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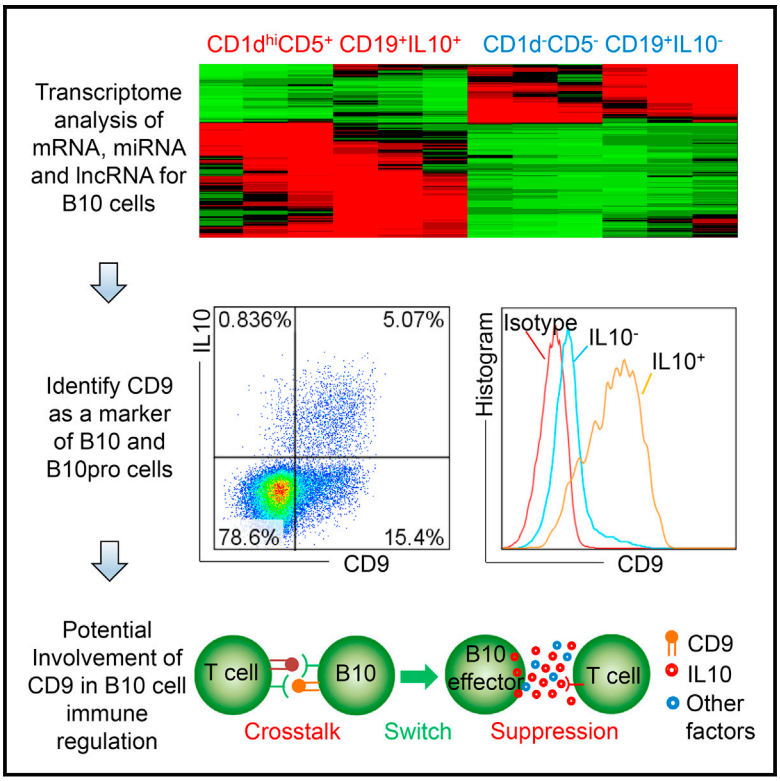
Report

Transcriptomics Identify CD9 as a Marker of Murine IL-10-Competent Regulatory B Cells

Graphical Abstract



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In Brief

The lack of unique marker(s) exclusively identifying regulatory B cells (Bregs) impedes further investigation into their origin, development, and role. Sun et al. profile mRNA and non-coding RNA expression of CD1d^{hi}CD5^{+}CD19^{+}IL-10^{competent} Bregs, identify key upstream regulators, and find CD9 is a functional marker of most IL-10^{competent} Breg cells.

Highlights

- RNA profiling identifies key factors correlated with Breg biogenesis and function
- IL-10^{competent} B cells and their progenitors are distinguished by CD9
- CD9 promotes suppressive function of IL-10^{competent} Breg cells

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Report

Transcriptomics Identify CD9 as a Marker of Murine IL-10-Competent Regulatory B Cells

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SUMMARY

Regulatory B cells (Breg) have immune suppressive functions in various autoimmune/inflammation models and diseases and are found to be enriched in diverse B cell subsets. The lack of a unique marker or set of markers efficiently identifying Breg cells impedes detailed investigation into their origin, development, and immunological roles. Here, we perform transcriptome analysis of IL-10-expressing B cells to identify key regulators for Breg biogenesis and function and identify CD9, a tetraspanin-family transmembrane protein, as a key surface marker for most mouse IL-10$^+$ B cells and their progenitors. CD9 plays a role in the suppressive function of IL-10$^+$ B cells in ex vivo T cell proliferation assays through a mechanism that is dependent upon B/T cell interactions. CD9$^+$ B cells also demonstrate inhibition of Th1-mediated contact hypersensitivity in an in vivo model system. Taken together, our findings implicate CD9 in the immunosuppressive activity of regulatory B cells.

INTRODUCTION

In addition to the normal antibody generating function, a subset of B cells, known as regulatory B cells (Bregs), can suppress several immune processes including allergy, autoimmunity, antigen presentation, and pro-inflammatory cytokine production (DiLillo et al., 2010; Mauri and Bosma, 2012). Breg regulation has been demonstrated in various autoimmune- and inflammation-induced mouse models (Mauri et al., 2003; Sattler et al., 2014; Yanaba et al., 2008; Yoshizaki et al., 2012), and aberrant regulation of Bregs has been reported in human diseases such as systemic lupus erythematosus (Blair et al., 2010), allergies (van de Veen et al., 2013), and autoimmune diseases and disorders (Kalampokis et al., 2013).

Bregs are found enriched in phenotypically diverse B cell subsets. In mice, reported markers of Bregs include CD1d, CD5, CD19, CD11b, CD21, CD23, CD32b, CD138, immunoglobulin M (IgM), IgD, TIM-1, and CX3CR1 (Ding et al., 2011; Mauri and Bosma, 2012; Shen et al., 2014; Stolp et al., 2014; Yanaba et al., 2008), whereas in humans Bregs markers have been reported to include CD1d, CD5, CD19, CD24, CD25, CD27, CD38, CD48, CD71, CD73, CD148, and IgM (Iwata et al., 2011; Lindner et al., 2013; Mauri and Bosma, 2012; Stolp et al., 2014; van de Veen et al., 2013). Mice and humans thus possess distinct sets of Breg markers, and there is a scarcity of unique markers that would exclusively and exhaustively identify Breg cells.

It has been suggested that signals triggering the B cell receptor (BCR)—CD40 ligation and Toll-like receptor engagement—may play important roles in the development and/or activation of Bregs (Blair et al., 2009; Lampropoulou et al., 2008). Nonetheless, the precise cellular origins of Bregs remain unknown, as do their developmental pathways. It has been proposed that Bregs may derive from a unique progenitor (Yanaba et al., 2009) or differentiate from distinct subsets of B cells triggered by a particular stimulus (Zhang, 2013). These two hypotheses are not mutually exclusive but need to be further investigated. Isolating unique markers identifying all Bregs may be a crucial first step in determining their ontology. In this study, we have investigated the transcriptome of B10 cells, an antigen-specific CD1d^{hi}CD5$^+$CD19$^+$IL-10^{competent} Breg cell (DiLillo et al., 2010; Yanaba et al., 2008) and identified CD9 as an important B10 cell marker.

RESULTS

Identification of Differentially Expressed mRNAs, miRNAs, and lncRNAs in B10 Cells

We sorted B10$^+$ cells (CD1d^{hi}CD5$^+$CD19$^+$IL-10$^+$) and B10$^-$ cells (CD1d$^-$CD5$^-$CD19$^+$IL-10$^-$) from mouse splenic B cells (Figure S1A) for RNA sequencing (RNA-seq) and microarray analysis. Together with the qRT-PCR results, the mapped reads of published B10 cellular markers, common B cell markers, and the lack of T cell markers, demonstrate high quality of the RNA-seq data (Figures S1B–S1D). We analyzed the rank change (RC) and fold change (FC) of mRNAs (Figure 1A; Table S1) between B10$^+$ and B10$^-$ cells and generated a highly reliable list of differentially expressed mRNAs (Figure 1B). 55 mRNAs overlap between the top 100-fold change mRNAs and the top 100

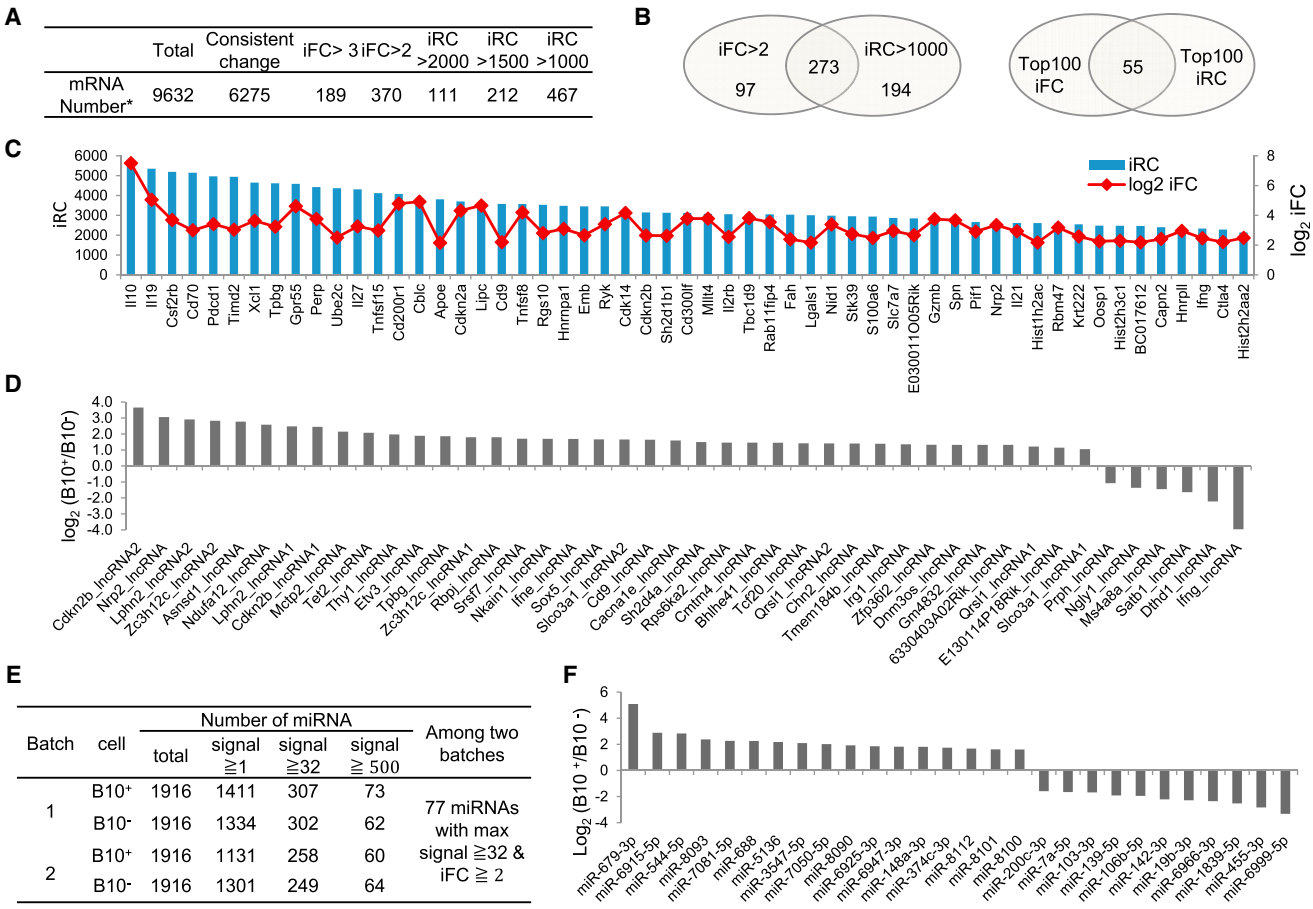

Figure 1. Differentially Expressed mRNA, lncRNA, and miRNA in B10 Cells

(A) Overall changes of gene expression level between B10$^+$ cells (CD1d^{hi}CD5$^+$CD19$^+$IL-10$^+$) and B10$^-$ cells (CD1d$^-$CD5$^-$CD19$^+$IL-10$^-$) determined by RNA-seq. *The number of mRNAs after removing mRNAs with FPKM <1 in both B10$^+$ and B10$^-$ cells in any batch, and mRNA with FPKM = 0 in any sample. See Supplemental Experimental Procedures for more information.

(B) The distribution of genes in the top iFC and iRC groups. The gene list is summarized in Table S1.

(C) The rank changes of the 55 genes existing in both the top 100 iFC and the top 100 iRC. IL-10 is ranked first in both rank lists.

(D) Fold change of the 44 novel lncRNAs identified from the lncRNA database (Pefanis et al., 2014, 2015). The lncRNAs are nomenclatured according to the gene closest to the lncRNA coding region.

(E) General changes of miRNA expression levels between B10$^+$ cells and B10$^-$ cells as determined by microarray analysis. Max signal, the maximum signal in all four samples of the two batches.

(F) The expression changes of the miRNAs with iFC ≥3 and max signal ≥32.

Please also see Figures S1 and S2 and Tables S1, S2, and S3.

rank change mRNAs, and *Il10* is ranked first by both methods (Figure 1C). We provide the full list of 273 differentially expressed mRNAs in Table S1.

Most of the mammalian genome has the potential to express various types of non-coding RNAs, ranging from miRNAs to lncRNAs (Fatica and Bozzoni, 2014; Hausser and Zavolan, 2014). As the RNA exosome complex is implicated in ncRNA half-life, we cross-referenced our database of lncRNAs isolated from RNA exosome knockout B cells (Pefanis et al., 2014, 2015) and found 38 upregulated lncRNAs and six downregulated lncRNAs from a library derived from B10$^+$ B cells (Figure 1D; Table S1). In addition, by microarray analysis we compared the miRNA expression levels between B10$^+$ and B10$^-$ cells. General changes in miRNA expression levels are summarized in Figure 1E; the expression changes of the miRNAs with iFC ≥3 and max signal ≥32 are shown in Figure 1F. Table S1 lists 77 differentially expressed miRNAs in B10$^+$ cells.

mRNA/miRNA Pairing, Prediction of Upstream Regulators, and Gene Ontology Term Enrichment Analysis

Using the Ingenuity Pathway Analysis (IPA) program (Krämer et al., 2014), we analyzed mRNA/miRNA pairing and predicted upstream regulators from the 273 differentially expressed mRNAs and the 77 differentially expressed miRNAs. We find there are 31 miRNAs targeting 134 mRNAs (Table S2). Notably, there are 56 upstream regulators differentially expressed between B10$^+$ and B10$^-$ cells in the total of 1,758 predicted

upstream regulators (Table S3), and four of the 56 are transcription regulators (STAT1, CDKN2A, MYB, and PRDM1).

Five interconnected stages and processes correlate tightly with Breg biogenesis and function (outlined in Figure S2). Based on the reported functions and features of various cell types in the literature, we divided the differentially expressed upstream regulators into eight groups by the IPA program and in Figure S2 predict the potential involvement of each group in the five stages according to gene ontology term enrichment analysis (Table S3). Of the upstream regulators, IL-10, IL-33, HAVCR1 (also named TIM1), and CD5 have been reported to be important molecules for Breg biogenesis and function (Ding et al., 2011; Sattler et al., 2014; Shen et al., 2014; Yanaba et al., 2008; Yoshizaki et al., 2012).

Comparison of Differentially Expressed mRNAs in B10 and Other Cell Subsets Suggests CD9 Is a Marker of IL-10^{competent} B Cells

Comparing the differentially expressed genes found in B10 cells with those found in other B cell subsets might provide important information on Breg origin and characterization. Based on data sets generated via The Immunological Genome Project (https://www.immgen.org/), differentially expressed genes were identified between B1a B cells and FO B cells derived from spleen or peritoneal cavity, between B1a and B1b B cells from peritoneal cavity, and between marginal zone (MZ) B cells and follicular (FO) B cells from spleen (Table S4). The expression of numerous differentially expressed genes in B10 cells undergoes similar change regardless of whether the B1a cells are derived from spleen, peritoneal cavity, or MZ B cells (Figure S3A). 19 genes (underlined) including *Actn1*, *Atxn1*, *Ccr6*, *Cd300lf*, *Cd9*, *Cfp*, *Csf2rb*, *Ctla4*, *Emb*, *Fcer2a*, *Fcrl5*, *Nek2*, *Nrp2*, *Ptpn22*, *Spn*, *Stom*, *Tbc1d9*, *Zfp318*, *and Zfp608* appear on at least three lists. *Cd9* and *Ccr1* are the only two genes appearing on all four lists, suggesting that CD9 and CCR1 may be good markers with which to identify Bregs from different B cell subsets.

Screening of Unique Markers Found by RNA-Seq Identifies CD9 as a Robust Marker of IL-10^{competent} B Cells

To find unique markers for Bregs, we examined surface expression levels of CD9, CCR1, and other proteins corresponding to the main cluster of differentiation (CD) antigens that showed differential mRNA expression levels in B10 cells (Figure 2A). As shown in Figure 2B, IL-10$^+$ B cells contain a higher percentage of cells expressing certain markers than do IL-10$^-$ B cells upon treatment with anti-CD40 antibody for 48 hr, consistent with our RNA-seq data. Notably, the difference in CD9 expression between IL-10$^+$ and IL-10$^-$ B cells at 48 hr is greater than that of any other tested marker(s) as well as the cells at 5 hr without CD40 antibody stimulation (Figure 2C). Moreover, most IL-10$^+$ B cells are CD9 positive at both 5 hr (87.03% ± 1.22%) and 48 hr (87.95% ± 1.25%). The same phenomenon was also found for peritoneal cavity B cells (Figure 2E). As CD9 is also a marker of all murine hematopoietic stem cells (Karlsson et al., 2013), an important question is whether the IL-10$^+$ B cells are derived from CD9$^+$ B cells, CD9$^-$ B cells, or both. To address this issue, we sorted CD9$^+$ and CD9$^-$ B cells from naive B cells, CD9$^-$IL-10$^-$ B cells, CD9$^+$IL-10$^-$ B cells, and CD9$^+$IL-10$^+$ B cells from cells treated with lipopolysaccharides (LPSs), phorbol 12-myristate 13-acetate (PMA), and ionomycin (LPI) for 5 hr (Figure 2F). The sorted cells were cultured with CD40 antibody for 48 hr and LPI plus monensin (LPI+M) were added for the final 5 hr. Figure 2F shows that the progenitors of IL-10^{competent} B cells exist as CD9$^+$ B cells. As CD9 is expressed in MZ B cells, B1 cells, and plasma cells (Won and Kearney, 2002), we evaluated the expression of CD9 and IL-10 in these cell populations (Figures S3C and S3D). Although IL-10$^+$ B cells are also enriched in MZ B cells compared to FO B cells (around 30% of MZ-B cells are IL-10$^+$, while only around 5% of FO B cells are IL-10$^+$ B cells), MZ B cells only represent approximately 20% of total IL-10$^+$ B cells, while FO B cells represent 40% of total IL-10$^+$ B cells. IL-10$^+$ B cells are indeed enriched in B1a and B1b cells compared to B2 cells. More than 40% of B1 cells are IL-10$^+$ B cells, and this population represents more than 88% of total IL-10$^+$ B cells. The IL-10$^+$ B1 cells and IL-10$^+$ MZ B cells are all enriched in CD9-expressing B cells. Next, in mouse B10 cells we compared CD9 expression with previously described markers CD1d, CD5, CD11b, CD21, CD23, CD32b, CD138, CX3CR1, IgD, IgM, and TIM1 (Figure 3) and further confirmed that CD9 is a surface marker for a variety of mouse IL-10^{competent} B cells.

Suppressive Function of CD19$^+$ CD9$^+$ B Cells

To check whether CD9$^+$ B cells have immune regulatory functions, we isolated CD19$^+$CD9$^+$ B cells and CD19$^+$CD9$^-$ B cells and co-cultured them with VPD450-stained CD4$^+$ CD25$^-$ T cells. CD9$^+$ B cells suppressed T cell proliferation more efficiently than CD9$^-$ B cells, based on VPD450 dilution in co-culture assays (B cell: T cell = 400:100) at both 48 and 72 hr (Figure 4A). Interestingly, as shown in Figure 4B, blocking the CD9 moiety with antibody impaired the suppression of T cell growth by CD9$^+$ B cells, suggesting that CD9 plays an important role in immune suppressive function of B10 cells. We further checked whether CD9 acts as a scavenger molecule for CD9$^+$ B cell suppressive activity by culturing T cells and CD9$^+$/CD9$^-$ B cells in separate compartments of a transwell plate. As shown in Figure 4C, CD9$^+$ B cells show inhibitory effects on proliferation of T cells, which are either separated from B cells by insert (T cells in receiver well) or mixed with the CD9$^+$ B cells in insert (T cells in insert well). Additional interesting corollary conclusions emerging from these experiments include (1) even without addition of CD40 antibody and LPS during the terminal 5 hr of culture with PMA and ionomycin, a certain number of CD9$^+$ B cells secreted IL-10, while fewer of the CD9$^-$ B cells secreted IL-10 (Figure 4D) and (2) more CD9$^+$ cells secreted IL-10 if the ratio of T cells to B cells in the co-culture increased, suggesting that IL-10 secretion by B cells may require interaction between B cells and T cells. These observations suggest that CD9$^+$ B cells may require both cross-talk with T cells and secretion of IL-10 to eventually suppress CD4$^+$ T cell proliferation. These observations have been modeled in Figure S4D and are discussed in greater detail below.

To ascertain whether results obtained in ex vivo assays recapitulate in vivo function, we adoptively transferred isolated CD9$^+$ B cells and CD9$^-$ B cells into CD19$^{-/-}$ mice to check the suppressive function of CD9$^+$ B cells on Th1-cell-mediated contact

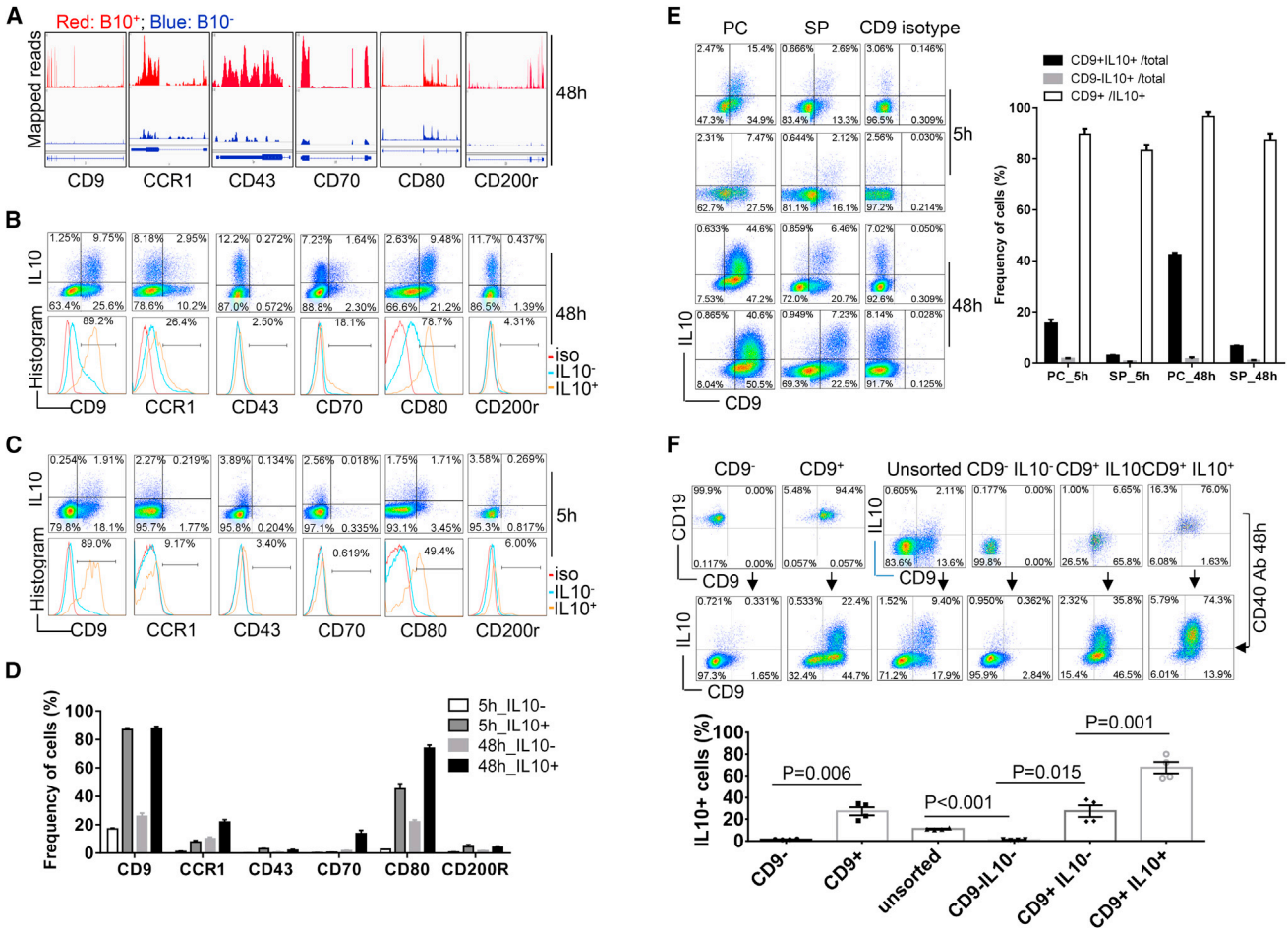

Figure 2. IL-10^{competent} B Cells and Their Progenitors Are Distinguished by CD9

(A–D) Screening markers for IL-10^{competent} B cells identified by RNA-seq. (A) The mapped reads of differentially expressed CD antigens as well as CCR1 determined by RNA-seq on B10^{+} and B10^{-} cells. (B and C) Representative expression of IL-10 and the molecules by total B cells (the dot plots), and expression of the molecules on CD19^{+}IL-10^{+} and CD19^{+}IL-10^{-} cells (the histogram plots) was determined by flow cytometry. The percentage population is shown for CD19^{+}IL-10^{+} cells. All B cells are enriched with CD19 micro-beads and then treated with LPI+M for 5 hr (C) or CD40 antibody for 48 hr plus LPI+M for the terminal 5 hr (B). (D) Statistical results of expression of various molecules on IL-10^{+} or IL-10^{-} B cells.

(E) Expression of IL-10 and CD9 on CD19^{+} B cells isolated from peritoneal cavity (PC) B cells or splenic (SP) B cells and treated with LPI+M for 5 hr or CD40 antibody for 48 hr followed by LPI+M for the terminal 5 hr. Two different CD9 antibodies were used. The bar graph shows the statistical frequency of subsets in total or IL-10^{+} B cells.

(F) Expression change of IL-10 and CD9 on the indicated sorted cell subsets after incubation with CD40 antibody for 48 hr followed by LPI+M for the terminal 5 hr. The bar graph shows the statistical frequency of IL-10^{+} in the sorted subsets. Cell subsets were sorted from naive B cells treated with (for IL-10 staining) or without (for CD19 and CD9 staining only) LPI for 5 hr. Statistical data are represented as mean with SEM p value was obtained by multiple t test.

Please also see Figure S3 and Table S4.

hypersensitivity. In comparison to CD19$^{-/-}$ mice injected with CD9^{-} B cells, the ear thickness both of CD19$^{-/-}$ mice injected with CD9^{+} B cells and of WT mice injected with PBS were thinner throughout the whole process, and the differences were most significant at 48 hr post oxazolone challenge. These observations provide evidence that CD9^{+} B cells possess immune regulatory function (Figure 4E).

Increased IL-10^{competent} B Cells in a CD9$^{-/-}$ Mouse Model

To ascertain whether CD9 is important for IL-10 production or B10 biogenesis, we employed flow cytometry to compare the expression levels of the main markers reported for Bregs from WT mice and CD9$^{-/-}$ mice. Consistent with a previous report (Cariappa et al., 2005), none of the checked markers was expressed at an appreciably different level on total B cells from CD9$^{-/-}$ and WT mice. Likewise, for IL-10^{+} B cells isolated from CD9$^{-/-}$ and WT mice, no major difference in the marker expression was noted. However, on IL-10^{+} B cells from CD9$^{-/-}$ mice, CD23 expression differed from the WT (Figure S4F). Further analysis indicated that more MZ B cells and approximately twice the number of IL-10^{competent} B cells exist in CD9$^{-/-}$ B cells compared with WT (Figures 4F, S4F, and S4G), suggesting that CD9 may play an important role in B10 maturation.

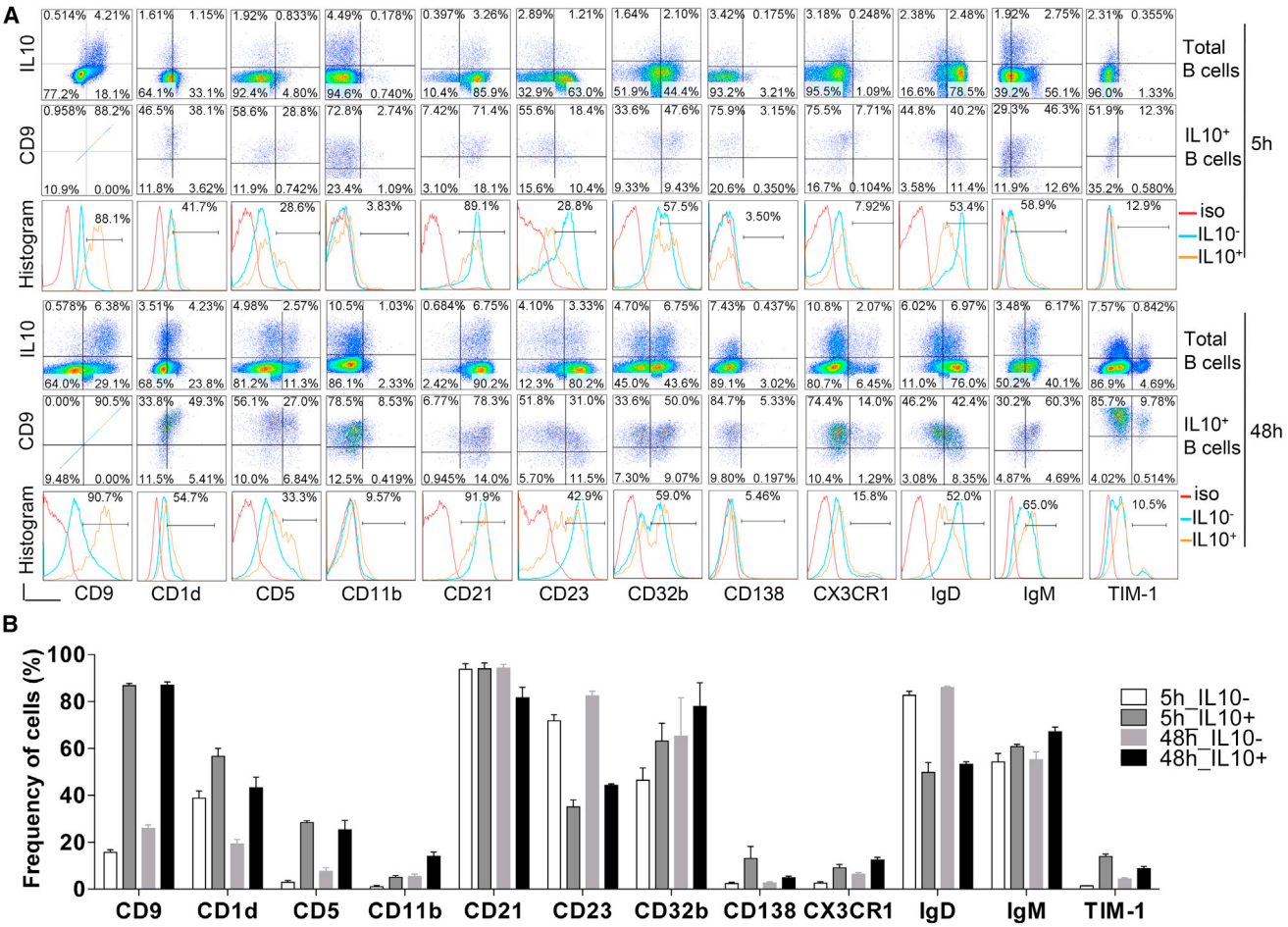

Figure 3. CD9 Is a Robust Surface Marker for Mouse IL-10^{competent} B Cells

(A) Representative expression of IL-10, CD9, and other reported Breg markers on total, IL-10$^+$, or IL-10$^-$ B cells stimulated with LPI+M for 5 hr or CD40 antibody for 48 hr plus LPI+M for the terminal 5 hr. The frequency of cells expressing specific markers on IL-10$^+$ B cells is shown in the histograms.

(B) The statistical frequency of subsets expressing specific markers on IL-10$^-$ or IL-10$^+$ B cells. Statistical data are represented as mean with SEM.

DISCUSSION

The phenotypic diversity found in Breg subsets makes the discovery of Breg origin a difficult endeavor. Unique cell surface markers, coupled with transcription factors, could provide distinct signatures (Mauri and Bosma, 2012). In this study, we profile the RNA expression of B10 cells, identify key upstream regulators, and find CD9 as a novel functional marker of most IL-10^{competent} B cells and their progenitors. The identified unique marker CD9 can separate B10 progenitors, IL-10$^+$ B cells from IL-10$^-$ B cells. Thus, the identification of CD9 allows the identification of IL-10$^+$ B cells without stimulation of IL-10 secretion, thereby providing a system to study unperturbed gene expression profiles of Bregs and Breg progenitors. This is especially important since Breg transcription factor activity and networks may otherwise be perturbed by stimulation with LPS, PMA, and ionomycin. Accordingly, the CD9 marker may assist in the identification of transcription factors driving Breg differentiation.

It remains unclear how and whether B10 cells regulate other cells of the immune system and whether the regulation occurs through direct cell-cell interaction or through the localized expression and supply of cytokines (DiLillo et al., 2010; Mauri and Blair, 2010). Ex vivo functional assay of CD19$^+$CD9$^+$ B cells with CD9 blocking antibody demonstrated that CD9 is involved in the suppressive function of CD19$^+$CD9$^+$ B cells (Figure 4B). However, following interaction with CD4$^+$ T cells in the insert of the transwell plate, CD9$^+$ B cells are able to suppress proliferation of T cells present in a physically separate receiver well of the transwell plate (Figure 4C). Thus, it is possible that CD9 may facilitate communication of B10 cells with T cells leading to secretion of IL-10 which then suppresses T cell proliferation (modeled in Figure S4D). Further experiments, particularly with CD9-conditional allele knockout mouse models, will be required to establish the true nature of CD9's function during B cell-mediated immunosuppression. A separate but untested possibility exists—that the regulatory function of CD19$^+$CD9$^+$ B cells results from the release of CD9 containing exosomes, since mouse

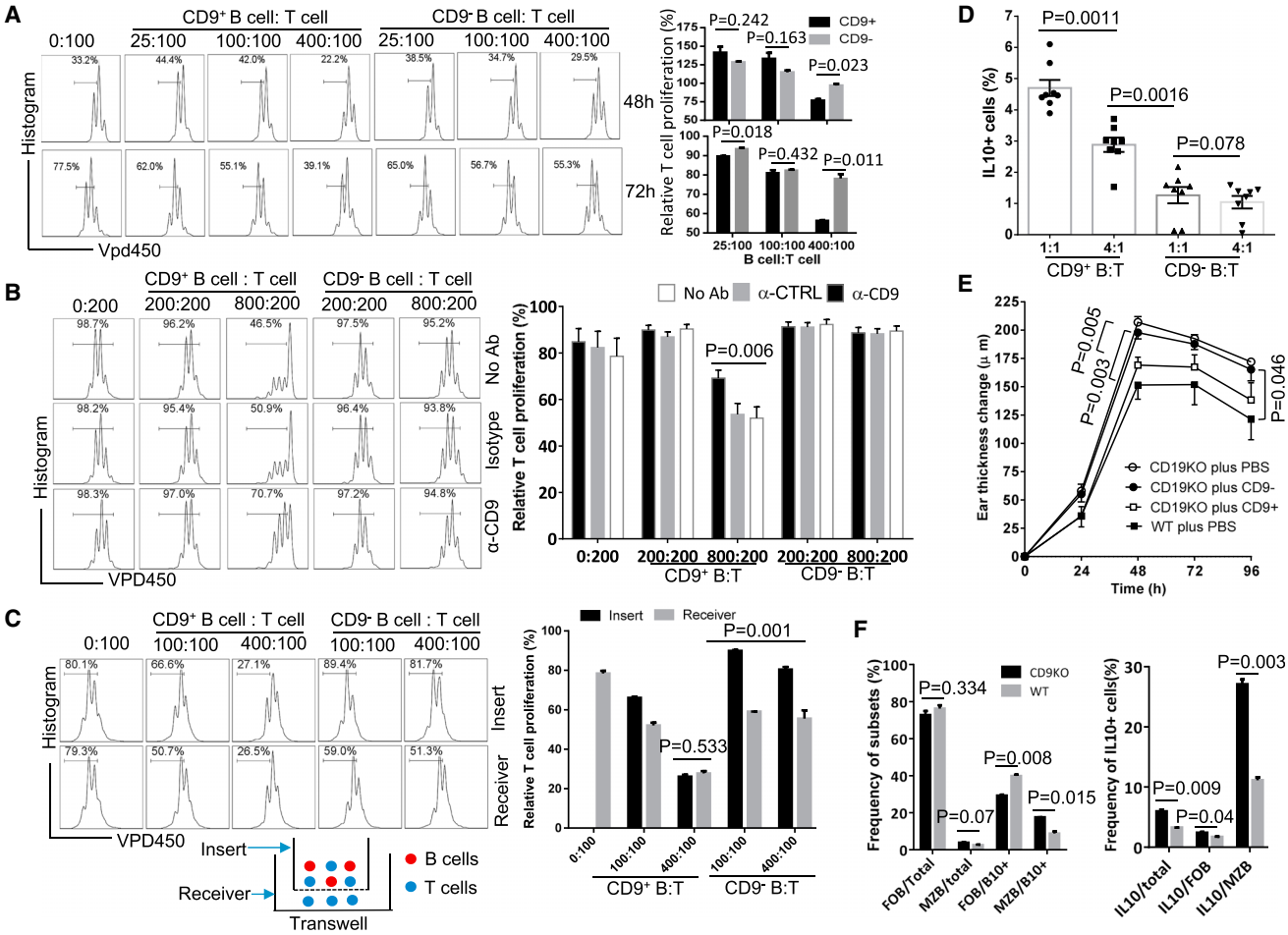

Figure 4. CD9 Is Involved in B10 Function and Biogenesis

(A) Representative and statistical proliferation of T cells co-cultured for 48 and 72 hr at the indicated initial ratio with $CD9^+$ or $CD9^-$ B cells. The statistical results are normalized by the T cells co-cultured without B cells.

(B) Representative and statistical proliferation of T cells co-cultured for 72 hr at the indicated initial ratio with $CD9^+$ or $CD9^-$ B cells in the presence of antibodies.

(C) Representative and statistical proliferation of T cells (100×10^3) in the insert or in the receiver well of the transwell plate. The insert was added at the indicated initial ratio with $CD9^+$ or $CD9^-$ B cells as shown in the bottom cartoon and incubated for 72 hr.

(D) Statistical analysis of IL-10 production by $CD9^+$ B cells and $CD9^-$ B cells treated with PMA, ionomycin, and monensinfor the terminal 5 hr of culture.

(A–D) $CD9^+$ or $CD9^-$ B cells were sorted from $CD19^+$ B cells treated with CD40 antibody and LPS for 5 hr. $CD4^+CD25^-$ T cells were sorted from naive $CD19^-$ cells and then stained by VPD450 before culturing in the plates pretreated with 5 μg/ml CD3e antibody. Medium was changed at 48 hr.

(E) Ear thickness change of CD19 knockout mice adoptively transferred with $CD9^+$ B cells or $CD9^-$ B cells in an in vivo B cell functional analysis assay. WT and CD19 KO mice injected with PBS were the controls for the experiment. The cells were sorted as described in Experimental Procedures.

(F) The frequency of MZ B and FO B in total B cells or IL-10^+ B cells and the frequency of IL-10^+ B cells in total, FO, and MZ B cells. Statistical data are represented as mean with SEM p values were obtained by multiple t test.

Please also see Figure S4.

exosomes contain an abundance of CD9 (Théry et al., 1999)—and the exosomes derived from mouse Tregs suppress pathogenic T helper 1 cells (Okoye et al., 2014).

Human $CD9^+$ B cells may not have functions similar to mouse $CD9^+$ B cells and may not represent most of the human IL-$10^{competent}$ B cells because proteomic analysis of exosomes from human B cells (Escola et al., 1998), lymphoma cells (Yao et al., 2015), and other human cells (Hegmans et al., 2004) did not identify CD9. Perhaps there is a CD9 equivalent developmental marker for B10 cells in humans, and it would be insightful to investigate the full range of tetraspanin family protein expression on the surface of human IL-10^+ B cells. Some identified regulators—such as CD80, CD5, HAVCR1, and IL-33—have been reported to be important Breg differentiation and function factors. CD80/CD86, as well as Toll-like receptors (TLRs) and CD40, are important co-stimulatory molecules involved in B cell differentiation (Mann et al., 2007). CD5 is a marker shared by many reported Breg subsets. HAVCR1 is a co-stimulatory molecule enriched in IL-10 producing Breg cells that can be induced by Havcr1 crosslinking using a Havcr1-specific antibody (Ding et al., 2011). These are all worthy of study, but the many regulators, including both protein factors

and non-coding RNAs, we have reported here need to be studied further to determine their true biological potential in Breg biology.

EXPERIMENTAL PROCEDURES

Mouse Strains and Spleens

C57BL/6J mice and CD19 knockout mice (B6.129P2(C)-*Cd19*$^{tm1(cre)Cgn}$/J) were purchased from The Jackson Laboratory. Spleens of CD9 knockout mice (Miyado et al., 2000) and WT mice were obtained from the animal facility of Osaka University Graduate School of Medicine in Japan. All animals were handled in strict accordance with the Columbia University "ethical treatment of research animals" guidelines.

Cell Isolation, Culture, Staining, and Sorting

B cells were enriched from mouse splenocytes or peritoneal cavity cells through CD19 positive selection or CD90.2 negative selection using MACS (Miltenyi Biotec) columns. B cells were cultured in RPMI-1640 medium containing 10% FBS. 1 μg/ml CD40 antibody, 10 μg/ml LPS, 50 ng/ml PMA, 500 ng/ml ionomycin, and 2 μM monensin were added as required for specific experiments. T cells were cultured with or without B cells in RPMI-1640 medium containing 10% FBS with 5 μg/ml CD3e antibody bound to the well surface of the plate. See Supplemental Experimental Procedures for more information on cell staining and sorting.

RNA Preparation, qRT-PCR, RNA Expression Profiling, and IPA Analysis

Total RNA was prepared from isolated B10$^+$ cells and B10$^-$ cells. A fraction of total RNA was used for miRNA analysis (GEO: GSE63374) by hybridization to microarrays with Sanger miRBase Release 20.0, a fraction for qRT-PCR validation of RNA-seq, and the remainder was used for further RNA sequencing (GEO: GSE63426). An independent repeat was performed to enhance the reliability. Reads were mapped to the mouse genome (mm9) and normalized expression level FPKM (fragments per kilobase of transcript per million mapped reads) of known genes and transcripts was obtained. Rank change (RC) and fold change (FC) of mRNAs between B10$^+$ and B10$^-$ cells were analyzed. The generated list of differentially expressed factors was analyzed by "Core Analysis" with the IPA (Ingenuity Pathway Analysis) program (QIAGEN) (Krämer et al., 2014).

The qRT-PCR primer pairs used for coding RNAs were obtained from PrimerBank (Spandidos et al., 2010), and the primer pairs for noncoding RNA were designed with DNASTAR Lasergene (Figure S1E).

Ex Vivo B Cell Functional Analysis

VPD450 (BD Biosciences) -stained CD4$^+$CD25$^-$ T cells (100 or 200 × 10^3) were co-cultured with different numbers of autologous CD19$^+$CD9$^+$ B cells or CD19$^+$CD9$^-$ B cells with or without the addition of 10 μg/ml blocking antibody of CD9 (BioLegend, MZ3 clone) or isotype control. The co-culture was incubated with plate-bound CD3e antibody for 72 hr. Medium was changed at 48 hr. T cell proliferation was detected by dilution of VPD450, and the intracellular IL-10 staining was performed after the cells were stimulated with PMA, ionomycin, and monensin for the terminal 5 hr of culture. For assays with transwells (Costar HTS Transwell −96 Well) shown in Figure 4C, VPD450-stained CD4$^+$CD25$^-$ T cells (100 × 10^3) were seeded in each well of both the receiver plate and the insert. The insert well was also loaded with different numbers of autologous CD19$^+$CD9$^+$ B cells or CD19$^+$CD9$^-$ B cells. T cell proliferation was measured after 72 hr in culture.

In Vivo B Cell Functional Analysis

The experiment was performed with mice experiencing an induced CHS reaction as described (Yanaba et al., 2008). See Supplemental Experimental Procedures for more information.

Statistics

Statistical significance was determined by Prism software (GraphPad).

ACCESSION NUMBERS

The accession numbers for the RNA-seq and microarray data reported in this paper are GEO: GSE63426 and GEO: GSE63374.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.09.070.

AUTHOR CONTRIBUTIONS

J.S. and U.B. designed and interpreted all the experiments, E.P. and I.I.I. assisted in designing some of them; J.S. and J.W. performed bioinformatics analysis with guidance from R.R.; J.S. performed all the experiments, J.C., G.R., and J.K.C. assisted in some of them; I.T. and Y.T. provided CD9$^{-/-}$ mice; J.S. and U.B. wrote the manuscript.

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