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B cells expressing IFN- γ suppress Treg-cell differentiation and promote autoimmune experimental arthritis

Susan A. Olalekan^{*}, Yanxia Cao[†], Keith M. Hamel[§], and Alison Finnegan^{*,†}

^{*}Department of Immunology/Microbiology

[†]Department of Internal Medicine, Division of Rheumatology, Rush University Medical Center, Cohn Research Building, Chicago, IL, USA

[§]Department of Medicine, Section of Rheumatology, Gwen Knapp Center for Lupus and Immunology Research, The University of Chicago, IL, USA

Abstract

Clinical efficacy in the treatment of rheumatoid arthritis with anti-CD20 (Rituximab)-mediated B-cell depletion has garnered interest in the mechanisms by which B cells contribute to autoimmunity. We have reported that B-cell depletion in a murine model of proteoglycan-induced arthritis (PGIA) leads to an increase in regulatory T (Treg) cells that correlates with decreased autoreactivity. Here, we demonstrate that the increase in Treg cells after B-cell depletion is due to an increase in the differentiation of naïve CD4⁺ T cells into Treg cells. Since the development of PGIA is dependent on IFN- γ and B cells are reported to produce IFN- γ , we hypothesized that B-cell-specific IFN- γ plays a role in the development of PGIA. Accordingly, mice with B-cell-specific IFN- γ -deficiency were as resistant to the induction of PGIA as mice that were completely IFN- γ -deficient. Importantly, despite a normal frequency of IFN- γ -producing CD4⁺ T cells, B-cell-specific IFN- γ -deficient mice exhibited a higher percentage of Treg cells compared with that in wild type (WT) mice. These data indicate that B-cell IFN- γ production inhibits Treg-cell differentiation and exacerbates arthritis. Thus, we have established that IFN- γ , specifically derived from B cells, uniquely contributes to the pathogenesis of autoimmunity through prevention of immunoregulatory mechanisms.

Keywords

autoimmunity; arthritis; B cells; IFN- γ ; regulatory T cells

Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease involving inflammation of the synovial tissue of multiple joints that leads to the destruction of cartilage and bone. Several different cell populations including T cells and B cells contribute

Address Correspondence to: Alison Finnegan, Ph.D., Department of Medicine, Division of Rheumatology, Rush University Medical Center, 1735 West Harrison Ave., Chicago, IL 60612, Tel: +1-312-942-7847, Fax: +1-312-942-8828, Alison.Finnegan@rush.edu.

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to synovial changes as the disease progresses, however the precise contribution of each of these immune cells in the pathogenesis of RA is unclear [1]. The involvement of B cells in the manifestation and progression of RA has gained renewed interest based on the success of B-cell depletion therapy [2, 3]. Administration of Rituximab, a chimeric monoclonal antibody specific for the CD20 surface antigen, transiently depletes B cells and maintains long-term humoral immunity since CD20 is not expressed on stem cells and plasma cells [3, 4]. In our model of RA, proteoglycan-induced arthritis (PGIA), depletion of B cells leads to a reduction in PG-specific antibodies, T-cell activation and IFN- γ production [5-9], resulting in alleviation of arthritis [5]. Further studies demonstrate that Treg cells are increased in B-cell depleted mice and the elimination of Treg cells results in exacerbation of disease [10].

CD4⁺CD25⁺Foxp3⁺ Treg cells play a crucial role in immunity by limiting responses to self and foreign antigens [11]. RA patients and healthy donors have similar numbers of CD4⁺CD25⁺ Treg cells [12, 13]. However, synovial fluid Treg cells from patients with RA are defective in their ability to suppress proinflammatory cytokine production by effector T cells [14]. High concentrations of TNF- α can block Treg mediated immunosuppression [15] and treatment of RA with anti-TNF- α drugs increases the number and activity of Treg cells [14, 16]. Together, this evidence of defective Treg cells suggests that highly inflammatory conditions affect Treg-cell suppressor function.

Beyond functioning as APCs and antibody producing cells, B cells are known to produce several proinflammatory cytokines including IFN- γ which is required for disease in PGIA [17-19]. The effects of IFN- γ on Treg cells are contradictory as some studies suggest IFN- γ is important for regulatory function whereas others indicate IFN- γ inhibits Treg cells [20-23]. The production of IFN- γ by B cells was originally observed in the Be1 subset of B cells [18]. Recently, a population of IFN- γ producing CD11a^{hi}/CD16/CD32^{hi}CD19⁺ innate B cells was identified early after *Listeria* infection [24]. B cells from some RA patients also express IFN- γ mRNA [25]. Since IFN- γ is necessary for the development of PGIA and negatively affects Treg-cell activity, we asked whether B-cell derived IFN- γ was contributing to arthritis by suppressing Treg cells.

In this study we report, that B-cell depletion leads to a reduction in antigen-specific T-cell priming and a reciprocal increase in Treg-cell differentiation. Arthritis was suppressed in mice with a B-cell-specific IFN- γ deficiency similar to mice with a complete IFN- γ deficiency. In B-cell-specific IFN- γ deficient mice, suppression of arthritis correlated with an increase in Treg cells and a reduction in antigen-specific T- and B-cell responses. In PGIA, B-cell production of IFN- γ plays a major role in the development of arthritis.

Results

Increase in Treg-cell compartment in rG1-primed mice

B-cell depletion in arthritic mice leads to an increase in the percentage of Treg cells; the resultant increase in Treg cells inhibits PGIA [10]. These data suggest that B cells may have a direct effect on Treg differentiation. We first determined whether B-cell depletion affects the CD4⁺ T- cell compartment early after immunization. Foxp3^{eGFP} mice were immunized with rG1/DDA and B cells were depleted on day 5 post-immunization. Spleens were

harvested 4 days later and assessed for B cells, T effector and Treg numbers and percentages as well as the number of antigen-specific, tetramer G1₇₀₋₈₀/I-A^d-positive CD4⁺ T cells. B cells were successfully depleted in anti-mCD20 treated groups (Fig. 1A-B). The percentage of splenic CD4⁺ T cells was higher in B-cell depleted mice as expected due to the loss of the mature B-cell compartment, but the total numbers of CD4⁺ T cells in both B-cell-depleted and control antibody (Ab)-treated mice were similar (Fig. 1A, C). However, when G1-specific T cells were assessed there was a significant ($p = 0.02$) reduction in tetramer-positive CD4⁺ T cells in B-cell depleted mice (Fig. 1D-E). Further analysis demonstrated that both the numbers and percentages of splenic Treg cells were increased in B-cell depleted mice suggesting that B-cell depletion influenced differentiation of Treg cells (Fig. 1F-G). B-cell depletion of rG1 immunized TCR-Tg5/4E8 mice also resulted in an increase in Treg cells as compared to control Ab treated mice (data not shown). These data demonstrate that B-cell depletion early after immunization leads to a reduction in antigen-specific T effectors and an augmentation in Treg differentiation.

Cytokine production in B-cell-depleted mice

PGIA is a Th1 disease, with arthritis suppressed in IFN- γ -deficient mice and after neutralization with anti-IFN- γ antibodies [19]. To determine whether the reduction in antigen-specific T cells corresponds to reduction in T-cell IFN- γ , we assessed IFN- γ production and antigen-specific T-cell proliferation. Mice were immunized with rG1/DDA, B cells were depleted on day 5 and spleens were harvested on day 9 post immunization. CD4⁺ T cells were assessed for intracellular IFN- γ by flow cytometry or were isolated by negative selection and re-stimulated for 4 days with rG1 *in vitro*. Although the frequency of IFN- γ producing CD4⁺ T cells was similar, the quantity of IFN- γ production, as detected by ELISA, was markedly lower in CD4⁺ T-cells from B-cell depleted mice compared to controls (Fig. 2A-C). Reciprocally, the production of IL-10 by Treg cells in B-cell depleted mice was enhanced compared to those from non-B-cell depleted mice (Fig. 2D-E) [26]. In the remaining B cells, there was a similar percentage and number of IL-10 producing Breg cells in B-cell depleted and control Ab treated mice (data not shown). In accordance with a reduction of IFN- γ secretion by CD4⁺ T cells along with the increase in suppressive IL-10 production by Treg cells, antigen-specific T-cell proliferation was reduced (Fig. 2F). CD4⁺T-cell from antigen stimulated mice proliferated in the media control indicating they were activated as naïve T cells under similar condition minimally proliferate (data not shown). These data indicate that B-cell depletion leads to a reduction in antigen-specific T-cell priming and a reciprocal increase in Treg cells that produce IL-10.

B-cell depletion induces Treg-cell differentiation in vivo

To determine if the increase in Treg cells observed after B-cell depletion was a result of an increase in naïve CD4⁺ T cells differentiating into Treg cells, we set up an adoptive transfer of CD90.2⁺CD4⁺CD62L⁺Foxp3⁻ T cells from TCR-Tg5/4E8Foxp3^{eGFP} mice into congenic CD90.1⁺ BALB/c recipient mice. Mice were immunized one day after CD4⁺Foxp3⁻ T-cell transfer and B cells were depleted 5 days later. Spleens were harvested 4 days following B-cell depletion and transferred CD90.2⁺ T cells were assessed for the total numbers of CD4⁺ T cells and frequency of CD4⁺Foxp3⁺ T-cell. In the B-cell depleted group there was a significant reduction in the percentages of CD4⁺ T cells and a trend in the reduction in the

number of CD4⁺ T cells in comparison to the control mAb treated group (Fig. 3A-B) suggesting that there was decreased T-cell activation in B-cell depleted mice. Importantly, the conversion of transferred, naïve CD4⁺ Foxp3⁻ T cells into CD4⁺ Foxp3⁺ Treg cells, as measured by induction of Foxp3, was increased in both percentage and numbers in B-cell depleted mice as compared to control Ab-treated mice (Fig. 3C-D). B-cell depletion in naïve mice did not lead to an increase in Treg cells numbers or percentages (Fig. 3E-F) indicating that T-cell activation was necessary for B cells to effectively inhibit CD4⁺ Foxp3⁻ T cells differentiation into CD4⁺ Foxp3⁺ Treg cells. These data demonstrate that B cells are an important component in suppressing the differentiation of Treg cells under conditions of T-cell activation.

B-cell-derived IFN- γ suppresses Treg-cell differentiation

We confirmed that IL-12 and IFN- γ suppress the differentiation of naïve CD4⁺CD25⁻CD62L⁺ T cells into Treg cells as previously described [21, 27]. There are several reports showing that B cells produce IFN- γ [18, 24, 25]. To determine if IFN- γ derived from B cells was contributing to the suppression of Treg differentiation we first confirmed that B cells produce IFN- γ . Sorted splenic B cells (99%) cultured in the presence of LPS, IL-12 and anti-CD40 produced IFN- γ (Fig. 4A). More importantly B cells from arthritic mice produced IFN- γ (Fig 4B-C). To assess the contribution of B-cell-derived IFN- γ to Treg differentiation we cultured IFN- γ ^{-/-} CD4CD25⁻CD62L⁺ T cells in the presence of WT or IFN- γ ^{-/-} B cells. In this way, the only source of IFN- γ would be from WT B cells. WT B cells suppressed Treg-cell differentiation more effectively than IFN- γ ^{-/-} B cells (Fig. 4D-E). These results demonstrate that B-cell derived IFN- γ contributes to the suppression Treg differentiation.

Development of PGIA requires B-cell IFN- γ expression

To determine whether B-cell expression of IFN- γ was required for PGIA, we constructed mice in which IFN- γ -deficient B cells coexisted with other APCs and T cells that express IFN- γ . To accomplish this, lethally irradiated CD45.1 WT mice were reconstituted with an approximate 70/30 mixture of CD45.2 bone marrow (BM) from B-cell deficient (B cell^{-/-}) and IFN- γ ^{-/-} mice. In these chimeric mice the APC and T-cell compartment are derived mostly from B cell^{-/-} BM and thus give rise to IFN- γ positive non-B cell APCs and T cells, whereas the B cells will arise from IFN- γ ^{-/-} BM (designated B cell: IFN- γ ^{-/-} mice). Positive control chimeras consisted of a mixture of BM from B-cell^{-/-} and WT mice (B cell: IFN- γ ^{+/+}) and negative control chimera were constructed using only BM from IFN- γ ^{-/-} mice injected into lethally irradiated CD45.1 recipient mice. At 3 months post-transplant, peripheral blood was assessed for the degree of reconstitution of CD45.1 mice with CD45.2 cells. Reconstitution from residual host BM was minimal as there was on the average 98% reconstitution of CD45.2 cells in chimeric mice (data not shown). Mice were immunized at 4 months post-transplant with rG1/DDA and subsequently evaluated for the incidence and severity of arthritis.

In the positive control group, 100% of the B cell: IFN- γ ^{+/+} mice developed severe arthritis. However, chimeric mice with IFN- γ -deficient B cells, B cell: IFN- γ ^{-/-}, displayed minimal disease severity with an arthritic score and incidence similar to complete IFN- γ ^{-/-} chimeric

mice (Fig. 5 A). These data demonstrate that B-cell production of IFN- γ is necessary for the development of arthritis.

To decipher the mechanism responsible for the requirement for IFN- γ producing B cells in arthritis we assessed the T effector and Treg-cell populations in spleens of chimeric mice. As suggested from *in vitro* experiment (Fig.4), the CD4⁺CD25⁺Foxp3⁺ T cells were significantly increased in B cell: IFN- γ ^{-/-} chimeric mice in comparison to B cell: IFN- γ ^{+/+} mice (Fig. 5B). In fact, the percentage of Treg cells from B cell: IFN- γ ^{-/-} spleens was nearly double that of WT controls, and ultimately phenocopied the percentages and total numbers of those observed in mice reconstituted completely with IFN- γ ^{-/-} BM. Importantly, when we examined the frequency of CD4⁺ T cells that express IFN- γ , there was not a significant difference between B cell: IFN- γ ^{+/+} and B cell: IFN- γ ^{-/-} mice (Fig. 5C). However, splenocytes from B cell: IFN- γ ^{-/-} chimeric mice cultured in the presence of rG1 produced IFN- γ although less than splenocytes from B cell:IFN- γ ^{+/+} (Fig 5D). These data indicate that other non-B cells are producing IFN- γ but that it is insufficient to induce arthritis. Earlier studies in PGIA showed that in the absence of IFN- γ there is an increase in T-cell production of IL-17 [28]. Therefore, we examined the production of IL-17 and found that only the IFN- γ ^{-/-} chimeric mice produced enhanced IL-17 in comparison to B cell: IFN- γ ^{+/+} or B cell: IFN- γ ^{-/-} mice (Fig. 5E). Although B cell: IFN- γ ^{-/-} chimeric mice produced less IFN- γ than B cell: IFN- γ ^{+/+} mice it was sufficient to inhibit the IL-17 response.

In assessing the recall response to rG1 we observed that splenocytes from B cell: IFN- γ ^{-/-} and IFN- γ ^{-/-} chimeric mice displayed a reduced proliferative capacity in comparison to B cell: IFN- γ ^{+/+} mice (Fig. 5F). The reduction in antigen-specific recall response in B cell: IFN- γ ^{-/-} chimeric mice (Fig. 5D) suggests that T-cell priming was defective. Since CD4⁺ T-cell help is required for Ab production we examined the serum levels of G1-specific IgG1 and IgG2a. Corresponding to the degree of arthritis, anti-G1 IgG1 and anti-G1 IgG2a levels were reduced in B cell: IFN- γ ^{-/-} and IFN- γ ^{-/-} chimeric mice compared to B cell: IFN- γ ^{+/+} chimeric mice (Fig. 5G). These data suggests that the inherent expression of IFN- γ by B cells is required for T-cell priming, Ab production, suppression of Treg cells, and ultimately the development of PGIA.

B-cell depletion therapy ameliorates disease via reduction of IL-6 and IL-6 is known to suppress Treg [29] thus reduction in IFN- γ may indirectly affect the development of PGIA through IL-6. However, there was no significant reduction of IL-6 in B cell: IFN- γ ^{-/-} chimeric mice (Fig.5H). These data indicates that IFN- γ produced from other non-B cell splenocytes was not sufficient to sustain PGIA and that IFN- γ from B cells was absolutely required for the perpetuation of arthritis.

Discussion

B-cell depletion suppresses arthritis via an increase in the number and function of Treg cells however the mechanism of B-cell regulation of Treg cells is not understood [10]. We report here that B-cell depletion early after T-cell priming *in vivo* increased the Treg cell population while simultaneously reducing the number of G1-specific T cells. These data led to the hypothesis that naïve CD4⁺ T cells were differentiating into Treg cells in the absence

of B cells. In adoptive transfer of CD4⁺Foxp3⁻ from TCR-Tg5/4E8Foxp3^{eGFP} we observed a significant increase in CD4⁺Foxp3⁻ T cells differentiation into CD4⁺Foxp3⁺ Treg cells and a decrease in CD4⁺T cells in B-cell depleted mice. The decrease in CD4⁺ T cells is likely due to a reduction in rG1-specific T-cell proliferation as B cells are important antigen-presenting cells. CD4⁺ T-cell survival may also be reduced in the absence of antigen stimulation. B-cell suppression of Treg differentiation requires antigen exposure as B-cell depletion in naïve mice did not increase Treg cells. This is in accordance with findings that Treg cells differentiate from naïve T cells in vivo by sub-immunogenic doses of antigen as well as endogenous expression of foreign antigen in a lymphopenic environment [30, 31]. We show here that B cells play a major role in regulating the generation of Treg cells from naïve T cells in vivo.

Our observation that the increase of Treg cells in B-cell-depleted mice correlated with a decrease in IFN- γ production prompted us to study the role of IFN- γ in Treg differentiation during arthritis. Some studies show that B cells produce IFN- γ in response to IL-12 and IL-18 or when primed by IFN- γ producing Th1 cells [18, 32]. CD11a^{hi}Fc γ RIII^{hi} B cells produced high levels of IFN- γ at an early time point in response to *Listeria monocytogenes* infection [24]. In addition, B cells obtained from a subset of RA patients express mRNA for IFN- γ [25]. We confirmed the production of IFN- γ in sorted in vitro stimulated B cells and in B cells from arthritic mice. In assessing the effects of B cells on Treg differentiation in vitro, we found that the presence of WT B cells suppressed Treg differentiation. However, IFN- γ ^{-/-} B cells were less effective than WT B cells in suppressing Treg differentiation suggesting that B-cell IFN- γ may play a role in inhibiting Treg cells in vivo. To address this question, we created mice with IFN- γ specifically deleted in the B cells while other APCs and T cells were sufficient in IFN- γ expression. Our results clearly demonstrate that while B cell: IFN- γ ^{+/+} chimeras develop robust arthritis, B cell: IFN- γ ^{-/-} chimeras are unable to develop disease similar to IFN- γ ^{-/-} chimeras. Furthermore, we identified a significant increase in the percentage and numbers of Treg cells, despite similar numbers of total CD4⁺ T cells in B cell: IFN- γ ^{-/-} chimeras compared to control. However, the significant impairment in the recall response to rG1 from B cell: IFN- γ ^{-/-} chimeras compared to B cell: IFN- γ ^{+/+} and similar to IFN- γ ^{-/-} chimeras indicate a reduced antigen-specific T-cell activation. Importantly, IFN- γ was produced by other splenocytes in B cell: IFN- γ ^{-/-}chimeras although it was not sufficient to induce arthritis. These findings indicate that during an immune response, the expression of IFN- γ by B cells is required for optimal Treg inhibition and/or Th-1 activation to promote arthritis.

Several reports indicate that IFN- γ inhibits the development of TGF- β induced Foxp3 expressing Treg cells [21, 27, 33-35]. However, there are also studies that show that IFN- γ is essential for Treg generation and function [20, 22, 23, 36]. Autocrine production of IFN- γ by alloantigen-reactive Treg cells is necessary for their role in preventing rejection of donor-specific grafts [20, 22]. Since IFN- γ can both enhance and inhibit Treg cells it is possible that IFN- γ is important at different stages of the immune response. Upregulation of IFN- γ mRNA by Treg cells is early and transient compared to CD4⁺CD25⁻ T cells indicating that IFN- γ production is activation dependant but short lived [22]. However, under highly inflammatory conditions, a strong IFN- γ milieu may inhibit differentiation of Treg cells

[35]. Our results confirm that the presence of IFN- γ creates a highly inflammatory environment that negatively affects Treg generation and function.

B cells are subdivided into different subsets based on the cytokines they produce. B cells that produce cytokines such as TNF- α , IFN- γ , IL-12, IL-4, IL-10, IL-6 and lymphotoxin are referred to as B effector cells [37, 38]. These effector B cells polarize CD4⁺ T cells into Th1/Th2 phenotypes [39]. Another subset of B cells functionally distinguished based on cytokine production is regulatory B (Breg) cells. Breg cells are phenotyped as CD1d^{high}CD5⁺IL10⁺ B cells and are negative regulators of autoimmunity [40, 41]. IL-10 is required for Foxp3 expression and the proper function of Treg cells and some studies have shown that IL-10 expression by Breg cells is important for the generation of Treg cells and thus the benefit of B-cell depletion is often associated with Breg cells [42-45]. However we did not detect a difference in the numbers of IL10 producing Breg cells between B-cell depleted and control Ab treated rG1 primed mice nor was there an increase in Breg cells production of IL-10 in IFN- γ ^{-/-} mice (data not shown). There are several other cytokine producing B-cell subsets that differentially modulate immune responses. Murine and human B cells secrete IL-17 in response to *Trypanosoma cruzi* infection [46]. Innate response activator B cells derived from B1b cells and uniquely characterized by their high production of GM-CSF protect against sepsis in mice [47]. A deficiency in B-cell-specific IL-35 exacerbates EAE by increasing Th1 and Th17 autoreactivity without affecting the Treg-cell compartment [48]. In salmonella infection, the absence of IL-35 producing B cells increased mice survival indicating that these cells suppress antimicrobial immune responses [48]. In addition, B-cell depletion ablates IL-6 producing B cells and reduces autoimmunity [49]. However, splenocytes from B cell: IFN- γ ^{-/-} produced similar amount of IL-6 to B cell: IFN- γ ^{+/+} indicating that the amelioration of arthritis observed in these mice was not due to a reduction of IL-6. Paradoxically, CpG- induced proB cells derived from IFN- γ deficient NOD mice failed to protect against the transfer of T1D in NOD mice as compared to the proB cells derived from WT NOD mice [50]. The identification of new cytokine secreting B cells suggests that there are potentially other cytokine producing B cells subsets that have not been characterized and these subsets might contribute to autoimmunity in a variety of ways. The resistance of B cell: IFN- γ ^{-/-} chimeric animals to induction of PGIA indicates the importance of B-cell cytokines and better understanding of the B-cell cytokines may lead to modulation of current therapies that will enhance patient response to treatment.

Thus, our results suggest that an intimate interaction between B cells and naïve CD4⁺ T cells is required for delivery of IFN- γ to activate naïve CD4⁺ T cells to effector status. B-cell depletion reduces CD4⁺ T-cell memory formation in lymphocytic choriomeningitis virus infection reducing the frequency of IFN- γ ⁺CD4⁺ T cells. Similarly, Be1 cells polarize naïve CD4⁺ T cells into Th1 cells *in vitro* [39, 51, 52]. Reciprocally, Treg cells suppress IFN- γ production and proliferation of Th1 cells without inhibiting the commitment to the Th1 lineage [53]. Our findings here contribute to a cyclical model of inflammation where modulation of IFN- γ dictates the degree of inflammation. B-cell IFN- γ may be necessary for Th1 priming and in the absence of Th1 cells Treg cells dominate. It is possible that these mechanisms work synergistically to suppress the induction of arthritis.

Materials and Methods

Mice

Wild type (WT) BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). BALB/c Foxp3^{eGFP}, IFN- γ -deficient (IFN- γ ^{-/-}), CD90.1 congenic and CD45.1 congenic mice were obtained from Jackson Laboratories. BALB/c B-cell-deficient JHD mice were provided by Dr. Mark Shlomchik (Yale University). BALB/c TCR transgenic mice (TCR-Tg5/4E8) are specific for an immunodominant peptide (74-80) in the human G1 domain of PG and cross-reacts with mouse G1 and were generated as described [54]. These TCR-Tg5/4E8 mice express the V β 4 TCR. In TCR-Tg5/4E8 mice 96% of the CD4⁺ T cells are V β 4 positive. TCR-Tg5/4E8Foxp3^{eGFP} mice were obtained by crossing TCR-Tg5/4E8 mice to Foxp3^{eGFP} mice. IFN- γ ^{-/-} Foxp3^{eGFP} mice were obtained by crossing IFN- γ ^{-/-} mice to Foxp3^{eGFP} mice. Genotyping of these mice was confirmed by PCR. Animal experiments were approved by the Institutional Animal Care and Use Committee (Rush University Medical Center, Chicago, IL).

Immunization, antigen and B-cell depletion

Recombinant human aggrecan G1-domain protein (rG1) was produced as previously described [54]. Arthritis was induced in age-matched female BALB/c mice 12-14 weeks (weeks) of age by intraperitoneal (i.p.) immunization with 50 μ g rG1 in 1 mg of dimethyldioctadecyl-ammonium bromide (DDA) adjuvant (Sigma Aldrich, St. Louis, MO) and boosted with 40 μ g of rG1/DDA at 3 and 6 weeks. Mice were scored in a blinded manner three times a wk to monitor the development of arthritis. Scoring of each paw was as follows: 0, normal; 1, mild erythema and swelling of several digits; 2, moderate erythema and swelling; 3, diffuse erythema and swelling; and 4, severe erythema and swelling of complete paw with ankylosis. For short-term activation in vivo, mice were immunized with 40 μ g rG1/DDA and spleens were harvested on day 9.

In B-cell-depletion experiments, WT, TCR-Tg5/4E8 and Foxp3^{eGFP} mice were immunized as described above and B cells were depleted on day 4 by a single intravenous (i.v.) injection of 250 μ g of rat anti-mouse CD20 mAb (1B12, IgG2a) (anti-mCD20) or control Ab, rat anti-human CD20 mAb (2B8) (anti-hCD20). Naïve Foxp3^{eGFP} mice were treated with anti-mCD20 or anti-hCD20 and sacrificed 4 days later. Monoclonal anti-mCD20 was generated as previously described [5]. These antibodies were provided by Biogen Idec, Cambridge, MA.

In vitro B-cell and Treg cultures

Splenic B cells were labeled with anti-CD19 (PE) (BD Biosciences, San Diego, California) and sorted using the MoFlo Legacy cell sorter (Beckman Coulter, Brea, CA). B cells (5×10^5) were cultured in the presence of anti-CD40 (30 μ g/ml), LPS (10 μ g/ml) and IL-12 (100 ng/ml) in RPMI 1640 media containing 5% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 50 μ M 2-ME and 2 mM L-glutamine (complete media). FBS (Gemini-Bio-Products, West Sacramento, CA) was from a single lot (A40D5D) and chosen for low background in T-cell proliferation assays. Supernatants were collected at 48 h. IFN- γ was measured using the IFN- γ Femto HS kit (eBioscience, San Diego, CA) Splenic CD4⁺CD25-

T cells IFN- γ ^{-/-} Foxp3^{eGFP} mice were purified by negative selection using microbeads (Miltenyi Biotec, San Diego, CA) and autoMACS (Miltenyi Biotec). CD4⁺ naïve T cells were prepared by positive selection of CD62L^{hi} CD4⁺ T cells by autoMACS. T cells (1×10^5 cells) were cultured with plate bound anti-CD3 (5 μ g/ml)(eBioscience), soluble anti-CD28 (5 μ g/ml)(BD Biosciences), TGF- β (5 ng/ml)(eBioscience), IL-12 (8 ng/ml)(R&D Systems, Minneapolis, MN) and IFN- γ (5 ng/ml)(BD Biosciences) in complete media in a 96-well flat bottomed Falcon plate (Fisher Scientific, Pittsburgh, PA). Activated B cells (2×10^5) (as described above) were cultured with naïve CD4⁺ T cells for 4 days and the phenotype of the T cells was determined by flow cytometry. Supernatant were assessed for cytokines by ELISA (IFN- γ [R & D Systems]) according to manufacturer's instructions. Data represent the mean $\pm$ SD.

Splenocytes (5×10^5 /well) from immunized mice were stimulated with rG1 (2 μ g/ml) in vitro. Cells were cultured in triplicates in 96-well Falcon plates (Fisher Scientific) in complete media and pulsed with [³H] thymidine on day 4 or supernatants were collected on day 4. Pulsed cells were examined for proliferation on day 5. Supernatants were assessed for cytokines by ELISA (IFN- γ , IL-17 [R & D Systems] and IL-6 [eBioscience]). Data represent the mean $\pm$ SD.

Flow cytometry

Spleen cells were obtained from immunized mice at the time of sacrifice and in vitro cultured T cells were analyzed by flow cytometry. Single cell suspensions of cells were washed in buffer (3% FBS in PBS) and blocked with Anti-Fc receptor antibody (2.4G2) for 10 min at 4°C. Cells were gated on lymphocytes and singlets. T effector and Treg cells were assessed using anti-CD4 (allophycocyanin-Cy7) (eBioscience), anti-B220 (PE-Cy7) (BD Biosciences). For intracellular staining, cells were stimulated with PMA (25 ng/ml) and ionomycin (500 ng/ml) (Sigma-Aldrich) and treated with golgiplug (BD Biosciences) for 4 h. After cell surface staining as above, cells were permeabilized using cytofix/cytoperm (BD Biosciences) and labeled with anti-IL-10 (PE) (BD Biosciences) and anti-IFN- γ (allophycocyanin) (BD Biosciences). For arthritic B-cell IFN- γ production, arthritic mice were sacrificed 10 days after the third i.p immunization. Cells were surface stained for CD4, CD19 and intracellularly stained with anti-IFN- γ (PE) (BD Biosciences) or rat IgG1 (PE) (BD Biosciences) as described above.

For the mixed bone marrow chimera cell populations, splenocytes were stained with anti-CD45.2 (perCP-Cy 5.5), anti-CD4 (APC-Cy7 or Pacific Blue), anti-CD25 (PE-Cy7), anti-Foxp3 (FITC) and anti-IFN- γ (allophycocyanin-Cy7).

Tetramer-positive cells were stained with anti-CD4 (perCP-Cy5.5) and anti-CD44 (allophycocyanin-Cy7), tetramer (allophycocyanin), anti-CD8 (PE-Cy7), anti-B220 (PE-Cy7) and anti-GR1 (PE-Cy7). PE-Cy7 was the dump gate.

After adoptive transfer, immunization and B-cell depletion, spleens were harvested and labeled with anti-CD4 (allophycocyanin-Cy7), anti-V β 4 (PE), CD90.2 (allophycocyanin). Antibodies were either from BD Biosciences or eBioscience. A BD LSR II cytometer was used for cytometry and data were analyzed using BD FACS Diva Software.

Isolation of tetramer-positive cells

Tetramer G1₇₀₋₈₀/I-A^d was produced by the Tetramer Facility at Emory University location under contract with NIH. G1₇₀₋₈₄/I-A^d tetramers were labeled with allophycocyanin. Peptide 74-80, ATEGRVRVNSAYQDK, is a dominant peptide in PGIA as T-cell receptor transgenic mice (TCR-Tg 5/4E8) specific for 74-80 peptide develop arthritis after immunization with rG1 [55]. The T cells from the TCR-Tg 5/4E8 mice recognize both the human and mouse peptide. Tetramer binding cells splenocytes were enriched by incubation with 20 ug/ml allophycocyanin allophycocyanin-tetramer G1₇₀₋₈₄/I-A^d for 4 h at 37°C; followed by anti-allophycocyanin microbeads for 15 min at 4°C prior to elution over magnetic columns (Miltenyi Biotec).

Adoptive cell transfer

CD4⁺ T cells were enriched from single cell suspensions of splenocytes from TCR-Tg (5/4E8)/Foxp3^{eGFP} mice by negative selection using microbeads (Miltenyi Biotec) followed by positive selection for CD62L⁺ cells using the AutoMACS (Miltenyi Biotec). CD4⁺CD62L⁺Foxp3⁻ T cells were sorted using the MoFlo Legacy cell sorter (Beckman Coulter). Sorted CD4⁺CD62L⁺Foxp3⁻ T cells were injected i.v into CD90.1 BALB/c mice on day 0. Mice were immunized on day 1 and B cells depleted on day 6. Spleens were harvested on day 10.

Generation of mixed bone marrow chimera

Female BALB/c mice (8-10 weeks of age) received acidified water starting one wk prior to irradiation and bone marrow reconstitution. Chimeric mice were generated by irradiating recipient mice with 950 Rad from a ¹³⁷Cs source delivered in two equal doses 4-5 h apart. After the second irradiation, mice were injected i.v. with bone marrow (BM) cells obtained from the assigned donor mice and allowed to reconstitute for 4 months before immunization. B cell: IFN- γ ^{-/-} were reconstituted with 1 $\times$ 10⁷ B cell^{-/-} and 6 $\times$ 10⁶ IFN- γ ^{-/-} BM cells per mouse. B cell: IFN- γ ^{+/+} were reconstituted with 1 $\times$ 10⁷ B cell^{-/-} and 6 $\times$ 10⁶ WT BM cells. IFN- γ ^{-/-} were reconstituted with 1.6 $\times$ 10⁷ IFN- γ ^{-/-} BM.

Statistical analysis

All significance was determined using computer-based statistics (PC statistical software from SPSS, Chicago, IL). The differences in mean values were analyzed using a two-tailed Student's t test. *p*-values of <0.05 were considered statistically significant.

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Abbreviations

RA	rheumatoid arthritis
PGIA	proteoglycan-induced arthritis
Ab	antibody

Treg cells	T regulatory cells
Breg cells	B regulatory cells
WT	wildtype
rG1	recombinant human aggrecan G1-domain protein
DDA	dimethyldioctadecyl-ammonium bromide
i.v.	intravenous
i.p.	intraperitoneal
wk	week
h	hour

Figure 1. Antigen-specific effector CD4⁺ T cells and T regulatory cells in rG1/DDA-primed mice after B-cell depletion

Foxp3^{eGFP} mice were immunized with rG1 on day 0 and treated with anti-mCD20 (or control Ab) on day 5. Spleens were harvested on day 9 and analyzed by flow cytometry. (A) Flow cytometry plot of splenic B220⁺ B cells and CD4⁺ T cells. (B) Percentage (left) and numbers (right) of B cells in spleen. (C) Percentage (left) and numbers (right) of CD4⁺ T cells in spleen. (D, E) Single cell suspensions of spleens were incubated with tetramer G1₇₀₋₈₄/I-A^d for 4 h and a MACS magnet was used to isolate tetramer-positive (antigen-specific) cells (D) CD44⁺tetramer⁺ cells were gated on CD4⁺ T cells. (E) The number of tetramer-positive CD4⁺ T cells. (F) Foxp3⁺ Treg cells were gated on total CD4⁺ T cells in (A). (G) The percentage (left) and numbers (right) of Foxp3⁺ Treg cells. Results are presented as mean ± SD of 5 mice and from single experiments representative of 3 independent experiments performed. * p<0.05, two-tailed Student's t test.

Figure 2. Antigen-specific responses in B-cell-depleted animals

Foxp3^{eGFP} mice were immunized with rG1 on day 0, treated with anti-mCD20 (or control Ab) on day 5 and spleens were harvested on day 9. For intracellular staining, single cell suspensions from spleens were stimulated with PMA and ionomycin for 4 h. Cells were surfaced stained for CD4 and permeabilized and stained for IFN- γ and IL-10. (A) Flow cytometry plots are based on gated CD4⁺ T cells. (B) Percentage (left) and number (right) of CD4⁺IFN- γ ⁺ T cells. (C) IFN- γ production by CD4⁺ T cells in response to rG1 (2 μ g/ml) restimulation in the presence of mitomycin C-treated naïve splenocytes cultured for 4 days. (D) Foxp3⁺IL-10⁺ Treg cells were gated on Foxp3⁺ Treg cells as shown in Fig. 1F. (E) Percentage (left) and number (right) of Foxp3⁺IL-10⁺ Treg cells. (F) Proliferation of CD4⁺ T cells in response to rG1 (2 μ g/ml) restimulation was measured by ³H-thymidine incorporation during the last 24 h of a 5-day culture. Results are presented as mean $\pm$ SD of 5 mice and from single experiments representative of 3 independent experiments performed. * $p < 0.05$, two-tailed Student's t test.

Figure 3. Treg-cell differentiation after B-cell depletion in vivo

CD4⁺Foxp3⁻ T cells were sorted from CD4⁺CD62L⁺ T cells isolated from naive Tg5/4E8Foxp3^{eGFP} mice and transferred into CD90.1 congenic mice at (8×10^5 cells/mouse) on day 0. Mice were immunized with rG1/DDA on day 1, treated with anti-mCD20 (or control Ab) on day 5 and spleens were harvested on day 10 and analyzed by flow cytometry. (A) The transferred CD90.2⁺CD4⁺ T cells in recipient mice were evaluated by flow cytometry and gated on CD90.2 and CD4. (B) The percentage (left) and number (right) of CD90.2⁺CD4⁺ T cells. (C, D) Cells that upregulated Foxp3^{eGFP} expression are shown by flow cytometry. Gates are based on CD90.2⁺CD4⁺ T cells in (A). (C) Gating to identify Vβ4⁺Foxp3^{eGFP} Treg cells. (D) Percentage (left) and number (right) of Vβ4⁺Foxp3^{eGFP} Treg cells in recipient mice. Results are presented as mean $\pm$ SD of 3-4 mice from single experiments representative of 2 independent experiments. (E, F) Naïve Foxp3^{eGFP} mice were treated with anti-mCD20 (or control Ab) and spleens were harvested 4 days later. (E) Foxp3^{eGFP} expression gated on CD4⁺ T cells. (F) Percentage (left) and numbers (right) of Foxp3^{eGFP} Treg cells. Results are presented as mean $\pm$ SD of 4 mice and from single experiments representative of 2 independent experiments. * $p < 0.05$, two-tailed Student's t test.

Figure 4. Treg-cell differentiation in the presence of B-cell-derived IFN-γ

(A) B cells were sorted from WT naïve BALB/c mice and activated with LPS, IL-12 and/or α-CD40 for 48h. IFN-γ production was measured by ELISA in supernatants. Results are shown as mean ± SD of triplicate wells from one experiment representative of 2 performed. (B, C) Splenocytes from naïve and arthritic mice were surface stained for CD19 and intracellularly stained for IFN-γ. (B) IFN-γ-producing cells were gated on CD19⁺ cells. (C) Percentage (left) and numbers (right) of IFN-γ-producing B cells and CD4⁺ T cells. Results are shown as mean ± SD of 5 mice from a single experiment representative of 2 performed. (D, E) CD4CD25-CD62L⁺ T cells were isolated from naïve IFN-γ^{-/-} Foxp3^{eGFP} mice and activated in vitro with plate-bound α-CD3 and soluble α-CD28 in the presence of TGF-β and activated (stimulated with LPS and IL-12, 24 hours before incubation with T cells) WT or IFN-γ^{-/-} B cells. Flow gates are based on CD4⁺ T cells. (D) Gating of CD4⁺Foxp3⁺ Treg cells. (E) Percentage of CD4⁺Foxp3^{eGFP} Treg cells. Results are presented as mean ± SD of triplicate wells and are from single experiments representative of 3 independent experiments. * p<0.05, two-tailed Student's t test.