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*J Immunol* 2003; 170:279-286; doi: 10.4049/jimmunol.170.1.279

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CD25+ Immunoregulatory CD4 T Cells Mediate Acquired Central Transplantation Tolerance

Jose Trani,* Daniel J. Moore,* Beth P. Jarrett,* Joseph W. Markmann,* Major K. Lee,* Andrew Singer,* Moh-Moh Lian,* Brian Tran,* Andrew J. Caton,† and James F. Markmann2*

Transplantation tolerance is induced reliably in experimental animals following intrathymic inoculation with the relevant donor strain Ags; however, the immunological mechanisms responsible for the induction and maintenance of the tolerant state remain unknown. We investigated these mechanisms using TCR transgenic mice (TS1) that carry T cells specific for an immunodominant, MHC class II-restricted peptide (S1) of the influenza PR8 hemagglutinin (HA) molecule. We demonstrated that TS1 mice reject skin grafts that have transgene-encoded HA molecules (HA104) as their sole antigenic disparity and that intrathymic but not i.v. inoculation of TS1 mice with S1 peptide induces tolerance to HA-expressing skin grafts. Intrathymic peptide inoculation was associated with a dose-dependent reduction in T cells bearing high levels of TCR specific for HA. However, this reduction was both incomplete and transient, with a full recovery of S1-specific thymocytes by 4 wk. Peptide inoculation into the thymus also resulted in the generation of immunoregulatory T cells (CD4+CD25+) that migrated to the peripheral lymphoid organs. Adoptive transfer experiments using FACS sorted CD4+CD25- and CD4+CD25+ T cells from tolerant mice revealed that the former but not the latter maintain the capacity to induce rejection of HA bearing skin allografts in syngeneic hosts. Our results suggest that both clonal frequency reduction in the thymus and immunoregulatory T cells exported from the thymus are critical to transplantation tolerance induced by intrathymic Ag inoculation. The Journal of Immunology, 2003, 170: 279–286.

The success of current clinical transplantation regimens is largely dependent on chronic immunosuppressive therapy to maintain graft acceptance in the face of ongoing anti-donor immune reactivity. Although great advances have been made in the production of more potent but less toxic pharmacologic agents, the ideal of transplantation biology, organ specific tolerance, remains elusive (1).

Our laboratory has previously reported that inoculation of donor Ags into the recipient thymus can promote donor-specific tolerance (2–5). Although the mechanism underlying this finding remains unknown, several hypotheses have been put forward to explain the phenomenon. We and others have previously proposed that clonal deletion of Ag-reactive T cells may be predominantly responsible (6–10). However, this mechanism cannot account for the observation that depletion of already mature peripheral T cells is not invariably required by these protocols (11–14). That the introduction of Ag into the thymus may exert a tolerogenic effect on an already established peripheral immune system requires further explanation.

The recent functional and phenotypic characterization of immunoregulatory CD4+CD25+ T cells invites the hypothesis that these cells may be responsible for the translation of tolerance from an Ag-inoculated thymus to a mature but naive peripheral immune system (15–20). These cells which are characterized by expression of high levels of CD25 and intermediate levels of CD45RB are known to be weakly reactive to antigenic stimulation and able to mediate suppression of CD25–naive T cells. Until now, determination of the relative contribution of clonal deletion and active immunoregulation in intrathymic tolerance has been problematic given the inability to follow the fate of Ag-reactive T cells. However, the availability of TCR transgenic mouse lines in which the transgenic T cell is specific for transplantation Ags affords such investigation.

In the present work, we used the TS1 line of TCR transgenic mice to probe the mechanisms responsible for the tolerant state that results from intrathymic Ag inoculation (21). The TCR α- and β-chain genes cloned to generate TS1 mice were derived from a T cell line specific for the immunodominant epitope of influenza A virus PR8 hemagglutinin (HA)3 (22, 23). As a result, TS1 mice possess a high percentage of T cells specific for the hemagglutinin molecule S1 determinant (aa 110–119) presented in the context of MHC class II IE3.

We have found the TS1 line to be especially well-suited for studies on induced transplantation tolerance for the following reasons: 1) TS1 mice have been backcrossed extensively to the BALB/c parental line and are nearly genetically uniform; 2) a clonotypic Ab (6.5) is available to detect S1-reactive T cells; 3) HA transgenic BALB/c mice are available as a source of HA-expressing tissues for transplantation (20); and 4) a portion of both CD4 and CD8 T cell subsets are specific for S1. In addition, thymic self-tolerance in TS1 mice expressing HA as a “neo-self Ag” has been extensively studied (20, 24, 25) and has revealed the participation of multiple pathways of self-tolerance, including the development of CD4+ and CD25+ T cells that demonstrate potent immunoregulatory activity. We hypothesized that such a population of

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1 This research was supported by Grant RO1AI48820 from the National Institutes of Health. D.J.M. is supported by the American Diabetes Association Physician Scientist Training Grant.

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1 Abbreviations used in this paper: HA, hemagglutinin; SP, single positive; int, intermediate.
immunoregulatory cells might also contribute to the tolerant state induced by central Ag exposure via intrathymic injection of Ag and may provide a valuable component of protocols designed to achieve transplantation tolerance.

**Materials and Methods**

**Animals**

TS1 transgenic mice were created by von Boehmer and colleagues (21), and have been described in detail previously. HA28 and HA104 mice were created by Caton and colleagues (20) and have also been previously described. TS1, HA28, and HA104 transgenic lines are maintained as hemizygotes and repeatedly backcrossed with BALB/c mice (Taconic Farms, Germantown, NY). The progeny of these matings are screened by PCR for the presence of the relevant transgene. All animals are maintained in a sterile pathogen-free environment in the University of Pennsylvania animal facility (Philadelphia, PA).

**Flow cytometric analysis**

Cells were either harvested directly from in vitro culture flasks or were obtained from in vivo experiments by sacrificing animals and harvesting thymus and lymph nodes followed by gentle single-cell suspensions. Cells were washed in cold PBS (Irvine Scientific, Santa Ana, CA), and 1 x 10^6 cells were stained per sample. The following Abs were used for analysis: anti-CD4 PE, anti-CD4 APC, anti-CD8 FITC, anti-CD8 PE, anti-CD25 FITC, anti-CD25-PE, anti-CD45RB PE (BD PharMingen, San Diego, CA), and anti-vp8 FITC (CalTag Laboratories, Burlingame, CA). In addition, 6.5 biotin (21) and Streptavidin-Red670 (Life Technologies) were used to detect the transgenic TCR. Flow cytometric analysis was performed on a FACSscan cytometer (BD Immunocytometry Systems, Rockville, MD). Data acquisition and analysis were accomplished with BD Biosciences CellQuest software (BD Biosciences, Mountain View, CA).

**Peptide synthesis**

All peptides were synthesized by the protein chemistry laboratory of the University of Pennsylvania. All peptide preparations are HPLC purified for >90% purity. The sequences of the S1 and SCR peptides are respectively: H-SFERRFIIPK-COOH and H-FIERKFPPFES-COOH.

**FACS purification of cell populations**

Cells were sorted on a BD FACSVantage SE (BD Biosciences) high-speed cell sorter. The dual laser Vantage is equipped with 5 W argon (Coherent Innova 305; Santa Clara, CA) and mixed gas argon-krypton (Coherent Innova 305; Santa Clara, CA). The progeny of these matings are screened by PCR for the presence of the relevant transgene. All animals are maintained in a sterile pathogen-free environment in the University of Pennsylvania animal facility (Philadelphia, PA).

The proliferation of CD25⁺ and CD25⁻ T lymphocytes was assessed by tritiated thymidine uptake. A total of 10,000 FACS-purified responder cells were cultured in 96-well plates in the presence of 100,000 irradiated (2200 rad) BALB/c splenocytes as stimulator cells and 0.1 μM S1 peptide. [³H]Thymidine was added 18 h before harvesting. Some cultures were supplemented with 5 ng/ml rIL-2 (BioSource International).

**Skin grafting**

Skin grafts were transplanted to mice according to the technique of Billingham and Medawar (27). Mice were anesthetized, and skin grafts were placed in a lateral thoracic position and stabilized with vaseline gauze and band-aids. The bandages were removed on the tenth day. Grafts were scored as rejected when >75% of the grafted tissue area had been lost. In adoptive transfer experiments to assess the graft-reactive potential of sorted cells, animals received 500,000 donor cells in all cases.

**Results**

**Rejection of HA-expressing grafts by TS1 mice**

In our first studies, we evaluated the rejection response of TS1 recipients to HA-expressing skin grafts using two distinct HA transgenic lines as skin graft donors (20, 24). The HA104 and HA28 mice carry the HA transgene controlled by the SV40 promoter that results in diffuse expression that is not tissue-specific. Skin grafts from each of the transgenic lines were rejected by TS1 recipients (Table I). The mean survival times of skin grafts from the two HA lines did not differ significantly. All grafts from each group were rejected by 40 days posttransplant.

To exclude formally a contribution of conventional minor antigenic disparities to rejection in this model, a number of control experiments were performed (Table I). As expected, we found that TS1 skin grafts transplanted to TS1 recipients are consistently accepted indefinitely, documenting that the TS1 line is adequately inbred. BALB/c mice grafted onto TS1 recipients and reciprocal grafts from TS1 to BALB/c are also accepted thus confirming that the TS1 line carries histocompatibility Ags of the BALB/c background. Because the TS1 line is maintained by backcross matings of TS1 transgene hemizygotes with normal BALB/c mice, the transgene negative littermate siblings of the TS1 transgene positive progeny provide ideal controls. Thus, we transplanted HA104 skin grafts to TS1-negative BALB/c littermates (TS1⁻) to assess the need for 6.5 transgenic T cells in HA graft rejection. In no case was rejection observed in this group that has now been followed for >200 days.

The rejection of HA104 skin by TS1 recipients provides a unique and reliable model of rejection for use in transplantation tolerance experiments. Rejection is reproducible and consistent in its evolution. However, the fact that complete rejection of HA104 skin grafts required almost 1 mo on average was somewhat surprising given the high frequency of HA-reactive cells in TS1 hosts. One possible explanation is that transgenic TCR expression leads to such severe perturbations in the T cell repertoire that immune system function is globally compromised (28, 29). To test this possibility, we examined the response of TS1 mice to allogeneic skin grafts that were MHC incompatible (B6 H-2b and C3H H-2k). We found that TS1 mice reject allografts promptly with a tempo similar to that of TS1-negative littermates, indicating the presence of a competent immune system (Table I). Collectively, data from these grafting experiments suggest that the rejection of HA104 skin by TS1 recipients is dependent on both the transgenic T cells of the TS1 recipient and expression of the HA transgene by the donor.

**Induction of transplantation tolerance in TS1 mice by intrathymic inoculation with S1 peptide**

Having established that HA104 skin graft rejection by TS1 recipients is dependent on HA-specific transgenic T cells, we next determined whether these cells could be tolerated by intrathymic exposure to the immunodominant epitope of HA (S1). TS1 mice were transplanted into syngeneic recipients and then treated with 2 × 10⁷ S1 peptide-loaded cells or sham inoculation as described in Table II. Purity checks on the sorted populations ranged from 97–99%.

Stimulation of sorted T lymphocytes

The proliferation of CD25⁺ and CD25⁻ T lymphocytes was assessed by tritiated thymidine uptake. A total of 10,000 FACS-purified responder cells were cultured in 96-well plates in the presence of 100,000 irradiated (2200 rad) BALB/c splenocytes as stimulator cells and 0.1 μM S1 peptide. [³H]Thymidine was added 18 h before harvesting. Some cultures were supplemented with 5 ng/ml rIL-2 (BioSource International).
were inoculated intrathymically with 250 µg of S1 peptide (125 µg/lobe) and grafted with skin from HA104 donors the next day. In control experiments, recipient mice were untreated, treated with S1 peptide i.v., or injected intrathymically with a control peptide (SCR) comprised of the same amino acids present in S1 but scrambled in their order. As detailed in Table I, intrathymic inoculation with S1 peptide led to indefinite survival of HA104 skin grafts. In contrast, prolongation of HA104 skin survival was not observed in untreated controls, TS1 mice injected with S1 i.v., or TS1 mice inoculated intrathymically with SCR. These results indicate a stable state of specific transplantation tolerance to HA is induced by intrathymic S1 injection.

Phenotypic alteration of thymocytes following intrathymic inoculation with S1 peptide

To examine the effect of S1 peptide Ag exposure on developing 6.5+ thymocytes, we inoculated 6- to 8-wk-old TS1 mice with varying doses of S1 peptide directly into both thymic lobes. Thymocytes were isolated 1 wk later and analyzed by three-color flow cytometry. A representative pattern of 6.5 TCR expression in CD4<sup>+</sup>CD8<sup>+</sup> thymocytes in S1 peptide-injected and saline-injected control TS1 mice is show in Fig. 1 with a quantitative summary in Table II. In general, the injection of increasing doses of Ag produced a graded reduction in the intensity of staining with anti-6.5 Ab. The greatest reduction we have detected has been at a dose of 250 µg. Further increases in injected levels did not result in increased reduction in staining intensity. A combination of a decrease in receptor levels and deletion of transgenic T cells may account for the disappearance of 6.5<sup>high</sup> cells. As there was no statistically significant change in the proportion of 6.5<sup>+</sup> T cells even with high dose peptide administration, we favor receptor down-regulation to explain these changes (Table II). To maintain high levels of intrathymic peptide, we have selected a dose of 250 µg for all additional experiments.

To define the period of time that this phenotypic alteration persists following intrathymic peptide administration, we evaluated mice at weekly intervals after treatment. We compared the percentage of CD4 single positive (SP) thymocytes that were 6.5<sup>high</sup> in control untreated or i.v. peptide-treated mice with TS1 mice treated with intrathymic S1 peptide 1–4 wk earlier (Fig. 2). Reduction in expression of high levels of the transgenic TCR on CD4 thymocytes was most pronounced at 1 and 2 wk (p ≤ 0.05 and p = 0.06, respectively, vs control mice), and then progressively returned to baseline at 3–4 wk. TS1 mice treated i.v. with peptide did not reveal statistically significant reductions in 6.5<sup>+</sup>CD4<sup>+</sup> thymocytes (see below). We also observed that CD8 SP thymocytes that have high levels of 6.5 (and are thought to be class II-restricted and S1-specific) are efficiently eliminated (data not shown) (21).

### Table I. Skin graft rejection and tolerance in TS1 recipients

<table>
<thead>
<tr>
<th>Donor</th>
<th>(n)</th>
<th>Recipient (Rx)</th>
<th>Survival Range (days)</th>
<th>Mean Survival Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA transgenic lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA104</td>
<td>(15)</td>
<td>TS1</td>
<td>21–38</td>
<td>26.7</td>
</tr>
<tr>
<td>HA28</td>
<td>(7)</td>
<td>TS1</td>
<td>24–35</td>
<td>28.6</td>
</tr>
<tr>
<td>Histocompatibility controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TS1</td>
<td>(5)</td>
<td>TS1</td>
<td>≥200</td>
<td>≥200</td>
</tr>
<tr>
<td>BALB/c</td>
<td>(6)</td>
<td>TS1</td>
<td>≥200</td>
<td>≥200</td>
</tr>
<tr>
<td>TS1</td>
<td>(6)</td>
<td>TS1-littermate (Balb/c)</td>
<td>≥200</td>
<td>≥200</td>
</tr>
<tr>
<td>HA104</td>
<td>(11)</td>
<td>TS1-littermate (Balb/c)</td>
<td>≥200</td>
<td>≥200</td>
</tr>
<tr>
<td>MHC incompatible grafts</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3H(H-2&lt;sup&gt;k&lt;/sup&gt;)</td>
<td>(4)</td>
<td>Balb/c</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Bl/6(H-&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>(4)</td>
<td>Balb/c</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>C3H</td>
<td>(4)</td>
<td>TS1</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Bl/6</td>
<td>(4)</td>
<td>TS1</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Tolerance following S1 peptide intrathymically</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA104</td>
<td>(8)</td>
<td>TS1 (i.v. S1 peptide)</td>
<td>12–26</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>(8)</td>
<td>TS1 (IT SCR peptide)*</td>
<td>13–30</td>
<td>20.5</td>
</tr>
<tr>
<td></td>
<td>(8)</td>
<td>TS1 (IT S1 peptide)</td>
<td>12–&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

* IT, intrathymic.
Table II. Percentage of cells expressing the transgenic TCR following intrathymic injection

<table>
<thead>
<tr>
<th>Peptide Dose (n)</th>
<th>6.5 Negative</th>
<th>6.5 Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (6)</td>
<td>60.5 ± 4.1</td>
<td>39.3 ± 5.8</td>
</tr>
<tr>
<td>1 µg S1 (2)</td>
<td>55.4 ± 5.2</td>
<td>44.7 ± 5.1</td>
</tr>
<tr>
<td>50 µg S1 (5)</td>
<td>65.2 ± 7.5</td>
<td>34.9 ± 18.4</td>
</tr>
<tr>
<td>100 µg S1 (2)</td>
<td>54.9 ± 12.0</td>
<td>45.2 ± 12.1</td>
</tr>
<tr>
<td>250 µg S1 (11)</td>
<td>72.9 ± 8.0</td>
<td>27.1 ± 11.6</td>
</tr>
</tbody>
</table>

The effect of intrathymic Ag inoculation on peripheral T cells

We next characterized the effect of intrathymic Ag injection on peripheral T cells. To evaluate the effect of peptide that might escape to the periphery during or immediately after thymic injection, we performed parallel experiments in mice receiving an i.v. injection of the S1 peptide. Thus, TS1 mice were inoculated either intrathymically (Fig. 3, left panels) or i.v. (Fig. 3, right panels) with 250 µg of S1 peptide and sacrificed at day 7 for analysis of the thymic (top) and lymph node cells (bottom) for CD4, CD8, and 6.5 expression. We observed a marked reduction in CD4 SP thymocytes that expressed high levels of 6.5 TCR following intrathymic but not i.v. delivery of Ag (compared with naive TS1, solid black line in each). In addition, we observed little change in the pattern of peripheral 6.5 expression from either intrathymic (Fig. 3, lower left panel) or i.v. (Fig. 3, lower right panel) treatment. The lack of peripheral alterations following intrathymic injection of 6.5 suggested that alterations at the thymic level must be exported to the periphery to promote the observed tolerogenic effect. We hypothesized that this conversion of the periphery might be accomplished by intrathymic generation of CD25⁺ regulatory T cells. Our phenotypic analysis of the thymocyte population also suggests this possibility in that TCR down-regulation is characteristic of the periphery to promote the observed tolerogenic effect. We hypothesized that this conversion of the periphery might be accomplished by intrathymic generation of CD25⁺ regulatory T cells. Our phenotypic analysis of the thymocyte population also suggests this possibility in that TCR down-regulation is characteristic of the periphery to promote the observed tolerogenic effect.

The in vitro response of TS1 6.5⁺CD4 T cells to S1 peptide

To assess this hypothesis, we undertook a detailed analysis of the response of peripheral T cells to stimulation with the target Ag S1.

FIGURE 2. Time course of CD4⁺, CD8⁺, 6.5⁺thymocyte deletion. The level of expression of the transgenic TCR in CD4 SP thymocytes was monitored at weekly intervals over 1 mo following peptide injection. Expression levels are diminished during the first 2 wk after treatment but return to normal levels by the third week. Values of p were generated from Student’s t test comparing percentages of 6.5⁺thymocytes between saline-injected controls (black line, n = 6) and intrathymic peptide recipients (gray line, n ≥ 4 at each time point).

FIGURE 3. Central and peripheral effects of intrathymic or i.v. Ag delivery. Among developing thymocytes, intrathymic (A) but not i.v. (B) peptide administrations results in a decrease in the percentage of CD4 SP T cells expressing high levels of 6.5 compared with naive TS1 (black line). In contrast, among peripheral lymphocytes, there is no effect on the percentage of transgenic TCR expressing (6.5⁺) cells following either intrathymic (C) or i.v. (D) treatment. The data shown are representative of five separate experiments.

TS1 lymph node cells were labeled with CFSE and cultured with 5 µM S1 peptide and irradiated syngeneic BALB/c spleen cells as a source of APCs. After 3 days of culture, cells were labeled with anti-CD4 and anti-6.5, and mitotic activity was measured by flow cytometry. The responder precursor frequency was calculated as described previously (26). To assess the response of tolerant mice, TS1 mice were injected intrathymically at 6–8 wk of age with either saline, 50 µg, or 250 µg of S1 peptide and analyzed 1 wk later. Mice injected intrathymically with a high dose of peptide demonstrated an abortive proliferative response in vitro (Fig. 4) as evidenced by a marked reduction in the precursor frequency of cells responding and lack of progression of cells to late division peaks. This pattern, characterized by a limited number of mitotic events and an absence of cells accumulating in later peaks, has been noted previously in the response of T cells which are cocultured with CD25⁺ regulatory cells (20). Thus, despite the absence of gross phenotypic alterations in the peripheral T cells, the response of 6.5 T cells from tolerant mice to S1 peptide in vitro is markedly abnormal.

Generation of immunoregulatory T cells following intrathymic Ag inoculation

To determine whether intrathymic peptide inoculation generates cells with a regulatory phenotype similar to that reported by Jordan et al. (20), we studied thymocytes of naive TS1 control mice and TS1 mice that had been rendered tolerant by intrathymic inoculation with S1 peptide 1 wk earlier. When CD4 SP 6.5⁺ thymocytes were examined selectively, we observed a dramatic increase in the percentage of CD25⁺ cells comparing tolerant with untreated control mice (Fig. 5). This increase was evident as early as 1 wk after injection and CD25 levels returned to normal at 3 wk. Moreover, in TS1 mice injected with S1 peptide intrathymically, the increase in CD25⁺ expression was confined to the 6.5⁺ population of CD4 SP thymocytes, indicating that it was the result of an Ag-specific interaction. Analysis of CD4 SP subpopulations found CD25⁺ cells to be first evident at the early SP stage. No change in the percentage of CD25⁺ T cells was evident in the 6.5⁻ CD4 SP population.
A similar analysis was performed on the peripheral T cells of TS1 mice that had received intrathymic S1 peptide. Paralleling the results observed using thymocytes, we found a significant increase in CD4$^{+}$/H11001, 6.5$^{+}$/H11001 T cells that expressed CD25 compared with control mice (Fig. 6). Again the effect was not observed in the population of peripheral T cells that do not express the transgenic 6.5 TCR. Simultaneous analysis for CD25 and CD45RB found the majority of CD25$^+$ cells to have intermediate staining for CD45RB (data not shown). As was the case for thymocytes, peripheral levels of CD25$^+$ cells also returned to normal levels after 1 mo. However, this finding does not preclude their continued involvement in the protection of the grafted tissue. Recent analyses have suggested that these cells may continue to reside and function within the graft (30); determination of the distribution of these cells throughout the animal following this therapy will require further investigation.

To verify that this cell population demonstrated the functional characteristics previously ascribed to CD25$^+$ regulatory cells, we sorted this cell population and the corresponding CD25$^-$ thymocyte fraction from treated mice and analyzed proliferation of these cells in vitro (18, 20, 24, 31, 32). As illustrated in Fig. 7, the uptake of tritiated thymidine by these cells is greatly diminished in comparison to the CD25$^-$ population. However, the addition of IL-2 to the culture medium enhanced proliferation to supranormal levels. The sorted CD25$^+$ cell fraction is likely to be enriched in 6.5-expressing T cells; this increase in responder cell number may

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Proliferation of 6.5 TCR$^{\text{high}}$ CD4 lymph node cell stimulated in vitro with following intrathymic peptide inoculation. The response of peripheral lymph node cells to S1 peptide was determined with CFSE following intrathymic peptide inoculation. Mice injected with 50 $\mu$g peptide were indistinguishable in their response (dark gray line) from the unmanipulated TS1 (black line). However, mice treated intrathymically with 250 $\mu$g of peptide demonstrated a profoundly reduced peripheral response (light gray line). The response of BALB/c mice to S1 peptide is undetectable given their low precursor frequency for this singular Ag (medium gray line). This figure is representative of three separate experiments. IT, intrathythmic.

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** Development of CD25$^+$ CD4 SP thymocytes in TS1 mice inoculated intrathymically with S1 peptide. At the indicated times after IT peptide inoculation, thymocytes were analyzed for 6.5 and CD25 expression within the CD4 SP pool. The percentage of cells positive for CD25 is indicated for each of three levels of TCR expression.

![FIGURE 6](http://www.jimmunol.org/)

**FIGURE 6.** CD25 expression in 6.5$^{\text{high}}$ and 6.5$^-$, CD4 lymph node T cells from intrathythmic S1-treated and control TS1 mice. The expression of the CD25 cell surface marker was detected on peripheral CD4$^+$ lymphocytes 2 wk after intrathythmic inoculation of S1 (gray line) as compared with saline-injected controls (black line). Among 6.5$^-$, nontransgenic T cells (A), there is no increase in CD25 levels; among transgenic, 6.5-responsive T cells, the frequency of CD25$^+$ cells increased from 6.8 to 21.2%.

![FIGURE 7](http://www.jimmunol.org/)

**FIGURE 7.** Response of sorted thymocytes to peptide stimulation. Thymocytes from animals receiving 250 $\mu$g S1 peptide IT were sorted into CD4$^+$ CD25$^+$ and CD4$^+$ CD25$^-$ fractions by FACS. Sorted cells were stimulated with 0.1 M S1 peptide and proliferation assessed by tritiated thymidine uptake following 3 days of culture. CD25$^+$ cells demonstrate minimal proliferation that is recovered by the addition of IL-2 (5 ng/ml), a phenotype characteristic of CD25$^+$ regulatory cell populations.
account for the augmented response engendered by the addition of IL-2.

**Detection of recent thymic emigrants in the periphery following thymic injection with Ag**

To demonstrate that the CD25\(^+/\), CD4\(^-\), 6.5\(^-\) T cells we identified in peripheral lymph nodes were generated intrathymically rather than as a result of encountering Ag that escaped to the periphery, we tracked the phenotype of recent thymic emigrants following thymic Ag inoculation. To accomplish this analysis, we labeled thymocytes in vivo with CFSE, as described by Graziano et al. (33). With this technique, CFSE-positive T cells could be identified subsequently in peripheral lymphoid organs with \(\sim 2\%\) of peripheral T cells CFSE\(^+\) at 4 and 7 days.

To examine the fate of CD25\(^+\) thymocytes generated by intrathymic S1 peptide inoculation, TS1 mice were injected intrathymically with S1 peptide to provide a peptide dose of 250 \(\mu\)g in 20 \(\mu\)l. Mice were sacrificed either 4 h or 4 days later and thymic and lymph node tissue suspensions prepared and labeled for four-color flow cytometry with CFSE and Abs for CD4, CD8, and 6.5. In lymph node cells, we found that CFSE-positive cells were not detectable at 4 h postinjection but comprised \(\sim 2\%\) of CD4 T cells at 4 days. By analyzing CFSE-positive cells, we were able to characterize the phenotype of T cells that had migrated to the periphery during the prior 4 days. We found that recent thymic emigrants in peptide injected tolerant mice revealed both a relative reduction in 6.5\(^+\) T cells (Fig. 8A) and an increase in the proportion that were CD25\(^+\) (Fig. 8, B and C).

**Characterization of CD4\(^+\), CD25\(^+\) T cell function in vivo**

To investigate the function of tolerant T cells in vivo, we developed an adoptive transfer model of skin graft rejection by transferring TS1 T cells to naive BALB/c hosts grafted with HA104 skin. Lymph node cells or thymocytes from naive or tolerant TS1 mice were transferred to BALB/c host that had been grafted with HA104 skin at least 4 wk earlier. The use of established grafts avoids the nonspecific inflammation accompanying the skin grafting procedure that could override the immunoregulatory effects of CD25\(^+\) cells. As shown in Table III, TS1 lymph node cells (5 \(\times\) 10\(^3\)) transferred to BALB/c hosts with established HA104 skin resulted in the prompt destruction of the skin. Similarly, transfer of thymocytes from TS1 hosts that had received an intrathymic injection with SCR control peptide 1 wk earlier also efficiently mediated rejection of established HA104 skin grafts. In contrast, thymocytes harvested from mice rendered tolerant by intrathymic inoculation with S1 peptide 1 wk earlier did not mediate graft rejection upon transfer to BALB/c hosts. To determine whether the presence of CD4 SP, CD25\(^+\) regulatory T cells was responsible for the lack of rejection, tolerant thymocytes were negatively sorted to remove CD25\(^+\) cells. The resulting population of CD4 SP, CD25\(^-\) thymocytes from intrathymic S1-injected mice mediated efficient graft rejection.

Parallel experiments were performed using lymph node cells from tolerant mice. Lymph node T cells from mice inoculated with S1 peptide 2 wk earlier were sorted into CD4\(^+\), CD25\(^-\), CD45RB\(^{hi}\) and CD4\(^-\), CD25\(^+\), CD45RB\(^{lo}\) fractions. Upon transfer to BALB/c hosts with established HA104 skin, CD25\(^-\) but not CD25\(^+\) cells mediated efficient graft rejection. To rule out the possibility that the lack of rejection observed following transfer of CD25\(^+\) T cells from tolerant mice was due to a lack of cell engraftment, lymph node cells from the recipient mice were analyzed by flow cytometry for 6.5 at 6 wk posttransfer. At this time, 6.5\(^+\) T cells comprised between 1.5–3\% of peripheral lymph node cells and there was no difference between the groups.

<table>
<thead>
<tr>
<th>Cell Donor</th>
<th>Cell Type</th>
<th>HA104 Graft Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>Thymocytes</td>
<td>&gt;200 (\times) 4</td>
</tr>
<tr>
<td>Naive TS1</td>
<td>Thymocytes</td>
<td>22, 26, 26, 26, 26</td>
</tr>
<tr>
<td>TS1 post IT SCR peptide(^a)</td>
<td>Thymocytes</td>
<td>18, 29, 29, 41, 51, 55, &gt;200</td>
</tr>
<tr>
<td>TS1 post IT S1 peptide</td>
<td>Thymocytes</td>
<td>&gt;200 (\times) 6</td>
</tr>
<tr>
<td>TS1 post IT S1 peptide</td>
<td>CD4(^+)CD25(^+) Thymocytes</td>
<td>20, 20, 24, 24, 24, 43, 56, &gt;100 (\times) 2</td>
</tr>
<tr>
<td>Naive TS1</td>
<td>Lymph node cells</td>
<td>17, 17</td>
</tr>
<tr>
<td>TS1 post IT S1 peptide</td>
<td>CD4(^+)CD25(^-) LNC</td>
<td>11, 11, 11, 11, 17, 17, 24, 24, 29 &gt;100</td>
</tr>
<tr>
<td>TS1 post IT S1 peptide</td>
<td>CD4(^+)CD25(^-) LNC</td>
<td>&gt;43, &gt;100 (\times) 10</td>
</tr>
</tbody>
</table>

\(^a\) IT, intrathymic.
Discussion

We have determined that TS1 TCR transgenic mice consistently reject skin grafts from donors expressing an HA transgene. Rejection in this model is dependent both on recipient T cells of the 6.5 TCR\(^+\) clonotype and HA expression by the graft. Transplantation tolerance can be induced in TS1 mice by intrathyMIC inoculation with S1, the immunodominant peptide of HA. The tolerant state is associated with substantial deletion and/or receptor down-regulation of S1-specific transgenic thymocytes but without a substantial reduction in the frequency of peripheral 6.5\(^+\) T cells. Despite this fact, the response of peripheral T cells to the S1 peptide is curtailed, exhibiting a pattern of proliferation that mimics that described in other cases of T cell suppression (34–36). The fact that transplantation tolerance occurs in this model by thymic manipulation but without a significant reduction in peripheral Ag-specific T cells represents an important extension of current work investigating the role of immunoregulation in graft rejection. Although a number of groups have reported the efficacy of CD25\(^+\) cells in prolonging graft survival, the specificity and activation requirements of these cells have heretofore been impossible to study. As these other routes of investigation rely on allograft challenge, knowledge of the role of Ag-reactive cells in the graft response and a definitive indication of immunoregulatory cells among graft-reactive lymphocytes has been lacking. The development of this transgenic model of graft rejection coupled with intrathymic tolerance induction permitted us to address this issue directly and to demonstrate the Ag specificity of the interaction for the first time.

The generation of immunoregulatory T cells may result directly from Ag contact in the thymus at the appropriate stage of thymocyte development. Cells with an immunoregulatory phenotype (CD4\(^+\), CD25\(^+\), CD45RB\(^{hi}\)) migrate from the thymus to the peripheral lymphoid organs within the first 2 wk following tolerance induction and may be responsible for exporting tolerance from the thymus to the periphery. Using an adoptive transfer approach, we demonstrated that unlike T cells from naive TS1 mice, T cells from TS1 mice rendered tolerant by intrathymic S1 peptide inoculation do not mount a productive response to HA104 skin upon transfer to BALB/c hosts. Elimination of CD4\(^+\)CD25\(^-\) T cells from the tolerant population restores a prompt rejection response, confirming a key regulatory role for this population in the tolerant state. These results suggest that, as with tolerance to self Ags, multiple mechanisms contribute to the development of acquired central tolerance following intrathymic inoculation with Ag, and that regulatory cells generated by the tolerant thymus may be primarily responsible for translation of tolerance to the peripheral immune compartment.

Additional insight into the generation of intrathymic tolerance is afforded by a number of transgenic models in which self-tolerance has been studied. A particularly incisive line of investigation resulted from intercrossing TS1 mice with the HA-expressing transgenic lines HA104 and HA28. These transgenic mice lines each express the HA Ag under the direction of the SV40 promoter, but they are distinguished by the amount of Ag produced from this construct. HA104 mice express high levels of the HA Ag in all tissues whereas HA28 mice have been found to express lower levels. When TS1 mice are crossed with HA104 mice, the resulting double transgenic progeny delete the majority of all 6.5-expressing thymocytes (25). In contrast, (TS1 × HA28)F\(_1\) mice delete few of these cells, and rather have a peripheral repertoire characterized by large numbers of 6.5\(^+\) CD4 T cells with an immunoregulatory phenotype (CD25\(^-\) CD45RB\(^{hi}\)) (20). Almost 50% of 6.5\(^{hi}\) lymph node cells from (TS1 × HA28)F\(_1\) mice express CD25 and an intermediate level of the CD45RB marker. Functional analysis of these CD25\(^+\), CD4\(^+\), 6.5\(^+\) T cells reveals not only a lack of responsiveness to the S1 peptide but also potent regulatory activity. In cell mixing experiments, CD25\(^+\), CD4\(^+\) T cells suppressed the response of naive 6.5\(^+\) CD4 TS1 T cells to the S1 peptide even under conditions of more than a 10-fold excess of naive cells. The functional activity and phenotypic characteristics of the CD4\(^+\), CD25\(^+\) T Cells described by Jordan et al. (20) are similar in many ways to those we have identified in a model of acquired central transplantation tolerance. Whether they constitutively express cell surface CTLA4 or TGF-\(\beta\) as has been described for other immunoregulatory T cells is under study (31, 37).

These additional models of central tolerance support the hypothesis that avidity is a primary determinant of Ag-specific cell fate following intrathymic peptide inoculation. Our current work provides further understanding of earlier findings using the intrathymic tolerance model. In virtually all of the initial transplant investigations of intrathymic tolerance induction, treatment of the recipient with a peripheral T cell-depleting agent such as anti-lymphocyte serum or anti-CD4 was required to achieve tolerance to a subsequent tissue graft. However, more recently, a growing number of cases have been reported in which peripheral T cell depletion has been found to be unnecessary (12–14, 38–42). Interestingly, in each case, either purified protein preparations or synthetic peptide, but not donor cells, has been used as the inoculated Ag. Although the immunological mechanisms underlying these apparently contradictory findings are unclear, it is tempting to speculate that peptide preparations may promote lower avidity interactions that generate CD25\(^+\) regulatory T cells while inoculated cells which often repeat the same determinant may fall at a higher end of the avidity spectrum.

The TS1 TCR model provides a unique opportunity for dissection of the events promoting rejection and tolerance to allografts. However, a recurring concern in using a TCR transgenic host for such studies is whether in the setting of an overabundance of a single clone of T cells, the response under study still recapitulates a physiological response. In fact, our observation that TS1 hosts reject MHC disparate allografts in 10 days compared with an average of 28 days for HA104 graft rejection was unexpected in light of the fact that the precursor frequency of T cells reactive for the allogeneic donor is likely to be many fold lower than the frequency of HA-specific T cells in TS1 recipients. Although differences in Ag density (HA vs Allo-MHC) on the graft could contribute to this result, an alternate hypothesis is that rejection of HA grafts by TS1 hosts is impeded by too high a frequency of responding T cells (43). In support of the latter possibility is our finding that HA104 graft rejection is typically more rapid following transfer of a small number of TS1 T cells to BALB/c hosts than in naive TS1 recipients.

Intrathymic inoculation with Ag may provide a unique opportunity to study the generation of immunoregulatory T cells. As far as we are aware, this technique is the first reported to permit induction of such cells in the thymus of adult mice. This innovation should permit further manipulations that define the events leading to the pathway of clonal deletion vs anergic regulatory T cell induction. Generation of transplantation tolerance based on immunoregulatory T cells may provide an ideal means to maintain graft survival without the need for chronic immunosuppression.

In summary, the current results indicate that immunoregulatory T cells may play a critical role in the generation and maintenance of acquired central transplantation tolerance. Our results delineate a simple means both to generate immunoregulatory T cells in normal adult mice by central tolerance induction and provide a relevant assay for their function in vivo, in the form of regulation of a response to an allograft. Further study of their mechanism of
action and the events that determine whether a developing T cell will undergo deletion or will assume an anergic regulatory cell role, has the potential to define new strategies to induce stable transplantation tolerance.

References