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Regulatory B Cells Control T Cell Autoimmunity Through IL-21-Dependent Cognate Interactions

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Summary

B cells regulate immune responses by producing antigen-specific antibody[1]. However, specific B cell subsets can also negatively regulate immune responses, validating the existence of regulatory B cells[2–4]. Human and mouse regulatory B cells (B10 cells) with the ability to express the inhibitory cytokine IL-10 have been identified[2–5]. Although rare, B10 cells are potent negative regulators of antigen-specific inflammation and T cell-dependent autoimmune diseases in mice[5–7]. How B10 cell IL-10 production and regulation of antigen-specific immune responses are controlled *in vivo* without inducing systemic immunosuppression are unknown. Using a mouse model for multiple sclerosis, we show here that B10 cell maturation into functional IL-10-secreting effector cells that inhibit *in vivo* autoimmune disease requires IL-21 and CD40-dependent cognate interactions with T cells. Moreover, the *ex vivo* provision of CD40 and IL-21 receptor signals can drive B10 cell development and expansion by four-million-fold and generate B10 effector cells producing IL-10 that dramatically inhibit disease symptoms when transferred into mice with established autoimmune disease. Thereby, the *ex vivo* expansion and reinfusion of autologous B10 cells may provide a novel and effective *in vivo* treatment for severe autoimmune diseases that are resistant to current therapies.

Keywords

Rodent; B cells; autoimmunity; cell differentiation; tolerance

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Reprints and permissions information isavailable at www.nature.com\reprints. T.F.T. is a consultant and shareholder for Angelica Therapeutics, Inc. R.S. and W.J.L. are inventors on patents and patent applications related to IL-21. The other authors have no financial conflicts of interest.

A subset of regulatory B cells has been functionally defined in humans and mice by their ability to express IL-10[5–7]. B cells that are competent to express IL-10 following 5 h of *ex vivo* phorbol ester and ionomycin stimulation are called B10 cells[6] to distinguish them from other regulatory B cells that modulate immune responses through other mechanisms[2,8]. B10 cells are found at low frequencies (1–5%) in naïve mice but expand with autoimmunity[9]. Spleen B10 cells are predominantly found within the minor CD1d^{hi}CD5^{+} B cell subpopulation along with B10 progenitor (B10pro) cells that are induced to become IL-10-competent during *in vitro* culture with agonistic CD40 monoclonal antibody (mAb) or lipopolysaccharide (LPS)[9,10]. The capacity of human and mouse B10 cells to produce IL-10 is central to their ability to negatively regulate inflammation and autoimmune disease, as well as innate and antigen-specific adaptive immune responses[5–7,9–12], but the physiologic signals controlling IL-10 production *in vivo* are unknown.

B10 cell immunoregulation is antigen-specific, and B cell antigen receptor (BCR) specificity dramatically influences B10 cell development[6,9]. Receptors or pathways that positively or negatively regulate BCR signaling can also modulate B10 cell numbers *in vivo*. For example, CD19-deficient (CD19$^{-/-}$) mice are essentially devoid of regulatory B10 cells, which leads to exacerbated inflammation and disease symptoms during contact hypersensitivity and in the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis[6,7]. IL-10 itself is not required for B10 cell development since B cells with the capacity to express IL-10 reporter genes develop normally in IL-10$^{-/-}$ mice[13]. B10 cell numbers are also normal in T cell-deficient nude mice and in mice deficient in expression of major histocompatibility complex class II (MHC-II) or CD40 molecules that are important for cognate B cell-T cell interactions[9]. Consequently, appropriate BCR signals are thought to select a subset of B cells to become IL-10-competent B10 cells. Innate pathogen-induced signals also influence regulatory B10 cell IL-10 production *in vivo*[3,13]. Little is otherwise known about how B10 cell IL-10 production is regulated, and it remains unclear how such rare B cells exert such potent *in vivo* effects and selectively inhibit antigen-specific T cell function during inflammation and autoimmunity.

To identify signals that regulate B10 cells *in vivo*, purified B cells were cultured with cytokines known to influence B cell function. Stimulation with IL-21, but not IL-4, -6, -10, -12, -23 or -27, induced 2.7- to 3.2-fold higher B10 cell frequencies and 4.4- to 5.3-fold more IL-10 secretion ($p<0.01$) at 48 and 72 h, respectively, while interferon-γ (IFN-γ) or transforming growth factor-β (TGF-β) reduced IL-10^{+} B cell frequencies by 56% ($p<0.05$; Fig. 1a). In fact, IL-21 induced B10 cells to produce IL-10 without a need for *in vitro* stimulation (Fig. 1b, Supplementary Fig. 1a) and induced IL-10 secretion at levels similar to LPS stimulation (Fig. 1a). IL-21 also induced a 3-fold increase in IL-10^{+} B cells within the spleen CD1d^{hi}CD5^{+} B cell subset that is enriched for B10pro and B10 cells, but it did not induce significant numbers of IL-10^{+} B cells among the CD1d^{lo}CD5^{-} subset (Fig. 1b). T cell-derived IL-21 plays multiple important roles in B cell effector function[14–18], and IL-21 is a potent inducer of T cell IL-10 production[19,20]. Both B10 and non-B10 cells expressed cell surface IL-21 receptor (IL-21R) at similar levels (Fig. 1c). Despite this, *ex vivo* B10 and B10+B10pro cell and CD1d^{hi}CD5^{+} B cell numbers were similar in IL-21R-deficient (IL-21R$^{-/-}$), wild type, MHC-II$^{-/-}$ and CD40$^{-/-}$ mice (Supplementary Fig. 1b–d, data not

shown). However, IL-21R expression was required for B10 cell expansion *in vivo* following myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}) immunizations to induce EAE (Fig. 1d). Thus, IL-21R-generated signals induced B10 cell expansion and IL-10 secretion *in vivo*.

Whether B10 cells require IL-21 to induce their regulatory function *in vivo* was determined by the adoptive transfer of IL-21R$^{-/-}$ B cells into CD19$^{-/-}$ mice before the induction of EAE. Because CD19$^{-/-}$ mice are B10 cell-deficient (Fig. 1d), their EAE disease severity is worse (Fig. 2a)[7,11]. The adoptive transfer of wild type CD1d^{hi}CD5^{+} B cells normalized EAE severity in CD19$^{-/-}$ mice. By contrast, the transfer of CD1d^{hi}CD5^{+} B cells from IL-21R$^{-/-}$ or IL-10$^{-/-}$ mice or wild type CD1d^{lo}CD5^{-} non-B10 cells did not alter disease. Because CD4^{+} T cells are a major source of IL-21, we determined whether cognate B10-T cell interactions also controlled B10 cell-mediated suppression of EAE. The transfer of CD1d^{hi}CD5^{+} B cells from MHC-II$^{-/-}$ or CD40$^{-/-}$ mice into CD19$^{-/-}$ mice before MOG immunizations did not reduce EAE disease severity (Fig. 2a, bottom right two panels). CD1d^{lo}CD5^{-} B cells from IL-21R$^{-/-}$, CD40$^{-/-}$ or MHC-II$^{-/-}$ mice were also without effect (data not shown). EAE is also exacerbated in wild type mice depleted of mature B cells by CD20 mAb[7,11]. However, transfer of CD1d^{hi}CD5^{+} B cells from CD20$^{-/-}$ mice but not MHC-II$^{-/-}$CD20$^{-/-}$ mice normalized disease severity in this model, and CD1d^{lo}CD5^{-} B cells from CD20$^{-/-}$ or MHC-II$^{-/-}$CD20$^{-/-}$ mice were without effect (Fig. 2b, data not shown). Similarly, the adoptive transfer of *in vitro* activated CD1d^{hi}CD5^{+} B cells from wild type mice significantly reduced EAE disease severity in wild type mice, whereas activated MHC-II$^{-/-}$ CD1d^{hi}CD5^{+} or wild type CD1d^{lo}CD5^{-} B cells had no effect (Fig. 2c, data not shown). Thus, regulatory B10 cell function required IL-10 expression, IL-21R signaling, as well as CD40 and MHC-II interactions, thereby potentially explaining antigen-specific B10 cell effector function[6].

To determine whether cognate B10-T cell interactions regulate antigen-specific T cell proliferation *in vivo*, B10 cell function was assessed in MOG_{35-55}-immunized CD19$^{-/-}$ mice following the adoptive transfer of dye-labeled CD4^{+} T cells from transgenic mice expressing antigen receptors (TCR^{MOG}) specific for MOG_{35-55} peptide[21]. CD1d^{hi}CD5^{+} B cells from naïve wild type mice significantly reduced TCR^{MOG} CD4^{+} T cell proliferation as measured by *in vivo* dye dilution (Fig. 3a). CD1d^{hi}CD5^{+} B cells obtained from mice with EAE were even more potent inhibitors of T cell proliferation, while CD1d^{lo}CD5^{-} B cells from wild type mice or CD1d^{hi}CD5^{+} B cells from IL-10$^{-/-}$, IL-21R$^{-/-}$, CD40$^{-/-}$, or MHC-II$^{-/-}$ mice were without effect. CD1d^{hi}CD5^{+} B cells from naïve or antigen-experienced wild type mice also significantly reduced TCR^{MOG} CD4^{+} T cell IFN-γ and IL-17 production in MOG_{35-55}-immunized CD19$^{-/-}$ mice, while CD1d^{hi}CD5^{+} B cells from IL-10$^{-/-}$, IL-21R$^{-/-}$, CD40$^{-/-}$ or MHC-II$^{-/-}$ mice did not (Fig. 3b). The ability of B10 cells to inhibit T cell IL-17 production is particularly important since pathogenic T_H17 T cells induce EAE and can produce IL-21[22]. The majority of T follicular helper cells isolated from mice with MOG_{35-55}-induced EAE also express IL-21[23], and CD19$^{-/-}$ mice have T follicular helper cells (Supplementary Fig. 2). Thus, B10 and T cells may require intimate interactions during reciprocal IL-10 and IL-21 production to optimally regulate antigen-specific disease (Fig. 3c).

To verify that T cell-derived IL-21 and CD40 signals drive B10 cell expansion and IL-10 production, B cells were cultured using optimized conditions that promote mouse B10 cell expansion *in vivo*[24] and B cell expansion *in vitro*[25]. B cells were cultured on monolayers of NIH-3T3 cells expressing the T cell ligand for CD40 (CD154) and BLyS in the presence of IL-4 for 4 days to induce B10pro cell maturation into IL-10-competent B10 cells. The B cells were then cultured on fresh NIH-3T3-CD154/BLyS cells with exogenous IL-21 for 5 days, which was essential to optimally expand B10 cells and induce IL-10 production (Fig. 4a). After the 9 day culture period, B cell and B10 cell numbers were increased by 25,000- and 4,000,000-fold, respectively, with 38% of the B cells actively producing IL-10 (Fig. 4b). The vast majority of IL-10^{+} B cells in the cultures expressed CD5 (Fig. 4c), facilitating their purification and underscoring the dramatic effect of IL-21 on B10 cell numbers *in vitro*.

In vitro-expanded CD5^{+} B10 cells retained their regulatory function. The transfer of CD5^{+} B10 cells dramatically reduced EAE disease severity in wild type mice, even when given after the appearance of disease symptoms, while CD5^{-} B cells were without effect (Fig. 4d). Although the *in vitro* expansion of B10 cells required both IL-21R and CD40 signals, MHC-II expression was not required (Fig. 4e). However, *in vitro*-expanded MHC-II$^{-/-}$CD5^{+} B10 cells and IL-10$^{-/-}$ CD5^{+} B cells did not regulate EAE disease severity (Fig. 4f), further documenting a requirement for IL-10 and cognate interactions in the regulation of T cell-mediated disease. B10 cells did not expand during *in vitro* cultures of B cells from CD19$^{-/-}$ mice or MD4 transgenic mice[26] that have a fixed BCR specific for egg lysozyme (Fig. 4e), further underscoring the importance of BCR specificity and signaling in B10 cell generation. Otherwise, *in vitro*-expanded B10 effector cells were potent regulators of both disease initiation and progression.

This study demonstrates that CD40 signals induce B10pro cell acquisition of IL-10 competence with IL-21 driving B10 cell expansion and effector cell generation. These critical checkpoints in B10 cell development may lead to localized IL-10 production that blunts antigen-specific T cell responses during cognate interactions (Fig. 3c) without untoward immunosuppression. Transient IL-10 production by B10 cells *in vivo* may further restrict the effects of IL-10 secretion[13]. B10 effector cells may also regulate T cell responses to autoantigens in addition to MOG once inflammation and tissue destruction are initiated by MOG_{35-55} immunization. Since human and mouse B10 cells are also potent regulators of macrophage and dendritic cell function[5,12], T cell induction of B10 effector cells may also contribute to EAE resolution by restraining monocyte and dendritic cell activation. These collective results may explain in part why EAE is exacerbated in the absence of IL-21 signaling[27]. By contrast, TGF-β and IFN-γ may counterbalance B10 cell expansion *in vivo* based on the current *in vitro* findings (Fig. 1a). Regulatory T cells provide an independent layer of regulation during EAE since their expansion, accumulation in the central nervous system, and suppressive activity are normal when B10 cells are absent[11,28]. The *in vitro* recapitulation of these collective signals induced a several million-fold expansion of B10 cells and their functional maturation into potent B10 effector cells that reversed established autoimmune disease (Fig. 4). In addition to BCR specificity, MHC-II expression remained an important checkpoint for B10 effector cell regulatory function during EAE (Fig. 4f), as first described for regulatory type II monocytes[29]. Since autoimmunity has multigenic

origins and autoantigens vary between patients and disease, *in vitro* expansion of the rare pool of human blood B10pro and B10 effector cells[5] may provide a potent future immunotherapy for individuals with severe autoimmune disease.

FULL METHODS

Mice

C57BL/6, IL-10$^{-/-}$ (B6.129P2-*Il10*^{tmlCgn}/J)[31], CD40$^{-/-}$ (B6.129P2-*CD40*^{tm1Kik}/J), and MD4 (C57BL/6-*Tg(TghelMD4)4Ccg*/J) mice were from the Jackson Laboratory (Bar Harbor, ME). MHC-II$^{-/-}$ (B6.129-*H2-Ab1*^{tm1Gru}*B2m*^{tmJae}N17) mice (Taconic Farms, Inc., Hudson, NY) were as described[32]. CD19$^{-/-}$ mice were backcrossed onto the C57BL/6 background for 14 generations[33,34]. IL-21R$^{-/-}$ mice were as described[18]. TCR^{MOG} transgenic mice[21] (Thy1.2^{+}, provided by Dr. V. K. Kuchroo, Harvard Medical School, Boston, MA) were crossed to C57BL/6.Thy1.1 mice to generate Thy1.1-expressing T cells. All mice were bred in a specific pathogen-free barrier facility and used at 6–12 wks of age. The Duke University Animal Care and Use Committee approved all studies.

Cell preparation

Single-cell suspensions from spleens and peripheral lymph nodes (paired axillary and inguinal) were generated by gentle dissection, with the cells passed through 70-mm cell strainers (BD Biosciences, San Diego, CA) followed by percoll gradient (70/37%) centrifugation. Lymphocytes were collected from the 37:70% interface and washed. MACS (Miltenyi Biotech, Auburn, CA) was used to purify lymphocyte populations according to the manufacturer's instructions. CD19 mAb-coated microbeads and CD4^{+} T cell isolation kits (Miltenyi Biotech) were used to purify B cells and CD4^{+} T cells, respectively. When necessary, the cells were enriched a second time using a fresh MACS column to obtain >95% cell purities.

Immunofluorescence analysis

FITC-, PE-, PE-Cy5-, PE-Cy7-, or APC-conjugated CD1d (1B1), CD4 (H129.19), CD5 (53–7.3), CD19 (1D3), B220 (RA3-6B2), and Thy1.1 (OX-7) mAbs were from BD Biosciences. PE-conjugated IL-21R (4A9) mAb was from BioLegend (San Diego, CA). Intracellular staining used mAbs reactive with IL-10 (JES5-16E3), IL-17 (17B7), and IFN-γ (XMG1.2) (all from eBioscience) and Cytofix/Cytoperm kits (BD Biosciences). Background staining was assessed using non-reactive, isotype-matched control mAbs (Caltag Laboratories, San Francisco, CA). For two- to six-color immunofluorescence analysis, single cell suspensions (10^6 cells) were stained at 4°C using predetermined optimal mAb concentrations for 20 min as described[35]. Blood erythrocytes were lysed after staining using FACS™ Lysing Solution (Becton Dickinson, San Jose, CA).

B cell intracellular IL-10 expression was visualized by immunofluorescence staining and analyzed by flow cytometry as described[6,30]. Briefly, isolated leukocytes or purified cells were resuspended (2×10^6 cells/ml) in complete medium (RPMI 1640 media containing 10% FCS, 200 μg/ml penicillin, 200 U/ml streptomycin, 4 mM L-Glutamine, and 5×10^{-5} M 2-mercaptoethanol, all from Gibco, Carlsbad, CA) with LPS (10 μg/ml, *Escherichia coli*

serotype 0111: B4, Sigma), PMA (50 ng/ml; Sigma), ionomycin (500 ng/ml; Sigma) and monensin (2 μM; eBioscience) for 5 h in 48-well flat-bottom plates. In some experiments, the cells were incubated for 48 h with an agonistic anti-mouse CD40 mAb (1 μg/ml; HM40-3 mAb; BD Pharmingen) as described[9]. For IL-10 detection, Fc receptors were blocked with mouse Fc receptor mAb (2.4G2; BD PharMingen), and dead cells were detected using a LIVE/DEAD® Fixable Violet Dead Cell Stain Kit (Invitrogen-Molecular Probes) before cell surface staining. Stained cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD PharMingen) according to the manufacturer's instructions and stained with PE-conjugated mouse anti-IL-10 mAb. Splenocytes from IL-$10^{-/-}$ mice served as negative controls to demonstrate specificity and to establish background IL-10 staining levels. For T cell intracellular cytokine staining, lymphocytes were stimulated *in vitro* with PMA (50 ng/ml; Sigma, St. Louis, MO) and ionomycin (1 μg/ml; Sigma) in the presence of Brefeldin A (BFA, 1 μl/ml; eBioscience) for 5 h before staining. Viable cells with the forward and side light scatter properties of lymphocytes were analyzed using a FACScan flow cytometer (Becton Dickinson) or BD FACSCanto™ II (BD Biosciences).

In vitro B cell cultures

Purified splenic B cells (1×10^6/ml) were cultured in RPMI 1640 medium containing 10% FBS, 1% HEPES, 1% L-Glutamate, 1% Pen/Strep, and 0.1% 2-mercapthoethanol, and either recombinant IFN-γ (10 ng/ml IL-4 (2 ng/ml), IL-6 (10 ng/ml) or IL-21 (100 ng/ml) (from e-Bioscience); TGF-β (10 ng/ml), IL-10 (10 ng/ml), or IL-12 (10 ng/ml) (from R&D systems, Minneapolis, MN); or IL-23 (20 ng/ml) and IL-27 (100 ng/ml) (Biolegend), or LPS (10 μg/ml) before B10 cell numbers and culture supernatant fluid IL-10 concentrations were determined. In separate experiments, purified spleen B cells were cultured with NIH-3T3 cells expressing CD154 and BLyS as described[25,36] with exogenous recombinant IL-4 (2 ng/ml) or IL-21 (10 ng/ml) added to the cultures. For adoptive transfer experiments, cultured $CD5^+$ and $CD5^-$ B cells were purified by cell sorting (FACSVantage SE, Becton Dickinson), with purities of 95–98%. After purification, 1×10^6 cells were immediately transferred i.v. into each recipient mouse. In some experiments, CD40 mAb (clone HM40-3; hamster, no azide/endotoxin-free, BD Pharmingen, San Jose, CA) was added to cultures where indicated.

EAE induction

EAE was induced in 6- to 8-week-old female mice by subcutaneous immunization with 100 μg of MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK; NeoMPS, San Diego, CA) emulsified in CFA containing 200 μg of heat-killed *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI) on day 0. Additionally, mice received 200 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) i.p. in 0.5 ml of PBS on days 0 and 2. Clinical signs of disease were assessed daily with a 0 to 6 point scoring system: 0, normal; 1, flaccid tail; 2, impaired righting reflex and/or gait; 3, partial hind limb paralysis; 4, total hind limb paralysis; 5, hind limb paralysis with partial forelimb paralysis; 6, moribund state, as described[37]. Moribund mice were given disease severity scores of 6 and euthanized.

Adoptive transfer experiments

B cells from naïve mice or mice with EAE (day 28) were first enriched using CD19 mAb-coated microbeads, stained for cell surface CD19, CD1d and CD5 expression, with $CD1d^{hi}CD5^{+}$ and $CD1d^{lo}CD5^{-}$ B cells purified by cell sorting as described[6,30] with purities of 95–98%. After purification, the $CD1d^{hi}CD5^{+}$ or $CD1d^{lo}CD5^{-}$ B cells (1×10^{6}) were immediately transferred i.v. into recipient mice, with B10 cells representing 13–20% and <0.1% of the transferred cells[6,30]. In some experiments, donor Thy1.1 $CD4^{+}$ T cells were isolated from pooled spleens and lymph nodes of TCR^{MOG} transgenic mice, then labeled with CFSE Vybrant™ CFDA SE fluorescent dye (5 mM; CFSE; Invitrogen) and transferred i.v. (5×10^{6}/mouse) into Thy1.2 congenic recipients. Five days after adoptive transfer, the TCR^{MOG} $CD4^{+}$ T cells were assessed by flow cytometry.

Statistical analysis

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

Supplementary Material

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Acknowledgments

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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. Fillatreau S. Novel regulatory functions for Toll-like receptor-activated B cells during intracellular bacterial infection. Immunol. Rev. 2011; 240:52–71. [PubMed: 21349086]
4. Mauri C. Regulation of immunity and autoimmunity by B cells. Curr. Opin. Immunol. 2010; 22:761–7657. [PubMed: 21087847]
5. Iwata Y, et al. 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]
6. Yanaba K, et al. 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]
7. 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]
8. Ray A, Basu S, Williams CB, Salzman NH, Dittel BN. A novel IL-10-independent regulatory role for B cells in suppressing autoimmunity by maintenance of regulatory T cells via GITR ligand. J. Immunol. 2012; 188:3188–3198. [PubMed: 22368274]
9. 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]

10. Haas KM, et al. 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. 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]
12. Horikawa M, Minard-Colin V, Matsushita T, Tedder TF. Regulatory B cell production of IL-10 inhibits lymphoma depletion during CD20 immunotherapy in mice. J. Clin. Invest. 2011; 121:4268–4280. [PubMed: 22019587]
13. Maseda D, et al. Regulatory B10 cells differentiate into antibody-secreting cells after transient IL-10 production in vivo. J. Immunol. 2012; 188:1036–1048. [PubMed: 22198952]
14. Spolski R, Leonard WJ. IL-21 and T follicular helper cells. Int. Immunol. 2009; 22:7–12. [PubMed: 19933709]
15. Linterman MA, Vinuesa CG. T follicular helper cells during immunity and tolerance. Prog. Mol. Biol. Transl. Sci. 2010; 92:207–248. [PubMed: 20800823]
16. Ettinger R, et al. IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. J. Immunol. 2005; 175:7867–7879. [PubMed: 16339522]
17. Ozaki K, et al. Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. J. Immunol. 2004; 173:5361–5371. [PubMed: 15494482]
18. Ozaki K, et al. A critical role for IL-21 in regulating immunoglobulin production. Science. 2002; 298:1630–1634. [PubMed: 12446913]
19. Spolski R, Kim HP, Zhu W, Levy DE, Leonard WJ. IL-21 mediates suppressive effects via its induction of IL-10. J. Immunol. 2009; 182:2859–2867. [PubMed: 19234181]
20. Pot C, et al. Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. J. Immunol. 2009; 183:797–801. [PubMed: 19570826]
21. Bettelli E, et al. Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis. J. Exp. Med. 2003; 197:1073–1081. [PubMed: 12732654]
22. Bettelli E, et al. Reciprocal developmental pathways for the generation of pathogenic effector T_H17 and regulatory T cells. Nature. 2006; 441:235–238. [PubMed: 16648838]
23. Chen G, et al. Regulation of the IL-21 gene by the NF-κB transcription factor c-Rel. J. Immunol. 2010; 185:2350–2359. [PubMed: 20639489]
24. Poe JC, et al. Amplified B lymphocyte CD40 signaling drives regulatory B10 cell expansion in mice. PLoS ONE. 2011; 6:e22464. [PubMed: 21799861]
25. Nojima T, et al. In-vitro derived germinal centre B cells differentially generate memory B or plasma cells in vivo. Nature Comm. 2011; 2:465.
26. Goodnow CC, et al. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. Nature. 1988; 334:676–682. [PubMed: 3261841]
27. Coquet JM, Chakravarti S, Smyth MJ, Godfrey DI. Cutting edge: IL-21 is not essential for Th17 differentiation or experimental autoimmune encephalomyelitis. J. Immunol. 2008; 180:7097–7101. [PubMed: 18490706]
28. Hoehlig K, et al. Activation of $CD4^+$ $Foxp3^+$ regulatory T cells proceeds normally in the absence of B cells during EAE. Eur. J. Immunol. 2012; 42:1164–1173. [PubMed: 22539290]
29. Weber MS, et al. Type II monocytes modulate T cell-mediated central nervous system autoimmune disease. Nat. Med. 2007; 13:935–943. [PubMed: 17676050]
30. Matsushita T, Tedder TF. Identifying regulatory B cells (B10 cells) that produce IL-10. Methods Mol. Biol. 2011; 677:99–111. [PubMed: 20941605]
31. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. Cell. 1993; 75:263–274. [PubMed: 8402911]
32. Grusby MJ, et al. Mice lacking major histocompatibility complex class I and class II molecules. Proc. Natl. Acad. Sci. U S A. 1993; 90:3913–3917. [PubMed: 8483910]

33. Sato S, Ono N, Steeber DA, Pisetsky DS, Tedder TF. CD19 regulates B lymphocyte signaling thresholds critical for the development of B-1 lineage cells and autoimmunity. J. Immunol. 1996; 157:4371–4378. [PubMed: 8906812]

34. Sato S, Steeber DA, Jansen PJ, Tedder TF. CD19 expression levels regulate B lymphocyte development: human CD19 restores normal function in mice lacking endogenous CD19. J. Immunol. 1997; 158:4662–4669. [PubMed: 9144478]

35. Zhou L-J, et al. Tissue-specific expression of the human CD19 gene in transgenic mice inhibits antigen-independent B lymphocyte development. Mol. Cell. Biol. 1994; 14:3884–3894. [PubMed: 7515149]

36. Tedder, TF.; Wagner, N.; Engel, P. Leukocyte Typing V: White Cell Differentiation Antigens. Schlossman, SF., et al., editors. Vol. Vol. 1. Oxford University Press; 1995. p. 483-504.

37. 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]

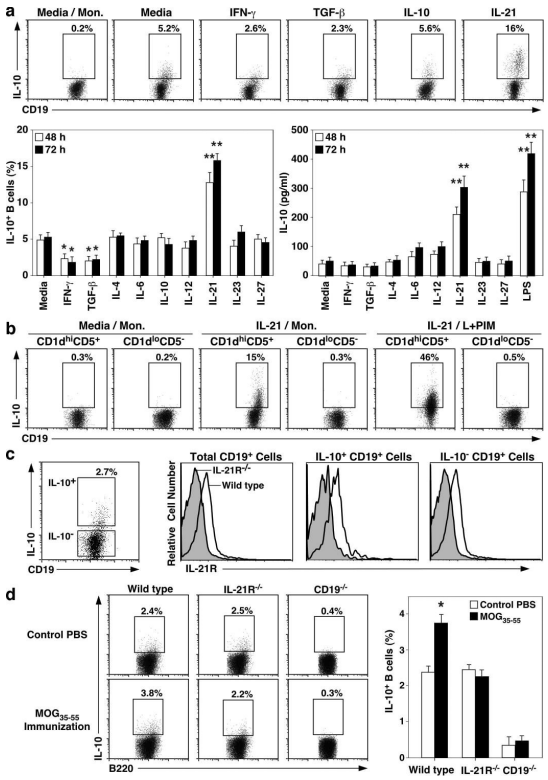

Figure 1.

IL-21 induces regulatory B10 cell function. **a**, IL-21 induces B10 cell IL-10 production and secretion. purified spleen $CD19^+$ B cells from wild type mice were cultured in medium alone or containing the indicated cytokines or LPS. To visualize IL-10-competent B cells, LPS, PMA, ionomycin and monensin (L+PIM) were added to the cultures 5 h before the cells were stained for cell surface CD19 and cytoplasmic IL-10 expression and analyzed by flow cytometry. Representative histograms show IL-10^+ cell frequencies within the indicated gates, with background staining shown for cells cultured with monensin (Mon.) alone. Bar graphs indicate mean (±s.e.m.) I L-10^+ B cell frequencies or culture supernatant fluid IL-10 concentrations at 48 or 72 h from three independent experiments using individual mice. **b**, IL-21 induces $CD1d^{hi}CD5^+$ B cell IL-10 production. Purified spleen $CD1d^{hi}CD5^+$ or $CD1d^{lo}CD5^-$ B cells from wild type mice were cultured with media alone or containing IL-21 for 48 h before IL-10^+ B cell frequencies were assessed as in (a). **c**, B10 cells express IL-21R. $CD19^+$ splenocytes purified from wild type mice were cultured with L +PIM for 5 h before cell surface CD19 and IL-21R, and cytoplasmic IL-10 staining to identify IL-10-competent B10 cells (dot plot, left panel). Representative IL-21R expression by IL-10^+ and IL-10^- B cells from wild type mice is shown in comparison with control B cells from IL-$21R^{-/-}$ mice (gray histograms). Results represent three independent experiments using individual mice. **d**, IL-21R expression is required for B10 cell expansion *in vivo* following MOG immunization. B10 cell numbers were assessed in wild type, IL-$21R^{-/-}$ or $CD19^{-/-}$ mice 7 days after saline (PBS) or $MOG_{35\text{-}55}$ immunization. Representative flow cytometry histograms are shown. Bar graphs indicate mean (±s.e.m.) B10 cell frequencies (≥3 mice per group). a and d, Significant differences between sample means are indicated; *, $p<0.05$; **, $p<0.01$.

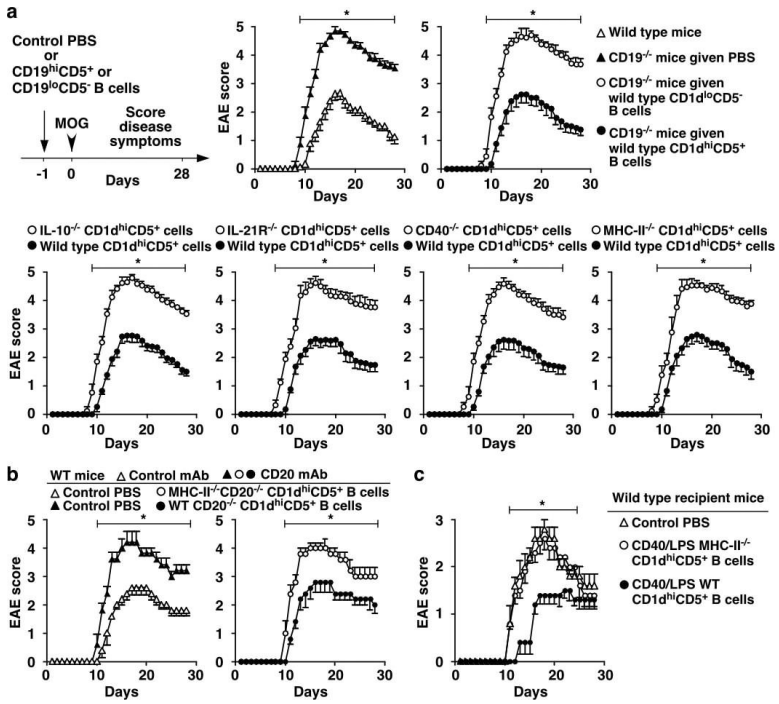

Figure 2.

B10 cells require IL-10, IL-21R, CD40, and MHC-II expression to regulate EAE severity. **a**, One day before $CD19^{-/-}$ or wild type (WT) mice were immunized with MOG_{35-55} on day 0, the $CD19^{-/-}$ mice received PBS or purified spleen $CD1d^{hi}CD5^{+}$ or $CD1d^{lo}CD5^{-}$ B cells from either wild type, IL-$10^{-/-}$, IL-$21R^{-/-}$, $CD40^{-/-}$, or MHC-$II^{-/-}$ mice. Mice were scored daily thereafter for disease severity. The top two graphs show data from the same experiment, but were separated to facilitate visualization of the overlapping curves. **b**, B10 cells require MHC-II expression to regulate EAE severity in wild type mice treated with CD20 or control mAb 7 days before MOG_{35-55} immunization on day 0. The mice also received PBS or purified $CD1d^{hi}CD5^{+}$ B cells from either $CD20^{-/-}$ or MHC-$II^{-/-}CD20^{-/-}$ mice 1 day before MOG_{35-55} immunization. The two graphs are from the same experiment, but were separated to facilitate visualization of the overlapping curves. **c**, Activated MHC-$II^{-/-}$ B10 cells do not reduce disease severity in wild type mice. Purified $CD1d^{hi}CD5^{+}$ B cells from wild type or MHC-$II^{-/-}$ mice were cultured with agonistic CD40 mAb for 48 h to induce B10pro cell maturation, with LPS added during the final 5 h of culture. Wild type mice were given either PBS or $CD1d^{hi}CD5^{+}$ B cells 1 day before MOG_{35-55} immunization on day 0. a–c, Values represent mean (±s.e.m.) symptom scores from ≥3 mice in each group, with similar results obtained in three independent experiments. Significant differences between sample means are indicated; *, $p<0.05$.

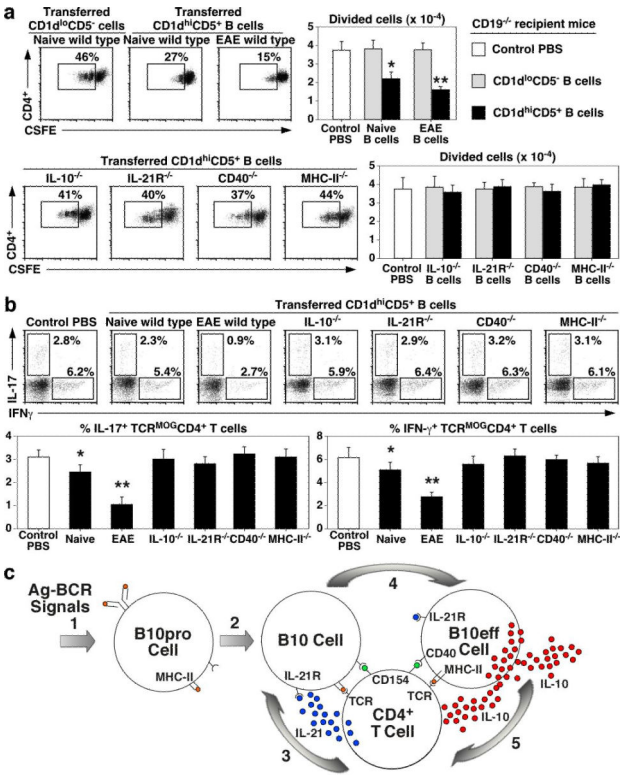

Figure 3.

B10 cell expansion and regulation of T cell-mediated autoimmunity. **a**, B10 cells require IL-10, IL-21R, CD40 and MHC-II expression to regulate antigen-specific T cell proliferation *in vivo*. $CD19^{-/-}$ recipient mice were given PBS as a control, or purified $CD1d^{hi}CD5^{+}$ or $CD1d^{lo}CD5^{-}$ B cells from naïve wild type (WT), $IL\text{-}10^{-/-}$, $IL\text{-}21R^{-/-}$, $CD40^{-/-}$ or $MHC\text{-}II^{-/-}$ mice, or wild type mice with EAE (day 28) 1 day before $MOG_{35–55}$ immunization on day 0. Four days after immunization, dye (CFSE)-labeled TCR^{MOG} $CD4^{+}Thy1.1^{+}$ T cells were transferred into $CD19^{-/-}$ recipient mice. Five days later, peripheral lymph node $CD4^{+}Thy1.1^{+}$ T cells were analyzed for proliferation, with representative flow cytometry analysis of CFSE dilution show. Bar graphs indicate mean (±s.e.m.) numbers of divided TCR^{MOG} T cells. **b**, B10 cells require IL-10, IL-21R, CD40 and MHC-II expression for their regulation of antigen-specific T cell cytokine production. Purified $CD1d^{hi}CD5^{+}$ B cells from the indicated mice were transferred into $CD19^{-/-}$ recipient mice 1 day before MOG35–55 immunization on day 0, with TCR^{MOG} $Thy1.1^{+}CD4^{+}$ T cells transferred on day 4. Fourteen days later, lymph node $Thy1.1^{+}CD4^{+}$ T cells were analyzed for IL-17 and IFN-γ production by intracellular cytokine staining, with representative flow cytometry results shown. (a–b) Bar graphs indicate mean (±s.e.m.) frequencies of divided or cytokine-expressing cells, with three mice in each group. Significant differences between sample means are indicated: *, $p<0.05$; **, $p<0.01$. **c**, Model for autoantigen (Ag)-specific B10 cell function. B cells capture autoantigens that trigger appropriate BCR signals (step 1) and promote IL-10-competent $B10_{pro}$ cell development. During immune responses (step 2), $B10_{pro}$ cells present peptides to antigen-specific T cells through cognate interactions that induce T cell activation and CD40/CD154 interactions. Activated T cells may produce IL-21 locally, which binds to proximal B10 cell IL-21R (step

3). IL-21R signals induce B10 cell IL-10 production and effector function (B10eff, step 4), which may negatively regulate antigen-specific T cell function (step 5).

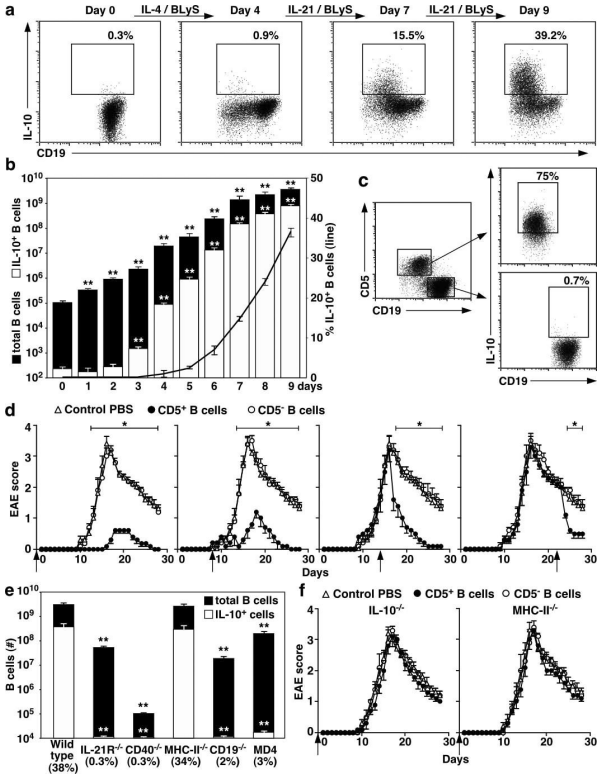

Figure 4.

IL-21 drives *ex vivo* regulatory B10 cell expansion. **a**, B10 cell development *in vitro*. Purified spleen B cells were cultured on NIH-3T3-CD154/BLyS cell monolayers with exogenous IL-4 for 4 days, then cultured on fresh NIH-3T3-CD154/BLyS cells with exogenous IL-21 for 3 or 5 days as indicated, isolated, cultured with monensin for 5 h and stained for cytoplasmic IL-10 expression. Representative IL-10^{+} B cell frequencies within the indicated gates are shown. Similar results were obtained in ≥10 experiments. **b**, IL-21 drives B10 cell expansion *in vitro*. B cells cultured as in (a) were harvested each day. Bar values represent mean (±s.e.m.) B cell and B10 cell numbers, or B10 cell frequencies (solid line) from three independent experiments. **c**, IL- 21-induced B10 cells express CD5. B cells cultured for 9 days as in (a) were stained for CD5 and CD19 expression. CD5^{+} or CD5^{-} B cells were then purified and cultured with monensin for 5 h before cytoplasmic IL-10 staining. Results represent three independent experiments. **d**, B10 effector cells inhibit EAE initiation and progression. CD5^{+}CD19^{+} or CD5^{-}CD19^{+} cells were isolated as in (c) and adoptively transferred into wild type mice on days −1, 7, 14 or 21 (arrows) before/after MOG immunization and EAE induction as in figure 2. **e**, B10 cell expansion *in vitro* requires IL-21R and CD40 expression, and *in vivo* BCR signaling. Purified spleen B cells isolated from wild type, IL-21R$^{-/-}$, CD40$^{-/-}$, MHC-II$^{-/-}$, CD19$^{-/-}$, or MD4 mice were cultured as in (a), with mean (±s.e.m.) cell numbers quantified after culture. Values represent means (±s.e.m.) of three independent experiments. IL-10^{+} B cell frequencies in the cultures are shown in parentheses. **f**, B10 effector cells require IL-10 and MHC-II expression to inhibit EAE. B cells from IL-10$^{-/-}$ or MHC-II$^{-/-}$ mice were cultured as in (a), separated into CD5^{+} or CD5^{-} cells as in (c) and adoptively transferred into wild type mice before MOG35–55 immunization as in (d). (d, f) Values represent mean (±s.e.m.) symptom

scores from ≥3 mice in each group, with similar results obtained in three independent experiments. (b, d, e) Significant differences between sample means are indicated: *, $p<0.05$; **, $p<0.01$.