Published in final edited form as:
J Immunol. 2012 February 1; 188(3): 1036–1048. doi:10.4049/jimmunol.1102500.

Regulatory B10 Cells Differentiate Into Antibody-Secreting Cells After Transient IL-10 Production *In Vivo*

Damian Maseda[†,§], **Susan H. Smith**[†,§], **David J. DiLillo**[†], **Jacquelyn M. Bryant**[†], **Kathleen M. Candando**[†], **Casey T. Weaver**[‡], and **Thomas F. Tedder**[†,*]

[†]Department of Immunology, Duke University Medical Center, Durham, NC 27710

[‡]Departments of Pathology and Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294

Abstract

Regulatory B cells that are functionally defined by their capacity to express IL-10 (B10 cells) downregulate inflammation and autoimmunity. In studies using well-defined IL-10-reporter mice, this rare B10 cell subset was also found to maintain a capacity for plasma cell differentiation. During a transient period of *il10* transcription, the *blimp1* and *irf4* transcription factors were induced in B10 cells while *pax5* and *bcl6* were downregulated as a significant fraction of B10 cells completed the genetic and phenotypic program leading to antibody-secreting cell differentiation *in vitro* and *in vivo*. B10 cell-derived IgM reacted with both self and foreign Ags, whereas B10 cells generated Ag-specific IgG in response to immunizations. Moreover, B10 cells represented a significant source of serum IgM and IgG during adoptive transfer experiments, and produced Ag-specific, polyreactive and autoreactive antibody specificities that were consistent with their expression of a diverse Ag receptor repertoire. Thereby, B10 cells not only limit inflammation and immune responses by the transient production of IL-10, but may also facilitate clearance of their eliciting Ags through an inherent capacity to quickly generate polyreactive and/or Ag-specific antibodies during humoral immune responses.

Introduction

B lymphocytes mediate humoral immunity through their production of secreted antibody, but are also central regulators of $CD4^+$ T cell activation by serving as APCs and providing co-stimulatory molecules and cytokines that regulate cellular immune responses during T cell expansion, memory formation, and cytokine production (1). However, B cells and specific B cell subsets can also negatively regulate immune responses (2). The absence or loss of these regulatory B cells exacerbates disease symptoms in diverse models of inflammation and autoimmunity, predominantly through the production of the regulatory cytokine, IL-10 (3–11).

A specific subset of regulatory B cells was recently found to inhibit inflammation, autoimmunity, and innate and adaptive immune responses through the production of IL-10 (8, 9, 12, 13), a potent and pleiotropic cytokine (14). We call these B cells “regulatory B10 cells” because IL-10 is required for their negative regulatory function (2) and additional B

[*]Correspondence: Thomas F. Tedder, Box 3010, Department of Immunology, Room 353 Jones Building, Research Drive, Duke University Medical Center, Durham, NC 27710; phone (919) 684-3578; Fax (919) 684-8982; thomas.tedder@duke.edu..
[§]Both authors contributed equally to these studies and share first authorship.

Disclosures Conflict-of-interest disclosure: T.F.T. is a consultant for MedImmune/AstraZeneca, Inc, and shareholder and consultant for Angelica Therapeutics, Inc. All other authors declare no competing financial interests.

cell subsets with unique regulatory properties also exist. For example, IL-12-producing B cells regulate intestinal inflammation (15). In mice, regulatory B10 cells are functionally identified by cytoplasmic IL-10 expression following *in vitro* stimulation with LPS, PMA, and ionomycin (L+PI), with monensin (L+PIM) included in the cultures to block IL-10 secretion (8, 9). Spleen B10 cells are found at low frequencies (1–5%), where they are predominantly found within the phenotypically unique $CD1d^{hi}CD5^{+}CD19^{hi}$ B cell subpopulation (8–10). Regulatory B10 cells share overlapping cell surface markers with multiple other phenotypically-defined B cell subsets (B1a, marginal zone, and marginal zone precursor cells), potentially consistent with their localization within spleen follicles and marginal zones (16). B10 cells are presumed to be functionally mature since they are competent to express IL-10 after 5 h of *ex vivo* stimulation, and they proliferate rapidly following *in vitro* or *in vivo* activation (12, 17). Additional B cells within the $CD1d^{hi}CD5^{+}$ B cell subpopulation acquire the ability to function like B10 cells during 48 h of *in vitro* stimulation with either agonistic CD40 mAb or LPS (17). These B10 progenitor (B10pro) cells are then able to express cytoplasmic IL-10 following L+PIM stimulation for 5 h. Regulatory B10 cell functions are Ag-restricted *in vivo* (8, 9), with B10pro and B10 cells requiring diverse Ag receptors (BCR) for their development (17). Spleen B10 cell numbers increase significantly during inflammation and autoimmunity, with the adoptive transfer of Ag-primed $CD1d^{hi}CD5^{+}$ B cells suppressing inflammation and disease in mouse models (8, 9, 11, 17, 18). Human blood B10 and B10pro cells that parallel their mouse counterparts are equally rare, and represent a subset of the circulating $CD24^{hi}CD27^{+}$ “memory” B cell subset (12). Thus, the capacity of human and mouse B10pro and B10 cells to express IL-10 is central to their regulatory function.

IL-10 reporter mice have been developed to examine regulatory T cell IL-10 expression and cell fates. In Tiger mice, an internal ribosomal entry site-GFP construct follows the genomic *il10* coding sequence, resulting in cytoplasmic GFP expression during *il10* transcription (19). Similarly, 10BiT mice express Thy1.1 under the control of *il10* BAC-transgene regulatory elements, leading to cell surface Thy1.1 expression following IL-10 production (20). In the current studies, IL-10 reporter expression was used to track regulatory B10 cell induction and fates in Tiger and 10BiT mice, with the findings that regulatory B10 cells only transiently express IL-10 prior to their terminal differentiation into clonally diverse antibody-secreting plasmablasts and plasma cells that contribute significantly to the serum antibody pool. Thereby, regulatory B10 cells not only limit inflammation and immune responses by the production of IL-10, but also contribute to humoral immunity.

Material and Methods

Mice

C57BL/6 and $Rag2^{-/-}$ mice were from NCI Frederick (Bethesda, MD). Tiger mice (19) were from The Jackson Laboratory (Bar Harbor, ME). A gene dose-dependent decrease in IL-10 production was not observed in homozygous Tiger mice, which occurs with T cells (19). Hemizygous 10BiT mice were as described (20). Mice were housed in a specific pathogen free barrier facility with end-point analyses carried out between 8–14 weeks of age. Mice were given (i.p.) sterile LPS in PBS (25 μg, *E. coli*, clone 0111:B4; Sigma, St. Louis, MO), CFA or IFA (200 μl of 1:1 emulsified mixture with PBS, Sigma, St. Louis, MO), Imject® Alum (200 μl of 1:1 emulsified mixture with PBS, Pierce, Rockford, IL), or alum with TNP_{29}KLH (50 μg/200 μl; Biosearch Technologies, Novato, CA). All studies and procedures were approved by the Duke University Animal Care and Use Committee.

B cell purification, cultures, and immunofluorescence analysis

B cells enriched (>95% $CD19^+$) from single cell tissue suspensions by MACS selection using CD19-microbeads (Miltenyi Biotec Inc., Auburn, CA) were cultured in complete medium (RPMI 1640 medium containing 10% FBS, 1% HEPES, 1% L-Glutamine, 1% Pen/Strep, and 0.1% 2-ME). Sterile LPS (10 μg/ml), goat $F(ab')_2$ anti-mouse IgM antibody (5 μg/ml, Jackson ImmunoResearch, West Grove, PA), and CD40 mAb (2 μg/ml, clone HM40-3; BD Pharmingen, San Jose, CA) were added to cultures where indicated.

Single cell leukocyte suspensions were stained with pre-determined optimal antibody concentrations as described (21) with cytoplasmic IL-10 expression assessed as described (22). Antibodies included anti-mouse IL-10 (JES5-2A5), CD138 (281–2), CD43 (S7), CD38 (90) and GL7 (Ly-77) mAbs from BD Pharmingen; CD16/CD32 (FcBlock), FITC-, PE-, PE.Cy5-, PE.Cy7-, Biotin- or APC-conjugated anti-mouse B220 (clone RA3-6B2), CD19 (eBio1D3), CD1d (1B1), CD5 (53–7.3), Thy1.1 (HIS51), Thy1.1 (OX-7), CD21/35 (eBio8D9) and CD23 (B3B4) mAbs from eBioscience, Inc. (San Diego, CA); anti-mouse IL-10 (JES5-16E3), CD19 (6D5) and CD16/32 (TruStain) from BioLegend (San Diego, CA); and goat anti-mouse IgM antibody (Southern Biotech, Birmingham, AL). In some instances, Streptavidin conjugated to PE.Cy5 or PE.Cy7 (eBioscience) was used to reveal biotinylated antibody binding. Anti-mouse IgG1, IgG2a, IgG3 and IgA antibodies were from Southern Biotech. Anti-mouse Blimp-1 mAb (3H2-E8) was from Novus Biologicals (Littleton, CO). Data were collected on a FACSCantoII™ flow cytometer (BD Biosciences, Franklin Lakes, NJ) and analyzed using Flowjo Software (TreeStar, Inc., Ashland, OR).

Adoptive transfers of syngeneic spleen B cell populations were as described (22). For some experiments, purified spleen $CD19^+$ B cells were first cultured overnight with LPS in complete medium, then washed twice and suspended in sterile PBS prior to i.v. injection through lateral tail veins.

Transcript quantification

RNA extracted from enriched spleen B cells was used to generate cDNA, with relative transcript levels determined by reverse transcriptase quantitative real-time PCR of triplicate samples as described (9). *Thy1.1* transcripts were amplified using forward (CGTTGGCGCACCAGGAGGAG) and reverse (TGGAGAGGGTGACGCGGGAG) primers. Other primers were as described: *gapdh* and *il10* (9); *xbp1* (23); *bcl6* (24); *blimp1*, *irf4*, and *pax5* (25). Cycle conditions were as follows: 1 denaturation step of 94° C for 2 minutes followed by 40 cycles of 94° C for 30 seconds, 60° C for 30 seconds, and 72° C for 1 minute. PCR products were controlled for purity by analyses of their melting curves. Expression threshold values (ΔCt) for each transcript were determined by normalizing to *gapdh* expression within each sample group.

ELISA and ELISPOT assays

Sera were collected weekly, with Ag-specific antibodies quantified by ELISA using DNP-BSA. Serum IgM and IgG levels, autoantibody levels, and TNP- or DNP-specific antibodies were quantified by ELISA as described (21, 26). ASC frequencies from cell sorter purified B10 and non-B10 cells were determined using ELISpot assays as described (27).

Ig sequences

Purified spleen B cells from three individual mice were stimulated with LPS (10 μg/ml), PMA (50 ng/ml), and ionomycin (1 μg/ml) for 5 h. IL-10-secreting cells were identified using the Mouse IL-10 Secretion Assay Kit (Miltenyi Biotech Inc., Auburn, CA). Individual $IL\text{-}10^+\lambda\text{-}CD19^+$ cells were sorted into single wells of 96-well PCR plates using a FACSAria II cell sorter (BD Biosciences). cDNA was synthesized with Ig H and L chain transcripts

amplified using nested PCR primers as described (28). PCR products were purified (QIAquick PCR Purification Kit, Qiagen, Valencia, CA) and cloned (StrataClone PCR Cloning Kit, Agilent Technologies, La Jolla, CA) before sequencing (Duke University DNA Analysis Facility). Productive Ig rearrangements were compared against germline Ig sequences according to the Ig Basic Local Alignment Search Tool (IgBLAST) database (National Center for Biotechnology Information, Bethesda, MD) and analyzed using the Immunogenetics V-query and Standardization tool (29) to determine V(D)J gene family usage. Mutation frequencies were determined using germline V, D and J sequences from IgBLAST. When light chain sequences obtained from adjacent wells were identical, only one sequence was reported. V_H-D-J_H and V_K-J_K transcript alignments and phylogenetic trees based on average percent identity were constructed using ClustalW2 (30).

Statistical analysis

Data are shown as means (±SEM). The two-tailed Student's t test was used to identify significant differences between sample means.

Results

B cell GFP IL-10 reporter expression in Tiger mice

Spleen GFP^+ or cytoplasmic IL-10^+ B cells were not observed in Tiger mice at frequencies significantly above background levels in monensin-treated wild type mice and their IL-$10^{-/-}$ littermates (Fig. 1A–B, not shown). However, GFP^+ and cytoplasmic IL-10^+ B cell frequencies increased significantly after *ex vivo* stimulation using L+PIM for 5 h. GFP^+ or IL-10^+ B cells represented between 2–3% of spleen B cells in both Tiger and wild type mice. Furthermore, 72±3% of IL-10^+ B cells from Tiger mice expressed readily measurable GFP in these assays. Likewise, the majority of GFP^+ B cells expressed IL-10 (Fig. 1C). In comparison with spleen, significantly fewer IL-10- or GFP-competent B10 cells were found within peripheral or mesenteric lymph nodes after L+PIM stimulation (Fig. 1D). Thus, GFP mimicked cytoplasmic IL-10 expression by most B10 cells during 5 h induction assays.

Agonistic CD40 signals provided during 48 h *in vitro* cultures render B10pro cells competent to express IL-10 when subsequently stimulated with L+PIM. Under these conditions, similar frequencies of cytoplasmic IL-10^+ (7.3±0.2%) and GFP^+ (6.3±0.1%) B10+B10pro cells were enumerated (Fig. 1E). By contrast, LPS induces both B10pro cell maturation and B10 cell IL-10 secretion during 48 h assays (17). Under these conditions, the frequency of GFP^+ B cells (9.3±0.1%) was consistently higher than the frequency of cytoplasmic IL-10^+ B cells (7.8±0.5%), while BCR ligation did not induce B10pro maturation into GFP-competent B cells. Thus, GFP expression was more durable than IL-10 expression following prolonged (48 h) LPS stimulation due to IL-10 secretion and/or relative differences in protein turnover.

B cell Thy1.1 IL-10 reporter expression in 10BiT mice

A small fraction of spleen $CD19^+$ B cells (0.16±0.02%) from 10BiT mice expressed cell surface $Thy1.1^+$ *ex vivo* relative to background staining in wild type mice (Fig. 2A–B). However, significantly increased $Thy1.1^+$ (0.9±0.1%, $p<0.01$) and IL-10^+ (1.8±0.4%, $p<0.05$) B10 cell frequencies were found after 5 h L+PIM stimulation. Only 30±2% of IL-10^+ B cells from 10BiT mice expressed measurable Thy1.1 in these assays, while 47±4% of the $Thy1.1^+$ B cells expressed IL-10 (Fig. 2A–C). Mesenteric lymph nodes had the highest frequencies of $Thy1.1^+$ B cells (2.1±0.2%) when observed directly *ex vivo* (not shown), as shown for T cells in mesenteric lymph nodes of 10BiT mice (20). Mesenteric lymph node $Thy1.1^+$ B10 cell frequencies were also higher following 5 h L+PIM stimulation, but the highest numbers of $Thy1.1^+$ B cells were in the spleen (Fig. 2D). To

determine whether the *il10* and *thy1.1* genes were transcribed with similar kinetics in 10BiT spleen B cells, their transcripts were measured after *in vitro* LPS stimulation. Both transcript levels rose congruently in $CD1d^{hi}CD5^{+}$ B cells and peaked at 24 h relative to $CD1d^{lo}CD5^{-}$ cells (Fig. 2E). Thus, the temporal delay in cell surface Thy1.1 expression relative to cytoplasmic IL-10 was likely due to Thy1.1 processing and cell surface transport during the 5 h assays.

CD40-induced B10pro cell maturation did not induce nascent cell surface Thy1.1 expression or change the kinetics of Thy1.1 expression induced by PIM stimulation. A normal portion of B cells cultured with CD40 mAb for 48 h expressed cytoplasmic IL-10 after L+PIM stimulation for 5 h, while Thy 1.1 expression was only modestly induced (Fig. 2F). However, a higher fraction of 10BiT B cells expressed Thy1.1 than expressed IL-10 after 48 h cultures with LPS plus 5 h PIM stimulation. Thus, cell surface Thy1.1 expression served as a more durable marker than IL-10 induction, with a large portion of the B10 cells having terminated IL-10 expression during the 48 h LPS cultures.

LPS drives B10 cell expansion in vivo

To evaluate B10 cell expansion *in vivo*, wild type mice were given complete and incomplete Freund's adjuvants, alum, or low-dose LPS, with spleen B10 cell numbers enumerated 3 days later by IL-10 staining after 5 h monensin or L+PIM treatment. Freund's adjuvants did not drive B10 cell expansion, while B10 cell numbers increased 2- to 3-fold after alum and LPS treatments (Fig. 3A). When Tiger mice were given LPS, *ex vivo* $IL\text{-}10^{+}$ or GFP^{+} B10 cell frequencies and numbers remained low, but expanded 2- to 4-fold relative to their frequencies in littermates given only PBS (monensin treatment, Fig. 3B). Following 5 h of *in vitro* L+PIM stimulation, there were 2- to 3-fold increases in $IL\text{-}10^{+}$ or GFP^{+} B10 cell frequencies and numbers relative to control mice, with most B10 cells expressing both IL-10 and GFP. Thus, GFP served as a reliable reporter for IL-10 expression in Tiger mice.

After 3 days of LPS-treatment *in vivo*, $Thy1.1^{+}$ and $IL\text{-}10^{+}$ B cell frequencies and numbers in 10BiT mice increased by 4- and 2-fold, respectively (Fig. 3C). However, the higher frequencies and numbers of $Thy1.1^{+}$ B cells relative to $IL\text{-}10^{+}$ cells demonstrated that Thy1.1 expression served as a more durable B cell marker than IL-10 expression since half of the cells had already lost the capacity to express IL-10 following *in vitro* L+PIM stimulation. Thus, ongoing and terminated IL-10 production *in vivo* was reported by B cell Thy1.1 expression in 10BiT mice.

B10 cells differentiate into ASCs following IL-10 production in vivo

After *in vivo* low-dose LPS treatment for 3 days, the phenotype of spleen $IL\text{-}10^{+}$, GFP^{+} or $Thy1.1^{+}$ B cells remained predominantly $IgM^{hi}CD1d^{hi}CD5^{+}CD19^{hi}CD23^{low}CD38^{hi}B220^{hi}$ (Fig. 3D), consistent with the *ex vivo* phenotype of B10 cells from untreated wild type mice (8, 10). However, variable frequencies of LPS-induced B10 cells also expressed the CD43 and GL7 activation markers (31), suggesting that LPS drives a subset of the reporter-positive B10 cells towards an antibody-secreting cell (ASC) phenotype.

Spleen ASCs are predominantly found within the rare $CD138^{hi}B220^{int/lo}$ B cell subset (27). However, CD138 staining is lost under the conditions used to visualize cytoplasmic $IL\text{-}10^{+}$ cells. Therefore, Tiger and 10BiT mice were used to determine whether *in vivo* LPS treatment induced B10 cells to differentiate into ASCs. In Tiger mice, GFP^{+} B cells expanded *in vivo* after LPS treatment, but predominantly remained $CD138^{low}$ (Fig. 4A). Rare GFP^{+} B cells (<2%) were found within the $CD138^{hi}B220^{int/lo}$ B cell subset in untreated Tiger mice, with LPS inducing significant numbers of GFP^{+} B cells (16%, $p<0.01$) that peaked 1 day after LPS treatment and subsequently declined (Fig. 4B). By contrast, a

significant portion of Thy1.1$^+$ B cells (17–40%) in 10BiT mice expressed CD138 after 2–3 days of LPS treatment (Fig. 4A). Before receiving LPS, 14% of CD138^{hi}B220^{lo} B cells expressed Thy1.1, with almost half of the CD138^{hi}B220^{lo} B cells expressing Thy1.1 2 days after LPS treatment (Fig. 4C). Thus, Thy1.1$^+$ B cells contributed significantly to the ASC pool following LPS treatment.

Since some pre-B cells, immature B cells and plasma cells express CD43, GL-7, and CD138 (32), an association between B10 cells and ASCs was more rigorously tested. Thy1.1$^+$ B10 cells purified from LPS-treated 10BiT mice spontaneously secreted IgM in ELISpot assays at 5.5-fold higher frequencies than Thy1.1$^-$ B cells (Fig. 4D). IgG-secreting cells were not detectable within the Thy1.1$^+$ or Thy1.1$^-$ B cell subsets under these conditions (not shown). Furthermore, Thy1.1$^+$ B cells from LPS-treated 10BiT mice expressed transcripts for the plasma cell-associated transcription factors *blimp1* (also known as *prdm1*), *xbp1* and *irf4* at 2- to 6-fold higher levels than Thy1.1$^-$ B cells (Fig. 4E). Likewise, *pax5* and *bcl6* transcripts were markedly reduced in Thy1.1$^+$ B cells relative to Thy1.1$^-$ B cells, suggesting that reporter-positive B10 cells adopt an ASC or plasma cell fate.

B10 cell Blimp-1 expression was also measured during IL-10 induction. CD1d^{hi}CD5$^+$ B cells (B10 cell-enriched) from wild type mice expressed significantly higher *il10* and *blimp1* transcript levels relative to CD1d^{lo}CD5$^-$ B cells after 5 h of L+PI stimulation (Fig. 4F). Similarly, CD1d^{hi}CD5$^+$ B cells cultured with CD40 mAb for 48 h expressed significant *il10*, *blimp1* and *irf4* transcripts relative to CD1d^{lo}CD5$^-$ B cells following 5 h of L+PI stimulation. Independently, *blimp1* transcripts were significantly increased in purified IL-10$^+$ B10 cells when compared with IL-10$^-$ B cells after 5 h of L+PI stimulation (Fig. 4G). Measurable B10 cell intracellular Blimp-1 protein expression was confirmed by immunofluorescence staining in comparison with non-B10 cells (Fig. 4H) using described methods (33). Intracellular Blimp-1 expression increased when purified B cells were cultured in the presence of LPS for 24 h, with ~2-fold higher Blimp-1 levels in IL-10$^+$ B cells than in IL-10$^-$ B cells (Fig. 4I). Thus, B10 cells expressed Blimp-1 before initiating the ASC differentiation program.

IL-10 is not required for B10 cell ASC differentiation

IL-10 induces human plasma cell differentiation *in vitro* (34–36). To determine whether autocrine IL-10 drives mouse B10 cell development or differentiation, the 10BiT transgene was bred into an IL-10$^{-/-}$ background to create 10BiT.IL-10$^{-/-}$ mice. Spleen Thy1.1$^+$ B cell frequencies were identical in both 10BiT and 10BiT.IL-10$^{-/-}$ mice after *in vitro* stimulation with agonistic CD40 mAb or LPS for 48 h (Fig. 5A). Identical frequencies of IgM ASCs were also found within the spleen Thy1.1$^+$ subsets of 10BiT and 10BiT.IL-10$^{-/-}$ mice following *in vivo* LPS treatment (Fig. 5B). ASC frequencies within the spleen CD1d^{hi}CD5$^+$ subset were also equivalent in LPS-treated IL-10$^{-/-}$ and wild type mice, with the B10 cell-enriched CD1d^{hi}CD5$^+$ B cells containing a higher frequency of ASCs when compared with CD1d^{lo}CD5$^-$ B cells. Thus, autocrine IL-10 was not required for either B10 cell development or ASC differentiation.

B10 cells differentiate into IgM and IgG ASCs

Although spleen B10 cells are predominantly cell surface IgM^{hi} (Fig. 3D), B10 cells co-expressing IgG2c, IgG3 and IgA were over-represented in the B10 cell subset relative to non-B10 cells (Fig. 5C). The relative contribution of B10 cells to the ASC pool was therefore assessed using GFP$^+$ B10 cells purified from Tiger mice. Spleen B cells were stimulated for 5 h with L+PI to induce GFP expression, sorted into GFP$^+$ and GFP$^-$ fractions, and cultured overnight with LPS prior to ELISPOT analysis. Consistent with the B10 cell ASC potential demonstrated in 10BiT mice (Fig. 4D), GFP$^+$ B10 cells were also a

major source of IgM ASCs (Fig. 5D). Thus, a large portion of B cells in both Tiger and 10BiT mice produced IL-10 prior to ASC differentiation.

To determine whether B10 cells produce Ag-specific antibody, Tiger mice were immunized with the T cell-dependent Ag 2,4,6-trinitrophenol-conjugated keyhole limpet hemocyanin (TNPKLH) in alum. Seven days later, spleen B cells were stimulated for 5 h with L+PI to induce GFP expression, with purified GFP^+ and GFP^- cells assessed for anti-TNP IgM and IgG ASC potential. GFP^+ B cells from both unimmunized and TNP-immunized Tiger mice produced TNP-reactive IgM, indicating that some reactivity was attributable to polyreactive or natural antibodies (Fig. 5E). TNP-reactive IgG was only produced by GFP^+ B cells from immunized mice. Thereby, B10 cells produced both polyreactive IgM and Ag-specific IgM and IgG.

B10 cells contribute to serum antibody levels

To determine whether B10 cells contribute to serum Ig, equal numbers of spleen GFP^+ B10 cells or GFP^- non-B10 cells were transferred from unimmunized Tiger mice into *Rag2*$^{-/-}$ hosts. Serum IgM and IgG were first detected in mice given GFP^+ cells after 1 and 4 days, respectively, and increased thereafter (Fig. 5F). In mice receiving non-B10 cells, IgM and IgG were detected after 4 and 6 days, respectively. At day 10 post-transfer, serum IgM levels from *Rag2*$^{-/-}$ mice that had received GFP^+ B10 cells were significantly higher than those of untreated *Rag2*$^{-/-}$ mice controls or *Rag2*$^{-/-}$ mice given non-B10 cells. Serum IgG levels in *Rag2*$^{-/-}$ recipients given either B10 or non-B10 cells were below the levels found in wild type mice (Fig. 5G). *Rag2*$^{-/-}$ recipients given B10 cells produced IgM but not IgG antibodies reactive with TNP, further confirming that B10 cells produce polyreactive IgM. Serum IgM from these mice also reacted with nuclear Ags, including single- and double-stranded DNA and histone proteins. IgM or IgG autoantibodies were not detected in sera from *Rag2*$^{-/-}$ mice given non-B10 cells. Thus, B10 cells contributed to the serum IgM and IgG pools, including IgM antibodies with autoreactive/polyreactive specificities.

B10 cells express diverse Ag receptors

PCR methods were used to obtain an unbiased representation of the IgH and IgL repertoires of single IL-$10^+\lambda^-$ $CD19^+$ cells from wild type mice. Both H and L chain transcripts revealed the utilization of diverse V_H and V_K family members (Fig. 6, Tables I–II). V_H1 (J558) was the most frequently observed V_H family, reflecting the predominance of this family within the Ig locus. Germline sequences without mutations encoded 84% of 50 representative V_H-D-J_H sequences and 91% of 69 representative V_K-J_K sequences. Thereby, B10 cells express diverse BCRs that were predominantly germline-encoded.

Discussion

These results demonstrate that the B10 cell subset not only regulates inflammatory immune responses through the production of IL-10, but also maintains a capacity for plasma cell differentiation. Following a transient period of IL-10 production, a significant fraction of B10 cells initiated the genetic and phenotypic program leading to ASC differentiation *in vitro* and *in vivo* (Figs. 4 and 5). B10 cells not only produced Ag-specific antibodies and represented a significant source of serum IgM and IgG (Figs. 5D–F), but also contributed polyreactive and autoreactive antibody specificities (Fig. 5G), consistent with the broad diversity of their expressed BCRs (Fig. 6). Hence, B10 cells do not define a distinct B cell lineage committed exclusively to IL-10-dependent immunoregulation. Instead, Ag-specific *in vivo* signals select B10pro cells, which develop into IL-10-competent B10 cells that secrete IL-10 in response to Ag exposure and/or TLR signaling before plasma cell differentiation (Fig. 7). Thus, B10 cells not only regulate acute inflammation and immune

responses by the transient production of IL-10, but may also have the capacity to clear their inducing Ags by producing polyreactive and/or Ag-specific antibody.

The BCR repertoire of spleen B10 cells was remarkably diverse, involving a wide spectrum of V_H, D and J_H elements, normal frequencies of noncoded nucleotide (N) insertions, as well as considerable complementarity-determining region 3 diversity (Fig. 6, Tables I–II). Regulatory B10 cell BCRs were predominantly germline-encoded with no somatic mutations in most clones. Thereby, spleen B10 cell V_H utilization was similar to that observed for conventional spleen B cells (37) and did not exhibit the skewed pattern associated with peritoneal cavity B-1a cells (38, 39). While different selective and/or developmental forces may ultimately shape the regulatory B10 cell BCR repertoire, the current findings demonstrate that IL-10 competent B cells are generated in response to diverse foreign and self Ags, including a T cell-dependent Ag. Some B10 cells also produced “natural” IgM antibody that was characteristically polyreactive (Fig. 5E and G). Consistent with their $IgM^{hi}IgD^{lo}$ phenotype (Fig. 3D) and ability to clonally expand rapidly *in vitro* (12, 17), it is likely that B10 cells contribute substantially to the short-lived plasma cell pool that develops rapidly following Ag encounter. Regulatory B10 cells also develop at normal frequencies in T cell-deficient mice (17), suggesting that many respond to T cell-independent Ags and are unlikely products of germinal center reactions. Germinal center-independent B cell isotype switching may apply to B10 cells as described (40, 41), although it remains possible that some B10 cells are recruited into germinal centers. Whether B10 cells re-enter the memory B cell pool after IL-10 production is also unknown since methods are not currently available to track B10 cells after they lose Thy1.1 expression. Regardless, B10 cell production of diverse antibody products following transient IL-10 production highlights their functional plasticity.

There were significant changes in B10 cell expression of the *blimp1*, *xbp1*, *irf4*, *pax5* and *bcl6* transcription factors following activation *in vivo*, which paralleled ASC differentiation (Fig. 4E). Upregulated B10 cell expression of *blimp1* and *irf4* (Fig. 4F–I) may be of considerable functional significance since these transcription factors cooperatively induce regulatory T cell differentiation and *il10* gene expression (42). The Blimp-1 transcriptional repressor is well known for its role in promoting plasma cell differentiation (43), with IRF4 required for *blimp1* expression (44). Blimp-1 may also exert its normal function as a transcriptional repressor and stop IL-10 expression during B10 cell differentiation into ASCs. Identifying the overlapping upregulation of *il10*, *blimp1*, and *irf4* by B10 cells highlights the potential importance of these transcription factors for regulatory B10 cell function, although other B cells also upregulate *blimp1* and *irf4* as they differentiate.

Based on their unique phenotypes and ability to proliferate rapidly following mitogenic stimulation, it is likely that mouse and human regulatory B10 cells represent subsets of Ag-experienced B cells (12, 17). Despite high IgM expression by most B10 cells (Fig. 3D), some B10 cells have undergone isotype switching (Fig. 5C). Furthermore, B10 cells do not develop in transgenic mice with fixed Ag receptors and genetic alterations that regulate BCR signaling significantly influence B10 cell numbers (17, 45–47). Since only a small subset of B cells have the capacity to produce IL-10 *in vivo* or *in vitro* (Figs. 1–2) and not all ASC expressed IL-10 before differentiation (Fig. 4A–C), specific *in vivo* signals must be required to induce IL-10 competence. This may explain why potent BCR ligation alone does not induce B10pro cells to mature into B10 cells *in vitro*, but may instead drive these cells towards different functional programs (Figs. 1E and 2F) (17). Since neither CD40 nor MyD88 expression are absolutely required for B10 cell development *in vivo* (17), it is likely that these signals and polyclonal mitogens such as LPS expand B10pro and B10 cells subsequent to Ag encounter. Consistent with this, murine cytomegalovirus infection leads to the development of IL-10-expressing $CD138^{hi}$ B cells by 7 days (48). *Salmonella* infection

also results in the rapid development of IL-10-expressing $CD138^{hi}$ B cells, which is maximal at day 1 post infection (49). Thereby, pathways that modify intrinsic BCR signals will drive IL-10 competence and B10 cell differentiation (16).

B10 cell antibody production *in vitro* and *in vivo* suggests that B10 cells contribute significantly to the serum IgM and IgG antibody pool after transient IL-10 secretion. The spleen marginal zone and B1a cell subsets also contribute significantly to antibody responses. In fact, spleen marginal zone B cells, by virtue of their preactivated state and topographical location, join B1 B cells to generate a wave of IgM producing plasmablasts during early responses to blood-borne antigens (50, 51). B10 cells also proliferate rapidly following *in vitro* or *in vivo* activation (12, 17) and rapidly convert to plasmablasts (Figs. 4–5). Since the regulatory B10 cell, B1a and marginal zone B cell subsets share overlapping cell surface markers, it is not currently possible to ascertain whether individual members of any one of these functionally- or phenotypically-defined subsets are the primary source of natural, polyreactive, autoreactive or antigen-specific antibody. Furthermore, B10pro cells cannot be identified apart from the $CD1d^{hi}CD5^{+}$ subset of B cells, so it is not possible to remove B cells that have the functional capacity to become IL-10 competent from either the $CD5^{+}$ B1a or the $CD1d^{hi}$ marginal zone subsets for functional studies. Thus, B1a, marginal zone and B10 B cells share the capacity to produce antibodies *in vivo* and contribute to early innate and subsequent adaptive immune responses.

B10 cell antibody secretion may also contribute to their immunosuppressive functions *in vivo*. Soluble antibodies can quickly reduce Ag load and promote Ag clearance by opsonization or complement-mediated phagocytosis. In addition, bound antibody can directly interfere with Ag recognition by other cell types, effectively reducing the availability of activation signals *via* Ag neutralization. Autoantibodies can also be important negative regulators of intestinal inflammation and suppress colitis (52, 53). B10 cells may thus exhibit two waves of protection that are first IL-10- and subsequently antibody-dependent. For example, B10 cell IL-10 production inhibits the initial pathology associated with experimental autoimmune encephalomyelitis induction (9, 18), while others have defined a subsequent wave of B cell-mediated immunosuppression in this model that is both Ag-specific and enhanced by CD40 signals (4, 54). Since B10 cells can produce autoantibodies (Fig. 5G), it is possible that their antibody products reduce inflammation and disease through a second wave of Ag clearance. Also, B10 cells primarily produced germline-encoded IgM antibodies that are likely to be of low affinity and non-pathogenic, which may be optimally suited to neutralize self-Ags, preempt pathogenic IgG production, and contribute to the suppression of autoimmunity (Fig. 7). Consistent with this, treatment of MRL^{lpr} mice with unmutated IgM autoantibodies confers protection against lupus nephritis (55). Further characterization of the B10 cell repertoire will be important for understanding both B10 cell development and expansion, particularly during autoimmune disease. Defining the BCR ligands and other signals important for B10 cell expansion and subsequent antibody production may also lead to new therapies for treating both inflammatory and autoimmune conditions.

Acknowledgments

We thank Drs. Eric Weimer and Garnett Kelsoe for help with the experiments, interpretation of the results and writing the manuscript.

These studies were supported by grants from the NIH, AI56363 and Southeastern Regional Center of Excellence for Emerging Infections and Biodefense (U54 AI057157).

References

1. LeBien TW, Tedder TF. B-lymphocytes: How they develop and function. Blood. 2008; 112:1570–1579. [PubMed: 18725575]
2. DiLillo DJ, Matsushita T, Tedder TF. B10 cells and regulatory B cells balance immune responses during inflammation, autoimmunity, and cancer. Ann. N. Y. Acad. Sci. 2010; 1183:38–57. [PubMed: 20146707]
3. Mizoguchi A, Bhan AK. A case for regulatory B cells. J. Immunol. 2006; 176:705–710. [PubMed: 16393950]
4. Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, Anderton SM. B cells regulate autoimmunity by provision of IL-10. Nat. Immunol. 2002; 3:944–950. [PubMed: 12244307]
5. Mauri C, Gray D, Mushtaq N, Londei M. Prevention of arthritis by interleukin 10-producing B cells. The Journal of experimental medicine. 2003; 197:489–501. [PubMed: 12591906]
6. Evans JG, Chavez-Rueda KA, Eddaoudi A, Meyer-Bahlburg A, Rawlings DJ, Ehrenstein MR, Mauri C. Novel suppressive function of transitional 2 B cells in experimental arthritis. J. Immunol. 2007; 178:7868–7878. [PubMed: 17548625]
7. Bouaziz J-D, Yanaba K, Tedder TF. Regulatory B cells as inhibitors of immune responses and inflammation. Immunological reviews. 2008; 224:201–214. [PubMed: 18759928]
8. Yanaba K, Bouaziz J-D, Haas KM, Poe JC, Fujimoto M, Tedder TF. A regulatory B cell subset with a unique $CD1d^{hi}CD5^{+}$ phenotype controls T cell-dependent inflammatory responses. Immunity. 2008; 28:639–650. [PubMed: 18482568]
9. Matsushita T, Yanaba K, Bouaziz J-D, Fujimoto M, Tedder TF. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. J. Clin. Invest. 2008; 118:3420–3430. [PubMed: 18802481]
10. Haas KM, Watanabe R, Matsushita T, Nakashima H, Ishiura N, Okochi H, Fujimoto M, Tedder TF. Protective and pathogenic roles for B cells during systemic autoimmunity in NZB/W F_1 mice. J. Immunol. 2010; 184:4789–4800. [PubMed: 20368280]
11. Watanabe R, Ishiura N, Nakashima H, Kuwano Y, Okochi H, Tamaki K, Sato S, Tedder TF, Fujimoto M. Regulatory B cells (B10 cells) have a suppressive role in murine lupus: CD19 and B10 cell deficiency exacerbates systemic autoimmunity. J. Immunol. 2010; 184:4801–4809. [PubMed: 20368271]
12. Iwata Y, Matsushita T, Horikawa M, DiLillo DJ, Yanaba K, Venturi GM, Szabolcs PM, Bernstein SH, Magro CM, Williams AD, Hall RP, St.Clair EW, Tedder TF. Characterization of a rare IL-10-competent B cell subset in humans that parallels mouse regulatory B10 cells. Blood. 2011; 117:530–541. [PubMed: 20962324]
13. Horikawa M, Minard-Colin V, Matsushita T, Tedder TF. Regulatory B lymphocyte production of IL-10 inhibits lymphoma depletion during CD20 immunotherapy in mice. J. Clin. Invest. 2011 in press.
14. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. Nat. Rev. Immunol. 2010; 10:170–181. [PubMed: 20154735]
15. Sugimoto K, Ogawa A, Shimomura Y, Nagahama K, Mizoguchi A, Bhan AK. Inducible IL-12-producing B cells regulate Th2-mediated intestinal inflammation. Gastroenterology. 2007; 133:124–136. [PubMed: 17631137]
16. Poe JC, Smith S-H, Haas KM, Yanaba K, Tsubata T, Matsushita T, Tedder TF. Amplified B lymphocyte CD40 signaling drives regulatory B10 cell expansion in mice. PLoS ONE. 2011; 6:e22464. [PubMed: 21799861]
17. Yanaba K, Bouaziz J-D, Matsushita T, Tsubata T, Tedder TF. The development and function of regulatory B cells expressing IL-10 (B10 cells) requires antigen receptor diversity and TLR signals. J. Immunol. 2009; 182:7459–7472. [PubMed: 19494269]
18. Matsushita T, Horikawa M, Iwata Y, Tedder TF. Regulatory B cells (B10 cells) and regulatory T cells have independent roles in controlling EAE initiation and late-phase immunopathogenesis. J. Immunol. 2010; 185:2240–2252. [PubMed: 20624940]

19. Kamanaka M, Kim ST, Wan YY, Sutterwala FS, Lara-Tejero M, Galan JE, Harhaj E, Flavell RA. Expression of interleukin-10 in intestinal lymphocytes detected by an interleukin-10 reporter knockin tiger mouse. Immunity. 2006; 25:941–952. [PubMed: 17137799]
20. Maynard CL, Harrington LE, Janowski KM, Oliver JR, Zindl CL, Rudensky AY, Weaver CT. Regulatory T cells expressing interleukin 10 develop from Foxp3^{+} and Foxp3^{-} precursor cells in the absence of interleukin 10. Nat. Immunol. 2007; 8:931–941. [PubMed: 17694059]
21. Sato S, Miller AS, Inaoki M, Bock CB, Jansen PJ, Tang MLK, Tedder TF. CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: altered signaling in CD22-deficient mice. Immunity. 1996; 5:551–562. [PubMed: 8986715]
22. Matsushita T, Tedder TF. Identifying regulatory B cells (B10 cells) that produce IL-10. Methods Mol. Biol. 2010; 677:99–111. [PubMed: 20941605]
23. Sha H, He Y, Chen H, Wang C, Zenno A, Shi H, Yang X, Zhang X, Qi L. The IRE1alpha-XBP1 pathway of the unfolded protein response is required for adipogenesis. Cell metabolism. 2009; 9:556–564. [PubMed: 19490910]
24. Saito M, Novak U, Piovan E, Basso K, Sumazin P, Schneider C, Crespo M, Shen Q, Bhagat G, Califano A, Chadburn A, Pasqualucci L, Dalla-Favera R. BCL6 suppression of BCL2 via Miz1 and its disruption in diffuse large B cell lymphoma. Proceedings of the National Academy of Sciences of the United States of America. 2009; 106:11294–11299. [PubMed: 19549844]
25. Todd DJ, McHeyzer-Williams LJ, Kowal C, Lee AH, Volpe BT, Diamond B, McHeyzer-Williams MG, Glimcher LH. XBP1 governs late events in plasma cell differentiation and is not required for antigen-specific memory B cell development. The Journal of experimental medicine. 2009; 206:2151–2159. [PubMed: 19752183]
26. Engel P, Zhou L-J, Ord DC, Sato S, Koller B, Tedder TF. Abnormal B lymphocyte development, activation and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. Immunity. 1995; 3:39–50. [PubMed: 7542548]
27. DiLillo DJ, Hamaguchi Y, Ueda Y, Yang K, Uchida J, Haas KM, Kelsoe G, Tedder TF. Maintenance of long-lived plasma cells and serological memory despite mature and memory B cell depletion during CD20 immunotherapy in mice. J. Immunol. 2008; 180:361–371. [PubMed: 18097037]
28. Rohatgi S, Ganju P, Sehgal D. Systematic design and testing of nested (RT-)PCR primers for specific amplification of mouse rearranged/expressed immunoglobulin variable region genes from small number of B cells. Journal of immunological methods. 2008; 339:205–219. [PubMed: 18926828]
29. Brochet X, Lefranc MP, Giudicelli V. IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. Nucleic Acids Res. 2008; 36:W503–508. [PubMed: 18503082]
30. Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD. Multiple sequence alignment with the Clustal series of programs. Nucleic acids research. 2003; 31:3497–3500. [PubMed: 12824352]
31. Laszlo G, Hathcock KS, Dickler HB, Hodes RJ. Characterization of a novel cell-surface molecule expressed on subpopulations of activated T and B cells. J. Immunol. 1993; 150:5252–5262. [PubMed: 8515058]
32. Sanderson RD, Lalor P, Bernfield M. B lymphocytes express and lose syndecan at specific stages of differentiation. Cell Reg. 1989; 1:27–35.
33. Yang Y, Tung JW, Ghosn EE, Herzenberg LA, Herzenberg LA. Division and differentiation of natural antibody-producing cells in mouse spleen. Proc. Natl. Acad. Sci., U. S. A. 2007; 104:4542–4546. [PubMed: 17360560]
34. Rousset F, Garcia E, Defrance T, Peronne C, Vezzio N, Hsu DH, Kastelein R, Moore KW, Banchereau J. Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. Proc. Natl. Acad. Sci. USA. 1992; 89:1890–1893. [PubMed: 1371884]
35. Briere F, Servet-Delprat C, Bridon JM, Saint-Remy JM, Banchereau J. Human interleukin 10 induces naive surface immunoglobulin D^{+} (sIgD^{+}) B cells to secrete IgG1 and IgG3. The Journal of experimental medicine. 1994; 179:757–762. [PubMed: 8294883]

36. Itoh K, Hirohata S. The role of IL-10 in human B cell activation, proliferation, and differentiation. J. Immunol. 1995; 154:4341–4350. [PubMed: 7722292]
37. Kantor AB, Merrill CE, Herzenberg LA, Hillson JL. An unbiased analysis of VH-D-JH sequences from B1-a, B1-b, and conventional B cells. J. Immunol. 1996; 158:1175–1186. [PubMed: 9013957]
38. Hardy RR, Carmack CE, Shinton SA, Riblet RJ, Hayakawa K. A single V_H gene is utilized predominantly in anti-BrMRBC hybridomas derived from purified Ly-1 B cells. Definition of the V_H11 family. J. Immunol. 1989; 142:3643–3651. [PubMed: 2497178]
39. Seidl KJ, MacKenzie JD, Wang D, Kantor AB, Kabat EA, Herzenberg LA, Herzenberg LA. Frequent occurrence of identical heavy and light chain Ig rearrangements. Int. Immunol. 1997; 9:689–702. [PubMed: 9184914]
40. Snapper CM, McIntyre TM, Mandler R, Pecanha LM, Finkelman FD, Lees A, Mond JJ. Induction of IgG3 secretion by interferon γ: a model for T cell-independent class switching in response to T cell-independent type 2 antigens. The Journal of experimental medicine. 1992; 175:1367–1371. [PubMed: 1373759]
41. Bergqvist P, Gardby E, Stensson A, Bemark M, Lycke NY. Gut IgA class switch recombination in the absence of CD40 does not occur in the lamina propria and is independent of germinal centers. J. Immunol. 2006; 177:7772–7783. [PubMed: 17114448]
42. Cretney E, Xin A, Shi W, Minnich M, Masson F, Miasari M, Belz GT, Smyth GK, Busslinger M, Nutt SL, Kallies A. The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. Nat. Immunol. 2011; 12:304–311. [PubMed: 21378976]
43. Martins G, Calame K. Regulation and functions of Blimp-1 in T and B lymphocytes. Annu. Rev. Immunol. 2008; 26:133–169. [PubMed: 18370921]
44. Sciammas R, Shaffer AL, Schatz JH, Zhao H, Staudt LM, Singh H. Graded expression of interferon regulatory factor-4 coordinates isotype switching with plasma cell differentiation. Immunity. 2006; 25:225–236. [PubMed: 16919487]
45. Scapini P, Lamagna C, Hu Y, Lee K, Tang Q, DeFranco AL, Lowell CA. B cell-derived IL-10 suppresses inflammatory disease in Lyn-deficient mice. Proceedings of the National Academy of Sciences of the United States of America. 2011 in press.
46. Ding Q, Yeung M, Camirand G, Zeng Q, Akiba H, Yagita H, Chalasani G, Sayegh MH, Najafian N, Rothstein DM. Regulatory B cells are identified by expression of TIM-1 and can be induced through TIM-1 ligation to promote tolerance in mice. J. Clin. Invest. 2011; 121:3645–3656. [PubMed: 21821911]
47. Matsumoto M, Fujii Y, Baba A, Hikida M, Kurosaki T, Baba Y. The calcium sensors STIM1 and STIM2 control B cell regulatory function through interleukin-10 production. Immunity. 2011; 34:703–714. [PubMed: 21530328]
48. Madan R, Demircik F, Surianarayanan S, Allen JL, Divanovic S, Trompette A, Yogev N, Gu Y, Khodoun M, Hildeman D, Boespflug N, Fogolin MB, Grobe L, Greweling M, Finkelman FD, Cardin R, Mohrs M, Muller W, Waisman A, Roers A, Karp CL. Nonredundant roles for B cell-derived IL-10 in immune counter-regulation. J. Immunol. 2009; 183:2312–2320. [PubMed: 19620304]
49. Neves P, Lampropoulou V, Calderon-Gomez E, Roch T, Stervbo U, Shen P, Kuhl AA, Loddenkemper C, Haury M, Nedospasov SA, Kaufmann SH, Steinhoff U, Calado DP, Fillatreau S. Signaling via the MyD88 adaptor protein in B cells suppresses protective immunity during *Salmonella typhimurium* infection. Immunity. 2010; 33:777–790. [PubMed: 21093317]
50. Oliver AM, Martin F, Kearney JF. $IgM^{high}CD21^{high}$ lymphocytes enriched in the splenic marginal zone generate effector cells more rapidly than the bulk of follicular B cells. J. Immunol. 1999; 162:7198–7207. [PubMed: 10358166]
51. Martin F, Oliver AM, Kearney JF. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. Immunity. 2001; 14:617–629. [PubMed: 11371363]

52. Mizoguchi A, Mizoguchi E, Smith RN, Preffer FI, Bhan AK. Suppressive role of B cells in chronic colitis of T cell receptor α mutant mice. The Journal of experimental medicine. 1997; 186:1749–1756. [PubMed: 9362534]

53. Genestier L, Taillardet M, Mondiere P, Gheit H, Bella C, Defrance T. TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses. J. Immunol. 2007; 178:7779–7786. [PubMed: 17548615]

54. Lampropoulou V, Calderon-Gomez E, Roch T, Neves P, Shen P, Stervbo U, Boudinot P, Anderton SM, Fillatreau S. Suppressive functions of activated B cells in autoimmune diseases reveal the dual roles of Toll-like receptors in immunity. Immunological reviews. 2010; 233:146–161. [PubMed: 20192998]

55. Jiang C, Zhao ML, Scearce RM, Diaz M. Activation-induced deaminase-deficient MRL/lpr mice secrete high levels of protective antibodies against lupus nephritis. Arthritis Rheum. 2011; 63:1086–1096. [PubMed: 21225690]

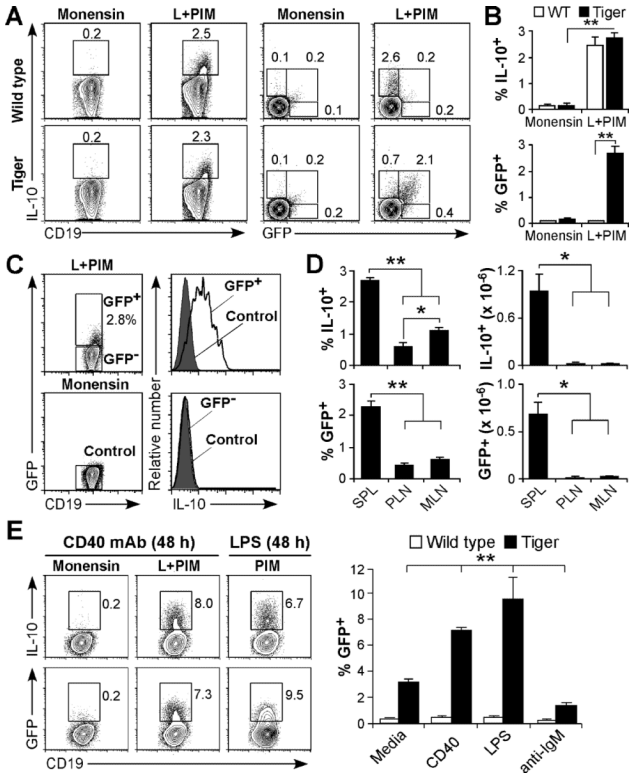

Figure 1. B cell GFP expression in Tiger mice parallels cytoplasmic IL-10 expression

(A) B cell IL-10 production relative to GFP expression in Tiger mice. Splenocytes were cultured for 5 h with L+PIM before cell surface CD19 and cytoplasmic IL-10 immunofluorescence staining with flow cytometry analysis. Cells cultured with monensin alone served as negative controls for IL-10 staining, with results similar to isotype control mAb staining (not shown). Representative contour plots show the IL-10$^+$, IL-10$^+$GFP$^+$ and GFP$^+$ cell frequencies within the indicated gates for CD19$^+$ B cells (n=5 mice). **(B)** Mean IL-10$^+$ and GFP$^+$ B cell frequencies (±SEM) in wild type and Tiger mice (n=5 mice/group) as identified in (A). **(C)** Representative IL-10 expression by GFP$^+$ B cells in Tiger mice. GFP$^+$ and GFP$^-$ CD19$^+$ B cells were assessed for IL-10 expression (thick lines) relative to control mAb staining (shaded histograms) after 5 h L+PIM stimulation (n=5 mice) as in (A). **(D)** Mean frequencies and numbers of IL-10$^+$ and GFP$^+$ B cells in tissues of Tiger mice among CD19$^+$ B cells from spleen (SPL), peripheral lymph nodes (PLN, inguinal), or mesenteric lymph node (MLN) (≥3 mice) as in (A). **(E)** GFP expression by B10+B10pro cells from Tiger mice. Spleen CD19$^+$ cells were cultured for 48 h in media alone or with agonistic CD40 mAb, LPS or anti-IgM antibody. Monensin, L+PIM or PIM were added during the final 5 h of culture, with IL-10$^+$ or GFP$^+$ B cells identified as in (A). Cultured spleen B cells from wild type mice served as background controls for GFP expression. Bar graphs show mean frequencies of GFP$^+$ B cells after culture (n≥3 mice/group). (B, D, E) Significant differences between cultures with media alone or between values are indicated: *p<0.05, **p≤0.01. All experiments were performed ≥3 times.

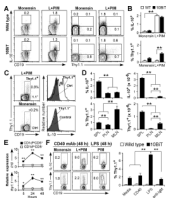

Figure 2. Divergent IL-10 and Thy1.1 expression by 10BiT B cells

(A) B cell IL-10 production relative to cell surface Thy1.1 expression in 10BiT mice. Splenocytes were stimulated for 5 h before IL-10 and CD19 staining as in figure 1A. Representative contour plots show the IL-10$^+$, IL-10$^+$Thy1.1$^+$ and Thy1.1$^+$ cell frequencies within the indicated gates for CD19$^+$ B cells. **(B)** Mean IL-10$^+$ and Thy1.1$^+$ B cell frequencies in wild type and 10BiT mice (n=5 mice/group) as in (A). **(C)** Representative IL-10 expression by Thy1.1$^+$ B cells in 10BiT mice. Thy1.1$^+$ and Thy1.1$^-$ CD19$^+$ B cells were assessed for IL-10 expression (thick lines) relative to control mAb staining (shaded histograms) after 5 h cultures with L+PIM (n=5 mice) as in (A). **(D)** Mean frequencies and numbers of tissue IL-10$^+$ or Thy1.1$^+$ B cells in spleen (SPL), lymph nodes (PLN), or mesenteric lymph node (MLN) of 10BiT mice (n≥3 mice) as in (A). **(E)** Relative *il10* and *thy1.1* transcript expression by B cells from 10BiT mice. Purified CD1d^{hi}CD5$^+$ (black boxes) and CD1d^{lo}CD5$^-$ (empty boxes) CD19$^+$ B cells were cultured alone or with LPS for 5, 24 and 48 h prior to RNA isolation and reverse transcriptase quantitative real-time PCR analysis. Values were normalized to the CD1d^{lo}CD5$^-$ population at each time point, with relative values shown as mean frequencies from 3 experiments. **(F)** Thy1.1 expression by B10+B10pro cells from 10BiT mice. Contour plots and bar graphs (representative of two experiments) show mean frequencies of Thy1.1$^+$ spleen CD19$^+$ B cells from wild type (background controls) and 10BiT mice (n≥3 mice/group) after 48 h cultures with the indicated stimuli as in figure 1E. (B, D, F) Significant differences between cultures with media alone or between the indicated values are indicated: **p≤0.01. Unless indicated, all experiments were performed ≥3 times.

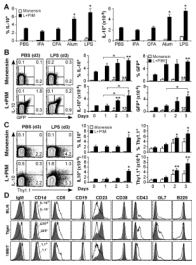

Figure 3. B10 cells expand after *in vivo* LPS treatment

(A) Alum and LPS drive B10 cell expansion *in vivo*. Spleen B10 cell numbers were quantified as in figure 1, three days after PBS, CFA, IFA, alum or LPS treatment. Values represent mean frequencies or numbers of IL-10$^+$ CD19$^+$ B cells from one of two experiments with similar results (n≥3 mice/group/experiment). **(B)** LPS drives GFP$^+$ B10 cell expansion in Tiger mice. Representative contour plots show IL-10 and GFP expression by spleen CD19$^+$ B cells 3 days after PBS or LPS treatment. B cells were cultured with monensin alone or L+PIM for 5 h before IL-10 and GFP analysis as in figure 1A. Bar graphs show mean frequencies or numbers of IL-10$^+$ or GFP$^+$ B cells from PBS- (d 3) or LPS-treated (days 1–3) mice (≥3 mice/group). **(C)** LPS treatment drives Thy1.1$^+$ B10 cell expansion in 10BiT mice. Representative contour plots and bar graphs indicate frequencies and total numbers of IL-10$^+$ or Thy1.1$^+$ B cells from 10BiT mice (3–4 mice per group) as assessed in (B). (A–C) Means significantly different from PBS-treated control mice are indicated: *p≤0.05, **p≤0.01. Data presented in Fig. 3B–C were pooled from 3 independent experiments. **(D)** *Ex vivo* cell surface phenotype of B cells from wild type, Tiger or 10BiT mice. Spleen B cells were isolated 3 days after LPS treatment, with subsequent L+PIM stimulation for 5 h before cell surface staining. Open histograms (thick lines) represent the IL-10$^+$, GFP$^+$ or Thy1.1$^+$ B cell subsets, while shaded histograms represent IL-10$^-$, GFP$^-$ or Thy1.1$^-$ B cells, as indicated. Similar results were obtained in 2 experiments.

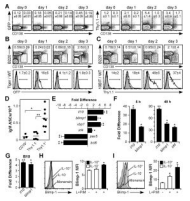

Figure 4. B10 cells differentiate into ASC *in vivo*

(A) Representative spleen GFP$^+$ or Thy1.1$^+$ cell frequencies versus CD138 expression among B220$^{hi/int}$ B cells in Tiger (left) and 10BiT (right) mice before (day 0) or 1–3 days following LPS treatment. Numbers within quadrants indicate means (n=3–5 mice). **(B)** Spleen CD138^{hi}B220$^{int/lo}$ B cells in Tiger mice express GFP after LPS treatment *in vivo*. Representative contour plots show CD138^{hi}B220^{lo} B cell frequencies in Tiger mice before (day 0) or 1–3 days following LPS treatment. Representative histograms indicate GFP expression by CD138^{hi}B220$^{int/lo}$ B cells at the same time points (heavy lines, lower panels) relative to CD138^{hi}B220$^{int/lo}$ B cells from wild type mice as negative controls (shaded lines). Mean CD138^{hi}B220$^{int/lo}$ B cell frequencies or percentages of reporter-positive cells within the indicated gates are shown with backgrounds subtracted (n=3–5 mice). **(C)** CD138^{hi}B220$^{int/lo}$ B cells in 10BiT mice express Thy1.1 before and after LPS treatment *in vivo*. Representative contour plots and histograms are shown as in (B). **(D)** Thy1.1$^+$ B10 cells secrete IgM *in vitro*. Purified spleen B cells from 10BiT mice given LPS 3 days earlier were sorted into Thy1.1$^+$ or Thy1.1$^-$ CD19$^+$ cell fractions and cultured on ELISpot plates overnight to enumerate IgM-secreting cells from 3–8 individual mice. (A–D) Data are pooled from 3 independent experiments. **(E)** Thy1.1$^+$ B10 cells express transcription factors associated with plasma cell differentiation. Spleen Thy1.1$^+$ or Thy1.1$^-$ CD19$^+$ B cells were purified from 10BiT mice given LPS 3 days earlier, with relative transcription factor expression measured by reverse transcriptase quantitative real-time PCR. Bars indicate mean fold differences between Thy1.1$^+$ B cells normalized to Thy1.1$^-$ B cells from 3 experiments (n=5 mice/experiment). **(F)** B10 cells from wild type mice express *blimp1* and *irf4*. Purified spleen CD1d^{hi}CD5$^+$ and CD1d^{lo}CD5$^-$ B cells were stimulated with L+PI for 5 h (B10 cells) or were cultured with CD40 mAb for 48 h with L+PI added during the final 5 h (B10+B10pro cells). Values indicate mean fold differences between CD1d^{hi}CD5$^+$ and CD1d^{lo}CD5$^-$ B cells (n=3 mice). **(G)** IL-10$^+$ B10 cells from wild type mice express *blimp1*. B cells were stimulated with L+PI for 5 h before IL-10$^+$ and IL-10$^-$ CD19$^+$ B cells were purified. Values indicate mean fold differences between IL-10$^+$ and IL-10$^-$ B cells (n=3 mice). (F–G) *il10*, *irf4* and *blimp1* transcripts were quantified as in (E). **(H)** Intracellular Blimp-1 expression by spleen IL-10$^+$, IL-10$^-$ or monensin only-treated B cells following 5 h of L+PIM stimulation. **(I)** Intracellular Blimp-1 levels in IL-10$^+$, IL-10$^-$ or monensin only treated cells following 24 h LPS stimulation with PIM added during the final 5 h. (H–I) Mean MFI values for the indicated populations are shown (n=3 mice). (D–I) Significant differences between means are indicated: *p≤0.05, **p≤0.01.

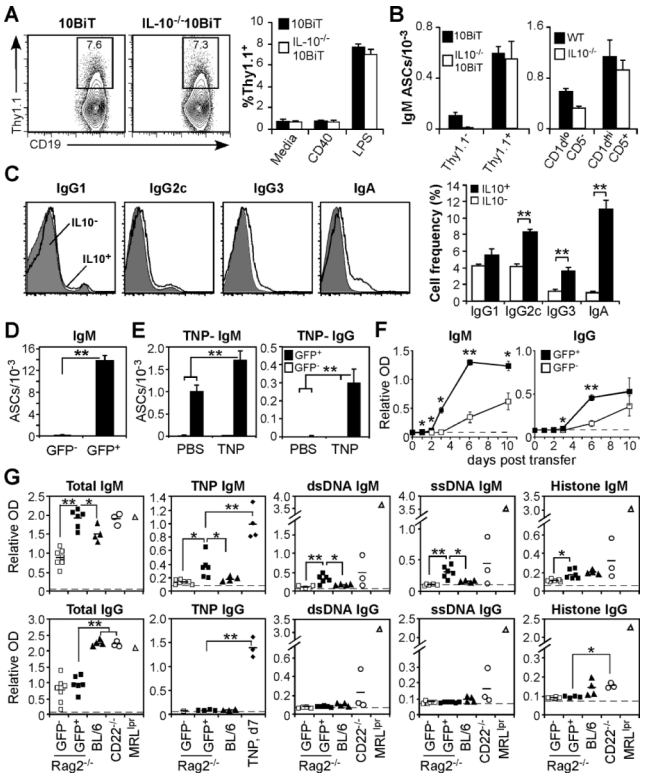

Figure 5. B10 cells produce Ag-specific antibody and autoantibodies

(A) IL-10 is not required for B10+B10pro cell development in 10BiT mice. Splenocytes from 10BiT or IL-10$^{-/-}$10BiT mice were cultured for 48 h with media alone, CD40 mAb, or LPS, with the frequency of Thy1.1^{+} B10+B10pro cells determined as in figure 1E. Representative contour plots show CD19^{+} B cells from LPS-stimulated cultures. Bar graphs indicate relative mean frequencies of Thy1.1^{+} cells among CD19^{+} B cells (n=3 mice/group). **(B)** IL-10 expression is not required for B10 cell differentiation into ASCs. 10BiT or wild type mice (open bars) and IL-10$^{-/-}$10BiT or IL-10$^{-/-}$ mice (filled bars) were given LPS 3 days before relative ASC frequencies were determined among Thy1.1^{+} or Thy1.1^{-} subsets from 10BiT mice and CD1d^{hi}CD5^{+} or CD1d^{lo}CD5^{-} subsets from wild type mice as in fig. 4D (n=3 mice/group, data represent 2 experiments). **(C)** B10 cell expression of cell surface IgG and IgA. Spleen B cells from wild type mice were stimulated with L+PIM for 5 h before staining for IL-10 and cell surface IgG and IgA. Bar graphs show mean frequencies of B cells expressing each isotype (n=8 mice/group) from 2 experiments. **(D)** B10 cells from Tiger mice can secrete IgM. Purified spleen CD19^{+} B cells from Tiger mice were stimulated for 5 h with L+PI before GFP^{+} and GFP^{-} B cells were isolated by cell sorting. After 18 h of culture with LPS, the cells were cultured on ELISpot plates for 5 h. Bar graphs show mean IgM ASC frequencies (n=3 mice/group). **(E)** B10 cells can secrete Ag-specific IgM and IgG. Tiger mice were immunized with TNP-KLH plus alum, or PBS plus alum. Spleen TNP-specific IgM and IgG ASCs were quantified 7 days later using ELISpot assays as in (B). Bar graphs indicate mean ASC frequencies from 2 PBS- and 3 TNP-immunized mice in 2 experiments. **(F)** B10 cells contribute to serum antibody titers *in vivo*. In 2 experiments, purified spleen B cells from 4 or 8 Tiger mice were pooled and cultured overnight (18 h) with LPS, followed by 5 h stimulation with L+PI to induce GFP expression. Cell sorter purified GFP^{+} (closed squares) and GFP^{-} (open squares) B cells were then transferred into 5 and 6 Rag2$^{-/-}$ recipients, respectively. Serum was collected at the indicated times, with antibody levels quantified by ELISA. Background IgM and IgG levels were determined using serum from untreated Rag2$^{-/-}$ mice (dashed lines). **(G)** Reactivity of antibodies

produced by B10 cells. Serum from Rag2$^{-/-}$ mice given GFP^{+} (closed squares) or GFP^{-} (open squares) B cells 10 days earlier (as in D) was analyzed for reactivity with the indicated Ags by ELISA. Positive and negative controls included pooled sera from two-month-old wild type mice before (closed triangles) and 7 days after (diamonds) TNP-KLH-immunization, 10-month-old CD22$^{-/-}$ mice (open circles), and a 6-month-old female MRL^{lpr} mouse (open triangles). Values indicate results from individual mice. (A–G) Means significantly different between groups are indicated: *p≤0.05, **p≤0.01.

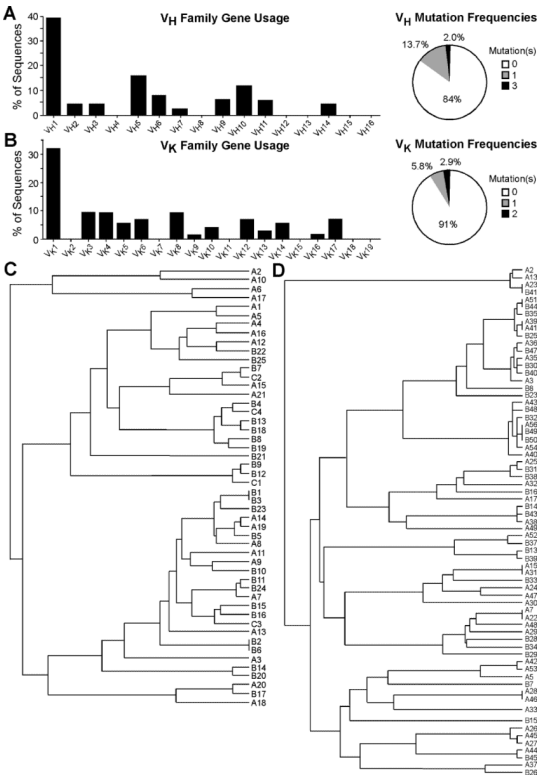

Figure 6. B10 cells utilize diverse V genes that are largely unmutated

(A) V_H family gene usage by 50 representative IL-10$^+$ B cells from 3 individual mice. Mutation frequencies within the V_H-D-J_H gene sequences are shown on the right. **(B)** V_K gene family usage by 69 representative IL-10$^+$ B cells. V_K-J_K mutation frequencies are shown on the right. **(C)** Phylogenetic tree showing relationships between the V_H-D-J_H amino acid sequences of individual B cells from mice named A–C with numbers indicating different B cells. Branches indicate the average distance between two sequences based on percent identity. **(D)** Phylogenetic tree showing the relationship between the V_K-J_K amino acid sequences of individual B cells.

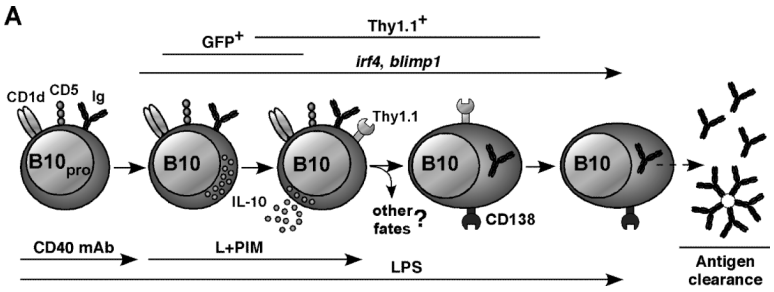

Figure 7. B10 cells regulate antibody production *in vivo*

Model for B10 cell maturation and antibody production. Transient B10 cell IL-10 production parallels GFP expression in IL-10 reporter mice, while cell surface Thy1.1 expression is observed later and accumulates over time. Although other B10 cell fates are possible, some spleen B10 cells differentiate into ASC cells that predominantly produce IgM. Antibody production by B10-derived B cells may constitute a second wave of humoral regulation during immune responses.

Table I

B10 cell V_H-D_H-J_H sequences.

Cell	V Gene	V_H	D	J_H	V End	P	N	P	D	P	N	P	J End	CDR3 Translation	Mutation Locations
A1	7183.20.37	5	DFL16.1	JH2	*TGT* GCAAGG				TATTACTACGGTAGTAGCTAC		A		TTGACTAC	ARYYYGSSYIDY	
A2	36–60.6.70	3	DSP2.x	JH2	*TGT* GCAAGAGA		TTCCC		ATAGTAACTAC	CCTTT			CTAC	ARDSHSNYPFY	
A3	J558.39.129	1	DSP2.9	JH1	*TGT* GCAA(GA)				TGGTTAC		CGGT		CTACTGGTACTTCGATGTC	ARWLPVYWYFDV	
A4	7183.a7.10	5	DST4.3	JH4	*TGT* GCAAGAC		CGGACGTGACGA		GG(GCTA)				TGGACTAC	ARPDVTRAMDY	
A5	7183.20.37	5	DST4.3	JH2	*TGT* GCAAGG	GGAA	G	G	CAGCTCAGGCTAC		C		CTTTGACTAC	ARGRQLRLPFDY	
A6	Q52.8.22	2	DSP2.2	JH3	*TGT* GCCAGACA		AGGG		GATTACGAC	T	CTC		CCTGGTTTGCTTAC	ARQGDYDSPWFAY	
A7	J558.50.143	1	DFL16.1	JH3	*TGT* GCAAGA	G			ACTACGGTAGTAGCTAC		GATACTTC		C	ARDYGSSYDTS	
A8	J558.53.146	1	DSP2.2	JH4	*TGT* GCAAGA		ACCCT		CTATGATTACG		GCC		CTATGGACTAC	ARTLYDYGPMDY	
A9	J558.55.149	1	DSP2.x	JH4	*TGT* GCA				(A)GTAA		AG		GCTATGGACTAC	AVKAMDY	
A10	36–60.8.74	3	DFL16.1	JH1	*TGT* GCAAGA		AATTTT	G	ATTACTACGGTAGTAG	TC	CT		TACTGGTACTTCGATGTC	ARNFDYYGSSPYWYFDV	
A11	J558.67.166	1	DSP2.2	JH4	*TGT* GCAAGA	T	ATGT		CTATGATTACGAC	G	GACG		TGCTATGGACTAC	ARYVYDYDGRAMDY	T40C, FWR1
A12	7183.4.6	5	DSP2.2	JH2	*TGT* GCAAGA		GATGAGG		GATTACGAC		CT		TTTGACTAC	ARDEGLRPFDY	*A78G*, FWR1; G89A, CDR1
A13	J558.75.177	1	DSP2.2	JH3	*TGT* GCAAGA		G		ATGATTACG		GTC	G	CTGGTTTGCTTAC	ARDDYGRWFAY	G102A, C103A, A104C, FWR2
A14	J558.53.146	1	DSP2.8	JH3	*TGT* GCAAGA		GGG		GGTAACTAC	GTA	GT	A	TGGTTTGCTTAC	ARGGNYVWFAY	C40A, FWR1
A15	J606.1.79	6	DSP2.7	JH3	*TGC* ACAG		AAAGGAC		CTATGGTAAC		C		CCTGGTTTGCTTAC	TERTYGNPWFAY	T263A, FWR3
A16	7183.7.10	5	DSP2.9	JH1	*TGT* GCAAGA		CAGGCG		TCTATGATGGTTA		AGA		GGTACTTCGATGTC	ARQASMMVKRYFDV	
A17	Q52.3.8	2	DSP2.9	JH3	*TGT* GCCAAACC	G			GATGGTTACTA(C)				TGGTTTGCTTAC	AKPDGYYWFAY	
A18	VGAM3.8-1-57	9	DQ52	JH2	*TGT* GTAAG		GAG	G	CTAACTGGGA				ACT AC	VRRLTGNY	
A19	J558.53.146	1	none	JH3	*TGT* GCAAGA		GA		none				TGCTTAC	ARDAY	*C58T*, FWR1
A20	VGAM3.8-3-61	9	DSP2.13	JH2	*TGC* GC(A)				CTAC		AGGGGCT		CTTTGACTAC	ALQGLFDY	*T288C*, FWR3
A21	J606.4.82	6	DFL16.1	JH2	*TGT* ACC(A)				CTACGGTAGTAGCT		GGGGAAGAC		TACTTTGACTAC	TTTVVAGEDYFDY	
B1	J558.53.146	1	DSP2.9	JH2	*TGT* GCAAGA		GGG		GATGGTTACTAC		CCCCTCTAC		TACTTTGACTAC	ARGDGYYPLYYFDY	
B2	J558.80.186	1	DST4	JH2	*TGT* GCAAGA		GACGAC		CAGGC				CTTTGACTAC	ARDDQAFDY	
B3	J558.53.146	1	DSP2.9	JH2	*TGT* GCAAGA		GGG		GATGGTTACTAC		CCCCTCTAC		TACTTTGACTAC	ARGDGYYPLYYFDY	
B4	VH10.3.91	10	DSP2.6	JH4	*TGT* GTGAGAGA	T	GATAGG		GGTTACGAC	G	GTGGA	T	ATTACTAT	VRDDRGYDGGYYY	
B5	J558.53.146	1	DFL16.1	JH3	*TGT* GCAAGA		GGGG		ACTACGGTAGTAG	TC	TC		TTTGCTTAC	ARGDYGSSLFAY	
B6	J558.80.186	1	DST4	JH2	*TGT* GCAAGA		GACGAC		CAGGC				CTTTGACTAC	ARDDQAFDY	

Cell	V Gene	V_H	D	J_H	V End	P	N	P	D	P	N	P	J End	CDR3 Translation	Mutation Locations
B7	J606.1.79	6	DFL16.2	JH4	*TGC* ACAGG		A	A	ATTACT		C		C	TGNYS	
B8	VH10.1.86	10	DSP2.8	JH2	*TGT* GT			C	GGTAA	T	G		TTGACTAC	VGNVDY	
B9	VH11.2.53	11	DSP2.x	JH1	*TGT* ATGAGA(TA)				TAGTAA(CTAC)				TGGTACTTCGATGTC	MRYSNYWYFDV	
B10	J558.55.149	1	DQ52	JH2	*TGT* GCAAGA		GGGGG		TAACTGGG		TCCT		CTTTGACTAC	ARGGNWVLFDY	
B11	J558.50.143	1	DFL16.1	JH3	*TGT* GCAAGA		G		ACTACGGTAGTAGCTAC	G	ATACTTC		C	ARDYGSSYDTS	
B12	VH11.2.53	11	DSP2.x	JH1	*TGT* ATGAGA(TA)				TAGTAA(CTAC)				TGGTACTTCGATGTC	MRYSNYWYFDV	
B13	VH10.3.91	10	DSP2.9	JH2	*TGT* GTGAGAG		GGG		TCTATGATGGTTACTAC		C		TTGACTAC	VRGVYDGYYLDY	
B14	SM7.3.54	14	DSP2.8	JH2	*TGT* G(CTAG)				G		GGGG		TTGACTAC	ARGVDY	
B15	J558.77.180	1	DSP2.6	JH2	*TGT* GCAATA		G		AC(GAC)				TAC	AIDDY	
B16	J558.72.173	1	DFL16.1	JH2	*TGT* GCAAGA		C		TACTACGGT		T		CTTTGACTAC	ARLLRFFDY	
B17	VGAM3.8-3-61	9	DSP2.9	JH2	*TGT* GCAAGA	TCT	GTCGT		TTAC(TAC)				TTTGACTAC	ARSWYYFDY	
B18	VH10.3.91	10	DQ52	JH2	*TGT* GTGAG		G		ACTGG			A	TTTGACTAC	VRTGFDYW	
B19	Vh10.2b	10	DSP2.5	JH4	*TGT* GTGAGAC		T		TCTACTATGG		G	G	CTATGCTATGGACTAC	VRLLLWGYAMDY	
B20	SM7.3.54	14	DFL16.1	JH4	*TGT* GCTAGA		AC		CGGTAGTAGC		CCCC		ATTACTATGCTATGGACTAC	ARTGSSPHYYAMDY	
B21	S107.3.62	7	DQ52	JH1	*TGT* GCAAGA	T	TTCTCA		AACTGG		T		CTACTGGTACTTCGATGTC	ARFLKLVYWYFDV	
B22	7183.4.6	5	DFL16.1	JH4	*TGT* GCAAGA		G	A	TATTACT		TTAGGG		GCTATGGACTAC	ARDITLGAMDY	
B23	J558.53.146	1	DFL16.1	JH2	*TGT* GCAAGA	T	TTGGG		TACTACGGTAGTA	T			CTTTGACTAC	ARFGYYGSIFDY	
B24	J558.50.143	1	DFL16.1	JH3	*TGT* GCAAGA	G			ACTACGGTAGTAG		TACGATAACTTC		C	ARDYGSSTITS	
B25	7183.14.25	5	DQ52	JH4	*TGT* AC(AA)				CTGGGA(C)				TATGGACTAC	TTGTMDY	
C1	VH11.2.53	11	DFL16.1	JH1	*TGT* AT		CCT		CTACGGTAGTAG(CTAC)				TGGTACTTCGATGTC	ILYGSSYWYFDV	
C2	J606.1.79	6	DFL16.1	JH4	*TGC* ACAGG	C		A	TACTACGGT		CG		TATGCTATGGACTAC	TGILRSYAMDY	
C3	J558.72.173	1	DFL16.1	JH4	*TGT* GCAAGA	TC			TTACTACGG		GACCCCC		TACTATGCTATGGACTAC	ARSYYGTPYYAMDY	T212G, FWR3
C4	VH10.3.91	10	DSP2.9	JH4	*TGT* GTGAGA(GA)				TGGTTACTA	T	TCCTT		CTATGCTATGGACTAC	VRDGYYSFYAMDY	

V_H-D_H-J_H genes from single IL-10^+ B cells were identified as in figure 6A–C. P, P nucleotide addition; N, N nucleotide addition; FWR, framework region.

Table II

B10 cell V_K-J_K sequences.

Cell	V Gene	J Gene	V End	P	N	P	J End	CDR3 Translation	Mutation Locations
A2	bt20	JK4	*TGT* TTGCAAAGTGATAACTTGCCT		C		TCACG	LQSDNLPLT	
A3	cr1	JK2	*TGC* TTTCAAGGTTCACATGTTCC	G			TACACG	FQGSHVPYT	
A5	cp9	JK2	*TGT* CAGCAGTATAGTAAGCTTCC	G			TACACG	QQYSKLPYT	
A7	kn4	JK5	*TGC* CATCAGCGGAGTAGTTA(C)				ACG	HQRSSYT	
A13	bt20	JK5	*TGT* TTGCAAAGTGATAACTTGCCT				CTCACG	LQSDNLPLT	
A15	21–12	JK5	*TGT* CAGCACAGTAGGGAGCTTCC	G			CTCACG	QHSRELPLT	
A17	8–34	JK2	*TGT* CAGCAGTCCTACAGCGCTCC	G			TACACG	QQSYSAPYT	
A22	kn4	JK5	*TGC* CATCAGCGGAGTAGTTA(C)				ACG	HQRSSYT	
A23	bt20	JK2	*TGT* TTGCAAAGTGATAACTTGCC	G			TACACG	LQSDNLPYT	
A24	21–10	JK5	*TGT* CAGCAAAATAATGAGGATCC	G			CTCACG	QQNNEDPLT	
A25	8–34	JK1	*TGT* CAGCAATATTATAGCTATCC				GACG	QQYYSYPT	*T88C*, FWR1
A26	fl12	JK2	*TGT* CAAAATGTGTTAAGTACTCCT				TACACG	QNVLSTPYT	*G313A*, J region
A27	fl12	JK1	*TGT* CAAAATGTGTTAAGTACTCC	G			TGGACG	QNVLSTPWT	
A28	ba9	JK2	*TGT* CTACAGTATGATGAGTTTCC	G			TACACG	LQYDEFPYT	
A29	at4	JK5	*TGC* CAGCAGTGGAGTAGTTACCC	G			CTCACG	QQWSSYPLT	
A30	21–2	JK1	*TGT* CAGCAAAGTAAGGAGGTTCC	G			TGGACG	QQSKEVPWT	
A31	21–12	JK5	*TGT* CAGCACAGTAGGGAGCTTCC	G			CTCACG	QHSRELPLT	
A32	8–28	JK2	*TGT* CAGAATGATCATAGTTATCC	G			TACACG	QNDHSYPYT	
A33	cf9	JK2	*TGT* GTACAGTATGCTCAGTTTCC	G			TACACG	VQYAQFPYT	
A34	cf9	JK2	*TGT* GTACAGTATGCTCAGTTTCC	G			TACACG	VQYAQFPYT	
A35	cr1	JK1	*TGC* TTTCAAGGTTCACATGTTCC		TC		GGACG	FQGSHVPRT	
A36	cr1	JK1	*TGC* TTTCAAGGTTCACATGTTCC	G			TGGACG	FQGSHVPWT	
A37	gn33	JK2	*TGT* CAACAGTATTGGAGTACTCC	G			TACACG	QQYWSTPYT	
A38	19–32	JK1	*TGT* CAGCAGGATTATAGCTCTCC	G			TGGACG	QQDYSSPWT	
A39	bb1	JK2	*TGC* TCTCAAAGTACACATGTTCC	G			TACACG	SQSTHVPYT	
A40	bd2	JK1	*TGC* TGGCAAGGTACACATTT		G		TGGACG	WQGTHLWT	
A41	bb1	JK2	*TGC* TCTCAAAGTACACATGTTCC	G			TACACG	SQSTHVPYT	
A42	ce9	JK1	*TGC* CAACAGGGTAATACGCTGCTTCCT			C	GGACG	QQGNTLPRT	

Cell	V Gene	J Gene	V End	P	N	P	J End	CDR3 Translation	Mutation Locations
A43	bd2	JK1	*TGC* TGGCAAGGTACACATTTTCCT		CA		GACG	WQGTHFPQT	
A44	n12–46	JK2	*TGT* CAACATTTTTGGGGTACTCC	G			TACACG	QHFWGTPYT	
A45	fl12	JK2	*TGT* CAAAATGTGTTAAGTACTCCT		CCG		TACACG	QNVLSTPPYT	
A46	ba9	JK2	*TGT* CTACAGTATGATGAGTTTCC	G			TACACG	LQYDEFPYT	
A47	21–5	JK2	*TGT* CAGCAAAGTAATGAGGATCC	G			TACACG	QQSNEDPYT	
A48	ap4	JK4	*TGC* CAGCAAAGGAGTAGTTACCCA				TTCACG	QQRSSYPFT	
A49	19–15	JK4	*TGT* CAGCAATATAACAGCTATCC			A	TTCACG	QQYNSYPFT	
A51	bb1	JK2	*TGC* TCTCAAAGTACACATGTTCC	G			TACACG	SQSTHVPYT	
A52	23–43	JK4	*TGT* CAACAGAGTAACAGCTGGCC			A	TTCACG	QQSNSWPFT	
A53	ce9	JK1	*TGC* CAACAGGGTAATACGCTTCCT		C	C	GACG	QQGNTLPPT	
A54	bd2	JK2	*TGC* TGGCAAGGTACACATTTTCC	G			TACACG	WQGTHFPYT	
A56	bd2	JK1	*TGC* TGGCAAGGTACACATTTTCC	G			TGGACG	WQGTHFPWT	
B7	cw9	JK1	*TGT* CTACAATATGCTAGTTATCCT		C	C	GACG	LQYASYPPT	
B8	bl1	JK2	*TGC* CTCCAAGTTACACATGTCCC	G			TACACG	LQVTHVPYT	
B13	23–39	JK5	*TGT* CAAAATGGTCACAGCTTTCC	G			CTCACG	QNGHSFPLT	
B14	19–32	JK4	*TGT* CAGCAGGATTATAGCTCTCC				CACG	QQDYSSPT	
B15	RF	JK1	*TGT* CAACAGCATAATGAAT ACCCG(T)				GGACG	QQHNEYPWT	
B16	8–24	JK1	*TGT* CAGCAACATTATAGCACTCC	G			TGGACG	QQHYSTPWT	
B23	bb1	JK5	*TGC* TCTCAAAGTACACATGTTCC	G			CTCACG	SQSTHVPLT	
B25	bb1	JK1	*TGC* TCTCAAAGTACACATGTTCCT		C	C	GACG	SQSTHVPPT	
B26	gm33	JK1	*TGT* CAACAGTATTGGAGTACTCCT			C	GGACG	QQYWSTPRT	
B28	ae4	JK4	*TGC* CATCAGTGGAGTAGTTACCCA				TTCACG	HQWSYPFT	
B29	kh4	JK4	*TGT* CAACAGTGGAGTAGTTACCCATT(C)				ACG	QQWSSYPFT	C289A, CDR3
B30	cr1	JK1	*TGC* TTTCAAGGTTCACATGTTCC	G			TGGACG	FQGSHVPWT	
B31	8–30	JK1	*TGT* CAGCAATATTATAGCTATCCT			C	GGACG	QQYYSYPRT	G151A, FWR3
B32	bd2	JK1	*TGC* TGGCAAGGTACACATTTTCCT			C	GGACG	WQGTHFPRT	
B33	21–4	JK2	*TGT* CAGCAAAGTAATGAGGATCC	G			TACACG	QQSNEDPYT	
B34	aa4	JK1	*TGC* CAGCAGTATCATAGTTACCCAC				GGACG	QQYHSYPRT	
B35	bb1	JK1	*TGC* TCTCAAAGTACACATGT		G		TGGACG	SQSTHVWT	
B37	23–43	JK5	*TGT* CAACAGAGTAACAGCTGGCCT		GC	G	CTCACG	QQSNSWPALT	
B38	n8–30	JK1	*TGT* CAGCAATATTATAGCTATCCT			C	GGACG	QQYYSYPRT	C224T, FWR3; *A322G*, J region

Cell	V Gene	J Gene	V End	P	N	P	J End	CDR3 Translation	Mutation Locations
B39	23–39	JK5	*TGT* CAAAATGGTCACAGCTTTCCT		CC		CACG	QNGHSFPPT	
B40	cr1	JK2	*TGC* TTTCAAGGTTCACATGTTCC	G			TACACG	FQGSHVPYT	
B41	bt20	JK2	*TGT* TTGCAAAGTGATAACTTGCC	G			TACACG	LQSDNLPYT	
B43	19–32	JK1	*TGT* CAGCAGGATTATAGCTCTCCT		C	C	GACG	QQDYSSPPT	
B44	bb1	JK2	*TGC* TCTCAAAGTACACATGTTCC	G			TACACG	SQSTHVPYT	
B45	n12–46	JK1	*TGT* CAACATTTTTGGGGTACTCC	G			TGGACG	QHFWGTPWT	
B47	cr1	JK1	*TGC* TTTCAAGGTTCACATGTTCC		T	C	GGACG	FQGSHVPRT	
B48	bd2	JK1	*TGC* TGGCAAGGTACACATTTTCCT		CA		GACG	WQGTHFPQT	*T66C*, FWR1; G217A, FWR3
B49	bd2	JK1	*TGC* TGGCAAGGTACACATTTTCC	G			TGGACG	WQGTHFPWT	
B50	bd2	JK1	*TGC* TGGCAAGGTACACATTTTCC	G			TGGACG	WQGTHFPWT	

V_K-J_K genes from single IL-10^+ B cells were identified as in figure 6D–F. P, P nucleotide addition; N, N nucleotide addition; FWR, framework region.