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CD4⁺CD25⁺ Regulatory T Cells Inhibit the Antigen-Dependent Expansion of Self- Reactive T Cells In Vivo¹

Tricia D. Zwar, Simon Read, Ian R. van Driel, and Paul A. Gleeson²

A deficiency of CD4⁺CD25⁺ regulatory T cells (CD25⁺ Tregs) in lymphopenic mice can result in the onset of autoimmune gastritis. The gastric H/K ATPase α (H/Kα) and β (H/Kβ) subunits are the immunodominant autoantigens recognized by effector CD4⁺ T cells in autoimmune gastritis. The mechanism by which CD25⁺ Tregs suppress autoimmune gastritis in lymphopenic mice is poorly understood. To investigate the antigenic requirements for the genesis and survival of gastritis-protecting CD25⁺ Tregs, we analyzed mice deficient in H/Kβ and H/Kα, as well as a transgenic mouse line (H/Kβ-tsA58 Tg line 224) that lacks differentiated gastric epithelial cells. By adoptive transfer of purified T cell populations to athymic mice, we show that the CD25⁺ Treg population from mice deficient in either one or both of H/Kα and H/Kβ, or from the H/Kβ-tsA58 Tg line 224 mice, is equally effective in suppressing the ability of polyclonal populations of effector CD4⁺ T cells to induce autoimmune gastritis. Furthermore, CD25⁺ Tregs, from either wild-type or H/Kα-deficient mice, dramatically reduced the expansion of pathogenic H/Kα-specific TCR transgenic T cells and the induction of autoimmune gastritis in athymic recipient mice. Proliferation of H/Kα-specific T cells in lymphopenic hosts occurs predominantly in the paragastric lymph node and was dependent on the presence of the cognate H/Kα Ag. Collectively, these studies demonstrate that the gastritis-protecting CD25⁺ Tregs do not depend on the major gastric Ags for their thymic development or their survival in the periphery, and that CD25⁺ Tregs inhibit the Ag-specific expansion of pathogenic T cells in vivo. The Journal of Immunology, 2006, 176: 1609–1617.

The CD4⁺CD25⁺ immunoregulatory T cells (CD25⁺ Tregs)³ have recently emerged as a key factor in the maintenance of a normal peripheral immune system. In particular, CD25⁺ Tregs play an indispensable role in maintaining self-tolerance and protecting from autoimmune diseases (1–3) as well as influencing immune responses to pathogens (4). The majority of CD4⁺CD25⁺ T cells in the periphery appear to act as immunoregulatory T cells (5, 6), and there is now considerable evidence that this CD25⁺ Treg population represents a stable population of peripheral lymphocytes (7). A defining feature of CD25⁺ Tregs is the expression of the transcription factor Foxp3, which is necessary for their genesis (8–10). Foxp3-null mice have a deficiency in the CD25⁺ Treg population and, as a consequence, develop a lethal autoimmune syndrome (10). A wide variety of animal models have clearly demonstrated the immunoregulatory activity of CD25⁺ Tregs in vivo (11); nonetheless, the pathways by which CD25⁺ Tregs suppress immune responses to self Ags remain poorly understood.

The CD25⁺ Treg population has a diverse repertoire of TCRs, and this repertoire appears to be distinct from conventional T cells (12, 13). There is increasing evidence that CD25⁺ Tregs preferentially recognize self Ags bound to MHC class II molecules. The interaction of CD25⁺ Tregs with self Ags is considered important not only for their thymic development, but also for their maintenance in the periphery (14–16). CD25⁺ Treg lineage commitment in the thymus has been proposed to be driven by TCR interactions with MHC/self peptides within a particular range of avidity (14, 17). A non-TCR signal may also contribute to CD25⁺ Treg cell lineage commitment (18); however, the importance of TCR-MHC interactions is highlighted by the recent finding that Foxp3⁺ thymocytes do not develop in the absence of MHC molecules (6). Although peripheral CD25⁺ Tregs were initially considered anergic, it is now clear that CD25⁺ Tregs proliferate in vivo, not only in lymphopenic conditions, but also after exposure to self proteins in peripheral tissues (15, 19, 20). The homing of CD25⁺ Tregs to specific sites and their proliferation following recognition of agonist TCR ligands are probably critical for their capacity to prevent autoimmune diseases, as illustrated by the finding that CD25⁺ Tregs from the pancreatic lymph nodes, but not other lymph nodes, were effective in preventing diabetes in an inflammation-induced diabetes mouse model (21).

Although in vitro studies suggest that CD25⁺ Tregs can inhibit T cell activation of conventional T cells, the mechanism by which they function in vivo remains poorly understood (22). Many models of autoimmunity involve transient states of lymphopenia as well as a reduction in the number of CD25⁺ Tregs, for example, neonatal thymectomy or the transfer of T cell subsets to T cell-deficient mice. As CD25⁺ Tregs appear to be important in maintaining homeostasis of naïve T cells (23), it is unclear in these lymphopenic models whether the absence of CD25⁺ Tregs may influence lymphopenic expansion of self-reactive T cells or subsequent events associated with Ag-specific activation within the
environment of the target tissue. The fact that depletion of CD25+ Tregs in adult mice does not result in spontaneous development of autoimmune disease (24, 25) indicates that the lymphopenic environment per se is a contributing factor in the development of autoimmune disease.

One of the most thoroughly investigated models of organ-specific autoimmunity is murine autoimmune gastritis that occurs after neonatal thymectomy or transfer of CD25+ T cells into a lymphopenic recipient (26). These models have been used extensively to investigate the pathogenesis and specificity of the immune response as well as the genetics of gastritis susceptibility (27). Work over the past decade has firmly established that a CD4+ T cell response to the gastric H/K ATPase is responsible for gastritis (28). The gastric H/K ATPase is abundantly expressed in parietal cells, and consists of two subunits, α (H/Kα) and β (H/Kβ) (29). Pathogenic self-reactive CD4+ T cells to both H/K ATPase subunits have been identified (30–32). Mice that transgenically expressed the H/Kβ in the thymus failed to develop gastritis after neonatal thymectomy (30), immunization with the mouse gastric H/K ATPase (33), or after adult thymectomy combined with cyclophosphamide treatment (34). Our recent analysis has revealed that the thymic expression of H/Kβ can result in tolerance to not only the H/Kβ, but also the H/Kα subunit of the H/K ATPase (35). We demonstrated that thymic expression of solely the H/Kα, as is the normal situation, was not able to tolerate H/Kα-specific T cells, presumably due to the inability of this subunit to be transported from the endoplasmic reticulum. However, thymic overexpression of H/Kβ, which allows the H/Kα to be transported out of the endoplasmic reticulum as the heterodimer, results in deletion of H/Kα-specific T cells (35). This recent observation provides insight into why the overexpression of the H/Kβ in the thymus can prevent autoimmune gastritis, as both anti-H/Kα and anti-H/Kβ T cells contribute to disease development (28).

The gastric H/K ATPase is highly abundant in the gastric mucosa, and dendritic cells (DCs) from the lymph nodes draining the stomach have been shown to acquire parietal cell-derived H/Kα epitopes in healthy untreated mice and activate H/Kα-specific T cells in vitro (36). Therefore, the H/K ATPase represents one of the few tissue-specific Ags that has been demonstrated to be presented in the local lymph node of the target organ.

The Ag specificity of the CD25+ Tregs that protect from autoimmune gastritis or any other autoimmune disease is not known. Whereas both H/K ATPase subunits are highly abundant in the gastric mucosa (29), H/Kβ appears to be absent from the thymus, or at best marginally expressed (37), whereas H/Kα is expressed in medullary thymic epithelial cells and thymic DCs (37, 38). Thus, there is a marked difference in the thymic expression of the two dominant gastric autoantigens that could influence CD25+ Treg development.

To investigate the antigenic requirements for the genesis and survival of gastritis-protecting CD25+ Tregs, we have analyzed mice deficient in H/Kβ (H/Kβ−/−) (39) and H/Kα (H/Kα−/−) (40), as well as a transgenic mouse line (H/Kβ-tsA58 Tg line 224) that has defects in gastric mucosal development and lacks the major differentiated cell types, including the acid-secreting parietal cells (41). Our studies demonstrate that the gastritis-protecting CD25+ Tregs do not depend on the major gastric Ags for their thymic development or their survival in the periphery. Furthermore, using an H/Kα-specific TCR transgenic T cell (A23) (42), we have shown that CD25+ Tregs are able to inhibit the Ag-specific expansion of pathogenic T cells in athymic mice. Proliferation of H/Kα-specific TCR transgenic T cells in lymphopenic hosts occurs predominantly in the para gastric lymph node and was dependent on the presence of the cognate H/Kα Ag. Significantly, this study has made the important observation that CD25+ Tregs can inhibit the Ag-dependent expansion of self-reactive pathogenic T cells in vivo.

Materials and Methods

Mice

BALB/c mice (6–12 wk old) were obtained from the Walter and Eliza Hall Institute and the University of Melbourne Department of Microbiology and Immunology animal facility. BALB/c nude (nu/nu) mice were obtained from University of New South Wales and from Animal Resources Centre. H/Kα-deficient (H/Kα−/−) (40) and H/Kβ-deficient (H/Kβ−/−) mice (39) were backcrossed at least 10 times onto the BALB/cCrSlc background. H/Kα−/− mice were intercrossed with H/Kβ−/− mice to generate H/Kα- and H/Kβ-deficient mice (H/Kβ−/−). A23 transgenic mice have been described (42) and were backcrossed onto Thy-1.1 congenic BALB/c background to generate Thy-1.1 A23 mice. H/Kβ-tsA58 transgenic line 224 has been described (41) and was backcrossed at least 10 times onto the BALB/cCrSlc background. DO11.10 TCR transgenic mice have been described (43). Mice were genotyped by PCR and flow cytometry.

Mice were housed under conventional facilities at the Monash University Medical School animal facility and the University of Melbourne Department of Biochemistry and Molecular Biology animal facility. Experiments were approved by the Monash University Ethics Committee and the University of Melbourne Ethics Committee.

Flow cytometry

Conjugated mAbs were purchased from BD Pharmingen, except for the anti-FOXP3 Staining Set, which was obtained from eBioscience. Flow cytometric analyses were performed on a FACSAir or a FACSort (BD Biosciences) using CellQuest software. Cells were analyzed for expression of cell surface markers, using a combination of the following Abs: anti-CD4 FITC (GR1.5), anti-CD4 allophycocyanin (RM4-5), anti-CD25 FITC (7D4), anti-CD25 PE (PC61), and anti-Thy-1.1 PerCP (OX-7). Vα2 expression was determined by labeling with anti-Vα2 biotin (B20.1), followed by streptavidin PE.

Preparation of donor cell populations

Single-cell suspensions were prepared from spleen, lymph nodes, and thymus by gently mashing tissues through 70-μm nylon cell strainers into PBS/2% FCS (PBS/FCS). Spleen cells were resuspended in 0.17 M NH4Cl and incubated for 5 min at room temperature to lyse RBC, then washed in PBS/FCS.

For isolation of CD25+ and CD25− cell populations, cells suspensions were incubated with anti-CD25 FITC or anti-CD25 PE mAb on ice for 15 min, then with anti-FITC Microbeads (5 μl/107 cells) or anti-PE Microbeads (10 μl/107 cells) (Miltenyi Biotec), respectively, at 4°C for 15 min. The labeled CD25+ cells and nonlabeled CD25− cells were then fractionated using an autoMACS separator (Miltenyi Biotec).

Preparation of A23 CD4+ T cells for adoptive transfer experiments was achieved by depletion of non-CD4+ cells and F4/80+ cells from lymph node cell suspensions of A23 TCR transgenic mice. Briefly, cell suspensions from pooled lymph nodes of A23 mice were resuspended in mixed anti-B220 (RA3-6B2), anti-CD8 (53-6.7), and F4/80 hybridoma supernatant, and incubated on a rotating wheel at 4°C for 15 min. Cells were then washed and resuspended in PBS/FCS containing M-450 sheep anti-rat IgG Dynabeads (Dynal Biotech) (1–2 beads per target cell), and rotated at 4°C for an additional 20 min. The suspension was placed in a Dynal Magnetic Particle Concentrator (Dynal Biotech), and after 2 min the supernatant containing nonlabeled cells was recovered. The purity of the resulting cell population was assessed by flow cytometry.

Adaptive transfer of cell populations

Cell populations were administered to athymic BALB/c (nu/nu) and irradiated (900 rad) BALB/cCrSlc and H/Kα-deficient mice by either i.p. or i.v. injection, as indicated. For i.p. injections, cells were administered in a volume of 500 μl of FCS/PBS, and for i.v. in a volume of 100 μl of FCS/PBS. A23 CD4+ T cells were identified in recipient mice based on CD4 and Thy-1.1 coexpression.

In vivo T cell proliferation assay

To assess in vivo proliferation, cell suspensions (2 × 106 cells/ml in complete DMEM) were labeled with 5 μM CFSE, as described (44). The reaction was quenched by addition of a large volume of ice-cold FCS/PBS, and the cells were pelleted, washed twice with PBS/FCS, and injected i.v.
into mice. CFSE labeling of gated CD4 "Thy-1.1" lymphocytes was analyzed to determine cell division.

Autoimmune gastritis assessment

Six to 8 wk after transfer of CD4 + T cells, sera were collected and the presence of autoantibodies was examined by ELISA, as described (30). Histological examination of the stomachs was also performed to assess the presence of mononuclear cell infiltrates. Autoimmune gastritis was graded on a scale of 0–3, according to the following criteria: 0, normal gastric mucosa; 1, mononuclear cell infiltrate, mainly restricted to submucosa, and no hypertrophy or disruption of gastric units; 2, mononuclear cell infiltrate extending into the mucosa and gastric unit hypertrophy and/or disruption of the gastric units; 3, mucosal mononuclear cell infiltrate or no infiltrate, gastric unit hypertrophy, and severe disruption of normal gastric units with depletion of parietal cells and zymogenic cells. Histological analyses were performed blind by two individuals, with agreement of scoring in the majority of cases.

Statistical analysis

Cell population data were analyzed by an unpaired Student’s t test, two tailed. Gastritis severity data were analyzed using a Mann-Whitney U test, one tailed.

Results

The Ag specificities of CD25 + Tregs that protect from the induction of autoimmune diseases have yet to be identified. Based on our current understanding of the thymic development of the CD25 + Tregs and their survival in the periphery (14–16), gastric Ags are likely to play a critical role in establishing a peripheral repertoire of autoimmune gastritis-protecting CD25 + Tregs. Splenic CD25 + Tregs from wild-type BALB/c mice can prevent the development of autoimmune gastritis in cell transfer studies to athymic BALB/c mice (11). To examine the autoantigen requirements of the gastritis-protecting CD25 + Tregs, we have used a number of genetically modified mouse lines, including gastric H/Kα and H/Kβ autoantigen-deficient mice as well as transgenic mice with a block in development of mature gastric epithelial cells (H/Kβ-tsA58 Tg line 224). The H/Kβ-tsA58 Tg line 224 lacks the major differentiated cell types of the gastric mucosa, including parietal and zymogenic cells, and, as a consequence, has very low levels of H/K ATPase in the stomach (41). Initially, we examined the peripheral CD25 + Treg population in these mouse lines. Analysis of lymphocytes showed that CD25 + Tregs represented ~10% of the total CD4 + T cells in the paragastric lymph node, inguinal lymph node, and spleen of wild-type BALB/c mice and H/K ATPase-deficient mice (Fig. 1). In addition, analysis of single-positive CD4 + thymocytes showed no difference in the percentage of CD4 + CD25 + cells in the three genotypes. The majority of the H/Kβ +/−CD4 + CD25 + cells were positive for Foxp3 (Fig. 1B), confirming their identity as immunoregulatory T cells. Therefore, the absence of gastric Ags does not influence the size of the thymic or peripheral CD25 + Treg population.

Gastric autoantigen-deficient mice contain CD25 + Treg populations that can protect against autoimmune gastritis

To determine whether the absence of gastric Ags in the thymus and periphery resulted in altered regulatory T cell activity, we have performed adoptive transfer studies using athymic recipients. As H/Kα and H/Kβ are the immunodominant Ags recognized by effector T cells, we determined whether the H/K ATPase was also critical for the generation and maintenance of gastritis-protecting CD25 + Tregs. Splenic CD25 + Tregs were purified from wild-type BALB/c, H/Kβ-deficient, or H/Kα-deficient mice by magnetic cell sorting. The populations of CD25 + Tregs were typically ~80% pure (Fig. 2A). Splenic CD25 + cells (2 × 10^6) from wild-type BALB/c mice, used as a source of pathogenic cells, were transferred to athymic BALB/c mice in the presence and absence of 2 × 10^6 CD25 + Tregs. Eight weeks after transfer, recipient mice were examined for the development of autoimmune gastritis, and the severity of gastritis was scored from 0 to 3, according to the criteria defined in Materials and Methods. As expected, all athymic BALB/c mice (6 of 6) that had received CD25 + splenocytes from wild-type mice showed an extensive mononuclear infiltrate and considerable disruption of the gastric mucosa (grades 2 and 3). In addition, H/K-ATPase-specific autoantibodies were detected in the serum of all six mice (data not shown). The cotransfer of wild-type CD25 + cells and wild-type CD25 + Tregs resulted in protection from the development of autoimmune gastritis, as none of the eight mice of this group had a mononuclear infiltrate in the gastric mucosa (Fig. 2B) or circulating H/K-ATPase-specific autoantibodies (data not shown). The CD25 + Tregs from H/Kβ-deficient mice were also effective in protection against gastritis, with none of the eight recipients developing disease. Similarly, CD25 + Tregs from H/Kβ-deficient mice also showed considerable protection against the induction of autoimmune disease by CD25 + T cells, as four of six mice did not develop autoimmune gastritis, one of six developed only mild gastritis, and only one of the six recipients developed severe autoimmune gastritis. Therefore, despite the lack of the immunodominant autoantigens, the H/K ATPase-deficient mice have CD25 + Tregs that can inhibit the induction of autoimmune gastritis in lymphopenic recipients.

It is possible that the self Ag(s) recognized by gastritis-protecting CD25 + Tregs is a gastric Ag other than the H/K ATPase. As the H/Kβ-tsA58 Tg line 224 lacks mature gastric cells, these mice would be expected to have reduced peripheral levels of a wide range of gastric-specific molecules. Given that CD25 + Tregs can proliferate in vivo in an Ag-dependent manner (15, 19, 20), the
absence of a number of gastric Ags could influence the repertoire of CD25⁺ Tregs in the adult individual. Therefore, we also examined whether the H/Kβ-tsA58 Tg line 224 has altered regulatory T cell activity. Splenic CD25⁺ Tregs (2 × 10⁶) from the BALB/c H/Kβ-tsA58 Tg line 224 mice were transferred together with CD25⁻ splenic cells (2 × 10⁶) from normal mice to athymic BALB/c and analyzed 8 wk later for autoimmune gastritis. Whereas all athymic BALB/c mice that received CD25⁻ Tregs alone showed an extensive mononuclear infiltrate with considerable disruption of the gastric mucosa (4 of 5, grade 2; 1 of 5, grade 1), the cotransfer of CD25⁺ Tregs from H/Kβ-tsA58 Tg line 224 mice resulted in protection from the development of autoimmune gastritis in five of five mice (Fig. 2C). Therefore, H/Kβ-tsA58 Tg line 224 clearly contains gastritis-protecting CD25⁺ Tregs, even though they lack mature gastric epithelial cells.

FIGURE 2. CD25⁺ Tregs from gastric Ag-deficient mice prevent autoimmune gastritis in athymic mice. A, Analysis of CD4⁺CD25⁺ T cell populations from spleens of wild-type (BALB/c) and H/K ATPase-deficient mice purified using an autoMACS separator. B, Development of autoimmune gastritis in athymic BALB/c mice (6–8 wk old) transferred with 2 × 10⁶ CD25⁺ splenocytes from wild-type BALB/c mice alone or cotransferred with 2 × 10⁶ CD4⁺CD25⁺ T cells from wild-type, H/Kβ-deficient, and H/Kαβ-deficient mice. C, CD4⁺CD25⁺ T cells were isolated from H/Kβ-tsA58 transgenic line 224 (Tg224). Athymic BALB/c recipient mice received injections i.p. of CD25⁻ splenocytes and CD25⁺ Treg cells. Eight weeks later, mice were killed, and their stomachs were examined for histological evidence of autoimmune gastritis. For statistical analysis, all groups were compared with the group of CD25⁻ cells alone.

The experiments described above were performed with a single dose of CD25⁺ Tregs routinely used in cell transfer studies to examine CD25⁺ Treg activity (45). It is likely that the gastritis-protecting CD25⁺ Tregs are polyclonal and recognize a number of different gastric Ags, which include the H/K ATPase. Thus, it is possible that the frequency of gastritis-protecting CD25⁺ Tregs is reduced in the H/K ATPase subunit-deficient mice. To explore this possibility further, a titration experiment was performed in which we examined the ability of reduced numbers of CD25⁺ Tregs to protect from the development of gastritis. CD25⁺ Tregs were purified from wild-type (80% purity) and H/Kαβ-deficient mice (90% purity) and cotransferred with wild-type CD25⁺ splenic cells (2 × 10⁷). As expected, 2 × 10⁶ wild-type CD25⁺ Tregs efficiently protected from the development of autoimmune gastritis. However, a reduction in the number of CD25⁺ Tregs over the range of 5 × 10⁵ to 1 × 10⁵ resulted in a dose-dependent increase in the percentage of mice that developed autoimmune gastritis. Titration of CD25⁺ Tregs from H/Kαβ-deficient mice, over the range of 2 × 10⁵ to 1 × 10⁶ cells, showed similar results to wild-type CD25⁺ Tregs (Fig. 3); therefore, CD25⁺ Tregs from H/Kαβ double-deficient mice appeared to be as effective as the CD25⁺ Treg repertoire from wild-type mice. This would suggest that the repertoire of gastritis-protecting CD25⁺ Tregs in the H/K ATPase-deficient mice is comparable to wild-type mice.

CD25⁺ Tregs from H/Kα-deficient mice suppress the induction of autoimmune gastritis by H/Kα-specific CD4 effector T cells

For the above studies, we had used polyclonal CD4⁺CD25⁺ T cells as the source of pathogenic effector T cells. To explore the requirements for the specificity of the effector and regulatory populations in more detail, we have used highly gastritogenic H/Kα-specific CD4⁺ T cells from TCR transgenic mice (A23) as a source of effector T cells. Thymocytes from A23 transgenic mice were used as a source of single-positive CD4⁺ T cells to ensure that the transferred population of T cells was naive. When 10⁵ thymocytes from A23 transgenic mice, equivalent to ~100 mature CD4⁺ T cells, were transferred to athymic BALB/c mice, seven of eight (88%) recipient mice developed autoimmune gastritis (Fig. 4A), a result consistent with previous reports (42). The cotransfer of 10⁵ A23 thymocytes and 10⁶ CD25⁺ Tregs from wild-type mice resulted in only one of five recipient athymic mice developing autoimmune gastritis, demonstrating that CD25⁺ Tregs from normal

FIGURE 3. Titration of CD25⁺ Tregs from H/K ATPase-deficient mice. CD25⁺ and CD4⁺CD25⁺ (Treg) cells were isolated from spleens of wild-type (BALB/c) and H/Kαβ-deficient mice. Athymic BALB/c recipient mice received injections i.p. of 2 × 10⁷ CD25⁺ cells from wild-type BALB/c mice together with 1 × 10⁵-2 × 10⁵ CD4⁺CD25⁺ cells, as indicated. Eight weeks later, mice were killed, and their stomachs were examined for histological evidence of autoimmune gastritis.
mice can inhibit the ability of the gastritogenic H/Kα-specific A23 T cells to cause disease. The cotransfer of $10^3$ A23 thymocytes and $10^6$ CD25$^+$ Tregs from H/Kα-deficient mice also prevented the induction of autoimmune gastritis, as four of five recipient mice showed no disease and only one of the five mice showed a low level gastritis. Thus, the transfer of A23 T cells alone resulted in an extensive mononuclear infiltrate, gastric hypertrophy, and disruption of the gastric units (Fig. 5). In contrast, a normal gastric mucosa was maintained in the presence of CD25$^+$ Tregs from either wild-type or H/Kα-deficient mice, with no evidence of a mononuclear infiltrate (Fig. 5). Therefore, CD25$^+$ Tregs inhibited the ability of A23 T cells to establish a detectable infiltrate within the gastric mucosa.

The inhibition of autoimmune gastritis in these transfer experiments was dependent on the presence of CD25$^+$ Tregs, rather than the presence of large number of T cells per se, as the cotransfer of an equivalent number (10$^6$) of OVA-specific CD4$^+$ CD25$^-$ T cells (DO11) with $10^3$ A23 thymocytes resulted in all mice developing autoimmune gastritis (4 of 4) (Figs. 4A and 5). As CD4$^+$ CD25$^-$ DO11 T cells did not cause autoimmune gastritis when transferred alone to athymic mice, the gastritis in the above experiments is clearly mediated by the gastritogenic H/Kα-specific A23 T cells. These results demonstrate that CD25$^+$ Tregs from H/Kα-deficient mice inhibited the ability of pathogenic H/Kα-specific T cells to induce autoimmune disease in lymphopenic recipients.

CD25$^+$ Tregs suppress the expansion of H/Kα-specific CD4$^+$ T cells in lymphopenic mice

The development of autoimmune gastritis following transfer of a small number of naïve A23 T cells to a lymphopenic recipient is a consequence of the expansion of the A23 T cell population and a gain of effector function of activated A23 T cells. CD25$^+$ Tregs may influence either or both of these events. To gain insight into how CD25$^+$ Tregs are able to suppress the development of autoimmune gastritis in this model, we determined the impact of CD25$^+$ Tregs on the expansion of Thy-1.1 A23 T cells in the experiment above. A23 T cells were identified in athymic recipients by expression of CD4$^+$ and the congenic marker Thy-1.1. The total number of Thy-1.1$^+$ CD4$^+$ T cells in the spleens of athymic mice that had received donor A23 T cells only ranged from $10^5$ to $6 \times 10^5$ (mean $2.6 \times 10^5 \pm 1.6 \times 10^3; n = 6$) (Fig. 4B). Nondonor (Thy-1.1$^-$) CD4$^+$ T cells were also detected in these mice (data not shown), consistent with the presence of extrathymically derived CD4$^+$ T cells in adult (>13-wk-old) athymic mice (46). Given that the starting population contained ~100 mature A23 thymocytes, considerable expansion of donor H/Kα-specific Thy-1.1 CD4$^+$ T cells had occurred over the 8-wk period. The mouse with the lowest number of splenic Thy-1.1$^+$ CD4$^+$ T cells did not develop autoimmune gastritis (Fig. 4B). In contrast, the transfer of A23 thymocytes with $10^6$ CD25$^+$ Tregs from either wild-type mice or H/Kα-deficient mice resulted in considerably fewer A23 T cells recovered from the spleens of recipient mice 8 wk after transfer (A23 T cells with CD25$^+$ Tregs from wild-type mice, $1.7 \times 10^7 \pm 0.9 \times 10^7; n = 3$; A23 T cells with CD25$^+$ Tregs from H/Kα-deficient mice, $3.4 \times 10^4 \pm 1.8 \times 10^4; n = 3$) (Fig. 4B).

The number of splenic A23 T cells represents a 15- and 8-fold reduction, respectively, in expansion of the A23 population compared with the expansion of A23 T cells in the absence of CD25$^+$ Tregs. The cotransfer of $10^3$ A23 thymocytes and $10^6$ CD4$^+$ CD25$^-$ DO11 T cells resulted in considerable expansion of the A23 T cell population ($2.3 \times 10^5 \pm 2.2 \times 10^5; n = 3$) (Fig. 4B) as well as some expansion of the DO11 CD4$^+$ T cell population ($7.1 \times 10^5 \pm 0.8 \times 10^5; n = 3$). Therefore, the reduced
expansion of the A23 CD4<sup>+</sup> T cell population in the presence of CD25<sup>+</sup> Tregs is due to the specific activity of the regulatory T cell population and not simply due to the cotransfer of a large number of additional lymphocytes.

In the paragastric lymph nodes of athymic mice that received A23 thymocytes alone, the Thy-1.1<sup>+</sup> population represented up to 65% of total CD4<sup>+</sup> T cells. There was considerable variation within the group (40 ± 20%; n = 8); the Thy-1.1<sup>+</sup> population of five of eight mice was >40% of the total CD4<sup>+</sup> T cells. In the two mice with the lowest percentage of Thy-1.1<sup>+</sup> T cells (25 and 5%) in the paragastric lymph nodes, the gastritis was either modest or not detectable. In the paragastric lymph nodes of athymic mice that had also received CD25<sup>+</sup> Tregs, the Thy-1.1<sup>+</sup> population represented <20% of total CD4<sup>+</sup> T cells (Fig. 4C). For example, in the nongastric athymic mice that received CD25<sup>+</sup> Tregs from wild-type mice, the Thy-1.1<sup>+</sup> population from the paragastric lymph nodes comprised 8.6 ± 8.6% (n = 4) of total CD4<sup>+</sup> T cells. Thus, there was a clear relationship between severity of gastritis and proportion of H/Kα-specific CD4<sup>+</sup> T cells detected in the local draining lymph node of the stomach.

Expansion of H/Kα-specific CD4<sup>+</sup> T cells in lymphopenic mice is dependent on the gastric H/Kα Ag

The above data suggest that the degree of expansion of the A23 T cell population in lymphopenic recipients is critical for the development of autoimmune disease. To determine whether the expansion was driven by the cognate Ag, H/Kα, or was independent of this Ag, we analyzed the behavior of A23 T cells in irradiated wild-type and H/Kα-deficient mice. Mice were irradiated to induce lymphopenia using a dose of radiation that reduced the number of splenic CD4<sup>+</sup> T cells to <5% of that of nonirradiated mice. A23 thymocytes (10<sup>3</sup>) were transferred to irradiated mice, and lymph node cells were analyzed 6 wk after transfer. In wild-type mice, the number of clonotypic Vα2<sup>+</sup> Thy-1.1<sup>+</sup> CD4<sup>+</sup> T cells recovered from the paragastric lymph node was 1.7 × 10<sup>4</sup> ± 0.9 × 10<sup>4</sup> (n = 5), whereas very few A23 T cells were detected in nongastric lymph nodes, such as the inguinal lymph node (<500) (Fig. 6). Furthermore, all five mice developed autoimmune gastritis (3 of 5 with grade 2.5; 1 of 5 with grade 1.5; and 1 of 5 with grade 0.5). Strikingly, very few Vα2<sup>+</sup> Thy-1.1<sup>+</sup> CD4<sup>+</sup> T cells were detected in either paragastric or inguinal lymph nodes (<500; n = 5) of irradiated H/Kα-deficient mice 6 wk after transfer of the A23 thymocytes (Fig. 6). Therefore, the expansion of the A23 T cell population in the absence of the gastric H/Kα was minimal.

Because the H/Kα Ag appears to be critical for expansion of the A23 T cell population in a lymphopenic environment, we directly analyzed the proliferation of A23 T cells in vivo. CFSE-labeled Thy-1.1<sup>+</sup> CD4<sup>+</sup> A23 T cells were injected into irradiated wild-type and H/Kα-deficient mice, and lymph nodes were removed 3 days later. The majority of the CFSE-labeled Thy-1.1<sup>+</sup> A23 T cells recovered from the paragastric lymph node of irradiated wild-type mice had divided, whereas very few of the Thy-1.1<sup>+</sup> A23 T cells recovered from the inguinal lymph node or spleen had divided (Fig. 7). CFSE-labeled A23 T cells recovered from the paragastric and inguinal lymph nodes of irradiated H/Kα-deficient mice remained mostly undivided (>80%) after 3 days. Taken together, these data indicate that the expansion of A23 T cells in the lymphopenic environment occurs predominantly in the paragastric lymph node and is dependent on the presence of the cognate H/Kα Ag.

Discussion

Although the importance of CD25<sup>+</sup> Tregs in maintaining self-tolerance and protection from autoimmune diseases is now well established, an understanding of the mechanism by which CD25<sup>+</sup> Tregs suppress conventional T cells in vivo remains rudimentary. The repertoire of CD25<sup>+</sup> Tregs is highly diverse, and a number of groups have shown that CD25<sup>+</sup> Tregs are positively selected in the thymus by self ligands presented by MHC class II molecules (17). Although the target Ags recognized by pathogenic effector T cells
have been identified in a number of autoimmune diseases, there have been limited studies analyzing the specificity and mode of action of CD25+ Tregs involved in protection from disease. The identification of the immunodominant Ags in autoimmune gastritis as the gastric H/K ATPase α and β subunits together with the availability of H/K ATPase-deficient mice has provided us with a defined system to investigate the role of the major gastric Ags on the genesis and survival of gastritis-protecting CD25+ Tregs.

Using a well-established lymphopenic model of autoimmune gastritis, our data have allowed a number of important conclusions concerning the function of CD25+ Tregs in vivo. First, thymic expression of the H/Kα and H/Kβ is not required for the production or maintenance of gastritis-protecting CD25+ Tregs. Second, analysis of H/Kα-specific CD4+ T cells demonstrated that the expansion of the self-reactive T cells in a lymphopenic environment was dependent on the H/Kα cognate Ag and occurred predominantly in the paragastric lymph node; expansion of this self-reactive CD4+ T cell population in a lymphopenic environment was dramatically reduced by the presence of CD25+ Tregs. Together, these findings demonstrate that CD25+ Tregs inhibit the Ag-specific expansion of tissue-specific T cells in vivo and indicate that a major site for this suppression is the local draining lymph node of the target organ.

There is a marked difference in the thymic expression of the two dominant gastric autoantigens, H/Kα expression is readily detected in thymic epithelial cells and APCs (37, 38), whereas H/Kβ appears to be absent from the thymus, or at best is marginally expressed (37, 47). Given the importance of thymic self Ags in the selection of CD25+ Tregs, it was possible that the H/K ATPase, and the H/Kα in particular, could drive selection of H/K ATPase-specific CD25+ Tregs that protect from autoimmune gastritis. However, CD25+ Treg populations purified from H/K ATPase subunit-deficient mice were as effective as the CD25+ Treg population from wild-type mice at suppressing gastritis. Titration studies of purified CD25+ Tregs showed no evidence of a reduction in the precursor frequency of the gastritis-protecting CD25+ Tregs in H/K ATPase-deficient mice. These data suggest that the CD25+ Tregs do not depend on the H/K ATPase Ags for their development in the thymus or maintenance in the periphery. It is likely, therefore, that the CD25+ Tregs that suppress autoimmune gastritis recognize gastric Ags other than the H/K ATPase, or alternatively may react with Ags of the gut flora that could also be presented with the gastric environment. Protection from other organ-specific autoimmune diseases by CD25+ Tregs is also considered to require endogenous tissue-specific Ags, for example, autoimmune ovarian disease (48, 49).

Analysis of the CD25− T-cell population indicates a low precursor frequency of pathogenic T cells in the normal polyclonal repertoire, as 4 × 10^6 polyclonal CD4+ effector cells induce autoimmune gastritis with a high incidence, whereas 4 × 10^5 CD4+ T cells induced gastritis with reduced incidence and severity (our unpublished data). The requirement for a large number of CD25− Tregs to protect against the development of autoimmune gastritis induced by polyclonal effector T cells suggests that the gastritis-protecting CD25+ Tregs also represent a small proportion of the CD25− Treg repertoire. The presence of a low precursor frequency of CD25+ Tregs that specifically protect against autoimmune gastritis would argue that the relevant CD25+ Tregs in this system recognize a small number of self Ags found in the local environment of the gastric mucosa. Recently developed techniques for expanding CD25+ Tregs in vitro (50, 51) could allow the CD25+ Treg population from paragastric lymph nodes to be isolated and the TCR specificity of these cells characterized.
We found that the CD25+ Tregs isolated from H/Ko-deficient mice could inhibit the ability of A23 T cells to cause disease in the cell transfer model. Given the requirement for self Ag in the selection and expansion of CD25+ Tregs in the periphery (14–16), it is likely that H/Ko-deficient mice are devoid of H/Ko-specific CD25+ Tregs. If the CD25+ Treg repertoire of H/Ko-deficient mice is devoid of H/Ko-specific CD25+ Tregs, then this would indicate that the Ag specificity of CD25+ Tregs can differ from the specificity of effector T cells. In vitro studies have also demonstrated that suppression by CD25+ Tregs is non-Ag specific (12, 52); however, the data in vivo are contradictory. A recent study using TCR transgenic T cells and immunization with Ags has indicated that regulation by CD25+ Tregs in vivo is Ag specific (53), and also in another study the effector function of CD25+ Tregs in myelin basic protein-specific TCR transgenic mice was shown to be Ag specific (54). Our study is the first to use regulatory T cells from normal mice and pathogenic T cells that are specific for an endogenous Ag, and suggests that suppression in vivo does not require the recognition of the same Ag as the effector T cells, but nonetheless most likely requires recognition of an Ag in the same local environment as the effector T cell population.

We have used the H/Ko-specific A23 T cells to explore how CD25+ Tregs inhibit autoimmune gastritis. The A23 T cell clone was isolated from a gastritic mouse that was thymectomized on day 3 after birth (32), and therefore represents a pathogenic T cell that could normally be present in the peripheral repertoire. We found that the expansion of the A23 T cell population in lymphopenic mice 8 wk after transfer was dramatically reduced when CD25+ Tregs were present. Previous studies have shown that the expansion of T cell populations in a lymphopenic environment is complex and can be regulated by competition between T cells (23, 55). Hence, it could be argued that the presence of additional T cells per se affected the expansion of the A23 T cell population. However, this was not the case, as the inhibition of the expansion of the A23 T population required the presence of CD25+ Tregs rather than simply a bulk population of T cells. An equivalent number of OVA-specific CD4+CD25− DO11 T cells had no apparent effect on the expansion of A23 T cells or on their ability to cause autoimmune gastritis. CD25+ Tregs may limit the expansion of A23 T cells in the lymphopenic environment by either inhibition of their proliferation and/or by the promotion of apoptosis following activation. Although it is likely that CD25+ Tregs directly inhibit proliferation of A23 T cells, this is difficult to demonstrate, as a ratio of 23-polyclonal CD25+ Tregs of ~1:10⁴ is required to protect against gastritis and >10⁵ CFSE-labeled A23 T cells are needed for in vivo analysis of proliferation. Consistent with this hypothesis, CD25+ Tregs have also been shown to inhibit the proliferation of graft-specific T cells in vivo and promote graft survival (56).

To assess the role of the gastric H/Ko on the homeostatic expansion of A23 T cells, we used irradiated H/Ko-deficient mice. Previous studies have indicated that expansion of most T cells in sublethally irradiated mice is equivalent to that in congenitally T cell-deficient mice (57). In our experiments, the presence of the gastric H/Ko was required for the peripheral expansion of A23 cell population. Significantly, this result shows that other self Ags in the periphery are unable to drive proliferation of A23 T cells even in the altered cytokine environment of a lymphopenic host. The proliferation of CFSE-labeled A23 T cells in vivo was restricted to the draining lymph node of the stomach, the paragastric lymph node. Proliferation in the paragastric lymph node is consistent with previous observations that have demonstrated that H/Ko is presented by DCs in this lymph node (36). The finding that cognate self Ag is necessary to drive the expansion of A23 T cells in a lymphopenic environment represents one of the few reports in which the Ag responsible has been identified.

The absence of mononuclear infiltrates in the gastric mucosa of lymphopenic mice that received both A23 cells and CD25+ Tregs suggests that CD25+ Tregs blocked expansion of the self-reactive T cells before migration to the target organ. As the expansion of A23 T cells is dependent on H/Ko, and as H/Ko is presented by DCs in the paragastric lymph node and can activate A23 T cells, the results collectively indicate that CD25+ Tregs function primarily within the paragastric lymph node.

Although our study, using a lymphopenic model of autoimmune disease induction, has shown that CD25+ Tregs can influence the early phase of an immune response, other in vivo studies have shown that CD25+ Tregs can suppress later stages of the immune response, such as development of effector functions and cytokine secretion by T cells (20, 58, 59). Regulation by CD25+ Tregs may be mediated by a variety of mechanisms, including direct cell contact with effector T cells or APC and/or by the secretion of suppressor cytokines that interfere with T cell activation, such as TGF-β or IL-10 (20, 60–65). The ability of CD25+ Tregs to mediate suppression by several mechanisms has the potential to allow regulation at various stages of the immune response. The precise mechanisms of regulation may depend on the tissue environment and the nature of the inflammation. Thus, it is likely that detailed understanding of the immunoregulatory mechanisms of CD25+ Tregs will require a variety of in vivo systems to be explored in detail.

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Disclosures
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References