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

Yaoyang Liu,*1 Aijing Liu,*4†,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 mice lupus B cells by promoting B cell anergy, both in vitro and in vivo. This phenomenon associated with a reduction in Ca⁺⁺ 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: 000–000.

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 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 Alkbustix 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 Care.

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) and maintained at the University of California Los Angeles.

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Address correspondence and reprints requests to Prof. Antonio La Cava, Department of Medicine, University of California, Los Angeles, 1000 Veteran Avenue 32–59, Los Angeles, CA 90095-1670. E-mail address: alacava@mednet.ucla.edu

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Abbreviations used in this article: NZB/W mouse, (New Zealand Black × New Zealand White)F₁ mouse; SLE, systemic lupus erythematosus; Tcell, effector T cell; Treg, regulatory T cell.

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i.v. injection of 2–5 × 10^6 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 presence of circulating Ab by ELISA. In the mice treated with anti-CD3 Ab, circulating T cells in peripheral blood were typically reduced by >85% 1 d after treatment, whereas they did not change in the control group. B cell frequency in all animals was not affected by treatment (not shown). Serum concentration of anti-CD3 or control Ab was <23.1 ± 2.0 ng/ml 1.5 d after treatment, which is when T cell depletion was confirmed by flow cytometry prior to i.v. injection of 1 × 10^7 Tregs. Serum IgG and anti-DNA Ab in recipient mice were monitored by ELISA.

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-B220, -CD23, -CD93, -CD66, -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^bright^ cells. Samples were acquired on an LSRII flow cytometer (Becton Dickinson, San Diego, CA), and analysis was done with FlowJo software (TreeStar, Ashland, OR).

ELISA

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

Intracellular Ca^{2+} measurement

Splenocytes were incubated with Indo-1 AM ester (Invitrogen, Carlsbad, CA) at room temperature for 30 min and then stained with anti-B220, -CD23, -CD93, and -IgM Ab. Ca^{2+} data were collected and displayed as the relative ratio of intensities of Indo fluorescence (Ca^{2+}-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).
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/2 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 (Fig. 3A). 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.

CTLA-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

FIGURE 3. Tregs modulate NZB/W B220+CD93+CD23+IgMlo/2 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/2 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 up-regulated 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.
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\textsubscript{Reg} promote B cell apoptosis. \textit{a}. T\textsubscript{Reg} were cocultured with anti-CD40/Ig-stimulated B cells at a 1:1 ratio for the indicated time points (see \textit{Materials and Methods}). Analogous results were obtained at 1:2 and 1:5 ratios (not shown). *\textit{P}<0.05. \textit{b}. Percent increase in B cell apoptosis in the presence of cocultured T\textsubscript{Reg} vs. B cells alone.
Supplementary Table I. IgG concentration (fg/cell) in supernatant of B cells cultured or not with T_{Reg}. 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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<th>hrs</th>
<th>B alone</th>
<th>B + T_{Reg}</th>
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<td>3</td>
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