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Autoantigen-Specific TGFβ-Induced Foxp3\(^+\) Regulatory T Cells Prevent Autoimmunity by Inhibiting Dendritic Cells from Activating Autoreactive T Cells\(^1\)

Richard J. DiPaolo, Carine Brinster, Todd S. Davidson, John Andersson, Deborah Glass, and Ethan M. Shevach\(^2\)

Several strategies are being designed to test the therapeutic potential of Ag-specific regulatory T cells to prevent or treat autoimmune diseases. In this study, we demonstrate that naïve CD4\(^+\)Foxp3\(^-\) T cells specific for a naturally expressed autoantigen (H\(^+/\)K\(^+\) ATPase) can be converted to Foxp3\(^+\) T regulatory cells (Tregs) when stimulated in presence of TGFβ. TGFβ-induced Tregs (iTregs) have all the characteristics of naturally generated regulatory T cells in vitro, and more importantly, are effective at preventing organ-specific autoimmunity in a murine model of autoimmune gastritis. H\(^+/\)K\(^+\) ATPase specific iTregs were able to inhibit the initial priming and proliferation of autoreactive T cells, and appear to do so by acting on H\(^+/\)K\(^+\) ATPase presenting dendritic cells (DC). DC exposed to iTregs in vivo were reduced in their ability to stimulate proliferation and cytokine production by H\(^+/\)K\(^+\) ATPase specific T cells. iTregs specifically reduced CD80 and CD86 expression on the surface of H\(^+/\)K\(^+\) ATPase presenting DC in vitro. These studies reveal the therapeutic potential of Ag specific iTregs to prevent autoimmunity, and provide a mechanism by which this population of regulatory T cells, and perhaps others, mediate their suppressive effects in vivo. The Journal of Immunology, 2007, 179: 4685–4693.

**Thymic**-derived CD4\(^+\)CD25\(^+\)Foxp3\(^+\) naturally occurring T regulatory cells (nTregs)\(^3\) play an important role in maintaining self-tolerance and preventing autoimmunity in mice and humans (1–3). Although many murine models have been developed to demonstrate that polyclonal nTregs can inhibit organ-specific autoimmunity, the precise mechanism(s) used by nTregs to suppress autoreactive T cells in vivo during the development of autoimmune pathology is not clear. Although nTregs have been shown to prevent IL-2 production and proliferation of responding T cells in vitro (4), the mechanism(s) used by nTregs in vivo is more complex and controversial, with possibilities ranging from the suppression of initial activation and proliferation, differentiation, migration, and effector functions of responding T cells, to effects on dendritic cells (DCs) and other cell types (1, 5). It is widely believed that nTregs are selected based on recognition of self-peptides, but it is not clear whether nTregs recognize organ-specific autoantigens, or whether they are activated by ubiquitous self-Ags and suppress organ-specific responses via bystander suppression. Recent studies using nTregs from TCR transgenic mice have indicated that organ-specific nTregs may be more efficient at preventing autoimmunity than a polyclonal population of nTregs with unknown Ag specificities (6–10).

Autoimmune gastritis (AIG) has proven to be an excellent model to examine the therapeutic potential of nTreg. AIG is a CD4\(^+\) T cell mediated autoimmune disease that develops in the absence of nTreg secondary to thymectomy on day 3 of life, and was one of the first diseases used to demonstrate the regulatory activity of CD4\(^+\)CD25\(^+\) T cells (11–13). The target autoantigen for the pathogenic CD4\(^+\) T cells has been defined as the H\(^+/\)K\(^+\) ATPase, the proton pump of the parietal cells located in the gastric mucosa (14, 15). We have generated a line of TCR transgenic mice (TxA23) whose receptors recognize a defined peptide of the H\(^+/\)K\(^+\) ATPase and these mice spontaneously develop severe AIG (16, 17). TxA23 T cells escape the thymus due to insufficient levels of H\(^+/\)K\(^+\) ATPase presentation in the thymus (18), and are activated in the gastric lymph node (LN) by DCs that have acquired the H\(^+/\)K\(^+\) ATPase from the parietal cells in the gastric mucosa (18, 19). Small numbers of thymocytes from TxA23 mice can transfer AIG, and polyclonal nTreg prevent disease by inhibiting the differentiation of the effector cells to pathogenic Th1 cells (20).

Recent studies have demonstrated that stimulation of naive polyclonal T cells in the presence of TGFβ results in the induction of Foxp3 expression and T regulatory activity in vitro and in vivo (21–24). In this study, we demonstrate that naïve H\(^+/\)K\(^+\) ATPase-specific CD4\(^+\)CD8\(^-\)Foxp3\(^-\) thymocytes from TCR transgenic mice can be easily expanded and induced to express Foxp3 when activated in the presence of TGFβ1. TGFβ-induced regulatory T cells (iTregs) resemble nTreg as they are anergic, suppressive, and have a reduced capacity to produce effector cytokines upon re-stimulation in vitro. Most importantly, after transfer, these autoantigen-specific iTregs maintained Foxp3 expression and inhibited the development of AIG. Autoreactive effector T cells within the gastric LN were not activated and did not undergo expansion in the presence of iTregs. DC isolated from gastric LN containing iTregs had a markedly reduced capacity to stimulate H\(^+/\)K\(^+\) ATPase T

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\(^3\) Abbreviations used in this paper: nTreg, naturally occurring T regulatory cell; DC, dendritic cell; AIG, autoimmune gastritis; LN, lymph node; iTreg, TGFβ-induced regulatory T cells; PCAb, parietal cell Abs; Treg, regulatory T cell.
cells, and DC cultured with iTregs and effectors in vitro expressed substantially reduced levels of CD80 and CD86. Taken together, these data demonstrate that it is possible to convert and expand autoantigen-specific effector T cells in vitro into potent autoantigen-specific iTregs that are capable of preventing autoimmunity by reducing the ability of DC to prime autoreactive T cells. A similar approach might be applicable to the treatment of autoimmune disease in man.

Materials and Methods

Mice

Female BALB/c and BALB/c nu/nu (4–8-wk-old) mice were purchased from the National Cancer Institute animal facility and housed under specified pathogen-free conditions. TxA23 TCR transgenic mice have been described previously (17). All mice were maintained in our animal facility and cared for in accordance with institutional guidelines.

Abs and reagents

Anti-CD25-PE (PC61), anti-Thy-1.1-PE (OX-7), and anti-Thy-1.2-FITC (53-2.1), anti-CD3 (145-2C11), anti-CD28 (37.51), anti-CD80-PE, and anti-CD86-PE were purchased from BD Pharmingen; anti-CD4-TC (Tri-color) was purchased from Caltag Laboratories; and anti-FoxP3-APC (FJK-16s) was purchased from eBioscience. Recombinant human TGFβ was purchased from R&D Systems. Human IL-2 was purchased from PeproTech. Anti-TGFβ1, ρ2, and ρ3 mAb (clone D1/11), anti-mlL-10R (clone 1B1.3a), and anti-CTLA4 (clone UC10-F10) were purified from a hybridoma supernatant pre pared on a protein G Sepharose column, and used in vitro at 50 μg/ml. CFSE was obtained from Molecular Probes and used at a final concentration of 10 μM for 10 min at 37°C in PBS. All flow cytometry was performed on BD FACSan or FACS-Calibur and analyzed using CellQuest or FlowJo software (BD Biosciences).

T cell proliferation, expansion, and in vitro assays

TxA23 thymocytes were isolated from a single cell suspension from thymus of TxA23 mice. Thymocytes were depleted of CD8+ by incubating for 10 min with anti-CD8 microbeads (Miltenyi Biotec). The negative fraction was kept using the AutoMACS deplete sensitive program. The CD8 depleted fraction was then sorted for CD4+ and CD25+ T cells after sorting. Sorted TxA23-Thy-1.1+IL-2+ thymocytes were cultured in 2 ml in a 24-well plate with T-depleted irradiated (1 × 10⁶; 3000 rads) BALB/c splenocytes, anti-CD3 (1 μg/ml), anti-CD28 (1 μg/ml), and th TL-2 (50 U/ml) with or without th TLGFB (5 ng/ml) in RPMI 1640 supplemented with 5% heat inactivated FCS (Atlanta Biologicals), penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (all from BioSource International). After 2 days, each well was expanded into two wells, and 1 ml of fresh medium and 50 U/ml IL-2 were added. Cells were used 7 days after the culture was initiated. After 7 days in culture with or without th TLGFB, 1 cells were washed twice and counted. For cytokine production, cells were put in 24-well plates that had been coated with anti-CD3 (1 μg/ml) and anti-CD28 (3 μg/ml). After washing the cells three times, T cells were added in medium containing GolgiStop for 4 h. Cells were then harvested, fixed in 4% paraformaldehyde for 5 min at 37°C, washed once, and permeabilized with PBS plus 0.5% BSA plus 0.1% Triton X-100 before staining. In the sup pression assays, CD4+ T cells (5 × 10⁶) from BALB/c mice were cultured with irradiated with T-depleted spleen cells (5 × 10⁶) and 0.25 μg/ml α-CD3 for 3 days in the presence of varying numbers of pretreated TxA23-derived CD4+CD25+ T cells. Proliferation was measured in triplicates by the incorporation of [3H]Thymidine over the last 6–8 h of the culture.

Adoptive transfers

CD25-depleted spleen cells were prepared from BALB/c mice as described previously (20). A typical depletion resulted in <1% of the CD4+ CD25+ expressing CD25. naive TxA23-Thy-1.1+IL-2+ T cells were isolated from a single cell suspension from thymus of TxA23 mice. Thymocytes were washed and purified over a T cell enrichment column (R&D Systems). The purified cells were then depleted of CD8+ and CD25+ cells after incubation for 10 min with anti-CD25-PE (PC61), washed in MACS buffer, and incubated for a further 10 min with anti-CD8 and anti-PE MA Abs (Miltenyi Biotec). The negative fraction was kept using the autoMACS deplete sensitive program. Before injection, all cells were washed twice in PBS, and the naive TxA23-Thy-1.1/IL-2-1 T cells and isolated from the thymus, and cells were diluted such that an i.p. injection of 0.5 ml per mouse resulted in the transfer of 50,000 ThxA23-Thy-1.1/IL-2-1 T cells and 20 × 10⁶ spleen cells with or without 200,000 TGFβ-treated TxA23-Thy-1.1/Thy-1.2+ T cells.

Detection of anti-parietal cell Abs (PCAb) and histologic evaluation of gastric pathology

PCAb were detected by immunofluorescence on cryostat sections of normal BALB/c stomach as described (44). In brief, sections were blocked with 2% FBS in 5% dry milk in PBS and incubated with a 1/50 dilution of serum for 1 h at room temperature. The presence of autoantibodies was visualized by adding FITC goat F(ab’)2 anti-mouse Ig (BioSource International). Slides were examined under a fluorescent microscope and given a score ranging from 0 to 4 depending on the extent of pathological change for FITC. For histologic evaluation, stomachs were washed, removed, and fixed in 4% formaldehyde, cut into 5-μM sections, and stained with H&E by American Histolabs. The extent of gastritis was graded on a scale of 1 to 6, depending on the extent of mononuclear cell infiltration and parietal and chief cell destruction. Eight individual sections from each stomach were scored blindly by two individuals. General descriptions for scores are as follows: 1.0, scattered lymphocytes throughout submucosa and muscularis; 1.5, one or two small dense blankets of lymphocytes; 2.0, two to four small dense clusters of lymphocytes in the submucosa/mucosa; 3.0, two to three areas with intermediate infiltration spanning 1/3 of mucosa; 4.0, big nodules of lymphocytic accumulation spanning 1/2 to all of mucosa, but no evidence of parietal cell loss; 5.0, heavy lymphocytic infiltration throughout mucosa, parietal and chief cell loss (25–50%) and replacement by foamy cells; and 6.0, heavy infiltration into gastric mucosa, total parietal and chief cell loss, no mucosal architecture, and many foamy cells.

Purification of DC from gastric LN and spleens

After isolation, the tissues were fragmented and digested for 30 min at 37°C in the presence of liberase blendzyme II (Roche) and DNase (2 μg/ml) (Roche) in complete medium (modified RPMI 1640 supplemented by 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 4.10⁻¹ M 2-ME, 1 mM essential amino acids, and 1 mM sodium pyruvate; all from Biofluids). To disrupt DC-T cell complexes, EDTA (1 mM, 0.1 M (pH 7.2)) was added and mixed for 5 min. Undigested fibrous material was removed by filtration through a 40-μM cell strainer. RBC were lysed using an ACR lysis buffer (BioSource International) and the cells were incubated for 15 min with 10 μg/ml purified murine and hamster IgG in PBS, EDTA 2 mM, and 0.5% BSA. For gastric LN, CD11c+ cells were then isolated by positive selection using anti-CD11c-coated magnetic column (Miltenyi Biotec). Thymocytes were typically >98% CD4+CD8+ T cells after sorting. Sorted TxA23-Thy-1.1+IL-2+ thymocytes were cultured in 2 ml in a 24-well plate with T-depleted irradiated (1 × 10⁶; 3000 rads) BALB/c splenocytes, anti-CD3 (1 μg/ml), anti-CD28 (1 μg/ml), and th TL-2 (50 U/ml) with or without th TLGFB (5 ng/ml) in RPMI 1640 supplemented with 5% heat inactivated FCS (Atlanta Biologicals), penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (all from BioSource International). After 2 days, each well was expanded into two wells, and 1 ml of fresh medium and 50 U/ml IL-2 were added. Cells were used 7 days after the culture was initiated. After 7 days in culture with or without th TLGFB, 1 cells were washed twice and counted. For cytokine production, cells were put in 24-well plates that had been coated with anti-CD3 (1 μg/ml) and anti-CD28 (3 μg/ml). After washing the cells three times, T cells were added in medium containing GolgiStop for 4 h. Cells were then harvested, fixed in 4% paraformaldehyde for 5 min at 37°C, washed once, and permeabilized with PBS plus 0.5% BSA plus 0.1% Triton X-100 before staining. In the suppression assays, CD4+ T cells (5 × 10⁶) from BALB/c mice were cultured with irradiated with T-depleted spleen cells (5 × 10⁶) and 0.25 μg/ml α-CD3 for 3 days in the presence of varying numbers of pretreated TxA23-derived CD4+CD25+ T cells. Proliferation was measured in triplicates by the incorporation of [3H]Thymidine over the last 6–8 h of the culture.

Results

Induction and expansion of organ-specific FoxP3+ cells from FoxP3− precursors with TGFβ

To determine the ability and efficiency of TGFβ to induce FoxP3 expression in naïve T cells specific for the H₂K⁺ ATPase autoantigen, we isolated CD4+ CD8+ CD25+ T cells from the thymus of ThxA23 transgenic mice by cell sorting. The naive thymocytes (>98% CD4+ CD25+ FoxP3−) were then cultured with anti-CD3, anti-CD28, and IL-2 with or without 5 ng/ml TGFβ. In the presence of TGFβ, >90% of the ThxA23 CD4+ T cells were induced to express FoxP3, as measured by intracellular staining for the FoxP3 protein. In contrast, very few of the T cells stimulated without TGFβ were FoxP3+ (Fig. 1A). Although TGFβ is reported to
inhibit T cell proliferation, in the presence of exogenous IL-2, we typically observed a 25-fold increase in the number of T cells after 7 days. This recovery was lower than cells cultured in the absence of TGFβ, where on average, a 100-fold expansion was observed (data not shown). After expansion, >98% of T cells in both groups expressed the transgenic TCR specific for the H2/K Ag ATPase peptide in the context of MHC class II I-A<sup>d</sup> (data not shown). These data demonstrate the ability to generate large numbers of Ag-specific Foxp3<sup>+</sup> T cells by activating and expanding Foxp3<sup>+</sup> T cells in vitro in the presence of TGFβ.

**TGFβ-iTreg exhibit a suppressor phenotype in vitro**

When restimulated with anti-CD3/CD28, T cells exposed to TGFβ produced very little IL-2, IL-4, IFN-γ, and TNF-α compared with the control population. Interestingly, the production of IL-10, which is sometimes considered to be a property of iTreg, was also suppressed in TGFβ-treated T cells (Fig. 1, B and C). TGFβ-treated TxA23 T cells were nonresponsive when restimulated with anti-CD3, but did respond to anti-CD3 stimulation in the presence of exogenous IL-2 and IL-2 alone (Fig. 1D). Most importantly, TGFβ-treated cells potently suppressed the responses of fresh CD4<sup>+</sup> CD25<sup>+</sup> T cells in the standard in vitro coculture assay (Fig. 1E). The suppressive activity of the TGFβ-treated cells was not abrogated by the addition of neutralizing Abs to IL-10 and TGFβ (Fig. 1F). Together, these data show that TxA23 TGFβ-iTregs have all the characteristics of nTregs in vitro.

**TGFβ-iTreg inhibit AIG**

Although the in vitro functional phenotype of Foxp3<sup>+</sup> TGFβ-iTreg closely resembles that of thymic-derived nTreg, it is still

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**FIGURE 1.** Converting organ-specific Foxp3<sup>+</sup> thymocytes into Foxp3<sup>+</sup> Tregs. A, CD4<sup>+</sup>CD25<sup>+</sup> thymocytes were sorted from TxA23 TCR transgenic mice, and Foxp3 expression was determined before and after culture with or without TGFβ. Cytokine production was determined by TxA23 T cells restimulated 7 days after culturing without TGFβ (B), or after converting into Foxp3<sup>+</sup> cells with TGFβ (C). The proliferation of TxA23 T cells cultured without (●) or with TGFβ-1 (○) and restimulated with APC alone, APC and anti-CD3, or APC and anti-CD3<sup>+</sup> IL-2, or IL-2 alone (D). In vitro suppression assays measuring the response of CD4<sup>+</sup> T cells in the presence of the indicated number of TxA23 T cells cultured without (●) or with (○) TGFβ (E), and the same suppression assay in the presence of Abs neutralizing TGFβ and IL-10 (F).
unclear whether Foxp3 expression is stable and whether the iTreg will also exhibit suppressor activity in vivo. To evaluate the ability of the autoantigen-specific iTreg to inhibit the transfer of AIG, we cotransferred the TGFβ-iTreg (Thy-1.1+) with CD4⁺CD25⁻ Foxp3⁻ thymocytes from TxA23 mice (Thy-1.1⁺/Thy-1.2⁻) to nu/nu recipients together with CD25-depleted splenocytes (Thy-1.2⁻) to minimize homeostatic proliferation (20). The hallmarks of AIG are the development of PCAbs, heavy mononuclear cell infiltrate, and destruction of parietal cells within the gastric mucosa. As expected, transfer of naïve TxA23 T cells and CD25-depleted splenocytes induced AIG in BALB/c nu/nu recipients (Fig. 2). In mice that did not receive iTregs, 9 out of 11 mice (82%) developed PCAbs 30 days after transfer (Fig. 2A), and 8 out of 10 mice (80%) exhibited severe inflammation of the gastric mucosa with extensive destruction of parietal cells within the gastric mucosa (Fig. 2B, a score of 5 or higher). In contrast, only 3 out of 12 (25%) mice that received TGFβ-iTreg developed PCAbs, and only 1 out of 10 (10%) mice showed any evidence of parietal cell destruction (Fig. 2, A and B). In the group that received the TGFβ-iTreg, 2 out of 10 mice had normal stomach pathology, and the remaining mice had low or intermediate levels of inflammation in the gastric mucosa with no evidence of parietal cell destruction (scores of 4 and lower). Five mice in each group were analyzed after 150 days. All mice in the group without regulatory T cells (Tregs) developed a severe destructive AIG, whereas only 1 out of 5 mice in the group with TGFβ-iTreg exhibited a pathology score of 5, indicating that iTreg-mediated protection is stable for long periods of time (data not shown). Thus, these data demonstrate that the in vitro induced and expanded autoantigen-specific TGFβ-iTreg are highly effective suppressors of the induction of AIG.

iTregs are long lived in vivo and maintain Foxp3 expression in vivo

As the cell populations used in these cotransfer studies expressed unique congenic markers, we analyzed the different cell populations in the gastric LN after transfer. As expected, both TxA23 TGFβ-iTreg (Thy-1.1⁺) and TxA23 effectors (Thy-1.1⁺/Thy-1.2⁻) accumulated in the gastric LN, which has been reported to be the site of H⁺/K⁺ ATPase presentation and T cell activation. Very few TGFβ-iTreg or TxA23 effectors were detectable in nongastric LN (data not shown). Importantly, Foxp3 expression was detectable in a majority of TGFβ-iTregs, and no differences were seen in the low percentages of Foxp3⁺ T cells in the TxA23 effector T cells or the cotransferred splenocytes in the presence or absence of iTreg (Fig. 3). Similar results were observed 50 days after transfer. We conclude from these studies that the Foxp3 expression in transferred iTregs is stable, that the iTreg do not induce Foxp3 expression in other cells, and that the iTregs and effector T cells coexist for an extended period of time.

iTregs reduce the number of total and autoreactive T cells in the gastric LN

To more fully understand the mechanisms by which TGFβ-iTreg mediate disease suppression, we harvested gastric LN at various time points after transfer and measured total cellularity, as well as the absolute numbers of TxA23 effectors T cells (Fig. 4, A and B). The number of total cells in a gastric LN before injection averaged between 300,000–400,000 cells. This number increased 10-fold within a period of a week following transfer in animals that received effectors without iTregs (Fig. 4B). A rapid expansion of CD4⁺Thy-1.1⁺/Thy-1.2⁺ effector T cells was also observed, increasing from an average of 150 TxA23 T cells per gastric LN 2 days posttransfer to close to 50,000 TxA23 T cells 7 days posttransfer (Fig. 4B). Conversely, only 2,000 TxA23 effector T cells were detected at day 7, and little increase in the total cellularity of the gastric LN was observed when H⁺/K⁺ ATPase-specific iTregs were cotransferred. As expected, very few TxA23 transgenic T cells were detectable in nongastric LN and no differences in cellularity of nongastric LN were observed between the two groups.

FIGURE 2. Coinjecting iTregs inhibits the transfer of AIG. Naïve TxA23 thymocytes and CD25-depleted splenocytes from BALB/c mice were transferred into BALB/c nu/nu recipients with or without 1 × 10⁶ TGFβ-cultured TxA23 T cells. A, Thirty days after transfer, serum from each mouse was collected and tested for the presence of PCAbs. B, Fifty days after transfer, mice were sacrificed and the amount of inflammation and extent of parietal cell destruction in the gastric mucosa was determined in each mouse (see Materials and Methods for scoring). The mean score of recipients that received or did not receive TGFβ-cultured TxA23 T cells was 5.0 ± 0.21 and the group that received TGFβ-cultured TxA23 T cells was 2.7 ± 0.27 (p = .0005; nonparametric Mann-Whitney U test).

FIGURE 3. iTregs colocalize in the gastric LN, maintain Foxp3 expression, and do not convert other cells to Foxp3⁺ T cells. Gastric LN cells were isolated 7 days after transfer, and were analyzed for surface expression of CD4, Thy-1.1, Thy-1.2, and intracellular staining for Foxp3. Histograms show Foxp3 expression on each of the populations: iTregs-Thy-1.1⁺/Thy-1.2⁺, TxA23 effectors-Thy-1.1⁺/Thy-1.2⁺, and splenic T cells-Thy-1.1⁺/Thy-1.2⁺.
iTregs inhibit the priming and expansion autoreactive T cells in the gastric LN

The 25-fold decrease in the number of effector T cells present 7 days after transfer in the mice that received the TGFβ-iTregs raised the possibility that the TGFβ-iTregs might inhibit the migration of the effector cells into the gastric LN. However, two days after injection, between 100 and 200 Thy1.1+/Thy1.2+ staining on cells in the gastric LN in mice injected with or without TGFβ-iTregs. B. The number of total gastric LN cells and the total number of TxA23 (CD4+Thy-1.1+/1.2+) effectors was determined per gastric LN at various time points after mice were injected with (○) or without (●) TxA23 TGFβ-iTregs. The data represent the average of combining between 5 and 15 mice per group at each time point.

iTregs prevent the initial priming and expansion of gastric-specific T cells in the gastric LN. A, CD69 expression was determined after gating on TxA23 effectors (CD4+Thy-1.1+/1.2+) in the gastric LN 48 h after transferring TxA23-Thy-1.1+/1.2+ naive T cells with CD25-depleted splenocytes either alone or with TxA23-TGFβ-iTregs (CD4+Thy-1.1+). CD69 expression on TxA23 effectors in nongastric (inguinal) LN is shown as a control. B. CFSE content was determined after gating on TxA23 effectors (CD4+Thy-1.1+/1.2+) from gastric LN 5 days after injection of mice with or without TxA23-TGFβ-iTregs. CFSE content of TxA23 effectors from nongastric LN is shown as a control.

iTregs were also in the gastric LN. The extent of division in the gastric LN of the iTreg-treated mice was similar to that seen in peripheral LN, which do not contain autoantigen-presenting DC. No decrease in the total number or in CFSE dilution was observed when TxA23 T cells that were cultured without TGFβ were co-transferred, ruling out a simple competition for an APC model of suppression (data not shown). Taken together, these data are most consistent with a model in which iTregs are inhibiting the initial priming and ensuing proliferation of autoreactive effector T cells very early in the response.

iTregs suppress presentation by DC in the gastric LN

One unique aspect of this model of AIG is that the H+/K+ ATPase protein can only be detected in a small number of DC in the gastric LN. H+/K+ ATPase containing DC are likely to have acquired this endogenous autoantigen from parietal cells in the stomach and migrated to the gastric LN. It has been demonstrated that Ag-bearing DC can activate T cells in the absence of an inflammatory signal (19, 25). To directly examine the effect of H+/K+ ATPase presentation, we isolated CD11c+ cells from the gastric LN of several mice (30 mice per group) 48 h after transferring either effector T cells without iTregs, effector T cells with iTregs, and from noninjected mice. As a sensitive readout to measure the effect of H+/K+ ATPase, iTregs on the ability of gastric LN DC to present endogenous H+/K+ ATPase, we tested the isolated gastric LN DC from each group to stimulate a TxA23 T cell line.

CD11c+ DC isolated from gastric LN of unmanipulated nu/nu mice stimulated the proliferation and cytokine production by the TxA23 T cells, and DC isolated from mice injected with effectors T cells without iTregs stimulated even more proliferation and cytokine production (Fig. 6A). This enhanced stimulation of TxA23 T cells by DC from gastric LN of mice that received effector T cells is likely due to the mutual activation between the effector T cells and DC in vivo. In contrast, DC isolated from the gastric LN

FIGURE 4. iTregs suppress the total number of cells, and the number of H+/K+ ATPase-specific TxA23 T cells in the gastric LN. A. Analysis of Thy-1.1 and Thy-1.2 staining on cells in the gastric LN in mice injected with or without TGFβ-iTregs. B. The number of total gastric LN cells and the total number of TxA23 (CD4+Thy-1.1+/1.2+) effectors was determined per gastric LN at various time points after mice were injected with (○) or without (●) TxA23 TGFβ-iTregs. The data represent the average of combining between 5 and 15 mice per group at each time point.

FIGURE 5. iTregs prevent the initial priming and expansion of gastric-specific T cells in the gastric LN. A, CD69 expression was determined after gating on TxA23 effectors (CD4+Thy-1.1+/1.2+) in the gastric LN 48 h after transferring TxA23-Thy-1.1+/1.2+ naive T cells with CD25-depleted splenocytes either alone or with TxA23-TGFβ-iTregs (CD4+Thy-1.1+). CD69 expression on TxA23 effectors in nongastric (inguinal) LN is shown as a control. B. CFSE content was determined after gating on TxA23 effectors (CD4+Thy-1.1+/1.2+) from gastric LN 5 days after injection of mice with or without TxA23-TGFβ-iTregs. CFSE content of TxA23 effectors from nongastric LN is shown as a control.
that were exposed to iTreg in vivo (Fig. 7). These data show that H\(^+\)/K\(^+\) ATPase-specific TxA23 T cells were then tested for their ability to stimulate an H\(^+\)/K\(^+\) ATPase peptide presentation by gastric LN (data not shown). This is likely due to the fact that we did not observe any effects of iTregs on MHC class II or DC. In these studies, two populations of DC, one from BALB/c, capable of presenting the H\(^+\)/K\(^+\) ATPase peptide to TxA23 effector T cells and iTregs, and one from C57BL/6, which are unable to present to the MHC class II I-A\(^d\)-restricted T cells, are cultured with peptide, alone or in the presence of the effectors and/or iTregs. This protocol allows us to distinguish whether the suppressive effects of the iTreg were mediated through functional alteration of peptide-presenting DC, or specific killing of the DC, and allows us to determine whether bystander effects mediated by soluble mediators modify the nonpresenting DC (C57BL/6) in the cocultures. After 20 h of coculture with TxA23 T cells, TxA23 iTregs, or both, no reduction in the relative proportion of BALB/c DC compared with C57BL/6 DC was observed, indicating that the iTregs were not killing H\(^+\)/K\(^+\) ATPase-specific T cells. The unfilled histogram represents the DC cultured without T cells, and the numbers represent the geometric mean fluorescence intensities.

\[\text{FIGURE 6. iTregs inhibit the endogenous presentation of gastric autoantigen by DC isolated from gastric LN. CD11c}^+\text{ DC were isolated from the gastric LN of 30 uninjected BALB/c nu/nu mice (○), 30 mice injected with TxA23 effector T cells only 48 h earlier (■), or 30 mice injected with TxA23 effector T cells and TGFβ-iTregs 48 h earlier (□). CD11c}^+\text{ cells were then tested for their ability to stimulate an H}\(^+\)/K\(^+\) ATPase peptide presentation by ATPase-specific TxA23 T cell line A, Proliferation of TxA23 T cells cocultured with live CD11c}^+\text{ cells isolated from gastric LN, and with CD11c}^+\text{ cells fixed immediately after isolation. B, The levels of cytokines in the supernatants were measured 48 h after stimulation with each population of live DC. Results are representative of two independent experiments.}\]

\[\text{iTregs modulate CD80 and CD86 expression}\]

We did not observe any effects of iTregs on MHC class II or various costimulatory molecules on the total population of DC from gastric LN (data not shown). This is likely due to the fact that H\(^+\)/K\(^+\) ATPase-specific iTreg act on H\(^+\)/K\(^+\) ATPase-presenting DC, and these DC are only a very small fraction of the total DC in the gastric LN (19). It has also not been proven possible to directly analyze the effects of iTreg on the small population of gastric LN DC that present the endogenous H\(^+\)/K\(^+\) ATPase due to technical limitations in detecting H\(^+\)/K\(^+\) ATPase-positive DC after disruption of the gastric LN. As an alternative, we have developed an in vitro assay in which the effects of the H\(^+\)/K\(^+\) ATPase-specific iTregs can be examined on H\(^+\)/K\(^+\) ATPase peptide presentation by splenic DC in vitro. In these studies, two populations of DC,
cells in the presence of TGF-β demonstrated that polyclonal nTreg prevented the transfer of AIG and previously developed a model to characterize nTreg-mediated suppression on a population level by mRNA, and by demonstrating that TGF-β protein rather than on a single cell level by intracellular staining for FoxP3 (26). We confirm and extend previous studies by measuring FoxP3 expression on a single cell level by intracellular staining for FoxP3 in the converted cells have the capacity to function as Tregs (21–24, 25). We considered a number of possible mechanisms for the iTreg-mediated suppression of the expansion of the effector cells. It is unlikely that the iTreg inhibited the initial migration of the effectors to the draining LN as small, but equal numbers, of effector cells could be detected in the node 2 days after transfer in the presence or absence of iTreg. Although activated Treg have been reported to express granzyme B and to kill certain responder populations (27–29), the TGF-β-iTreg did not express granzyme B, no evidence of effector cell death could be detected, and low numbers of effector cells could still be identified 50 days after transfer. The major effect of the iTreg was to inhibit the priming of autoreactive T cells within the gastric LN. A reduction in the number of CD69+ T cells, suppression of proliferation, and a 40-fold reduction in the number of autoreactive effectors was observed within the first 5 days. As these observations were most consistent with a failure of priming of the effectors, we isolated DC from the gastric LN 2 days after transfer and measured their ability to present the endogenous H+/K+ ATPase. DC that had been exposed to iTregs in vivo had a reduced capacity to present endogenous Ag compared with those from noninjected mice, and mice injected with only effector T cells. These same iTregs were able to reduce the surface expression of CD80 and CD86 on the surface of H+/K+ ATPase-presenting DC in vitro. Thus, it appears that one mechanism by which iTregs exert their function in vivo is by reducing the ability of DC to prime autoreactive T cells, hence stopping the autoimmune reaction before it even starts.

The concept that Tregs can mediate their suppressive effects in vivo by affecting Ag-presenting DC is supported by two recently published studies in which it was shown that Ag-specific nTregs interacted with Ag presenting DC, and that the presence of nTregs led to fewer long-lasting interactions between effector T cells and DC (30, 31). Our data support and help to clarify this model using TGF-β-iTregs, and show that they exert their negative effects by decreasing the stimulatory capacity of DCs, rather than by competing with the effectors for Ag or by acting directly on the effector cells to prevent their interaction with the DC. A number of in vitro studies have suggested that Tregs can inhibit the maturation of bone marrow-derived or splenic DC by modulating the expression of costimulatory or inhibitory molecules and the production of inflammatory cytokines (32–36). Other experiments have suggested that Tregs can also inhibit T cell responses by fully activated DC in vitro and mature DC in vivo (37, 38).

We could not directly analyze the mechanism of action of the iTregs on the small subset of H+/K+ ATPase-bearing DC in the gastric LN due to their limited number and lack of ability to detect Ag containing DC. We therefore examined the effects of iTregs on splenic DC and demonstrated that Ag-stimulated iTregs specifically down-regulated the expression of CD80/CD86 on Ag-presenting splenic DC. The magnitude of the decrease in CD80/CD86 expression observed could be sufficient to account for the failure of the effector cells to expand in vivo, and for the reduced ability of the DC to activate TxA23 T cells in vitro. Although one might conclude from our studies that the target for iTreg-mediated suppression in vivo is exclusively the DC, it should be emphasized that the magnitude of reduction in CD80/CD86 expression was...
significantly greater when all three cell types were present in the cocultures. Thus, it is possible that Treg-mediated suppression of DC function is optimal after a signal from the effector cells is sent to the DC to render them more susceptible to suppression, or a signal is sent to the Treg to render them more capable of suppressing, or that direct Treg-T effector interactions also play a role.

In our previous studies with polyclonal Treg cells, and in other similar studies with both polyclonal and Ag-specific Treg cells, Tregs blocked the differentiation, migration, and/or effector functions (20, 39–42) rather than effector cell expansion. It remains possible in these models that the DC also was the primary target of the Tregs and suppression was secondary to Treg-mediated suppression of IL-12 (or other cytokine) production by the DC. Polyclonal nTregs may not traffic selectively to sites of autoantigen expression in the draining LN of target organs, but may only be recruited following activation of T effector cells. Although the early expansion of T effector cells may escape inhibition, later steps in the differentiation to pathogenic Th1 cells would be blocked. Thus, Treg-mediated inhibition of the stimulatory capacity of autoantigen presenting DC, rather than direct Treg suppression of effector T cell function leading to their inactivation, may be their primary mechanism of action in vivo. This hypothesis is consistent with studies demonstrating fully competent autoreactive T cells following deletion of Tregs (43).

From a therapeutic standpoint, recent studies have focused on generating and expanding Ag-specific Tregs as a novel cellular immunotherapy to prevent or treat autoimmunity. However, the isolation and expansion of autoantigen-specific Treg from non-transgenic mice or humans with autoimmune disease represents a much more difficult task, as their frequency is likely to be low and their Ag specificity unknown. Many approaches to expanding Ag specific populations from small numbers of CD4+CD25+ T cells are being developed, including stimulation with peptide-MHC complexes coated to beads (7), peptide-pulsed DC (9), and retroviral transduction of cells with FoxP3 (44). Our results demonstrate that an alternative approach to the generation of Treg for therapy would be to isolate and convert potential effector cells to Treg cells in vitro with TCR stimulation in the presence of TGFβ. It is unlikely that this protocol is unique to the treatment of AIG, as it was recently shown that BDC2.5 T cells activated and expanded in the presence of TGFβ could inhibit the induction of diabetes induced by the transfer of Th1 effector T cells, although the mechanism of action of the iTreg in these studies was unclear (23). We are now carefully evaluating whether conventional memory T cells can be induced to express FoxP3. Nevertheless, it may be feasible to isolate organ-specific CD4+FoxP3+ T cells from the peripheral blood or tissue fluids from patients with autoimmune disease and convert them to iTreg in the presence of their target Ag and TGFβ. It is likely that such autoantigen-specific iTreg would function as bystander suppressor cells and target the subpopulation of DC in draining LN that present a number of different target organ-derived Ags. We also have not examined the therapeutic use of iTreg to reverse or stop the progression of ongoing autoimmune disease. However, the capacity of iTreg to inhibit the presentation of autoantigens by DC raises the possibility that the process of epitope spreading might also be susceptible to iTreg-mediated down-regulation. It remains to be determined if the suppressive mechanisms used by iTregs are the same or different from those used by the nTregs.

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References


