

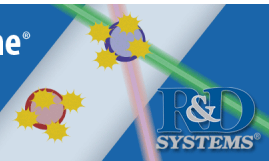


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CD4⁺CD25⁺ Regulatory T Cells Inhibit the Maturation but Not the Initiation of an Autoantibody Response¹

Michele L. Fields,² Brian D. Hondowicz,² Michele H. Metzgar, Simone A. Nish, Gina N. Wharton, Cristina C. Picca, Andrew J. Caton, and Jan Erikson³

To investigate the mechanism by which T regulatory (Treg) cells may control the early onset of autoimmunity, we have used an adoptive transfer model to track Treg, Th, and anti-chromatin B cell interactions in vivo. We show that anti-chromatin B cells secrete Abs by day 8 in vivo upon provision of undeveloped, Th1- or Th2-type CD4⁺ T cell help, but this secretion is blocked by the coinjection of CD4⁺CD25⁺ Treg cells. Although Treg cells do not interfere with the initial follicular entry or activation of Th or B cells at day 3, ICOS levels on Th cells are decreased. Furthermore, Treg cells must be administered during the initial phases of the Ab response to exert full suppression of autoantibody production. These studies indicate that CD25⁺ Treg cells act to inhibit the maturation, rather than the initiation, of autoantibody responses. *The Journal of Immunology*, 2005, 175: 4255–4264.

The CD4⁺CD25⁺ T regulatory (Treg)⁴ cells have been shown to block several organ-specific autoimmune diseases in which T cells are the primary effector lymphocytes (reviewed in Ref. 1). However, the mechanism of suppression by Treg cells in vivo is still unclear. In some cases, the presence of Treg cells is associated with decreased Th cell proliferation, cytokine production, and/or altered chemokine receptor expression (2, 3).

How Treg cells inhibit autoimmune diseases in which B cells and autoantibody production are prominent mediators of pathology is even less well understood. Indeed, while Treg cells can block LPS-induced B cell proliferation in vitro (4), comparatively little is known about the effects of Treg cells upon B cells in vivo. It has been argued that B cells are capable of attracting Treg cells via CCL4, which in turn may inhibit formation of germinal centers and Ab-forming cells (AFCs) (4). Furthermore, day 3 thymectomy (a protocol that depletes Treg cells) results in organ-specific autoimmunity (5, 6), and can also accelerate autoantibody production in lupus-prone mice (7). Moreover, in several induced models of autoantibody production, injection of Treg cells decreased or abrogated in vivo autoantibody levels (8–10). Although these studies were able to follow markers of the autoimmune response such as pathology and/or autoantibody production, they were not able to track the in vivo fates of the participating autoreactive B, Th, and Treg cells.

We have used an Ig transgenic (Tg) model to track B cells that produce Abs specific for DNA and chromatin (in this study simply

referred to as anti-chromatin B cells) (11). Such Abs are a hallmark of systemic lupus erythematosus and arise in several mouse models of lupus (12). Anti-chromatin B cells persist in the peripheral repertoire of healthy mice, but with a reduced $t_{1/2}$ (13). Furthermore, they appear developmentally arrested, and localize to the T-B interface within the splenic white pulp as do other Ag-engaged B cells in the absence of T cell help (13, 14). Upon provision of T cell help, the anti-chromatin B cells respond by producing autoantibodies (9).

In Fas/Fas ligand (FasL)-deficient *lpr/lpr* or *gld/gld* mice, anti-chromatin B cells localize within B cell follicles, in contrast to their Fas/FasL-sufficient counterparts (15). This localization is dependent on CD4⁺ T cells and the CD40-CD154 (CD40L) pathway (9). However, before 10 wk of age, anti-chromatin Abs are not detected in the serum (15, 16). We have hypothesized that in young mice, T cell help for anti-chromatin B cells is held in check by Treg cells (9).

To examine how Treg cells influence the Th/B cell interactions in vivo, we have devised a strategy in which Th cells, Treg cells, and anti-chromatin B cells can be identified and tracked independently in an adoptive transfer mouse model. Using this approach, we demonstrate that the presence of Treg cells at the initiation of the Th-B cell response does not alter the primary events characterizing a productive Th-B cell interaction, but does curtail the later survival of Th and B cells and the maturation of the autoantibody response.

Materials and Methods

Mice

All transgenic mice were bred and maintained in specific pathogen-free conditions at the Wistar Institute under the supervision of the Institutional Animal Care and Use Committee. Mouse genotypes (V_H3-H9, HACII, HA28, or TS1 Tg) were determined by PCR amplification of tail DNA, as described (11, 17, 18). V_H3-H9/HACII mice were bred to be deficient in the Igκ locus (V_H3-H9/HACII/Igκ^{-/-} mice) to increase the frequency of V_H3-H9/Vλ1 cells (9). In some experiments, either the Treg cells (TS1 × HA28 mice) (19) or the Th cells (TS1 mice) (18) were derived from mice that were Thy-1.1 heterozygous (20, 21), so they could be tracked in vivo. CB17 mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were used at age 6–16 wk and were age and sex matched in experiments. Males and females were used with no apparent differences.

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⁴ Abbreviations used in this paper: Treg, T regulatory; AFC, Ab-forming cell; AP, alkaline phosphatase; HA, hemagglutinin; LN, lymph node; sAv, streptavidin; Tg, transgenic.

Purification of Th and Treg cells

Axillary, brachial, popliteal, cervical, and inguinal lymph nodes (LNs) were harvested and dispersed using sterile glass slides. Cells from TS1 or TS1 $\times$ HA28 LN preparations were stained with anti-CD25 FITC (7D4) and anti-CD4 PE or allophycocyanin (GK1.5 or RM4-5) (BD Pharmingen) and sorted using a Cytomation MoFlo. Purity was consistently above 90% for CD4⁺CD25⁺ cells and above 97% for CD4⁺CD25⁻ cells.

Th1 and Th2 cell cultures

TS1 BALB/c LNs were harvested and dispersed using sterile frosted glass slides, and depleted of CD8⁺ cells using anti-CD8 Dynabeads, followed by magnetic removal (Dynalect). Efficacy of CD8⁺ T cell removal was checked using flow cytometric staining for CD3⁺CD4⁻ cells, and was consistently >90%. A total of 0.5×10^6 CD8-depleted lymphocytes was cultured in 24-well plates along with 5×10^6 irradiated, RBC-depleted BALB/c splenocytes, 1 μ M HA S1 peptide (residues 110–120), and IL-2 (22). Additionally, Th1 cultures received anti-IL-4 (clone 11B11, cell supernatant) and rIL-12 (obtained as a generous gift from G. Trinchieri (National Institutes of Health, Bethesda, MD) or purchased from R&D Systems, or PeproTech) (22). At least 35% of T cells derived from these cultures were IFN- γ ⁺ by intracellular cytokine staining. Th2 cultures received IL-4 (clone X-4, cell supernatant) and anti-IFN- γ (clone XMG, cell supernatant) (22). Th2 cultures made IL-4 (14–44.5% of cells) and IL-10 (4–10% of cells). Cells were cultured for 9 days, receiving fresh medium containing IL-2 at days 3 and 5, and rested in the absence of IL-2 at day 7 (22). At day 9, cells were harvested and an aliquot was tested for cytokine production, as described below. More than 95% of live cells were CD4⁺ (our unpublished data).

Intracellular cytokine and Foxp3 staining

For cytokine staining, cells were placed into culture with PMA and ionomycin (Sigma-Aldrich) in the presence of brefeldin A (Cytofix/Cytoperm kit; BD Pharmingen) for 4–6 h. After harvest, cells were stained for surface CD4 expression, fixed, permeabilized, and stained for intracellular cytokines, using anti-IL-2 PE, anti-IL-4 PE, anti-IL-6 PE, anti-IL-10 PE, anti-IL-17 PE, anti-IFN- γ FITC, and/or anti-TNF- α -FITC (BD Pharmingen) (23). Isotype controls were used for all stains. To determine the percentage of cells producing a certain cytokine, the value obtained with the isotype control was subtracted from the value obtained with each specific Ab. For intracellular Foxp3 staining, cells were fixed and permeabilized using the reagents provided with the anti-Foxp3 PE (FJK-16s) Ab (eBioscience). All experiments also included staining with the appropriate isotype control (IgG2a PE (eBR2a)) that was included with the purchase of the anti-Foxp3 Ab.

T cell injections

Before injection, cells were purified by centrifugation with Lympholyte M (Cedarlane Laboratories). A total of $1\text{--}2 \times 10^6$ nondifferentiated Th cells, Th1, or Th2 cells were injected i.v., followed by the injection of $0.5\text{--}2 \times 10^6$ Treg cells to achieve a final ratio of Th:Treg cells between 1:1 and 4:1. All cells were resuspended in sterile PBS. Mice also received 1000 hemagglutinating units (24) of purified PR8 influenza virus i.v. (25).

Anti-chromatin B cell injections

Splenocytes from V_H3-H9 Tg/HACII/Ig κ ^{-/-} mice were depleted of RBC, and an aliquot was stained by flow cytometry to determine the frequency of anti-chromatin B cells (B220⁺ Ig λ 1⁺). CB17 recipient mice were injected with splenocytes containing $4\text{--}10 \times 10^6$ anti-chromatin B cells. Control CB17 mice receiving no exogenous T cells plus B cells ("B cells alone" mice) were given spleen preparations from V_H3-H9 Tg Ig κ ^{-/-} or V_H3-H9 Tg/HACII/Ig κ ^{-/-} mice (either source gave identical results) containing the same number of anti-chromatin B cells as were given to experimental mice on that day.

Determination of cell recovery

By flow cytometry, the frequency of IgM⁺Ig λ 1⁺ B cells, CD4⁺Thy-1.1⁺ T cells, or CD4⁺6.5⁺ T cells in the spleen was determined and multiplied by the total number of live splenocytes to determine the absolute number of cells. The percentage of recovery of transferred B or T cells was determined by dividing the absolute number of cells recovered by the number of cells injected.

Chromatin ELISAs

ELISA plates (ThermoLabSystems) were coated with 2 μ g/ml chromatin (a generous gift of M. Monestier, Temple University, Philadelphia, PA)

overnight at 4°C. The remaining steps were conducted at room temperature. All washes were conducted at least eight times in 1 $\times$ PBS/0.05% Tween 20. Following the coating step, plates were washed, blocked with 1% BSA/PBS/azide for at least 1 h, and washed again. Sera were then added at increasing dilutions (typically 1/100 to 1/6400) and incubated for a minimum of 1 h. Plates were washed and incubated with developing Ab (anti-IgM^a biotin; BD Pharmingen), for at least 1 h. Finally, plates were washed, incubated with streptavidin (sAv) alkaline phosphatase (AP) (Southern Biotechnology Associates) for at least 1 h, washed, and developed for 14–18 h. The plates were developed with ImmunoPure *p*-nitrophenyl phosphate (Pierce) as the substrate. Absorbances were read at dual wavelength, 405/650 nm, using a microplate reader. OD values were recorded and background values were subtracted out (background was defined as the OD values generated by a hybridoma supernatant of irrelevant specificity, typically 0.07). Points derived from the linear range of the ELISAs were used for generating graphs.

Flow cytometry

A total of $0.5\text{--}2 \times 10^6$ cells was prepared from spleens or LNs and surface stained as per standard protocol (26). The following Abs were used: anti-B220 FITC (RA3-6B2); anti-CD3 FITC (145-2C11); anti-CD4 PE or allophycocyanin (GK1.5 or RM4.5); anti-IgM^a PE, biotin, or FITC (DS-1); anti-Ig λ 1 biotin (R11-153); anti-CD93 allophycocyanin (AA4.1); anti-CD80 FITC (16-10A1); anti-CD86 PE (GL1); anti-CXCR5 PE (2G8); anti-CD103 bio (M290); anti-CD154 biotin (CD40L, MR1); anti-CD95L biotin (MFL3); anti-Thy-1.1 PE (OX-7); and anti-ICOS biotin (7E.17G9) (BD Pharmingen). The 6.5 biotin (anti-clonotype) (18) was grown as a supernatant and biotinylated. Secondary reagents were: sAv PE, sAv CyChrome, sAv PerCPcy5.5, or sAv allophycocyanin (BD Pharmingen). All plots show log₁₀ fluorescence.

Immunostaining

Spleens were frozen, sectioned, and stained (13). Immunohistochemistry protocols used anti-CD22 FITC or biotin (Cy34.1), or anti-IgM^a FITC or biotin (DS-1) (BD Pharmingen). Secondary reagents were anti-FITC AP, anti-FITC HRP, sAv AP, or sAv HRP (Southern Biotechnology Associates). For immunofluorescent staining, the following Abs were used: anti-Thy-1.1 PE (OX-7), anti-B220 FITC (RA3-6B2), anti-CD4 bio (GK1.5), or anti-IgM^a PE (DS-1). Biotinylated CD4 was visualized using sAv AlexaFluor 350 (Molecular Probes).

Real-time PCR

Tg⁻ BALB/c or BALB/c *lpr/lpr gld/gld* LN cells were sorted into populations of CD4⁺CD25⁻ or CD4⁺CD25⁺ cells. CD4⁺ cells from *lpr/lpr gld/gld* mice were also negatively sorted against B220⁺ cells. Cells were washed twice with PBS before RNA was isolated using the Qiagen RNeasy mini kit (Qiagen). A total of 0.2 μ g of RNA was used to make cDNA from the SuperScript First Strand Synthesis kit (Invitrogen Life Technologies). The cDNA was diluted 1/25 and amplified according to Applied Biosystems.

Statistical analyses

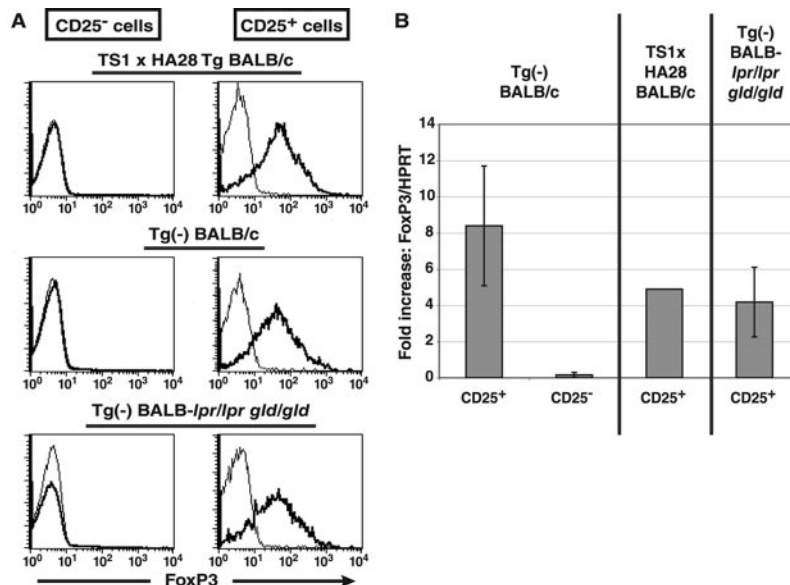
Statistical significance was determined via the unpaired, two-sample Student's *t* test provided by Microsoft Excel software, unless otherwise noted. Significance was ascribed when *p* < 0.05.

Results

CD4⁺CD25⁺ cells in BALB-*lpr/lpr gld/gld* mice express Foxp3

We have previously documented an increased number of CD4⁺CD25⁺ cells in Fas-deficient mice, even at early ages (6–8 wk) (27). To determine whether these cells are regulatory, we examined their expression of Foxp3, a lineage-specific transcription factor for Treg cells (28). CD4⁺CD25⁺ T cells from TS1 $\times$ HA28 Tg mice, which have been shown to have regulatory function in vitro and in vivo (9, 19), expressed high levels of Foxp3 as measured by intracellular staining (Fig. 1A). Similarly, CD4⁺CD25⁺ cells from Tg⁻ BALB/c and Tg⁻ BALB-*lpr/lpr gld/gld* (deficient in both Fas and FasL) mice also express Foxp3, whereas the CD4⁺CD25⁻ cells do not (Fig. 1A). Furthermore, real-time PCR amplification of mRNA from purified CD4⁺CD25⁺ T cells from non-Tg BALB/c or BALB-*lpr/lpr gld/gld* mice demonstrated that both cell populations express high levels of Foxp3 (Fig. 1B).

FIGURE 1. $CD4^+CD25^+$ T cells from Fas/FasL-deficient mice express Foxp3. **A**, Intracellular Foxp3 expression from cells obtained from LNs was determined in $B220^-CD4^+CD25^-$ (left column) or $B220^-CD4^+CD25^+$ (right column) cells by flow cytometry. Thin black lines show staining with an isotype control (IgG2a) Ab, and bold black lines show staining with anti-Foxp3; $n = 3$. **B**, Real-time PCR was used to determine Foxp3 or hypoxanthine phosphoribosyltransferase message expression from RNA transcripts obtained from purified $B220^-CD4^+CD25^-$ or $B220^-CD4^+CD25^+$ cells from Tg $^-$ BALB/c, TS1 $\times$ HA28 BALB/c, or Tg $^-$ BALB-*lpr/lpr gld/gld* LNs. Data are expressed as the fold increase in amount of Foxp3 message compared with hypoxanthine phosphoribosyltransferase message. There is no significant difference between the values for Tg $^-$ BALB/c and Tg $^-$ BALB-*lpr/lpr gld/gld* B220 $^-$ $CD4^+CD25^+$ cells ($p = 0.14$). Sample sizes: Tg $^-$ BALB/c, $n = 3$; TS1 $\times$ HA28 BALB/c, $n = 1$; Tg $^-$ BALB-*lpr/lpr gld/gld*, $n = 4$.



Strategy to track anti-chromatin B cells and anti-hemagglutinin (HA) Th and Treg cells

To begin to investigate the mechanism(s) by which Treg cells affect T-B cell interactions, we have devised a system to track autoimmune B cells as well as Th and Treg cells in vivo. The V_{H3-H9} H chain, when paired with the $V_{\lambda 1}$ L chain, forms an Ab that binds to DNA and chromatin (29, 30). Thus, in V_{H3-H9} H chain Tg mice, autoreactive anti-chromatin B cells can be tracked via IgG1 staining (13). To test the activation potential of anti-chromatin B cells in response to Th cells, V_{H3-H9} mice were bred with HAcII Tg mice, which express the influenza HA Ag under the control of a MHC class II promoter (31). $V_{H3-H9}/HAcII$ mice were bred to be deficient in the Ig κ locus (Ig $\kappa^{-/-}$ mice) to greatly enrich the population of anti-chromatin B cells ($V_{H3-H9}/V_{\lambda 1}$) in donor preparations. Anti-HA Th cells were obtained from TS1 TCR Tg mice (18), and Treg cells from TS1 $\times$ HA28 Tg mice (19). In some experiments, either the Th or Treg cells were derived from Thy-1.1 heterozygous mice to facilitate in vivo tracking. To examine the effects of $CD4^+CD25^+$ Treg cells upon Th/anti-chromatin B cell interactions, purified HA-reactive Th and/or Treg cells and anti-chromatin B cells (Ig ^{a}) were transferred into CB17 (Ig ^{b}) mice, and both early (day 3) and later (day 8) stages of the immune response were studied (Fig. 2A).

Anti-chromatin Ab formation is blocked by Treg cells, but early proliferation and recovery of anti-chromatin B cells are unaffected

Anti-chromatin B cells produced significant amounts of autoantibodies 8 days after transfer of $CD4^+CD25^-$ Th cells (Fig. 2B).

Strikingly, the coinjection of $CD4^+CD25^+$ Treg cells blocked the production of these Abs, even when Treg cells were outnumbered by Th cells 4:1 (Fig. 2B). To examine how Treg cells block autoantibody production, cells were transferred as described above and their phenotype was examined at day 3.

Although few transferred anti-chromatin B cells remained at day 3 in the absence of T cell help, consistent with the short $t_{1/2}$ of these cells in vivo (13), significantly more were detected in the presence of Th cells (Fig. 3A; 23.0 vs 6.1% recovery, $p < 0.01$). At this time point, no difference in B cell recovery was observed between mice given Th cells alone or Th and Treg cells (Fig. 3A; 23.0 vs 20.5% recovery, $p = 0.51$). Anti-chromatin B cells transferred in the presence of Treg cells without Th cells had similarly low levels of recovery as those given B cells only (Fig. 3A; 7.7 vs 6.1%, $p = 0.45$).

To test whether the presence of Treg cells altered the proliferative response of the transferred anti-chromatin B cells, CFSE-labeled anti-chromatin B cells were transferred either in the presence of Th cells, Th + Treg cells, or Treg cells alone. CFSE-labeled anti-chromatin B cells transferred without exogenous T cells or with only Treg cells underwent minimal proliferation (Fig. 3, B and C). In contrast, in the presence of Th cells, many anti-chromatin B cells proliferated, and this was not significantly diminished with the addition of Treg cells (Fig. 3, B and C).

Early in the immune response, Th cell proliferation is not suppressed by Treg cells

To determine whether Treg cells affected the proliferative response of Th cells in the presence of anti-chromatin B cells, Th cells were

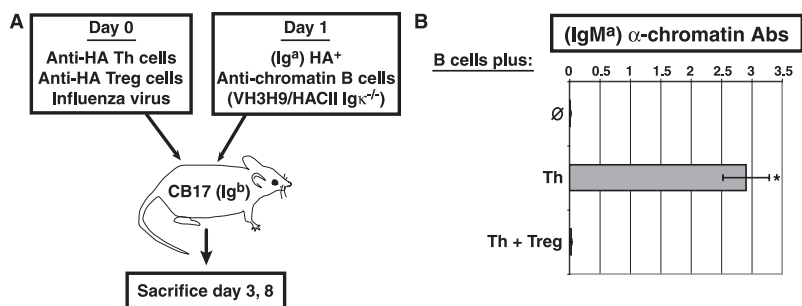


FIGURE 2. Treg cells block autoantibody production from anti-chromatin B cells. **A**, Experimental design. CB17 mice received Th cells $\pm$ Treg cells and influenza virus (day 0). The following day, HA $^+$ anti-chromatin B cells were transferred (from $V_{H3-H9}/HAcII$ Ig $\kappa^{-/-}$ donors). Mice were analyzed on day 3 or 8. **B**, Sera collected on day 8 were tested for IgM ^{a} anti-chromatin Abs by ELISA. Values indicate OD values obtained in the ELISA. *, Indicates a significant difference ($p < 0.05$) between Th and either B cells transferred alone ($\emptyset$) or with both Th and Treg cells. One to two million Th cells and 0.5–2 million Treg cells were injected. Sample sizes: $\emptyset$, $n = 7$; Th, $n = 6$; Th + Treg, $n = 5$.

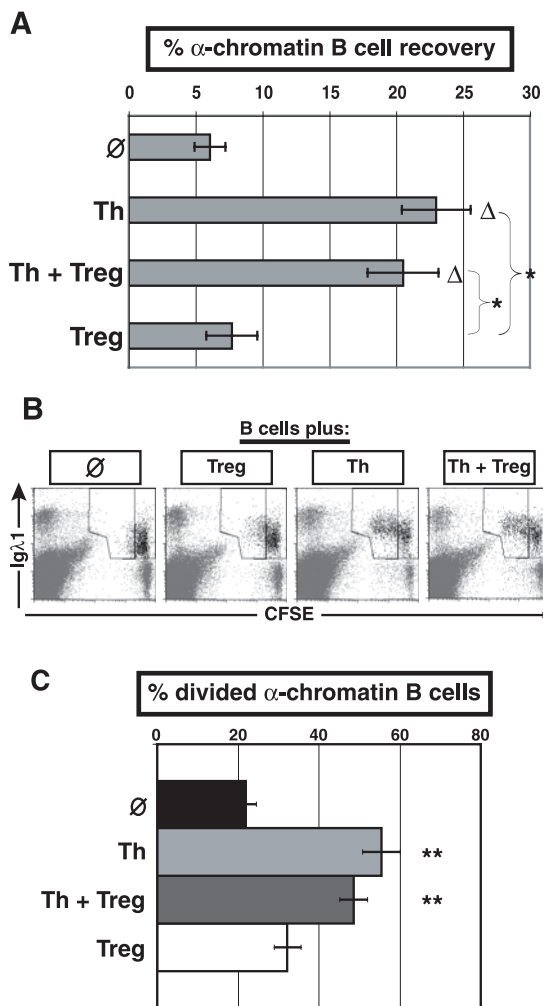


FIGURE 3. Anti-chromatin B cell proliferation in the presence of Treg cells. **A**, Splenocytes from mice at day 3 were stained to detect transferred B cells ($\text{IgM}^{\text{a+}}\text{Ig}\lambda 1^+$), and their recoveries were calculated. Sample sizes: B cells alone ($\emptyset$), $n = 9$; Th, $n = 12$; Th + Treg, $n = 12$; Treg, $n = 5$. Significant differences between experimental groups ($p < 0.05$) are marked with *, and a significant difference compared with control mice given B cells alone is marked with Δ . **B**, Splenocytes from anti-chromatin B cell donor mice were labeled with CFSE before transfer. On day 3, flow cytometry was used to determine the proliferation history of the transferred anti-chromatin cells ($\text{Ig}\lambda 1^+\text{CFSE}^+$) in the spleens of recipient CB17 mice. Dot plots are gated on live cells and are representative of three separate experiments. Cells were gated on divided (dimmer CFSE) vs undivided (brightest CFSE) cells. **C**, Based on the CFSE staining, the number of divided anti-chromatin B cells ($\text{Ig}\lambda 1^+\text{CFSE}^+$) was determined and compared in the presence or absence of Treg cells; $n = 3$. **, Denotes a significant difference compared with both anti-chromatin B cells transferred without exogenous T cells or virus, and with anti-chromatin B cells transferred with Treg cells only. There is no significant difference between the Th and Th + Treg conditions ($p > 0.05$).

labeled with CFSE and injected into CB17 mice, in the presence or absence of Treg cells. Th cells recovered from both the LN and spleen had proliferated robustly in vivo such that by day 3, most had undergone six or seven divisions (Fig. 4, A and B). The majority of Th cells from both the spleen and LN proliferated, and the number of Th cells that underwent division was not affected by Treg cells (Fig. 4, A and B). Unlike for the B cells, it was possible to differentiate the number of discrete divisions that had occurred in the Th cell population (Fig. 4B). This analysis showed that the

presence of Treg cells resulted in a slight decrease in Th cell proliferation such that fewer cells reached the maximum number of divisions (Fig. 4B).

Thy-1.1 was also used to measure Th cell recovery, which slightly declined in the presence of Treg cells (Fig. 4, C and D; 1.86×10^6 vs 1.55×10^6 cells recovered, $p = 0.048$). Although the changes in Th cell proliferation and recovery are statistically significant, the effects are modest. The number of Treg cells recovered in the spleen was the same when comparing mice that received only Treg cells vs mice that received Th and Treg cells (Fig. 4E; 0.82×10^6 vs 1.04×10^6 cells recovered, $n = 4$, $p = 0.58$).

The early activation phenotype of B and Th cells is unaltered by Treg cells with the exception of Th cell ICOS expression

To examine the effects of Treg cells on the phenotype of anti-chromatin B cells, maturation and activation markers were measured at day 3 of the response in the presence or absence of Treg cells (Fig. 5A). B220, CD93 (AA4.1), and CXCR5 were examined to determine maturation status; CXCR5 is also a critical follicular homing molecule (14). CD80 and CD86 served as markers of activation and potential costimulation. When anti-chromatin B cells were transferred in the presence of Treg cells alone, the few remaining B cells displayed a small shift in activation markers (Fig. 5A). In the presence of Th cells, the anti-chromatin B cells expressed higher levels of B220, CD80, CD86, and CXCR5, and a lower frequency were $\text{CD}93^+$ (Fig. 5A). Together this indicates that anti-chromatin B cells transferred with Th cells have a more mature phenotype compared with anti-chromatin B cells transferred in the absence of exogenous Th cells. The coadministration of Treg cells did not alter the expression levels of these markers (Fig. 5A).

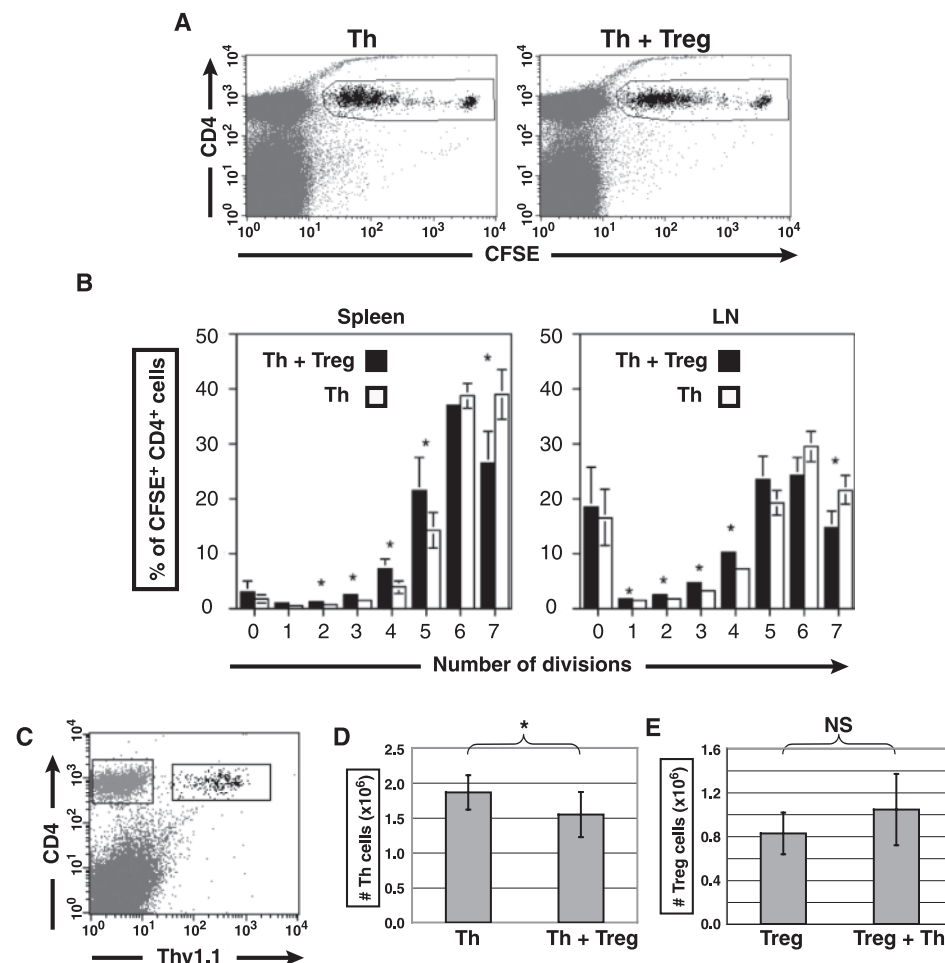
To examine the activation status of the transferred Thy-1.1⁺ Th or Treg cells, the following markers were assessed: CXCR5, CD154 (CD40L), CD178 (FasL), ICOS, and CD103 (Fig. 5, B and C). CD154 and ICOS are important for the induction or maintenance of the Th-B cell response, and CD178 can induce killing of target cells, but also may exert a positive signal (32–35). CD103, a TGF- β -inducible integrin, has been used as a marker for exposure to TGF- β produced by Treg cells (36). These markers were all elevated on the transferred Th cells compared with the endogenous (non-Thy-1.1⁺) T cells in the recipient mice (Fig. 5B). The additional presence of Treg cells did not alter the phenotype of the Th cells for most markers, with one notable exception (Fig. 5B). ICOS was consistently decreased on Th cells in the presence of Treg cells (ICOS levels were on average 35% lower; $n = 5$). Although this decrease appears modest, changes in ICOS expression levels correlate with differences in T cell effector functions (37).

When the Treg cells were tracked by Thy-1.1, they also appeared activated relative to endogenous T cells in that levels of CXCR5, CD154 (CD40L), CD178 (FasL), and ICOS were elevated (Fig. 5C). This activation was slightly, but consistently enhanced in the presence of Th cells (Fig. 5C).

Cytokine production is affected by the presence of Treg cells

The potential of the transferred Th and Treg cells to produce cytokines was assessed at day 3 of the immune response using Thy-1.1 to mark either the Th or Treg cells. When transferred with anti-chromatin B cells (but no Treg cells), Th cells developed into a mixed population, with cells able to produce IL-2, IL-10, and IFN- γ upon in vitro restimulation with PMA and ionomycin (Fig. 5D). The in vivo coadministration of Treg cells resulted in a decrease in the frequency of Th cells able to produce both IL-10 and IFN- γ upon ex vivo restimulation (Fig. 5D).

FIGURE 4. Th cell proliferation in the presence of Treg cells. *A*, Flow cytometry was used to determine the proliferation history of transferred $CD4^+CFSE^+$ Th cells in the spleens and LN of recipient CB17 mice at day 3. Shown are dot plots for Th cells (recovered from the LN) transferred either alone (*left*) or in the presence of Treg cells (*right*), and are representative of three separate experiments. *B*, The number of divisions that the Th cells underwent in the spleen and LN was determined and was compared in the presence or absence of Treg cells; $n = 3$. *, Represents a significant difference between the Th alone and Th + Treg conditions. *C*, Dot plot demonstrates how $CD4^+Thy-1.1^+$ transferred cells were detected using flow cytometry. *D*, Transferred Th cells were derived from $Thy-1.1^+$ animals. The absolute number of splenic $CD4^+Thy-1.1^+$ or $CD4^+CFSE^+$ transferred cells was determined and compared in the presence or absence of Treg cells ($n = 10$ for each condition). *, Indicates significant difference ($p < 0.05$). *E*, In experiments in which Treg cells were derived from $Thy-1.1^+$ animals, their recovery was also tracked on day 3 and compared in the presence or absence of Th cells ($n = 4$ for each condition). NS, $p > 0.05$.



The Treg cells produced small amounts of IL-2 and IFN- γ , but high levels of IL-10 when restimulated (Fig. 5E). The co-injection of Th cells increased the levels of IFN- γ and IL-10 produced by Treg cells (Fig. 5E), consistent with the hypothesis that the presence of Th cells further activates the Treg cells. Although the $Thy-1.1^+$ Treg cells were sorted to at least 90% purity before transfer, it remains a possibility that contaminating $CD4^+CD25^-$ cells contribute some of the observed cytokine production.

Treg cells do not alter the follicular localization of Th cells or anti-chromatin B cells

Previous studies have documented the orchestrated movements of B and T cells during the first few days of a T cell-dependent B cell response (reviewed in Refs. 38 and 39). To determine whether and how Treg cells may interfere with this process, the splenic localization of the transferred Th, Treg, and anti-chromatin B cells at day 3 was examined (Fig. 6). Upon transfer with HA⁺ anti-chromatin B cells, $Thy-1.1$ -marked Th cells were concentrated in the T cell areas of the spleens, with some cells also localizing within the B cell follicles (Fig. 6B). The additional presence of Treg cells did not affect the localization of the Th cells (Fig. 6B). When the $Thy-1.1$ marker was used to track Treg cells, the vast majority was visible in the T cell area in the absence of Th cells (Fig. 6C). Notably, the presence of Th cells increased the frequency of Treg cells localizing in B cell follicles (Fig. 6C), consistent with their slight increase in CXCR5 expression (Fig. 5B). Although follicular entry for Th cells is important for their helper function (39), the localization of Treg cells in the B cell follicle has not been documented previously.

Few anti-chromatin B cells were detected in the absence of exogenous Th cells or in the presence of only Treg cells (Fig. 6D). Strikingly, the localization of anti-chromatin B cells was similar in mice given Th cells alone, or mice given Th cells plus Treg cells: in both groups, the anti-chromatin B cells were dispersed in the B cell follicles (Fig. 6D). Follicular re-entry after Ag and Th cell engagement is an important step for a B cell toward further differentiation (38).

Treg cells block autoantibody production induced by Th1 or Th2 cells

Because polarized Th cells have lower requirements for stimulation through APCs and may produce higher levels of cytokines that could interfere with Treg cells (40, 41), we tested the ability of Treg cells to suppress Th1/Th2 B cell help. One possibility is that under autoimmune conditions, undifferentiated Th cells arise early and are able to be blocked by Treg cells, but then at later ages, polarized T cell subsets arise that are more resistant to regulation. However, similar to their nondeviated counterparts, both Th1- and Th2-induced autoantibody production was blocked by the addition of Treg cells (Fig. 7A).

By day 8, autoreactive B and Th cells are dramatically decreased in the presence of Treg cells

Although the effects of Treg cells upon anti-chromatin B and Th cells appear subtle at day 3, they become more evident by day 8. Anti-chromatin B cells were readily identified by flow cytometric staining in mice that received nondifferentiated Th, Th1, or Th2 cells (with no differences between these groups) (Fig. 7, B and D).

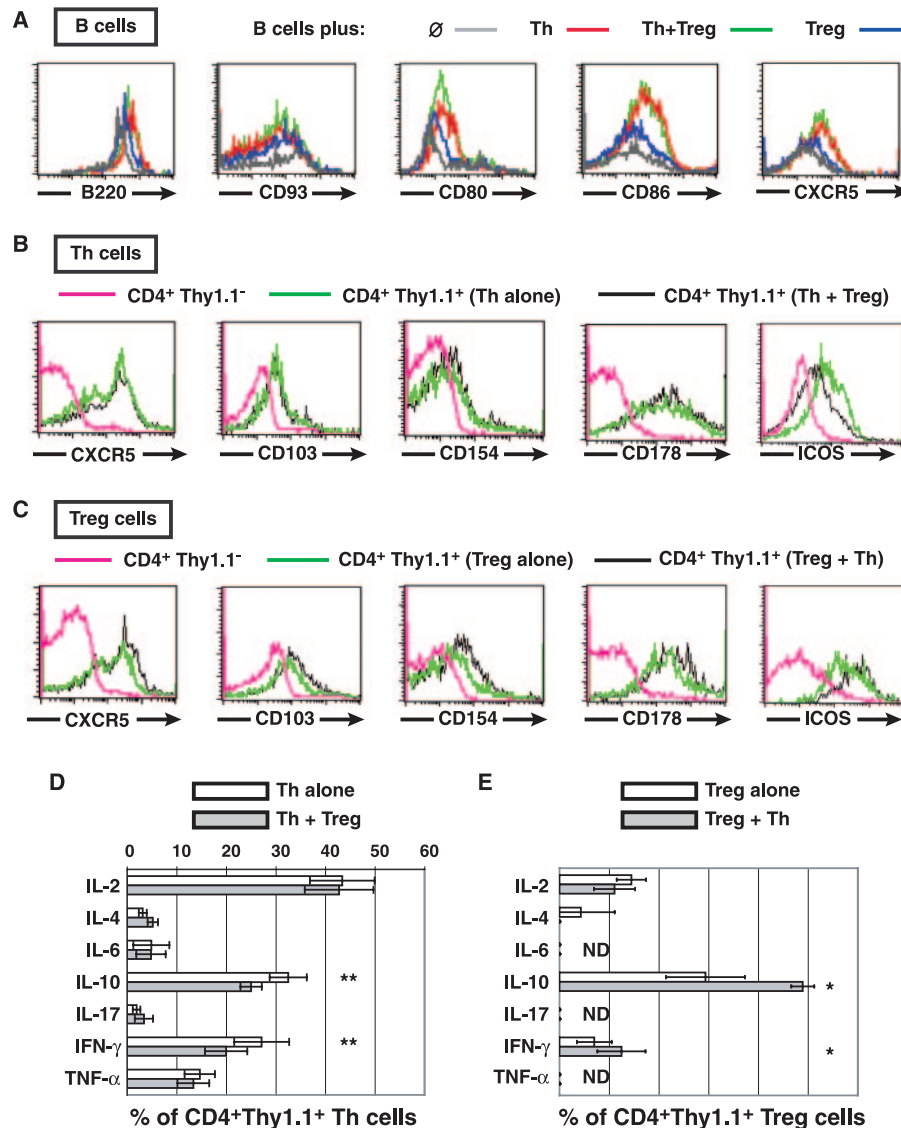


FIGURE 5. Effects of Treg cells upon anti-chromatin B and Th cell activation. Mice were analyzed on day 3 to determine: *A*, the levels of maturation and activation markers (B220, CD93, CD80, CD86, and CXCR5) on IgM⁺ IgA1⁺ splenocytes; *B*, expression levels of CXCR5, CD103, CD154 (CD40L), CD178 (FasL), and ICOS on CD4⁺Thy1.1⁺ Th cells in the presence (black line) or absence (green line) of Treg cells; or *C*, these same markers on CD4⁺Thy1.1⁺ Treg cells in the presence (black line) or absence (green line) of Th cells. For *B* and *C*, the pink line shows levels on endogenous (CD4⁺Thy1.1⁺) cells in the presence of B cells alone. All histograms are on a logarithmic scale and are representative of at least three separate mice per condition, and per stain. *B*, Mean geometric means for the following markers on Th cells (Th cells transferred alone listed first, Th cells in presence of Treg cells listed second): CXCR5, 96.0 vs 109 ($n = 5$; $p = 0.30$); CD103, 25.5 vs 28.3 ($n = 5$; $p = 0.06$); CD154, 12.0 vs 15.0 ($n = 3$; $p = 0.10$); CD178, 118.8 vs 111.5 ($n = 4$; $p = 0.76$); ICOS, 197.4 vs 136.6 ($n = 5$; $p = 0.03$). *C*, Mean geometric means for the following markers on Treg cells (Treg cells transferred alone listed first, Treg cells in presence of Th cells listed second): CXCR5, 78.4 vs 133.5 ($n = 3$; $p = 0.04$); CD103, 43.7 vs 65.8 ($n = 3$; $p = 0.05$); CD154, 12.4 vs 18.6 ($n = 3$; $p = 0.27$); CD178, 188.7 vs 357.7 ($n = 4$; $p = 0.06$); ICOS, 108.3 vs 222.7 ($n = 3$; $p = 0.08$). *D*, Splenic Thy1.1-marked Th cells were tested for cytokine production at day 3. **, Indicates $p < 0.05$ for both one- and two-tailed paired t test. *E*, Splenic Thy1.1-marked Treg cells were similarly tested for cytokines on day 3. *, Indicates $p < 0.05$ for one-tailed paired t test. Sample sizes: *D*, IL-2, $n = 5$; IL-4, $n = 3$; IL-6, $n = 3$; IL-10, $n = 6$; IL-17, $n = 3$; IFN- γ , $n = 6$; TNF- α , $n = 3$. *E*, $n = 3$ for all except where indicated (ND, not done).

Many fewer of these cells were detected in mice that received anti-chromatin B cells in the absence of Th cells (Fig. 7, *B* and *D*). Furthermore, immunohistochemical analyses demonstrated that the transferred anti-chromatin B cells (IgM^a) were concentrated in extrafollicular foci in the presence of Th cells (Fig. 8). Their localization, and the finding that they costain with CD138 (syndecan-1, a marker for plasma cells; data not shown) indicate that they are AFCs. In contrast, minimal IgM^a cells were observed in mice that received Treg cells either alone or with Th cells (Fig. 8), consistent with the serum Ab and B cell recovery data (Fig. 7, *A* and *D*).

To track the recovery of transferred Th cells at day 8, the 6.5 clonotypic Ab that recognizes anti-HA TS1 T cells (18) was used (Fig. 7C). Nondifferentiated Th, Th1, or Th2 cells were identified in the spleens of recipient mice, with Th2 cell recovery being lower than that of nondifferentiated Th or Th1 cells (Fig. 7, *C* and *E*). Importantly, the presence of Treg cells resulted in a dramatic reduction in the numbers of Th cells recovered from all groups by day 8 (Fig. 7E).

Timing of Treg cell injection affects autoantibody response

Because many of the early events associated with a productive Th-B cell collaboration were not altered in the presence of Treg

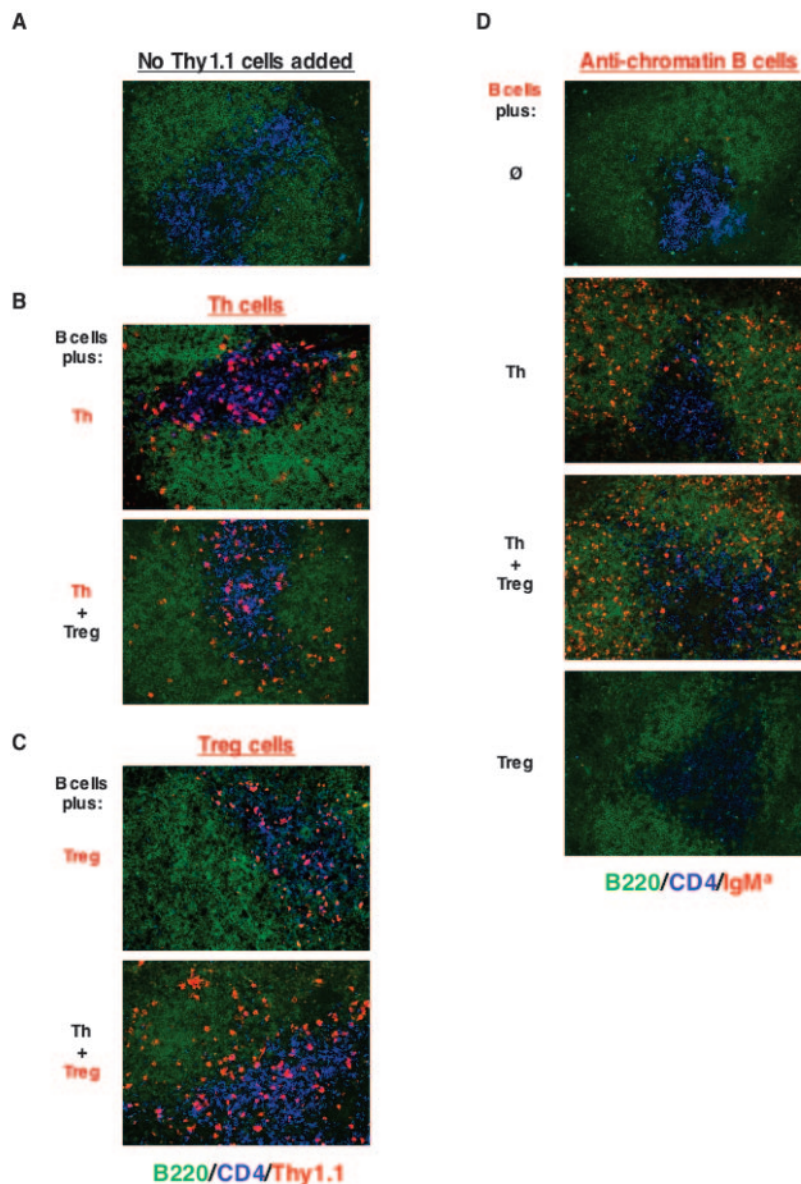


FIGURE 6. Localization of Th cells, Treg cells, and anti-chromatin B cells, day 3. Day 3 spleens were sectioned and stained for the following markers: A–C, B220 (green), CD4 (blue), and Thy-1.1 (red); D, B220 (green), CD4 (blue), and IgM^a (red). A, No Thy-1.1⁺ cells were added. Thy-1.1⁺ Th cells (B) or Thy-1.1⁺ Treg cells (C) were identified. D, IgM^a was used to detect transferred anti-chromatin B cells. Pictures show follicles representative of at least three mice per condition.

cells, but later events such as autoantibody production were dramatically blunted, we determined when the Treg cells were required to mediate their suppressive effects. To examine this issue, mice were injected with Th cells and anti-chromatin B cells as before, but the injection of Treg cells was postponed by 1 or 2 days relative to Th cell injection, and on day 8, recipient mice were analyzed for serum autoantibody titers as well as B and T cell recoveries.

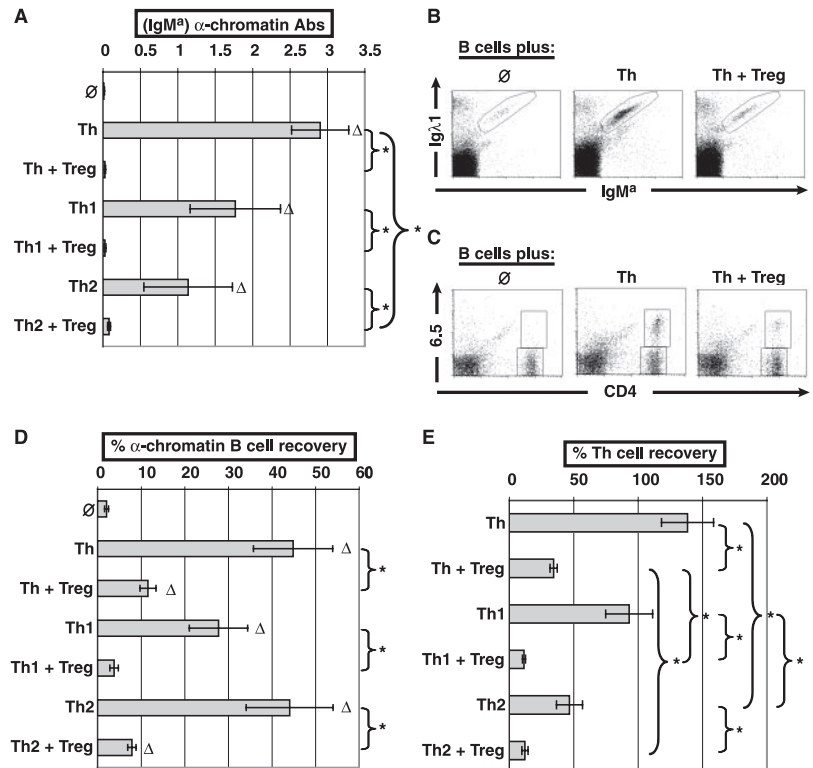
Delayed injection of Treg cells led to significant, stepwise increases in serum autoantibody titers (with mean suppression levels decreasing from 97%, to 57%, and then 22% for each successive day; Fig. 9A). Histological analyses also showed incremental increases in the frequency and size of AFC clusters in the spleens of recipient mice with each delayed time point (data not shown). Furthermore, anti-chromatin B cell (Fig. 9B) and Th cell (Fig. 9C) recoveries were not significantly affected by Treg cells when the injection of Treg cells was delayed. Although delayed injection of Treg cells may compromise their expansion relative to the Th cells, we have shown that Treg cells can still fully suppress even when outnumbered by Th cells 4:1. Thus, the presence of Treg cells during the initial stages of the Th/B cell interactions is required for

full inhibition of autoantibody production, and Th and B cell recovery.

Discussion

We have hypothesized that autoreactive B and T cells are held in check by the counterbalancing effects of Treg cells (9), a proposal that is bolstered in this study by the description of CD4⁺CD25⁺Foxp3⁺ Treg cells in young Fas-deficient mice before autoantibody production. To begin to address the mechanisms by which Treg cells function, we have examined the ability of Treg cells to interfere with anti-chromatin B and Th cell interactions. Our approach has been to use a third-party adoptive transfer model to track the fates of Treg, Th, and anti-chromatin B cells in vivo. Using this strategy, we showed that in the presence of Treg cells, both Th cells and anti-chromatin B cells express the characteristic activation and localization features of a productive immune response, but this response is interrupted and autoantibody production does not ensue. Hence, Treg cells are able to suppress humoral responses to systemic self Ags such as DNA/chromatin, in addition to their clear importance in cell-mediated, organ-specific autoimmune diseases (reviewed in Refs. 1 and 42). Treg cells retain this

FIGURE 7. Treg cells suppress anti-chromatin Ab production and recoveries of B and T cells by day 8 in vivo. **A**, Sera collected on day 8 were tested for IgM⁺ anti-chromatin Abs by ELISA, and OD values from the assays are given. Significant differences between experimental groups ($p < 0.05$) are marked with *, and a significant difference compared with mice given B cells alone ($\emptyset$) is marked with Δ . Sample sizes: $\emptyset$, $n = 7$; Th, $n = 6$; Th + Treg, $n = 5$; Th1, $n = 4$; Th1 + Treg, $n = 4$; Th2, $n = 5$; Th2 + Treg, $n = 3$. **B**, Splenocytes were stained to detect transferred B cells (IgM⁺IgA1⁺), or **C**, T cells (CD4⁺6.5⁺). Based on the flow cytometric data, the recoveries of transferred B cells (**D**) or T cells (**E**) were calculated. **D**, Sample sizes: $\emptyset$, $n = 11$; Th, $n = 16$; Th + Treg, $n = 11$; Th1, $n = 8$; Th1 + Treg, $n = 4$; Th2, $n = 6$; Th2 + Treg, $n = 3$. **E**, Sample sizes: Th, $n = 10$; Th + Treg, $n = 11$; Th1, $n = 7$; Th1 + Treg, $n = 3$; Th2, $n = 8$; Th2 + Treg, $n = 3$.



suppressive capability even in the face of in vitro deviated Th1 and Th2 cells. Interestingly, even though many of the early steps in Th-B cell activation and migration appear unaltered by Treg cells, the Treg cells are required at the initiation of the Th-anti-chromatin B cell collaboration for optimal suppression.

Although day 8 autoantibody production and B cell recovery are severely curtailed, coinjection of Treg cells with Th cells did not alter the proliferation, activation, or follicular entry of the anti-chromatin B cells at day 3. At this early time point, the phenotype of the anti-chromatin B cells in the presence of Th cells, with or without Treg cells, is highly reminiscent of that observed in young *lpr/lpr* mice in which Th cell activation is high, but autoantibodies are not detected (15, 16). These findings lend support to the hypothesis that in young *lpr/lpr* mice, autoimmunity is held in check by Treg cells (9), resulting in an abortive B cell response marked by the follicular entry of autoreactive B cells in the absence of autoantibody production (15, 16).

Early studies involving Treg cells described their ability to inhibit the proliferation of Th cells in vitro. More recently, this finding has been corroborated by some in vivo experiments (3), while other reports have shown only modest inhibition of Th cell expansion (2). In this study, we show that at day 3 in vivo Th cell proliferation and recovery are only slightly decreased by the presence of Treg cells. However, the presence of Treg cells results in a sharp decline of Th cell recovery by day 8.

To begin to understand how Treg cells may be mediating the decline of the Th cells, we measured intracellular cytokine production and the expression of activation markers by the Treg cells at day 3. The coinjection of Th cells increased the frequency of Treg cells capable of secreting IL-10, a critical cytokine for Treg cell activity in some settings (43, 44). Interestingly, in the presence of Th cells, many Treg cells are positioned in B cell follicles and express slightly, but consistently higher levels of CXCR5. The follicular localization of Treg cells juxtaposes them with the Th and anti-chromatin B cells, possibly facilitating their suppressive

interactions. Future studies using vital microscopy are needed to verify the active engagement of these cell types.

As predicted, a high frequency of Th cells secreted IL-2 after transfer, consistent with their having undergone extensive proliferation. Interestingly, when Treg cells were coinjected, the majority of Th cells still underwent division, and the IL-2 production by the Th cells was unaffected. However, Th cell production of IFN- γ and IL-10, two cytokines known to promote B cell help and isotype switching (45, 46), was slightly, but significantly decreased. In a diabetes model, a reduction in IFN- γ levels produced by Th cells was also linked to the presence of Treg cells (2). Although the difference in cytokine production in Th cells is small, we have shown that even a small decline in IFN- γ may have profound effects on Th cell activity (47).

Activation markers crucial to initiate and/or sustain a Th-B cell cognate interaction (CD154 and CD178) were unaffected by the Treg cells, as was CD103. Likewise, Th cell CXCR5 expression and follicular entry at day 3 were not curtailed. Strikingly, however, Treg cell suppression was consistently associated with lower

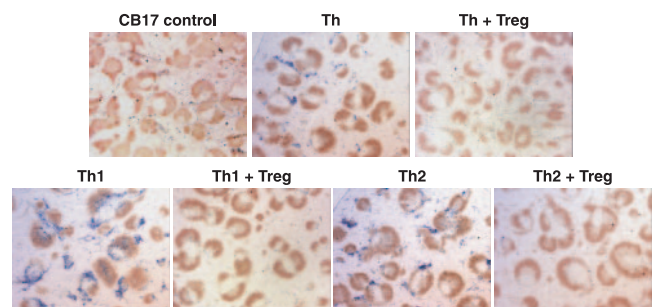


FIGURE 8. Treg cells suppress anti-chromatin AFC production. Spleen sections were stained with anti-CD22 (brown) and anti-IgMa (blue). Pictures are representative of at least three mice per condition.

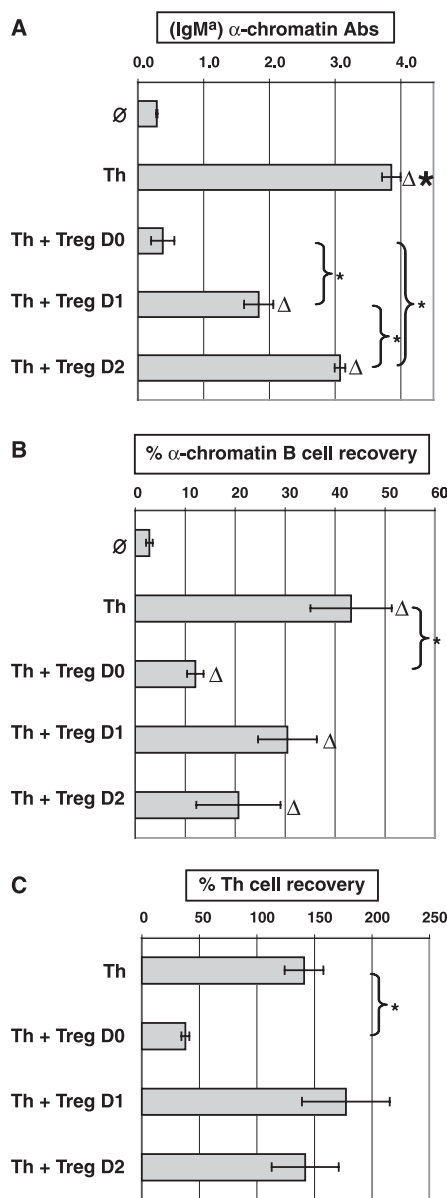


FIGURE 9. Effects of delaying Treg cell injection. Mice received Treg cells, either together with Th cells (Treg D0), together with anti-chromatin B cells (Treg D1), or 1 day following B cell injection (Treg D2). Mice were sacrificed on day 8. Significant differences between experimental groups are marked with *, and a significant difference compared with mice given only B cells ($\emptyset$) is marked with Δ . A, Sera were tested for IgM^a anti-chromatin Abs by ELISA. $n = 3$ for all sera. The bar graph depicting serum anti-chromatin Ab levels from mice given Th cells without Treg cells is marked with a large * to indicate that this value is significantly different from all other values shown ($p < 0.05$). Splenocytes were stained to detect transferred B cells (IgM^aIg λ ⁺, B) or T cells (CD4⁺6.5⁺, C), and their recoveries were calculated (B and C). B, Sample sizes: $\emptyset$, $n = 13$; Th, $n = 18$; Th + Treg D0, $n = 13$; Th + Treg D1, $n = 3$; Th + Treg D3, $n = 3$. C, Sample sizes: Th, $n = 12$; Th + Treg D0, $n = 13$; Th + Treg D1, $n = 3$; Th + Treg D3, $n = 3$.

ICOS expression levels on the Th cells. Notably, in a nonautoreactive Th/B cell model, the use of a blocking ICOS Ab also correlated with a decrease in Ab production, but not a block in follicular entry of the responding cells (48). Furthermore, blockade of ICOS in vitro and/or in vivo was shown to decrease production of IFN- γ and IL-10, but not IL-2, by Th cells (49, 50), similar to the cytokine profile reported in this work. A potential role for

ICOS-B7 homologous protein interactions in autoimmunity has been described in a variety of autoimmune settings (49, 51, 52). Thus, we hypothesize that alterations in ICOS expression on Th cells may be a consequence of exposure to Treg cells, which in turn limits the expansion and differentiation of the autoantibody response.

Importantly from a therapeutic stance, it has been shown that Treg cells can suppress autoimmune responses and pathology even when injected 14 days after initiation of the autoimmune process (8, 53, 54). However, we find in the model presented in this study that Treg cells are required during the first day of the immune response to mediate their full suppressive effects.

We hypothesize that in the preautoimmune state of young Fas/FasL-deficient mice, T cell help is available for anti-chromatin B cells. The provision of this help mediates some phenotypic changes in the anti-chromatin B cells, including their migration into B cell follicles. However, Treg cells prevent their terminal differentiation into plasma cells. A key question is what changes occur in older autoimmune mice that result in autoantibody production. Future studies will focus on the mechanisms leading to the disruption of T cell regulation with an emphasis on the role of inflammation in inhibiting Treg suppression (55).

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Disclosures

The authors have no financial conflict of interest.

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