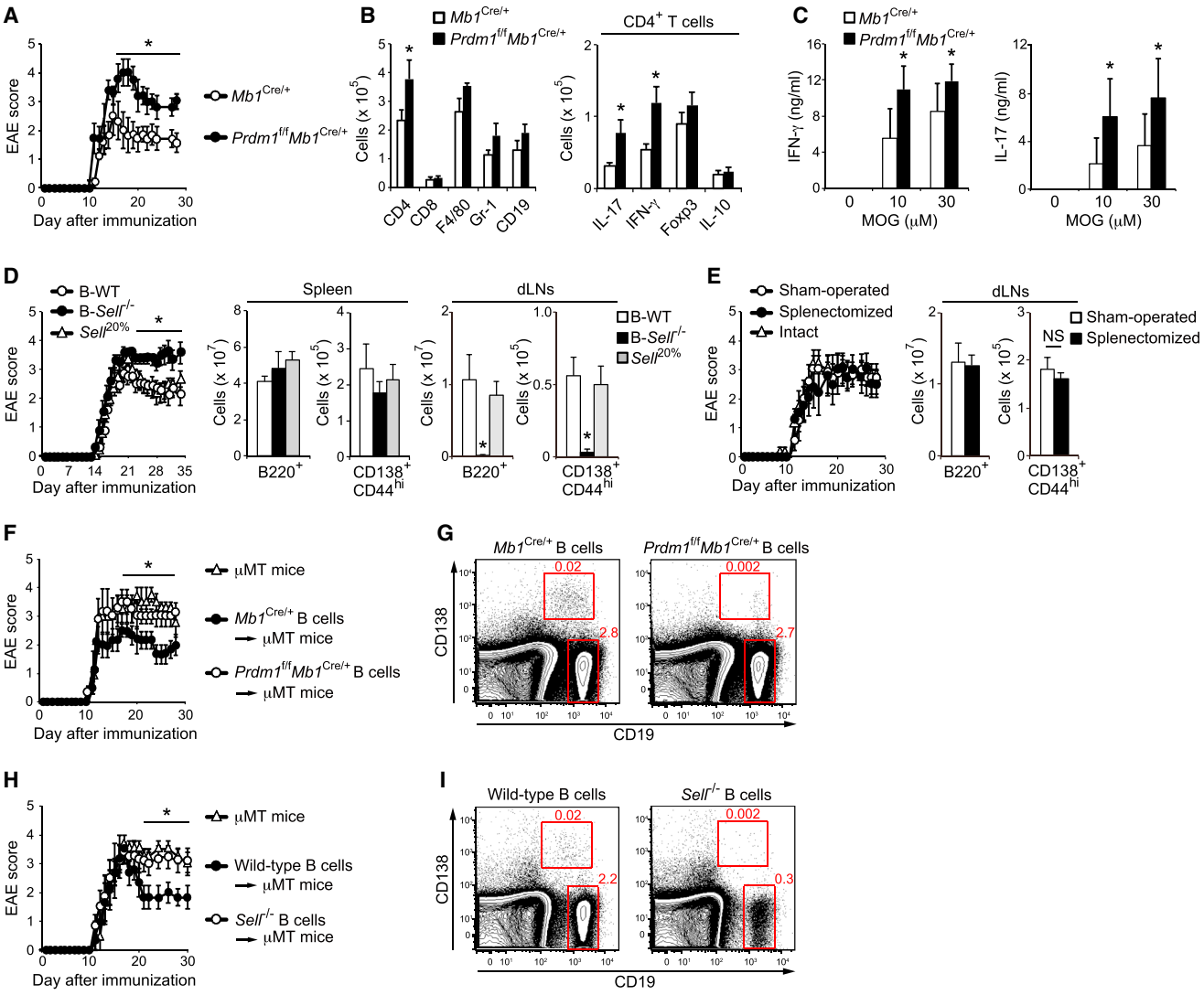

Figure 2. Plasmablasts in the dLNs Negatively Regulate EAE Inflammation

(A) Clinical EAE scores for *Mb1*$^{Cre/+}$ and *Prdm1*$^{f/f}$*Mb1*$^{Cre/+}$ mice immunized with MOG_{35-55}. The EAE score is shown as mean ± SEM for six to seven mice.

(B) Absolute number of cells from spinal cords harvested from *Mb1*$^{Cre/+}$ and *Prdm1*$^{f/f}$*Mb1*$^{Cre/+}$ mice 14 days after MOG_{35-55} immunization. Data are presented as mean ± SEM for five mice.

(C) ELISA of IFN-γ and IL-17 by cells isolated from the dLNs of *Mb1*$^{Cre/+}$ and *Prdm1*$^{f/f}$*Mb1*$^{Cre/+}$ mice 14 days after EAE induction followed by stimulation with MOG_{35-55} for 48 hr. Data are presented as mean ± SD.

(A–C) *p < 0.05 versus *Mb1*$^{Cre/+}$ mice (Mann-Whitney U test).

(D) Clinical EAE scores for B-*Sell*$^{-/-}$ (chimeric mice generated by transplanting a mixture of BM cells from μMT (80%) and *Sell*$^{-/-}$ (20%) mice and two control chimera groups: wild-type mice lethally irradiated and reconstituted with 80% μMT plus 20% wild-type bone marrow (B-WT) or reconstituted with 80% wild-type plus 20% *Sell*$^{-/-}$ bone marrow (*Sell*$^{20\%}$). The absolute number of B220$^+$ and CD138$^+$CD44^{hi} cells harvested from spleen and dLNs of chimeras 14 days after MOG_{35-55} immunization is shown on the right. Data are shown as mean ± SEM for five to ten mice. *p < 0.05 versus B-WT mice (Mann-Whitney U test).

(E) Clinical EAE scores for sham-operated, splenectomized, and intact wild-type mice immunized with MOG_{35-55}. Absolute number of B220$^+$ and CD138$^+$CD44^{hi} cells from dLNs of sham-operated and splenectomized mice 14 days after MOG_{35-55} immunization is shown on the right. Data are shown as mean ± SEM for five to seven mice. NS, not significant (Mann-Whitney U test).

(F) Clinical EAE scores for μMT mice immunized with MOG_{35-55} after injecting splenic B cells harvested from *Mb1*$^{Cre/+}$ and *Prdm1*$^{f/f}$*Mb1*$^{Cre/+}$ mice 28 days after EAE induction. The EAE score is shown as mean ± SEM for five to six mice. *p < 0.05 versus *Prdm1*$^{f/f}$*Mb1*$^{Cre/+}$ B cells (Mann-Whitney U test).

(G) Flow cytometry of cells from dLNs of μMT mice immunized for 12 days with MOG_{35-55} after injecting splenic *Mb1*$^{Cre/+}$ and *Prdm1*$^{f/f}$*Mb1*$^{Cre/+}$ B cells.

(H) Clinical EAE scores for μMT mice immunized with MOG_{35-55} after injecting splenic B cells harvested from wild-type and *Sell*$^{-/-}$ mice. The EAE score is shown as mean ± SEM for five to nine mice. *p < 0.05 versus *Sell*$^{-/-}$ B cells (Mann-Whitney U test).

(I) Flow cytometry of cells from dLNs of μMT mice immunized for 12 days with MOG_{35-55} after injecting splenic wild-type and *Sell*$^{-/-}$ B cells. CD19$^+$ and CD138$^+$ cells are gated and their percentages are shown (G and I).

Data are representative from three (A–D and F) or two (E and G–I) independent experiments. See also Figure S2.

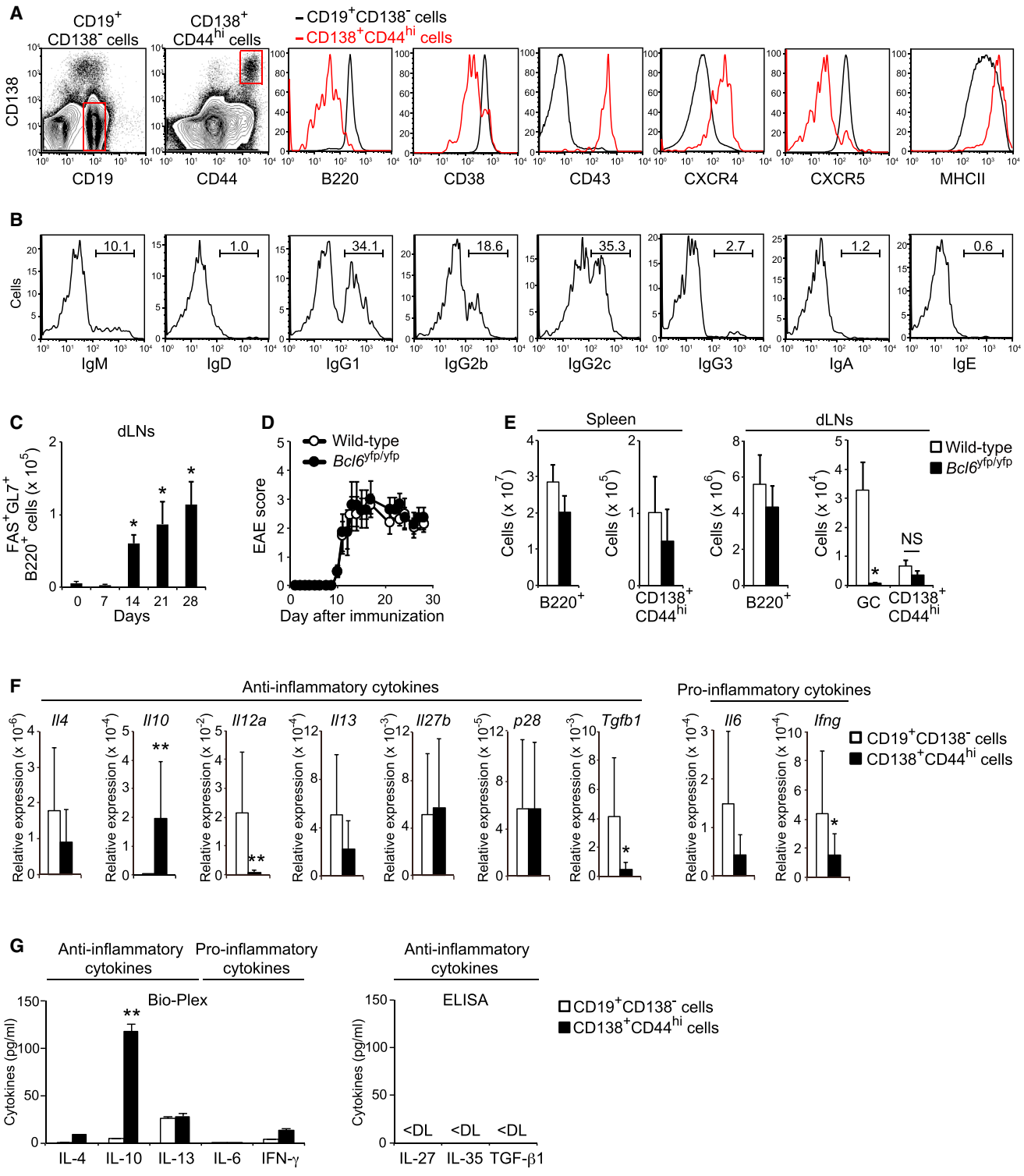

Figure 3. EAE Induces Generation of GC-Independent Plasmablasts that Produce IL-10 Preferentially

(A) Flow cytometry of $CD19^+CD138^-$ (black histogram, gated as in the left-most panel) and $CD138^+CD44^{hi}$ cells (red histogram, gated as in second panel from the left) harvested from dLNs of wild-type mice 14 days after MOG_{35-55} immunization.

(B) Flow cytometry of $CD138^+CD44^{hi}$ cells harvested from dLNs of wild-type mice 14 days after MOG_{35-55} immunization. Percentages of Ig^+ cells are shown.

(C) Absolute number of $FAS^+GL7^+B220^+$ (GC) B cells from dLNs harvested from wild-type mice before and 7, 14, 21, and 28 days after MOG_{35-55} immunization. Data are presented as mean ± SEM for five to six mice. *$p < 0.05$ versus day 0 (Mann-Whitney U test).

(D) Clinical EAE scores for wild-type and $Bcl6^{yfp/yfp}$ mice immunized with MOG_{35-55}. The EAE score is shown as mean ± SEM for seven mice.

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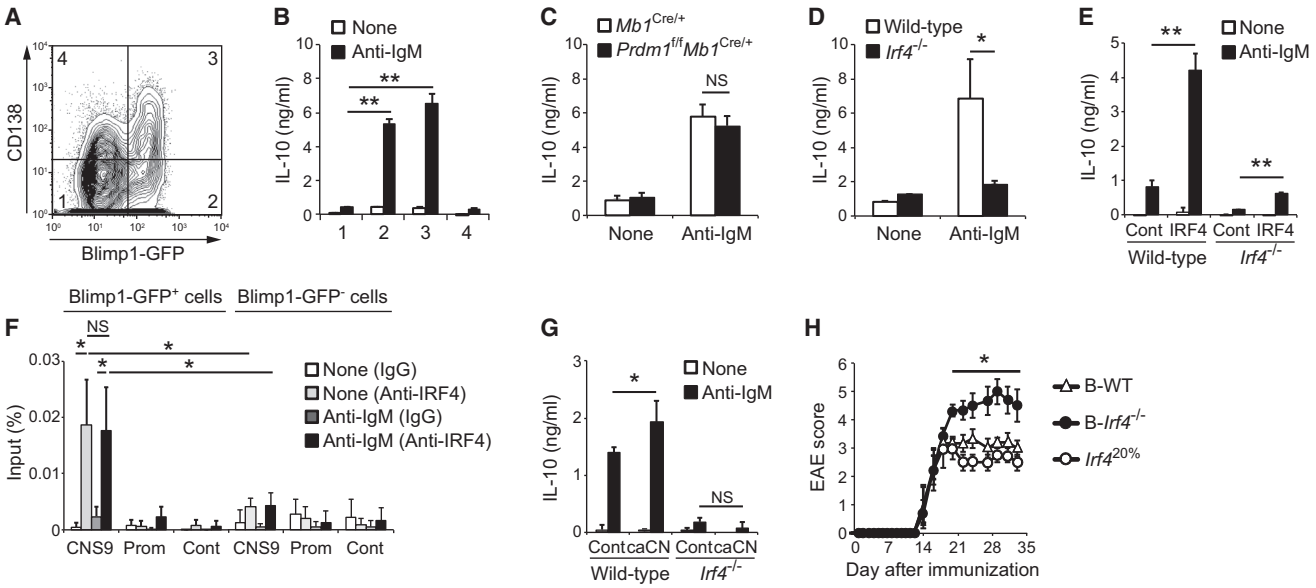

Figure 4. IRF4 Is Essential for Plasmablast IL-10 Production

(A) Flow cytometry of B cells isolated from spleen of *Prdm1*$^{gfp/+}$ mice and cultured with LPS for 48 hr. Four populations—GFP$^-$CD138$^-$ (fraction 1), GFP$^+$CD138$^-$ (fraction 2), GFP$^+$CD138$^+$ (fraction 3), and GFP$^-$CD138$^+$ (fraction 4) cells—were sorted and assayed in (B).

(B) ELISA of IL-10 secreted by the sorted B cells after stimulation with anti-IgM for 24 hr.

(C) ELISA of IL-10 secreted by B cells isolated from peripheral LNs of *Mb1*$^{Cre/+}$ and *Prdm1*$^{f/f}$*Mb1*$^{Cre/+}$ mice and cultured with LPS for 48 hr followed by stimulation with anti-IgM for 24 hr.

(D) ELISA of IL-10 secreted by B cells isolated from peripheral LNs of wild-type and *Irf4*$^{-/-}$ mice and cultured with LPS for 48 hr followed by stimulation with anti-IgM for 24 hr.

(E) ELISA of IL-10 secreted by GFP$^+$ cells sorted from LPS-activated wild-type and *Irf4*$^{-/-}$ B cells retrovirally transduced with GFP alone (Cont) or IRF4 followed by stimulation with anti-IgM for 24 hr.

(F) ChIP analysis of GFP$^+$ plasmablasts and GFP$^-$ B cells sorted from LPS-activated *Prdm1*$^{gfp/+}$ B cells, stimulated with anti-IgM for 30 min, and then precipitated with anti-IRF4 Ab or goat IgG. Input DNA and precipitated DNA were quantified by RT-PCR with PCR primers specific for CNS9 and promoter (Prom) regions of *Il10* or 3′ region of *Cd19* (Cont). Data shown are pooled from two independent experiments.

(G) ELISA of IL-10 secreted by GFP$^+$ cells sorted from LPS-activated wild-type and *Irf4*$^{-/-}$ B cells retrovirally transduced with GFP alone (Cont) or constitutively active calcineurin (caCN) followed by stimulation with anti-IgM for 24 hr. NS, not significant.

(H) Clinical EAE scores for chimeric mice in which only B cells lacked IRF4 (B-*Irf4*$^{-/-}$; wild-type mice lethally irradiated and reconstituted with 80% μMT plus 20% *Irf4*$^{-/-}$ bone marrow) and two control chimera groups: wild-type mice lethally irradiated and reconstituted with 80% μMT plus 20% wild-type bone marrow (B-WT) or reconstituted with 80% wild-type plus 20% *Irf4*$^{-/-}$ bone marrow (*Irf4*$^{20\%}$). The EAE score is shown as mean ± SEM for five to six mice. *$p < 0.05$ versus B-WT mice (Mann-Whitney U test).

(B–G) Data are presented as mean ± SD. *$p < 0.05$, **$p < 0.001$ (Student's t test).

Data are representative of three (A–E and G) or two (H) independent experiments. See also Figures S3 and S4.

cell generation (Figures 4C, S3A, and S3B), suggesting that Blimp1 in developing plasmablasts is dispensable for IL-10 production. Importantly, B cells lacking Blimp1 fail to fully differentiate into plasma cells, but rather initiate this differentiation pathway (Kallies et al., 2007; Kallies and Nutt, 2007; Shapiro-Shelef et al., 2003). Therefore, we next focused on the functional importance of IRF4 because it is a critical factor in the early phase of plasma cell differentiation and one of the downstream targets of TLR and BCR signaling (Mittrücker et al., 1997; Oracki et al., 2010). LPS-activated *Irf4*$^{-/-}$ B cells had impaired IL-10 secretion after BCR ligation (Figure 4D). Reciprocally, retroviral expression of IRF4 in wild-type B cells substantially increased IL-10 production and partially rescued it in *Irf4*$^{-/-}$ B cells (Figure 4E). Furthermore, chromatin immunoprecipitation (ChIP) analysis revealed that IRF4 in plasmablasts bound to the *Il10* CNS9 region, which controls *Il10* expression and is located approximately 9.1 kbp upstream of the transcription start site (Lee et al., 2009), though this binding frequency was unaffected by BCR stimulation (Figure 4F). These results imply that IRF4 not only induces plasmablast generation but also directly regulates *Il10* expression. Nuclear factor

(E) Absolute number of each B cell subset from spleen and dLNs harvested from wild-type and *Bcl6*$^{yfp/yfp}$ mice 28 days after MOG_{35-55} immunization. Data are presented as mean ± SEM for six mice. *$p < 0.05$ versus wild-type mice (Mann-Whitney U test). NS, not significant.

(F and G) Quantitative RT-PCR (F) and ELISA and Bio-Plex cytokine (G) analysis of CD19$^+$CD138$^-$ and CD138$^+$CD44^{hi} cells harvested from dLNs of wild-type mice 14 days after MOG_{35-55} immunization. For ELISA and Bio-Plex suspension assay, the isolated CD19$^+$CD138$^-$ and CD138$^+$CD44^{hi} cells were stimulated with PMA and ionomycin for 5 hr (G). Data are presented as mean ± SD. Abbreviations: <DL, below detection limit. *$p < 0.05$, **$p < 0.001$ versus CD19$^+$ cells (Student's t test).

Data are representative from three (A, B, and F) or two (C–E and G) independent experiments.

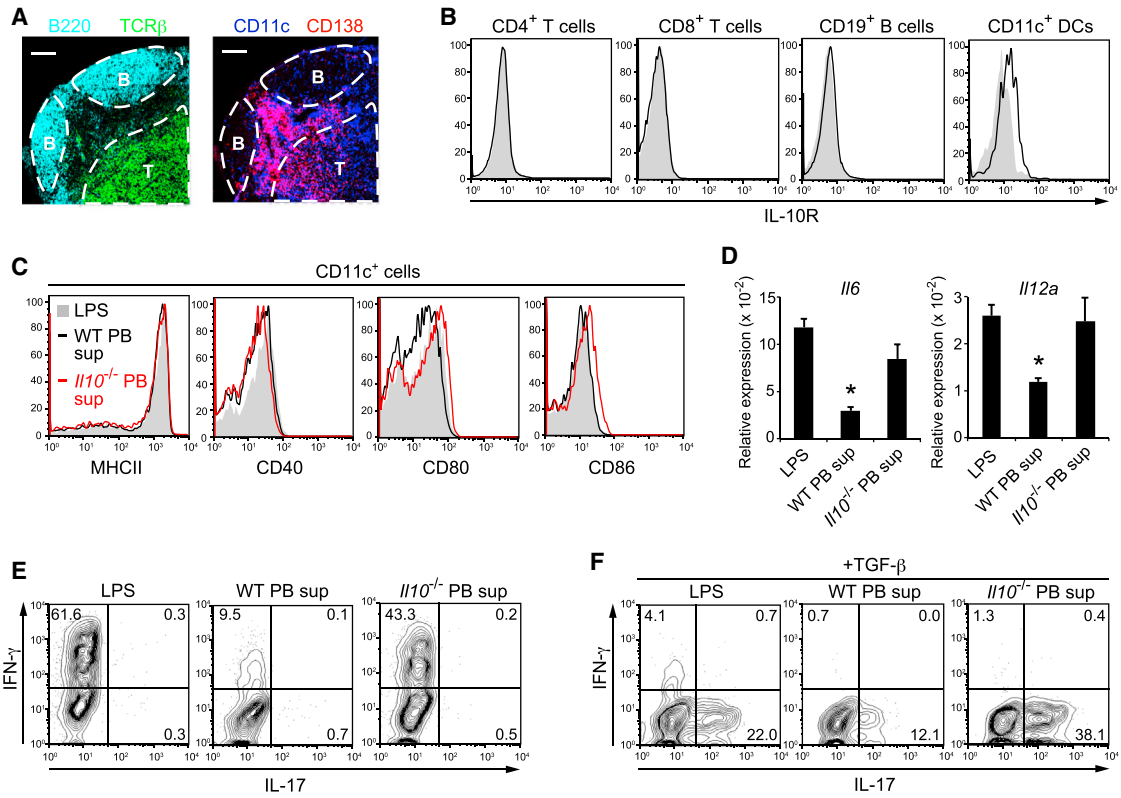

Figure 5. Plasmablasts Inhibit Dendritic Cell Function to Generate Autoreactive T Cells

(A) Histological analysis of dLNs harvested from wild-type mice 14 days after MOG_{35-55} immunization. Sections were stained with B220 and TCR-β Abs (left) or with CD11c and CD138 Abs (right). Original magnification, ×10; scale bars represent 100 μm.

(B) Flow cytometry of IL-10R expression by cells harvested from dLNs of wild-type mice 14 days after MOG_{35-55} immunization. Cells were stained with an IL-10R mAb (open histogram) or isotype control (shaded histogram).

(C) Flow cytometry of $CD11c^+$ cells harvested from dLNs of wild-type mice 14 days after MOG_{35-55} immunization followed by stimulation with LPS alone (shaded histogram) or supernatants from wild-type plasmablasts (PB) (WT PB sup; black histogram) and *Il10*$^{-/-}$ plasmablasts (*Il10*$^{-/-}$ PB sup; red histogram) activated with LPS and then anti-IgM.

(D) Quantitative RT-PCR analysis of *Il6* and *Il12a* transcripts in $CD11c^+$ cells harvested from dLNs of wild-type mice 14 days after MOG_{35-55} immunization, stimulated with LPS or WT and *Il10*$^{-/-}$ PB sup, normalized to the expression of *Gapdh*. Data are presented as mean ± SD. *$p < 0.05$ versus DC treated with *Il10*$^{-/-}$ PB sup (Student's t test).

(E and F) Cytokine profiles of TCR^{MOG}-expressing naive $CD4^+$ T cells cocultured with dLN $CD11c^+$ cells stimulated with LPS or WT and *Il10*$^{-/-}$ PB sup in the absence (E) or presence (F) of TGF-β together with MOG_{35-55} for 72 hr. Percentages of IFN-γ^+ and/or IL-17^+ cells are shown.

Results represent one of three similar experiments. See also Figure S5.

of activated T cells (NFAT), which is activated by Ca^{2+} and the calmodulin-dependent phosphatase calcineurin, is also vital for BCR-induced IL-10 production (Matsumoto et al., 2011). Retroviral expression of a constitutively active form of calcineurin A (caCN) markedly increased BCR-induced IL-10 production in an IRF4-dependent manner (Figure 4G), suggesting that NFAT-dependent IL-10 production requires IRF4. Moreover, we found that IRF4 has a B cell regulatory role in vivo because B-cell-specific *Irf4*-deficient chimeric mice lacking $CD138^+CD44^{hi}$ cells in the dLNs became susceptible to EAE (Figures 4H and S4). Together, these data indicate that IRF4 is essential for B cell IL-10 production to suppress EAE.

Plasmablast-Derived IL-10 Inhibits Dendritic Cell Function to Generate Pathogenic T Cells

We next elucidated the mechanisms by which plasmablasts suppress EAE. Immunohistochemical analysis of the dLNs in EAE-induced mice revealed that $CD138^+$ plasmablasts were mainly colocalized with $CD11c^+$ dendritic cells (DCs) in the extrafollicular region between T cell zones and B cell follicles (Figure 5A). Given that DCs, but not T and B cells, expressed detectable amounts of IL-10 receptor (IL-10R) (Figure 5B), we next examined whether DC function is affected by plasmablast-derived IL-10. When DCs were stimulated with supernatants derived from wild-type plasmablasts activated with LPS and then anti-IgM, the expression of MHCII, CD40, CD80, and CD86 was unchanged, but *Il6* and *Il12* mRNA was significantly decreased. This effect was not observed with *Il10*$^{-/-}$ plasmablasts (Figures 5C and 5D). Consistent with these results, Th1 cell differentiation of MOG-specific T cells was markedly prevented by supernatants from wild-type, but not *Il10*$^{-/-}$, plasmablasts when cocultured with DCs (Figure 5E). Very similar results were obtained with TGF-β-mediated Th17 cell generation (Figure 5F). Furthermore, we also observed equivalent results

when DCs were cocultured with Blimp1-GFP$^+$ plasmablasts (Figure S5). Thus, these results suggest that IL-10-producing plasmablasts inhibit DC functions to generate autoreactive T cells. This does not exclude the possibility that other cell types will be affected in vivo by plasmablast IL-10.

Human Plasmablasts Are IL-10-Producing B Cells

Our findings that plasmablasts represent the IL-10-producing B cells in mice led us to test whether this also applies to humans. B cells were isolated from peripheral blood of healthy donors and cultured with CpG (a TLR9 agonist) and/or cytokine cocktails including IL-2, IL-6, and interferon-alpha (IFN-α), which are known to provide conditions for effective plasmablast differentiation (Jego et al., 2003; Joo et al., 2012). Indeed, we detected CD27^{hi}CD38$^+$ putative plasmablasts after culture with CpG, while concomitant treatment with CpG and cytokine cocktails induced a greater frequency of an additional population of CD27^{int}CD38$^+$ cells as well as CD27^{hi}CD38$^+$ cells (Figure 6A). In particular, IFN-α was considerably effective for CD27^{int}CD38$^+$ differentiation and essentially the same results were obtained with IFN-β instead of IFN-α (Figure S6A). We found that IL-10 production was greatly induced in culture with a mixture of CpG and cytokine cocktails (Figure 6B). Both CD27^{hi}CD38$^+$ and CD27^{int}CD38$^+$ populations had a progressive loss of CD20, CD180, and Pax5 (Figures 6C, S6B, and S6C). Inversely, they had higher expression of IRF4, Blimp1, and XBP1 proteins and their transcripts (Figures 6C and S6C) and showed morphological maturation into plasma cells, as displayed by larger size with abundant cytoplasm, eccentric nuclei, and perinuclear haloes (Figure 6D). Consistent with these observations, both CD27^{hi}CD38$^+$ and CD27^{int}CD38$^+$ cells substantially secreted IgM (Figure 6E). Given the lack of a human mature plasma cell marker CD138 (Figure S6B), CD27^{int}CD38$^+$ cells as well as CD27^{hi}CD38$^+$ cells can be considered as plasmablasts whereas the CD27^{hi} cells apparently are more mature than CD27^{int} cells in view of their phenotypes. To determine which populations produce IL-10, we purified four fractions based on CD27 and CD38 expression after culture. ELISA assay showed that CD27^{int}CD38$^+$ plasmablasts selectively secreted IL-10 (Figure 6F). As a further test of this finding, we conducted IL-10 secretion assay by using IL-10 capture and detection antibodies, which allow us to detect live IL-10-secreting cells and found that the majority of IL-10$^+$ B cells consisted of CD27^{int}CD38$^+$ cell fraction (Figure 6G). Of note, this IL-10$^+$CD27^{int}CD38$^+$ population substantially secreted IgM, as assessed by ELISPOT assay (Figure 6H), suggesting that IL-10-producing B cells are Ig-secreting CD27^{int}CD38$^+$ plasmablasts.

We next addressed the issue of why CD27^{int}, but not CD27^{hi}, plasmablasts produce IL-10. Given that freshly prepared peripheral blood B cells consist of three major populations, i.e., CD24^{lo}CD27$^-$CD38$^-$ (naive mature), CD24^{hi}CD27$^-$CD38^{lo} (naive immature), and CD24^{hi}CD27$^+$CD38$^-$ (memory) cells (Figure 6I), the origin of each might be different. To test this hypothesis, they were sorted and then cultured. Memory B cells were predominantly differentiated into CD27^{hi}CD38$^+$ plasmablasts, whereas naive immature B cells and mature B cells, albeit to a lesser degree, became CD27^{int}CD38$^+$ plasmablasts (Figure 6I). Naive B-cell-derived CD27^{int} plasmablasts produced considerably more IL-10 (Figures 6J, S6D, and S6E). Collectively, these findings establish that human plasmablasts that arise from naive and especially immature B cells, but not memory B cells, are the major IL-10-producing B cells.

DISCUSSION

Our findings identify plasmablasts as the IL-10-producing B cells that can suppress autoimmunity. This was the case for EAE, where they were developed in the dLNs under the control of Blimp1 and IRF4 and disease progression was enhanced by their deletion. Furthermore, human plasmablasts also preferentially secreted IL-10, and these cells were derived from naive but not memory B cells.

It was previously thought that splenic B cells secrete the IL-10 that limits EAE. Instead, we found that CD138$^+$ plasmablasts in the dLNs were the major producers of this cytokine during EAE. This was the case when assessed by *Il10*$^{Venus/+}$ reporter mice or quantitative RT-PCR. In accordance with previous reports using several other IL-10 reporter lines injected with LPS or infected with *Salmonella* (Madan et al., 2009; Maseda et al., 2012; Shen et al., 2014), we also observed Venus expression in splenic CD138$^+$ cells in *Il10*$^{Venus/+}$ mice. However, amounts were very low and the frequency of positive cells was unaffected by EAE induction. By contrast, IL-10$^+$ plasmablasts in the dLNs were newly generated within extrafollicular foci, implying negative feedback regulation to protect excessive inflammation.

Our finding of severe EAE pathogenesis in the absence of plasmablasts due to B-cell-specific deletion of Blimp1 or IRF4 supports the idea that plasmablasts possess regulatory activity in vivo. This regulatory function is dependent on the dLNs and independent of the spleen. On the other hand, results from adoptive transfer studies were interpreted to mean that splenic B cells, especially the CD1d^{hi}CD5$^+$ B cell population, could suppress EAE through some unknown mechanism (Matsushita et al., 2008). Although we also observed that adoptive transfer of splenic B cells normalized EAE, plasmablast generation in the dLNs was required. CD1d^{hi}CD5$^+$ B cells extensively differentiate into plasmablasts in culture (Maseda et al., 2012) and their adoptive transfer from mice lacking IL-21R, CD40, and MHCII, which are indispensable for plasma cell differentiation (McHeyzer-Williams et al., 2012), into *Cd19*$^{-/-}$ mice does not resolve EAE development (Yoshizaki et al., 2012). Therefore, this population might serve as plasmablast precursors in an adoptive transfer setting.

The finding of in vitro BCR-dependent IL-10 production specifically in Blimp1$^+$ cells provides further evidence for the importance of plasmablasts and can explain the previously demonstrated need for TLR signaling for BCR-mediated IL-10 expression (Matsumoto et al., 2011). This idea is also supported by the observation of impaired IL-10 secretion in the absence of IRF4, which resulted in defective plasmablast differentiation (Figure S3A). Thus, IRF4 is required for IL-10 expression along with plasmablast differentiation in vitro and in vivo. Importantly, TLR and BCR signals induce the expression of IRF4 (De Silva et al., 2012) and therefore operate upstream of both plasmablast differentiation and IL-10 production. We detected deposition of IRF4 at the CNS9 region upstream enhancer in the *Il10* locus. This is in agreement with published studies that demonstrated

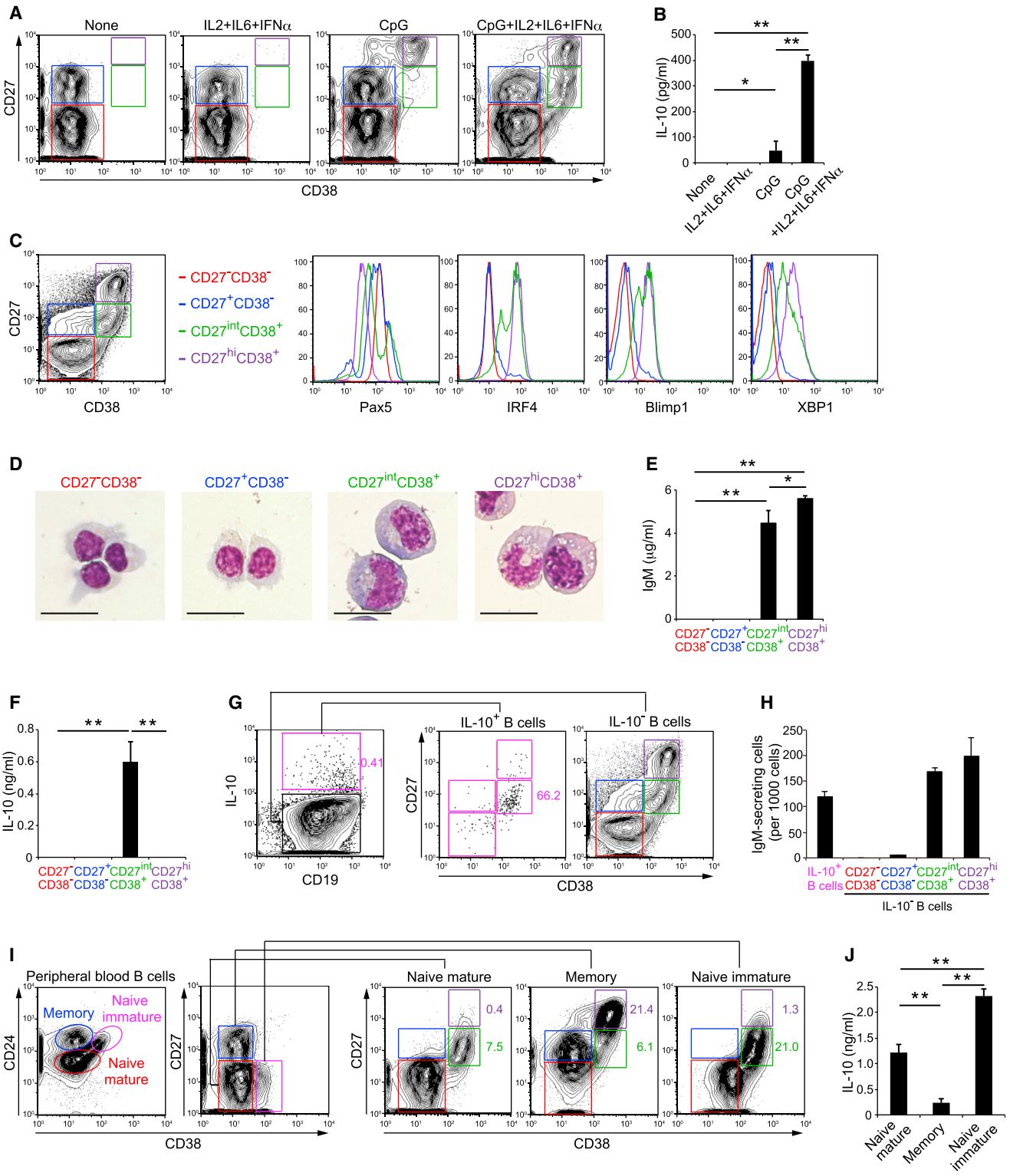

Figure 6. Human Plasmablasts Are IL-10-Producing B Cells

(A) Flow cytometry of B cells isolated from healthy blood donors and cultured with IL-2, IL-6 plus IFN-α (IL2, IL6, IFNα), and/or CpG for 96 hr. Four populations—$CD27^{-}CD38^{-}$ (red), $CD27^{+}CD38^{-}$ (blue), $CD27^{int}CD38^{+}$ (green), and $CD27^{hi}CD38^{+}$ (purple) cells—are gated.

(B) ELISA of IL-10 secreted by B cells isolated from peripheral blood of healthy donors and cultured with IL-2, IL-6 plus IFN-α, and/or CpG for 96 hr.

(C) Flow cytometry of B cell populations indicated in the left panel.

(legend continued on next page)

IRF4 binding to the same element in various types of cells (Cretney et al., 2011; Lee et al., 2009; Li et al., 2012). NFAT bound to the same region in Th2 cells, which was essential for IL-10 transcription (Lee et al., 2009). Taking into account our previous finding that B-cell-mediated IL-10 production requires NFAT activation (Matsumoto et al., 2011), it seems likely that IRF4 serves as an NFAT transcription partner to produce IL-10 in plasmablasts.

Unexpectedly, Blimp1-deficient B cells secreted IL-10 in our in vitro experiments despite impaired CD138$^+$ cell differentiation. Considering that the initiation of plasma cell differentiation takes place in vitro in the absence of Blimp1 (Kallies et al., 2007), it seems possible that IL-10 production is initiated already in the early preplasmablastic stage of plasma cell development, which is independent of Blimp1 (Kallies et al., 2007). However, we could detect little Venus-positive CD138$^-$ B cell population in mice during EAE (data not shown). Given that GFP$^+$CD138$^-$ cells in *Prdm1*$^{gfp/+}$ mice were effectively generated in vitro (Figure 4A), but not in vivo (Figure 1B), it seems likely that no or few preplasmablasts as is detected in culture exist in vivo.

We now have evidence that naive B-cell-derived plasmablasts represent the most significant IL-10 producers in humans. Although activation of human peripheral blood B cells with CpG caused CD27^{hi}CD38$^+$ plasmablast generation, our results establish that additional treatment with cytokines including IL-2, IL-6, and, especially, IFN-α drove the differentiation of CD27^{int}CD38$^+$ plasmablasts that predominantly secrete IL-10. Given that IFN-α enhances CD38$^+$ expression on naive B cells (Giordani et al., 2009) and can induce plasma cell differentiation (Jego et al., 2003), IFN receptor signals seem likely to be key for IL-10-producing plasmablast generation. Indeed, patients with SLE have high serum IFN-α concentrations (Kim et al., 1987) and increased CD27^{int}CD38$^+$ cells in peripheral blood (Arce et al., 2001), suggesting that IL-10$^+$ plasmablast expansion might be the result of the inflammatory conditions. Furthermore, the treatment with IFN-β, another type I IFN approved for MS therapy, enhances B cell IL-10 secretion after BCR and CD40 ligation (Ramgolam et al., 2011). Although the precise mechanism by which IFN-β suppresses MS remains unclear, one of the possible explanations is that IFN-β might promote generation of IL-10-producing plasmablasts. Noteworthy, in clinical trials, MS patients who received Atacicept, a transmembrane activator and calcium-modulating cyclophilin-ligand interactor (TACI)-Ig fusion protein to deplete antibody-secreting cells, had exacerbated inflammatory symptoms (Hartung and Kieseier, 2010). This would be consistent with an inhibitory function for human plasmablasts. We have provided evidence that IL-10-producing plasmablasts effectively stem from naive immature B cells. This might support a recent study that human IL-10-competent B cells were enriched in immature CD24^{hi}CD38^{hi} B cells after culture with CD40 stimulation (Blair et al., 2010). We found that memory B-cell-derived plasmablasts failed to secrete IL-10, suggesting that the immediate precursor of developing plasmablasts would dictate the balance between cells that promote autoimmunity by antibody production or have regulatory capacity that protects from overt pathology.

In conclusion, our findings have identified plasmablasts in the dLNs as the IL-10-producing B cells that suppress autoimmunity. We also established a phenotype for human plasmablasts that predominantly secreted IL-10. Our study might lead to better understanding of the nature of autoimmune diseases and provide a basis for exploring new therapeutic strategies.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice were purchased from CLEA Japan. *Bcl6*$^{yfp/yfp}$ (Kitano et al., 2011), *Il10*$^{Venus/+}$ (Atarashi et al., 2011), *Irf4*$^{-/-}$ (Mittrücker et al., 1997; Suzuki et al., 2004), *Mb1*$^{Cre/+}$ (Hobeika et al., 2006), μMT (Kitamura et al., 1991), and *Prdm1*$^{gfp/+}$ (Kallies et al., 2004) mice have been described previously. *Il10*$^{-/-}$, *Prdm1*$^{f/f}$, *Sell*$^{-/-}$, and TCR^{MOG} transgenic mice were purchased from the Jackson Laboratory. We generated *Prdm1*$^{f/f}$*Mb1*$^{Cre/+}$ mice by crossing of *Prdm1*$^{f/f}$ mice with *Mb1*$^{Cre/+}$ mice. Mice were bred and maintained under specific-pathogen-free conditions and used at 6 to 12 weeks of age. Animal care and experiments were conducted according to the guidelines established by the animal committee of Osaka University.

Generation of Mixed Bone Marrow Chimeras

Mixed bone marrow chimeras were produced as described previously (Fillatreau et al., 2002). In brief, recipient wild-type mice received 800 cGy of X-ray irradiation. One day later, the recipients were reconstituted with a mixed inoculum of 80% μMT bone marrow cells supplemented with 20% bone marrow cells from *Irf4*$^{-/-}$ or *Sell*$^{-/-}$ mice. Control groups received 80% μMT and 20% wild-type bone marrow cells or 80% wild-type and 20% bone marrow cells from *Irf4*$^{-/-}$ or *Sell*$^{-/-}$ mice. Chimeric mice were left to fully reconstitute their lymphoid system for at least 12 weeks before EAE induction.

Induction and Assessment of EAE

EAE was induced by subcutaneous immunization with 200 μg of MOG$_{35-55}$ (MBL) emulsified in complete Freund's adjuvant (CFA) containing 500 μg of

(D) May-Grünwald-Giemsa staining of sorted B cell populations after culture with IL-2, IL-6, IFN-α plus CpG for 96 hr. Original magnification, ×400; scale bars represent 20 μm.
(E and F) ELISA of IgM (E) and IL-10 (F) secreted by the indicated B cell populations after culture with IL-2, IL-6, IFN-α plus CpG for 96 hr and then cultured for an additional 24 hr.
(G) Flow cytometry of B cells cultured with IL-2, IL-6, IFN-α plus CpG for 96 hr and labeled with IL-10 capture and detection antibodies to detect IL-10$^+$ B cells. Percentage of IL-10$^+$ B cells and IL-10$^+$CD27^{int}CD38$^+$ cells are shown.
(H) ELISPOT of IgM secreted by the indicated B cell populations after culture with IL-2, IL-6, IFN-α plus CpG for 96 hr followed by an additional 24 hr culture.
(I) Flow cytometry of three B cell populations freshly isolated from peripheral blood of healthy donors and then cultured with IL-2, IL-6, IFN-α plus CpG for 96 hr. Three major populations such as CD24^{lo}CD27$^-$CD38$^-$ (naive mature; red), CD24^{hi}CD27$^-$CD38^{lo} (naive immature; pink), and CD24^{hi}CD27$^+$CD38$^-$ (memory; blue) cells in peripheral blood B cells before culture are gated (left two panels). Percentages of CD27^{int}CD38$^+$ and CD27^{hi}CD38$^+$ cells after culture are shown (right three panels).
(J) ELISA of IL-10 secreted by naive mature, naive immature, and memory B cells isolated from healthy blood donors and cultured with IL-2, IL-6, IFN-α plus CpG for 96 hr.
Data shown are representative of three independent experiments. See also Figure S6.
(B, E, F, H, and J) Data are presented as mean ± SD. *$p < 0.05$, **$p < 0.001$ (Student's t test).

heat-killed mycobacterium tuberculosis H37RA (Difco). Mice also received 200 ng of pertussis toxin (List Biological Laboratories) i.p. in 0.2 ml of PBS on the same day and 2 days later. Clinical signs of EAE were assessed daily with a 0–6 scoring system (0, no signs; 1, tail limpness; 2, impaired righting reflex; 3, hind limb weakness; 4, hind limb paralysis; 5, hind limb paralysis with fore limb weakness; 6, death).

Isolation of Mouse B Cells and Adoptive Transfer

For B cell isolation, splenic B cells were purified by negative selection of $CD43^+$ cells with anti-CD43 magnetic beads (Miltenyi Biotec). The enriched B cell population was >95% positive for B220 staining. The B cells (2×10^7 cells) from spleen of $Mb1^{Cre/+}$ and $Prdm1^{f/f}Mb1^{Cre/+}$ mice 28 days after MOG_{35-55} immunization or wild-type and $Sell^{-/-}$ mice were transferred intravenously into μMT mice 24 hr before EAE induction.

Isolation and Stimulation of Human B Cells from Healthy Blood Donors

Mononuclear cells were isolated from peripheral blood of healthy donors by centrifugation over Ficoll-Paque PLUS (GE Healthcare). B cells were enriched by positive selection of $CD19^+$ cells with anti-human CD19 magnetic beads (Miltenyi Biotec) and were routinely >95% positive for CD19 staining. The purified B cells (5×10^5 cells/ml) were cultured for 96 hr with IL-2 (10 ng/ml; R&D), IL-6 (10 ng/ml; R&D), and CpG ODN 2006 (CpG; 1 μg/ml; InvivoGen) in the presence of IFN-α (1,000 U/ml; PBL Biomedical Laboratories) or IFN-β (1,000 U/ml; PBL Biomedical Laboratories). This study was approved by the ethics committees of Research Institute for Microbial Diseases, Osaka University. Healthy volunteers were recruited after obtaining informed consent.

ELISA and ELISPOT

MOG-specific IgG in serum was measured by ELISA with a plate coated with 10 μg/ml MOG_{35-55} and then detected with goat anti-mouse IgG and HRP-conjugated anti-goat IgG Abs (SouthernBiotech). For measurement of cytokine release by autoantigen-reactive lymphocytes, single-cell suspensions of the dLNs prepared from mice 14 days after EAE induction were cultured with a range of MOG_{35-55} doses for 48 hr. For measurement of IL-10 production by mouse B cells, purified B cells (1×10^6 cells/ml) were cultured for 48 hr with 10 μg/ml of LPS (Sigma-Aldrich) and then stimulated with 10 μg/ml of anti-mouse IgM $F(ab)'_2$ (Jackson Immunoresearch). In some experiments, $CD19^+CD138^-$ and $CD138^+CD44^{hi}$ cells harvested from dLNs of wild-type mice 14 days after EAE induction were stimulated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) plus 1 μM ionomycin (Sigma-Aldrich) for 5 or 24 hr. For measurement of IL-10 and IgM production by human peripheral blood B cells, purified B cells (5×10^5 cells/ml) were cultured for 96 hr with IL-2, IL-6, IFN-α, and CpG. IFN-γ, IgM, IL-4, IL-6, IL-10, IL-13, IL-17a, IL-27, IL-35, and TGF-β1 in the culture medium were detected by ELISA and Bio-Plex suspension assay according to the manufacturer's protocol (Biolegend, BIO-RAD, Bethyl Laboratories, or R&D). IgM secretion by human B cells was detected by ELISPOT according to the manufacturer's protocol (R&D).

Statistical Analysis

We performed statistical evaluation with Prism software (GraphPad). A two-tailed, unpaired Student's t test was applied for statistical comparison of two groups. In case of unequal variance, t test with Welch's correction was used. Comparisons of two nonparametric data sets were done by the Mann-Whitney U test. A p value of less than 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.10.016.

AUTHOR CONTRIBUTIONS

M.M. planned and performed most of the experiments, analyzed data, and prepared the manuscript; A.B. assisted with experiments; T.Y. contributed to morphological characterization of cultured cells; Y.O. provided technical contributions to ChIP assays; H.K. and K.T. provided $Il10^{Venus/+}$ reporter mice; H.N. and S.S. collected human blood and provided reagents; A.K. and S.L.N. provided $Prdm1^{gfp/+}$ mice and edited the manuscript; Y.B. and T.K. supervised the project; T.K. contributed to manuscript writing; and Y.B. designed the study, performed some experiments, interpreted data, and wrote the manuscript.

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