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Regulatory CD4+ T Cells Promote B Cell Anergy in Murine Lupus

Yaoyang Liu,*1 Aijing Liu,*‡1 Noriko Iikuni,∗ Huji Xu,‡ Fu-Dong Shi,§ and Antonio La Cava∗

To prevent autoimmunity, anergy of autoreactive B cells needs to be maintained, together with the suppression of hyperactive B cells. We previously reported that CD4+CD25+Foxp3+ regulatory T cells (Tregs) can directly suppress autoantibody-producing autoreactive B cells in systemic lupus erythematosus. In this article, we show that Tregs can also reduce the production of autoantibodies in (NZB × NZW)F1 mouse lupus B cells by promoting B cell anergy, both in vitro and in vivo. This phenomenon associated with a reduction in Ca2+ flux in B cells, and CTLA-4 blockade inhibited the effects of Tregs on anergic lupus B cells. These findings identify a new mechanism by which Tregs can control production of autoantibodies in lupus B cells and, more generally, B cell activity in physiopathological conditions. The Journal of Immunology, 2014, 192: 4069–4073.

To prevent autoimmunity, autoreactive B cells are silenced through several mechanisms of immune tolerance, which include: 1) clonal deletion (resulting in apoptotic death), 2) receptor editing (resulting in new Ab specificities), and 3) anergy (allowing B cells to persist in an Ag-unresponsive state). Relevant to autoimmunity, anergic B cells are neither deleted nor edited, so they can be reactivated to produce Ab (1). This implies that the escape of silenced autoreactive B cells from anergy could result in the production of autoantibodies and subsequent autoimmunity (2).

Of note, the majority of B cells generated in the bone marrow are autoreactive (3). Most of those autoreactive B cells undergo receptor editing or are deleted, whereas a fraction becomes anergic (2). Anergic B cells maintain their pathogenic potential because of the reversibility of their state of hyporesponsiveness to self-antigen stimulation. Therefore, the functional suppression of autoantibody production via B cell anergy represents an active process that operates on a large scale, and under a constant immune monitoring. Recently, it was shown that transitional (T)3 B220+CD93+ CD23+IgMlo/− B cells are in an anergic state (2), do not proliferate or mobilize Ca2+, or mount an immune response after Ag-receptor stimulation (2).

We and others previously reported that B cell functions can be modulated by regulatory T cells (Tregs) including in autoimmune diseases such as systemic lupus erythematosus (SLE) (4, 5). SLE is characterized by the presence of elevated titers of autoantibodies (6). Of note, B cell anergy is impaired in SLE patients, but not in healthy individuals (7, 8).

In this article, we extend the above findings by showing that Tregs can promote B cell anergy in vitro and in vivo. These studies identify a new mechanism by which the deficiency of Tregs in SLE could facilitate autoantibody production and autoimmunity.

Materials and Methods

Mice

Female (New Zealand Black × New Zealand White)F1, (NZB/W) mice and BALB/c mice crossed to NZW mice to obtain non-autoimmune, matched CW control mice (9) were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained at the University of California Los Angeles. Each experiment (including the Ca2+ flux studies) included six to eight individual mice per group. Monitoring for the development of proteinuria was assessed using Albustix reagent strips (Bayer, Pittsburgh, PA), according to the manufacturer’s instructions. Animals were treated according to the National Institutes of Health guidelines for the use of experimental animals, under protocols approved by the Institutional Animal Research Committee.

Cell cultures

B cells, Tregs, and effector T cells (Teffs) were separated from NZB/W splenocytes, using magnetic bead kits (Miltenyi Biotec, Auburn, CA) on an AutoMACS separator (Miltenyi Biotec). Each cell fraction was >97% pure prior to experimental use. B cells were cultured in HL-1 medium (Lonza, Walkersville, MD) in a 5% CO2 incubator at 37˚C with Teffs, with or without Tregs, including, or not, anti–CTLA-4 blocking Ab (Novus, Littleton, CO). In some experiments, Tregs were cocultured with B cells at ratios of both 1:1 and 1:4 of B/Treg. In the experiments in which culture supernatants were collected for anti-dsDNA Ab measurements by ELISA, B cells were stimulated with 10 μg/ml CD40 Ab (eBioscience, San Diego, CA) and 4 μg/ml anti-mouse Ig (BioLegend, San Diego, CA).

In vivo cell depletion and adoptive transfer experiments

Mice were injected i.p. with 300 μg anti-CD25 Ab (clone PC61.5; eBioscience) on day 0 and day 3 for Treg depletion. A total of >95% of circulating Tregs were eliminated by day 1 until day 3 (not shown). Control mice that received isotype control Ig (clone eBRG1; eBioscience) under the same protocol maintained intact frequency of circulating CD25+ cells (not shown). In the adoptive transfer experiments, 1 × 10⁷ purified Tregs were injected 1.5 d after the second injection of anti-CD25 Ab or isotype control Ab (i.e., when the injected Ab was no longer detectable in serum by ELISA). Serum concentration of anti-dsDNA Ab was assessed by ELISA, and cell phenotypes by flow cytometry.

For in vivo Treg suppression, sorted B cells were coinjected, or not, conjected, with Tregs (1:1) into mice that had been previously irradiated with 900 cGy (divided into two doses, 3 h apart), before receiving a single
i.v. injection of 2–5 × 10⁶ cells in saline. After transfer, mice were monitored for B cell apoptosis by ex vivo flow cytometry. For depletion of T cells, mice with elevated anti-DNA Ab titers (measured by ELISA) were divided into two groups, one receiving a single i.v. dose of 300 µg depleting anti-CD3 Ab (clone 17A2; BioLegend), the other group receiving a similar amount of irrelevant isotype control Ab (clone RTK4530; BioLegend). After treatment, mice were monitored daily for the relative ratio of intensities of Indo fluorescence (Ca²⁺-bound Indo-1 fluorescence ratio) was acquired as a function of time, and kinetics curves were generated using FlowJo software (TreeStar, Ashland, OR).

Flow cytometry

Phenotypic analyses were performed with combinations of fluorochrome-conjugated mAb (including Fab obtained with the Pierce Fab Preparation Kit) using standard techniques. After Fc blocking, anti-mouse mAb used for staining included FITC-, PE-, PerCP-, and allophycocyanin-conjugated anti-CD220, -CD23, -CD93, -CD86, -IgM, -CD4, -CD25, and -Foxp3 (the last-named for intracellular staining, using the eBioscience Anti-mouse Foxp3 Staining Set), or isotype control Ab. All Abs were from eBioscience. Gating for T3 B cells was done on B220⁺CD93⁺CD23⁺IgM⁺ cells. Samples were acquired on an LSRII flow cytometer (Becton Dickinson, San Diego, CA), and analysis was done with FlowJo software (TreeStar).

ELISA

Anti-dsDNA Ab titers were quantified by ELISA, as previously described (10).

Intracellular Ca²⁺ measurement

Splenocytes were incubated with Indo-1 AM ester (Invitrogen, Carlsbad, CA) at room temperature for 30 min and then stained with anti-CD220, -CD23, -CD93, and -IgM Ab. Ca²⁺ data were collected and displayed as the relative ratio of intensities of Indo fluorescence (Ca²⁺-bound Indo-violet at 405 nm/free Indo-blue at 485 nm). For each experiment, collection of baseline measurement at 37°C was followed by stimulation with 50 µg/ml rat F(ab')₂ anti-mouse IgM (Southern Biotech, Birmingham, AL) or 500 ng/ml ionomycin (Sigma-Aldrich, St. Louis, MO). The indo-1 fluorescence ratio was acquired as a function of time, and kinetics curves were generated using FlowJo software (TreeStar).

Statistical analyses

Statistical analyses were performed using Prism 4 software (GraphPad, San Diego, CA). Parametric testing was done using the unpaired t test; non-parametric testing was used when data were not normally distributed. The p values < 0.05 were considered significant.

Results

Tregs suppress lupus B cell production of Ab in vivo

The suppressive capacity of Tregs on B cells was evaluated in vivo. NZB/W mice with elevated circulating anti-DNA Ab were depleted of endogenous T cells and then transferred with syngeneic Tregs (see Materials and Methods). In the absence of Th cells (confirmed by flow cytometry; not shown), Tregs suppressed in vivo the B cell production of IgG and anti-DNA autoantibodies (Fig. 1). Mice that had not been depleted of T cells before the transfer of Tregs also had reduced serum titers of total IgG and anti-DNA Ab, as shown before (4), albeit at a lesser extent than T cell-depleted animals (not shown). Age was not influential on these events because similar results were obtained when Tregs derived from young or old mice (Fig. 1). Thus, Tregs can directly suppress B cells in vivo, without an intermediate suppression of Th cells, and independently of age. Of interest, the suppression of different Ig isotypes suggested that multiple Ab specificities were inhibited by Tregs (Fig. 2).

Two non–mutually exclusive possibilities could explain these results: 1) that Tregs had favored apoptosis of B cells, or 2) that they had favored anergy in B cells. To explore these possibilities, we analyzed the effects of Tregs on B cells in vitro and in vivo.

We found that Tregs induced apoptosis in vivo in B cells (Supplemental Fig. 1A), confirming previous studies that had shown an ability of Tregs to promote B cell apoptosis in vitro in coculture experiments (4). Of note, the extent of B cell apoptosis in the presence of Tregs (Supplemental Fig. 1B) could not fully explain the reduced production of Ab from B cells (Supplemental Table I). In particular, the decreased production of IgG by B cells in the presence of Tregs in cocultures (Supplemental Table I) was higher than what was expected from the sole reduction of B cell frequency caused by apoptosis (Supplemental Fig. 1B). Because of this finding, we explored the possibility that, in addition to apoptosis, Tregs could induce anergy in B cells. Early in cocultures with Tregs, B cell apoptosis appeared to be the main mechanism of Treg suppression (39 and 41.5% of B cells underwent apoptosis by this finding, we explored the possibility that, in addition to apoptosis, Tregs could induce anergy in B cells. Early in cocultures with Tregs, B cell apoptosis appeared to be the main mechanism of Treg suppression (39 and 41.5% of B cells underwent apoptosis by the presence of Tregs in cocultures (Supplemental Fig. 1A), confirming previous studies that had shown an ability of Tregs to promote B cell apoptosis in vitro in coculture experiments (4). Of note, the extent of B cell apoptosis in the presence of Tregs (Supplemental Fig. 1B) could not fully explain the reduced production of Ab from B cells (Supplemental Table I). In particular, the decreased production of IgG by B cells in the presence of Tregs in cocultures (Supplemental Table I) was higher than what was expected from the sole reduction of B cell frequency caused by apoptosis (Supplemental Fig. 1B). Because of this finding, we explored the possibility that, in addition to apoptosis, Tregs could induce anergy in B cells. Early in cocultures with Tregs, B cell apoptosis appeared to be the main mechanism of Treg suppression (39 and 41.5% of B cells underwent apoptosis by 3 and 6 h, respectively; Supplemental Fig. 1B), and no additional reduction of IgG production by B cells was observed aside from that expected from apoptosis of B cells (Fig. 1B, Supplemental Table I).

FIGURE 1. Tregs suppress lupus B cell production of Ab in vivo without requirement of Th cells. Mice with elevated titers of anti-dsDNA Ab (monitored by ELISA) were depleted of endogenous T cells by i.v. injection of anti-CD3 Ab (see Materials and Methods). At 1.5 d after treatment, those mice were injected with saline (no Tregs) or 1 × 10⁷ Tregs from young (10–12 wk) or old (26–30 wk) NZB/W mice. Production of total IgG and anti-dsDNA Ig was monitored ex vivo by ELISA, at the indicated time points. *p < 0.01, **p < 0.03, ***p < 0.05 versus control (saline). p not significant between young and old donor Tregs.

FIGURE 2. Tregs directly suppress production of multiple Ab isotypes in lupus B cells. NZB/W B cells were stimulated with anti-CD40/Ig in cocultures with syngeneic Tregs (1:10). Ab production in supernatants was assessed by ELISA after 16 h of coculture. *p < 0.03, **p < 0.04.
However, after 9, 12, and 15 h of coculture of B cells with Tregs, an additional unaccounted decrease of IgG was observed (18.5%, 21.3%, and 18.7%, respectively; Supplemental Table I), in addition to the IgG reduction expected from the 52.3%, 56.2%, and 58.4% apoptosis of B cells at the respective time points (Supplemental Fig. 1B).

**Tregs promote lupus B cell anergy in vitro**

To address whether the above findings could be explained with the induction of anergy in B cells by Tregs, we evaluated the frequency of anergic B cells induced by Tregs in vitro. An increased frequency of anergic B cells (B220+CD93+CD23+IgMlo/T3 B cells) was seen in the presence of Tregs (Fig. 3A)—first modest in early cocultures, and then significantly different at later time points (Supplemental Fig. 1B). This finding was consistent with the observed changes in IgG production (Supplemental Table I). Also consistent with a Treg-mediated promotion of anergy in B cells, T3 B cells had high Ca2+ flux when cocultured with Teffs (as expected), but not in the presence of Tregs (Fig. 3B).

**Tregs promote lupus B cell anergy in vivo**

These findings prompted in vivo studies. NZB/W mice that had been depleted of Tregs were reconstituted with Tregs from young NZB/W mice (Fig. 4A). The expression of CD86—an indicator of loss of anergy in B cells (11)—increased after Treg depletion, being reversed by Treg replacement (Fig. 4B). Ca2+ mobilization was elevated after Treg depletion, and it appeared similar to controls after adoptive transfer of Tregs (Fig. 4C). In addition, depletion of Tregs associated with increased anti-dsDNA Ab levels, and this was reversed by adoptive transfers of Tregs (not shown). Altogether, these findings indicate an ability of Tregs to promote B cell anergy in vivo.

**Characteristics of anergic lupus B cells**

A low expression of CD86 is associated with an anergic phenotype in T3 B cells (11, 12), whereas an increase in CD86 expression enables B cells to respond to antigenic stimulation (13). In the comparison between NZB/W lupus mice and non-autoimmune CW control mice, we found that the expression of CD86 on T3 B cells was higher in the former mice (Fig. 5A). In addition, the stimulation of T3 B cells from NZB/W mice was associated with higher Ca2+ mobilization than in CW mice (Fig. 5B), suggesting a tendency of T3 B cells in NZB/W lupus mice to escape anergy. CTX-4 blockade abrogates the effects of Tregs on anergic lupus B cells

We had observed that Tregs promoted anergy in B cells and prevented their cell surface upregulation of CD86 in vivo (Fig. 4). Not surprisingly, CD86 expression was decreased on T3 B cells

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**FIGURE 3.** Tregs modulate NZB/W B220+CD93+CD23+IgMlo/T3 B cell frequency and intracellular Ca2+ mobilization in vitro. (A) B cells from NZB/W mice were cocultured (1:5) with Teffs, with or without Tregs (1:1 with Teffs) for 3, 6, 9, 12, and 15 h, and then analyzed by flow cytometry. Similar results were obtained in the absence of Teffs (not shown). *p < 0.05. (B) Ca2+ flux in T3 B cells alone (light gray) or cultured for 6 h with Teffs with (gray) or without (dark gray) Tregs. After addition of 50 μg/ml anti-IgM F(ab’)2, ratiometric quantitation of Indo-1 was measured for 300 s. Representative experiment of three that gave similar results.

**FIGURE 4.** Tregs influence NZB/W B220+CD93+CD23+IgMlo/T3 B cell frequency and intracellular Ca2+ mobilization in vivo. (A) Adoptive transfer of Tregs (+) restores frequency of peripheral T3 B cells in mice depleted of Tregs (−). (B) Mean fluorescence intensity (MFI) for surface CD86 on T3 B cells from control mice, in mice depleted of Tregs (−), and in mice reconstituted with Tregs (+). (C) Ca2+ flux in T3 B cells after ex vivo BCR stimulation in control mice (light gray) and mice depleted (dark gray) or reconstituted with Tregs (gray). For this experiment, 50 μg/ml anti-IgM F(ab’)2 was added prior to ratiometric quantitation of Indo-1, which was measured for 300 s. *p < 0.05.
when they were cocultured with Tregs (Fig. 6A). Of interest, an involvement of CTLA-4 in the control of B cell anergy by Tregs was observed. CTLA-4 blockade associated with reduced T3 B cell frequency in the presence of Tregs (Fig. 6B), and blockade of CTLA-4 restored the decreased expression of CD86 on T3 B cells cocultured with Tregs (Fig. 6A). Furthermore, blockade of CTLA-4 partly reversed the inhibition of anti-dsDNA Ab production by B cells cocultured with Tregs (Fig. 6C). Finally, CTLA-4 blockade facilitated Ca2+ mobilization in B cells cocultured with Tregs (Fig. 6D).

Discussion

Anergy is a key mechanism by which autoreactive B cells are silenced, and the maintenance of the state of unresponsiveness to Ag stimulation in anergic cells is considered a critical factor in the prevention of autoimmunity (14). Anergic (T3) B cells contain autoreactive cells (2), and a loss of anergy in this compartment facilitates the production of autoantibodies and the development of autoimmune disease (2, 8).

SLE is a systemic autoimmune disease in which the presence of multiple autoantibodies (produced by hyperactive B cells) plays a central role in the disease manifestations and underlying pathological features. In this study, we observe that in lupus mice, in vitro and in vivo, Tregs can modulate the frequency of anergic B cells. The observation that reduction of IgG and anti-DNA Ab in the presence of Tregs occurred rapidly (Fig. 3A) might relate to the abnormally elevated production of Ab in SLE (15) (as Tregs suppress Ab-producing B cells) and the accelerated Ab catabolism (16) (which expedites Ab elimination). Moreover, we found that blockade of CTLA-4 in anergic B cells was associated with upregulated CD86 expression and increased Ca2+ mobilization. One possible explanation could be that CD86 on anergic B cells might engage CD28 to promote Teffs proliferation and/or that CTLA-4 on Tregs might provide inhibitory signals, favor a reduced CD86 expression, and/or impair T cell interactions with anergic B cells (13, 17, 18). CD86 could as well modulate cytokine/cell surface molecules, thereby influencing B cell interactions and survival (13, 19, 20). Although more investigation is required to address in detail the above possibilities, the results reported in this article show that the size of the anergic B cell pool can be regulated by Tregs, and that CTLA-4 has an important role in such regulation.

It has been reported that anergic B cells have a short half-life, although the underlying reasons remain elusive (21). A short lifespan of anergic B cells could serve to keep a balanced pool of these potentially harmful cells, if autoreactive. Conversely, a longer lifespan for anergic B cells would increase their risk of losing anergy (22). Autoreactive lupus B cells with an anergic phenotype could have an abnormally prolonged lifespan, being neither activated nor deleted (23). Of interest, a loss of anergy in T3 B cells has been observed in autoimmune diseases such as collagen-induced arthritis and rheumatoid arthritis (24–26), suggesting that the generation and survival of anergic B cells requires a fine control to avert autoimmunity.

FIGURE 5. Anergic B cell characteristics. (A) B220+CD93+CD23+ IgMlo T3 B cells from NZB/W lupus mice have higher CD86 expression in flow cytometry than those from non-autoimmune control CW mice. *p < 0.05 MFI, mean fluorescence intensity. (B) Higher Ca2+ flux in T3 B cells from NZB/W lupus mice than in cells from non–lupus control CW mice. T3 B cells from spleens were stained for Indo-1, and Ca2+ flux was measured after BCR stimulation in NZB/W mice (dark gray) and in CW controls (light gray). A total of 50 µg/ml anti-IgM F(ab’)2 was added prior to ratiometric quantitation of Indo-1, which was measured for 300 s. Representative experiment of six that gave similar results.

FIGURE 6. The effects of Tregs on T3 B cells involve CTLA-4. (A and B) CTLA-4 blockade influences expression of CD86 on T3 B cells. B cells were cultured with Teffs and Tregs with or without anti-CTLA-4 Ab. Cells were harvested after 6 h and stained with anti-B220, -CD93, -CD23, -IgM, -CD86 for flow cytometry, MFI, mean fluorescence intensity. (C) Treg suppression of anti-dsDNA Ab production is influenced by CTLA-4 expression. NZB/W B cells were stimulated with anti-CD40/Ig, cocultured for 12 h with Teffs in the presence, or not, of Tregs and/or anti-CTLA-4 Ab. Supernatants were collected for anti-DNA Ab measurement by ELISA. (D) Ca2+ flux in T3 B cells after BCR stimulation in cocultures of B cells with Teffs and Tregs with (dark gray) or without (light gray) CTLA-4 blockade. A total of 50 µg anti-IgM F(ab’)2 was added prior to ratiometric quantitation of Indo-1, which was measured for 300 s. *p < 0.05.
Although the induction of B cell anergy by Tregs might as well occur in normal B cells, the findings reported in this article that Tregs could induce anergy in lupus B cells indicate that Tregs can control lupus B cells (and their production of autoantibodies) not only through deletion of autoreactive B cells (4, 5) but also via anergy. These data are consistent and expand the observation that an absence of Tregs in Foxp3-deficient mice is associated with impaired B cell anergy (27). The findings also help to further explain how the deficit of Tregs in SLE (28) could play a key role in the inefficient suppression of autoantibodies that characterizes the disease.

Disclosures
The authors have no financial conflicts of interest.

References
Supplementary Figure 1. T_{Reg} promote B cell apoptosis. 

a. T_{Reg} were cocultured with anti-CD40/Ig-stimulated B cells at a 1:1 ratio for the indicated time points (see Materials and Methods). Analogous results were obtained at 1:2 and 1:5 ratios (not shown). *P<0.05. 

b. Percent increase in B cell apoptosis in the presence of cocultured T_{Reg} vs. B cells alone.
Supplementary Table I. IgG concentration (fg/cell) in supernatant of B cells cultured or not with T<sub>Reg</sub>. B cells were stimulated with anti-CD40/Ig for the indicated time points (see Materials and Methods for details), and supernatant was collected for sensitive ELISA quantitation of IgG content.

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