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Protection against Autoimmunity in Nonlymphopenic Hosts by CD4+CD25+ Regulatory T Cells Is Antigen-Specific and Requires IL-10 and TGF-β

Xiaopei Huang, Jiangao Zhu, and Yiping Yang

CD4+CD25+ regulatory T cells (T_{Reg}) play a critical role in the control of autoimmunity. However, little is known about how T_{Reg} suppress self-reactive T cells in vivo, thus limiting the development of T_{Reg}-based therapy for treating autoimmune diseases. This is in large part due to the dependency on a state of lymphopenia to demonstrate T_{Reg}-mediated suppression in vivo and the unknown Ag specificity of T_{Reg} in most experimental models. Using a nonlymphopenic model of autoimmune pneumonitis and T_{Reg} with known Ag specificity, in this study we demonstrated that these T_{Reg} can actively suppress activation of self-reactive T cells and protect mice from fatal autoimmune pneumonitis. The protection required T_{Reg} with the same Ag specificity as the self-reactive T cells and depended on IL-10 and TGF-β. These results suggest that suppression of autoimmunity by T_{Reg} in vivo consists of multiple layers of regulation and advocate for a strategy involving Ag-specific T_{Reg} for treating organ-specific autoimmunity, because they do not cause generalized immune suppression. The Journal of Immunology, 2005, 175: 4283–4291.

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self-reactive T cells. Using this approach, we demonstrate that CD4+CD25+ T\textsubscript{Reg} can suppress self-Ag-driven activation of autoreactive T cells in vivo and prevent autoimmunity in a nonlymphopenic environment through both IL-10 and TGF-β in an Ag-specific manner.

**Materials and Methods**

**Mice**

B10.D2 and BALB/c mice (H-2\text{d}) were purchased from The Jackson Laboratory. The C3-HA transgenic mice were provided by Dr. D. Pardoll (Johns Hopkins University, Baltimore, MD) and have been described previously (28, 29, 31). The 6.5 TCR-HA transgenic mice on the BALB/c background were bred in our laboratory. The DO11.10 TCR-OVA transgenic mice in BALB/c background that express a TCR recognizing an I-Ad-restricted OVA epitope were purchased from The Jackson Laboratory (33). RAG-2\textsuperscript{-/-} mice were purchased from Taconic Farms. C3-HA high or C3-HA low mice (Johns Hopkins University, Baltimore, MD) and have been described previously (34) and transferred into recipients in 0.2 ml of B10.D2 genetic background. The background that express a TCR recognizing an I-Ed-restricted HA epitope were bred in our animal facility. All mice used in these studies were between 8 and 12 wk of age. Experimental procedures were performed in accordance with protocols approved by the Animal care and use committee of Duke University Medical Center.

**Adoptive transfer**

Naive clonotypic HA- and OVA-specific T cells were prepared from TCR-HA and TCR-OVA transgenic mice on a RAG-2\textsuperscript{-/-} background as described previously (34) and transferred into recipients in 0.2 ml of HBSS. For transfer of clonotypic CD4+CD25+ T cells, single-cell suspensions were prepared from TCR-HA × C3-HA double-transgenic mice. CD4+ T cells were enriched through depleting non-CD4 cells by a mixture of biotinylated mAbs, followed by anti-biotin MicroBeads (Miltenyi Biotech). Enriched CD4+ T cells were then subject to cell sorting gated on CD4+CD25+ cells with a high speed cell sorter FACS Vantage (BD Biosciences). The purity of FACS sorted populations of cells was >98%.

**In vivo Ab blocking**

For in vivo cytokine blocking experiments, mice were injected with 0.5 mg of anti-TGF-β (1D11) Ab (R&D Systems), anti-IL-10R (OX-7), anti-IFN-γ (XMG1.2), and anti-IFN-γ (RM4-5) Ab. Anti-GITR mAb (108619) was purified and conjugated in our laboratory. Cells were stained with the desired mAbs and subjected to FACS analysis using FACS Calibur (BD Biosciences). To assess the production of IFN-γ or IL-10 intracellularly, cells were incubated in the presence of 10 μg/ml 1-E4-HA peptide and 5 μg/ml brefeldin A containing Golgi-Plug (BD Pharmingen) for 6 h at 37°C. After washing, cells were stained with CD4 and Thy1.1 and permeabilized to detect intracellular IFN-γ or IL-10 using the Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer’s instructions.

**Real-time RT-PCR**

Total RNA was extracted from 5 × 10\textsuperscript{5} sorted cells using TRIsolv (Invitrogen Life Technologies). Oligo(dT)\textsubscript{12}-primed cDNA was generated with ImProm-II reverse transcriptase (Promega). The expression of Foxp3 and hypoxanthine phosphoribosyltransferase (HPRT) was quantified by real-time PCR using the TaqMan Universal PCR MasterMix (Applied Biosystems) as well as the following primers and internal fluorescent probes: Foxp3, 5'-GCC CCT TCT CCA GGA CAG A-3', 5'-GCT GAT CAT GCC TGG GTT GT-3', and 5'-FAM ACT TCA TGC ATC AGC TCT GCC ACC GTA TACC A-3'; or HPRT, 5'-TGA AGA GCT ACT GTA ATG ATC AGT CAA C-3', 5'-AGC AAG CTT GCC ACC TTA ACC A-3', and 5'-FAM-TGC TTT CCC TGG TTA AGC AGT ACA GCC C-TAMRA-3'. The normalized value for Foxp3 mRNA expression in each sample was calculated as the relative quantity of Foxp3 derided by the relative quantity of HPRT (x100). The expression of Foxp3 was calculated using the relative quantity of HPRT by the delta-delta Ct method. The expression of Foxp3 was calculated using the relative quantity of HPRT by the delta-delta Ct method.

**Flow cytometry**

The following mAbs were purchased from BD Pharmingen: anti-CD4 (RM4-5), anti-Thy1.1 (OX-7), anti-CD44 (1M7), anti-IL-10 (JES5), anti-CD25 (7D4), anti-CTLA4 (UC10), anti-TGF-β (A75-3-1), anti-TCR-OVA (KJ1-26), and anti-IFN-γ (XMG1.2). Anti-GITR mAb (108619) was purchased from R&D Systems. Anti-TCR-HA Abs (6.5) were purified and conjugated in our laboratory. Cells were stained with the desired mAbs and subjected to FACS analysis using FACS Calibur (BD Biosciences). To assess the production of IFN-γ or IL-10 intracellularly, cells were incubated in the presence of 10 μg/ml 1-E4-HA peptide and 5 μg/ml brefeldin A containing Golgi-Plug (BD Pharmingen) for 6 h at 37°C. After washing, cells were stained with CD4 and Thy1.1 and permeabilized to detect intracellular IFN-γ or IL-10 using the Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer’s instructions.

**Proliferation assay**

Naive 6.5 CD4+ cells (2.5 × 10\textsuperscript{5}) from TCR-HA mice were cultured in the presence of irradiated (3000 rad) B10.D2 splenocytes (2.5 × 10\textsuperscript{5}) and 10 μg/ml of the I-Ed-HA peptide. For in vitro suppression experiments, sorted 6.5 CD4+CD25+ (2.5 × 10\textsuperscript{5}) or 6.5 CD4+CD25− (2.5 × 10\textsuperscript{5}) cells were added to the wells. Incorporation of [3H]thymidine (1 μCi/well) during the last 18 h of a 66-h culture was measured by scintillation counting.

**FIGURE 1.** A model of fatal autoimmune pneumonitis. A, Nontransgenic B10.D2, C3-HA\textsuperscript{high}, or C3-HA\textsuperscript{low} mice (n = 10 for each group) were adoptively transferred with naive HA-specific T cells (Thy1.1+) and monitored for survival. Data represent the Kaplan-Meier survival curve, indicating the percent survival over time after T cell transfer. B, In some experiments mice were harvested 4 days after transfer of T cells and examined for pulmonary pathology by H&E staining and for T cell infiltration by immunohistochemistry using Thy1.1 mAb. C, CFSE-labeled naive HA-specific T cells (Thy1.1+) were used for transfer. Four days later, lymphocytes from hilar LNs were analyzed for in vivo divisions (by CFSE dilution) and function (by intracellular IFN-γ staining). For CFSE dilution, events were gated on CD4+ Thy1.1 T cells; for intracellular IFN-γ staining, events were gated on CD4+ T cells. D, C3-HA\textsuperscript{high} mice (n = 10 for each group) were adoptively transferred with 5 × 10\textsuperscript{5}, 2.5 × 10\textsuperscript{5}, 1.2 × 10\textsuperscript{5}, 6 × 10\textsuperscript{4}, 3 × 10\textsuperscript{4}, or 1 × 10\textsuperscript{4} of naive HA-specific T cells and monitored for survival. Representative data of three independent experiments are shown.
Dendritic cell (DC) preparation

Bone marrow-derived DCs were generated as previously described (34). Briefly, bone marrow cells were cultured in the presence of murine GM-CSF (1000 U/ml) and IL-4 (500 U/ml) for 5 days. On day 5, cells were harvested and transferred onto a new plate in the above-described DC medium with addition of TNF (500 U/ml). On day 7, DCs were harvested and loaded with HA and OVA peptides for immunization.

Histopathology and immunohistochemistry

Paraffin sections (5 μm) were stained with H&E according to standard procedures. Random sections were examined for histopathology in a blinded fashion. Detection of donor-derived Thy1.1 cells was performed by immunohistochemistry as previously described (31).

Results

A model of fatal autoimmune pneumonitis

To determine whether CD4<sup>+</sup>CD25<sup>+</sup> T<sub>Reg</sub> can inhibit self-Ag driven activation of autoreactive T cells in vivo in the absence of homeostatic signals, we first further characterized a previously described mouse model of autoimmune pneumonitis using C3-HA transgenic mice (30). Adoptive transfer of 1.2 × 10<sup>6</sup> naive HA-specific T cells (Thy1.1<sup>+</sup>) into C3-HA<sup>high</sup> mice that express high levels of HA in a variety of organs, including lung, led to 100% mortality in recipients within 5–6 days after transfer (Fig. 1A). Pulmonary pathology revealed that these animals developed severe pneumonitis characterized by perivascular and alveolar infiltration (Fig. 1B). Immunohistochemistry with anti-Thy1.1 Ab indicated that the pulmonary infiltrate was mainly composed of donor-derived, self-reactive T cells (Fig. 1B). Four days after transfer of CFSE-labeled naive HA-specific T cells, vigorous proliferation was detected in spleen and hilar lymph nodes (LNs) including at least six divisions in C3-HA<sup>high</sup> mice (Fig. 1C). Extensive proliferation resulted in activation of self-reactive T cells and development of effector function, indicated by their ability to produce IFN-γ (Fig. 1C). Neither mortality nor T cell activation was observed when naive HA-specific T cells were transferred into nontransgenic B10.D2 mice or C3-HA<sup>low</sup> mice (29), suggesting that induction of fatal pneumonitis was Ag specific and was dependent on the levels of self Ag expression in the target organ (Fig. 1, A and B). The mortality of C3-HA<sup>high</sup> mice was also related to the number of clonotypic T cells transferred in a dose-dependent manner (Fig. 1D).

Protection against pulmonary lethality in C3-HA<sup>high</sup> × TCR-HA double-transgenic mice

We next looked at whether the observed mortality can be induced by intercrossing TCR-HA transgenic mice with C3-HA<sup>high</sup> mice.
Although there was a reduction in the number of TCR-HA × C3-HA^high^ double-transgenic litters compared with that of TCR-HA or C3-HA^high^ single-transgenic ones, the double-transgenic mice survive well after birth. Furthermore, these double-transgenic mice became refractory to naive HA-specific T cell-induced pulmonary lethality (Fig. 2A). No mortality was observed even with a dose of 5 × 10^6^ HA-specific T cells (Fig. 2A). When CFSE-labeled naive HA-specific T cells were transferred into these double-transgenic mice, very little activation of self-reactive T cells, characterized by poor proliferation and no effector function, was detected in hilar LNs (Fig. 2B). These results suggested that protection against lethal pneumonitis in C3-HA^high^ × TCR-HA double-transgenic mice was probably due to lack of self-reactive T cell activation.

Abundant clonotypic CD4^+^CD25^+^ T cells in C3-HA^high^ × TCR-HA double-transgenic mice

We next observed that in double-transgenic mice, there was a large population of clonotypic HA-specific CD4^+^ T cells (6.5^CD4^+^) that expressed CD25 (Fig. 3). These 6.5^CD4^+^CD25^+^ T cells (referred to as 6.5^CD25^+) were abundant in secondary lymphoid tissues, such as spleen and peripheral LNs (Fig. 3A). They expressed high levels of CTLA-4, GITR, IL-10, and TGF-β compared with 6.5^CD25^- T cells from the same mouse (Fig. 4A). Thus, the phenotype of 6.5^CD25^+^ T cells suggested that they were TReg. Indeed, when the expression of the TReg-specific marker, Foxp3 (8–10), was analyzed by real-time PCR, high levels of Foxp3 mRNA were detected in sorted 6.5^CD25^+^ T cells (Fig. 4B). The level of Foxp3 expression in 6.5^CD25^+^ T cells was comparable to that in naturally occurring polyclonal CD4^+^CD25^+^ TReg cells from B10.D2 mice (Fig. 4B).

To study whether 6.5^CD25^+^ T cells were derived from the thymus, we analyzed the presence of 6.5^CD25^+^ T cells in the CD4^+^CD8^-^ fraction of thymocytes. Similar to the peripheral lymphoid organs, 6.5^CD25^+^ T cells were also abundant in the thymus of C3-HA^high^ × TCR-HA double-transgenic mice (Fig. 3A). These results suggest that 6.5^CD25^+^ T cells may also have arisen as a result of the intrathymic expression of HA (28, 29). To investigate whether the development of 6.5^CD25^+^ T cells required endogenous TCR chains, we bred C3-HA^high^ × TCR-HA mice onto a RAG-2-deficient background (Fig. 3B). Consistent with previous reports, the small amount of 6.5^CD25^+^ T cells that developed in TCR-HA single-transgenic mice required endogenous TCR chains, because they did not arise in TCR-HA RAG^-/-^ mice (Fig. 3) (12, 13, 18). However, the development of 6.5^CD25^+^ T cells was intact in C3-HA^high^ × TCR-HA mice bred onto a RAG^-/-^ background (Fig. 3B), arguing against a requirement for the rearrangement of endogenous TCRs for the development of the 6.5^CD25^+^ T cells in C3-HA^high^ × TCR-HA mice.

Clonotypic CD4^+^CD25^+^ T cells suppress naive TCR-HA T cell response in vitro

To confirm that 6.5^CD25^+^ TReg possess regulatory function, we analyzed their suppressive activity in vitro. Purified 6.5^CD25^+^ cells potently suppressed the proliferation of naive HA-specific T cells.
cells, whereas 6.5+CD25- cells failed to do so (Fig. 4C). Separation of 6.5+CD25+ TReg from naive HA-specific T cells by a Transwell abrogated suppression of these cells (Fig. 4C). In addition, the suppressive activity of 6.5+CD25+ TReg did not require IL-10 or TGF-β, because the addition of anti-IL-10 or anti-TGF-β Ab did not ablate suppression (Fig. 4C). Thus, similar to the property of naturally occurring polyclonal CD4+CD25+ TReg (17, 18), these results indicate that the suppressive activity of 6.5+CD25+ TReg is cell contact dependent and cytokine independent in vitro.

Clonotypic CD4+CD25+ TReg protect against lethal autoimmune pneumonitis in vivo

We next examined the capacity of 6.5+CD25+ TReg cells to inhibit fatal autoimmune pneumonitis in C3-HAhigh mice. Purified 6.5+CD25+ TReg cells were then transferred into C3-HAhigh recipients, which were subsequently challenged i.v. with naive HA-specific T cells (Thy1.1+) to induce lethal autoimmune pneumonitis. Adoptive transfer of 6.5+CD25+ TReg completely protected C3-HAhigh mice from pulmonary lethality (Fig. 5A) compared with 100% mortality in the C3-HAhigh mice that received only naive HA-specific T cells (Fig. 1A). However, transfer of 6.5+CD25- T cells generated from the same group of mice failed to prevent mortality (Fig. 5A). The protection from fatal pneumonitis by 6.5+CD25+ TReg cells was accompanied by a notable reduction in perivascular and alveolar infiltration compared with no reduction of infiltration by transfer of 6.5+CD25- T cells (Fig. 5B). Immunohistochemistry with anti-Thy1.1 Ab indicated that transfer of 6.5+CD25+ TReg cells inhibited the infiltration of donor-derived self-reactive T cells (Thy1.1+). When CFSE-labeled naive HA-specific T cells were used for the challenge experiment, transfer of 6.5+CD25+ TReg cells, but not 6.5+CD25- T cells, suppressed proliferation as well as the development of effector function by HA-specific T cells (Fig. 5C). Taken together, these results showed that the clonotypic CD4+CD25+ TReg possessed the capacity to protect mice from lethal autoimmune pneumonitis through suppressing the activation of self-reactive T cells in nonlymphopenic mice.

Clonotypic TReg-mediated protection in vivo is Ag specific

To test whether polyclonal TReg can also suppress naive HA-specific T cell-induced pneumonitis, we purified 6.5+CD25+ TReg cells from C3-HAhigh × TCR-HA double-transgenic mice or polyclonal naturally occurring CD4+CD25+ TReg from nontransgenic B10.D2 mice. The levels of Foxp3 expressed by these TReg were comparable to those expressed by 6.5+CD25+ TReg cells (Fig. 4B). There Foxp3+ TReg were then transferred into C3-HAhigh recipients, which were subsequently challenged i.v. with naive HA-specific T cells (Thy1.1+). Transfer of 6.5+CD25+ TReg or
polyclonal CD4+CD25+ TReg cells did not protect mice from fatal pneumonia (Fig. 5A). Neither reduction of pulmonary infiltrate (Fig. 5B) nor suppression of naive HA-specific T cell activation (Fig. 5C) was noted in mice transferred with 6.5+CD25+ TReg or polyclonal CD4+CD25+ TReg. These results suggested that suppression of self-reactive T cell activation and prevention of autoimmunity by TReg in vivo required TReg cells with the same Ag specificity.

To test whether differential expansion of clonotypic 6.5+CD25+ TReg vs polyclonal CD4+CD25+ TReg in C3-HAhigh mice contributes to the observed Ag specificity in TReg-mediated suppression in vivo, we first transferred purified 6.5+CD25+ TReg or polyclonal CD4+CD25+ TReg cells and followed their expansion over time in vivo (Table I). Transfer of 6.5+CD25+ TReg and CD4+CD25+ TReg cells led to 3- to 4-fold and 2- to 2.5-fold expansion, respectively, by day 15 in both spleen and hilar LNs in C3-HAhigh mice, consistent with previous observations that TReg are not anergic in vivo and can undergo limited expansion in response to Ag stimulation (35–38). Because we observed that expansion of 6.5+CD25+ TReg was slightly more efficient than that of polyclonal CD4+CD25+ TReg, we examined whether transfer of escalated doses of polyclonal CD4+CD25+ TReg protected C3-HAhigh mice from HA-specific, T cell-induced pneumonia. As shown in Fig. 5D, no protection was observed even with a dose of 5 x 10^6 of CD4+CD25+ TReg (8-fold higher than 6.5+CD25+ TReg), suggesting that the slight difference in expansion did not contribute to the observed Ag specificity in TReg-mediated suppression in vivo.

To further confirm the requirement for Ag specificity in TReg-mediated suppression in vivo, we tested whether 6.5+CD25+ TReg could suppress the activation of naive T cells with a different specificity. BALB/c mice were transferred with a mixture of naive HA-specific (2 x 10^5; Thy1.1+) and naive OVA-specific (2 x 10^5) T cells i.v. and challenged with mature DCs loaded with both HA and OVA peptides in the presence or the absence of 6.5+CD25+, HA-specific TReg. Naive HA-specific T cells and OVA-specific T cells were detected by anti-Thy1.1 Ab and the clonotypic Ab to OVA-TCR, KJ1-26. In the absence of 6.5+CD25+ TReg, immunization with DCs loaded with both HA and OVA peptide rapidly expanded both HA-specific and OVA-specific T cells compared with the controls using DCs not loaded with peptide (Fig. 6). However, cotransfer of 6.5+CD25+ TReg significantly (p < 0.001) abrogated the expansion of HA-specific T cells (Fig. 6). In contrast, the expansion of OVA-specific T cells was not affected by the presence of 6.5+CD25+ TReg cells (Fig. 6). These results indicate that TReg-mediated suppression in vivo in nonlymphopenic hosts is Ag specific.

**Clonotypic TReg-mediated protection in vivo is mediated by IL-10 and TGF-β**

Although IL-10 and TGF-β did not seem to play a role in 6.5+CD25+ TReg-mediated suppression in vitro (Fig. 4C), 6.5+CD25+ TReg cells from C3-HAhigh × TCR-HA double-transgenic mice secreted high levels of IL-10 and TGF-β (Fig. 4A). Thus, it was of interest to determine whether IL-10 and TGF-β

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**Table I. In vivo expansion of 6.5+CD25+ TReg vs polyclonal CD4+CD25+ TReg in C3-HAhigh mice**

<table>
<thead>
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<th>Days after Transfer</th>
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<th>8</th>
<th>15</th>
</tr>
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<td>Spleen</td>
<td>6.5+CD25+</td>
<td>0.1 ± 0.03</td>
<td>0.3 ± 0.02</td>
<td>0.4 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>CD4+CD25+</td>
<td>0.1 ± 0.03</td>
<td>0.15 ± 0.03</td>
<td>0.2 ± 0.04</td>
</tr>
<tr>
<td>Hilar LNs</td>
<td>6.5+CD25+</td>
<td>0.14 ± 0.04</td>
<td>0.28 ± 0.04</td>
<td>0.56 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>CD4+CD25+</td>
<td>0.16 ± 0.06</td>
<td>0.45 ± 0.07</td>
<td>0.30 ± 0.08</td>
</tr>
</tbody>
</table>

* A total of 1 x 10^6 of purified 6.5+CD25+ TReg from C3-HAhigh × TCR-HA double transgenic mice or polyclonal CD4+CD25+ TReg from Thy1.1+ B10.D2 mice were transferred into C3-HAhigh mice (Thy1.2+). At 1, 4, 8, and 15 days after transfer, cells from spleen and hilar LNs were stained with CD4 and 6.5 for detection of 6.5+CD25+, TReg, or with CD4 and Thy1.1 for detection of polyclonal CD4+CD25+ TReg by FACS. Events were gated on CD4+ T cells and data represent mean percentages of TReg SD in total CD4+ T cells.

**FIGURE 6.** Clonotypic TReg cell-mediated suppression in vivo is Ag specific. BALB/c mice were transferred with a mixture of 2 x 10^5 of naive HA-specific T cells (HA) and 2 x 10^5 of naive OVA-specific T cells (OVA) with or without coinjection of 2 x 10^5 sorted HA-specific TReg cells (6.5+CD25+) i.v. These mice were then immunized with 1 x 10^6 mature DCs (DC) or DCs loaded with 10 μg/ml L-A2- OVA peptide (DC/Pep). Four days later, splenocytes were harvested and stained for FACS analysis. A. In vivo expansion of HA-specific (CD4+Thy1.1+) or OVA-specific (CD4+KJ1–26+) T cells. B. Quantitative analysis of Ag-specific T cells. Representative data of two independent experiments are shown.
played a role in the protection of mice from lethal autoimmune pneumonitis. 6.5°CD25° TReg cells were transferred into C3-HAhigh recipients, and mice were challenged with naive HA-specific T cells. Before transfer, mice were treated with 0.5 mg of anti-IL-10, anti-TGF-β, or control Ab. Surprisingly, mice treated with anti-IL-10 or anti-TGF-β Ab succumbed with 100% mortality within 8 days after transfer of naive HA-specific T cells (Fig. 7A). In vivo blocking of IL-10 or TGF-β was associated with increased proliferation and gain of effector function by the self-reactive T cells in the presence of 6.5°CD25° TReg cells (Fig. 7B). These results suggest that in addition to Ag-specific regulation by TReg, both IL-10 and TGF-β were required for TReg-mediated suppression in vivo and protection from the lethal autoimmune pneumonitis.

![FIGURE 7](image)

**FIGURE 7.** IL-10 and TGF-β are required for the protection by clonotypic CD4+CD25+ TReg cells. A, Naive HA-specific T cells (Thy1.1°) were transferred into C3-HAhigh mice pretreated with sorted 6.5°CD25° cells. Six hours before the transfer of naive HA-specific T cells, these mice were treated with 0.5 mg of anti-IL-10, anti-TGF-β Ab, or irrelevant rat IgG (Control Ab) i.v. and monitored for survival. B, CFSE-labeled naive HA-specific T cells were used for transfer. Four days later, lymphocytes from hilar LNs were analyzed for in vivo divisions and function of HA-specific T cells. For CFSE dilution, events were gated on CD4°Thy1.1° T cells; for intracellular IFN-γ staining, events were gated on CD4+ T cells. Representative data of two independent experiments are shown.

**Discussion**

In this study we have characterized HA-specific CD4+CD25+ TReg. In vitro, these Ag-specific TReg were phenotypically and functionally indistinguishable from the naturally occurring CD4+CD25+ TReg. Using a murine model of autoimmune pneumonitis, we showed that these HA-specific TReg can protect mice from fatal autoimmune pneumonitis in vivo. This was accomplished by inhibiting self-HA-driven activation of naive HA-specific T cells. In this nonlymphopenic model, the TReg-mediated suppression in vivo was Ag specific and critically dependent on both IL-10 and TGF-β.

Despite accumulating evidence that CD4+CD25+ TReg play a crucial role in the control of autoimmunity (1, 2), very little is known about how they function in vivo. This is largely due to the limitations in current models for TReg-mediated suppression in vivo. One of them is that most models heavily rely on a state of lymphopenia to demonstrate TReg-mediated suppression (39–43). The presence of homeostatic signals in the lymphopenic environment has made it difficult to discern whether TReg-mediated suppression on naive or effector T cells in vivo is due to competition for space or cytokines (24) or to active suppression of self-Ag-driven activation of autoreactive T cells. Using a nonlymphopenic model of autoimmune pneumonitis, in this study we provide direct evidence that CD4+CD25+ TReg can inhibit self-Ag-driven activation of autoreactive T cells in vivo in the absence of homeostatic signals. Furthermore, our findings that only 6.5°CD25+ TReg, but not 6.5°CD25− T cells, inhibit activation of naive HA-specific T cells suggest that this is a process of active suppression, rather than intracranial T cell competition for available space or cytokines.

Another limitation is that in most in vivo models, the Ag specificity of the CD4+CD25− TReg cells is unknown. Thus, whether CD4+CD25+ TReg cells suppress the activation of self-reactive T cells with the same Ag specificity or different ones in a bystander fashion remains to be defined despite the idea that TReg-mediated suppression in vitro is non-Ag specific (18, 25). Some early studies suggest that Ag specificity may play a role in TReg-mediated regulation in vivo (26, 27). Using a model of HA-specific TReg, we have provided evidence that suppression of self-reactive T cells and prevention of autoimmune pneumonitis by TReg in vivo is Ag specific. Similarly, HA-specific TReg have been shown to specifically inhibit HA-specific allograft rejection (44, 45) or tumor rejection (46). Whether this reflects the mode of action for naturally occurring TReg in a nonlymphopenic setting remains to be confirmed, because TReg development in these transgenic TCR mice might be different from that of naturally occurring TReg cells in nontransgenic mice, which might influence the function of these transgenic TReg cells. Thus, it will be important to develop a model that allows us to study how naturally occurring TReg suppress autoimmunity in a nonlymphopenic host. Furthermore, the delineation of how TReg are generated in the thymus will also help us to better understand their function.

How TReg cells suppress activation of naive T cells with the same Ag specificity remains to be defined. We could envision three potential mechanisms for the observed Ag specificity in TReg-mediated suppression in vivo: 1) TReg could compete with self-reactive T cells for the available MHC/peptide ligands on DCs; 2) if cell-cell contact is also necessary for suppression in vivo, then the binding to DCs that present the same MHC/peptide ligand could serve as a mechanism to bring TReg and self-reactive T cells together; and 3) TReg could down-regulate the function of DCs that present the same MHC/peptide ligand to self-reactive T cells. Thus, future work should delineate these potential mechanisms responsible for TReg-mediated suppression in vivo.
In addition to the requirement for Ag specificity, we found that TReg-mediated suppression in vivo was critically dependent on immunosuppressive cytokines, IL-10 and TGF-β. These data suggest that the mechanisms by which TReg-mediated suppression in vivo is far more complex and require multiple layers of regulation. How, then, do IL-10 and TGF-β contribute to Ag-specific immune regulation by TReg in vivo? Our data indicate that both IL-10 and TGF-β may contribute to TReg-mediated suppression in vivo by inhibiting the expansion and effector functions of self-reactive T cells. These suppressive cytokines could exert an effect on self-reactive T cells directly or through DCs indirectly. These observations are consistent with the findings of a previous study that IL-10 may play a role in the control effector T cell expansion by CD4+CD25+ TReg (47). Furthermore, it has been shown that TGF-β signaling is required for in vivo expansion of CD4+CD25+ TReg (48, 49), suggesting that TGF-β may also contribute to TReg-mediated suppression in vivo by controlling the number of available Ag-specific TReg. Consistent with previous observations on naturally occurring TReg cells (22, 23, 35, 47), we have shown that clonotypic 6.5 CD25+ TReg cells produce high levels of TGF-β and IL-10. However, many other cell types may also produce soluble IL-10 and/or TGF-β, including endogenous polyclonal TReg cells in our system (50–53). Thus, it is not clear what type of cells produce IL-10 and/or TGF-β, which are required for the observed immune suppression by Ag-specific TReg cells. Thus, future studies will focus on identifying the cellular source of IL-10 and TGF-β as well as defining the precise roles of IL-10 and TGF-β in Ag-specific TReg cell-mediated suppression in vivo.

In conclusion, we have demonstrated in a model of autoimmune pneumonitis that CD4+CD25+ TReg cells can suppress self-Ag-driven activation of self-reactive T cells and protect mice from fatal pneumonitis in vivo. In this nonlymephocytic model, the suppression and protection by TReg are Ag specific and dependent on both IL-10 and TGF-β. These results indicate that TReg can actively suppress autoimmune in an Ag-specific manner and suggest an appealing therapeutic strategy using Ag-specific TReg for treating autoimmune diseases, because they would not cause generalized immune suppression.

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Disclosures

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References


