Inventory of Supplemental Information

1. **Supplemental Figures and Legends**

   Figure S1. Transcriptome analysis of B10 cells by RNA-seq. This provides the cell sorting strategy, mapped reads of markers for B cells/T cells/B10 cells, mapped reads of RNAs for qRT-PCR validation, and validation of some upstream regulators. Such data are very important for **Figure 1**.

   Figure S2. Differentially expressed upstream regulators correlated with Breg biogenesis and function. This provides a model to show how the predicted differentially expressed upstream regulators are involved in Breg biogenesis and function. It gives more information for understanding the importance of **Figure 1**.

   Figure S3. Differentially expressed mRNAs and expression of CD9 plus IL10 in various B cell subsets. This provides information of mRNA and protein expression in various B cell subsets such as B1 cells, Marginal Zone B cells, and Follicular B cells. It support **Figure 2**.

   Figure S4. Involvement of CD9 in B10 function and maturation. This provides information of cell preparation, frequency of T cells and B cells in the co-culture, model, and other important information for **Figure 4**.

2. **Supplemental Tables and Legends**

   Table S1. Expression profiles of mRNA, lncRNA and miRNA. Contains 3 sheets and supports **Figure 1**.

Table S2. Pairing between 273 mRNAs and 77 miRNAs. Contains 2 sheets and supports **Figure 1**

Table S3. **Predicted upstream regulators and gene ontology analysis.** Contains 4 sheets and supports **Figure 1**

Table S4. Differentially expressed mRNAs in various B cell subsets. Contains 6 sheets and supports **Figure 1 and Figure 2**.

3. **Supplemental Experimental Procedures**

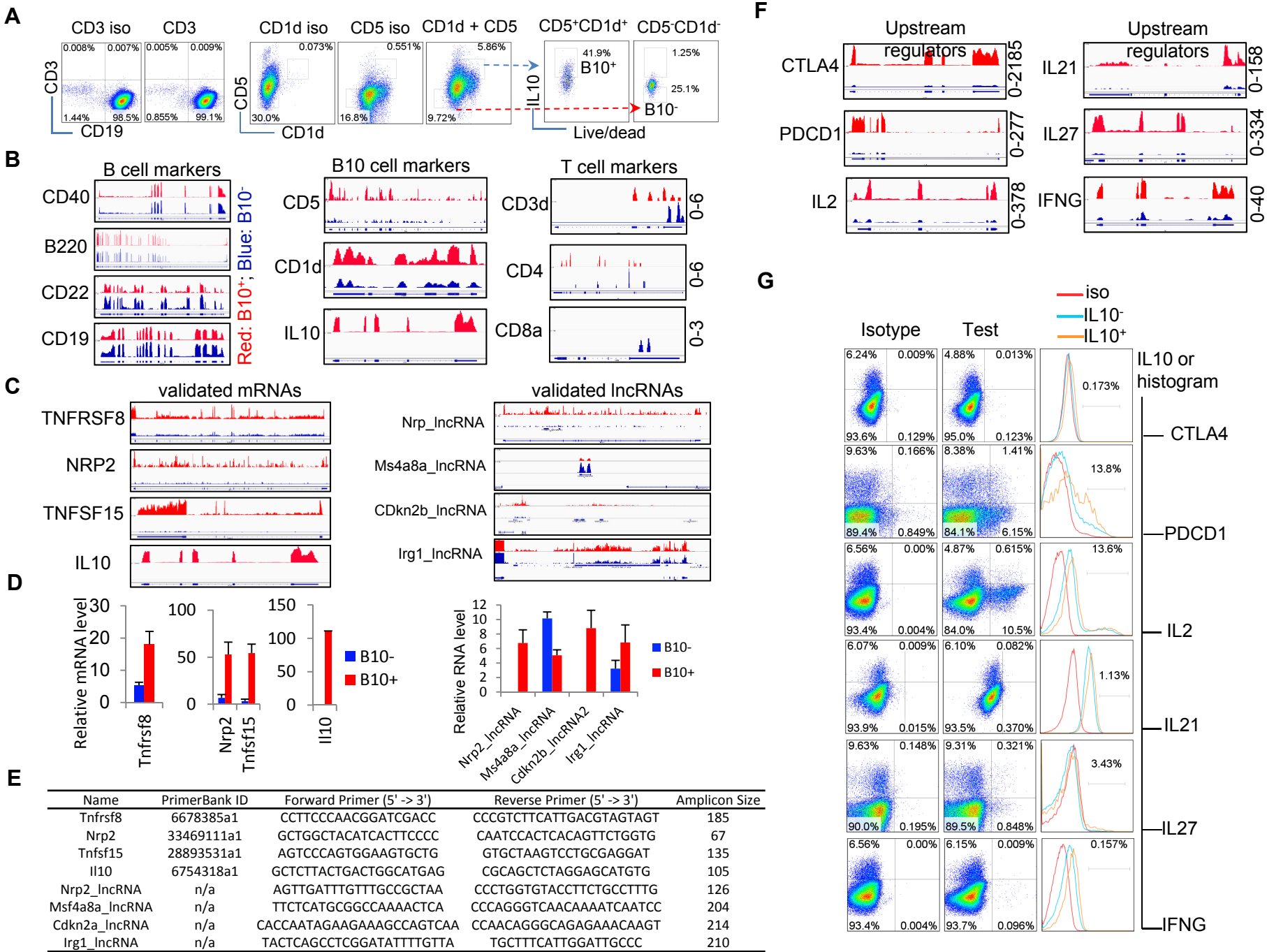
Cell Staining and Preparation

In vivo B cell functional analysis

Additional Bioinformatics Analysis

4. **References**

Figure S1. Transcriptome analysis of B10 cells and its validation.



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Name	PrimerBank ID	Forward Primer (5' -> 3')	Reverse Primer (5' -> 3')	Amplicon Size
Tnfrsf8	6678385a1	CCTTCCCAACGGATCGACC	CCCGTCTTCATTGACGTAGTAGT	185
Nrp2	33469111a1	GCTGGCTACATCACTTCCCC	CAATCCACTCACAGTTCTGGTG	67
Tnfsf15	28893531a1	AGTCCCAGTGGAAGTGCTG	GTGCTAAGTCCTGCGAGGAT	135
Il10	6754318a1	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG	105
Nrp2_lncRNA	n/a	AGTTGATTTGTTTGCCGCTAA	CCCTGGTGTACCTTCTGCCTTTG	126
Msf4a8a_lncRNA	n/a	TTCTCATGCGGCCAAAACTCA	CCCAGGGTCAACAAAATCAATCC	204
Cdkn2a_lncRNA	n/a	CACCAATAGAAGAAAGCCAGTCAA	CCAACAGGGCAGAGAAACAAGT	214
Irg1_lncRNA	n/a	TACTCAGCCTCGGATATTTTGTTA	TGCTTTCATTGGATTGCCC	210

Figure S2. Differentially expressed upstream regulators correlated with Breg biogenesis and function

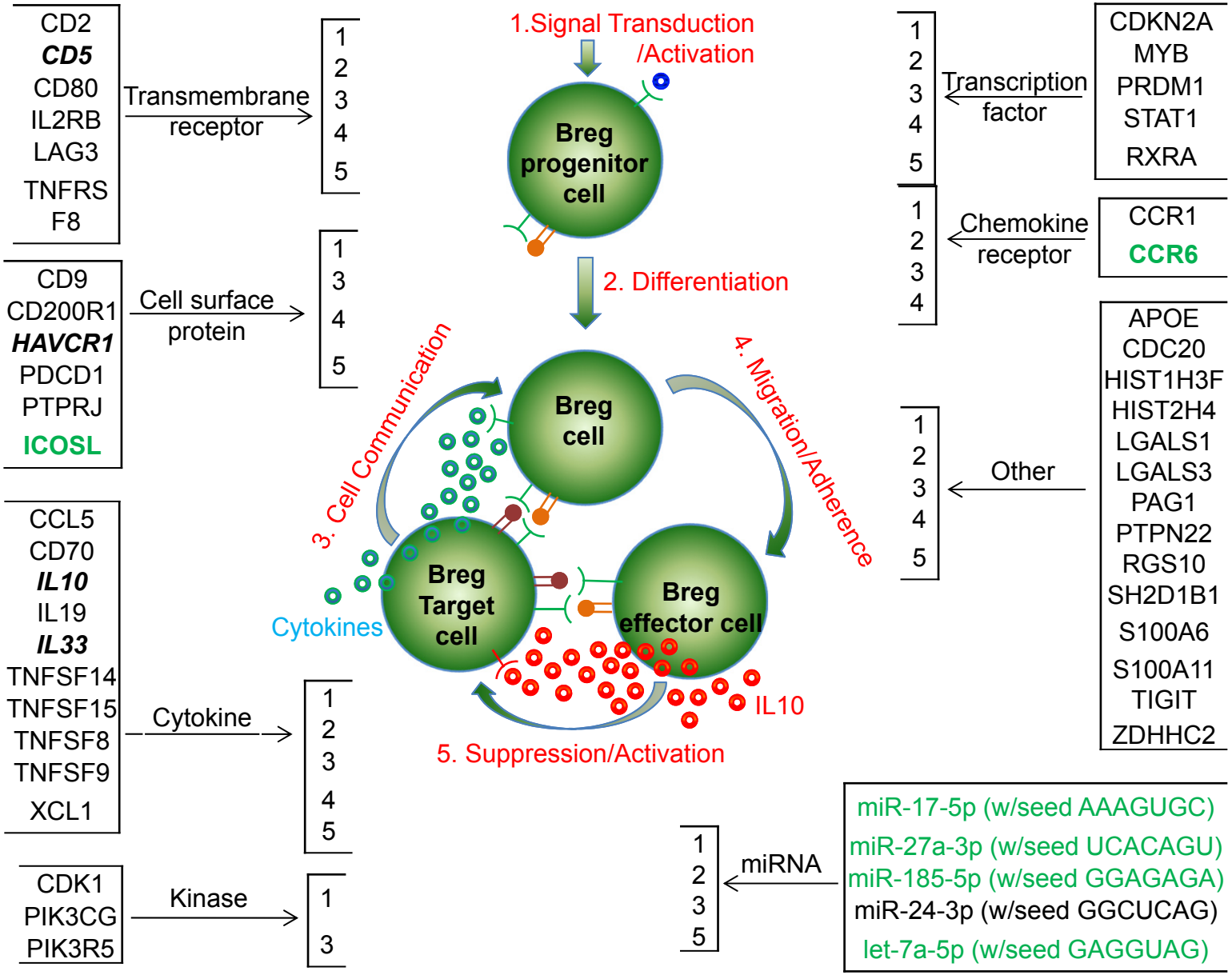


Figure S3. Differentially expressed mRNAs and expression of CD9 plus IL10 in various B cell subsets.

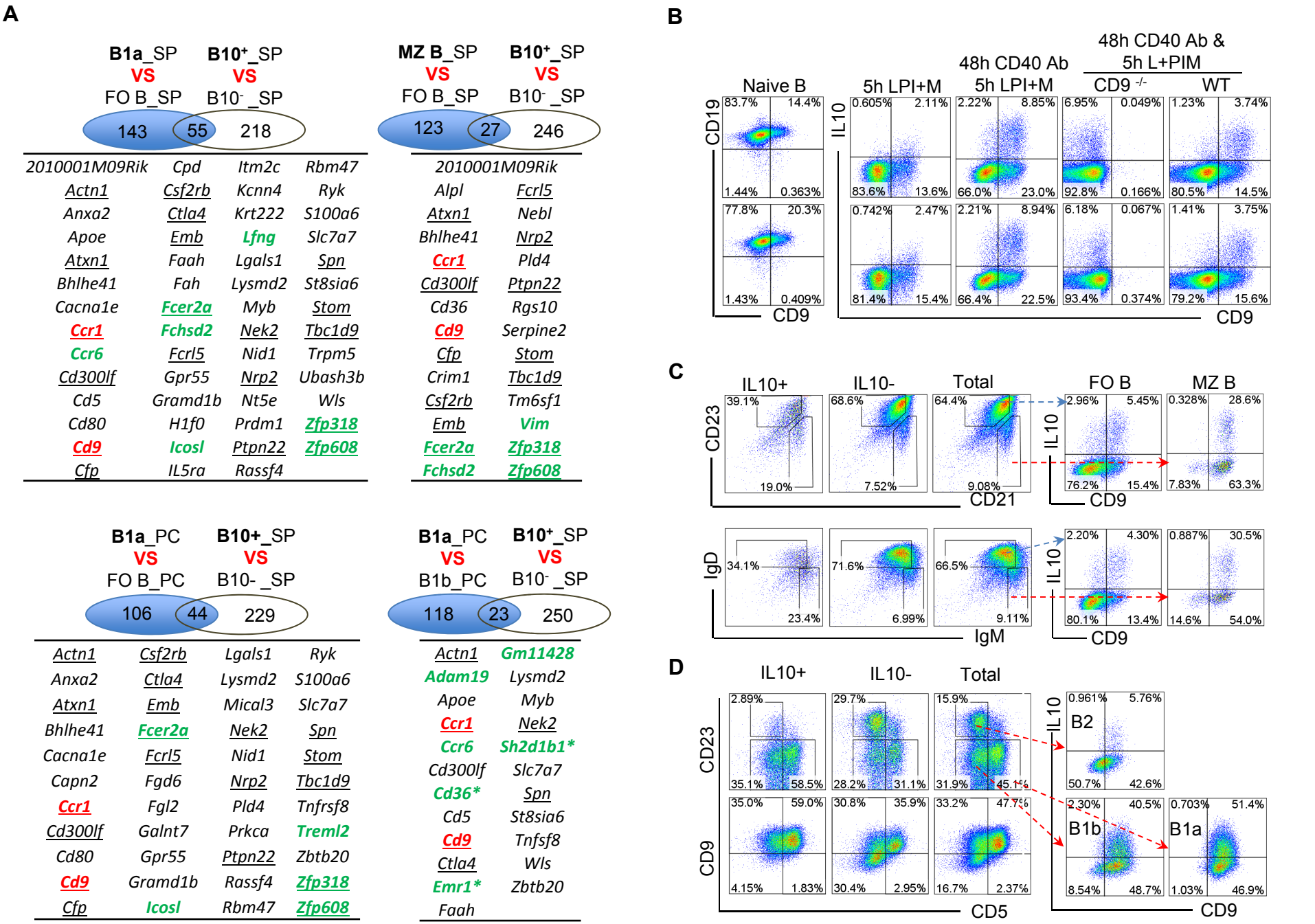
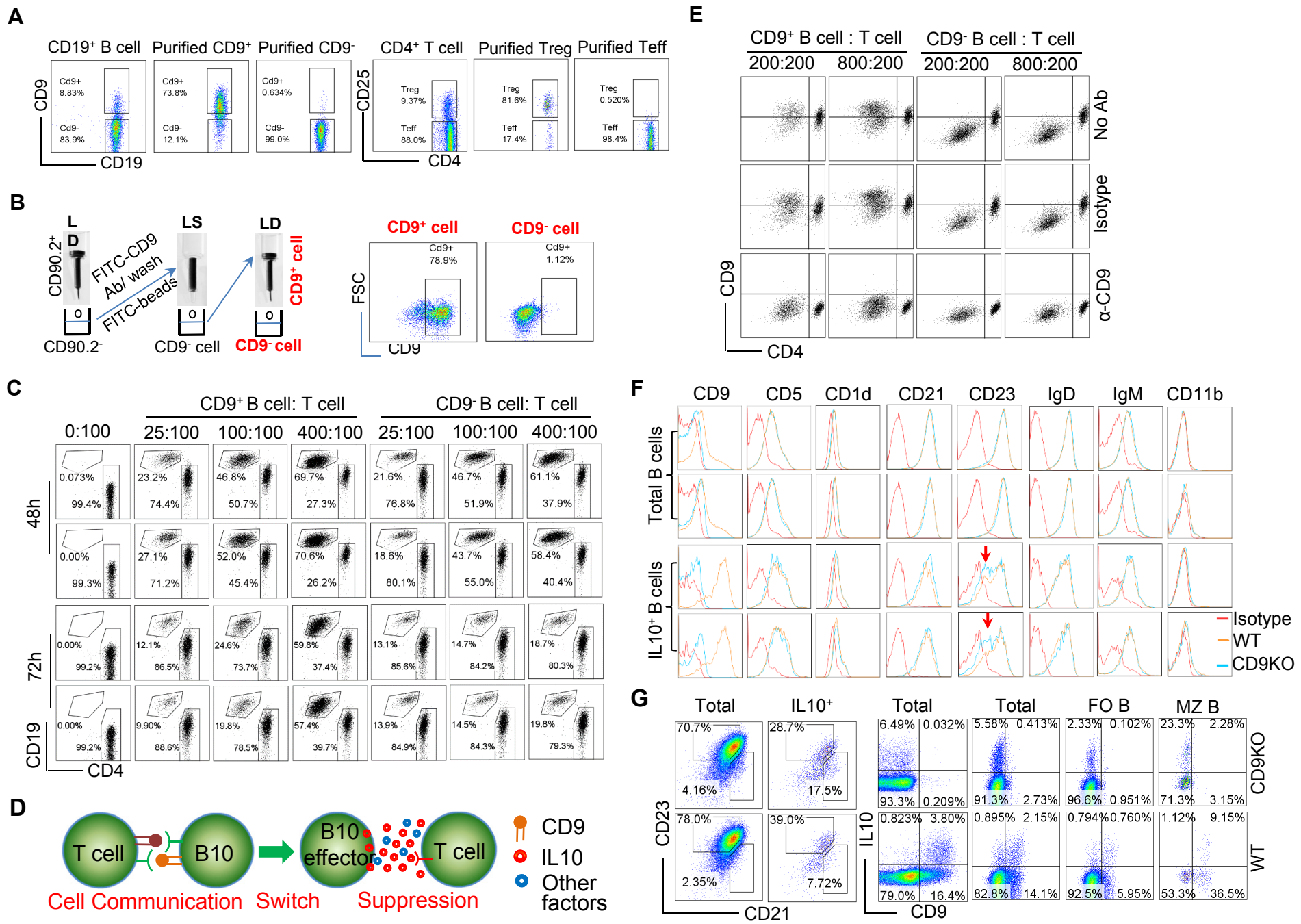


Figure S4. Involvement of CD9 in B10 function and maturation.



Supplemental figure legends

Figure S1. Transcriptome analysis of B10 cells by RNA-seq. (A) Cell sorting of $B10^+$ cells ($CD1d^{hi}CD5^+$ $CD19^+IL10^+$) and $B10^-$ cells ($CD1d^-CD5^-$ $CD19^+IL10^-$) from splenic B cells incubated with CD40 antibody for 48h and LPI for the terminal 5h. B cell purity (around 99%) is presented as frequency of $CD19^+$ B cells. Total RNA was prepared with Trizol® and rRNA was depleted via the Ribo-Zero™ rRNA Removal Kit for transcriptome analysis. (B) Mapped reads of markers for B cells, B10 cells, and T cells. Numbers on the right y-axis of T cell markers represent the reads level. (C) Mapped reads of 4 mRNAs and 4 lncRNAs for qRT-PCR validation. The 4 mRNAs include low (Tnfrsf8), middle (Nrp2 and Tnfsf15), and high (IL10) differentially expressed genes. (D) Validation of the expression of 4 mRNAs and the 4 lncRNAs in (C) by qRT-PCR. (E) Primers used for qRT-PCR validation. (F) Mapped reads of several upstream regulators usually expressed by T cells from RNA-seq data of $B10^+$ and $B10^-$ B cells. Numbers on the right y-axis represent the reads level. In (B), (C), and (F), red is from $B10^+$ cells and blue is from $B10^-$ cells. (G) Representative expression of IL10 and the upstream regulators from (F) on total B cells stimulated with CD40 antibody for 48h plus LPI+M for the terminal 5h. The frequency of cells expressing specific marker on $IL10^+$ B cells is shown in the histograms.

Figure S2. Differentially expressed upstream regulators correlated with Breg biogenesis and function. Five steps tightly correlating with Breg biogenesis and function are highlighted in the center. First, Breg progenitor cells receive signals from the environment and are driven to differentiate and mature into Bregs. Then, the matured Bregs communicate through intercellular cross-talk and/or signal transduction pathways

with the environment--especially their target cells--to determine whether Bregs should secrete cytokines like IL10, or migrate to the target region, or secrete chemokines to attract target cells. Depending on the outcome of the decision tree, the effector Breg cells can suppress their target cells (e.g., T_h cells) or activate their target cells (e.g., Treg cells) through cytokine secretion or cell-cell interactions. Based on the functions/features in various types of cells (not merely Bregs) reported in the literature and the tags put on the factors by IPA, we have arranged the 51 differentially expressed upstream regulators (excluding IFNG, IL2, IL21, IL27, and CTLA4 based on their expression in Figure S1) identified from the 273 mRNAs and 77 miRNAs into 9 groups at the periphery of the figure, and their predicted association with each of the 5 steps involved in Breg biogenesis and function is presented as a number 1 through 5 according to the gene ontology term enrichment analysis. Green colored factors are down-regulated in $B10^+$ cells. Factors both bold and italicized are reported in the literature as being important for Breg biogenesis and function. “w/seed” means the other miRNAs with the same seed sequence. Please also see Table S3.

Figure S3. Differentially expressed mRNAs and expression of CD9 plus IL10 in various B cell subsets. (A) Overlapping of the differentially expressed mRNAs in “splenic B1a cells versus splenic follicular B cells (FO B)”, “peritoneal cavity B1a cells versus peritoneal cavity FO B cells”, “splenic marginal zone B cells (MZ B) versus splenic FO B cells”, or “peritoneal cavity B1a cells versus peritoneal cavity B1b cells” with the mRNAs differentially expressed by “$B10^+$ cells versus $B10^-$ cells”. Green colored factors are downregulated in $B10^+$ cells. The underlined mRNAs appear on 3 lists and the red colored mRNAs appear on all 4 lists. The asterisk (*) indicates the gene expresses less

in B1a cells than in B1b cells but expresses more in $B10^+$ cells than $B10^-$ cells. The information on B cell subsets and their differentially expressed genes can be found in Table S4. (B) CD9 expression on naive B cells, B cells treated with LPI+M for 5h, B cells treated with CD40 antibody for 48h plus LPI+M for the terminal 5h culture, $CD9^{-/-}$ and WT B cells treated with CD40 antibody for 48h plus LPI+M for the terminal 5h culture. (C) Expression of IL10 and CD9 on the indicated gated MZ B and FO B cell subsets after the CD19-microbeads enriched splenocytes were incubated with CD40 antibody for 48h followed by LPI+M for the terminal 5h. CD21 and CD23, or IgD and IgM were used as markers for setting up the MZ B and FO B cell gates. (D) Expression of IL10 and CD9 on the indicated gated B1a, B1b and B2 cell subsets after the CD19-mirobeads enriched cells from peritoneal cavity were incubated with CD40 antibody for 48h followed by LPI+M for the terminal 5h. CD23 and CD5 were used as markers for setting up the B1a, B1b and B2 cell gates.

Figure S4. Involvement of CD9 in B10 function and maturation. (A) Cell sorting for T cell proliferation assay in Figures 4A-4D. $CD4^+CD25^-$ T cells were sorted from $CD19^-$ splenocytes without stimulation, while $CD19^+CD9^-$ B cells and $CD19^+CD9^+$ B cells were sorted from $CD19^+$ splenocytes which were treated with CD40 antibody and LPS for 5h. (B) Cell sorting for the CHS assay in Figure 4E. $CD90.2^-$ cells were prepared with CD90.2 microbeads and LD column from splenocytes cultured with CD40 antibody for 48h and LPS for the last 5h and then stained with CD9-FITC antibody. $CD9^+$ B cells were enriched with FITC-microbeads and LS column. The $CD9^-$ B cells (the flowthrough) were re-purified over LD columns to remove residual $CD9^+$ B cells. The purity of the final purified $CD9^+$ B cells and $CD9^-$ B cells as assayed by flow cytometry is shown. (C) Frequency of T cells

and B cells in the co-culture at 48h and 72h. VPD450 stained $CD4^+CD25^-$ T cells (100×10^3) were co-cultured for 48h and 72h at the indicated initial ratio with $CD9^+$ or $CD9^-$ B cells. (D) A model for B10 cell function. B10 cells communicate with target cells or other environmental cells via CD9 tetraspanin and then switch to B10 effector cells which express IL10 or secret other factors such as exosomes for suppression of T cells. Please note that this model is just a hypothesis based on the in vitro data presented. To obtain direct evidence that CD9 is physiologically required for the IL10 expression by B10 cells, potential experiments could involve CD9 reporter mice whose CD9 has been disrupted and replaced by GFP permitting comparison of the function of GFP^+ $CD9^{-/-}$ B cells with $CD9^+$ WT B cells in vitro and *in vivo*. Another option would be to check the GFP expression in B cells in IL10 reporter mice with or without a CD9 knockout background under the immunizations or immune disease models. (E) Expression of CD9 and CD4 on the co-cultured cells in the presence of blocking antibodies. VPD450 stained $CD4^+CD25^-$ T cells (200×10^3) were co-cultured for 72h at the indicated initial ratio with $CD9^+$ or $CD9^-$ B cells in the presence of antibodies. (F) Selected CD antigen expression on total B cells ($CD19^+$) or $IL10^+$ B cells from two CD9 KO and two WT spleens. Frequency of $CD23^+$ B cells is lower in $IL10^+$ B cells from CD9 KO spleen than from WT spleen (red arrows). (G) The frequency of MZ B cells and FO B cells in total B cells or $IL10^+$ B cells, and the CD9 and IL10 expression on Total, MZ B cells ($CD21^{hi}CD23^-CD19^+$) and FO B cells ($CD21^{lo}CD23^{hi}CD19^+$) from CD9 KO and WT spleens. Total cells were stained with two different CD9 antibodies.

Supplemental Tables (provided in separate Excel files):

Table S1. Expression profiles of mRNA, lncRNA and miRNA. Contains 3 sheets and supports Figure 1.

Table S2. Paring between 273 mRNAs and 77 miRNAs. Contains 2 sheets and supports Figure 1

Table S3. **Predicted upstream regulators and GO analysis.** Contains 4 sheets and supports Figure 1

Table S4. Differentially expressed mRNAs in various B cell subsets. Contains 6 sheets and supports Figure 1 and Figure 2.

Supplemental Experimental Procedures and References

Cell Staining and Preparation

For intracellular IL10 staining, B cells were cultured with LPS, PMA, ionomycin plus monensin (LPI+M) for 5h before cell harvest. For RNA-seq and microarray, $B10^+$ ($CD1d^{hi}CD5^+CD19^+IL10^+$) cells and $B10^-$ ($CD1d^-CD5^-CD19^+IL10^-$) cells were sorted by FACSAria™ (Figure S1A) from $CD19^+$ spleen B cells treated with CD40 antibody for 48h and LPI for the last 5h of culture and stained with IL10 Secretion Assay (catalog no. 130-090-490, Miltenyl Biotec) as described previously (Matsushita and Tedder, 2011) based on the principle that IL10 is captured by the catch reagent linked on lymphocytes where IL10 is secreted and then stained with IL10 antibody. The same method was used to isolate $CD9^-IL10^-$, $CD9^+IL10^-$, $CD9^+IL10^+$ B cells from the B cells cultured for only 5h with

LPI. For ex-vivo B cell functional analysis, $CD4^+CD25^-$ T cells were isolated by FACSAria™ from unstimulated $CD19^-$ cells while $CD19^+CD9^-$ and $CD19^+CD9^+$ B cell subsets were isolated from $CD19^+$ cells stimulated with CD40 antibody and LPS for 5h (Figure S4A). For *in vivo* B cell functional analysis, $CD9^-$ and $CD9^+$ B cells subsets were isolated with FITC-microbeads and LS/LD column from CD90.2 depleted cells cultured with CD40 antibody for 48h and LPS for the last 5h and then stained with CD9-FITC antibody (Figure S4B).

In vivo B cell functional analysis

The experiment was performed with mice experiencing an induced CHS reaction as described (Yanaba et al., 2008). Briefly, groups of 5 mice of the same age (6-8 weeks) were used. 3 days after arrival from the commercial supplier, $CD19^{-/-}$ mice were intravenously injected with 2 million isolated $CD9^-$ B cells, $CD9^+$ B cells or PBS (0.1ml/10g body weight)/mouse with 29G needles. Two days later, the mice were sensitized with 25 ul of oxazolone (100 mg/ml, Sigma) in acetone/olive oil (4:1 v/v) applied evenly for 2 consecutive days on a shaved abdomen. On day 5, sensitized mice were challenged by application of 10 ul of oxazolone solution (1mg/ml) in acetone/olive oil (4:1) to both ears (5 ul on the dorsal side and 5 ul on the ventral side). The thickness of the central portion of each ear lobe was measured at 0, 24, 48, 72, and 96 hours after challenge using a constant-force, calibrated digital thickness gauge (Mitsutoyo Corp., Tokyo, Japan).

Additional Bioinformatics Analysis

To identify differentially expressed mRNA in B10 cells, indices of rank change (iRC) and fold change (iFC) are used to describe the differential expression of transcripts between

$B10^+$ and $B10^-$ cells. Here, rank change (RC) is calculated as the absolute value of the difference between the rank number of a certain transcript in $B10^+$ cells and $B10^-$ cells in each batch. iRC is defined as the smaller RC in the two independent batches. iFC was calculated as 2^x where the power x is the smaller absolute value of logarithm base 2 expression fold change (log_2FC) in the two batches. The genes with contradictory changes between the two batches were removed before the calculation of iFC.

To obtain the annotations of the 56 differentially expressed upstream regulators, we used Generic GO Term Finder (http://go.princeton.edu/cgi-bin/GOTermFinder) with the "56 upstream regulators" as input, "process" as "ontology aspect", and MGI as annotation. Because miRNAs cannot be analyzed by GOTermFinder, we put together the differentially expressed target molecules (which exist in dataset "56 upstream regulators") of the 5 miRNAs as the input for the GOTermFinder analysis in the same manner as mentioned above.

References

Matsushita, T., and Tedder, T.F. (2011). Identifying regulatory B cells (B10 cells) that produce IL-10 in mice. Methods in molecular biology *677*, 99-111.

Yanaba, K., Bouaziz, J.D., Haas, K.M., Poe, J.C., Fujimoto, M., and Tedder, T.F. (2008). A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. Immunity *28*, 639-650.