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J Immunol 2003; 170:1240-1248; doi: 10.4049/jimmunol.170.3.1240
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Role for IL-10 in Suppression Mediated by Peptide-Induced Regulatory T Cells In Vivo

Anette Sundstedt, Emma J. O’Neill, Kirsty S. Nicolson, and David C. Wraith

Regulatory CD4+ T cells were induced in the Tg4 TCR transgenic mouse specific for the N-terminal peptide (Ac1-9) of myelin basic protein by intranasal administration of a high-affinity MHC-binding analog (Ac1-9[4Y]). Peptide-induced tolerant cells (PItol) were anergic, failed to produce IL-2, but responded to Ag by secretion of IL-10. PItol cells were predominantly CD25- and CTLA-4+ and their anergic state was reversed by addition of IL-2 in vitro. PItol cells suppressed the response of naive Tg4 cells both in vitro and in vivo. The in vitro suppression mediated by these cells was not reversed by cytokine neutralization and was cell-cell contact-dependent. However, suppression of proliferation and IL-2 production by PItol cells in vivo was abrogated by neutralization of IL-10. These results emphasize an important role for IL-10 in the function of peptide-induced regulatory T cells in vivo and highlight the caution required in extrapolating mechanisms of T regulatory cell function from in vitro studies. The Journal of Immunology, 2003, 170: 1240–1248.

Immunological tolerance is a state of unresponsiveness that is specific for a particular Ag. Active tolerance mechanisms are required to prevent inflammatory responses to the many innocuous airborne and food Ags that are encountered at mucosal surfaces in the lung and gut. Tolerance to self is required to prevent autoimmune disease and this largely involves the eradication of cells bearing self-reactive receptors. Many self-reactive T cells are deleted through negative selection in the thymus (1), but it is now clear that this process is imperfect. As a result, cells specific for self Ags, such as myelin basic protein (MBP), are commonly found in the peripheral blood of healthy individuals (2–4), despite expression of such proteins in the thymus (5, 6). The question arises as to how these cells can appear in the normal T cell repertoire without triggering autoimmune disease. First, these cells tend to recognize their Ag with low avidity and are unlikely to become activated under normal circumstances (7). Furthermore, regulatory cells suppress the activity of autoreactive cells and hence maintain a disease-free state (8, 9). The best evidence for such regulatory cells comes from mice transgenic for TCRs specific for self-Ags such as MBP. We and others (10) have noted that these mice suffer from spontaneous experimental autoimmune encephalomyelitis (EAE) when bred onto the recombination-activating gene (RAG)-deficient (RAG−/−) background. Regulatory cells are found in the peripheral lymphoid tissues of RAG+/− mice and lie within both the CD25− and CD25+ populations. These cells clearly depend on rearrangement of their endogenous TCR chains for their generation. Further evidence points to the general importance of the population of CD4+ CD25+ cells (9) that appear to be naturally generated during thymic selection (11). In addition, cells of the CD45RBlow phenotype have been shown to serve as regulators in models of disease transfer in lymphopenic recipients (8).

There is now clear evidence that Ag-specific regulatory cells can be induced by appropriate administration of Ags or their fragments. Mucosal administration has been a favored route largely because the immune system has evolved mechanisms to tolerate the presence of Ags encountered at these surfaces. Mucosal administration of intact proteins tends to induce TGF-β secreting T (Th3) cells that are thought to have evolved to help IgA production (12). Th3 cells specific for myelin Ags have been shown to suppress autoimmunity in models of autoimmune encephalomyelitis. In addition, the repeated administration of superantigens in suitable transgenic mice leads to the generation of IL-10-secreting cells that have a regulatory function in vitro (13, 14). These cells may be analogous to T regulatory-1 cells that may be generated by repeated Ag stimulation in the presence of excess IL-10 (15) or vitamin D3 and dexamethasone (16) in vitro.

Previous work has shown that intranasal (i.n.) administration of peptides recognized by CD4+ T cells can induce peripheral tolerance to self Ags (17–20). Intranasal administration of peptides could even induce tolerance in mice transgenic for an Ag-specific TCR (21). In this study, we characterize the cells arising from tolerance induction in the Tg4 transgenic model and demonstrate that peptide-induced regulatory cells suppress the response of naive T cells in vitro and in vivo. The suppressive properties of these cells are dependent on IL-10 for the suppression of T cell responses in vivo.

Materials and Methods

Reagents

mAbs directed to murine CD25, CD28, CD38, CD45RB, and CTLA-4 were purchased from BD PharMingen (San Diego, CA). The polyclonal antiserum specific for mouse glucocorticoid-induced TNFR (GITR) and the inducible costimulator protein (ICOS)-Fc fusion protein were purchased from R&D Systems (Abingdon, U.K.). The mAb specific for ICOS-l was kindly provided by Dr. A. Coyle (Millenium, Boston, MA).
The anti-IL-10R Ab (1B1.3a, rat IgG1) was kindly provided by Dr. F. Powrie with permission from DNAX (Palo Alto, CA). For in vivo experiments, this Ab was purified into endotoxin-free PBS (Life Technologies, Paisley, U.K.), filter sterilized, and stored at −20°C before its use. Control rat IgG was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). CFSE was purchased from Molecular Probes (Leiden, Holland).

Peptides

The acetylated N-terminal peptide of murine MBP (Ac1-9, AcASQKRPSQR) and the high MHC-affinity analog with a tyrosine substituting the wild-type lysine in position 4 (Ac1-9[4Y]) were synthesized using standard fluorenyl methoxycarbonyl chemistry on an AMS 422 multiple peptide synthesiser (Abimed Analyes-Technik, Langenfeld, Germany). For in vivo experiments, peptides were purified into endotoxin-free PBS (Life Technologies), filter sterilized, and stored at −20°C before use.

Animals and treatments

The Tg4 transgenic mouse expresses TCR α- and β-chains derived from the 1934.4 T cell hybridoma specific for the N-terminal, acetylated CD4+ T cell epitope of MBP (Ac1-9). This mouse strain was established as previously described (22) and back-crossed onto the B10.PL (H-2u) background. Mice were primed by a single i.n. dose of 100 µg Ac1-9[4Y]. Tolerance was induced by giving 10 doses of Ac1-9[4Y] at regular intervals over a period of 5 wk.

Cell separation

Spleens were taken from mice treated i.n. with Ac1-9[4Y] at different times before analysis. Purified CD4+ T cells (>95% CD4+ as determined by FACS analysis) were obtained by positive selection using magnetic beads coated with anti-CD4 mAb (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions.

CD4 T cell proliferation assays

Three days after the last Ag treatment, spleens were harvested and CD4 T cells were purified by positive selection as described. CD4+ T cells were cultured in IMDM medium supplemented with 5 × 10−5 M 2-ME, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Life Technologies), and 5% FCS (Sigma-Aldrich, Poole, U.K.) at 5 × 105 cells/well at 37°C in the presence of different concentrations of Ac1-9. Irradiated CD4-depleted splenocytes at 1 × 105 cells/well were used as APCs. After indicated times, cells were pulsed with 0.5 µCi [3H]thymidine overnight and the incorporation of radioactivity was measured on a liquid scintillation beta-counter (1450 Microbeta; Wallac, Milton Keynes, U.K.).

Transwell experiments

CD4+ T cells were cultured in IMDM medium at 5 × 105 cells/well in 24-well plates in the presence of 10 µg/ml of Ac1-9. Irradiated CD4-depleted splenocytes at 5 × 105 cells/well were used as APCs. In some of the cultures, the different CD4+ populations were separated by a semipermeable membrane (Falcon Transwell; BD Biosciences, Bedford, MA). When Transwells were used, APC and peptide were added to both chambers.

Cytokine protein levels

Blood samples, drawn 2 h after i.n. treatment of mice with Ac1-9[4Y], or cell culture supernatants were tested for cytokine content by specific ELISAs (BD PharMingen) according to instructions from the manufacturer.

FACS analysis (flow cytometry)

Cells were stained by direct immunofluorescence for two-color flow cytometry. Data were analyzed using CellQuest software (BD PharMingen).

CFSE staining and cell transfer

Labeling of naive and tolerant splenocytes with CFSE was performed as described previously (23). A total of 5 × 107 CFSE-labeled splenocytes were transferred i.v. into the tail vein of naive or tolerant syngeneic recipients. One day after the transfer, mice received one i.n. treatment of Ac1-9[4Y] or PBS, and T cell proliferation was monitored 2 days later in the spleen. Some mice received 0.5 mg anti-IL-10R Ab or isotype control IgG by i.p. injection 2 h before the peptide. Chimerism of CFSE- T cells in the spleen of recipient mice varied between 1 and 3% in all instances.

Tolerant cell transfer

Splenocytes (5 × 107 cells) from naive or tolerant mice were transferred by i.p. injection into recipient Tg4 mice. Some mice received 0.5 mg anti-IL-10R Ab by i.p. injection after 24 h. All mice were challenged with Ac1-9[4Y] (100 µg) i.n., and serum samples were obtained under terminal ether anesthesia after a further 2 h.

Statistical analysis

For statistical analysis, a Mann-Whitney U test was used for nonparametric data.

Results

Pltol are anergic but display an altered cytokine profile

Splenocytes from the Tg4 TCR transgenic mouse respond spontaneously to challenge with the N-terminal Ac1-9 peptide of MBP in vitro (22). Previous work has shown that repeated administration of the high-affinity, MHC-binding analog of Ac1-9 (Ac1-9[4Y]) leads to profound unresponsiveness among Tg4 T cells (21). This was confirmed by the experiments shown in Fig. 1. Fig. 1, A and B, compares the proliferation of splenocytes from either naive mice, mice primed by a single i.n. dose of Ac1-9[4Y], or mice rendered tolerant by 10 i.n. doses of Ac1-9[4Y]. Primed Tg4 cells responded more rapidly (Fig. 1A) and at a lower dose of Ag (Fig. 1B) when compared with naive cells. However, peptide-induced tolerant (Pltol) Tg4 cells failed to proliferate in response to Ac1-9.

The anergic phenotype of Pltol cells could be explained if these cells were unable to secrete IL-2. Comparison of tolerant and naive cells showed that Pltol cells failed to secrete significant amounts of IL-2 when compared with naive cells (Fig. 1C). However, as shown previously (21), Pltol cells secreted IL-10 at high levels when compared with naive cells (Fig. 1D). Pltol cells only secreted significant amounts of IL-10 when stimulated with the Ac1-9 peptide Ag. Thus, although these cells were apparently anergic, they retained the capacity to respond to Ag when stimulated through the TCR but only by production of IL-10. As demonstrated previously (21), Pltol cells did not secrete measurable amounts of IL-4, IL-5, or TGF-β, and only produced marginal levels of IFN-γ (data not shown).

Cell surface phenotype of Pltol cells

The cell surface phenotype of naive Tg4 cells was compared with that of primed and Pltol cells. The expression of various markers was measured 3 (CD25, CD38, CD45RB) or 9 days (CTLA-4, ICOS-1) after administration of Ag. Priming of Tg4 cells by administration of Ac1-9[4Y] did not significantly increase the proportion of CD25+ cells within the CD4 subset (Table I). However, the proportion of CD25+ cells doubled in Pltol Tg4 mice, although still at <10% of the total CD4+ population. A similar doubling of the proportion of CD38+ cells was noted in Pltol mice. The proportion of CD45RB+ cells did not increase significantly in Pltol when compared with naive mice. By contrast, both priming and tolerance induction led to a marked increase in the proportion of both CTLA-4- and ICOS-1-positive cells. Expression of these markers was measured at a time point when any transient changes following T cell activation would have subsided (24). The proportion of CTLA-4+ cells increased 10-fold after a single administration of peptide and increased further to reach 63% in Pltol mice.

Pltol cells suppress activation of naive Tg4 cells in vitro: role of cytokines in suppression

Naive Tg4 cells were stimulated in vitro either alone or in the presence of an equal number of Pltol cells. As shown in Fig. 2A, naive Tg4 cells proliferated well in response to Ac1-9, whereas Pltol cells failed to respond. Addition of an equal number of Pltol...
FIGURE 1. Proliferation and cytokine production by CD4 T cells from either naive, primed, or tolerant Tg4 mice. Mice were either primed by a single i.n. dose or rendered tolerant by 10 i.n. doses of Ac1-9[4Y]. Naive mice received 10 i.n. doses of PBS (untreated controls). Splenocytes were harvested 3 days after the final treatment and CD4+ T cells were purified. CD4+ T cells were cultured at 5 × 10^4 cells/well with 1 × 10^5 cells/well of irradiated CD4-depleted splenocytes in vitro with 10 μg/ml Ac1-9[4K] unless otherwise indicated. After 3 days, unless otherwise stated, supernatants were harvested for cytokine ELISA (C and D) or cultures pulsed with [3H]thymidine (A and B). The results are representative of groups of at least three mice per treatment group and the experiment was repeated four times with similar results. Error bars indicate the SEM of triplicate cultures.

IL-2 overcomes the in vitro suppression of Tg4 cells by PItol cells

Addition of IL-2 alone induced a low level of proliferation in both naive (Δcpm = 13,184, stimulation index = 21.0) and tolerant (Δcpm = 16,204, stimulation index = 27.7) CD4+ T cells. However, simultaneous addition of Ag to the lower chamber of a transwell led to the secretion of high levels of IL-2 from naive Tg4 cells. However, this was totally suppressed by addition of an equal number of PItol cells to the lower chamber of a transwell, thus separating the cells from naive effectors by a semipermeable membrane. We believe that the reduced level of IL-2 measured in the upper chamber of a transwell culture chambers were used to test whether cell-cell contact would be necessary for effective suppression of naive Tg4 cells by PItol cells in vitro. Secretion of IL-2 was used as the readout for activation of naive T cells. As shown in Table II, addition of Ag to the lower chamber of a transwell led to the secretion of high levels of IL-2 from naive Tg4 cells. However, this was totally suppressed by addition of an equal number of PItol cells to the lower chamber of the culture. Despite secretion of high levels of IL-10 by the tolerant cells (Fig. 1D), the PItol cells, although optimally stimulated with peptide, were not capable of effective suppression when placed in the upper chamber of a transwell, thus separating the cells from naive effectors by a semipermeable membrane. We believe that the reduced level of IL-2 measured in the

cells led to a substantial (93%) reduction in the proliferative response of naive Tg4 cells at the optimal dose of Ac1-9 (10 μg/ml). Because PItol cells secrete high levels of IL-10, it might be predicted that this cytokine would be responsible for this suppression. However, addition of excess anti-IL-10R Ab failed to significantly reverse suppression. A similar limited effect on suppression was seen by addition of an anti-TGF-β Ab, while the two Abs together had a marginally additive effect. Thus, although both IL-10 and TGF-β may contribute to the in vitro suppression of naive cell proliferation by PItol cells, they are certainly not sufficient. Other mechanisms must be involved in this process.

**Table I. Phenotypic characteristics of naive, primed, and PItol CD4+ T cells**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Naive Frequency (%)</th>
<th>Primed Frequency (%)</th>
<th>PItol Frequency (%)</th>
</tr>
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<tbody>
<tr>
<td>CD25^+</td>
<td>4.1 ± 0.8 (n = 11)</td>
<td>5.4 ± 3.0 (n = 12)</td>
<td>8.7 ± 3.4 (n = 16)</td>
</tr>
<tr>
<td>CD38^+</td>
<td>21.1 ± 6.7 (n = 9)</td>
<td>33.3 ± 7.8 (n = 8)</td>
<td>48.0 ± 4.0 (n = 12)</td>
</tr>
<tr>
<td>CD45RBlow</td>
<td>9.1 ± 2.5 (n = 9)</td>
<td>4.0 ± 1.9 (n = 9)</td>
<td>16.1 ± 6.6 (n = 13)</td>
</tr>
<tr>
<td>CTLA-4^+</td>
<td>2.7 ± 0.8 (n = 6)</td>
<td>24.3 ± 5.1 (n = 5)</td>
<td>62.9 ± 13.5 (n = 9)</td>
</tr>
<tr>
<td>ICOS-1^+</td>
<td>1.9 ± 0.6 (n = 4)</td>
<td>8.5 ± 3.2 (n = 6)</td>
<td>15.8 ± 6.0 (n = 6)</td>
</tr>
</tbody>
</table>

^a Expression of different markers among gated CD4+ T cells was measured at day 3 after last treatment. CTLA-4 and ICOS-1 expression was measured at day 9. Results are presented as mean values ± SE.
Table II. Suppression mediated by Ptol cells is cell-cell contact-dependent

<table>
<thead>
<tr>
<th></th>
<th>Without Ag IL-2 (pg/ml)</th>
<th>With Ag IL-2 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive Tg4 cells</td>
<td>55</td>
<td>2386</td>
</tr>
<tr>
<td>Tolerant Tg4 cells</td>
<td>56</td>
<td>57</td>
</tr>
<tr>
<td>Naive + tolerant (1:1)</td>
<td>57</td>
<td>61</td>
</tr>
<tr>
<td>Naive + tolerant (1:1) (Transwell)</td>
<td>57</td>
<td>1562</td>
</tr>
<tr>
<td>APC alone</td>
<td>57</td>
<td>57</td>
</tr>
</tbody>
</table>

*CD4+ T cells were cultured as described in Materials and Methods. Day 3 of culture, supernatants were harvested and analyzed for IL-2 content by ELISA. Results are presented as mean values from cultures containing pooled cells from at least two mice. SDS were routinely <10% of the mean. The experiment was repeated three times with similar results.

FIGURE 2. The influence of cytokines on the in vitro suppression of naive Tg4 cells by Ptol cells. Splenocytes from naive (10 i.n. doses of PBS) or tolerant mice (10 i.n. doses of Ac1-9[4Y]) were collected 3 days after the final treatment and CD4+ T cells were purified. CD4+ T cells were cultured at 5 \times 10^5 cells/well with 1 \times 10^7 cells/well of irradiated CD4-depleted splenocytes and varying concentrations of Ac1-9[4K]. Mixed cultures contained 5 \times 10^4 naive and tolerant cells. A, Naive and tolerant CD4+ cells were cultured with or without the indicated Abs at 10 \mu g/ml. B, Naive and tolerant CD4+ cells were cultured with or without IL-2 at 20 U/ml. Proliferation was assessed by [3H]thymidine incorporation (cpm) on day 4. Error bars indicate the SEM of triplicate cultures. The experiment was repeated three times with similar results.

FIGURE 3. In vitro suppression of naive Tg4 cells by Ptol cells is partly reversed by CD28 stimulation. Splenocytes from naive (10 i.n. doses of PBS) or tolerant mice (10 i.n. doses of Ac1-9[4Y]) were collected 3 days after the final treatment and CD4+ T cells were purified. CD4+ T cells were cultured at 5 \times 10^5 cells/well with 1 \times 10^7 cells/well of irradiated CD4-depleted splenocytes and 10 \mu g/ml Ac1-9[4K]. Mixed cultures contained 5 \times 10^5 naive and tolerant CD4+ cells and were cultured with or without the indicated Abs and fusion proteins at 10 \mu g/ml. IL-2 production was assessed by specific ELISA on day 3. Error bars indicate the SEM of triplicate cultures. The experiment was repeated three times with similar results.

The role of costimulatory molecules in suppression of naive Tg4 cells by Ptol cells in vitro

Various cell surface molecules have been implicated in regulation of CD4 T cell growth and some have been shown to play a role in the function of regulatory T cells. Thus, various costimulatory pathways could contribute to the suppressive properties of Ptol cells in vitro. Either Abs or fusion proteins specific for costimulatory pathways were introduced into cocultures of naive Tg4 and Ptol cells in vitro. As shown in Fig. 3, Abs to GITR did not have a significant effect on either naive Tg4, Ptol cells, or their cocultures. This is in contrast to the functional importance of the GITR receptor in suppression mediated by the CD4+CD25+ T cell subset (25, 26). Parallel experiments in this laboratory have shown that this reagent will abrogate the suppressive properties of IL-10-producing Tg4 cells grown in the presence of vitamin D3 and dexamethasone (M. Harber, unpublished observations) according to the method of Barrat and coworkers (16). The ICOS-Fc fusion protein did not affect IL-2 production by either the Ptol cells or their cocultures with naive Tg4 cells. If anything, the fusion protein reduced IL-2 production by naive Tg4 cells. Abs to CTLA-4 had no effect on the response of Ptol cells, but significantly increased the response of naive cells. Therefore, the minor reversal of suppression achieved by addition of anti-CTLA-4 to the cocultures may be accounted for by the effect of this Ab on the Ag-specific response of naive cells rather than a direct effect on Ptol cells. Anti-CD28 Abs significantly increased the response of naive but not tolerant Tg4 cells to Ag in vitro. The level of suppression of naive T cell activation mediated by the Ptol cells was also significantly reduced by addition of anti-CD28 Abs even though the additional costimulation had no effect on tolerant cells alone. Therefore, this significant reversal of suppression in cocultures by anti-CD28 could be accounted for by the direct effect of anti-CD28 on the naive T cell population. Alternatively, it could be that anti-CD28 affects tolerant cells so that they lose their suppressive capacity without restoring their ability to produce IL-2. Either way these results demonstrate that the suppressive effect of Ptol Tg4 cells on naive cell activation, mediated by cell-cell contact, may be overcome by a dominant costimulatory signal through CD28.
PI-tol cells suppress the proliferation of CFSE-labeled Tg4 cells in vivo

We wished to establish models of T cell-mediated suppression in vivo that would avoid any manipulation of cells in vitro. The first of these involved the transfer of CFSE-labeled Tg4 cells into either naive or tolerant Tg4 mice. Two populations of splenocytes obtained from either naive or PI-tol Tg4 mice were labeled with CFSE and transferred into either naive or PI-tol recipients by i.v. injection with each mouse receiving $5 \times 10^7$ cells. One day after cell transfer, the recipient mice were challenged with a single dose of Ac1-9[4Y] by the i.n. route. A single mouse in each group was left untreated to determine baseline CFSE levels. The spleens from these recipient mice were harvested on day 3 after transfer, disaggregated to yield a single-cell suspension, and counterstained with anti-CD4-tricolor Ab. FACS analysis of these splenocyte populations was performed gating on the CD4-tricolor, CFSE double-positive cells. Fig. 4A shows the response of naive cells transferred into naive recipients; the cells divided readily in response to Ag with only 14% remaining undivided. Fig. 4B shows that the same cells transferred into PI-tol recipients also divided; however, a greater proportion of these cells remained undivided (50%) or underwent lower numbers of divisions. Fig. 4C and D shows the results of equivalent transfers of CFSE-labeled PI-tol cells. There was no measurable evidence of cell division when tolerant cells were transferred into tolerant recipients (Fig. 4D). However, this same population of tolerant cells did divide to some extent when transferred into naive recipients (Fig. 4C). However, a large proportion of the cells (66%) remained undivided.

The role of IL-10 in suppression of proliferation of naive Tg4 cells in vivo

The CFSE-labeled cell transfer model was used to investigate the role of IL-10 in mediating suppression in vivo. Splenocytes from naive Tg4 mice were labeled with CFSE and then transferred into either naive or PI-tol recipients by i.v. injection ($5 \times 10^7$ cells/mouse). One day after cell transfer, the recipient mice were challenged with a single dose of Ac1-9[4Y] by the i.n. route and splenocytes analyzed by FACS after a further 48 h. Fig. 5A shows the CFSE staining of naive cells transferred into naive recipients. In this experiment, only 13% of cells remained in the undivided fraction after 48 h. The CFSE staining of naive cells transferred into PI-tol recipients (Fig. 5B) shows that transfer of naive cells into a PI-tol recipient-suppressed proliferation of the CFSE-labeled naive cells with 59% of transferred cells remaining in the undivided fraction. Fig. 5C shows the CFSE staining of naive cells transferred into tolerant recipients then treated with anti-IL-10R Ab 2 h before antigenic challenge. The suppression of proliferation was significantly reversed when IL-10 was neutralized (Fig. 5C). PI-tol mice treated with control IgG showed a comparable level of suppression as the PI-tol recipients albeit slightly reduced (Fig. 5D). Taken together these observations emphasize the role of IL-10 in the maintenance of tolerance in PI-tol mice. The fact that an anti-IL-10R Ab can reverse suppression in vivo differs significantly from the lack of effect of the same Ab on suppression measured in vitro.

Suppression of IL-2 production by transfer of PI-tol cells in vivo

We have investigated the use of a second in vivo measure of T cell activation. A time course of cytokine secretion was established in naive Tg4 mice treated with an i.n. dose of Ac1-9[4Y] and this demonstrated that high serum levels of IL-2 could be measured peaking at the 2 h time point (data not shown). This assay was used to confirm the observation that PI-tol cells do not secrete IL-2 and to test whether suppression of IL-2 production could be observed following transfer of PI-tol cells into naive Tg4 mice. Splenocytes were transferred on day 0 by i.p. injection from either naive (control transfer) or PI-tol (tolerant transfer) mice into two groups of
naive Tg4 recipients. These recipient mice were then challenged with peptide, delivered by the i.n. route on day 2, and serum samples were obtained 2 h later. Two additional groups were included as controls: a group of naive mice that were primed with only a single dose of peptide and a PItol group that received 10 doses of peptide and were given the final dose 2 h before the collection of serum. The results (Fig. 6A) demonstrate that the suppression of IL-2 production among PItol cells in vitro is also maintained in vivo. Furthermore, the transfer experiment demonstrated that PItol cells were capable of transferring suppression of IL-2 production by naive Tg4 cells in vivo. The results of the control and tolerant cell transfer groups were compared using the Mann-Whitney U test demonstrating that the medians of these two groups were significantly different (p = 0.0079).

**FIGURE 5.** Suppression of proliferation of naive Tg4 T cells in PItol mice is reversed by neutralization of IL-10. Splenocytes obtained from naive mice were labeled with CFSE and 5 x 10^7 cells transferred into either naive or PItol recipients by i.v. injection. Recipient mice received a single dose of Ac1-9[4Y] i.n. after 24 h. Splenocytes were harvested 48 h later and counter stained with anti-CD4-tricolor Ab. FACS analysis was performed gating on live CD4-tricolor, CFSE double-positive cells. A, Naive cells transferred into naive recipient. B, Naive cells transferred into PItol recipient. C, Naive cells transferred into PItol recipient injected with anti-IL-10R Abs. D, Naive cells transferred into PItol recipient injected with control IgG. Percentages indicate the fraction of cells in the nondividing peak (M1). The results are representative examples of experimental groups containing three mice. The experiment has been repeated three times with similar results.

**FIGURE 6.** Transfer of tolerance with spleen cells from PItol mice is IL-10-dependent. Splenocytes (5 x 10^7 cells) from PItol or naive mice were transferred by i.p. injection into recipient Tg4 mice. Mice were rested for 48 h before antigenic challenge. Further controls included naive and PItol Tg4 mice. A, All mice received a single dose of Ac1-9[4Y] i.n. and serum samples were obtained 2 h later and analyzed for IL-2 content. B, Recipients of transferred cells received either anti-IL-10R Abs or control IgG. The results shown are the mean serum IL-2 levels for each group of at least three mice with error bars depicting the SEM.

**Discussion**

IL-10-producing T cells have been ascribed a role in the regulation of immune responses to both self Ags and infectious pathogens (27). Although IL-10 was once thought to be a cytokine produced only by Th2 cells, there is now evidence that a variety of both CD4
and CD8 cells secrete IL-10. IL-10-producing CD4+ cells play a significant role in preventing the immune pathology associated with the immune response to infection (28). Thus, IL-10−/− mice suffer severe inflammatory responses to such pathogens as *Listeria monocytogenes* (29), *Plasmodium chabaudi* (30), and *Toxoplasma gondii* (31). Interestingly, these IL-10-producing cells also tend to secrete IFN-γ rather than the cytokines normally associated with Th2 cells (28). Evidence that IL-10 can also play a role in protecting against autoimmune disease first arose through the work of Powrie and colleagues (32). They showed that protection from inflammatory bowel disease, following transfer of CD45RBlow cells, could be inhibited with anti-IL-10R Abs in lymphopenic recipients of CD45RBhigh cells. This observation is consistent with the fact that IL-10−/− mice are predisposed to inflammatory bowel disease.

Recent observations have revealed that IL-10-secreting cells may be induced both in vitro and in vivo. Culture of naïve T cells in the presence of Ag and IL-10 leads to the generation of IL-10-secreting T cells (T regulatory-1 cells) capable of inhibiting inflammatory conditions when transferred back into the intact animal (15). These cells appear to be distinct from the IL-10/IFN-γ-producing subset in that they produce IL-10 and IL-5 but only low levels of IFN-γ. Furthermore, there is evidence that cells with a similar cytokine profile can be induced in vivo. Thus, repeated administration of the superantigen staphylococcal enterotoxin A in TCR Vβ3 transgenic mice led to the generation of CD4+ cells that secreted IL-10 but failed to produce significant amounts of IL-2, IFN-γ, or TNF-α (13). Cells rendered tolerant by in vivo administration of superantigen were profoundly unresponsive and did not respond to Ag in the presence of IL-2 (14), a normal characteristic of anergic CD4+ T cell clones (33). Similarly, oral administration of IL-10 and Ag generated lymphocytes producing decreased amounts of IL-2 and IFN-γ and increased amounts of IL-10 and TGF-β (34).

Previous studies from this laboratory showed that i.n. administration of peptide Ags could induce a state of tolerance in non-transgenic mice (17, 35). A similar state of tolerance could be induced in the Tg4 TCR transgenic mouse and the tolerant cells failed to secrete IL-2 but produced high levels of IL-10 (21). Recent studies have emphasized the role of CD4+CD25+ cells in immune regulation (36, 37), but questions have arisen as to whether these cells function by secreting cytokines such as IL-10 and TGF-β (9). Therefore, to further characterize the PItol cells, we have analyzed their cell surface phenotype. CD4+ T cells from the tolerant Tg4 mouse were predominantly CD25+. This alone distinguishes these cells from the “natural”, thymus-derived CD25+ population of regulatory/suppressor T cells. The most notable feature of the PItol cells was their expression of CTLa-4. Previous work from this laboratory showed that i.n. administration of the Ac1-9[4Y] peptide in Tg4 mice led to a rapid up-regulation of both CD28 and CTLa-4 (24). Although the cell surface expression of CD28 returned to baseline within 6 days, a significant proportion of CD4+ (10–20%) expressed CTLa-4 10 days after peptide administration. The fact that ~63% of PItol cells expressed CTLa-4 at stable levels in this study indicates that tolerance induction correlates with an increase in the proportion of CTLa-4+ cells. This peptide-induced tolerance model is a further example of various models of peripheral tolerance in which tolerance correlates with expression of CTLa-4 (38–40). Previous studies had shown that anti-CTLa-4 Abs would interfere with tolerance induction in vivo (41, 42). More recent studies have revealed that CTLa-4−/−CD4+ T cells were resistant to the induction of tolerance by high dose i.v. peptide (43). CTLa-4 plays a role in controlling cell cycle progression in these tolerant T cells and it is likely that this molecule contributes to the anergic state of PItol cells.

Recent experiments have revealed a role for ICOS in regulating EAE (44–46). Abs blockade of ICOS molecules during disease induction led to enhanced EAE and disease was exacerbated in ICOS knockout mice (44). We were particularly interested in studying the expression of ICOS because ICOS costimulation has been shown to induce IL-10 expression (47). Whereas ~63% of PItol cells expressed CTLa-4 constitutively, only ~16% of these cells expressed ICOS. This would indicate that ICOS expression is not required for the function of PItol cells. Indeed, blockade of the interaction between ICOS and its ligand in vitro did not reverse suppression mediated by the PItol cells. However, one cannot exclude the possibility that this molecule might be involved in the generation of such cells.

Previous in vitro experiments with the Tg4 model were limited to the demonstration of anergy and IL-10 production. We wished to know whether the tolerant cells might be capable of suppression. These cells were found to be potent suppressors of naïve T cell proliferation and IL-2 production. However, interestingly, the suppressive effect of PItol cells could not be reversed by blockade of the IL-10R. Previous studies had shown that supernatants from cells rendered tolerant with superantigen were capable of suppressing the response of naïve cells to Ag (14). The suppression mediated by supernatants could be blocked by addition of Abs to IL-10 and TGF-β. By contrast, we noted that supernatants from tolerant cells had no significant effect on APCs. Thus, supernatants from PItol cells failed to block the up-regulation of MHC class II, CD80, and CD86 costimulatory molecules on purified immature dendritic cells and had no effect on T cell proliferation (data not shown). This intriguing difference between regulatory cells induced with peptide vs superantigen cannot be explained by the amount of IL-10 produced since the levels were similar in both studies. The apparent requirement for cell-cell contact was further confirmed using transwell cultures, indicating that PItol cells were totally dependent on cell-cell contact for suppression of naïve cells in vitro. Therefore, despite the high level of IL-10 secreted by PItol cells in response to Ag, this cytokine does not account for the suppression of naïve cells observed in vitro.

A further difference between the PItol cells and TCR transgenic cells rendered tolerant by repeated stimulation with superantigen lies in their response to Ag and IL-2. PItol cells appear to be conventionally anergic. In other words, the unresponsive state of these cells and their capacity to suppress naïve cells in vitro could be reversed by the addition of exogenous IL-2 to cultures. However, previous studies by Schwartz and colleagues (14) showed that superantigen-treated cells were profoundly unresponsive and the addition of IL-2 at 10 U/ml did not increase their proliferative response, suggesting that responsiveness to IL-2 was impaired. Indeed, anergic cells were shown to display altered signaling via the common γ-chain of the IL-2R, which consequently resulted in diminished phosphorylation of several downstream proteins (48). This suggests to us that different degrees of unresponsiveness can be induced in T cells despite the fact that the two populations of regulatory cells both secrete IL-10 in response to Ag. It seems likely that this depends on the nature and strength of the tolerogen. It may well be that superantigens induce a “deeper state” of unresponsiveness involving impairment of more signaling pathways when compared with that induced by peptide Ags. However, most importantly, the IL-10 signaling pathway appears to be resistant to Ag-induced anergy.

We have established two separate models to test the function of PItol cells in vivo. The first transfer model involved transfer of either naïve or tolerant cells that had been labeled with CFSE. The
in vivo proliferation of naive cells was suppressed when these cells were transferred into tolerant mice, thus confirming the ability of tolerant cells to suppress the proliferation of naive cells in vitro. It was noticeable that tolerant cells transferred into naive mice underwent some rounds of division, whereas their proliferation was totally blocked in tolerant recipients. It seems likely that the proliferation of tolerant cells in naive mice resulted from a partial reversal of tolerance due to the secretion of IL-2 by naive T cells. This would be consistent with the reversal of tolerance by addition of IL-2 to tolerant cells in culture. Furthermore, the anergic state of PItol cells when transferred into tolerant mice was not reversed by injection of anti-IL-10R Abs (data not shown). This further emphasizes the requirement for IL-2 in reversal of anergy. However, most notably, the suppression observed when naive cells were transferred into tolerant mice was reversed by coinjection of an Ab directed to the IL-10R. This shows that IL-10 plays a significant role in the tolerance mediated by PItol cells in vivo and is in marked contrast to the results arising from experiments in vitro.

Naive Tg4 cells secrete IL-2 in response to peptide Ag and serum levels of the cytokine peak within 2 h. This observation was used as a read-out for transfer of tolerance. The results shown in Fig. 6 indicate that the transfer of PItol cells leads to the suppression of IL-2 production in vivo. However, most significantly, this suppression was reversed by coinjection of an Ab directed to the IL-10R. Therefore, we have used two independent methods to confirm the role of IL-10 in peripheral tolerance mediated by soluble peptide administration.

The results arising from this study reveal the limitations of in vitro models of suppression. A number of publications have demonstrated that the suppressive function of CD4+CD25+ natural regulatory T cells is cytokine-independent but requires cell-cell contact (37, 49, 50). These studies were based on assays conducted in vitro and the results clearly need to be carefully considered in light of the current findings. In other words, the fact that Abs to IL-10 or TGF-β do not inhibit the suppressive properties of regulatory cells in vitro does not necessarily mean that these cytokines are not involved in vivo. Various groups have clearly shown a role for cytokines in the function of regulatory cells in vivo (32, 51, 52). This depended on the type of model and often involved the cotransfer of naive and tolerant cells into lymphopenic mice. Interestingly, the dependence on IL-10 depended on the disease model with suppression of autoimmune colitis being IL-10-dependent and autoimmune gastritis being IL-10-independent. A clue as to why cytokines are required in some but not other models arises from a study by Annacker et al. (53). These authors showed that both CD25+ and CD25−CD45RBlow populations contain regulatory cells. The CD25+CD45RBlow cells were able to prevent disease arising from expansion of CD45RBhigh cells in lymphopenic mice and the suppression of cell expansion was dependent on IL-10. As it stands there is evidence that both CD25+ and PItol cells (predominantly CD25−) can suppress the response of neighboring T cells in vitro in a cell-cell contact-dependent and cytokine-independent fashion. The need for IL-10 production in vivo varies depending on the nature of the model, but we can conclude from the results of this study that PItol cells are dependent on IL-10 for suppression in vivo. Further work from this laboratory has shown that PItol cells are distinct both in origin and function to the natural CD4+CD25+ cells that make up ~5% of the T cell repertoire in the RAG−/− Tg4 mouse (A. Sundstedt, E. O’Neill, K. Nicolson, S. Parry, and D. Wraith, manuscript in preparation).

PITol cells arise through repeated Ag encounter in an environment where there is little inflammation; hence, the majority of APCs are in a resting state. A number of recent studies have shown that Ags presented to T cells by dendritic cells that are either immature or intermediate in maturity drive the generation of IL-10-secreting regulatory T cells (54–56). It is likely that peptides delivered in a highly soluble form bind to such dendritic cells and that this form of Ag presentation in vivo leads to the generation of IL-10-secreting PItol cells. Similarly, addition of IL-10 or the combination of vitamin D3 and dexamethasone (57–59) would block the spontaneous maturation that occurs when immature dendritic cells are cultured in vitro. This could, in part, explain why repeated exposure to Ag in the presence of such agents leads to the generation of IL-10-secreting regulatory cells. There is now evidence that soluble peptide administration leads to the generation of IL-10-secreting regulatory cells in humans, induction of these cells correlating with effective desensitization of the immune response to allergens (60–63). Therefore, cells with the properties of PItol cells can be induced in both mouse and man and these cells can moderate the immune response to both autoantigens and allergens. We believe that these cells function in the suppression of hypersensitivity conditions in general. Therefore, the findings of this paper are of central importance to the safe and effective use of peptides for immunotherapy of both allergic and autoimmune conditions.

Acknowledgments
We thank Pauline Lowrey for peptide synthesis and Drs. Anthony Coyle and Fiona Powrie for the kind gift of reagents.

References


