Immunity
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A Regulatory B Cell Subset with a Unique CD1d^{hi}CD5^{+} Phenotype Controls T Cell-Dependent Inflammatory Responses

Koichi Yanaba,[1,3] Jean-David Bouaziz,[1,3] Karen M. Haas,[1] Jonathan C. Poe,[1] Manabu Fujimoto,[2] and Thomas F. Tedder[1,*]
[1]Department of Immunology, Duke University Medical Center, Durham, NC 27710, USA
[2]Department of Dermatology, Kanazawa University Graduate School of Medical Science, Ishikawa 920-8641, Japan
[3]These authors contributed equally to this work.
*Correspondence: thomas.tedder@duke.edu
DOI 10.1016/j.immuni.2008.03.017

SUMMARY

B cells mediate multiple functions that influence immune and inflammatory responses. In this study, T cell-mediated inflammation was exaggerated in CD19-deficient (*Cd19*$^{-/-}$) mice and wild-type mice depleted of CD20^{+} B cells, whereas inflammation was substantially reduced in mice with hyperactive B cells as a result of CD19 overexpression (hCD19Tg). These inflammatory responses were negatively regulated by a unique CD1d^{hi}CD5^{+} B cell subset that was absent in *Cd19*$^{-/-}$ mice, represented only 1%–2% of spleen B220^{+} cells in wild-type mice, but was expanded to ~10% of spleen B220^{+} cells in hCD19Tg mice. Adoptive transfer of these CD1d^{hi}CD5^{+} B cells normalized inflammation in wild-type mice depleted of CD20^{+} B cells and in *Cd19*$^{-/-}$ mice. Remarkably, IL-10 production was restricted to this CD1d^{hi}CD5^{+} B cell subset, with IL-10 production diminished in *Cd19*$^{-/-}$ mice, yet increased in hCD19Tg mice. Thereby, CD1d^{hi}CD5^{+} B cells represent a unique subset of potent regulatory B cells.

INTRODUCTION

B cells play a central role in humoral immunity, but they also regulate CD4^{+} T cell responses to foreign and self-antigens (Bouaziz et al., 2007; Xiu et al., 2008), function as antigen-presenting cells (Constant et al., 1995), produce cytokines (Harris et al., 2000), provide costimulatory signals (Linton et al., 2003), and promote naive CD4^{+} T cell differentiation into T helper 1 (Th1) or Th2 subsets (Harris et al., 2000). Abnormal B cell function can also drive the development of autoimmunity (Sato et al., 1996). B cells play protective roles as well because both B cell-deficient and CD19-deficient (*Cd19*$^{-/-}$) mice develop a severe nonremitting form of experimental autoimmune encephalomyelitis (EAE), a model of human multiple sclerosis (Fillatreau et al., 2002; Matsushita et al., 2006). In addition, B cells play a protective role during T cell-mediated inflammation because *Cd19*$^{-/-}$ mice have augmented contact hypersensitivity (CHS) responses (Watanabe et al., 2007). CHS is an inflammatory immune reaction that is mediated by T cells in sensitized individuals after subsequent contact with the sensitizing antigen. During CHS sensitization, skin antigen-presenting Langerhans cells migrate to draining lymph nodes and prime antigen-specific T cells (Kripke et al., 1990). Subsequent antigen challenge at a separate skin site results in cutaneous infiltration of antigen-primed T cells and in edema or spongiosis that peaks 24–48 hr after challenge and then decreases (Wang et al., 2000). Although antigen-specific IgM production by peritoneal B-1 cells is important during CHS initiation (Itakura et al., 2005), this does not explain enhanced inflammation in *Cd19*$^{-/-}$ mice because they are B-1a cell-deficient and generate modest responses to most transmembrane signals and antigens (Sato et al., 1995).

B cell deficiency delays the emergence of regulatory T cells and interleukin-10 (IL-10) production in the central nervous system (CNS) during EAE (Mann et al., 2007). Furthermore, IL-10 produced by B cells can downregulate autoimmune disease in EAE (Fillatreau et al., 2002), collagen-induced arthritis (Mauri et al., 2003), and inflammatory bowel disease (Mizoguchi et al., 2002). IL-10-deficient (*Il10*$^{-/-}$) mice also have enhanced CHS responses (Berg et al., 1995). Neutralizing IL-10 by monoclonal antibody (mAb) treatment also enhances CHS responses, whereas systemic IL-10 administration reduces CHS responses (Ferguson et al., 1994; Schwarz et al., 1994). IL-10 is secreted by multiple cell types, including T cells, monocytes, macrophages, mast cells, eosinophils, and keratinocytes, and can suppress both Th1 and Th2 polarization and inhibit macrophage antigen presentation and proinflammatory cytokine production (Asadullah et al., 2003). Thus, B cells and IL-10 play important inhibitory roles during T cell-mediated inflammatory responses.

The generation of regulatory B cells has been reported in mouse models of chronic inflammation, although their existence in normal mice remains unknown (Mizoguchi and Bhan, 2006). Furthermore, it is unknown whether multiple B cell populations or a distinct B cell subset regulates inflammatory responses, whether regulatory B cells produce IL-10 or other cytokines directly, or whether regulatory B cells have potent activities in vivo. Therefore, the importance of B cells during T cell-mediated inflammation was examined with CHS responses as a model for inflammation in *Cd19*$^{-/-}$ and human CD19 transgenic (hCD19Tg) mice and in wild-type (WT) mice with intact immune systems that were depleted of B cells in vivo (Uchida et al., 2004a). CHS was chosen as a model because a balance between B cells and dendritic cells regulates CD4^{+} T cell expansion in response to antigens in vivo (Bouaziz et al., 2007; Xiu et al., 2008) but skin Langerhans cells are the exclusive antigen-presenting cells during CHS (Bursch et al., 2007). Thereby, using

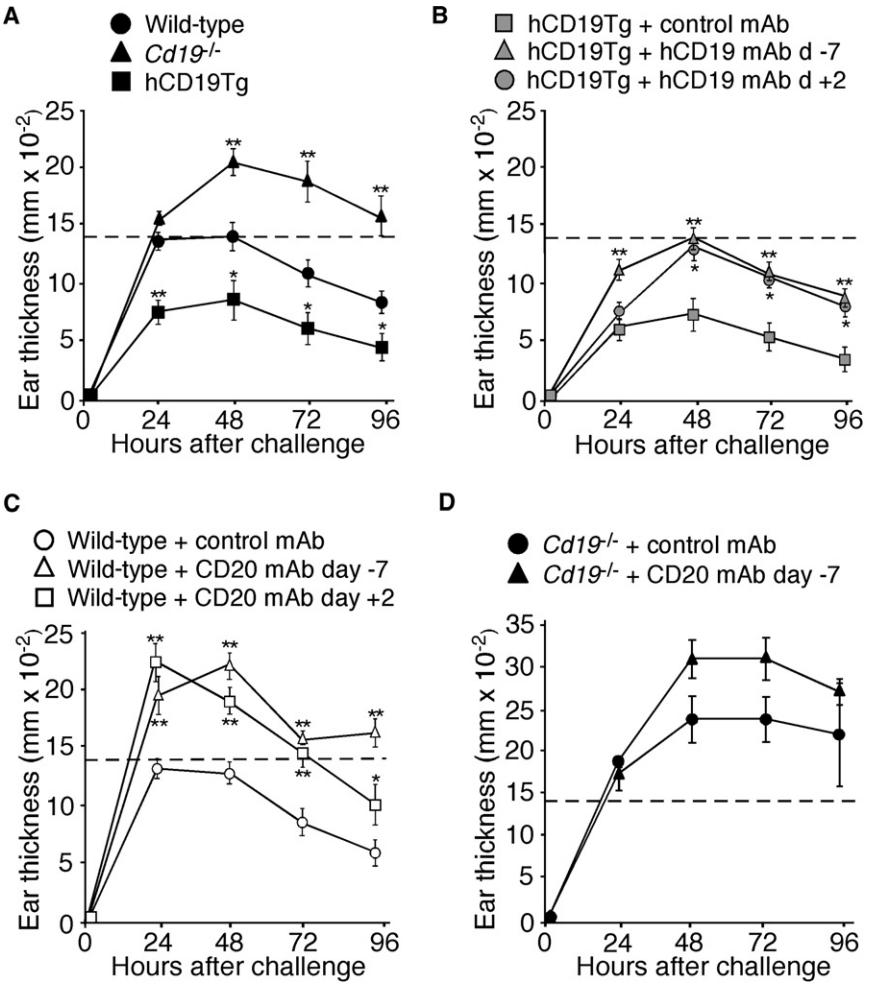

Figure 1. B Cell Regulation of T Cell-Mediated Inflammatory Responses

CHS responses in (A) WT, hCD19Tg, and *Cd19*$^{-/-}$ mice, (B) hCD19Tg mice treated with human CD19 or control mAb 7 days before or 2 days after initial oxazolone sensitization, and (C) WT or (D) *Cd19*$^{-/-}$ mice treated with CD20 or control mAb 7 days before or 2 days after first sensitization with oxazolone. Increased ear thickness was measured before and after oxazolone challenge. Values represent means (± SEM) from ≥ four mice per group. Horizontal dashed lines represent the average increase in ear thickness at 48 hr after oxazolone challenge in WT mice. Significant differences between sample means are indicated; *, $p < 0.05$; **, $p < 0.01$. Similar results were obtained in at least two independent experiments.

CHS as a model minimized the potential stimulatory roles for B cells during immune responses. This allowed the identification of a potent regulatory B cell subset identified by IL-10 production and a unique CD1d^{hi}CD5^{+} phenotype. These rare CD1d^{hi}CD5^{+} B cells negatively regulated antigen-specific T cell-dependent inflammation during CHS responses in vivo.

RESULTS

Mice with Altered B Cells Differentially Regulate Inflammation

To assess T cell-mediated inflammation responses in mice with altered B cell signaling, *Cd19*$^{-/-}$, hCD19Tg, and WT mice were sensitized and challenged with 4-ethyoxymethylene-2-phenyl-oxazoline-5-one (oxazolone). B cells from hCD19Tg mice are hyperresponsive to transmembrane signals, proliferate more to mitogens, generate elevated humoral immune responses to T dependent antigens, and spontaneously produce IgG autoantibodies as they age (Inaoki et al., 1997). Thus, CD19 functions as a general "rheostat" that defines signaling thresholds critical for expansion of the peripheral B cell pool (Tedder, 1998). In WT mice, ear inflammation peaked at 24 hr after challenge and then decreased gradually (Figure 1A). Ear swelling was significantly diminished in hCD19Tg mice compared with WT mice throughout the observation period (39% ± 13%, 48 hr, $p < 0.05$). By contrast, ear swelling was enhanced and prolonged in *Cd19*$^{-/-}$ mice (58% ± 8%, 48 hr, $p < 0.05$) as reported (Watanabe et al., 2007). Despite this, blood, spleen, and lymph node-regulatory CD25^{+}Foxp3^{+}CD4^{+} T cell numbers were identical in WT, hCD19Tg, and *Cd19*$^{-/-}$ mice (not shown). Thus, enhanced or reduced B cell function inversely paralleled T cell-mediated inflammatory responses.

B Cell Depletion Enhances CHS Responses

Whether B cells were directly responsible for decreased T cell-mediated inflammatory responses in hCD19Tg mice was determined by depleting B cells in hCD19Tg mice with human CD19 mAb as described (Yazawa et al., 2005). CD19 mAbs depleted the majority of circulating B cells within 1 hr of treatment, with > 95% depletion of spleen and lymph node B cells within 2 days. Mice treated with human CD19 mAb 7 days before primary sensitization showed significantly enhanced CHS responses compared with control mAb-treated mice ($p < 0.01$, Figure 1B). Mice treated with human CD19 mAb 2 days after primary oxazolone sensitization had comparable CHS responses with control mAb-treated mice at 24 hr after oxazolone elicitation but developed augmented CHS responses by 48 hr. Ear swelling 48 hr after oxazolone challenge in mice treated with human CD19 mAb 7 days before or 2 days after primary oxazolone sensitization was increased by 102% ± 8% or 89% ± 12%, respectively. Thus, B cell depletion in hCD19Tg mice restored CHS responses to levels observed in WT mice.

B cells were depleted from mice with intact immune systems by the use of CD20 mAb to examine whether normal B cells regulate T cell-mediated inflammation in WT mice. Mature spleen CD20^{+} B cells in WT mice are eliminated within 2 days after a single CD20 mAb treatment (Uchida et al., 2004a). Mice depleted of B cells 7 days before or 2 days after primary oxazolone sensitization exhibited significantly enhanced CHS responses when compared

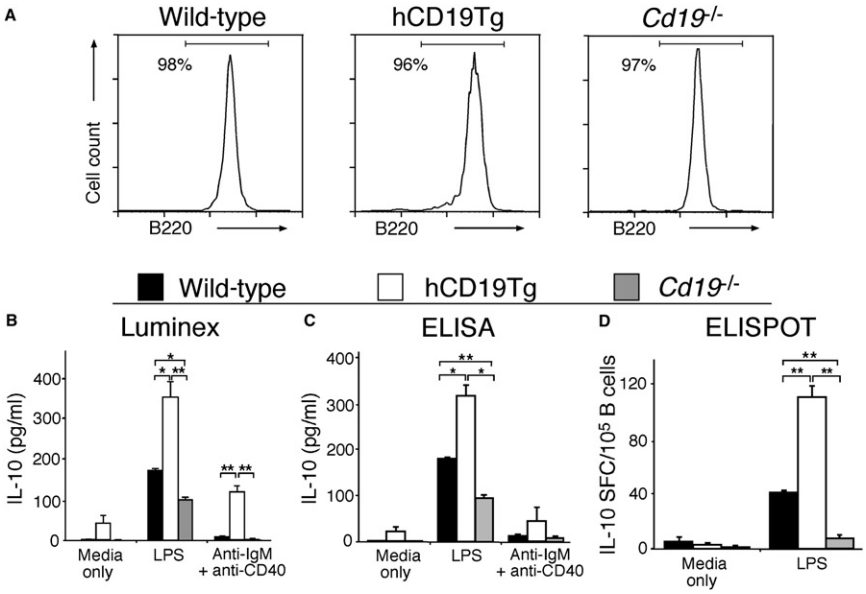

Figure 2. IL-10 Production by Purified Spleen B Cells from WT, hCD19Tg, and *Cd19*$^{-/-}$ Mice

(A) Representative flow-cytometry histograms showing B cell purities after B220 mAb-coated microbead isolation. (B) Luminex and (C) ELISA determinations of IL-10 secretion by B220^{+} cells cultured in media alone or containing LPS, or CD40 mAb plus IgM antibody are shown (mean ± SEM, n ≥ 3). (D) IL-10-secreting B cell frequencies determined by ELISPOT assay are shown. B220^{+} cells were incubated in the absence or presence of LPS for 24 hr. Values represent mean numbers (± SEM) of spot-forming cells per 10^5 B220^{+} cells from ≥ three mice of each group.

(B–D) Significant differences between sample means are indicated; *, $p < 0.05$; **, $p < 0.01$. Results represent one of two independent experiments producing similar results.

with control mAb-treated mice: 91% ± 10% and 72% ± 11% increase, respectively, at 48 hr after oxazolone challenge ($p < 0.01$, Figure 1C). Thus, B cell depletion augmented T cell-mediated inflammatory responses in both WT and hCD19Tg mice.

Whether CD19 deficiency completely eliminates B cell negative regulation was determined by depleting B cells from *Cd19*$^{-/-}$ mice with CD20 mAb. B cell depletion further increased CHS severity in *Cd19*$^{-/-}$ mice, but the difference was not statistically different from control mAb-treated mice (Figure 1D). This suggests that some regulatory B cells still exist in *Cd19*$^{-/-}$ mice, but at levels below those found in WT and hCD19Tg mice. Moreover, anti-human CD19 as well as anti-mouse CD20 mAbs do not eliminate all peritoneal B cells (Hamaguchi et al., 2005; Yazawa et al., 2005). Furthermore, CD20 mAb treatment does not reduce serum or natural antibody levels (DiLillo et al., 2008). Thereby, induced B cell depletion eliminates most B cell negative regulation but does not eliminate the peritoneal B-1 cell population that also appears important for CHS initiation (Itakura et al., 2005).

B Cell Cytokine Expression in WT, hCD19Tg, and *Cd19*$^{-/-}$ Mice

B cells produce multiple cytokines that influence immune responses (Harris et al., 2000). Therefore, B cells from WT, hCD19Tg, and *Cd19*$^{-/-}$ mice were purified (>95% purities, Figure 2A), with cytokine production quantified and compared with T cell-mediated inflammatory responses observed in each mouse line. Whereas B cells cultured without mitogens did not produce cytokines, lipopolysaccharide (LPS)-stimulated B cells from WT, hCD19Tg, and *Cd19*$^{-/-}$ mice produced tumor necrosis factor-α, IL-1β, IL-10, and IL-6 protein as determined with Luminex assays (Figure 2B, not shown). IgM antibody plus CD40 mAb stimulation also induced the production of these cytokines, but at lower amounts than LPS. Only WT B cells secreted transforming growth factor-β1, but only at very low amounts after IgM antibody plus CD40 mAb stimulation (not shown). Neither LPS nor IgM antibody plus CD40 mAb stimulation induced detectable IL-4, -5, -12, -13, -17, or -23 secretion (not shown). Nonetheless, increased IL-10 production by hCD19Tg but reduced IL-10 production by *Cd19*$^{-/-}$ B cells was the only cytokine change that was inversely proportional to inflammatory responses in these mice. In Luminex and standard ELISAs, B cells from hCD19Tg mice showed increased IL-10 production compared with WT mice (LPS stimulation, 1.8-fold; $p < 0.01$), whereas B cells from *Cd19*$^{-/-}$ mice exhibited reduced IL-10 production (65% of WT, $p < 0.05$; Figures 2B and 2C). With ELISPOT assays, IL-10-producing B cell frequencies were 2.7-fold higher in hCD19Tg mice than WT mice ($p < 0.01$) but 74% lower in *Cd19*$^{-/-}$ mice than in WT mice ($p < 0.01$; Figure 2D). Thus, frequencies of IL-10-producing B cells were inversely proportional to the inflammatory responses of hCD19Tg and *CD19*$^{-/-}$ mice.

IL-10-Producing B Cells Localize in the Spleen and Peritoneal Cavity

Reciprocal IL-10 production by B cells from hCD19Tg and *Cd19*$^{-/-}$ mice was verified directly by intracellular cytokine staining. Cytoplasmic IL-10 production was not detected in resting B cells from WT, hCD19Tg, or *Cd19*$^{-/-}$ mice (Figure 3A). After LPS, phorbol 12-myristate 13-acetate (PMA), and ionomycin stimulation for 5 hr, the frequencies of spleen IL-10-producing B cells was 7.4-fold higher in hCD19Tg mice than in WT mice ($p < 0.01$), whereas the frequency of IL-10-producing B cells was 85% lower in *Cd19*$^{-/-}$ mice than in WT mice ($p < 0.01$; Figure 3B). Interestingly, IL-10 production by non-B cells after LPS, PMA, and ionomycin stimulation was also increased in hCD19Tg mice (Figure S1 online). Peritoneal IL-10-producing B cell frequencies were 3-fold higher in hCD19Tg mice than in WT mice ($p < 0.01$) but 80% lower in *Cd19*$^{-/-}$ mice ($p < 0.01$; Figure 3C). Even though *Cd19*$^{-/-}$ and hCD19Tg mice have reduced numbers of splenic B cells compared with WT mice (Haas et al., 2005), the numbers of IL-10-producing splenic and peritoneal B cells were 2.1-fold- and 3.1-fold-higher in hCD19Tg mice than in WT mice, respectively ($p < 0.01$). Splenic

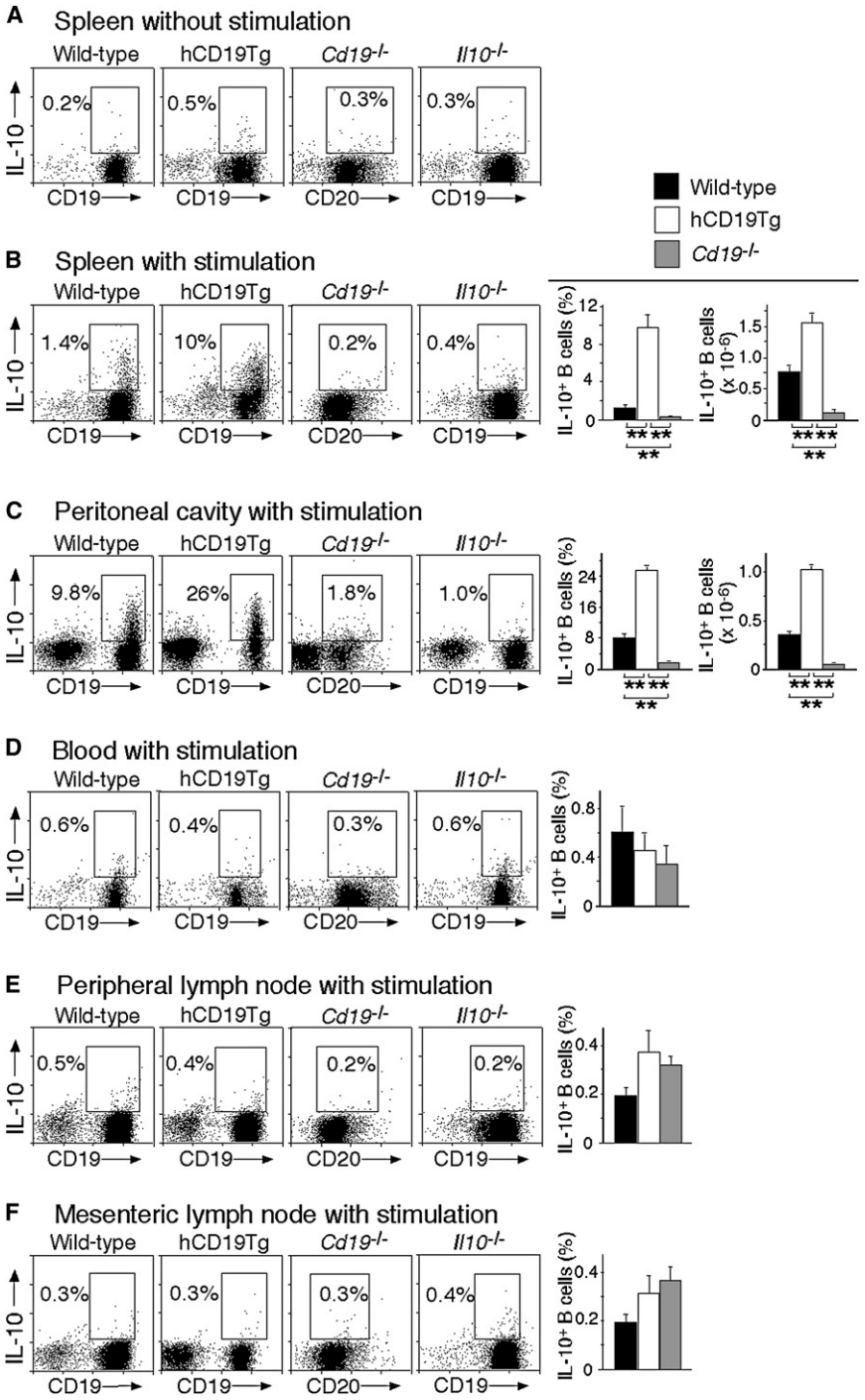

Figure 3. Cytokine Production by B Cells from WT, hCD19Tg, and *Cd19*$^{-/-}$ Mice

(A) Splenocytes without stimulation, or (B) spleen, (C) peritoneal cavity, (D) blood, (E) peripheral lymph node, and (F) mesenteric lymph node lymphocytes after culture with LPS, PMA, ionomycin, and monensin for 5 hr. B cells were stained with B220 mAb and/or CD19 or CD20 mAbs. After permeabilization, the cells were stained with IL-10 mAb. B220 staining was used as the initial gate for identifying B cells (except peritoneal cavity). All data are representative of three independent experiments. Representative histograms for one mouse show frequencies of IL-10-producing cells among total B cells within the indicated gates. Bar graphs indicate mean (± SEM) percentages and numbers of B cells that produced IL-10 in one representative experiment with three mice per group. Significant differences between sample means are indicated; **, $p < 0.01$.

Spleen IL-10-Producing B Cells Are CD1d^{hi}CD5^{+}

Whether IL-10-producing B cells represent a known B cell subset was determined by immunofluorescence staining. Because B cell cytoplasmic IL-10 was only visualized after combined LPS, PMA, ionomycin, and monensin treatment for 5 hr (Figure 3), the effect of this treatment and cell permeabilization on phenotypes was determined. Untreated, treated, or permeabilized B cells from WT and hCD19Tg mice expressed identical IgM, IgD, CD19, CD5, CD1d, CD21, CD24, CD23, CD11b, CD43, and B220 densities (Figure 4A, not shown). These cell-surface molecules were therefore used to categorize IL-10-producing B cells. Spleen IL-10-producing B cells in WT and hCD19Tg mice were part of a CD19^{hi} subset (Figures 3B and 3C). Spleen IL-10-producing B cells exhibited the CD5^{+}CD19^{hi} phenotype characteristic of B-1a cells, but they unexpectedly expressed cell-surface CD1d at high amounts in both WT and hCD19Tg mice (Figure 4B). By contrast, *Cd19*$^{-/-}$ mice did not have detectable spleen CD1d^{hi}CD5^{+} or IL-10-producing B cells (Figures 3B and 4C, Table 1). On average, B cells with a CD1d^{hi}CD5^{+} phenotype represented 2.3% and 12.2% of spleen B220^{+} cells in WT and hCD19Tg mice, respectively (Figure 4C). CD1d^{hi}CD5^{+} splenic B cell numbers were 38% higher in hCD19Tg mice than in WT mice (Table 1). Within the CD1d^{hi}CD5^{+} B cell subset, an average of 18% and 58% expressed IL-10 in WT and hCD19Tg mice, respectively. CD1d^{lo}CD5^{+} B cells did not express IL-10 (Figure 4C). When CD1d^{hi}CD5^{+} or the remaining spleen B cells were purified and then stimulated, the vast majority of IL-10-producing B cells were found within the CD1d^{hi}CD5^{+} subset and peritoneal IL-10-producing B cell numbers were 80% and 78% lower in *Cd19*$^{-/-}$ mice than in WT mice, respectively ($p < 0.01$). By contrast, naive or stimulated B cells from blood, peripheral and mesenteric lymph nodes, and Peyer's patches exhibited little, if any, IL-10 production in WT, hCD19Tg, or *Cd19*$^{-/-}$ mice (Figures 3D–3F, not shown). Intracellular staining of B cells from *Il10*$^{-/-}$ mice served as background controls. Thus, IL-10-producing B cells represent a distinct subset that was dramatically reduced in *Cd19*$^{-/-}$ mice but preferentially expanded in hCD19Tg mouse spleen and peritoneal cavity.

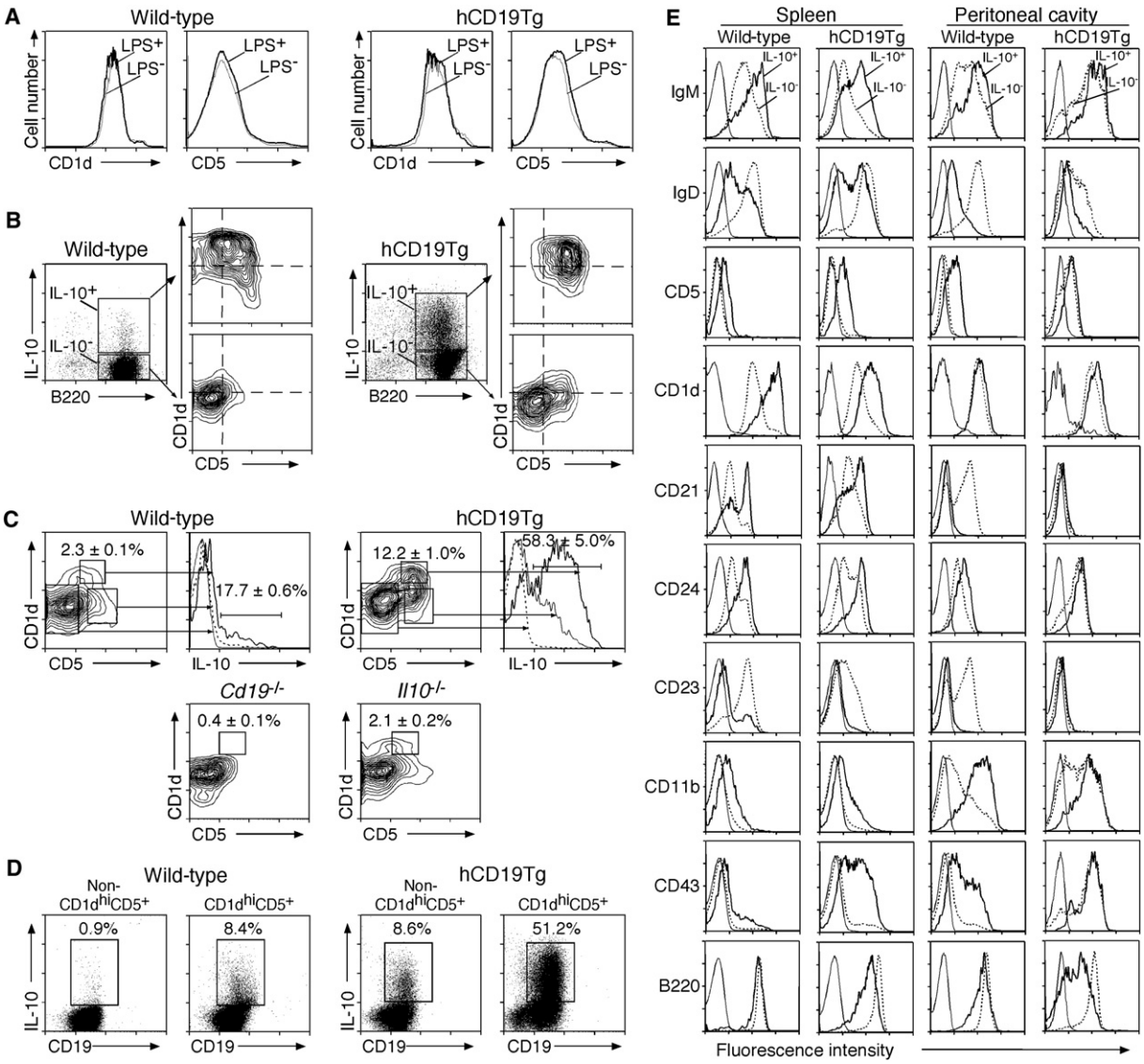

Figure 4. Phenotypes of IL-10-Producing B Cells

(A) CD1d and CD5 expression by CD19^{+} B220^{+} splenocytes from WT and hCD19Tg mice does not change after LPS, PMA, ionomycin, and monensin treatments. CD1d and CD5 expression on CD19^{+}B220^{+} cells before (thin line) or after 5 hr incubation with LPS, PMA, ionomycin, monensin, and permeabilization (thick line) was determined by immunofluorescence staining.

(B) IL-10-producing spleen B cells from WT and hCD19Tg mice expressed both CD1d and CD5. Purified CD19^{+} splenocytes were cultured with LPS, PMA, ionomycin, and monensin for 5 hr before permeabilization and staining with CD1d, CD5, B220, and IL-10 mAbs.

(C) Spleen IL-10-producing B cells represent a CD1d^{hi}CD5^{+} subset distinct from B-1a cells in WT and hCD19Tg mice. Histograms demonstrate cytoplasmic IL-10 expression by permeabilized CD1d^{hi}CD5^{+}, CD1d^{lo}CD5^{+}, and CD1d^{lo}CD5^{-} B cells from WT and hCD19Tg mice after LPS, PMA, and ionomycin stimulation. CD1d and CD5 expression by B cells from *Cd19*$^{-/-}$ and *Il10*$^{-/-}$ mice is also shown. Percentages indicate mean (± SEM) CD1d^{hi}CD5^{+} or IL-10^{+} cell frequencies among CD1d^{hi}CD5^{+} B cells as indicated for each group of three mice.

(D) In vitro stimulation does not induce the CD1d^{hi}CD5^{+} phenotype of IL-10-secreting B cells. Splenic CD1d^{hi}CD5^{+} or CD1d^{lo}CD5^{-} B cells were purified from three WT or hCD19Tg mice by cell sorting and pooled before LPS, PMA, and ionomycin stimulation for 5 hr, with subsequent assessment for cytoplasmic IL-10 production by immunofluorescence staining. Percentages indicate IL-10^{+} cell frequencies.

(E) Cell-surface molecule expression by IL-10^{+} (thick line) or IL-10^{-} (dashed line) B cells from WT and hCD19Tg mice. CD19^{+} splenocytes or peritoneal cells were cultured with LPS, PMA, ionomycin, and monensin for 5 hr before permeabilization and staining for IL-10. Thin lines represent isotype-matched control mAb staining. All results represent ≥ two independent experiments with three mice in each group.

of B cells from WT and hCD19Tg mice (Figure 4D), further excluding the possibility that LPS, PMA, and ionomycin treatment induced their phenotype. Whether the CD1d^{hi}CD5^{+} B cell phenotype was induced by oxazolone sensitization in vivo was also assessed. B cell CD1d and CD5 expression and CD1d^{hi}CD5^{+} B cell numbers were similar in oxazolone sensitized and naive WT mice (Figure S2), making it unlikely that the CD1d^{hi}CD5^{+} B phenotype was induced by oxazolone sensitization.

Table 1. Spleen B Cell Subsets in Wild-Type, hCD19Tg, *Cd19*$^{-/-}$, and *Il10*$^{-/-}$ Mice

	B Cell Subset Numbers (× 10^{-5})			
Mouse Genotype	CD1d^{hi}CD5^{+}	B-1a	MZ	Follicular
hCD19Tg	18 ± 2*	32 ± 4	16 ± 1**	68 ± 4*
Wild-type	13 ± 1	30 ± 3	42 ± 3	362 ± 42
Il10$^{-/-}$	12 ± 1	31 ± 2	42 ± 1	324 ± 17
Cd19$^{-/-}$	0.4 ± 0.1**	8 ± 1**	12 ± 1**	148 ± 6*

B cell subsets were as follows: CD1d^{hi}CD5^{+}, B-1a (CD5^{+}B220^{lo}), MZ (CD1d^{hi}CD21^{hi}B220^{hi}), and follicular (CD21^{int}CD23^{+}B220^{hi}). Values (± SEM, n ≥ 4 mice) were significantly different from those of wild type mice; *, p < 0.05; **, p < 0.01.

The phenotype of IL-10-producing spleen B cells was further verified by determining the phenotypes of IL-10^{+} and IL-10^{-} populations. Most splenic IL-10-producing B cells expressed IgM, CD1d, CD19, and CD24 at high levels (Figures 4B and 4E, not shown). Approximately half of splenic IL-10-producing B cells expressed high-density CD21 (44.3% ± 2.6% and 54.8% ± 1.6% in WT and hCD19Tg mice, respectively). Peritoneal IL-10-producing B cells were CD19^{hi}IgM^{hi}IgD^{lo}CD5^{+}CD23^{-}CD11b^{+}CD43^{+}B220^{lo}, a phenotype shared by B-1a cells. Thus, splenic IL-10-producing B cells shared features common to marginal zone (MZ), T2-MZ precursor, and B-1a B cells but were localized within a unique CD1d^{hi}CD5^{+} subset.

Increased B Cell IL-10 Expression during Inflammation

Whether B cell IL-10 production might regulate T cell-mediated inflammation was determined by assessment of IL-10 production by B cells during CHS responses in WT, hCD19Tg, and *Cd19*$^{-/-}$ mice. Spleen and draining axillary and inguinal lymph node B cells were purified 2 days after ear challenge with oxazolone, with IL-10 mRNA expression quantified by real-time polymerase chain reaction (PCR). Relative IL-10 transcripts in B cells from spleen and peripheral lymph nodes of unchallenged hCD19Tg mice were significantly increased relative to B cells from WT mice (spleen 4.5-fold, p < 0.01, lymph node 1.5-fold, p < 0.05; Figure 5A).

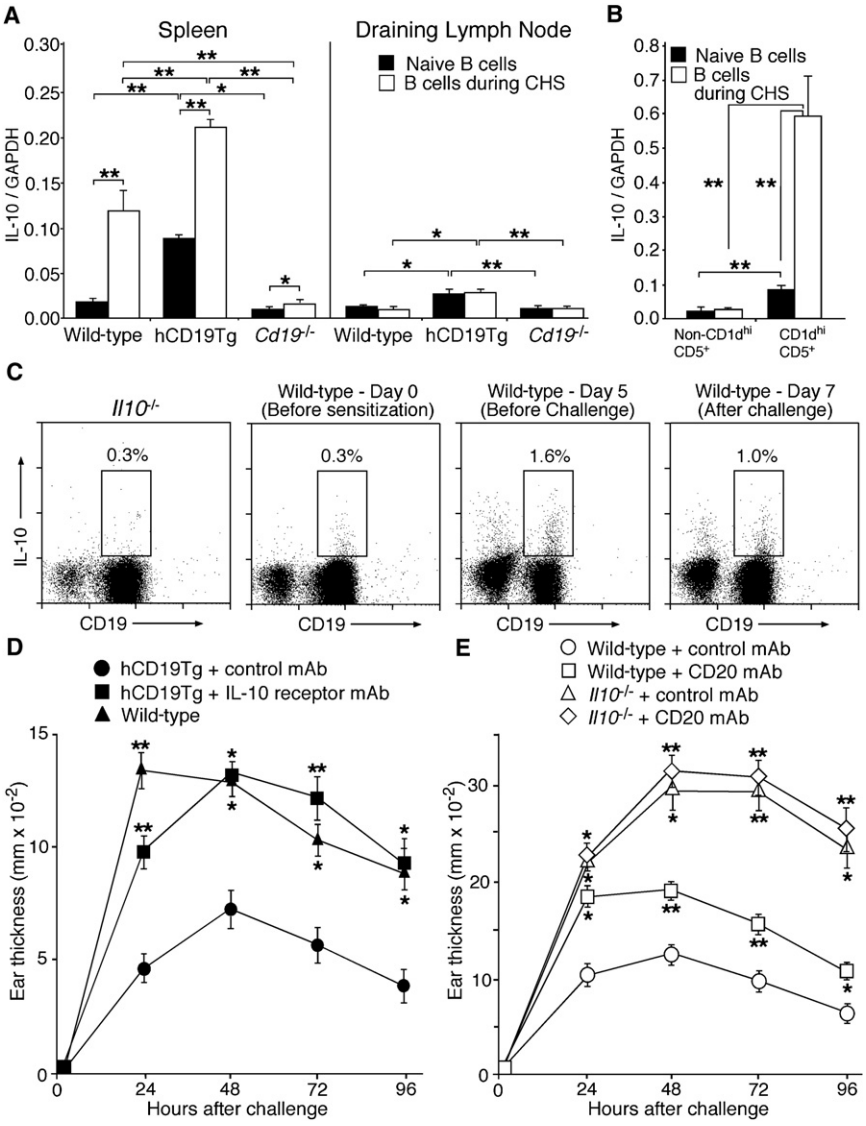

Figure 5. IL-10 Production by CD1d^{hi}CD5^{+} B Cells Correlates with Suppression of T Cell-Mediated Inflammation

(A) IL-10 production by WT, hCD19Tg, and *Cd19*$^{-/-}$ B cells during CHS responses. B220^{+} cells were purified from the spleen and draining lymph nodes of naive mice (filled bars) or 2 days following oxazolone challenge (open bars).

(B) Splenic CD1d^{hi}CD5^{+} or non-CD1d^{hi}CD5^{+} B cells were purified from naive or oxazolone-challenged WT mice (as in A) by cell sorting.

(A and B) Values represent mean ratios of IL-10 transcripts normalized to GAPDH transcript levels (± SEM) in triplicate samples of pooled RNA from three mice as quantified by real-time PCR. Results are representative of at least two independent experiments with three mice in each group. Significant differences between sample means are indicated; *, p < 0.05; **, p < 0.01.

(C) IL-10 production by circulating B cells from WT mice during CHS responses. Blood mononuclear cells from three mice were pooled and cultured with LPS, PMA, ionomycin, and monensin for 5 hr before staining with B220 and/or CD19 or CD20 mAbs. Values represent the percentage of IL-10-producing cells among total B cells with results from *Il10*$^{-/-}$ mice shown as a control.

(D) CHS responses in hCD19Tg mice treated with control or IL-10 receptor-specific mAb 1 hr before and 47 hr after oxazolone challenge.

(E) CHS responses in WT and *Il10*$^{-/-}$ mice after B cell depletion. Mice were treated with CD20 or control mAb 7 days before the first sensitization with oxazolone (mean ± SEM, n ≥ 4). Significant differences between the mean CHS responses between groups and control mAb-treated WT mice are indicated; *, p < 0.05; **, p < 0.01.

During CHS responses, spleen B cells from both WT and hCD19Tg mice expressed more IL-10 transcripts than naive B cells (6-fold, $p < 0.01$ and 2.2-fold, $p < 0.01$, respectively), and hCD19Tg B cells produced higher IL-10 transcripts than WT B cells (1.8-fold, $p < 0.01$; Figure 5A, left panel). IL-10 transcripts in spleen B cells from challenged *Cd19*$^{-/-}$ mice increased significantly during CHS responses, but only up to 16% of the amount seen in WT B cells ($p < 0.01$). By contrast, B cell IL-10 mRNA expression in draining lymph nodes did not change during CHS response (Figure 5A, right panel). In similar experiments, IL-10 transcripts produced by the spleen CD1d^{hi}CD5^{+} B cell subset were increased 7.1-fold during CHS responses in comparison with naive CD1d^{hi}CD5^{+} B cells, whereas IL-10 transcripts were lower in non-CD1d^{hi}CD5^{+} B cells with or without sensitization and challenge (Figure 5B). Thus, B cell IL-10 production in the spleen but not lymph nodes was increased during CHS responses, with the amount of IL-10 production by B cells being inversely proportional to the severity of inflammatory responses.

IL-10 production by blood B cells was assessed to determine whether IL-10-producing B cells enter the circulation during CHS responses. IL-10-producing B cells were not observed in *Il10*$^{-/-}$ or naive mice before oxazolone sensitization (Figures 3D and 5C). However, circulating IL-10-producing B cells were found in the blood after sensitization, with the percentage of circulating IL-10-producing B cells peaking before challenge and gradually decreasing after challenge (Figure 5C). Thus, IL-10-producing B cells enter the circulation during CHS responses.

IL-10 Inhibits T Cell-Mediated Inflammatory Responses in hCD19Tg and WT Mice

Blocking IL-10 function in vivo with an IL-10 receptor-specific mAb enhances CHS responses in WT mice (Ferguson et al., 1994). Therefore, whether the enhanced CHS responses observed in hCD19Tg mice were dependent on IL-10 was assessed with a function-blocking mAb against the IL-10 receptor. Blocking IL-10 receptor function 1 hr before oxazolone challenge significantly augmented CHS responses in hCD19Tg mice when compared with control mAb-treated mice (Figure 5D, $p < 0.05$ at 48 hr). In fact, blocking IL-10 receptor function restored CHS responses to the degree normally observed in WT mice. Thus, the suppression of T cell-mediated inflammation observed in hCD19Tg mice was IL-10 dependent.

The relative contribution of WT IL-10-producing B cells to CHS suppression was assessed by comparison of the effects of CD20 mAb-induced B cell depletion in WT and *Il10*$^{-/-}$ mice. B cell depletion significantly augmented CHS responses in WT mice (Figure 5E). Remarkably, CHS responses were increased similarly by B cell depletion and IL-10 deficiency 24 hr after challenge. Subsequently, CHS responses were higher in *Il10*$^{-/-}$ mice, suggesting that B cell IL-10 production contributed most substantially to early inhibition of CHS responses, whereas IL-10 production by the remaining peritoneal B cells or other IL-10-producing subsets regulated later stages of the CHS response. Equally important was that B cell depletion did not affect CHS severity in *Il10*$^{-/-}$ mice, arguing that the inhibitory role of B cells in CHS regulation in WT mice is due to IL-10 production. Thus, B cells suppressed T cell-mediated inflammation in an IL-10-dependent manner, with a more substantial contribution during early inflammation.

CD1d^{hi}CD5^{+} B Cells Inhibit Inflammatory Responses

The ability of CD1d^{hi}CD5^{+} B cells to regulate CHS responses was assessed with adoptive-transfer experiments. Spleen CD1d^{hi}CD5^{+} B cells and non-CD1d^{hi}CD5^{+} B cells were purified from either oxazolone-sensitized (5 days after primary sensitization) or unsensitized WT mice (Figure 6A, left panels). Purified B cells were then transferred into oxazolone-sensitized *Cd19*$^{-/-}$ mice that were challenged with oxazolone 48 hr after the transfer. Transferring CD1d^{hi}CD5^{+} B cells into *Cd19*$^{-/-}$ mice significantly reduced (43% at 48 hr, $p < 0.05$) ear swelling (Figure 6A, middle panel). Ear swelling was not inhibited in recipients given CD1d^{hi}CD5^{+} B cells from nonsensitized mice or non-CD1d^{hi}CD5^{+} B cells from sensitized mice. Likewise, splenic CD1d^{hi}CD5^{+} B cells purified from oxazolone-sensitized *Il10*$^{-/-}$ mice did not affect ear swelling in *Cd19*$^{-/-}$ recipients (Figure 6A, right panel). Thus, sensitized splenic CD1d^{hi}CD5^{+} B cells inhibited CHS responses in an IL-10-dependent manner.

Transfer of naive WT CD1d^{hi}CD5^{+} B cells into *Cd19*$^{-/-}$ mice 2 days before sensitization allowed sufficient time for the *Cd19*$^{-/-}$ mice to develop alloantigen-specific immune response against the cell-surface CD19 antigen that is abundantly expressed on WT B cells. Therefore, whether the transfer of IL-10-producing CD1d^{hi}CD5^{+} B cells before sensitization and before challenge played a role in CHS responses in WT mice was assessed. Spleen CD1d^{hi}CD5^{+} B cells were purified from oxazolone-sensitized CD20-deficient mice (Figure 6B, left panels) and transferred into WT recipients that were depleted of B cells using CD20 mAb. CD20-deficient mice had normal numbers of CD1d^{hi}CD5^{+} IL-10-producing B cells compared to WT mice, and CD20-deficient B cells were not depleted by CD20 mAb (not shown). When B cells were depleted in WT mice, CHS responses were increased. However, the adoptive transfer of sensitized CD1d^{hi}CD5^{+} CD20-deficient B cells just before challenge normalized CHS responses in B cell-depleted mice (Figure 6B, middle panel). The adoptive transfer of naive CD1d^{hi}CD5^{+} CD20-deficient B cells into B cell-depleted mice 2 days before oxazolone sensitization also normalized subsequent CHS responses in B cell-depleted mice (Figure 6B, right panel). The transfer of non-CD1d^{hi}CD5^{+} CD20-deficient B cells from naive or sensitized mice into recipients before sensitization or challenge, respectively, did not reduce oxazolone-induced ear swelling. Thus, IL-10 secretion by CD1d^{hi}CD5^{+} B cells regulated T cell-mediated inflammation in vivo.

Whether IL-10 secretion by CD1d^{hi}CD5^{+} B cells was antigen specific was addressed by the adoptive transfer of splenic CD1d^{hi}CD5^{+} B cells purified from 2,4-dinitrofluorobenzene (DNFB)-sensitized mice (Figure 6C, left panels). When transferred into oxazolone-sensitized *Cd19*$^{-/-}$ recipients, neither CD1d^{hi}CD5^{+} nor non-CD1d^{hi}CD5^{+} B cells purified from DNFB-sensitized mice affected ear swelling in oxazolone-challenged *Cd19*$^{-/-}$ recipients (Figure 6C, right panel). These findings suggest that IL-10-producing CD1d^{hi}CD5^{+} B cell function is antigen specific rather than a result of inflammatory stimuli.

DISCUSSION

This study demonstrates that a phenotypically distinct CD1d^{hi}CD5^{+}CD19^{hi} B cell subset regulates T cell-mediated inflammatory responses through IL-10 secretion. For convenience,

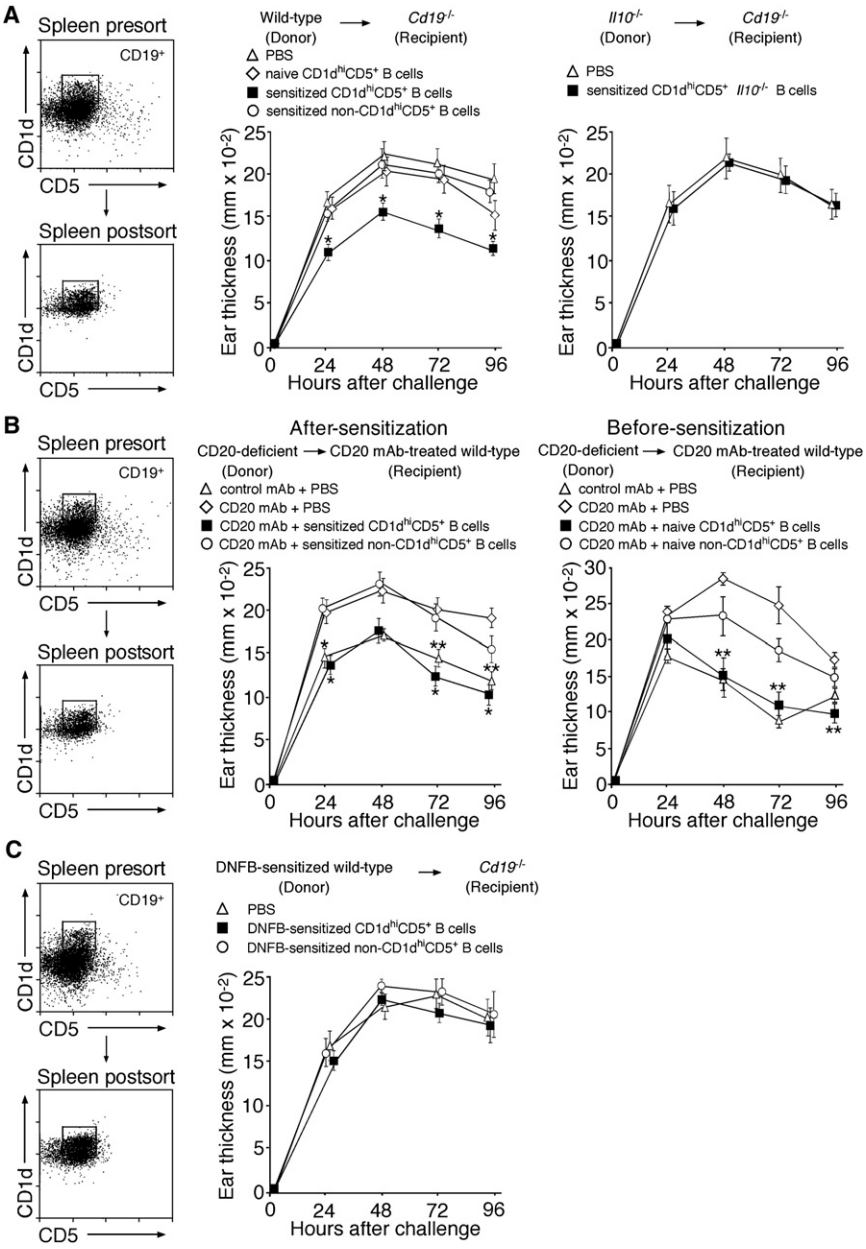

Figure 6. CD1d^{hi}CD5^{+} B Cells Inhibit T Cell-Mediated Inflammation

(A) Splenic CD1d^{hi}CD5^{+} or non-CD1d^{hi}CD5^{+} B cells from oxazolone-sensitized or naive mice were purified (representative results, left panel). Purified cells from WT (middle panel) or *Il10*$^{-/-}$ (right panel) mice were transferred into oxazolone-sensitized *Cd19*$^{-/-}$ mice. Recipient mice were challenged 48 hr after transfer with increased ear thickness measured. Significant differences between PBS-treated mice versus other groups are indicated; *, $p < 0.05$.

(B) CD1d^{hi}CD5^{+} or non-CD1d^{hi}CD5^{+} splenic B cells were purified from sensitized or naive CD20-deficient mice and transferred into sensitized (middle panel) or naive WT (right panel) mice that had been treated with CD20 or control mAb 7 days earlier as in (A). For sensitized mice, the transfer was performed 2 days before challenge (middle panel). For naive mice, the transfer was performed 2 days before sensitization (right panel). Significant differences between CD20 mAb-treated mice versus other groups are indicated; *, $p < 0.05$; **, $p < 0.01$.

(C) Adoptive transfer of CD1d^{hi}CD5^{+} or non-CD1d^{hi}CD5^{+} B cells purified from DNFB-sensitized mice does not alter oxazolone-induced CHS responses in *Cd19*$^{-/-}$ recipients. The same experimental protocol was used as in (A), except that adoptively transferred B cells were purified from DNFB-sensitized WT mice.

Values represent means (± SEM) from ≥ four mice of each group.

we have designated splenic IL-10-producing CD1d^{hi}CD5^{+} B cells as "B10 cells." These rare IL-10-producing B cells represented only 1%–2% of spleen B220^{+} cells and 7%–8% of peritoneal B cells in WT mice but were not normally detectable in blood or lymph nodes. B10 cell numbers were expanded substantially in the spleen (~10% of B220^{+} cells) and peritoneal cavity (~25%) but not blood or lymph nodes of hCD19Tg mice, and they were rare in *Cd19*$^{-/-}$ mice. Likewise, the abilities of B cells from hCD19Tg, WT, and *Cd19*$^{-/-}$ mice to inhibit CHS responses paralleled their capacity to secrete IL-10. Spleen and blood B cell IL-10 expression was also enhanced in WT and hCD19Tg mice during CHS responses, but not in *Cd19*$^{-/-}$ mice. Furthermore, blocking IL-10 receptor function normalized CHS responses in hCD19Tg mice. The adoptive transfer of spleen CD1d^{hi}CD5^{+} B cells from WT and CD20-deficient mice normalized CHS responses in *Cd19*$^{-/-}$ mice and CD20 mAb-treated mice, respectively, whereas spleen CD1d^{hi}CD5^{+} B cells from *Il10*$^{-/-}$ mice were without effect. Thus, CD1d^{hi}CD5^{+} B cell production of IL-10 regulated T cell-dependent CHS responses.

The existence of B10 cells explains increased CHS responses in WT mice depleted of B cells and in *Cd19*$^{-/-}$ mice that were deficient in B10 cells. Likewise, the increase in B10 cell numbers in hCD19Tg mice explains their modest CHS responses. That B cell depletion in *Cd19*$^{-/-}$ mice did not reduce CHS severity excludes the possibility that *Cd19*$^{-/-}$ B cells abnormally produce proinflammatory mediators during CHS responses. Similarly, B cell depletion increased CHS responses in WT mice to the degree observed in *Cd19*$^{-/-}$ mice, arguing that B10 cells were directly involved in regulating the magnitude of CHS responses. However, B cell depletion or blocking IL-10 receptor function normalized CHS responses in hCD19Tg mice but did not induce CHS responses to the degree observed in *Cd19*$^{-/-}$ mice. IL-10 production by non-B cells was increased in hCD19Tg mice but decreased in *Cd19*$^{-/-}$ mice. In addition, hCD19Tg mice have developmental abnormalities within their immune systems as a result of congenital CD19 overexpression and hyperresponsive B cells, including abnormal B-1 subset development (Haas et al., 2005),

altered antibody and autoantibody production (Sato et al., 1996), and alterations within their T cell compartments. Thus, cell types in addition to B10 cells may also regulate CHS responses, particularly in hCD19Tg mice.

That B10 cells were found exclusively within the relatively rare spleen CD1d^{hi}CD5^{+}CD19^{hi} B cell subset distinguishes the current results from previous studies (Mizoguchi and Bhan, 2006) but also unifies most of the current studies regarding IL-10 production by B cells. Some spleen B cells and peritoneal CD5^{+} B-1a cells are known to produce IL-10 (Brummel and Lenert, 2005; Evans et al., 2007; Fillatreau et al., 2002; Harris et al., 2000; Mauri et al., 2003; Spencer and Daynes, 1997). Specifically, spleen B cells with a CD21^{+}CD23^{-} MZ phenotype can produce IL-10 in response to CpG (Brummel and Lenert, 2005) stimulation. Spleen CD1d^{+}CD21^{+}CD23^{+} B cells with a T2-MZ precursor phenotype also produce IL-10 and can inhibit collagen-induced arthritis (Evans et al., 2007). Spleen CD5^{+} B cells also produce IL-10 after IL-12 stimulation, whereas CD5^{-} B cells do not (Spencer and Daynes, 1997). Thus, spleen B10 cells share some phenotypic markers with both CD1d^{hi}CD21^{hi} MZ B cells and CD5^{+}CD19^{hi}B220^{lo} B-1a cells. However, the frequency of spleen CD1d^{hi}CD5^{+} B cells in WT mice (2.3% ± 0.1%) was significantly lower than the frequencies of spleen B-1a (6.2% ± 0.3%, $p < 0.01$) and MZ (6.9% ± 0.4%, $p < 0.01$) B cells. IL-10 secretion was predominantly localized within the spleen CD1d^{hi}CD5^{+} B cell subset in WT mice, whereas other spleen B cells including B-1a and follicular B cells did not secrete IL-10. Because fractionating such small B cell subsets with absolute purity is technically difficult, it is possible that some IL-10-producing B cells exist that are not CD1d^{hi}CD5^{+}, although these cells may also represent B10 cells at different states of maturation. Alternatively, it is possible that B10 cells may represent an activated MZ or B-1a subset, although B10 cells did not selectively produce other cytokines (our unpublished data). Thus, the B10 spleen subset as currently identified represents a relatively rare but functionally potent population of regulatory B cells.

IL-10 production probably explains the potent ability of B10 cells to regulate T cell-mediated inflammatory responses. The adoptive transfer of 2×10^6 WT CD1d^{hi}CD5^{+} B cells normalized the CHS responses of both *Cd19*$^{-/-}$ mice and mice depleted of B cells. This is remarkable given that not all CD1d^{hi}CD5^{+} B cells produced IL-10 after LPS stimulation. Moreover, B10 cells may be antigen specific because the adoptive transfer of CD1d^{hi}CD5^{+} B cells from antigen-sensitized mice into *Cd19*$^{-/-}$ recipients inhibited CHS responses, and CD1d^{hi}CD5^{+} B cells from unsensitized mice or from mice sensitized with a different antigen were without effect. It is not known whether splenic or peritoneal IL-10-producing B cells affect immune responses centrally or whether this depends on B10 cell migration into draining lymph nodes or peripheral tissues. However, IL-10 transcripts were not increased in B cells from lymph nodes draining the sites of antigen challenge. Furthermore, B cell infiltration is not observed in the challenged ears of WT and *Cd19*$^{-/-}$ mice during CHS responses (Watanabe et al., 2007). Nonetheless, IL-10-producing B cells were found in the circulation during CHS responses and may thereby migrate in small numbers to local sites of inflammation. B cell depletion in tight-skin mice, a model for human systemic sclerosis, reduces IL-10, IL-4, IL-6, and transforming growth factor-β production in the skin, whereas B cell transcripts are not found in the lesional skin (Hasegawa et al., 2006). Similarly, B cell-deficient and *Cd19*$^{-/-}$ mice have augmented EAE responses (Fillatreau et al., 2002; Matsushita et al., 2006), although CNS B cells are rare during EAE (McGeachy et al., 2005). Thus, splenic and peritoneal IL-10-producing B cells may alter the peripheral production of IL-10 and other cytokines by non-B cells circulating through draining lymph nodes or peripheral tissue, thereby influencing systemic and local inflammatory responses.

Functional and lineage relationships among spleen B10, B-1a, and MZ B cells and peritoneal B-1a, B-1b, and peritoneal IL-10-producing B cells are possible. However, their only common features identified thus far are shared phenotypic markers. Given that B10 and B-1a cell frequencies are increased in hCD19Tg mice, and B10 and B-1a cells in *Cd19*$^{-/-}$ mice are rare (Haas et al., 2005; Sato et al., 1996), it is possible that B10 cells and B-1a cells represent different branches of a common lineage. By contrast, B-1b cell frequencies are increased in *Cd19*$^{-/-}$ mice (Haas et al., 2005). Phenotypically and histologically defined MZ B cells are also reduced in *Cd19*$^{-/-}$ mice, whereas organized MZs are difficult to identify in hCD19Tg mice by immunohistochemistry staining (Haas et al., 2005). Likewise, spleen B cells with a CD1d^{hi}CD21^{hi}B220^{+} MZ phenotype were reduced in hCD19Tg mice, and CD1d^{hi}CD5^{+} B cells numbers were increased relative to WT mice. Moreover, only ~50% of B10 cells exhibited the CD21^{hi} phenotype of MZ B cells. Nonetheless, increased numbers of splenic IL-10-producing B cells and an expanded population of MZ-like CD1d^{hi} B cells that express CD5 have been identified in mouse lupus models (Duan et al., 2007). Thus, B10 cells may regulate autoimmune disease because hCD19Tg mice develop autoimmunity with age (Sato et al., 1996). Notably, IL-10 production was not required for CD1d^{hi}CD5^{+} B cell generation because this subset was present in *Il10*$^{-/-}$ mice. Regardless, it is exciting to speculate that each B cell subset has different functions, with B10 cells producing IL-10 and regulating T cell function, whereas B-1a cells produce natural and autoantibodies, B-1b cells produce adaptive immune responses to T cell-independent antigens (Haas et al., 2005), and MZ B cells provide protection early during pathogen challenge (Martin et al., 2001).

B cell depletion in mice resulted in enhanced CHS responses, suggesting that B10 cells regulate T cell responses. B cell depletion also significantly delays the onset of collagen-induced arthritis in DBA/1J mice (Yanaba et al., 2007), skin sclerosis in tight-skin mice (Hasegawa et al., 2006), and diabetes in nonobese diabetic mice (Xiu et al., 2008). By contrast, B cell depletion early in the course of disease worsens EAE, whereas B cell depletion at the height of disease ameliorates EAE (our unpublished data). This suggests the dominance of different B cell functions, including B10 cell function, during disease progression. Similarly, B cell depletion in humans with Rituximab, a chimeric human CD20 mAb, was recently found to exacerbate ulcerative colitis (Goetz et al., 2007) and may induce psoroasis development (Dass et al., 2007). By contrast, B cell depletion with Rituximab may benefit rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis patients (Edwards and Cambridge, 2006; Hauser et al., 2008). Thus, the benefit of B cell-depletion therapy is likely to vary according to disease and the relative involvement of different B and T cell subsets.

Moreover, the identification and functional characterization of the rare IL-10-producing CD1d^{hi}CD5^{+} B10 cell subset in this study may lead to the identification of B10 cells in humans that similarly regulate inflammatory responses. Further defining the role of B10 cells and other B cell subsets in disease and regulatory function in vivo may provide new insights and therapeutic approaches for treating inflammatory and organ-specific autoimmunity in addition to other diseases.

EXPERIMENTAL PROCEDURES

Mice and Immunotherapy

WT C57BL/6 and *Il10*$^{-/-}$ (B6.129P2-*Il10*^{tm1Cgn}/J) mice (Kuhn et al., 1993) were from the Jackson Laboratory (Bar Harbor, ME). CD20-deficient, *Cd19*$^{-/-}$, and human CD19Tg (h19-1 line) mice were as described (Sato et al., 1996, 1997; Uchida et al., 2004b). Specifically, *Cd19*$^{-/-}$ and hCD19Tg mice were backcrossed with C57BL/6 mice for 14 and seven generations, respectively.

B cells were depleted with sterile CD20 and human CD19 mAbs (250 μg) or isotype-matched control mAbs (250 μg) injected in 200 μl PBS through lateral tail veins. All mice were bred in a specific pathogen-free barrier facility and used at 8–12 weeks of age. The Duke University Animal Care and Use Committee approved all studies.

Abs and Immunofluorescence Analysis

Mouse CD20-specific mouse mAb MB20-11 (IgG2c) was used as described (Uchida et al., 2004b). The mouse anti-human CD19 (hCD19) mAb FMC63 (IgG2a, provided by Dr. Heddy Zola, Child Health Research Institute, Adelaide, South Australia) was used as described (Yazawa et al., 2005). Other mAbs included the following: B220 mAb RA3-6B2 (provided by Dr. Robert Coffman, DNAX Corp., Palo Alto, CA); CD19 (1D3), CD5 (53-7.3), CD1d (1B1), CD21/35 (7G6), CD23 (B3B4), CD24 (M1/69), CD25 (PC61), CD43 (S7), and CD11b (M1/70) (from BD PharMingen, San Diego, CA); IgM (11/41) (from eBioscience, San Diego, CA); and IgD (11-26) (from Southern Biotechnology Associates, Birmingham, AL). Intracellular staining for Foxp3 (FJK-16 s, eBioscience) used the Cytofix/Cytoperm kit (BD PharMingen). Single-cell suspensions of spleen, peripheral lymph node (cervical, paired axillary, and inguinal), and mesenteric lymph node were generated by gentle dissection. Peritoneal-cavity leukocytes were isolated with 10 ml of cold (4°C) PBS injected into the peritoneum of sacrificed mice followed by gentle massage of the abdomen. Intestinal Peyer's patches were isolated as described (Venturi et al., 2003). Peripheral blood mononuclear cells were isolated from heparinized blood after centrifugation over a discontinuous Lymphoprep (Axis-Shield PoC As, Oslo, Norway) gradient. Viable cells were counted with a hemocytometer, with relative lymphocyte percentages determined by flow-cytometry analysis. Single-cell leukocyte suspensions were stained on ice with predetermined optimal concentrations of each antibody for 20–60 min and fixed as described (Sato et al., 1996). Cells with the light-scatter properties of lymphocytes were analyzed by 2–4-color immunofluorescence staining and FACScan or FACSCalibur flow cytometers (Becton Dickinson, San Jose, CA). Background staining was determined with unreactive isotype-matched control mAbs (Caltag Laboratories, San Francisco, CA) with gates positioned to exclude ≥ 98% of unreactive cells.

Contact Hypersensitivity Reaction

CHS reactions were induced with oxazolone as described (Tedder et al., 1995). In brief, mice were sensitized with 25 μl of oxazolone (100 mg/ml, Sigma, St. Louis, MO) in acetone/olive oil (4:1 v/v) applied evenly for 2 consecutive days on a shaved hind flank. On day 5, sensitized mice were challenged by application of 10 μl of oxazolone solution (10 mg/ml) in acetone/olive oil (4:1) to the right ear (5 μl on the dorsal side and 5 μl on the ventral side). In certain experiments, 25 μl of 0.5% DNFB (Sigma) in acetone/olive oil (4:1) was used as the sensitization agent. An identical amount of acetone/olive oil (4:1) was administered to the left ear. In some experiments, mice were treated with IL-10 receptor (1B1.3a; BD PharMingen) or control mAb (250 μg) 1 hr before and 47 hr after oxazolone challenge. The thickness of the central portion of each ear lobe was measured at 24, 48, 72, and 96 hr after challenge with a constant-force, calibrated digital thickness gage (Mitsutoyo Corp., Tokyo, Japan). Each ear lobe was measured three times at each time interval in a blinded fashion, with the mean of these values used for analysis.

B Cell Isolation and Stimulation

B220 mAb- or CD19 mAb-coated microbeads (Miltenyi Biotech, Auburn, CA) were used to purify B cells by positive selection following the manufacturer's instructions. For cytokine production, 4 × 10^5 purified B cells were cultured either with LPS (10 μg/ml, *Escherichia coli* serotype 0111: B4, Sigma) or with mouse IgM-specific goat F(ab')$_2$ antibody (20 μg/ml, Cappel, Aurora, OH) plus CD40 mAb (1 μg/ml, HM40-3; BD PharMingen) in 0.2 ml of complete medium in a 96-well flat-bottom plate for 48 hr. Culture supernatant fluid was collected after 48 hr to assess cytokine production.

Flow Cytometric Analysis of Intracellular IL-10 Synthesis

In brief, isolated leukocytes or purified cells were resuspended (1 × 10^6 cells/ml) with LPS (10 μg/ml), PMA (50 ng/ml; Sigma), ionomycin (500 ng/ml; Sigma), and monensin (2 μM; eBioscience) for 5 hr. For IL-10 detection, Fc receptors were blocked with mouse Fc receptor-specific mAb (2.4G2; BD PharMingen) before cell-surface staining and then fixed and permeabilized with the Cytofix/Cytoperm kit (BD PharMingen) according to the manufacturer's instructions. Permeabilized cells were stained with phycoerythrin-conjugated IL-10 mAb (JES5-16E3; BD PharMingen). Leukocytes from *Il10*$^{-/-}$ mice served as negative control to demonstrate specificity and background-staining levels.

Cell Sorting and Adoptive Transfers

Splenic B cells were purified with CD19 mAb-coupled microbeads (Miltenyi Biotech). In addition, CD1d^{hi}CD5^{+} B cells were selected with a FACSVantage SE flow cytometer (Becton Dickinson) with purities of ~85%–95%. After isolation, 2 × 10^6 CD1d^{hi}CD5^{+} or non-CD1d^{hi}CD5^{+} B cells were transferred intravenously into *Cd19*$^{-/-}$ or B cell-depleted recipient mice before CHS induction.

Statistical Analysis

All data are shown as mean ± standard error of the mean (SEM). The significance of differences between sample means was determined with the Student's t test.

SUPPLEMENTAL DATA

Additional Experimental Procedures and two figures are available at http://www.immunity.com/cgi/content/full/28/5/639/DC1/.

ACKNOWLEDGMENTS

These studies were supported by NIH grants CA105001, CA96547, AI56363, and CA098492. J.D.B. is supported by grants from Association pour la Recherche contre le Cancer (ARC), Foundation René Touraine, and the Philippe Foundation. T.F.T. is a paid consultant for MedImmune and Angelica Therapeutics, Inc. K.M.H. and J.C.P. are paid consultants for Angelica Therapeutics.

Received: November 6, 2007
Revised: January 15, 2008
Accepted: March 6, 2008
Pulished online: May 15, 2008

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