$CD4^+CD25^+$ T Cells Prevent the Development of Organ-Specific Autoimmune Disease by Inhibiting the Differentiation of Autoreactive Effector T Cells

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Thymic-derived, naturally occurring, $CD4^+CD25^+$ regulatory T cells (nTreg) are potent suppressors of immune responses. A detailed understanding of which components of the development and activation of pathogenic effector T cells are inhibited by nTreg during the course of T cell-mediated, organ-specific autoimmunity is as yet unknown. We have analyzed the effects of polyclonal nTreg on the development of autoimmune gastritis. The nTreg inhibited the development of disease, but failed to inhibit the migration of effector cells into the gastric lymph node or stomach. Notably, nTreg did not inhibit the expansion of autoreactive T cells in the gastric lymph node. The primary effect of nTreg appeared to be inhibition of differentiation of autoantigen-specific T cells to Th1 effector cells, as reflected by a decrease in Ag-stimulated IFN-γ production and a reduction in T-bet expression. ***The Journal of Immunology,*** **2005, 175: 7135–7142.**

Studies over the past 10 years have demonstrated that potentially autoreactive T cells can be regulated by a distinct lineage of $CD4^+CD25^+$ T cells that naturally develops in the thymus (nTreg)[2] (1, 2). The nTreg prevent autoimmune disease induced after transfer of $CD4^+CD25^-$ T cells to immunodeficient recipients and prevent the spectrum of autoimmune diseases that develops after thymectomy on the third day of life (d3Tx) (3). The development of autoimmune diseases after d3Tx has been shown to be secondary to a deficiency of nTreg that migrate from the thymus later in ontogeny than conventional $CD4^+CD25^-$ T cells. Although the ability of nTreg to inhibit organ-specific autoimmunity has been well documented, the mechanisms by which they prevent autoimmune disease in vivo are poorly characterized.

The nTreg inhibit T cell activation in vitro by preventing the induction of IL-2 gene transcription and T cell proliferation (4). However, the contribution of IL-2 to the expansion and differentiation of $CD4^+$ T cells in vivo is controversial (5). Furthermore, several groups have shown that nTreg, although demonstrating an anergic phenotype in vitro, efficiently expand after Ag stimulation in vivo (6–8). These studies have raised important questions about the relevance of in vitro studies of the suppressive effects of nTreg in vivo (9). It remains unknown whether nTreg prevent autoimmune disease by inhibiting the priming and expansion of effector cells, by acting on APC, by preventing migration from the draining lymph node (LN) into the target organ, or by preventing the differentiation of naive precursors into pathogenic effector cells. One additional difficulty in the interpretation of studies of nTreg function in vivo is that many of the studies were performed after transfer of effectors and suppressors into immunodeficient recipients, in which both cell populations undergo lymphopenia-induced proliferation.

We have chosen autoimmune gastritis (AIG) as an appropriate model for analyzing the suppressive properties of nTreg in vivo because it is one of the most common diseases that develop in mice after d3Tx or transfer of $CD4^+CD25^-$ T cells to *nu/nu* recipients on a BALB/c background (10). It is also one of the few spontaneous models of organ-specific autoimmune disease in which the target Ag, the proton pump of the gastric parietal cell (H/K-ATPase), has been defined (11, 12). In addition, it represents a good animal model for chronic gastritis in man leading to pernicious anemia in which the T and B cell responses in humans also target H/K-ATPase (13–15). We previously generated TCR-transgenic (TCR-Tg) mice expressing TCRs derived from T cell clones isolated from the gastric lymph node of d3Tx mice in the process of developing AIG (10, 16, 17). The TCR from one of these Tg mice, TxA23, is specific for the peptide H/K-ATPase α-$chain_{630-641}$, presented in association with $I\text{-}A^d$. The TxA23 strain spontaneously develops AIG very early in life, and the effector cells from these mice express a Th1 phenotype. Low numbers of thymocytes from TxA23 TCR-Tg mice can transfer AIG to *nu/nu* recipients.

We have developed a novel cell transfer system in which the effects of nTreg on preventing the induction of AIG can be analyzed throughout the course of the disease process. Importantly, we are able to examine the effects of nTreg on Ag-specific priming, differentiation, and migration into the organ targeted for autoimmune destruction under conditions where lymphopenia-induced proliferation is at least partially controlled. An additional advantage of this model is that the target Ag is expressed and presented at physiological levels, as opposed to being introduced artificially as a transgene, and immunization is not required for disease induction. Although the cotransfer of polyclonal nTreg prevented the induction of autoimmune gastritis, it did not prevent the migration of autoantigen-specific T cells to the draining LN or stomach, nor did it prevent their expansion. The major effect of nTreg was to inhibit the quality of priming, namely, the ability of

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Received for publication June 28, 2005. Accepted for publication September 14, 2005.

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[2] Abbreviations used in this paper: nTreg, thymic-derived, naturally occurring, $CD4^+CD25^+$ regulatory T cell; AIG, autoimmune gastritis; CD25D, CD25 depleted; CD25E, CD25 enriched; d3Tx, thymectomy on the third day of life; gLN, gastric LN; HPRT, hypoxanthine phosphoribosyltransferase; LN, lymph node; PCAb, parietal cell Ab; Tg, transgenic.

0022-1767/05/$02.00

the autoantigen-specific T cells to differentiate into pathogenic Th1 effector cells that drive the disease process. The implications of these results for the potential therapeutic use of polyclonal nTreg for the treatment of organ-specific autoimmunity and their potential Ag specificity (or lack thereof) will be discussed.

Materials and Methods

Mice, Abs, and reagents

Female BALB/c and BALB/c *nu/nu* (4–8 wk old) were purchased from the National Cancer Institute animal facility and housed under specific pathogen-free conditions. TxA23 TCR-Tg mice have been described previously (17). All mice were maintained in our animal facility and cared for in accordance with institutional guidelines. Anti-CD25-PE (PC61), anti-CD25-FITC (7D4), anti-Thy-1.1-PE (OX-7), and anti-Thy-1.2-FITC (53-2.1) were purchased from BD Pharmingen, and anti-CD4-Tri-Color was purchased from Caltag Laboratories. H^+/K^+-ATPase α-chain peptide (PITAKAIAASVG, aa 630–641) was obtained through the National Institute of Allergy and Infectious Diseases Peptide Synthesis Unit. CFSE was purchased from Molecular Probes. All flow cytometry was performed on a FACScan or FACSCalibur and analyzed using CellQuest software (all from BD Biosciences).

Cell purification and injection

Naive TxA23 T cells were isolated from a single-cell suspension from thymi of TxA23.Thy-1.1 mice. Thymocytes were ACK lysing buffer (BioSource) lysed, 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 an additional 10 min with anti-CD8 and anti-PE MACS microbeads (Miltenyi Biotec). The negative fraction was kept using the autoMACS deplete-sensitive program.

The $CD4^+CD25^+$-depleted and $CD4^+CD25^+$-enriched spleen cells were prepared from BALB/c mice. The RBC were removed by Ficoll centrifugation. A portion of the cells was depleted of CD25 by incubation with anti-CD25-PE (PC61) for 10 min, washed, and incubated for an additional 10 min with anti-PE microbeads, then isolated using the autoMACS deplete-sensitive program. The negative fraction was used as CD25-depleted (CD25D) spleen cells, and the positive fraction, which contained the $CD25^+$ T cells, was added to the portion of unseparated cells, and is referred to as CD25-enriched (CD25E) spleen cells. A typical depletion resulted in $<1\%$ of the $CD4^+$ cells expressing CD25, whereas a typical enrichment resulted in $>10\%$ of the $CD4^+$ cells expressing CD25. Before injection, all cells were washed twice in PBS, and the naive TxA23.Thy-1.1 T cells, isolated from thymus, were diluted into each spleen population such that an i.p. injection of 0.5 ml/mouse resulted in the transfer of 50,000 TxA23.Thy-1.1 T cells and 20×10^6 spleen cells. In some cases $CD4^+CD25^+$ T cells were isolated as described above, but from splenocytes of BALB/c.Thy-1.1 congenic mice, and TxA23 thymocytes were isolated as described above, but from TxA23.Thy-1.1/Thy-1.2 heterozygous mice.

CFSE labeling

After purifying TxA23.Thy-1.1 $CD4^+CD8^-CD25^-$ thymocytes or after separating $CD25^+$ splenocytes from BALB/c.Thy-1.1 mice, cells were washed twice in PBS. Cells were resuspended at 20×10^6/ml in 5 μM CFSE (Molecular Probes) in PBS for 15 min at room temperature. An equal volume of FBS was added, and cells were incubated an additional 5 min at room temperature. After two additional washes in PBS, cells were mixed for injection.

Activation and cytokine measurement of cells from gastric LN (gLN)

Single-cell suspensions were prepared from gLN and washed in PBS containing 5% FCS and penicillin/streptomycin (100 U/ml and 10 μg/ml, respectively). The number of TxA23.Thy-1.1 cells was determined by cell counts and FACS analysis, and 1000 TxA23 T cells were added to a 96-well, U-bottom tissue culture plate with 1×10^5 T-depleted splenocytes that had been irradiated (3000 rad) with or without 50 μg/ml H/K-ATPase α-chain$_{630-641}$ peptide 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). Forty microliters of supernatants were removed at 24 and 48 h and were tested using mouse Th1/Th2 cytokine CBA (BD Biosciences).

Detection of parietal cell Ab (PCAb) and histologic evaluation of gastric pathology

Anti-PCAb were detected by immunofluorescence on cryostat sections of normal BALB/c stomach as previously described (18). Briefly, 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 0–4 depending on the extent of parietal cell staining positive 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 1–6, depending on the extent of mononuclear cell infiltration and parietal and chief cell destruction. Four to six different stomach sections 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 or three areas with intermediate infiltration spanning one-third of musosa; 4.0, big nodules of lymphocytic accumulation spanning half to all of mucosa; 4.5, big nodules of lymphocytic accumulation spanning half to all of mucosa beginning to show evidence of parietal and chief cell destruction ($<25\%$); 5.0, heavy lymphocytic infiltration throughout mucosa, parietal and chief cell loss (25–75%), and replacement by foamy cells; and 6.0, total parietal and chief cell loss, no mucosal architecture, and many foamy cells.

Isolation of stomach cell infiltrates

The method for isolating cells from stomach tissue, with only minor changes, was previously described (19). Briefly, after removing the stomachs, the gLN were removed. The stomach was then opened with an incision along its length from the antrum to the fundus, stomach contents were removed, and the stomach was rinsed in PBS. The stomach tissue was then repeatedly injected with 10 ml of cold PBS/5% FCS/penicillin/streptomycin using a 6-ml syringe with a 27-gauge needle, injecting within the mucosa so as to swell and rupture the tissue. After several injections, the stomachs and single-cell suspension were gently vortexed and passed through a 70-μm pore size nylon filter. Cells were then washed in PBS/5% FCS/penicillin/streptomycin and used for analysis.

RNA isolation and real-time PCR analysis

Cells were washed in PBS, pelleted, and frozen at −80 C. Total RNA was isolated with TRIzol (Invitrogen Life Technologies). Total RNA was quantitated by A260/A280 and stored at −80°C until use. Fifty to 100 ng of total RNA was used to prepare cDNA with SuperScript II (Invitrogen Life Technologies) with 0.25 μg of random primers (Invitrogen Life Technologies) according to the manufacturer's protocol. Hypoxanthine phosphoribosyltransferase (HPRT) primers were purchased from Integrated DNA Technologies. The VIC-HPRT probe was purchased from Applied Biosystems. HPRT primer and probe sequences were previously described (20). T-bet primers and probe were a gift from Dr. J. Zhu (National Institute of Allergy and Infectious Diseases, Bethesda, MD). Quantitative real-time PCR, with 1–3 μl of cDNA was performed with TaqMan Universal PCR Master Mix (Applied Biosystems) in a 25-μl reaction for 40 cycles. All PCRs were performed in either duplicate or triplicate on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using the default cycling conditions.

Results

Cotransfer of normal splenocytes limits lymphopenia-induced proliferation of TxA23 cells

We generated Thy-1.1 congenic TxA23 TCR-Tg mice to follow a population of autoantigen-specific T cells during the induction and/or suppression of AIG. To reduce the expansion and potential differentiation that may take place after transferring cells into a lymphopenic host, naive $CD4^+CD8^-CD25^-$ thymocytes from TxA23.Thy-1.1^+ TCR-Tg mice were coinjected with a large number of splenocytes from BALB/c mice (21–24). Splenocytes were either depleted of or enriched for $CD4^+CD25^+$ T cells (Fig. 1, *C* and *D*) to examine the effects of nTregs on the disease course in this setting. It should be noted that CD25-depleted splenocytes themselves will induce AIG upon transfer to *nu/nu* recipients and that cotransfer of nTreg will prevent disease induction (1). The

A

Group	TxA23xThy1.1 CD4+ Thymocytes	BALB/c Splenocytes	BALB/c CD4+CD25+
TxA23+ Splenocytes (CD25 Depleted)	50,000	20 x 10^6	<80,000
TxA23 + Splenocytes (CD25 Enriched)	50,000	20 x 10^6	~1 x 10^5

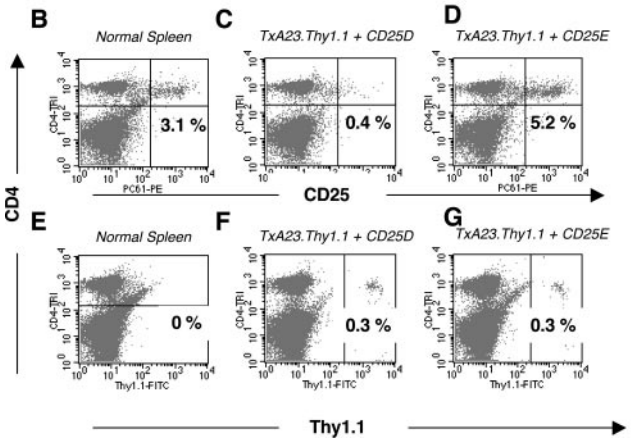

FIGURE 1. Experimental design. $CD4^+CD8^-CD25^-$ thymocytes from TxA23.Thy-1.1 TCR-Tg mice were cotransferred with splenocytes from BALB/c mice that had either been depleted (CD25D) of or enriched for $CD4^+CD25^+$ T cells (CD25E) into *nu/nu* recipients. *A*, Numbers of each cell type injected in each group. *B–D*, Preinjection FACS analysis of CD4 and CD25 from normal BALB/c splenocytes, CD25D splenocytes, and CD25E splenocytes. *E–G*, Preinjection FACS analysis of CD4 and Thy1.1 from normal BALB/c splenocytes, CD25D splenocytes, and CD25E splenocytes, illustrating the small number of congenically labeled TxA23 T cells.

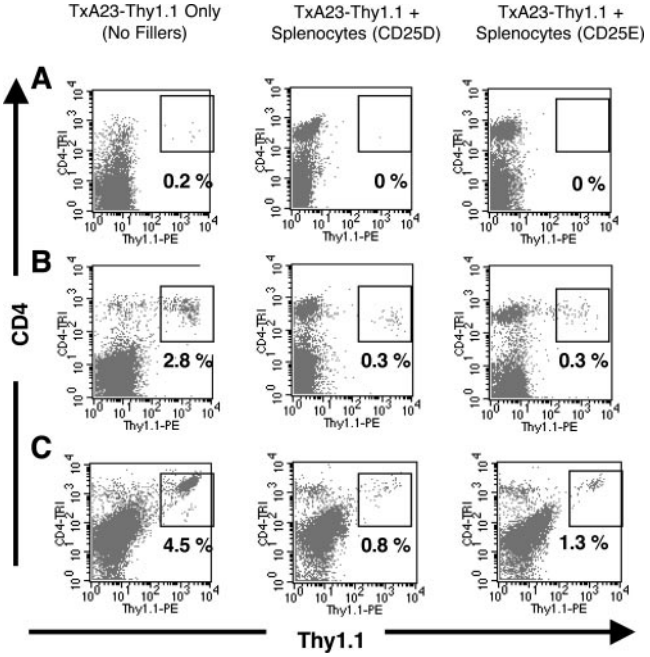

FIGURE 2. Cotransferring either CD25D or CD25E splenocytes limits the amount of proliferation after transferring TxA23.Thy-1.1 into lymphopenic *nu/nu* mice. TxA23.Thy-1.1 T cells (5×10^4) were transferred into *nu/nu* recipients either alone or with 20×10^6 splenocytes depleted of or enriched for $CD4^+CD25^+$ T cells. Shown are the percentages if TxA23 ($CD4^+$/Thy-1.1^+) T cells in the inguinal LN (*A*), gLN (*B*), and stomach infiltrate (*C*) in each group 12 days after transfer.

small number of TxA23.Thy-1.1^+ cells in this model thus served as an indicator population for a disease process also mediated by the $CD4^+CD25^-$ effector cells present in the cotransferred splenocytes (Fig. 1, *F* and *G*).

Cotransferring the splenocytes in this transfer system limited the amount of lymphopenia-induced proliferation, evident by the lack of TxA23 T cells in non-Ag-containing LN, such as inguinal LN, and by the fact that the number of cells in gLN were decreased by 10-fold throughout the response (Fig. 2; data not shown). Importantly, in both groups that were coinjected with splenocytes, TxA23.Thy-1.1^+ cells were identified primarily at the sites of expression of H/K-ATPase, the gLN and stomach (Fig. 2, *B* and *C*). Thus, the presence of nTreg did not prevent the early migration of the effector cells to sites of autoantigen expression. We then examined the extent of proliferation of TxA23.Thy-1.1^+ cells using CFSE to determine cell division. CFSE profiles were indistinguishable both 6 and 12 days after transfer whether nTregs were present or absent (Fig. 3). From these data we concluded that the cotransfer of a small number of Thy-1.1 congenic self-reactive T cells with a large number of splenocytes limited the amount of lymphopenia-induced expansion, and that the localization and proliferation of TxA23.Thy-1.1^+ T cells within gLN were not inhibited by the presence of nTregs.

The nTreg protect mice from AIG induced by TCR-Tg effectors

Both groups of recipients were monitored for the development of AIG by histologic examination of the stomach for inflammatory cell infiltration and parietal cell destruction as well as examination of the serum for the presence of anti-PCAb. Recipients of TxA23 T cells and nTreg-depleted splenocytes developed severe AIG 42–50 days after transfer. The mean pathology score of this group was 5.2 ± 0.6, which is indicative of heavy mononuclear cell infiltrate and extensive destruction of parietal cells. In addition, in the absence of nTregs, 15 of 15 mice exhibited heavy mononuclear cell infiltration into the gastric mucosa, 10 of 15 exhibited extensive destruction of parietal and chief cells, and PCAb were detected in the serum of seven of eight mice (Fig. 4). In contrast, recipients of TxA23 T cells and nTreg-containing splenocytes had a mean pathology score of 3.8 ± 1.4, indicating that most mice in this group had heavy cellular infiltration into the gastric mucosa, but little or no destruction of parietal or chief cells. Only three of 14 mice in this group exhibited parietal cell destruction, and only one of nine mice developed PCAb. Thus, the cotransfer of splenocytes containing nTreg was able to protect recipients from the two hallmarks of AIG, production of PCAb and destruction of parietal/chief cells within the gastric mucosa. It should be noted that although AIG was prevented by coinjection of nTreg, mice in both groups exhibited extensive mononuclear cell infiltration into the gastric mucosa. Thus, the regulation of AIG by nTreg appeared to be at the level of preventing tissue destruction rather than preventing the migration of self-reactive T cells and inflammatory cells into the target organ. To ensure that disease was not simply being delayed, several mice were examined 100 days after injection, and mice remained disease free in the presence of nTreg (data not shown).

The nTreg cells do not prevent expansion of autoreactive T cells

To quantitatively examine the effect of nTreg on the expansion and survival of autoreactive T cells over time, gLN were removed over the course of 52 days, and both the total number of cells in the gLN and the number of TxA23.Thy-1.1^+ cells were determined. Surprisingly, the absolute number of TxA23 T cells in the gLN at each time point did not differ between control animals and recipients coinjected with nTreg. TxA23 T cells from both groups went

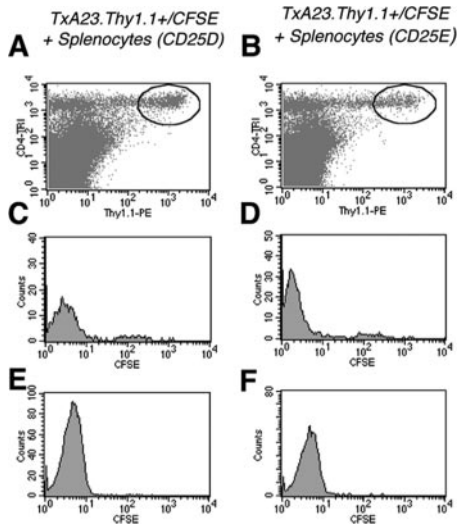

FIGURE 3. Cotransfer of nTreg does not inhibit the proliferation of TxA23 T cells in the gLN. TxA23.Thy-1.1$^+$ T cells (5×10^4) were labeled with CFSE and cotransferred with either 20×10^6 CD25D or CD25E splenocytes into *nu/nu* recipients. *A* and *B*, CD4$^+$ and Thy-1.1$^+$ cells were gated from gLN 6 days (*C* and *D*) and 13 days (*E* and *F*) after transfer, and CFSE profiles of CD4$^+$/Thy-1.1$^+$ gated cells were determined in each group. Similar results were observed in three independent experiments.

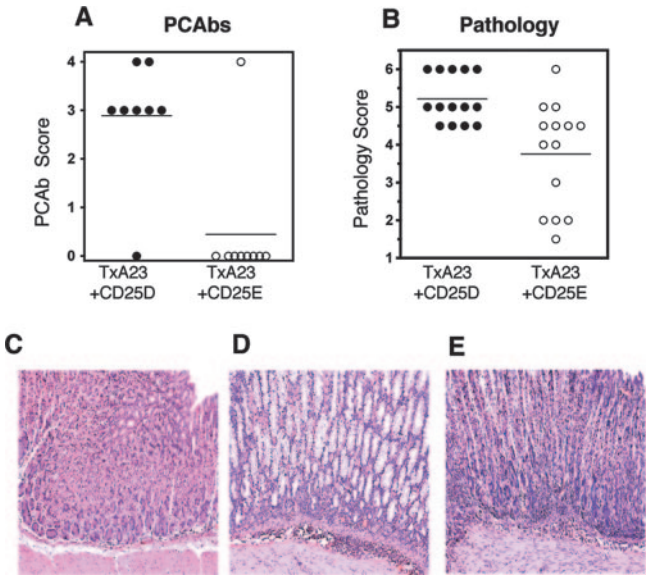

FIGURE 4. CD4$^+$CD25$^+$ T cells protect *nu/nu* recipients from AIG. Experimental groups are described in Fig. 1. *A*, Twenty-eight days after transfer, the recipient mice were bled, and serum was tested for the presence of autoantibodies (PCAbs). *B*, Forty-two to 50 days after transfer, stomach sections were scored for the extent of mononuclear cell infiltration into the gastric mucosa as well as parietal and chief cell destruction. The mean score of recipients that received TxA23 T cells and CD25-depleted splenocytes was 5.2 ± 0.6, indicating heavy infiltration and significant destruction of parietal and chief cells. The group that received TxA23- and CD25-enriched splenocytes had a mean pathological score of 3.7 ± 1.4, indicating heavy infiltration without parietal and chief cell destruction ($p = 0.0027$, by nonparametric Mann-Whitney *U* test; see *Materials and Methods* for description of scores). *C*, H&E-stained section of stomach from a normal *nu/nu* mouse. *D*, H&E-stained section of a recipient with a score of 5.0, illustrating heavy infiltration into the gastric mucosa with marked parietal cell destruction and the presence of replacement foamy cells. *E*, H&E-stained section from a mouse with a score of 4.0, illustrating heavy infiltration into the gastric mucosa without parietal cell destruction.

through a period of expansion and contraction over the 52-day period (Fig. 5*A*). TxA23 T cells in both groups also showed similar phenotypic signs of activation, in terms of increased CD69 expression and decreased CD62L expression (data not shown). Although the expansion and contraction of TxA23 T cells within the gLN was not altered by the presence of nTreg, the total cellularity of the gLN was decreased when nTreg were cotransferred (Fig. 5*B*). Marked differences in the total cellularity of the gLN were observed beginning ~10 days after transfer. A 3-fold reduction of total cells at the peak of the response was seen in recipients of nTreg. The decrease in cellularity of the gLN was not cell type specific, because the infiltrate on day 28 was composed of CD4$^+$, CD19$^+$, CD8$^+$, and CD11b$^+$ cells; cotransfer of nTreg reduced the numbers of all cell types detected (data not shown). Thus, although nTreg did not suppress the Ag-driven expansion of TxA23 T cells in the gLN, they did inhibit the recruitment and/or expansion of other cell types.

The nTreg localize to and proliferate in gLN and stomach

Having established that cotransfer of nTreg prevented the development of AIG, we next determined whether the nTreg were also trafficking to gLN and stomach. In these experiments we transferred TxA23.Thy-1.1$^+$/Thy-1.2$^+$ T cells along with splenocytes from BALB/c mice (Thy-1.2$^+$/Thy-1.2$^+$) that were depleted of or enriched (5×10^5) for nTreg (Thy-1.1$^+$/Thy-1.1$^+$) and had been isolated from BALB/c.Thy-1.1 mice. This allowed us to distinguish nTreg from both TxA23 T cells and from the splenocytes used as filler cells. The nTregs could be detected in both gLN (Fig. 6, *A* and *B*) and stomach infiltrates (Fig. 6, *B* and *D*). The nTreg were also easily detectable in other LN and in the spleen (data not shown). The number of nTreg in gLN and stomach increased over time, and the ratio of nTreg to TxA23 T cells increased between days 7 and 30 (data not shown). Thus, proliferation and/or homing of the nTregs are influenced in part by the presence of self-reactive T cells responding to Ag in the gLN.

The nTreg prevent IFN-γ production and Th1 differentiation

Our failure to detect an inhibitory effect of nTreg on the expansion of TxA23 cells in the gLN or their migration to the stomach raised the possibility that the regulatory cells were inhibiting the differentiation of TxA23 cells to pathogenic effectors. To address this issue, gLN were removed from mice in each group at various times and stimulated in vitro with the H/K-ATPase α-chain$_{630-641}$ peptide, and cytokine production in culture supernatants was measured. After peptide stimulation, the amount of IFN-γ produced in cultures of gLN from animals that received nTreg was markedly reduced (Fig. 7). Remarkably, the reduction in IFN-γ production by cells from the gLN was observed as early as 7 days after transfer (Fig. 7*A*) and was maintained at each time point between 7 and 42 days (Fig. 7*B* and data not shown). It should be noted that early in the course of the response, IFN-γ production was dependent on the addition of the target peptide to the culture, although later in the response, IFN-γ production by the group without nTregs was observed even in the absence of addition of exogenous peptide. The peptide-independent response may be due to TxA23 cells responding to their target peptide, which has been shown to be processed and presented by dendritic cells in the gLN after destruction of gastric parietal cells, or to the response of T cells from spleen that have become primed to H/K-ATPase or other gastric Ags released during the course of disease (25). In either case, IFN-γ production was suppressed in animals that received nTreg. To address whether suppression of IFN-γ was occurring only during the

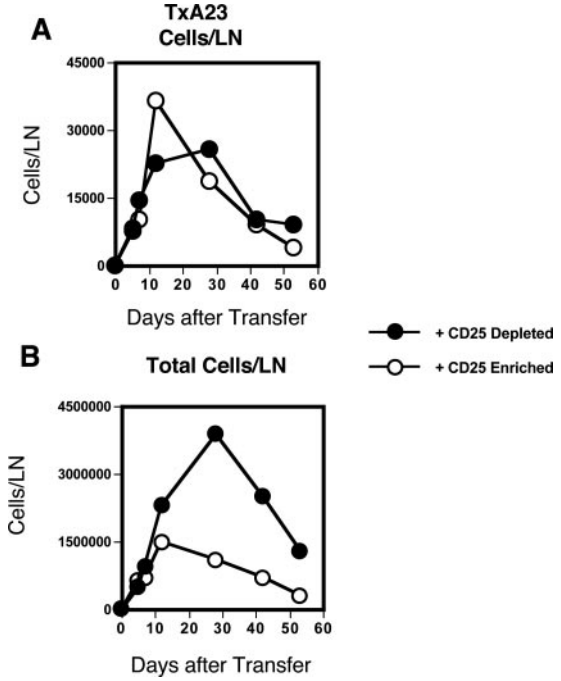

FIGURE 5. $CD4^+CD25^+$ T cells do not inhibit the expansion/survival of TxA23 T cells in the gLN throughout the course of disease, but reduce the total cellularity of gLNs. The total number of TxA23.Thy-1.1 cells (*A*) and the number of total cells (*B*) in the gLN after transferring naive TxA23 are indicated. Thy-1.1 T cells with either CD25D (●) or CD25E (○) splenocytes at various time points after injection are shown.

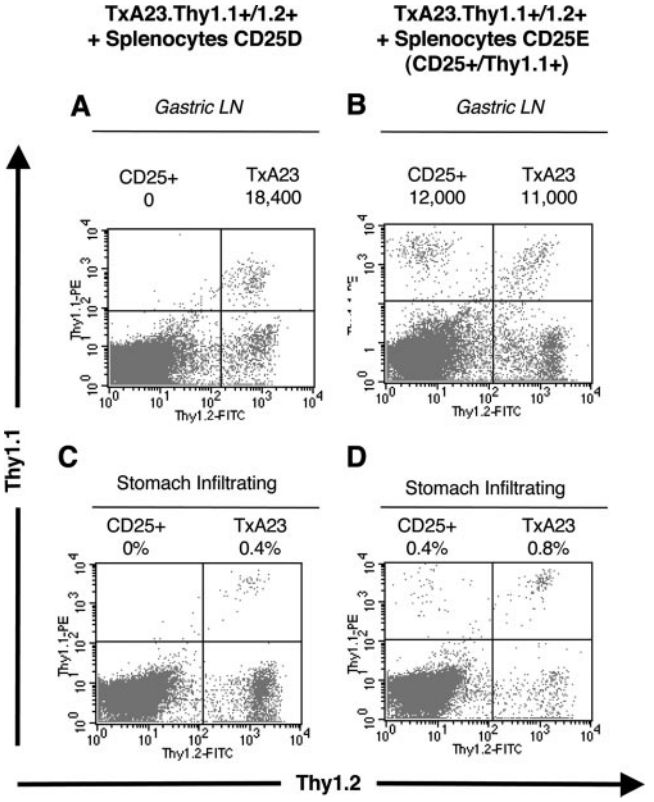

FIGURE 6. The nTregs colocalize with TxA23 T cells in gLN and stomach. TxA23 T cells (Thy-1.1^+/Thy-1.2^+; 5×10^4) were transferred to BALB/c *nu/nu* recipients with splenocytes (Thy-1.2^+/Thy-1.2^+; 20×10^6) that were either depleted of $CD4^+CD25^+$ T cells (*A* and *C*) or contained $CD4^+CD25^+$ T cells isolated from BALB/c.Thy-1.1 congenic mice (*B* and *D*). Cells from the gLN (*A* and *B*) and stomach (*C* and *D*) were isolated and analyzed 30 days after transfer. After determining the average number of total cells in the gLN and the percentage of Thy1.1^+/Thy1.2^- ($CD25^+$) and Thy1.1^+/Thy1.2^+ (TxA23) cells, the average number per gLN was calculated (*A* and *B*). For stomachs, the percentages are given.

restimulation in vitro in the presence of nTregs, in a single experiment TxA23.Thy-1.1 T cells were sorted from the gLN 28 days after transfer. TxA23.Thy-1.1 cells sorted from gLN expressed less IFN-γ mRNA ex vivo and failed to produce IFN-γ upon restimulation in vitro (data not shown), indicating that suppression of IFN-γ production was also detectable directly ex vivo and was acting on TxA23 T cells. Interestingly, transfer of nTreg did not have a consistent effect on the ability of TxA23 T cells to produce IL-2 upon in vitro stimulation. It is also important to note that the nTreg did not simply cause a switch from a Th1 to a Th2 cytokine response, because we did not observe an increase in IL-4 or IL-5 in the suppressed group.

We next examined the expression of IFN-γ and the Th1-specific transcription factor, T-bet, mRNA by real-time PCR in cells infiltrating the stomach 28 and 42 days after transfer. The expression of T-bet has been shown to be an important step in Th1 development and IFN-γ production (26, 27). Cellular infiltrates of the stomach were similar in terms of number of cells and cells types in each group (see Fig. 2*C* and Fig. 6, *C* and *D*). Fig. 8 shows that the expression levels of both IFN-γ and T-bet mRNA were reduced in cells isolated from the group containing nTregs (nondestructive infiltrates) when directly compared with cells isolated from stomachs that did not contain nTregs (destructive infiltrates). Taken together, these studies of mRNA expression indicate that nTreg inhibit the differentiation of autoreactive T cells during the course of disease in vivo, rather than merely inhibiting the production of IFN-γ during the in vitro restimulation assay.

Discussion

The nTreg have now been shown to be potent suppressors of the immune response to auto- and alloantigens, tumor Ags, and pathogen-derived Ags (28–35). Despite their well-documented suppressive effects in vivo, a detailed understanding of which components of the development and activation of pathogenic effector T cells are inhibited during the course of T cell-mediated, organ-specific autoimmunity is as yet unknown. We have developed an experimental model in which a small number of self-reactive T cells responding to a well-defined, naturally expressed and presented autoantigen can be examined for their location, number, and function during the course of an autoimmune response in the presence or the absence of polyclonal nTreg. Although the biochemical basis for nTreg-mediated suppression is not addressed in these studies, our data shed light on the fate of the autoantigen-specific effector cells during the generation of $CD4^+$ T cell-mediated autoimmune disease.

Cotransfer of polyclonal nTreg with a small number of naive autoantigen-specific T cells, under conditions where lymphopenia-induced proliferation was at least partially controlled, was quite effective in inhibiting the development of AIG in *nu/nu* recipients demonstrated by suppression of parietal cell destruction and lack of autoantibody production (PCAbs). Importantly, nTreg failed to inhibit the migration of the autoreactive T cells into the gLN or into the target organ and did not inhibit the Ag-driven expansion of autoreactive T cells in the gLN. The primary effect of the regulatory T cells appeared to be inhibition of the differentiation of the self-specific T cells to Th1 effector cells, as reflected by a decrease in Ag-stimulated IFN-γ production and reduction in T-bet expression. It is also likely that the decrease in cellularity of the gLN in

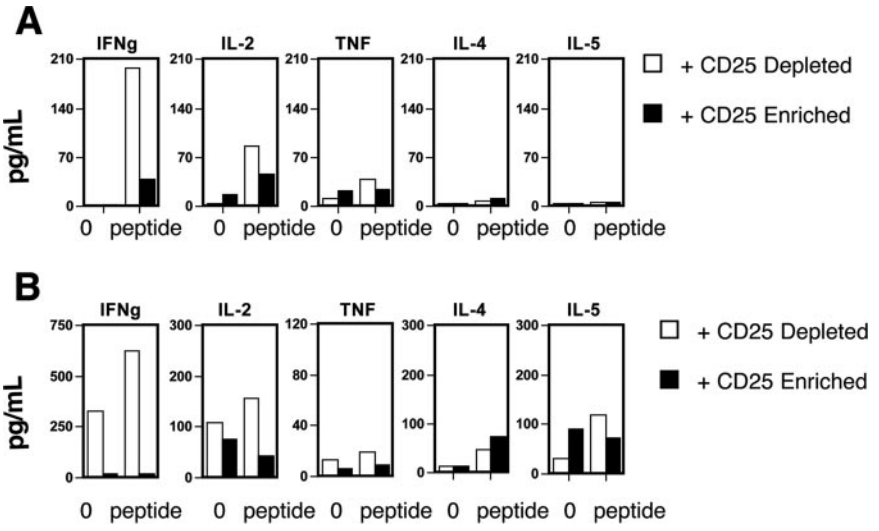

FIGURE 7. The nTregs inhibit IFN-γ production in the gLN. Gastric LN were removed after 7 (*A*) or 14 (*B*) days, and equal numbers of TxA23.Thy-1.1$^+$ T cells from either CD25D (□) or CD25E (■) gLN were cultured in vitro with T-depleted irradiated splenocytes in the presence or the absence of H/K-ATPase α-chain$_{630-641}$ (50 μg/ml). Cytokines were measured in the culture supernatants after 48 h of culture.

recipients of nTreg reflects, in part, decreased cytokine/chemokine production by the effector T cells secondary to the suppressive effects of the nTreg.

These results are both similar and distinct from other studies of nTreg suppression of organ-specific autoimmunity. Sarween et al. (36) reported that polyclonal nTreg inhibited the development of diabetes induced by transfer of OVA-specific CD4$^+$ TCR-Tg T cells into RAG2$^{-/-}$ mice expressing OVA driven by the rat insulin promoter. Similar to our results, they observed a minimal effect on the numbers of Tg T cells in the draining LN and a reduction in the amount of IFN-γ production, but little or no effect of IL-2 production. The ability of polyclonal nTreg to inhibit diabetes induction was associated with an inhibition of T cell infiltration into pancreatic islets, whereas in our studies autoreactive T cells were able to infiltrate the target organ, but not cause destructive pathology. Kohm et al. (37) demonstrated that supplementation of normal mice with nTreg rendered them resistant to myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis that was associated with a decrease in the number of IL-2-producing cells, but no reduction in the number of cells secreting IFN-γ and TNF-α. The primary protective effect of the nTreg was thought to be the prevention of migration of the effectors into the target organ. Our experiments differ from both these studies in that we did not need to immunize, so priming, and presumably suppression, of autoreactive T cells was probably limited to the LN draining the organ targeted for autoimmunity. We were also able to precisely localize and quantitate the numbers of regulatory and effector T cells with congenic markers throughout the course of disease induction or prevention. It should be noted that we did not rely on the expression of CD25 as a marker of regulatory T cells in the recipient mice, but identified the regulatory cells solely by the expression of congenic markers.

An interesting finding in our studies was that the polyclonal nTreg rapidly migrated to the gLN and the target organ in the presence of the H/K ATPase-reactive T cells. Recent studies using TCR transgenic models have described transgenic CD4$^+$CD25$^+$ T cells in pancreatic LN during suppression of diabetes (38, 39). Our studies differ in that the nTreg are not expressing a TCR transgene and, despite having a polyclonal TCR repertoire, still localized to the gLN. In one of these studies the homeostatic balance between the CD4$^+$CD25$^+$ T cells and the effector cells could be disrupted in prediabetic mice by treatment with anti-ICOS, presumably secondary to the loss of expression of ICOS-regulated genes such as IL-10 (38). We have not yet evaluated nTreg from the gLN or stomach for the expression of ICOS, but we have previously shown that nTreg from IL-10-deficient mice are fully competent in their ability to suppress AIG (18, 40). Similarly, it has been reported that polyclonal nTreg proliferate in the mesenteric LN and colon of SCID mice during prevention of the induction of inflammatory bowel disease induced by transfer of CD4$^+$CD45RB^{high} T cells. Interpretation of this finding is complicated, because lymphopenia-induced proliferation may play a role in this model (7).

Another approach used to define the effects of nTreg on responder cells in vivo was described by Klein et al. (9), who cotransferred equal numbers of CD25$^-$ and CD25$^+$ T cells from mice expressing the same Tg TCR into normal mice, followed by immunization of the recipients with the cognate Ag in IFA. For the first 4 days after transfer, both populations expanded in an equivalent manner. However, 6–8 days after transfer, the expansion of the CD25$^+$ T cells continued, but additional accumulation of effector T cells was suppressed. In contrast to our studies and those by Sarween et al. (36), the remaining effector cells appeared to be fully competent in their capacity to produce IL-2 or IFN-γ after restimulation in vitro, indicating that the nTreg did not inhibit commitment to cytokine secretion. A major limitation of these studies is that they cannot be conducted for longer periods of time because the transferred cells migrate out of the draining LN. It is therefore difficult to compare their results with the results of our longer time-course studies on induction of AIG.

The numerous examples in animal models that nTreg can prevent the induction or reverse the course of organ-specific autoimmunity have raised the possibility of their therapeutic potential in the treatment of autoimmune disease in man. Most of the initial studies in animals used polyclonal populations of nTreg from normal donors. Although these studies established that polyclonal populations of nTregs could prevent autoimmune disease, little was known about how they affected disease development. More recently, nTreg from TCR-Tg mice have been used immediately after removal from the donor or after expansion in vitro with stimulatory Abs on beads or with Ag-pulsed dendritic cells in the presence of exogenous IL-2 (39, 41). Very few studies have directly compared the in vivo effectiveness of polyclonal with monoclonal populations. In one study, Hori et al. (32) did show equivalent effectiveness of polyclonal and monoclonal populations of nTreg in preventing experimental autoimmune encephalomyelitis that develops spontaneously in mice expressing a Tg anti-MBP TCR on a RAG$^{-/-}$ background. In contrast, Tang et al. (39) demonstrated that expanded monoclonal nTreg were at least 4-fold more efficient

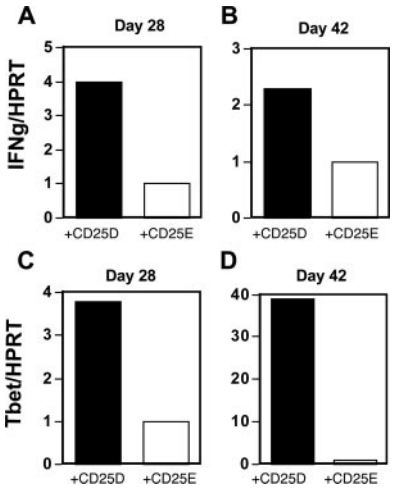

FIGURE 8. Reduced IFN-γ and T-bet message in cells infiltrating the stomach in the presence of nTregs. RNA was prepared from cells infiltrating the stomach in mice receiving 5×10^4 TxA23 with either 20×10^6 CD25D (■) or CD25E (□) splenocytes 28 and 42 days after transfer into *nu/nu* mice. IFN-γ mRNA (*A* and *B*) and T-bet mRNA (*C* and *D*) compared with HPRT mRNA were determined by real-time PCR analysis. In all cases the relative amount of IFN-γ mRNA and T-bet mRNA compared with HPRT mRNA in the TxA23$^+$ CD25E was normalized to 1.0.

than polyclonal nTreg in inhibiting the ability of diabetogenic Tg BDC2.5 T cells to induce diabetes after transfer into NOD.RAG$^{-/-}$ mice. Kanagawa et al. (42) also demonstrated that CD4$^+$CD25$^+$ T cells from BDC2.5 mice expressing high levels of clonotypic TCR were more efficient at preventing diabetes transfer than clonotype low or negative CD4$^+$CD25$^+$ T cells. One assumption behind the use of nTreg expressing a receptor for an organ-specific peptide is that they would preferentially home to the target organ, be activated by their target autoantigen, and be capable of rapidly mediating local bystander suppression in the absence of systemic immunosuppression. Thus, they would be potentially more effective in preventing disease after transfer of activated effectors or in reversing disease in its earliest stages. A comparison of the use of polyclonal vs monoclonal and freshly isolated vs in vitro-expanded nTreg in preventing or treating various autoimmune diseases deserves further study.

A second assumption in these studies is that nTreg must be activated through their TCR to exert their suppressive effects. This assumption is based on in vitro studies that demonstrated that nTreg-mediated suppression required activation of the nTreg via their TCR (43). The source of the TCR signal during in vivo activation of polyclonal populations of nTreg is not known. It has been hypothesized that the selection of nTregs occurs during thymic development due to the higher avidity of their TCRs for self-Ags; however, whether this pathway of positive selection results in a peripheral TCR repertoire that has a higher overall avidity for ubiquitous self-peptides or for organ-specific Ags is unknown (44). In the AIG model, suppression of the Ag-specific induction of IFN-γ production could be observed as early as 7 days after transfer of the polyclonal nTreg. It seems highly unlikely that this suppression is secondary to the activation and expansion of the limited number of gastric Ag-specific nTreg cells present within the polyclonal CD4$^+$CD25$^+$ population. An alternative possibility is that nTreg are able to control various responses because they are continuously recognizing and being partially activated by ubiquitous self-peptides and simply need a second signal, such as IL-2, provided by the responding effector T cells to activate their suppressive activity.

Cellular therapy with nTreg represents a novel therapeutic approach to the treatment of autoimmune disease in man. However, a number of major unanswered questions remain concerning their mechanism of action. Although the great majority of published studies have demonstrated their ability to prevent the development of autoimmunity, their effectiveness in treating and reversing ongoing autoimmune disease remains a major topic for future studies. The use of well-characterized animal models of organ-specific autoimmune disease that closely resemble human disease, such as AIG, should greatly facilitate resolution of some of the major issues.

Acknowledgments

We thank Dr. Bill Paul, John Andersson, and the members of Dr. Paul's and Dr. Shevach's laboratories for helpful discussions. We thank Tracy Lader, Stacey Miller, Cristina Corsino, Equar Desta, and Kwame Amoo for their hard work in helping us maintain the mice used in these experiments. We also thank Jinfang Zhu for T-bet primers, probe, and helpful suggestions, and Dean Follmann for help with statistical analysis of the data.

Disclosures

The authors have no financial conflict of interest.

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