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Autoantigen-Specific TGFβ-Induced Foxp3+ Regulatory T Cells Prevent Autoimmunity by Inhibiting Dendritic Cells from Activating Autoreactive T Cells

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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 naive 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 H9252 dendritic cell; AIG, autoimmune gastritis; LN, lymph node; iTreg, TGFβ-induced regulatory T cells; PCAb, parietal cell Abs; Treg, regulatory T cell.

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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 specific 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 obtained from R&D Systems. Human IL-2 was purchased from PeproTech. Anti-TGFβ1, β2, and β3 mAb (clone D11), anti-IL-10R (clone 1B1.3a), and anti-CTLA4 (clone UC10-4E10) were purified from a hybridoma supernatant. Macs microbeads were purchased from Miltenyi Biotec. All cytokines were from BioSource International. 2,4-dinitrophenol (DNP) was from Sigma.

T cell purification, 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 then kept using the AutoMACS deplete sensitive program. The CD8 depleted fraction was then sorted for CD4+ and CD25+ T cells after sorting. Sorted TxA23-Thy1.1/IL2 thymocytes were cultured in 2 ml in a 24-well plate with T-depleted irradiated (1 × 10^6; 3000 rads) BALB/c splenocytes, anti-CD3 (1 μg/ml), anti-CD28 (1 μg/ml), and thu-IL-2 (50 U/ml) with or without thuTGFβ1 (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 thuTGFβ1, cells were washed twice and counted. For cytoxicity 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 wells 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 × 10^6) from BALB/c mice were cultured with irradiated with T depleted spleen cells (5 × 10^6) and 0.25 μg/ml α-CD3 for 3 days in the presence of varying numbers of pretreated TxA23-derived CD4+CD8+T cells. Proliferation was measured in triplicates by the incorporation of [3H]ThdR over the last 6–8 h of the culture.

Adaptive transfers

CD25-depleted spleen cells were prepared from BALB/c mice as described previously (20). A typical depletion resulted in ~1% of the CD4+ cells expressing CD25. Naive TxA23-Thy-1.1/Thy-1.2 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 MAbs (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/Thy-1.2 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 TxA23-Thy-1.1/Thy-1.2+T cells and 20 × 10^6 spleen cells with or without 200,000 TGFβ-treated TxA23-Thy-1.1/Thy-1.2+T cells.

Detection of anti-parialietal 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 of 0 to 4 depending on the extent of papillary and parietal cell loss as described previously for FITC. For histologic evaluation, stomachs were removed, washed in PBS, 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 glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 10 mM HEPES, 4.10^-3 M 2-ME, 1 mM essential amino acids, and 1 mM sodium pyruvate; all from Biofluids). To disrupt DC-T cell complexes, EDTA (1 μM, 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 ACK lysis buffer (BioSource International) and the cells were incubated for 15 min with 10 μg/ml purified mouse 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 (Milenyi Biotec). For the spleen, CD11c+ cells were isolated by positive selection using AutoMacs. DC were collected and analyzed for their phenotype. They were stained with the PE-conjugated anti-CD11c (HL3 clone) Ab. The purity was around 80–85% for gastric LN and >95% for spleens. A typical isolation from 30 gastric LN yielded 200,000–400,000 CD11c+ cells. For gastric LN DC presentation assays, 25,000 TxA23 T cells were added to a 96-well U-bottom microtiter plate with 25,000 CD11c+ cells for 72 h. [3H]Thymidine (1 μCi/well) was added during the last 12 h of a 72-h culture, after which [3H]Thymidine incorporation was measured by scintillation counting. For assays with splenic DC, 250,000 DC from BALB/c and C57BL/6 were cocultured with 50 μg/ml H/K+ ATPaspe peptide, and 250,000 TxA23 T cells isolated from spleens and LN of TxA23 transgenic mice and/or 250,000 TxA23 iTregs (generated as described above) in a 24-well plate. After 20 h of coculture, cells were washed in 0.1 M EDTA, stained with the indicated Abs, and analyzed by FACS.

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 H/K+ ATPaspe autoantigen, we isolated CD4+CD8−CD25+ T cells from the thymus of TxA23 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 TxA23 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 TGF-β. The expansion was determined by TxA23 T cells restimulated 7 days after culturing without TGF-β (B), or after converting into Foxp3+ cells with TGF-β (C). The proliferation of TxA23 T cells cultured without (●) or with TGF-β (○) and restimulated with APC alone, APC and anti-CD3, or APC and anti-CD3 IL-2, or IL-2 alone (D). In vitro suppression assays measuring the response of CD4+ T cells in the presence of the indicated number of TxA23 T cells cultured without (●) or with (○) TGF-β (E), and the same suppression assay in the presence of Abs neutralizing TGF-β and IL-10 (F).

**TGF-β-iTreg exhibit a suppressor phenotype in vitro**

When restimulated with anti-CD3/CD28, T cells exposed to TGF-β produced very little IL-2, 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 TGF-β-treated T cells (Fig. 1, B and C). TGF-β-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, TGF-β-treated cells potently suppressed the responses of fresh CD4+ CD25− T cells in the standard in vitro coculture assay (Fig. 1E). The suppressive activity of the TGF-β-treated cells was not abrogated by the addition of neutralizing Abs to IL-10 and TGF-β (Fig. 1F). Together, these data show that TxA23 TGF-β-iTregs have all the characteristics of nTregs in vitro.

**TGF-β-iTreg inhibit AIG**

Although the in vitro functional phenotype of Foxp3+ TGF-β-iTreg closely resembles that of thymic-derived nTreg, it is still
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 naive 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 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 of 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. Naive TxA23 thymocytes and CD25-depleted splenocytes from BALB/c mice were transferred into BALB/c nu/nu recipients with or without 1 x 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+ effectors present in the gastric LN of both groups, indicating that the early migration of the effector TxA23 cells was not inhibited by the TGFβ-iTregs. An alternative explanation for the TGFβ-iTreg-mediated inhibition of effector cell expansion between days 2 and 7 was that the TGFβ-iTregs were inhibiting the priming and proliferation of these cells. We therefore examined CD69 expression, an early activation marker on T cells that have recently engaged their TCR, on the effector TxA23 T cells (Thy-1.1+/Thy-1.2+). The percentage of CD69+ effector T cells was 75–90%, in the absence of iTregs, and decreased to 40–50% in the presence of iTregs (Fig. 5A). This observation is consistent with the possibility that the iTregs interfere with the initial activation of autoreactive T cells. CD69 was expressed on $>$20% of TxA23 effectors in the inguinal LN in both groups of mice, which lack H2Kd ATPase-presentation cells, showing that the reduction of CD69 induced on effector TxA23 T cells was specific to the gastric LN.

We also examined the effects of the iTreg on the initial proliferation of the TxA23 effector cells (Thy-1.1+/Thy-1.2+) by analyzing CFSE dilution 5 days after transfer. In the absence of TGFβ-iTregs, ~40,000 TxA23 effector cells were present in the gastric LN, of which $>$99% had undergone eight or more divisions (Fig. 5B). In contrast, <2,000 TxA23 effector T cells were detected in the gastric LN in animals that had received TGFβ-iTregs, and a significant proportion of the T cells remained undivided (Fig. 5B). In fact, there was an ~50-fold increase in the fraction of effector T cells that had not divided (14.4% vs 0.3%) when TGFβ-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 H2Kd ATPase protein can only be detected in a small number of DC in the gastric LN. H2Kd 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 these Ag-bearing DC can activate T cells in the absence of an inflammatory signal (19, 25). To directly examine the effect of H2Kd 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 H2Kd ATPase iTregs on the ability of gastric LN DC to present endogenous H2Kd 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 H2Kd ATPase-specific TxA23 T cells in the gastric LN. At various time points after injection, gastric LN were harvested, counted, and analyzed for expression of Thy-1.1 and Thy-1.2. 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+/Thy-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+/Thy-1.2+) in the gastric LN 48 h after transferring TxA23-Thy-1.1+/Thy-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+/Thy-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.
of mice that received iTregs and effectors stimulated less proliferation and cytokine production than either the group that received only effectors or the control group. The same pattern of stimulation (although reduced in magnitude) was observed when DC were fixed immediately after isolation, indicating that the reduction in stimulation was not due to soluble factors released by DC during the in vitro culture (Fig. 6A). Similarly, the production of IL-4 and IFN-γ by the TxA23 T cells was reduced when stimulated by DC that were exposed to iTregs in vivo (Fig. 7B). These data show that the presence of iTregs not only prevented the enhancement of stimulation by DC from mice that received effectors, but also reduced the basal level of stimulation of H+/K+ ATPase-specific TxA23 T cells observed by gastric LN DC from noninjected mice. Taken together, these data show that H+/K+ ATPase-specific iTregs act on autoantigen-presenting DC, and actively reduce their ability to stimulate H+/K+ ATPase specific T cells.

**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, 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 also 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-presenting DC (Fig. 7A). The CD11c+ DC expressed high levels of CD80 and CD86 when cultured in the presence of peptide alone, and we observed a substantial decrease in the surface expression of CD80 and CD86 on the BALB/c DC in the presence of the iTreg. This reduction was not observed when TxA23 effector T cells were cocultured with DC, but the reduction was significantly greater when both the naive TxA23 effector T cells and the TxA23 iTregs were cultured together with the DC (Fig. 7B). This reduction was dependent on the presence of the H+/K+ ATPase peptide, and minimal effects on the expression of CD80/CD86 were observed on the C57BL/6 cells present in the cocultures (data not shown). Interestingly, the suppressive effects of the iTregs on CD80 and CD86 expression were selective, as CD40 levels were slightly increased under all conditions and the levels of MHC class II, PD-L1, PD-L2, and ICOS-L were similar in all groups (Fig. 8).
cells in the presence of TGFβ/H9252 failed to inhibit the expansion of the effector cells either in the CD40 (A), ICOSL (B), PDL1 (C), PDL2 (D), and MHC class II (E) were not influenced by the presence of iTregs.

FIGURE 8. iTregs do not influence the expression of CD40, ICOSL, PDL1, PDL2, or MHC class II on the surface of H+/K+ presenting DC. As in Fig. 7, 2.5 × 10^3 DC were cocultured with 50 μg/ml H+/K+ ATPase peptide with or without 2.5 × 10^3 naive TxA23 T cells and/or 2.5 × 10^3 TGFβ-induced TxA23 iTregs for 20 h. Although CD80 and CD86 levels were reduced by the presence of iTregs (Fig. 7), the expression levels of CD40 (A), ICOSL (B), PDL1 (C), PDL2 (D), and MHC class II (E) were not influenced by the presence of iTregs.

Discussion

The first aim of this study was to evaluate the capacity of TGFβ to convert autoantigen-specific effector T cells into Foxp3+ Tregs. Previous experiments have shown that stimulation of CD4+ T cells in the presence of TGFβ induces Foxp3 expression and that the converted cells have the capacity to function as Tregs (21–24, 26). We confirm and extend previous studies by measuring Foxp3 expression on a single cell level by intracellular staining for FoxP3 (26). We confirm and extend previous studies by measuring Foxp3 expression and that iTregs do not influence the expression of CD40, ICOSL, PDL2, or MHC class II on the surface of H+/K+ presenting DC. As in Fig. 7, 2.5 × 10^3 DC were cocultured with 50 μg/ml H+/K+ ATPase peptide with or without 2.5 × 10^3 naive TxA23 T cells and/or 2.5 × 10^3 TGFβ-induced TxA23 iTregs for 20 h. Although CD80 and CD86 levels were reduced by the presence of iTregs (Fig. 7), the expression levels of CD40 (A), ICOSL (B), PDL1 (C), PDL2 (D), and MHC class II (E) were not influenced by the presence of iTregs.

The second aim of these studies was to determine the stability of TGFβ to convert autoantigen-specific effectors T cells into Foxp3+ Tregs. Previous experiments have shown that stimulation of CD4+ T cells in the presence of TGFβ induces Foxp3 expression and that the converted cells have the capacity to function as Tregs (21–24, 26). We confirm and extend previous studies by measuring Foxp3 expression on a single cell level by intracellular staining for Foxp3 protein rather than on a population level by mRNA, and by demonstrating that TGFβ induces the intracellular expression of Foxp3 in almost all of the expanded T cells. This population of relatively pure Foxp3+ iTregs has all the in vitro properties of thymic-derived nTregs. Together, these data suggest that activating and expanding self-Ag-specific naive CD4+ T cells in the presence of TGFβ is an effective and efficient way to generate large numbers of organ-specific CD4+Foxp3+ iTregs.

The second aim of these studies was to determine the stability of Foxp3 expression in vivo and to evaluate the capacity of the iTreg to suppress the induction of autoimmunity, in this case, AIG. We previously developed a model to characterize nTreg-mediated suppression during the induction of AIG. In those studies, we demonstrated that polyclonal nTreg prevented the transfer of AIG and failed to inhibit the expansion of the effector cells either in the gastric LN or the stomach, but did inhibit the differentiation of the autoreactive T cells into the Th1 effector cells responsible for pathogenicity (20). In the current study, we demonstrated that Ag-specific TGFβ/iTregs were also highly effective at preventing AIG. As has been observed using TCR transgenic nTreg, Ag-specific iTregs were more efficient at preventing autoimmunity than polyclonal nTregs used in our previous studies. TGFβ/iTregs were long lived in vivo, maintained FoxP3 expression, and protected mice from disease.

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 TGFβ/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-prepresenting 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 TGFβ/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 cultures. 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 nontransgenic 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


