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IL-2 Overcomes the Unresponsiveness but Fails to Reverse the Regulatory Function of Antigen-Induced T Regulatory Cells

Per O. Anderson,*† Anette Sundstedt,²§ Zihni Yazici,§ Sophie Minaee,§ Richard Woolf,§ Kirsty Nicolson,§ Nathaniel Whitley,§ Li Li,*‡ Suling Li,†‡ David C. Wraith,§ and Ping Wang³#†

Intranasal administration of peptide Ac1–9[4Y], based on the N-terminal epitope of myelin basic protein, can induce CD4+ T cell tolerance, and suppress experimental autoimmune encephalomyelitis induction. The peptide-induced regulatory T (PI-TReg) cells failed to produce IL-2, but expressed IL-10 in response to Ag and could suppress naive T cell responses in vitro. Analysis of Jak-STAT signaling pathways revealed that the activation of Jak1, STAT3, and STAT5 were induced in tolerant T cells after Ag stimulation in vivo. In addition, the expression of suppressor of cytokine signaling 3 was induced in tolerant T cells, suggesting that cytokines regulate the tolerant state of the PI-TReg cells. Stimulation of PI-TReg cells in vitro with IL-10 induced Jak1 and STAT3 activation, but not STAT5, suggesting that IL-10 is important, but not the only cytokine involved in the development of T cell tolerance. Although IL-2 expression was deficient, stimulation with IL-2 in vitro induced Jak1 and STAT5 activation in PI-TReg cells, restored their proliferative response to antigenic stimulation, and abrogated PI-TReg-mediated suppression in vitro. However, the addition of IL-2 could not suppress IL-10 expression, and the IL-2 gene remained inactive. After withdrawal of IL-2, the PI-TReg cells regained their nonproliferative state and suppressive ability. These results underline the ability of the immune system to maintain tolerance to autoantigens, but at the same time having the ability to overcome the suppressive phenotype of tolerant T cells by cytokines, such as IL-2, during the protective immune response to infection. The Journal of Immunology, 2005, 174: 310–319.

T he function of the immune system is to respond to pathogens and fight infection. However, it is equally important to remain nonresponsive, i.e., tolerant, to self tissues and harmless environmental proteins, which are the Ags most frequently encountered by the immune system. Most self-reactive T cells are deleted in the thymus by negative selection, but autoreactive T cells are nevertheless present in the periphery of healthy individuals (1, 2). These cells must be controlled to avoid the immunopathological responses seen in autoimmune diseases. Several mechanisms have been proposed by which peripheral tolerance may be maintained, including functional inactivation (anergy) (3) or peripheral deletion of autoreactive T cells (4). Furthermore, there is increasing evidence indicating that peripheral tolerance against self Ags is controlled by regulatory T (TReg) cells in vivo (5–7).

In vitro anergy (clonal anergy) can be induced by stimulating CD4+ T cell clones through the TCR, in the absence of costimulation, or with low affinity peptides in the presence of costimulation. The anergic T cells fail to proliferate or secrete IL-2 even when given an optimal stimulation through both the TCR and co-stimulatory receptors (8). However, clonal anergy can be reversed by the addition of IL-2 (9). Although it has been discovered that the calcium/calcineurin/NF-AT pathway is intact in anergic T cells (10, 11), the mechanisms causing the block in proliferation and suppression of IL-2 production are still not well understood.

T cell anergy can also be induced in vivo following repetitive administration of antigenic peptide via intranasal (i.n.) (12) and oral (5) routes, or through repetitive i.v. injection of staphylococcal enterotoxin A (13). In contrast to clonal anergy, in vivo tolerance, induced by high affinity Ag, is not the result of an insufficient proliferative response due to weak TCR stimulation or the lack of costimulation. In this case, the tolerogenic stimulus is also the priming stimulus (reviewed in Ref. 8). In contrast to what is evident in clonal anergy, in vivo tolerized T cells produce high levels of IL-10 (12, 14), and addition of IL-2 may not break tolerance (15). These data suggest an important role for IL-10 signaling in the maintenance of the anergic state in parallel with a possible defect in IL-2R signaling (16, 17).

In the transgenic 4 (Tg4) TCR model of experimental autoimmune encephalomyelitis, repetitive i.n. administration of Ac1–9[4Y], a high-affinity antigenic peptide derived from myelin basic protein (MBP), has been shown to induce protection against subsequent experimental autoimmune encephalomyelitis induction. Ac1–9[4Y]-specific CD4+ T cells from tolerized mice were nonresponsive to in vitro Ag stimulation, failed to produce IL-2, but secreted high levels of IL-10 (12, 18). These peptide-induced tolerant T (PI-TReg) cells could suppress the response of naive CD4+ TReg cells to Ag stimulation experimentally or by adoptive transfer, restored the regulatory function of Ag-induced TReg in vivo, and secreted high levels of IL-10 in vitro and in vivo. This suggests that the mechanism of suppression of PI-TReg cells does not rely on IL-2 but on IL-10. In this model, the PI-TReg cells also expressed high levels of suppressor of cytokine signaling 3 but only in vivo.

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T cells in vitro and in vivo (18). Neutralization of IL-10 in vivo abrogated protection against disease, suggesting that IL-10 production by PI-TReg cells plays an important role in peptide-induced tolerance (12). However, the anergic state of the PI-TReg cells in our model could be reversed by the addition of IL-2 and Ag in vitro. More interestingly, IL-2 addition also abrogated the suppressive capacity of the PI-TReg cells in vitro.

To understand the roles of IL-10 and IL-2 in the induction/maintenance and reversal of tolerance, we set out to characterize the signaling pathways induced by these cytokines, and the consequences of IL-2 stimulation on PI-TReg cell function in vitro. We analyzed the expression of IL-10, IL-2, TGF-β, and most Th1 and Th2 cytokines during the course of tolerance induction in vivo. We also examined the activation of the Jak/STAT pathways, the expression of the suppressor of cytokine signaling (SOCS) molecules in vivo, and IL-10/IL-2-induced Jak/STAT activation in vitro. Jak1, STAT3, and STAT5 were all induced in PI-TReg cells in response to Ag stimulation in vivo. Furthermore, in our vitro results demonstrated that IL-2R and IL-10R signaling pathways were intact and distinct in PI-TReg cells. IL-2-induced Jak1/STAT5 activation, whereas IL-10 induced STAT3 activation. Stimulation of PI-TReg cells with IL-2 and Ag in vitro induced proliferation by the PI-TReg cells, but also abrogated the suppressive capacity of the PI-TReg cells. However, the proliferating PI-TReg cells still produced high levels of IL-10, and were unable to transcribe the IL-2 gene. Upon removal of IL-2, the PI-TReg cells regained their suppressive capacity. Our data suggest that intact signaling pathways for both IL-2 and IL-10 in PI-TReg cells are important in regulating tolerance to self Ags, while maintaining the ability to respond to pathogen.

Materials and Methods

Peptides

The wild-type, acetylated, N-terminal peptide of murine MBP (Ac1–9, AcASQKRP5QR) and its high-affinity MHC-binding analog, with a tyrosine substituting the wild-type lysine at position 4 (Ac1–9[4Y]), were synthesized as peptide amides using standard Fmoc chemistry on an AMS 422 multiple peptide synthesizer (Abimed Analyse-Technik).

Animals and Ag-specific tolerance induction

The Tg4 TCR mouse was described previously (19). It expresses the αβTCR (Vβ4, Vβ8.2) of the Ac1–9-specific T cell hybridoma 19344.4 derived from an encephalitogenic T cell clone (20). Mice were bred onto the B10.PL (H-2b) background and maintained at the School of Medical Sciences, University of Bristol, and were between 6 and 12 wk of age when used for experiments. The screening of TCR Tg mice was performed using two-color immunofluorescent staining of PBLs with anti-CD4 and anti-Vß8 mAb. In the Tg4 mice, >95% of all CD4+ T cells were Vß8+. The modified acetylated N-terminal peptide of murine MBP Ac1–9[4Y] was dissolved in PBS at 4 mg/ml, and doses of 25 μg were administered i.n. under light halothane anesthesia at 3- to 4-day intervals.

Cytokine protein levels

Blood samples drawn at various time points after i.n. treatment with Ac1–9[4Y] and supernatants from in vitro cell cultures were tested for IL-2, IFN-γ, and IL-10 content by specific ELISAs, according to instructions from the manufacturer (BD Pharmingen).

Cell preparation

Spleens from Tg4 mice were used as a source of CD4+ T cells. 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), according to the manufacturer’s instructions.

FACS analysis (flow cytometry)

Cells were stained with mAb directed against murine CD25 (BD Pharmingen) for flow cytometry. Alternatively, cells were incubated with cognate Ag for the stated time, and BD GolgiStop was added for the last 6 h; then cells were permeabilized and subsequently stained for cell surface CD4 or CD25 and intracellularly with anti-IL-10 PE, anti-IFN-γ FITC for double cytokine staining, or PE anti-cytokine Abs for cytokine expression in CD25+ cells. Gates were selected using suitable isotype control PE- and FITC-conjugated Abs (all BD Pharmingen). Labeled cells were analyzed by FACS using CellQuest software (BD Pharmingen).

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 above. 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 Invitrogen Life Technologies), and 5% FCS (Sigma-Aldrich), at 5 × 105 cells/well at 37°C in the presence of different concentrations of Ac1–9. Irradiated CD4-depleted splenocytes at 1 × 106 cells/well were used as APCs. After the indicated times, cells were pulsed with 0.5 μc of 3Hthymidine for 16 h, and the incorporated radioactivity was measured on a liquid scintillation beta counter (1450 Microbeta; PerkinElmer Wallac).

In vitro T cell cultures

Purified CD4+ T cells from naive and tolerant mice, before and 2 h after in vivo peptide stimulation, were plated into six-well tissue culture plates using 5 × 106 cells/5 ml of RPMI 1640 supplemented with glutamine, gentamicin, HEPES, and 0.5% FCS. Cells were cultured in low-serum RPMI 1640 for 4 h before cytokine addition to reduce background phosphorylation of STAT3 and STAT5. Cells were then stimulated with murine IL-2 (rmlL-2) (50 ng/ml; R&D Systems) or with mIL-10 (100 ng/ml; R&D Systems) for 20 min. After stimulation, cells were transferred to prechilled tubes, washed once with 5 ml of ice-cold PBS containing phosphatase inhibitors, and processed for total cellular extracts, as described below.

Preparation of cellular extracts

Total cellular extracts for immunocomplex protein kinase assays and Western blots were made from 10 × 106 purified CD4+ T cells according to the method of Hibi et al. (21). Briefly, the cells were lysed in 600 μl of cold lysis buffer consisting of 20 mM Tris (pH 7.7), 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, and 0.5% Nonidet P-40, to which protease and phosphatase inhibitors had been freshly added. After incubating for 30 min on ice, the extracts were spun for 10 min in an Eppendorf centrifuge at 4°C to pellet cellular debris. The supernatants were removed and stored at −70°C.

SDS-PAGE and Western blot analysis

Western blotting was conducted using standard techniques. Briefly, proteins from cellular lysates were separated on 10% SDS-PAGE NOVEX gels (Invitrogen Life Technologies). Following electrophoresis, proteins were transferred to nitrocellulose membranes (Invitrogen Life Technologies), blots were blocked with 5% nonfat dry milk in TBS, 0.1% Tween 20 for 1 h at room temperature. Blots were then incubated overnight at 4°C with one of the following Abs: anti-phospho-STAT3 (B-7), STAT3, and STAT5 purchased from Santa Cruz Biotechnology; anti-phospho-STAT5 (Ty952p) purchased from New England Biolabs; and phospho-Jak1 and phospho-Jak2 from Zymed Laboratories. The blots were then washed with TBS-Tween 20 (0.1%), followed by incubation with one of the following secondary Abs: rabbit anti-mouse HRP, swine anti-rabbit HRP, or rabbit anti-goat HRP (DakoCytoimmun). Finally, the blots were developed using the ECL detection kit (Amersham Biosciences).

Rnase protection assay (RPA)

Cytokine-specific, 32P-labeled (Amersham Biosciences) RNA probes were generated through the in vitro translation (Riboquant Transcription kit; BD Pharmingen) of the mouse cytokine probe sets mCK1 and mCK3b (BD Pharmingen), according to the manufacturer’s protocol. Total RNA, purified from in vivo stimulated naive and tolerant CD4+ T cells (6 μg/sample), was hybridized overnight with the labeled cytokine probes using the Riboquant RPA kit purchased from BD Pharmingen, and subsequently RNase treated. Protected fragments were resolved on 5% PAGE gels.

Quantitative transcript analysis

Total RNA was extracted from purified CD4+ T cells using TRIzol (Invitrogen Life Technologies), according to the manufacturer’s instructions, and was reverse transcribed using random hexamer primers (Amersham Biosciences). Quantitative real-time PCR was performed using a Roche LightCycler (Roche Diagnostics) using SYBR Green 1 (Roche Diagnostics) for the sequence-independent detection of DNA, according to the
RESULTS

Repetitive in vivo Ag stimulation results in a switch in the cytokine expression profile in naive T cells

Previous work from this laboratory showed that cells from mice rendered tolerant by peptide administration were unable to proliferate in response to antigenic challenge in vitro (12, 18). Furthermore, these cells failed to secrete IL-2, but produced a significant amount of IL-10 when restimulated in vitro. Fig. 1A shows the result of an experiment in which the proliferative response of either naive or tolerant CD4\(^+\) cells was measured in a time course. The response of naive cells reached a peak at 3 days after antigenic challenge in vitro, whereas tolerant CD4\(^+\) cells failed to respond. Tg4 mice were challenged with soluble peptide Ag, and secretion of cytokines was measured in vivo by assessing serum concentrations of IL-2 and IL-10. As shown in Fig. 1, B and C, naive mice responded to soluble Ag by secreting significant quantities of IL-2, reaching a peak at 2 h after antigenic challenge. By contrast, tolerant mice failed to secrete IL-2, but generated significant serum concentrations of IL-10 with similar kinetics to the IL-2 measured in naive mice.

We have previously shown that Tg4 mice must be treated with soluble peptide repeatedly to become fully tolerant and for tolerant cells to suppress the proliferation of naive cells (12). Between 5 and 10 doses of Ag were required to achieve effective tolerance. This was further investigated using an RPA to measure the expression levels of cytokine genes in Tg4 mice receiving different number of doses of soluble peptide Ag (Fig. 2). Mice were treated every third or fourth day, i.e., twice per week. Cells from naive lymphotoxin mice did not transcribe mRNA for any cytokine other than β. The levels of lymphotoxin β remained unchanged throughout the treatment process. We chose to investigate the levels of mRNA at 2 h after each treatment, given that cytokine production in vivo reached a peak at this time point (Fig. 1, B and C). Naive Tg4 cells clearly responded to Ag, resulting in transcriptional activation of the IL-2, TNF-α, TNF-β, and TGF-β genes at 2 h after the first treatment (Fig. 2). During the course of tolerance induction, Tg4 cells expressed varying amounts of IL-2, IFN-γ, and IL-10, but did not transcribe message for either IL-4, IL-5, or IL-13.

After 10 treatments, at a time when the Tg4 T cells were anergic to Ag stimulation in vitro (Fig. 1A), the level of mRNA for cytokines detected after the first treatment, i.e., IL-2 and IFN-γ, was markedly reduced, while IL-10 remained the dominant cytokine (Fig. 2A). It is important to emphasize that the cells from mice treated 10 times with peptide did not spontaneously secrete IL-10 at the time of mRNA isolation. Indeed, the profile of cytokine secretion was the same at the 0-h time point for cells from mice treated 10 times with soluble peptide when compared with the 0-h profile for naive cells. This indicates that despite their apparently anergic state, as measured by proliferation, tolerant Tg4 cells remained Ag specific and responded to Ag by secreting IL-10 rather than IL-2.

Activation of Jak-STAT pathways in naive and tolerant Tg4 cells upon in vivo stimulation with Ag

Following the discovery of a distinct cytokine gene profile in naive and tolerant T cells in response to Ag stimulation, we
further characterized the Jak-STAT signaling pathways 2 h after Ag stimulation in vivo. Because both naive and tolerant mice responded to Ag rapidly, with cytokine levels peaking at 2 h, we wished to know whether Jak-STAT signaling pathways in T cells of Tg4 mice would be different. As shown in Fig. 3A, Jak1 was phosphorylated in both naive and tolerant cells, while Jak2 phosphorylation was only observed in naive Tg4 cells (Fig. 3A). This result is consistent with previous findings demonstrating that Jak2 is not involved in IL-10 signaling (22). Interestingly, despite the distinct profile of cytokine gene expression, STAT3 and STAT5 were both phosphorylated with similar kinetics in both naive and tolerant cells after antigenic challenge in vivo (Fig. 3B). By contrast, the phosphorylated forms of STAT4 and STAT6 were not detected in either naive or tolerant cells after antigenic challenge in vivo (data not shown).

### Induction of SOCS in naive and tolerant T cells

Recently, the SOCS adapter proteins were noted for their ability to inhibit the Jak-STAT pathway (23, 24), and in more recent findings, to regulate the MAPK pathway (25, 26). The function of SOCS molecules is associated with their inducible expression by different cytokines and their interaction with different signaling molecules (27). To investigate the involvement of SOCS molecules in the regulation of T cell activation and tolerance, the induction of these molecules was assessed in purified CD4+ T cells from naive and tolerant mice. Samples were collected before, 30 min, 2 h, and 12 h after antigenic challenge in vivo. The expression of SOCS molecules was analyzed by a real-time PCR technique. Both CIS and SOCS1 mRNA levels were low in naive and tolerant T cells before peptide stimulation (Fig. 4). After antigenic challenge, the level of CIS mRNA peaked at 2 h in both naive and tolerant T cells, and displayed a 6- to 10-fold increase relative to unstimulated cells. Expression levels returned to baseline by 12 h following stimulation. The basal level of SOCS1 mRNA expression was similar in both groups before stimulation (Fig. 4). However, at 30 min after stimulation, SOCS1 was induced up to 3-fold in naive T cells, but remained at a basal level in tolerant T cells. Two hours after stimulation, the expression of SOCS1 was increased by 5-fold in Ag-stimulated naive T cells and was induced in tolerant cells to a lesser extent. SOCS1 levels returned to baseline after 12 h.

SOCS3 mRNA was induced in tolerant T cells, but not in naive T cells, and reached a peak at 2 h after antigenic challenge in vivo. Interestingly, the expression of SOCS2 was detected in both naive and tolerant T cells before antigenic challenge. Antigenic challenge decreased the expression of SOCS2 in both groups (Fig. 4).
IL-10 induces STAT3, but not STAT5 phosphorylation in tolerant T cells

Although expression of IL-10 has been detected in various models used for studying tolerance in vivo, little is known about the selective induction of IL-10 and the regulation of IL-10 signaling in tolerant T cells. We have shown an exclusive production of IL-10 in Ag-stimulated tolerant T cells, which is paralleled by the induction of STAT3 and STAT5 phosphorylation. To examine the ability of IL-10 to induce phosphorylation of STAT3 and STAT5, isolated CD4\(^+\) T cells from naive and tolerant mice were stimulated with rIL-10 in vitro. Addition of IL-10 to either naive or tolerant cells led to phosphorylation of STAT3, but not STAT5 (Fig. 5, A and B), as shown previously (28). The basal level of STAT3 phosphorylation was increased in PI-TReg cells previously activated in vivo, but was further increased by addition of IL-10 in vitro. The data suggest that the activation of STAT3 in tolerant cells might be due to IL-10 signaling. Furthermore, these results suggest that the activation of STAT5 in tolerant T cells is due to signaling by unknown cytokines.

IL-2R signaling is intact in tolerant T cells

Previous experiments had shown that a mechanism for unresponsiveness in tolerant T cells was down-regulation of IL-2R signaling (16). STAT5 is known to be induced by ligation of the IL-2R, among other cytokine receptors (29). We noted that STAT5 was phosphorylated in tolerant T cells after antigenic challenge in vivo (Fig. 3B). However, because IL-2 was not detected in tolerant mice, we assume that this signaling was due to ligation of a different cytokine receptor. To investigate IL-2R signaling further, isolated naive and tolerant T cells were stimulated with rIL-2 in vitro for 20 min. Please note that the cells were maintained in low serum cultures for 4 h before cytokine stimulation to reduce background levels of STAT3 and STAT5 phosphorylation. Cytosolic
proteins from IL-2-stimulated cells were analyzed by Western blotting with anti-phospho-Jak or anti-phospho-STAT Abs. Jak1 and STAT5 were activated to a similar degree in both resting naive and tolerant T cells after IL-2 stimulation (Fig. 5, A and C). IL-2 did not induce phosphorylation of STAT3, suggesting that the activation of STAT3 in vivo resulted from stimulation with IL-10 in tolerant T cells and another cytokine in naive T cells.

**Biological consequences of signaling through the IL-2R**

Our data suggest that tolerant Tg4 cells responded to ligation of the IL-2R via the Jak-STAT pathway. The biological consequences of such signaling were therefore investigated. Tolerant Tg4 cells failed to proliferate in response to Ag in vitro (Fig. 1A). The effect of adding IL-2 to tolerant T cells was investigated. As shown in Fig. 6A, tolerant cells were profoundly unresponsive to antigenic challenge in vitro. However, addition of IL-2 to tolerant cells in culture fully restored their response to Ag (Fig. 6A). Addition of IL-2 also permitted secretion of IFN-γ by IL-10-negative CD4+ T cells from tolerant mice (Fig. 6C), suggesting that IL-2 reversed the regulatory function of PI-TReg cells and promoted the activation of bystander naive T cells. The inability of PI-TReg cells to produce IFN-γ after IL-2 stimulation in vitro indicates that the IL-10-producing PI-TReg cells were in effect suppressing the normal function of these bystander cells. Most interestingly, addition of IL-2 greatly enhanced the ability of tolerant cells to secrete IL-10 (Fig. 6B).

It is notable that the addition of IL-2 alone was sufficient to induce phosphorylation of STAT5 in tolerant cells (Fig. 5A). As with naive cells, however, addition of IL-2 was not sufficient to elicit proliferation; ligation of the TCR was also required. Ag-induced activation of T cells is known to induce cell surface expression of CD25, thereby providing the high affinity IL-2R (30, 31). Indeed, Ag-induced activation of Tg4 cells increased the expression of CD25 correlating with the peak of proliferation among naive cells (Figs. 1A and 7B). In addition to the IL-10-expressing T cells, a subset of IL-10-negative T cells was also induced to express CD25 (Fig. 7A), which may suggest that these CD25-positive and IL-10-negative T cells were either suppressed by PI-TReg cells in vivo or were simply anergic T cells that did not produce either IL-10 or IL-2 (Fig. 8). Although CD25 was induced in both

**FIGURE 6.** Effect of IL-2 on proliferation and cytokine production by Ag-stimulated PI-TReg cells in vitro. Naive CD4+ T cells and PI-TReg cells, purified from the spleens of mice 3 days after the ninth i.n. peptide administration, were cultured in vitro together with varying doses of Ac1–9, in the presence of CD4+ splenocytes as APCs. The effect of rmIL-2 (20 U/ml) stimulation on proliferation and cytokine production was examined. On day 3 of culture, the proliferative responses were assessed by measuring [3H]thymidine incorporation (A), and culture supernatants were checked for the presence of IL-10 using cytokine-specific ELISAs (B). IL-10- and IFN-γ-producing cells, after Ac1–9 or Ac1–9 plus rmIL-2 stimulation, were measured by intracellular staining and FACS analysis (C).
IL-10-positive and IL-10-negative T cells from tolerant mice, addition of IL-2 did not further enhance the expression of CD25 induced by Ag exposure among naive T cells.

Intracellular cytokine staining of T cells from PI-TReg cells had previously shown that the proportion of cells actively producing IL-10 in response to the Ac1–9 Ag, at any given time, was low (<10% when cells were stimulated with Ag alone in vitro) (32). After in vitro stimulation with IL-2 and Ag, the population of IL-10-producing T cells did not change (Figs. 6A and 7A), indicating that the IL-10-producing PI-TReg population is stable under the IL-2 treatment in vitro. To examine whether the suppression of IL-2 expression in tolerant T cells could be overcome by addition of IL-2 in vitro, relative levels of IL-2 and IL-10 transcripts were quantified by RT-PCR. The results demonstrate that neither IL-2 nor Ag plus IL-2 could activate IL-2 transcription in tolerant T cells (Fig. 8). Moreover, a reduced IL-2 expression was detected in a mixed culture of naive and tolerant T cells after stimulation with Ag and IL-2 (Fig. 8, lane 9), compared with the IL-2 expression in the culture of naive T cells stimulated with Ag and IL-2 (Fig. 8, lane 10). This result is consistent with proliferation results (Fig. 9B), and suggests that IL-2 could not reverse the regulatory function of PI-TReg cells. Thus, IL-2 gene expression remains suppressed despite effective IL-2R signaling in PI-TReg cells.

**IL-2 abrogates suppression, but does not reverse the tolerant phenotype**

We wished to investigate how ligation of the IL-2R would affect the suppressive properties of tolerant T cells. Fig. 9A shows the time course of cocultures containing naive and tolerant cells with and without IL-2. Tg4 cells stimulated with Ag alone reached a peak of proliferation at day 3. Proliferation was sustained through day 4 by addition of IL-2, indicating that the reduction in proliferation of naive cells at day 4 was due to the lack of IL-2. Tolerant cells remained anergic in the absence of IL-2, but addition of this cytokine led to a partial recovery of their proliferative capacity. Coculture of naive cells with tolerant cells substantially inhibited proliferation of the former cell population. This was completely overridden, however, by addition of IL-2. Addition of IL-2 restored proliferation to the level achieved by naive cells in the presence of IL-2. Interestingly, the reversal of suppression was both more marked and more rapid than the reversal of anergy among tolerant cells alone.
FIGURE 8. IL-2 and Ag stimulation of PI-TReg cells does not induce IL-2 gene transcription. Total splenocytes, purified from the spleens of naive or tolerant mice 3 days after the ninth i.n. peptide administration, were cultured in vitro either alone (lanes 1–3 and 10; tolerant, lanes 4–6) or in mixed cultures of naive and tolerant splenocytes at a 1:1 ratio (lanes 7–9) in the presence (lanes 3, 6, 9, and 10) or absence of IL-2 (20 U/ml) (lanes 1, 2, 4, 5, 7, and 8) and Ac1–9[4K] (10 ng/ml) (lanes 2, 3, 5, 6, 8, 9, and 10). After 3 days of culture, CD4+ T cells were purified using MACS columns (see Materials and Methods), and total RNA was extracted using TRIzol reagent. Semiquantitative PCR was performed on cDNA using gene-specific primers.

Addition of IL-2 to cultures of peptide-induced tolerant cells reversed their anergic phenotype (Fig. 6). This did not, however, represent a complete reversion to the naive phenotype of the cells because IL-10 production was both maintained and enhanced in the presence of IL-2. Addition of IL-2 to cocultures did, however, abrogate suppression mediated by these cells. To assess the long-term stability of the suppressor phenotype, spleen cells from tolerant Tg4 mice were stimulated in the presence of Ag and IL-2 for 3 days. At this time, tolerant CD4 cells were purified and washed to remove traces of IL-2. These IL-2-matured cells were then introduced into cocultures with naive Tg4 cells. As shown in Fig. 9B, IL-2-matured tolerant cells were anergic in the absence of IL-2 and recovered their capacity to suppress proliferation of naive T cells. Taken together, these data indicate that while PI-TReg cells may lose their capacity to suppress neighboring cells in the presence of IL-2, they nevertheless retain their suppressive phenotype such that they are able to suppress immune responses once IL-2 levels have fallen below a critical level.

Discussion

Previous work from this laboratory showed that the i.n. administration of soluble peptide to the Tg4 mouse led to a state of induced tolerance (12, 18). PI-TReg cells were predominantly CD25+ and could be generated from mice lacking natural CD4+CD25+ TReg cells (our unpublished observations). CD25+ TReg cells from mice treated with soluble peptide secreted low levels of IL-2 and proliferated poorly in response to Ag in vitro. We noted that CD25+ TReg cells from mice treated with peptide produced IL-10 in vitro, and therefore measured the kinetics of cytokine production in vivo. Remarkably, both naive and tolerant mice responded rapidly to Ag administered in soluble form in vivo. Thus, naive mice generated IL-2, while tolerant mice produced IL-10, with both cytokines reaching a peak within 2 h of Ag administration. This compares with the 24–48 h required for IL-2 to reach peak levels when naive cells were stimulated in vitro and the 72–96 h necessary to reach peak levels of IL-10 in culture supernatants in vitro. Presumably, the accelerated response of cells in the intact lymph node reflects the close proximity between APC and T cells in vivo. The characteristic feature of PI-TReg cells was their relative lack of IL-2 production and, conversely, high level of IL-10 secretion, the opposite being true for naive Tg4 cells. It is important to note, however, that PI-TReg cells remained essentially sensitive to activation through the TCR. PI-TReg cells only secreted IL-10 in response to Ag either in vitro or in vivo. The dominance of IL-10 in the cytokine secretion profile of PI-TReg cells implies that this cytokine could play an important role in either the generation or maintenance of these cells, and we therefore compared cytokine signaling events in naive and PI-TReg cells.

Given the dramatic difference in the response of Tg4 cells in vivo when compared with in vitro conditions, we chose to test responses in vivo and at early time points. Phosphorylation of Jak1 and STAT3 in PI-TReg cells, upon encountering Ag in vivo, suggested to us that IL-10 activates the Jak1/STAT3 pathway. Surprisingly, STAT5, which is not involved in IL-10 signaling, was also activated in Tg4 cells in response to Ag challenge in vivo, despite the deficiency in IL-2 protein expression. This suggests that the function of STAT5 in the regulation of PI-TReg cells and the maintenance of tolerance in vivo is distinguishable from its role in the regulation of IL-2-mediated cell cycle progression. A recent report has shown that STAT5 knockout mice develop autoimmune in several organs (33). This autoimmunity correlates with decreased numbers of CD4+CD25+ regulatory T cells that evidently undergo apoptosis at increased rates in the absence of STAT5 (33). These data suggest that STAT5 activation is required for the survival of CD4+CD25+ regulatory T cells. The fact that activation of STAT5 can increase the number of CD4+CD25+ T cells in IL-2 knockout mice (34) suggests that STAT5 may act...
downstream of other cytokines in regulating T cell tolerance. Such a mechanism would explain the up-regulation of STAT5 in activated PI-TReg cells despite the lack of IL-2 produced by these cells.

In support of our findings that STAT3 activation and IL-10 expression were induced in PI-TReg cells in vivo, recent work has revealed the central role of STAT3 signaling in immune tolerance (35). Disruption of STAT3 signaling in APCs led to activation of T cells in response to an otherwise tolerogenic encounter with Ag. STAT3-deficient APCs expressed higher levels of class II MHC and costimulatory molecules, activated T cells more readily, responded more rapidly to inflammatory stimuli, and failed to secrete IL-10 in response to such stimuli. STAT3 therefore lies at the heart of an autocrine pathway in APCs that would be maintained by IL-10 secreted by PI-TReg cells.

Although there was no apparent difference between the response of naive CD4+ T cells or PI-TReg cells to IL-2 and IL-10 in Jak/STAT signaling in vitro, there was a clear increase in SOCS3 expression among PI-TReg cells. SOCS3 can inhibit STAT activation in response to many cytokines, including growth hormone, leptin, IL-2, IL-4, and IL-10 (27). SOCS3 expression could therefore contribute to the tolerant state of TReg cells by inhibiting the response to IL-2. In addition, two recent studies by Yu et al. (36) and Banerjee et al. (37) have demonstrated an inhibitory effect of SOCS3 on T cell activation. Yu et al. showed that forced overexpression of SOCS3 in Th cells was able to inhibit anti-CD3/anti-CD28-induced proliferation, whereas antisense neutralization of SOCS3 promoted TCR-induced proliferation (36). The study by Banerjee et al. (37) demonstrated an association between SOCS3 and calcineurin, which correlated with the suppression of NF-ATp activation and IL-2 transcription in response to T cell activation. Thus, SOCS3 might regulate tolerance through the direct inhibition of IL-2 gene transcription. Finally, given the fact that IL-10 induces expression of SOCS3 (38), the induction of SOCS3 in tolerant T cells, possibly through IL-10 signaling, could be essential for negatively regulating TCR signaling and maintaining the anergic phenotype. Alternatively, negative feedback for IL-10 signaling could limit the inhibitory effect of IL-10 during a protective immune response.

Phosphorylation of STAT3 and STAT5 correlated with culture in the presence of IL-10 and IL-2, respectively. It appeared, however, that both naive and PI-TReg cells were equally sensitive to these cytokines, indicating that signaling pathways for IL-2 and IL-10 in PI-TReg cells are intact despite their regulatory phenotype. In fact, there was no detectable difference in the Jak/STAT response of naive vs. PI-TReg cells to either IL-2 or IL-10 in vitro, demonstrating that the primary control over the regulatory state of naive vs. PI-TReg cells lies in cytokine production rather than T cell responsiveness to cytokines. PI-TReg cells responded normally to IL-2 in terms of Jak/STAT signaling. The addition of IL-2 to PI-TReg cells reversed the proliferative anergy of the cells and also permitted IFN-γ production by a distinct population of anergic cells. This did not, however, represent a reinstatement of the naive T cell phenotype because IL-2 did not induce the expression of IFN-γ in IL-10-producing cells, but enhanced IL-10 production by the cells and failed to reverse the ability of the cells to secrete endogenous IL-2. The effect of IL-2 on PI-TReg cells required ligation of the TCR, and this correlated with up-regulation of CD25 on the IL-10-producing cells. Most of the tolerant T cells were IL-10 negative (Figs. 6 and 7), and a subset of these IL-10-negative cells also expressed CD25 in responding to Ag and IL-2 in vitro (Fig. 7). However, failure in production of IL-2 suggests that these cells were only partially reversed from tolerant status. Most importantly, however, PI-TReg cells retained their capacity to suppress naive cells in vitro once IL-2 had been removed from the proliferating cultures. This shows that the suppressive phenotype of PI-TReg cells is a stable phenotype. As such, these cells are similar to Ag-induced TReg cells expanded in vitro either in the presence of IL-10 (6) or dexamethasone and vitamin D3 (39). Both of these IL-10-secreting TReg cells require IL-2 for their expansion in vitro, and yet suppress naive cells following removal of IL-2. These cells are, however, distinct from cells rendered anergic by repeated encounter with superantigens in vivo that were defective in signaling through the common γ-chain of the IL-2R (16).

PI-TReg cells resemble the natural CD4+ CD25+ population of TReg cells in being regulated by IL-2 (40). Recent evidence suggests that CD4+ CD25+ TReg cells expand in vivo in response to Ag in an IL-2-dependent fashion. Thus, IL-2−/− mice are deficient in CD4+ CD25+ TReg cells (41). Furthermore, CD4+ CD25+ TReg cells were shown to depend on expression of the IL-2Rβ chain to function as effective regulatory cells in certain model systems (42). Most importantly, however, even the addition of IL-2 to PI-TReg cells did not induce IL-2 gene transcription in these cells. Therefore, while the ability of these cells to expand through proliferation, to respond to Ag through IFN-γ secretion, and to suppress naive cell activation was reversed by addition of exogenous IL-2, these cells remained unable to sustain themselves through production of their own IL-2. It has also been shown that high-dose IL-2 could break the anergic state and suppressive function of CD4+ CD25+ TReg cells in vitro (43). Interestingly, IL-2 overcame the suppressive properties of CD25+ cells in a similar fashion to the effect of this cytokine on PI-TReg cells. Furthermore, Takahashi et al. (43) showed that removal of IL-2 from CD25+ cultures led to reinstatement of their suppressive properties. However, it remains to be seen whether CD4+ CD25+ TReg cells maintain the ability to produce IL-10, which, we believe, is essential for PI-TReg cells to function as differentiated regulatory T cells.

We believe that the features of both CD4+ CD25+ and PI-TReg cells are consistent with an immune system designed to discriminate self from nonself Ags. TCRs are highly cross-reactive, and it is unlikely that any self Ag-specific TCR would not potentially respond to a foreign Ag expressed by an infectious agent (44, 45). TReg cells would also express cross-reactive TCR, and this begs the question of how an immune system containing such suppressor cells could still function effectively? There are a number of mechanisms by which the suppressive function of regulatory cells may be overcome in the context of a strong microbial stimulus. Ligation of TLRs on dendritic cells will result in up-regulation of MHC and costimulatory molecules (46). The effect of this is to alter the balance of signaling through CD28 and CTLA-4 on the regulatory cells themselves. Studies in both CD25-positive (47) and T regulatory type 1 cells (18) have indicated that CD28 costimulation overcomes the suppressive properties of these cells. In addition, TLR activation of dendritic cells leads to the secretion of cytokines, such as IL-6, that appear to sensitize T cells, making them resistant to suppression (48). Furthermore, as shown in this study for PI-TReg cells, the suppressive activity of both types of regulatory cells can be overcome by IL-2 secreted in response to a highly potent infectious agent.

In summary, a number of mechanisms dictate that regulatory cell activity is overcome in the face of strong microbial challenge. As revealed in this work, however, the regulatory phenotype of TReg cells is not necessarily reversed under these conditions. As such, the regulatory function of these cells may be overcome during infection, but the ability of the cells to suppress autoimmune responses would be reinstated once the infection had been cleared.
The maintenance of cytokine responsiveness among T_{Reg} cells enables these cells to be overridden transiently during infection while retaining their regulatory function.

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References


