Cutting Edge: Regulatory T Cells Directly Suppress B Cells in Systemic Lupus Erythematosus

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In systemic lupus erythematosus (SLE), adaptive CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) suppress Th cells that help autoantibody (autoAb)-producing B cells. It is not known whether naturally occurring Tregs can directly suppress B cells in SLE without an intermediate suppression of Th cells. This aspect is important for its implications in the natural course of SLE, because most if not all of the clinical and pathologic effects in SLE are associated with a dysregulated production of autoAbs. In this study, we show that natural Tregs can inhibit B cell activity in vitro and in vivo in SLE through cell contact-mediated mechanisms that directly suppress autoAb-producing B cells, including those B cells that increase numerically during active disease. These results indicate that one way by which natural Tregs attempt to limit humoral autoimmunity in SLE is by directly targeting autoreactive B cells. The Journal of Immunology, 2009, 183: 0000–0000.

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ystemic lupus erythematosus (SLE) is an autoimmune disease characterized by aberrant B cell responses to multiple nuclear and cytoplasmic Ags (1). The abnormal production of autoantibodies (autoAbs) is responsible for most of the clinical and pathologic manifestations of the disease. In particular, the presence of elevated titers of autoAbs that form immune complexes with Ag and precipitate in tissue can lead to local damage, chronic inflammation, and, eventually, loss of organ function. Thus, a reduction of the production of autoAbs has been envisioned as a potential therapeutic modality to improve the management of SLE (2).

Both B cells and CD4⁺ Th cells contribute to the development and maintenance of autoAb responses in SLE (3). One subpopulation of lymphocytes that can suppress the activity of Th cells is the CD4⁺CD25⁺Foxp3⁺ regulatory T cell (Treg) subset (4, 5). Tregs are key players in the maintenance of peripheral immune tolerance through suppression of the proliferation and release of proinflammatory cytokines from immune cells (6, 7). Moreover, depletion of Tregs in rodents can lead to dysregulated Ab production (8), and the transfer of Tregs into autoimmune animals can reduce Ab responses (9). Although it is known that activated Tregs can directly suppress Ab production from nonautoimmune B cells (10, 11), it is not known whether Tregs can directly suppress autoAb responses from B cells in humoral-mediated autoimmune diseases such as SLE. This aspect is particularly important, because SLE is a disease primarily driven by exaggerated B cell production of autoAbs with multiple specificities.

We and others have previously shown that the recovery of the numerical deficit of Tregs in SLE is associated with a reduced production of autoAbs in vitro and in vivo (3, 4). In those studies, Th cells represented the key intermediates for adaptive Treg-mediated suppression of autoAb-producing B cells (12). In this study we address whether natural Tregs can directly suppress autoreactive B cells in SLE.

Materials and Methods

Mice

Female (New Zealand Black × New Zealand White)F1 (NZB/W) mice were purchased from The Jackson Laboratory and maintained at the University of California, Los Angeles, CA (UCLA). The animals were treated according to National Institutes of Health guidelines for the use of experimental animals. Protocols were approved by the UCLA Animal Research Committee.

SLE patients and controls

SLE patients fulfilled the American College of Rheumatology criteria for the classification of SLE. Healthy volunteers were matched for gender, age, and ethnicity with the SLE patients. The study was conducted according to the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of UCLA. Each individual gave informed consent for participation in the study, and SLE patients with comorbid conditions were excluded from the investigation.

Cell preparations

Mouse cells. Murine Tregs and B cells were isolated by multistep magnetic sorting from splenocytes on an autoMACS instrument (Miltenyi Biotec) using a mouse regulatory T cell isolation kit and a mouse B cell isolation kit (Miltenyi Biotec), respectively, according to the manufacturer’s instructions. The purity of sorted cells was routinely ≥97%. Tregs and B cells were cultured in HL-1 complete medium (BioWhittaker) in a 5% CO₂ incubator at 37°C. B cells were unstimulated or stimulated with 10 μg/ml anti-CD40 Ab (eBioscience) and 4 μg/ml anti-mouse Ig (BD Biosciences) or LPS (20 μg/ml) (Sigma-Aldrich).
selected experiments, cocultures of Tregs and B cells included 20 μg/ml Fas-Fc (R&D Systems) or 5 μg/ml TNFRII-Fc (eBioscience, ImmunoX). All studies with T cells included stimulation with 2 μg/ml anti-CD3e and 1 μg/ml anti-CD28 anti-mouse mAbs (both from BD Biosciences). At different time points, supernatants were analyzed for Ig production by ELISA and cells were stained for flow cytometry.

**Human cells.** PBMCs were separated by ficoll gradient before the isolation of Tregs and B cells. Tregs were sorted through FACSVantage (BD Biosciences) as human CD4 Tregs and B cells. Tregs were sorted through FACSVantage (BD Biosciences) as B cells were stimulated with 1 μM sodium (Invitrogen) supplemented with 2% human AB serum (Sigma-Aldrich). B cells were stimulated with 1 μg/ml LPS or 5 μg/ml anti-CD40 Ig (eBioscience), 20 ng/ml IL-4, 40 ng/ml IL-10, and 0.25 ng/ml IL-2 (all from R&D Systems). The T cells were stimulated with a Dynabeads CD3/CD28 T cell expander (Invitrogen) at 0.5 bead/cell.

**Adoptive transfers.** Preliminary experiments defined the conditions used for the adoptive transfer experiments. Briefly, mice with proteinuria at concentrations >100 mg/dl as measured by Albustest reagent strips (Bayer) were divided into three groups of six mice each. One group of mice received a single i.v. dose of 300 μg of depleting anti-mouse CD3 rat Ab (17A2; BioLegend) for the depletion of endogenous CD3+ T cells, another group received 600 μg of the same depleting anti-CD3 Ab, and the third group received 300 μg of the isotype control rat Ab (RTK4530; BioLegend). The Abs were side free. After treatment, the mice were monitored daily for circulating CD3+ T cells in the PBMCs by flow cytometry and for the presence of free Abs in the plasma by ELISA. Because three mice in the group treated with 600 μg of anti-CD3 Ab died within a week after treatment and none of the mice treated with 300 μg of anti-CD3 Ab or control Ab died by the same time, we analyzed the animals that had been treated with the lower dose in comparison to the mice of the control group. We then conformed the experiment with the lower dose of Ab on additional six mice. In these mice treated with depleting anti-CD3 Ab, the numbers of circulating T cells in peripheral blood were typically reduced by 86 ± 11% 1 day after treatment, whereas they did not change significantly in the control group (not shown). B cell numbers in the treated animals were not affected by the treatment (not shown). The serum concentration of rat anti-CD3 or control Ab was 23.7 ± 1.9 ng/ml until 2 days after treatment. Adoptive transfer experiments were then performed on groups of eight mice each and repeated three times. In these experiments, the mice received a single dose of 1 × 10^6 purified Tregs 1.5 days after injection of anti-CD3 Abs or control Abs, and the concentrations of serum IgG and anti-DNA Ab were assessed ex vivo by ELISA.

**Flow cytometry.** Phenotypic analyses were performed with combinations of fluorochrome-conjugated mAbs using standard techniques. After Fc blocking, the anti-mouse mAbs used for staining included FITC-, PE-, PerCP-, and allophycocyanin-conjugated anti-CD19 (MB19-1), anti-CD4 (GK1.5), anti-CD25 (PC61.5), and anti-Foxp3 (FJK-16-1) mAbs, or an isotype control Abs. Fluorochrome-conjugated anti-human mAbs were anti-CD4 (RPA4), anti-CD25 (BC96), anti-CD127 (eBioRDR5), anti-Foxp3 (PC9101), anti-CD19 (HIB19), and anti-IgD and an isotype control Ab. All Abs were from eBioscience except for the anti-IgD Ab, which was from Beckman Coulter. Samples were acquired on a FACSCalibur flow cytometer (BD Biosciences) at the UCLA Translational Immunology Cell Core and analyzed using FlowJo software (Tree Star).

**Apoptosis assay.** Staining with annexin V was performed in conjunction with the dye 7-aminoactinomycin D (7-AAD) by using an annexin V apoptosis kit (BD Biosciences) and following the manufacturer’s instructions. Gated CD19+ B cells were analyzed by flow cytometry; gated Tregs stained negative. Anti-perforin (eBioOMAK-D) and anti-granzyme B (16G6) mAbs (both from eBioscience) were used at 20 μg/ml and EGTA (Sigma-Aldrich) at 3 mM.

**Cytotoxicity assay.** Cytotoxicity was assayed with the CytoTox 96 nonradioactive cytotoxicity assay (Promega) following the manufacturer’s instructions.

**ELISA.** To assess the concentration of rat anti-mouse CD3 mAbs in the mice used in the adoptive transfer experiments, we used an approach similar to what we previously reported for monitoring allospecific IgG titers in mouse serum (13), with some modifications. Briefly, mouse anti-rat IgG (Southern Biotech) was used as the capture Ab and the same peroxidase-labeled Ab (Southern Biotech) was used as the indicator. Sera from treated mice or purified anti-CD3 Abs as the standard were titrated 2-fold and the concentration of rat Ig in serum was calculated from the standard curves.

Human IgG or mouse IgG and anti-DNA Abs were quantified by ELISA, as previously described (3, 14).

**Statistical analysis.** Statistical comparisons by the paired t test or the Mann-Whitney U test were done using GraphPad Prism 4 software. Values of p < 0.05 were prespecified as significant.

**Results and Discussion.**

**In SLE, Tregs directly suppress B cells in vitro.** Our group and others have shown that adaptive Tregs in SLE can suppress CD4+ Th cells that stimulate autoAb-producing B cells (3, 4). The possibility that Tregs may directly target B cells for the suppression of autoAbs in SLE has not been explored. For this report we studied whether natural Tregs in NZB/W lupus mice could directly suppress B cells without the need of intermediate suppression of Th cells. We initially compared the ability of Tregs and CD4+ CD25+ Th cells to influence the production of Abs from stimulated B cells in premorbid NZB/W mice. Tregs suppressed IgG production whereas Th cells did not (Fig. 1a), suggesting the ability of lupus Tregs to directly inhibit Ab production from B cells. Because there is a possibility that Tregs could be functionally different in young and old NZB/W lupus mice, we next addressed whether the age of the mice affected the ability of Tregs to directly inhibit Ab production in B cells. In experiments in which Tregs from young (10–12 wk old) or old (30 wk old) NZB/W mice were cocultured with B cells, the suppression of IgG from B cells was observed with Tregs from both young and old mice (Fig. 1b). These data indicated that the age of the mice did not affect the ability of Tregs to suppress B cells. Moreover, a dose-dependent increase in the capacity of Tregs to suppress B cells (from 1:1 to 5:1 ratio) was found for the Tregs of both young (p < 0.04) and old mice (p < 0.03) (not shown).

Because Abs in SLE have multiple specificities and belong to different Ig classes and subclasses (15), we tested next whether NZB/W Tregs could suppress different Ig isotypes. It was found that Tregs suppressed IgG1, IgG2a, IgG2b, IgG3, and IgM by anti-CD40-lg-stimulated B cells (p < 0.04 for all comparisons between B cells vs B cells plus Tregs; data not shown). However, most of the clinical and pathologic manifestations of SLE at its onset depend on the presence of elevated titers of autoAbs produced by hyperactive B cells. Therefore, we studied whether Tregs could suppress B cells that produced high titers of anti-DNA Abs. It was found that Tregs efficiently suppressed autoAb-producing B cells, even after B cell stimulation (Fig. 1c). This ability of Tregs to suppress B cell production of anti-DNA autoAbs was present in both young and old mice (Fig. 1d).

Taken together, these results identify the capacity of natural Tregs to directly suppress B cells in SLE and an intact ability of Tregs from old NZB/W mice to suppress anti-DNA production in B cells.

**In SLE, Tregs directly suppress B cells in vivo.** We also addressed the possibility of a direct suppressive capacity of Tregs on B cells in vivo in the absence of Th cells. Old NZB/W mice with elevated serum titers of anti-DNA Ab (monitored by ELISA) were depleted of endogenous T cells before the adoptive transfer of syngeneic Tregs from young or old
mice (see Materials and Methods). In the absence of endogenous Th cells, Tregs from both young and old mice efficiently suppressed in vivo the production of IgG (p < 0.04) and anti-DNA autoAbs (p < 0.04) 1 week after transfer (as compared with mock-treated controls; data not shown). Mice that had not been depleted of T cells before the transfer of Tregs from young or old mice had reduced serum titers of total IgG and anti-DNA Abs, albeit to a lesser extent than what was found in T cell-depleted animals (not shown). These results suggest that the suppression of humoral responses by Tregs can occur in vivo in the presence or absence of Th cells.

Mechanisms of suppression of B cells by Tregs in SLE

One possibility was that Tregs could induce cell death to suppress B cells. Preliminary experiments indicated that Tregs were cytotoxic for B cells (not shown), so we investigated whether apoptosis occurred in B cells that had been cocultured with Tregs. Typically, cells that are in early apoptosis are annexin V+ and 7-AAD− because annexin V staining precedes the loss of membrane integrity (which accompanies the latest stages of cell death), whereas cells that are in late apoptosis or are dead are positive for both annexin V and 7-AAD. When Tregs were separated from B cells, early and late apoptosis did not occur (Fig. 2a), indicating the requirement for physical contact between Tregs and B cells in the mechanisms of B cell inactivation.

Tregs from young and old NZB/W mice were cocultured with B cells from old mice and apoptosis was assessed by flow cytometry. The results indicated that the ages of the mice donors of Tregs did not associate with differences in the ability of Tregs to induce apoptosis of autoAb-producing B cells, and a Treg dose-dependent increase in the induction of apoptosis in B cells was observed (Fig. 2b). Parallel DNA fragmentation studies in B cells confirmed these findings (not shown). Altogether, these results show that Tregs can directly suppress B cells by inducing apoptosis. This capacity of the Tregs could attempt to limit B cell autoreactivity, but it might be limited by the reduced number of Tregs in SLE-prone backgrounds and may become insufficient after the loss of immune tolerance.
Perforins and granzymes are cytotoxic molecules released from Tregs to induce immune cell death (10). Because exocytosis of intracellular granules depends on Ca\(^{2+}\), we examined whether the presence of Ca\(^{2+}\) was required for the induction of B cell death. The addition of EGTA to chelate Ca\(^{2+}\) ions protected B cells from death in coculture experiments (Fig. 2c). Moreover, the addition of Abs to perforin or granzyme to the cocultures resulted in a reduction of Treg-induced B cell death (Fig. 2c), and a reduced suppression by Tregs on IgG production by B cells was also observed in the presence of Abs to perforin or granzyme (not shown). To address whether other pathways such as the Fas/FasL or TNF/TNFR pathways were involved in the suppression of B cells, Tregs and B cells from old NZB/W mice (10:1 ratio) were cocultured for 16 h in the presence of Fas-Fc or TNFRII-Fc, respectively. The blockade of Fas/FasL or TNF/TNFR interactions did not abrogate Treg-mediated suppression of IgG production from unstimulated or stimulated B cells (p values were not significant in all comparisons; data not shown). These data suggest that Treg-mediated suppression of B cells in SLE may not occur through Fas/FasL and TNF/TNFR pathways. However, as for nonautoimmune Tregs (10), NZB/W Tregs can use granule exocytosis pathways that involve perforin and granzyme to inhibit lupus B cells.

**Tregs directly suppress B cells in human SLE**

To address whether a direct B cell suppression by Tregs also occurs in human SLE (16), Tregs from SLE patients were cocultured with autologous B cells. The results showed a significant suppression of autologous Tregs on IgG responses (Fig. 3a) and the induction of apoptosis in B cells (Fig. 3b) in SLE patients but not in matched controls, an effect that might be secondary to lupus B cell hyperactivity.

In SLE, some B cell subsets have been proposed to play key roles and/or have stronger associations with the disease. Particularly, the aberrant activation of human lupus B cells has recently been linked to phenotypic markers that may associate with disease activity. For example, increased numbers of peripheral CD27\(^{\text{high}}\)IgD\(^{-}\)memory CD19\(^{+}\)B cells in SLE patients have been associated with disease activity and with increased renal disease and autoAbs (17). Also, the number and frequency of peripheral CD19\(^{+}\)CD27\(^{\text{high}}\)B cells have been found to positively correlate with serologic abnormalities in SLE patients with active lupus (18).

To understand whether Tregs could inhibit those subsets of B cells in SLE patients, we cocultured Tregs with autologous CD19\(^{+}\)CD27\(^{\text{high}}\)IgD\(^{-}\)B cells or with autologous CD19\(^{+}\)CD27\(^{\text{high}}\)B cells. We found that Tregs induced the apoptosis of both CD19\(^{+}\)CD27\(^{\text{high}}\)IgD\(^{-}\) and CD19\(^{+}\)CD27\(^{\text{high}}\)IgD\(^{-}\)B cell subsets and that Tregs inhibited both subsets also after B cell stimulation (Fig. 3c). These effects were dose dependent, and an increase in the ratio of Tregs to B cells (from 1:1 to 5:1) resulted in a 25–30% increment of cell death in B cells (not shown). Thus, in human SLE Tregs can suppress B cells, including those that associate with active disease and with the production of autoAbs.

In summary, this is the first report indicating that Tregs can mediate a direct suppression of B cells in a chronic systemic condition that is primarily driven by aberrant production of autoAbs. This mechanism could attempt to directly control humoral autoimmunity (possibly also if effector Th cells are resistant to suppression by Tregs) (19).

Because we observe that the lupus Tregs are functional in aged mice and appear capable of suppressing B cells that expand in active SLE, we propose that quantitative rather than qualitative deficits of Tregs may be primarily responsible for the inability of Tregs to control SLE, at least in terms of B cell suppression. In other words, numerically deficient, functional Tregs might attempt to counteract the development of B cell autoimmunity in SLE but would be overwhelmed by the unbalanced immune homeostasis that develops with the progression of the disease.

**Disclosures**

The authors have no financial conflict of interest.

**References**