

Negative feedback control of the autoimmune response through antigen-induced differentiation of IL-10-secreting Th1 cells

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Regulation of the immune response to self- and foreign antigens is vitally important for limiting immune pathology associated with both infections and hypersensitivity conditions. Control of autoimmune conditions can be reinforced by tolerance induction with peptide epitopes, but the mechanism is not currently understood. Repetitive intranasal administration of soluble peptide induces peripheral tolerance in myelin basic protein (MBP)-specific TCR transgenic mice. This is characterized by the presence of anergic, interleukin (IL)-10-secreting CD4⁺ T cells with regulatory function (IL-10 T reg cells). The differentiation pathway of peptide-induced IL-10 T reg cells was investigated. CD4⁺ T cells became anergic after their second encounter with a high-affinity MBP peptide analogue. Loss of proliferative capacity correlated with a switch from the Th1-associated cytokines IL-2 and interferon (IFN)- γ to the regulatory cytokine IL-10. Nevertheless, IL-10 T reg cells retained the capacity to produce IFN- γ and concomitantly expressed T-bet, demonstrating their Th1 origin. IL-10 T reg cells suppressed dendritic cell maturation, prevented Th1 cell differentiation, and thereby created a negative feedback loop for Th1-driven immune pathology. These findings demonstrate that Th1 responses can be self-limiting in the context of peripheral tolerance to a self-antigen.

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Abbreviations used: i.n., intranasal; MBP, myelin basic protein; MFI, mean fluorescence intensity; rhIL-2, recombinant human IL-2.

Antigens administered in a tolerogenic form have long been known to result in down-regulation of immune responses. In recent years, the potential of antigen-driven immunotherapy for the treatment of allergic and autoimmune diseases has been investigated in several experimental models. Administration of antigenic peptides via the intranasal (i.n.) route induces tolerance, and thus inhibits the development of both autoimmunity (Metzler and Wraith, 1993; Staines et al., 1996; Tian et al., 1996; Karachunski et al., 1997) and allergy (Hoynes et al., 1993). Possible mechanisms of tolerance induction include elimination of peptide-specific T cells by activation-induced cell death/apoptosis (Critchfield et al., 1994; Chen et al., 1995; Liblau et al., 1996) or modification of their function via induction of anergy (Kearney et al., 1994), TCR/coreceptor down-regulation (Schonrich et al., 1991), immune deviation (Guery et al., 1996), or secretion of immunoregulatory cytokines such as IL-10 and TGF- β (Miller et al.,

1992; Sundstedt et al., 1997). Most immune cells, including monocytes, macrophages, DCs, NK cells, B cells, and T cells, are capable of secreting IL-10 under specific circumstances (Moore et al., 2001). Among these, IL-10-secreting CD4⁺ T cells are the best characterized because of their recently recognized role in immune regulation (O'Garra et al., 2004). Two phenotypically distinct CD4⁺ T regulatory (T reg) cell types have been described—naturally occurring FoxP3⁺ T reg cells that form an inherent part of the naive T cell repertoire (Sakaguchi et al., 1995) and induced, FoxP3⁻ IL-10-secreting T reg cells (for review see Roncarolo et al., 2006). Numerous subtypes of induced IL-10-secreting T reg cells with variable cytokine profiles have been generated

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in both murine and human systems. However, in contrast to T helper cells, the differentiation of induced T reg cells remains poorly defined.

i.n. administration of a soluble peptide induces peripheral tolerance in TCR transgenic (Tg4) mice specific for the acetylated N-terminal peptide Ac1-9 of murine myelin basic protein (MBP). Increasing the affinity of the peptide for I-A^u greatly enhances the tolerogenicity of the peptide in the Tg4 mouse (Liu et al., 1995). After a single i.n. dose of a high-affinity analogue of the MBP epitope, Ac1-9[4Y], with a tyrosine substituting the lysine at position four, T cell deletion is only transient and incomplete (Burkhart et al., 1999). Instead, Tg4 CD4⁺ T cells become anergic and exhibit a shift in cytokine secretion profile toward IL-10 after repeated i.n. treatment with peptide (Burkhart et al., 1999). Evidence for the generation of CD4⁺ T cells with a regulatory phenotype in this model stems from both in vitro and in vivo suppression assays (Sundstedt et al., 2003). Thus, i.n. treatment with MBP Ac1-9[4Y] induces active tolerance in the form of IL-10-secreting T reg cells (IL-10 T reg cells) rather than deletion. A role for IL-10 in suppression in vivo and in experimental autoimmune encephalomyelitis protection was demonstrated by anti-IL-10 (Burkhart et al., 1999) and anti-IL-10R (Sundstedt et al., 2003) antibody administration. IL-10 has important immunosuppressive and antiinflammatory effects on immune responses to both foreign and self-antigens (Moore et al., 2001) that are primarily mediated by its inhibitory activities on the function of APCs (de Waal Malefyt et al., 1991). Although the role of IL-10 in suppression of experimental autoimmune encephalomyelitis in the Tg4 model is not known, the effect of IL-10 on antigen presentation and inflammation is a likely mechanism. Naturally occurring FoxP3⁺ T reg cells form a part of the Tg4 CD4⁺ T cell repertoire and may rely on IL-10 to mediate suppression, as previously shown in other inflammatory settings (Asseman et al., 1999). Even so, peptide-induced IL-10 T reg cells were found to be distinct in origin from naturally occurring T reg cells in that they do not express Foxp3 (Vieira et al., 2004). Genetic depletion of FoxP3⁺ T reg cells from the CD4⁺ T cell repertoire in the RAG-deficient Tg4 mouse gives rise to spontaneous EAE. However, the onset of disease can be prevented by repetitive treatment with i.n. peptide, correlating with the generation of IL-10 T reg cells (Nicolson et al., 2006).

It has been proposed that induced IL-10 T reg cells arise from fully differentiated T effector cells that have lost the ability to secrete their hallmark cytokines as a result of chronic antigenic stimulation (O'Garra et al., 2004). Alternatively, induced IL-10 T reg cells could arise directly from naive precursors without a T effector phase. In this study, we investigate the ontogeny of induced IL-10 T reg cells generated by repeated i.n. peptide treatment. By following the differentiation pathway taken by CD4⁺ T cells over the course of tolerance induction, we demonstrate that peptide-induced IL-10 T reg cells are of Th1 origin and that IL-10 T reg cells complete the negative feedback loop of pathogenic Th1 responses in autoimmunity.

RESULTS

Serum cytokine levels associated with tolerization

Peripheral tolerance is induced in the Tg4 TCR transgenic mouse by repeated i.n. administration of antigenic peptide (Burkhart et al., 1999). This results in the generation of IL-10-secreting T reg cells capable of suppressing immune responses in vivo (Sundstedt et al., 2003). To investigate the differentiation pathway of peptide-induced IL-10 T reg cells, we first sought to determine changes in the serum cytokine profile over the course of tolerization in Tg4 *Rag1*^{-/-} mice. Serum concentrations of IL-2, IFN- γ , IL-17, IL-4, IL-5, and IL-10 were assessed at 2 h after successive i.n. peptide administration (Fig. 1). The 2-h time point represents the peak of cytokine secretion, as determined previously (Sundstedt et al., 2003). As shown in Fig. 1, distinct patterns of serum cytokine levels emerged. The Th1 cytokines IL-2 and IFN- γ followed a similar profile, with the mean serum levels peaking after the second i.n. peptide treatment and decreasing to near background thereafter. IL-17 secretion was delayed relative to the Th1 cytokines with a peak after the fourth i.n. peptide administration. In marked contrast, the Th2 cytokines IL-4 and IL-5 were secreted at only very low levels over the entire course of i.n. peptide treatment. Secretion of the immunoregulatory cytokine IL-10 peaked after the fifth i.n. peptide treatment, and although its level subsequently decreased, it remained above those of all other cytokines tested. These results suggest that repeated peptide administration leads

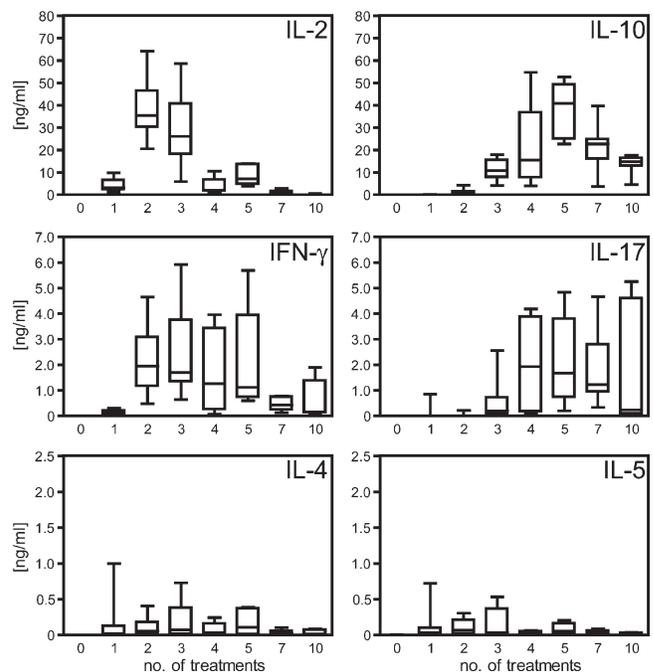


Figure 1. Changes in serum cytokine profile over the course of tolerance induction. Tg4 *Rag1*^{-/-} mice were bled before treatment and after each successive i.n. MBP Ac1-9[4Y] administration. Serum concentrations of IL-2, IL-10, IFN- γ , IL-17, IL-4, and IL-5 were measured by cytometric bead assay. Results show the mean serum cytokine concentration of six to eight mice per treatment group on pooled data from two separate experiments.

to suppression of the Th1-associated cytokines, IL-2 and IFN- γ , produced as an early response to antigen in vivo, while sparing IL-10 production.

CD4⁺ T cell tolerance encompasses anergy, down-regulation of IFN- γ , and increased IL-10 production

Although the serum cytokine profile provides an overall picture of the immune response to i.n. peptide administration, it does not allow discrimination of cytokine production by different cellular components of the immune system. We therefore investigated the contribution of CD4⁺ T cells to the aforementioned changes in serum cytokine concentrations. Tg4 CD4⁺ T cells were previously shown to respond to the first i.n. peptide treatment by initial proliferation and expansion accompanied by a cytokine burst (Burkhart et al., 1999). Indeed, splenocytes from Tg4 *Rag1*^{-/-} mice, after successive i.n. MBP Ac1-9[4Y] treatments, showed a fourfold increase in their number at 2 h after the second treatment compared with untreated mice; the number of splenocytes decreased with subsequent i.n. peptide treatments (Fig. S1 A). The number of CD4⁺ T cells in the peptide-treated Tg4 *Rag1*^{-/-} spleens increased with a similar pattern to that of whole splenocytes during the course of tolerance induction (Fig. S1 B). Given that CD4⁺ T cells from Tg4 mice treated repeatedly with i.n. peptide appear profoundly unresponsive (Sundstedt et al., 2003), we determined the kinetics of anergy induction as well as changes in the CD4⁺ T cell cytokine profile over the course of tolerization in Tg4 *Rag1*^{-/-} mice. As shown in Fig. 2 A, CD4⁺ T cells from mice either left untreated or treated once with i.n. peptide proliferated vigorously in response to antigen restimulation in vitro. However, the level of CD4⁺ T cell proliferation declined dramatically upon subsequent i.n. peptide treatments (Fig. 2 A). The addition of recombinant human IL-2 (rhIL-2) to the unresponsive CD4⁺ T cells led to recovery of proliferation (Fig. 2 B), thereby demonstrating that the cells were in a state of clonal anergy. Supernatants from the CD4⁺ T cell proliferation assays were collected and analyzed for IL-2, IFN- γ , IL-4 and IL-10 levels by ELISA. Fig. 2 C shows changes in CD4⁺ T cell cytokine secretion after successive i.n. peptide treatments. CD4⁺ T cells from mice either left untreated or treated once with i.n. peptide secreted high levels of IL-2 and IFN- γ when restimulated with antigen in vitro. Levels of both cytokines decreased dramatically after just two i.n. peptide treatments (Fig. 2 C, top and middle, respectively). IL-10 was only detected in cultures of CD4⁺ T cells from mice treated three times or more, i.e., after the decrease in IL-2 and IFN- γ secretion (Fig. 2 C, bottom). A similar inverse relationship between IL-2 and IL-10 was also observed in the analysis of serum cytokines (Fig. 1). Although the addition of rhIL-2 restored proliferation (Fig. 2 A), it did not have an effect on endogenous IL-2 secretion and resulted in only a minor increase in IFN- γ production by CD4⁺ T cells isolated after the tenth i.n. peptide treatment (Fig. 2 D, top and middle, respectively). In contrast, the secretion of IL-10 was greatly augmented by the presence of rhIL-2 (Fig. 2 D, bottom). IL-4 remained

below the detection level throughout the course of i.n. peptide treatment, irrespective of the addition of rhIL-2 (not depicted). These results support the findings of previous studies showing that IL-2 reverses the anergy of peptide-induced IL-10 T reg cells from mice treated with 10 doses of i.n. peptide without changing their cytokine secretion pattern (Anderson et al., 2005).

Peptide-induced IL-10 T reg cells differentiate via the Th1 pathway

To determine which cells were secreting IL-10, the proportion of CD4⁺ T cells producing IL-2, IFN- γ , IL-17, and/or IL-10 at the single-cell level was determined by intracellular

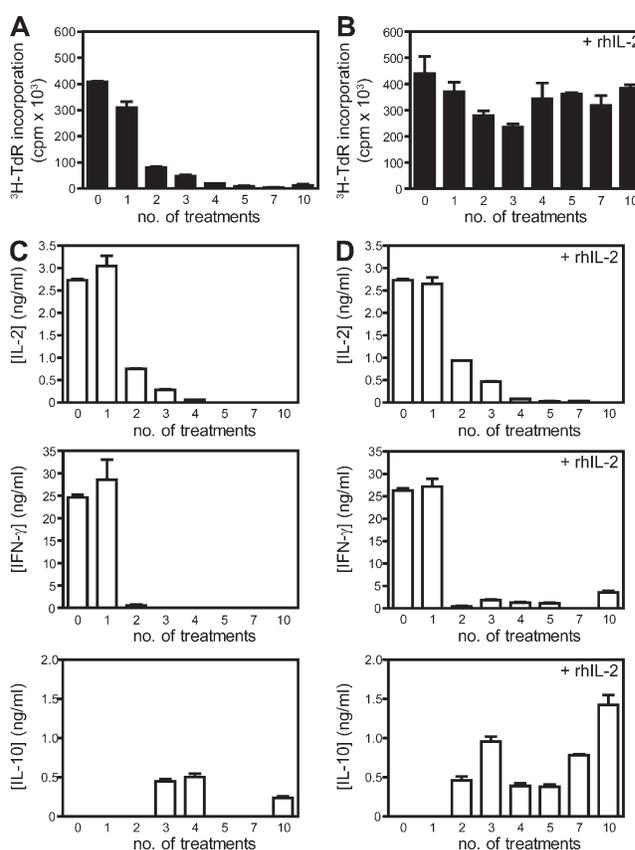


Figure 2. Anergy-associated down-regulation of Th1 cytokines and increase in IL-10 production by CD4⁺ T cells during tolerization.

Splenic CD4⁺ T cells were positively selected from untreated or i.n. MBP Ac1-9[4Y]-treated Tg4 *Rag1*^{-/-} mice at 2 h after each peptide administration. 5×10^4 CD4⁺ T cells per well were cultured with 100 μ g/ml of MBP Ac1-9[4K] in the presence of 10^5 autologous irradiated CD4 depleted splenocytes as APCs and, where indicated, 20 U/ml of rhIL-2. (A and B) Proliferative responses were measured at 72 h by ³[H]thymidine incorporation in the absence or presence of rhIL-2. Results are depicted as the mean thymidine incorporation \pm SEM. (C and D) Cytokine responses of CD4⁺ T cells from the cultures described in A and B were measured by ELISA at 24 h (IL-2) and 72 h (IFN- γ and IL-10) after in vitro restimulation in the absence or presence of rhIL-2. Results are depicted as the mean cytokine production in ng/ml of supernatant \pm SEM. Data in each panel are representative of three individual experiments.

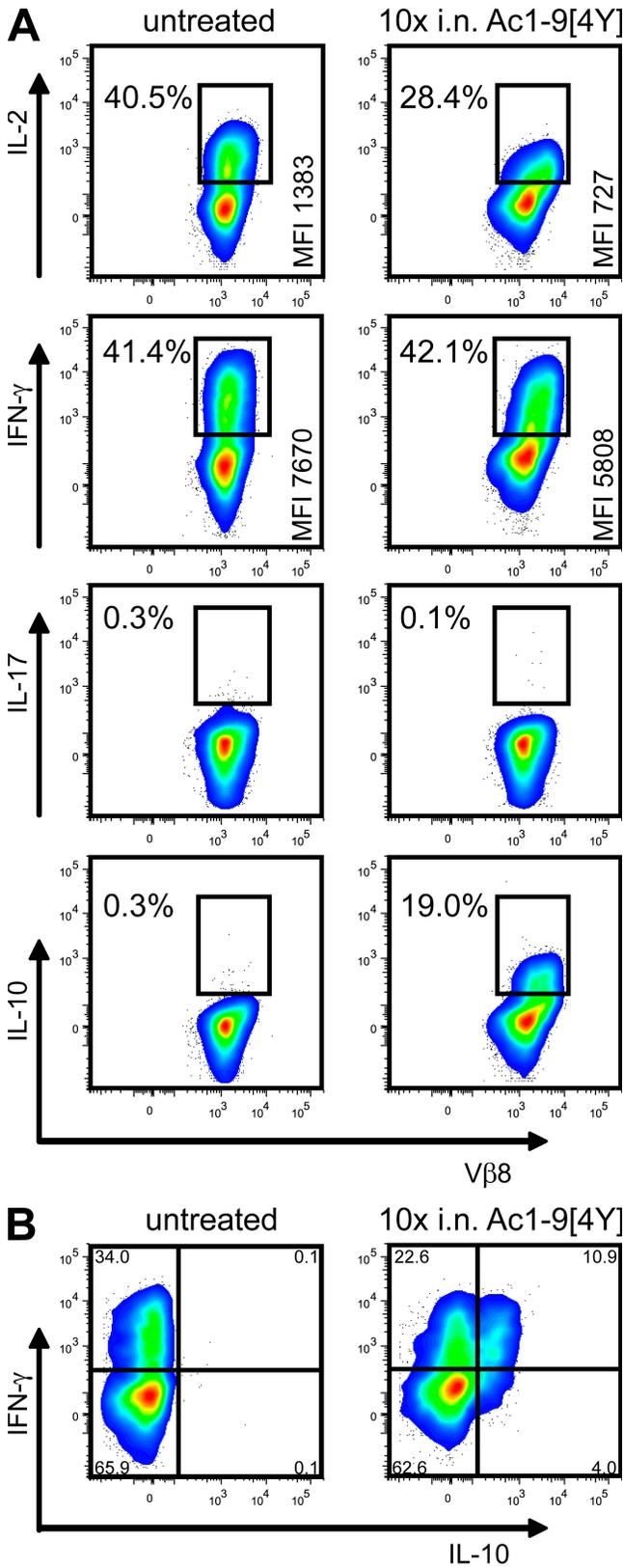


Figure 3. Th1 differentiation pathway of IL-10 T reg cells from tolerized mice. Tg4 *Rag1*^{-/-} mice were treated with a minimum of 10 i.n. doses of MBP Ac1-9[4Y]. Splenic CD4⁺ T cells were positively selected

cytokine staining. Previous work had shown that the combination of PMA and ionomycin was a sufficiently potent stimulus to induce synthesis of cytokines whose transcription had been inhibited through anergy induction (Macian et al., 2002). We have taken advantage of this phenomenon to investigate the cytokine history of peptide-induced IL-10 T reg cells. After antigenic stimulation in vitro, ~41% of CD4⁺ T cells from untreated mice produced IL-2 (mean fluorescence intensity [MFI] = 1,383) or IFN- γ (MFI = 7,670) when the cells were treated with PMA and ionomycin, whereas production of IL-17 and IL-10 was not induced (Fig. 3 A). A lower proportion of CD4⁺ T cells from mice treated with 10 doses of i.n. peptide produced IL-2 (MFI = 727), whereas a similar percentage produced IFN- γ (MFI = 5,808) upon restimulation with PMA and ionomycin, albeit with lower MFI. In addition, up to 19% of these CD4⁺ T cells produced IL-10. Interestingly, the majority of IL-10-secreting CD4⁺ T cells coproduced IFN- γ (Fig. 3 B). A similar pattern of cytokine secretion was observed when peptide-induced T reg cells were activated in vivo with a dose of i.n. peptide 2 h before PMA and ionomycin stimulation in vitro (unpublished data). No IL-17 was detected in CD4⁺ T cells from either untreated or peptide-treated mice (Fig. 3 A), suggesting that the IL-17 detected in serum (Fig. 1) originated from a different cellular source, such as NKT cells, monocytes, and/or granulocytes (Hue et al., 2006; Michel et al., 2007). These results demonstrate that the peptide-induced IL-10 T reg population is composed of cells capable of secreting IL-10 in response to antigen whose Th1 origin was revealed by the induction of IL-2 and IFN- γ synthesis after stimulation with PMA and ionomycin.

Gene expression associated with the differentiation of peptide-induced IL-10 T reg cells

Further evidence in favor of the Th1 origin of peptide-induced IL-10 T reg cells was provided by the analysis of lineage-determining transcription factors. Changes in the relative mRNA expression of Th1 and Th2 master regulators *t-bet* and *gata-3* by CD4⁺ T cells were monitored by quantitative real-time RT-PCR over the course of tolerization. As shown in Fig. 4 A, the relative expression of *t-bet* increased 25-fold after the first i.n. peptide treatment and reached a plateau of ~100-fold after the second i.n. peptide treatment. Conversely, the relative expression of *gata-3* decreased after the first i.n. peptide treatment in comparison to the level seen in untreated mice (Fig. 4 B). In addition to T-bet, a previous

from either untreated or peptide-treated mice 3 d after the last treatment and cultured with 10 μ g/ml of MBP Ac1-9[4K] in the presence of irradiated B10.PL splenocytes as APCs. RhIL-2 (20 U/ml) was added on d 1 and 3 to CD4⁺ T cell cultures from peptide-treated and untreated mice, respectively. (A and B) Intracellular cytokine staining for IL-2, IFN- γ , IL-17, and IL-10 was performed on d 7 of culture after additional restimulation with PMA and ionomycin. Depicted are density plots of V β 8⁺ T cells with cytokine gates based on isotype controls. Data are representative of at least three individual experiments.

study revealed expression of the anergy-associated gene *egr-2* and the co-stimulatory molecule *icos* in Tg4 CD4⁺ T cells from i.n. peptide-treated mice (Anderson et al., 2006). Expression of Egr-2 was strikingly up-regulated after the second i.n. peptide administration (Fig. 4 C), correlating with reduced proliferation (Fig. 2 A). Up-regulation of *icos* was delayed by one i.n. peptide treatment relative to *egr-2* and T-bet (Fig. 4 D). Instead, *icos* expression appeared to be more closely associated

with IL-10 secretion (Fig. 2, C and D). To assess T-bet expression by IL-10 T reg cells, we used a cytokine capture assay to enrich IL-10-secreting CD4⁺ T cells. The IL-10-secreting fraction comprised CD25^{high}, CD69^{high} to CD69^{intermediate}, CD62L^{low}, and CD44^{high} CD4⁺ T cells characteristic of activated, antigen-experienced CD4⁺ T cells (unpublished data). Essentially all IL-10-secreting CD4⁺ T cells expressed T-bet, as also found in in vitro-differentiated Th1, but not Th2,

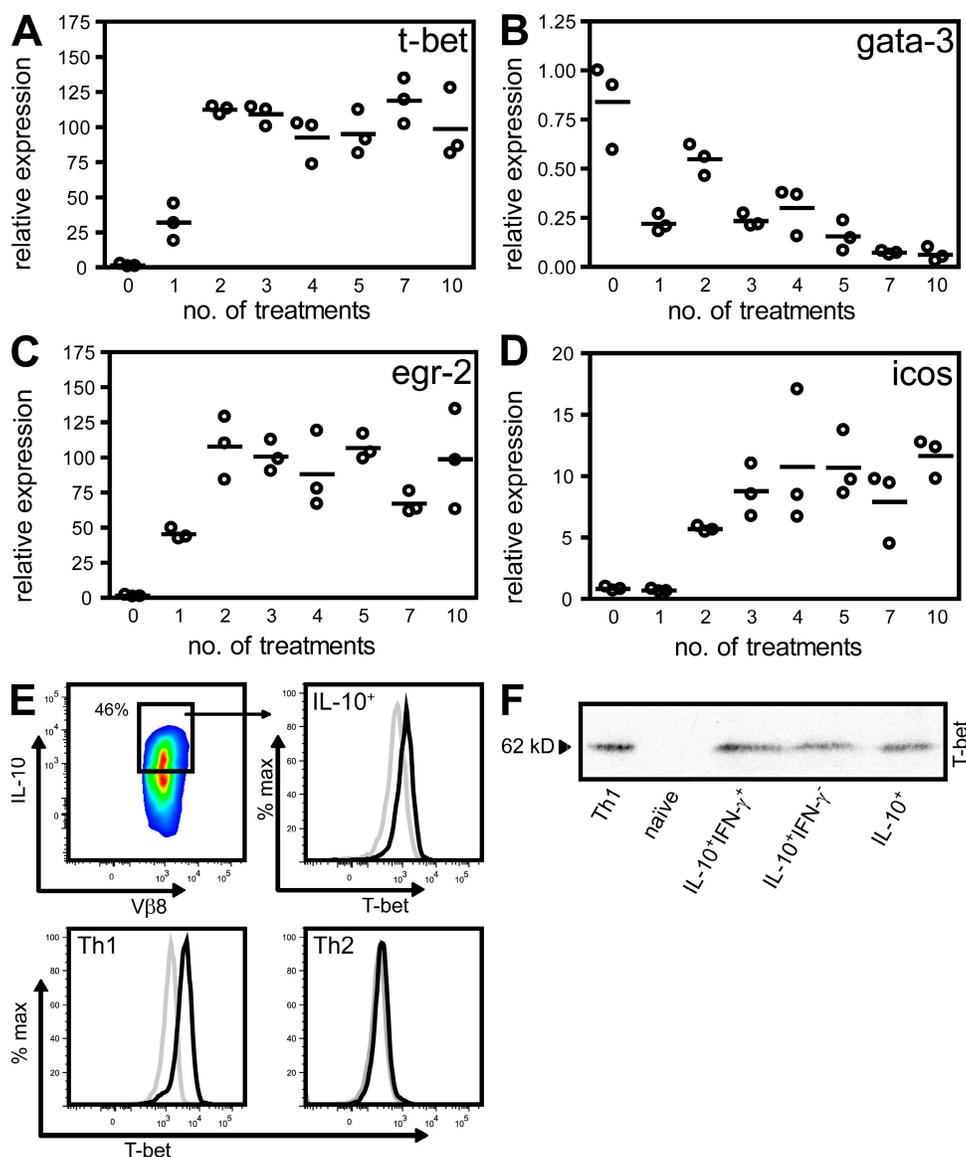


Figure 4. Expression of genes associated with differentiation of IL-10 T reg cells. (A–D) Tg4 *Rag1*^{-/-} mice were treated with a minimum of 10 i.n. doses of MBP Ac1-9[4Y]. Total RNA was extracted from splenic CD4⁺ T cells positively selected from either untreated or peptide-treated mice at 2 h after each peptide treatment. The RNA was reverse transcribed and expression levels of the transcription factors *t-bet*, *gata-3*, *egr-2*, and the co-stimulatory molecule *icos* were determined by quantitative real-time PCR after normalization to β 2-microglobulin expression. Results show the relative expression of the gene of interest from three accumulated experiments at each time point. (E) Enriched IL-10-secreting CD4⁺ T cells from Tg4 mice treated with a minimum of 10 i.n. doses of MBP Ac1-9[4Y] and in vitro-differentiated Th1 and Th2 cells were stained for surface Vβ8 and intracellular T-bet (black line) or isotype control (gray line). The depicted density plot of IL-10-enriched CD4⁺ T cells is gated on Vβ8⁺ T cells. The histogram of the IL-10⁺ cell fraction is gated on Vβ8⁺IL-10⁺ cells and Th1/Th2 cell histograms on Vβ8⁺ T cells. Data are representative of at least three individual experiments. (F) FACS-sorted IL-10⁺, IL-10⁺IFN-γ⁺, and IL-10⁺IFN-γ⁻ Tg4 CD4⁺ T cells were analyzed for T-bet expression by Western blot, with Th1 and naive (CD4⁺CD62L⁺) cells used as positive and negative controls for T-bet detection, respectively. Data are representative of two individual experiments.

cells (Fig. 4 E). Because a significant proportion of IL-10–secreting CD4⁺ T cells from mice treated with 10 doses of i.n. peptide coproduced IFN- γ , CD4⁺ T cells from peptide-treated mice were sorted into IL-10⁺, IL-10⁺ IFN- γ ⁺, and IL-10⁺ IFN- γ ⁻ CD4⁺ T cell fractions to reveal T-bet expression by each subset (Fig. 4 F). The Western blot results show T-bet protein expression in the sorted, IL-10–secreting T cell fraction as a whole, and importantly, in both the IL-10⁺ IFN- γ ⁺ and IL-10⁺ IFN- γ ⁻ CD4⁺ T cell subpopulations. This clearly demonstrates that IL-10–secreting CD4⁺ T cells retain T-bet expression despite losing the ability to secrete IFN- γ , thus further confirming their Th1 origin.

Negative feedback regulation of Th1 responses by IL-10 T reg cells is dependent on IL-10

The role of IL-10 in the induction of tolerance has been addressed by assessing the effect of repeated i.n. peptide treatment on CD4⁺ T cells from Tg4 *IL-10*^{-/-} mice. A comparison of in vitro proliferative responses from peptide-treated Tg4 or Tg4 *IL-10*^{-/-} mice revealed that CD4⁺ T cells from both mouse strains were anergic (Fig. 5 A). This correlated with a marked reduction in IL-2 production, which is consistent with clonal anergy (Fig. 5 B). Despite their anergic state, however, CD4⁺ T cells from peptide-treated Tg4 *IL-10*^{-/-} mice secreted IFN- γ at levels equivalent or higher than CD4⁺ T cells from untreated Tg4 mice

(Fig. 5 C). Furthermore, increased levels of IFN- γ , but not IL-2, were sustained in sera from peptide-treated Tg4 *IL-10*^{-/-} mice (Fig. S2). In contrast, IL-10, as opposed to IFN- γ , was detected in CD4⁺ T cell cultures from peptide-treated, control Tg4 mice (Fig. 5, C and D). These results suggest that treatment with i.n. peptide in the absence of IL-10 results in priming rather than tolerance induction, demonstrating the role for IL-10 in negative feedback regulation of Th1 responses.

Differentiation of peptide-induced IL-10 T reg cells is independent of extrinsic IL-10

Previous studies have shown that exogenous IL-10 encourages differentiation of IL-10–secreting T reg cells in vitro (Groux et al., 1996). We used an adoptive transfer model to investigate whether differentiation of peptide-induced IL-10 T reg cells depends on IL-10 secretion by other cells such as APCs. As shown in Fig. 6, transfer of Tg4 CD4⁺ T cells into B10.PL recipients followed by repeated peptide treatment led to the differentiation of IFN- γ –producing Tg4 cells, up to 8% of which coproduced IL-10 after activation with PMA and ionomycin. Transfer of Tg4 CD4⁺ T cells into IL-10–deficient B10.PL recipient mice allowed the differentiation of a population of cells with a similar proportion coproducing IFN- γ and IL-10, as well as a markedly distinct population (22%) producing IL-10 only. Neither group of control mice

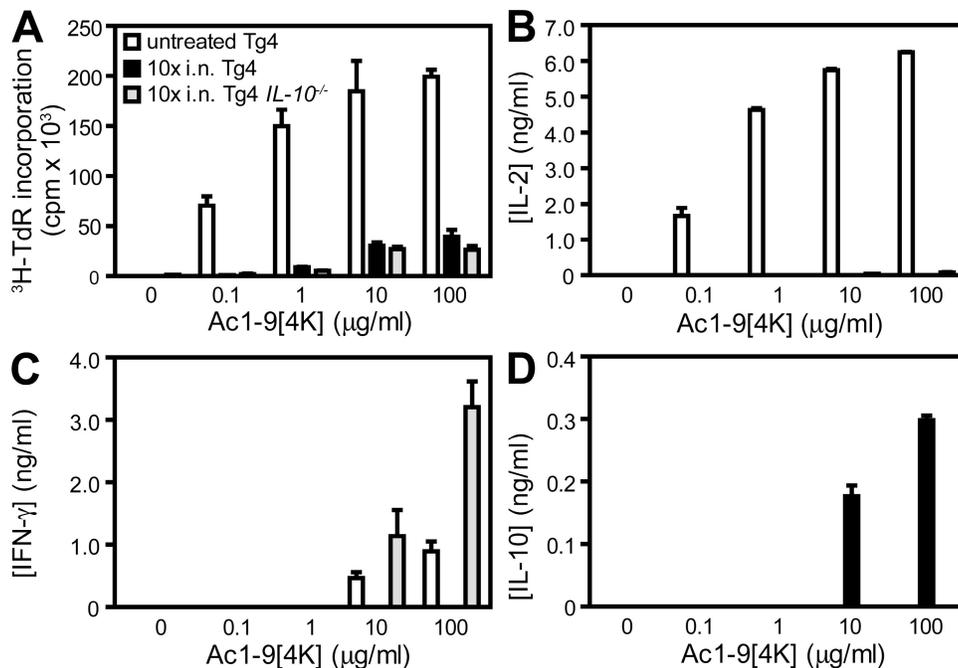


Figure 5. Role of IL-10 in down-regulation of Th1 cytokines in tolerance. Tg4 and Tg4 *IL-10*^{-/-} mice were treated with a minimum of 10 i.n. doses of MBP Ac1-9[4Y]. Splenic CD4⁺ T cells were positively selected from either untreated Tg4 or peptide-treated Tg4 and Tg4 *IL-10*^{-/-} mice 3 d after the last peptide treatment. 5×10^4 CD4⁺ T cells per well were cultured with 10^5 irradiated B10.PL splenocytes as APCs in the presence of a 10-fold titration of MBP Ac1-9[4K], ranging from 0.1 to 100 $\mu\text{g/ml}$. (A) Proliferative responses were measured at 72 h by [³H]thymidine incorporation. Results are depicted as the mean thymidine incorporation \pm SEM. (B–D) Cytokine responses of CD4⁺ T cells from the above cultures were measured by ELISA at 24 h (IL-2) and 72 h (IFN- γ and IL-10) after in vitro restimulation. Results are expressed as the mean cytokine production in nanogram/milliliter of supernatant \pm SEM. Data are representative of at least three individual experiments.

produced significant amounts of IL-10, and levels of IFN- γ were reduced (Fig. 6, bottom). IL-4 was not detected in either set of recipients (unpublished data). These data suggest that there is no requirement for IL-10 from non-T cells for the differentiation of IL-10 T reg cells. In fact, the ability of non-T cells to secrete IL-10 may limit the differentiation of IL-10 T reg cells.

Peptide-induced IL-10 T reg cells suppress DC function in an IL-10-dependent manner

Although IL-10 derived from non-T cells does not appear to be required for peptide-induced IL-10 T reg cell differentiation, our previous studies showed that IL-10 plays an essential role in T cell regulatory activity (Burkhart et al., 1999; Sundstedt et al., 2003). A potential negative feedback function for IL-10 T reg cells was investigated by studying their effect on DC activity. IL-10 is known to inhibit the function of macrophages and DCs by inhibiting their expression of MHC class II and co-stimulatory molecules and their proinflammatory cytokine secretion (Moore et al., 2001). The direct effect of peptide-induced IL-10 T reg cells on DC maturation was evaluated *in vitro*. As shown previously, splenic DCs undergo maturation when cultured *in vitro*,

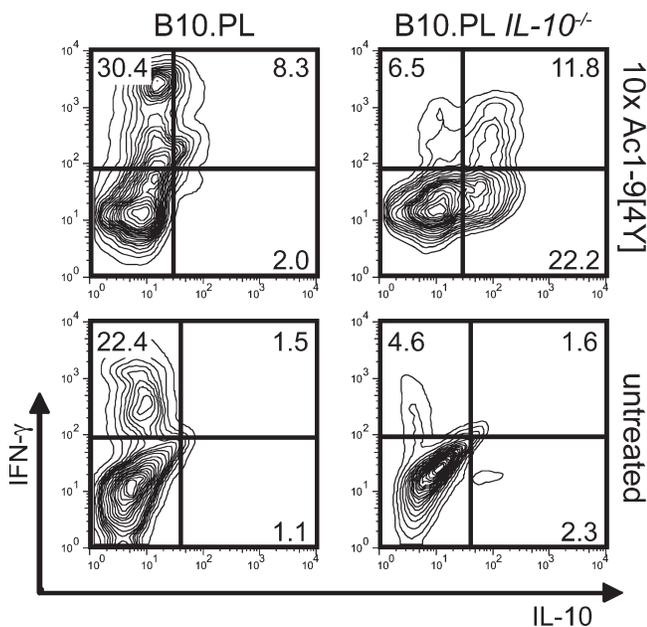


Figure 6. Redundancy of non-T cell-derived IL-10 in IL-10 T reg cell differentiation. Negatively selected Tg4 CD4⁺ T cells at 2×10^7 per mouse were adoptively transferred into either B10.PL or B10.PL *IL-10*^{-/-} recipients. 7 d later, mice were treated with a minimum of 10 i.n. doses of MBP Ac1-9[4Y] at 3–4 d intervals or left untreated as controls. Spleens from peptide-treated recipient mice were collected 3 d after the last peptide treatment. Tg4 CD4⁺ T cells were selected by culturing with 10 μ g/ml of Ac1-9[4K] and rIL-2 for 7 d and stained for surface ∇ B8 and intracellular IFN- γ or IL-10 after additional restimulation with PMA and ionomycin. The depicted contour plots are gated on ∇ B8⁺ T cells with quadrants based on isotype controls. Data are representative of at least three individual experiments.

demonstrating maximal increase in MHC class II and the co-stimulatory molecules CD80 and CD86 at 24 h after stimulation (Moser et al., 1995). Expression of CD80 and CD86, as well as CD40, was further enhanced by co-culture of splenic DCs with CD4⁺ T cells from untreated Tg4 mice in the presence of antigen (Fig. 7 A). This was, however, suppressed by cognate interaction with IL-10 T reg cells from peptide-treated Tg4 mice. CD80 and CD86 expression was suppressed, and this suppression was largely reversed with anti-IL-10R, but not with isotype control antibody (Fig. 7 A, top and middle). The presence of anti-IL-10R antibody, however, fully reversed suppression of CD40 expression (Fig. 7 A, bottom). In contrast to CD4⁺ T cells from peptide-treated Tg4 mice, only very low level suppression of CD80, CD86, and CD40 expression was observed in splenic DCs co-cultured with CD4⁺ T cells from peptide-treated Tg4 *IL-10*^{-/-} mice (Fig. 7 A).

IL-12 secretion is associated with DC maturation (Macatonia et al., 1995; Heufler et al., 1996) and optimal secretion of this cytokine is reported to depend on cognate interaction with activated T cells (Heufler et al., 1996; Koch et al., 1996; Winzler et al., 1997). IL-12 production by splenic DCs cultured with either naive CD4⁺ T cells or peptide-induced IL-10 T reg cells was investigated. Fig. 7 B shows the level of IL-12 secretion by splenic DCs after 24 h co-culture *in vitro*. In contrast to splenic DCs co-cultured with naive CD4⁺ T cells, which displayed increased IL-12 production in the presence of antigen, IL-12 production by splenic DCs co-cultured with IL-10 T reg cells from peptide-treated Tg4 mice was abrogated in an antigen-specific manner. Whereas IL-10 T reg cells alone induced a slight reduction in IL-12 production, the addition of antigen almost completely inhibited production of the cytokine. Importantly, this effect was shown to be specific for IL-10, as it was reversed in the presence of anti-IL-10R antibody, whereas the addition of a control antibody had no effect on IL-12 production. Furthermore, CD4⁺ T cells from Tg4 *IL-10*^{-/-} mice did not suppress DC IL-12 secretion.

To demonstrate the *in vivo* relevance of peptide-induced IL-10 T reg cell–DC interactions, the functional properties of DCs isolated from either naive Tg4 mice or mice rendered tolerant by i.n. peptide treatment were further evaluated. Compared with splenic DCs from naive mice, DCs from tolerant mice were less effective at inducing naive CD4⁺ T cell proliferation *in vitro* (Fig. 8 A). Furthermore, splenic DCs from peptide-treated mice failed to support secretion of Th1-associated cytokines by these cells (Fig. 8 B). The defective induction of IL-2 and IFN- γ secretion by naive CD4⁺ T cells in the co-cultures correlated with markedly reduced IL-12 secretion (Fig. 8 B). The suppressive properties of IL-10 T reg cells therefore extend to DC function as well as phenotype. Collectively, our results reveal a classical negative feedback loop whereby chronically activated Th1 cells differentiate into IL-10 T reg cells capable of regulating DC function, thus preventing further generation of Th1 cells.

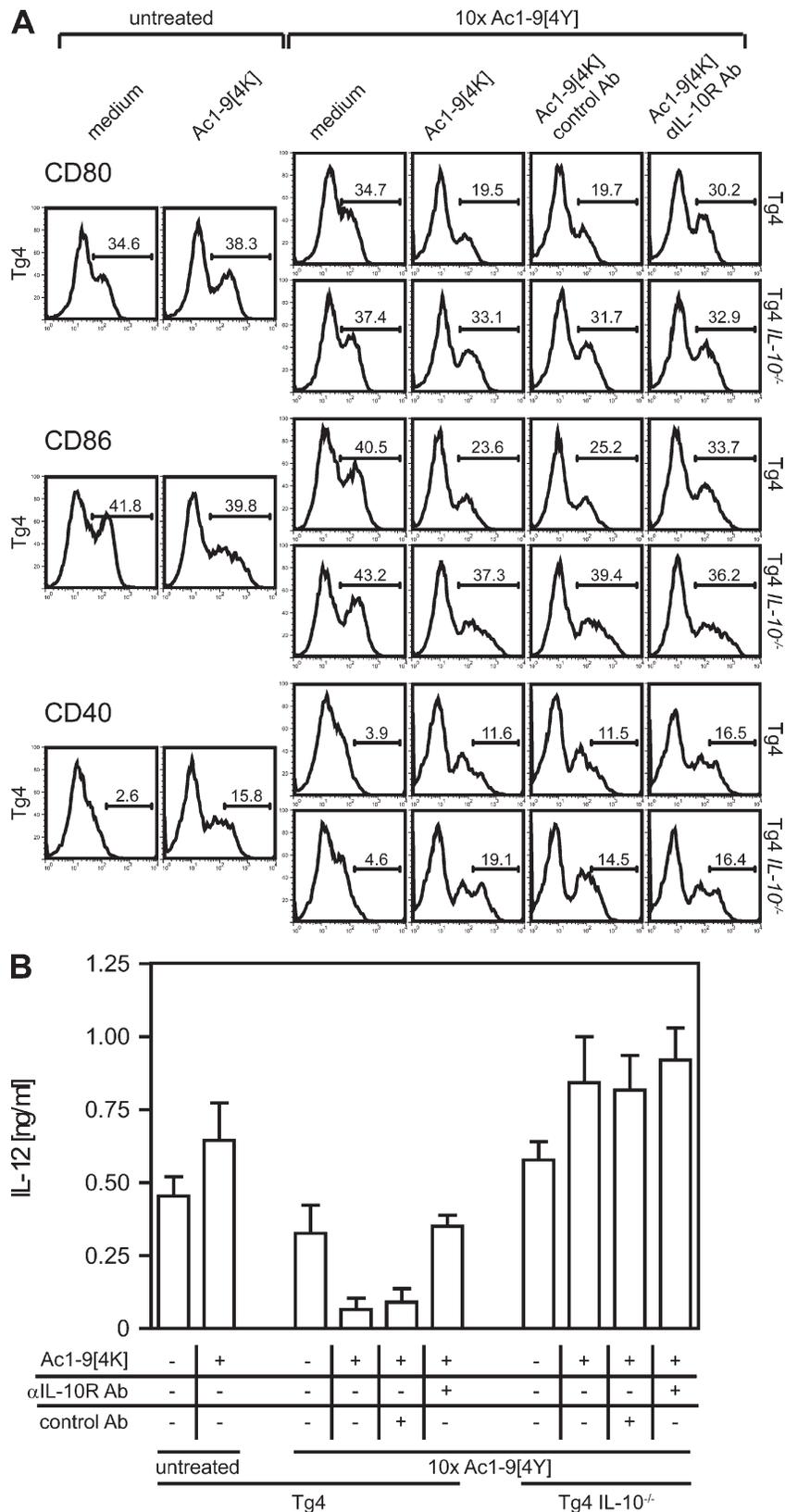


Figure 7. IL-10R signaling requirement for IL-10 T reg cell-mediated suppression of DC function. Tg4 and Tg4 *IL-10^{-/-}* mice were treated with a minimum of 10 i.n. doses of MBP Ac1-9[4Y]. Splenic DCs were isolated from untreated B10.PL mouse spleens and 5×10^4 DCs were cultured either alone or with equal numbers of CD4⁺ T cells positively selected from untreated or peptide-treated Tg4 or Tg4 *IL-10^{-/-}* mice. 100 μ g/ml of Ac1-9[4K] and/or

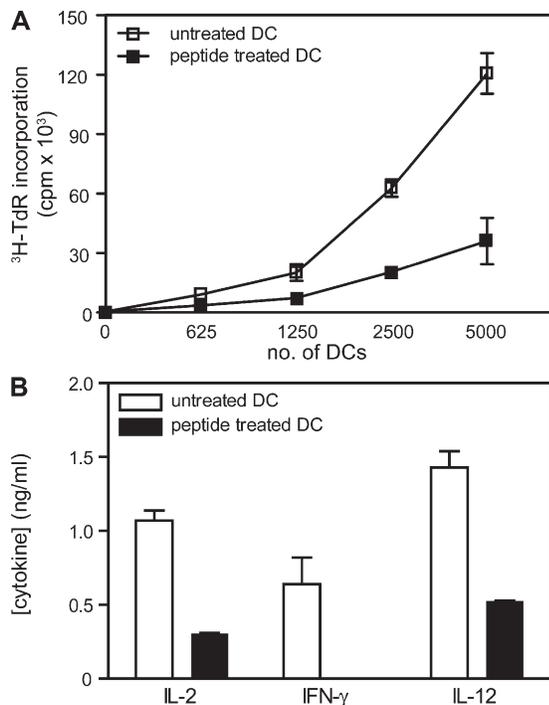


Figure 8. Role of IL-10 in negative feedback regulation of Th1 responses. Tg4 mice were treated with a minimum of 10 i.n. doses of MBP Ac1-9[4Y]. Splenic DCs were isolated from both untreated and peptide-treated Tg4 mice that were administered 100 μg of MBP Ac1-9[4Y] 3 h previously. CD4⁺ T cells positively selected from untreated Tg4 mouse spleens were cultured at 5×10^4 per well in the presence of a twofold titration of irradiated DCs at a ratio ranging from 80:1 to 10:1 and 10 $\mu\text{g}/\text{ml}$ of Ac1-9[4K]. (A) Proliferative responses were measured at 72 h by ^3H thymidine incorporation. Results are depicted as the mean thymidine incorporation \pm SEM. (B) Cytokine responses of CD4⁺ T cell from the 10:1 cultures were measured by ELISA at 24 h (IL-2) and 72 h (IFN- γ and IL-12) after in vitro restimulation. Results are depicted as the mean cytokine production in ng/ml of supernatant \pm SEM. Data are representative of two individual experiments.

DISCUSSION

Our results demonstrate how IL-10 T reg cells can be generated from Th1 precursors coincident with anergy induction under conditions involving repeated exposure to self-antigen. Peptide-induced IL-10 T reg cells block DC function in an IL-10-dependent manner and thereby complete a negative feedback loop of Th1 responses.

By studying the effect of chronic antigen exposure on a homogenous T cell population in RAG gene-deficient mice, we were able to exclude the influence of FoxP3⁺ T reg cells and B cells on IL-10 T reg cell generation (Dieckmann et al., 2002; Fillatreau et al., 2002; Mauri et al., 2003). Although FoxP3⁺ T reg cells and IL-10-secreting B cells can promote

IL-10 T reg cell generation, the results of this and other studies (Levings et al., 2005; Nicolson et al., 2006) demonstrate that the differentiation of IL-10 T reg cells can occur independently. The induction of IL-10 T reg cells in this model requires repeated administration of peptide antigen. This has allowed us to study the kinetics of IL-10 T reg cell generation. The initial response to soluble peptide was marked by secretion of the Th1 cytokines IL-2 and IFN- γ , followed by the induction of anergy and a switch to IL-10 secretion. The development of anergy after the second peptide administration is consistent with previous studies showing that naive cells are resistant to anergy induction (Hayashi et al., 1998; Andris et al., 2004). The induction of anergy in our study correlates with a high level of *egr-2* expression throughout the course of i.n. peptide treatment. *Egr-2* has previously been associated with T cell anergy (Harris et al., 2004; Safford et al., 2005). Recent studies have shown that *Egr-2* regulates the expression of the cell cycle inhibitor p21cip1 (Anderson et al., 2006) and that deletion of the *Egr-2* gene in T cells leads to loss of homeostatic control (Zhu et al., 2008). In addition, anergized CD4⁺ T cells from i.n. peptide-treated Tg4 mice have membrane proximal signaling defects affecting MAP kinase, PKC and calcium driven pathways (Anderson et al., 2006). As shown in other models of peripheral tolerance (Blackman et al., 1991; Macian et al., 2002), anergy can be overridden by treatment with agents capable of circumventing this membrane proximal block in signaling. Although exogenous IL-2 promotes proliferation of IL-10 T reg cells, it stimulates expression of Th1 cytokines to only a small degree. Macian et al. (2002) have shown that PMA and ionomycin treatment stimulated IFN- γ production in anergic Th1 cells. We adopted this approach to investigate which cytokines were suppressed as a consequence of the anergy induced by repetitive i.n. peptide administration. IL-10 T reg cells from i.n. peptide-treated mice produced IL-2 and IFN- γ at low or undetectable levels when stimulated with antigen. The same cells reexpressed IL-2 and IFN- γ , however, when stimulated with PMA and ionomycin. Interestingly, the majority of IL-10 T reg cells coproduce IFN- γ when activated with PMA and ionomycin, confirming their Th1 origin. The expression of Th1-associated transcription factor T-bet is maintained over the course of i.n. peptide treatment and can be detected in the IL-10-secreting fraction, both IFN- γ ⁺ and IFN- γ ⁻, of CD4⁺ T cells from i.n. peptide-treated mice. Because of the lack of IFN- γ expression by IL-10 T reg cells upon antigen stimulation in vivo (Anderson et al., 2005), the observed T-bet expression is most likely regulated by an IFN- γ independent mechanism such as TCR activation. Although anergy abrogates the production of IL-2 and IFN- γ , it permits the production of IL-10. Recent evidence has

10 $\mu\text{g}/\text{ml}$ of anti-IL-10R or isotype control antibody were added where indicated. (A) After 24 h, DCs were stained for surface CD11c, CD80, CD86, and CD40. Results are depicted histograms for each surface protein after gating on CD11c⁺ cells, with x and y axes showing the fluorescence intensity and percentage of max, respectively. (B) Supernatant cultures described in A were analyzed for IL-12 by ELISA at 24 h after in vitro restimulation. Results are depicted as the mean IL-12 production in nanogram/milliliter of supernatant \pm SEM. Data are representative of at least two individual experiments.

shown that induced T reg cells demonstrate enhanced activation of the p38–MAPKAP–K2/3 pathway (Ohkusu–Tsukada et al., 2004; Adler et al., 2007). The use of a p38–specific inhibitor in T cells anergized in vivo by superantigen exposure allowed recovery of IL-2, but inhibited IL-10 secretion. The p38 pathway can, therefore, differentially regulate both the cell cycle and IL-10 transcription.

The role of cytokines in the generation of IL-10 T reg cells is unknown. IL-2 and IL-12 have been reported to induce IL-10 expression in human T cells (Jeannin et al., 1996). A role for IL-2 signaling in IL-10 expression is supported by the in vitro enhancement of IL-10 secretion after addition of IL-2 in this study. Although exogenous IL-2 may increase IL-10 secretion by permitting proliferation rather than directly promoting its production, there is evidence for IL-2–driven enhancement of IL-10 production by other T reg cell populations, including FoxP3⁺ T reg cells (Barthlott et al., 2005) and cytotoxic T reg cells (Tsuji–Takayama et al., 2008). The role of IFN- γ in the generation of IL-10 T reg cells remains unclear. Generation of human IL-10–secreting Th1 cells was not affected by blocking antibodies against IFN- γ (Windhagen et al., 1996). Conversely, anti-IFN- γ suppressed the generation of IL-10 T reg cells after stimulation with a B7H1–Ig fusion protein (Ding et al., 2006). Furthermore, Shaw et al. (2006) proposed a model in which IFN- γ secretion by Th1 cells promotes IL-10 production among neighboring T cells by up–regulating ICOS–L expression on APCs. An association between IL-10 secretion and ICOS expression among CD4⁺ T cells has been shown previously (Lohning et al., 2003; Kohyama et al., 2004). As generation of IL-10 T reg cells by i.n. peptide administration is also associated with the induction of ICOS expression, such a mechanism could operate in our model. Although it is well established that growth of T cells in the presence of exogenous IL-10 leads to the generation of IL-10–secreting regulatory (Tr1) cells (Roncarolo et al., 2006), the role of exogenous IL-10 in generation of IL-10–secreting cells of Th1 origin is debatable. We have noted that IL-10 secretion by non–T cells is not required for and appears to suppress the generation of such cells in the face of chronic antigen encounter. Furthermore, IL-10⁺IFN- γ ⁺ CD4⁺ T cells are generated during chronic infection with *Toxoplasma gondii*, even when IL-10 signaling is blocked by anti–IL-10R (Jankovic et al., 2007). These data imply that the induction of IL-10 transcription in IL-10–secreting cells of Th1 origin is not dependent on exogenous IL-10.

T cell populations secreting both IFN- γ and IL-10 are found in both mouse and man (Trinchieri, 2007; O’Garra and Vieira, 2007; Haringer et al., 2009) and have been implicated in regulating the immune response to persistent infections with intracellular pathogens (Trinchieri, 2001; Anderson et al., 2007; Jankovic et al., 2007). The common feature of these infections is the development of a chronic stage of disease characterized by IL-10 secretion whereby low levels of the pathogen prevail and provide protection against subsequent reinfection. Under these circumstances, IL-10 plays a

role in protecting against excessive inflammation–associated pathology, suggesting an involvement in a negative feedback loop of Th1 responses. Here, we show for the first time that the generation of self–peptide–specific IL-10–secreting cells of Th1 origin depends primarily on repeated antigen encounter through the T cell receptor, and a similar mechanism could explain why such cells arise during the course of chronic infection.

The IL-10 T reg cells generated by repeated peptide administration are immune suppressive in vitro, inhibit T cell activation in vivo (Sundstedt et al., 2003), and, most importantly, suppress inflammatory autoimmune disease of the CNS in an IL-10–dependent fashion (Burkhart et al., 1999; O’Neill, et al., 2006). As yet, however, the mechanism of suppression mediated by IL-10 T reg cells has not been defined. In this study, IL-10 T reg cells were shown to suppress DC maturation and potently block IL-12 secretion. Our results support previous observations showing that IL-10 inhibits APC–dependent induction of Th1 cells by APC–derived IL-12 (Hino and Nariuchi, 1996; De Smedt et al., 1997). As previously suggested, bidirectional signaling between T cells and APCs, primarily via CD40–CD40L interaction, optimizes secretion of IL-12 (Heufler et al., 1996; Koch et al., 1996; Winzler et al., 1997). This appears to be overridden by IL-10 secretion in the case of IL-10 T reg cells. Indeed, DCs isolated from peptide–treated mice are unable to support Th1 cell differentiation. This demonstrates that DCs subjected to IL-10 conditioning in vivo do not immediately revert to an immunostimulatory phenotype when isolated from IL-10 T reg cells.

The influence of IL-10 T reg cells on DC function reveals a classical, negative feedback loop that has evolved to control the immune response to antigen and limit immune pathology. Repeated antigen encounter leads to anergy induction among Th1 cells and a shift in cytokine production from IFN- γ to IL-10. The resulting IL-10 T reg cells suppress DC maturation and IL-12 secretion, resulting in cessation of the ongoing Th1 response. We propose that i.n. administration of peptide antigens mimics the course of chronic infection for the induction of IL-10 T reg cells. This important finding not only advances our understanding of the mechanisms involved in the generation of peripheral tolerance but also demonstrates how self–limitation of Th1 responses could be used by antigen–directed therapy in the treatment of autoimmune diseases.

MATERIALS AND METHODS

Mice. All experimental mice were maintained under specific pathogen–free conditions. B10.PL (*H2^b*) mice were originally obtained from The Jackson Laboratory. The Tg4 TCR transgenic mouse expressing V β 8.2 TCR specific for MBP Ac1–9 in the context of I–A^u was described previously (Liu et al., 1995) and backcrossed for over 12 generations onto the B10.PL (*H2^b*) background. In Tg4 TCR transgenic mice, 95–100% of all CD4⁺ T cells express V β 8, whereas the diversity of V α chains is unknown. Generation of Tg4 *Rag1*^{–/–} mice was also previously described (Nicolson et al., 2006). Tg4 *IL-10*^{–/–} mice were generated by crossing Tg4 mice with a B10.129P2(B6)–*IL10*^{tm1Cgn/J} strain originally obtained from The Jackson Laboratory and bred

to provide a homozygous Tg4 *IL-10*^{-/-} (*H2^o*) line. The non-TCR transgenic *IL-10*^{-/-} (*H2^o*) littermates, hereafter termed B10.PL *IL-10*^{-/-} mice, were bred for experimental controls. All experiments were approved by the UK Home Office and performed according to animal welfare codes directed by the University of Bristol ethical review committee.

Peptides. The acetylated N-terminal peptide Ac1-9[4K] (AcASQKRP-SQR) of murine MBP and its higher MHC affinity analogue Ac1-9[4Y] (AcASQYRPSQR) with a tyrosine substituting the lysine at position four were synthesized on an AMS 422 multiple peptide synthesizer (Abimed Analyes-Technik) using standard fluorenyl methoxycarbonyl chemistry.

i.n. administration of MBP Ac1-9[4Y]. MBP Ac1-9[4Y] was administered i.n. at 80 µg of peptide in 20 ml of PBS under light isoflurane anesthesia at 3–4-d intervals over a period of 5 wk. CD4⁺ T cells from mice treated with a minimum of 10 i.n. doses of MBP Ac1-9[4Y] are referred to as IL-10 T reg cells.

Adoptive transfer. For adoptive transfer, recipients received 2 × 10⁷ CD4⁺ T cells by i.v. injection.

Cell separation. Purified CD4⁺ T cells were isolated from spleens by magnetic separation using mouse CD4 (L3T4) MicroBeads (positive selection), CD4⁺ T cell Isolation kit (negative selection), or CD4⁺CD62L⁺ T cell Isolation kit according to the manufacturer's instructions (Miltenyi Biotec). For T-bet flow cytometry staining of IL-10-secreting CD4⁺ T cells, splenocytes from mice treated with a minimum of 10 i.n. doses of MBP Ac1-9[4Y] were restimulated with 10 µg/ml of MBP Ac1-9[4K] overnight with additional PMA and ionomycin stimulation for the last 3 h of culture. IL-10-secreting CD4⁺ T cells were enriched by IL-10 Secretion Assay according to the manufacturer's instructions (Miltenyi Biotec). For Western blot analysis, CD4⁺ T cells from peptide-treated mice activated with a dose of i.n. Ac1-9[4Y] for 2 h, followed by an additional 3 h PMA and ionomycin stimulation *in vitro*, were dually labeled for IL-10 and IFN-γ secretion before sorting on FACS Vantage cell sorter (BD) into IL-10⁺, IL-10⁺IFN-γ⁺, and IL-10⁺IFN-γ⁻ T cell populations (gating on live [7AAD⁻; BD] Vβ8⁺ T cells). Splenic DCs were isolated from B10.PL spleens using collagenase IV (Worthington) and DNase I (Roche) followed by magnetic separation with CD11c MicroBeads (Miltenyi Biotec).

T cell proliferation assays and cultures. CD4⁺ T cells were cultured at 5 × 10⁴ per well in complete RPMI medium in round-bottomed 96-well plates at 37°C and 5% CO₂ humidified atmosphere in the presence of 10⁵ per well autologous, irradiated CD4-depleted splenocytes or B10.PL splenocytes as APCs. Alternatively, splenic DCs were used as APCs ranging from an 80:1 to a 10:1 ratio of CD4⁺ T cells to DCs. MBP Ac1-9[4K] ranging from 0.1 to 100 µg/ml and 20 U/ml of rhIL-2 were added to cultures where indicated. After 72 h, wells were pulsed with 0.5 µCi [³H]thymidine overnight and the incorporated radioactivity (corrected cpm) was measured on a liquid scintillation β-counter (1450 Microbeta; Wallac). For Th1 and Th2 cell lines, mIL-12 (PeproTech) and anti-IL-4 antibody (clone 11B11; BD) or mIL-4 (PeproTech) were added at a final concentration of 5 ng/ml and 10 µg/ml or 10 ng/ml of culture, respectively. RhIL-2 was added at 20 U/ml on day 3 to both Th1 and Th2 cultures.

Cytokine protein levels. Multiplex Fluorescent Bead Immunoassay was used to measure the amount of IL-2, IL-4, IL-5, IL-10, IL-17, and IFN-γ in mouse serum samples according to the manufacturer's instructions (Bender MedSystems). Fluorescence intensity was measured on a FACSCanto flow cytometer (BD) and analyzed using the manufacturer's own software. Conventional sandwich ELISA was performed according to the manufacturer's instructions to assay the quantity of IL-2, IL-4, IL-10, IFN-γ (paired antibodies), and IL-12 (OptEIA kit; BD) in cell culture supernatants. Optical densities were measured at 450/595 nm on a SpectraMax 190 microplate reader and the amount of cytokines present quantified with standard curves using SoftMax Pro software (both from Molecular Devices).

Flow cytometry. Staining for intracellular cytokine expression was performed using the CytoFix/CytoPerm PMA kit with GolgiStop (BD). CD4⁺ T cell cultures were restimulated with PMA and ionomycin (Sigma-Aldrich) at a final concentration of 5 ng/ml and 500 ng/ml of culture, respectively, for 3 h in the presence of GolgiStop. Cells were surface stained with anti-Vβ8 FITC, and intracellularly with anti-IL-2 APC, anti-IL-4 PE, anti-IL-10 APC, anti-IL-17 PE, and anti-IFN-γ PE antibodies, or isotype controls (BD). For T-bet staining, enriched IL-10-secreting cells were stained with surface anti-Vβ8 and intracellularly with either anti-T-bet Alexa Fluor 647 or mouse IgG1 isotype control antibody, using permeabilization buffers recommended by the manufacturer (eBioscience). For DC co-stimulatory molecule staining, splenic DCs were cultured either alone or with an equal number of CD4⁺ T cells from naive or peptide-treated mice in the presence of 100 µg/ml of Ac1-9[4K] and, where indicated, with 10 µg/ml of anti-IL-10R (clone 1B1.3a) or isotype control (BD) antibody. After 24 h, DCs were surface stained with anti-CD11c FITC, anti-CD40 PE and biotinylated anti-CD80 and anti-CD86 (BD) paired with Streptavidin Tricolor (Invitrogen). Fluorescence intensity was measured on a FACS-Calibur or BD LSR II flow cytometer (BD) and analyzed using FlowJo (Tree Star, Inc.) FACS analysis software.

Western blot analysis. Total protein isolated from FACS-sorted IL-10⁺, IL-10⁺IFN-γ⁺, and IL-10⁺IFN-γ⁻ CD4⁺ T cells, naive (CD4⁺CD62L⁺) T cells and *in vitro*-differentiated Th1 cells with RIPA lysis and extraction buffer supplemented with protease inhibitor cocktail, PMSF, and sodium orthovanadate (Santa Cruz Biotechnology, Inc.) was run on a 12.5% SDS-PAGE gel at 50,000 cells per lane. Protein was transferred onto Hybond C nitrocellulose blotting membrane (GE Healthcare), blocked for 1 h with PBS containing 0.1% Tween-20 and 5% BSA (Sigma-Aldrich), incubated overnight with 0.4 µg/ml anti-T-bet antibody (Santa Cruz Biotechnology) at 4°C, followed by 1 µg/ml rabbit anti-mouse IgG-HRP secondary antibody (Abcam) for 1 h at room temperature. The T-bet protein was detected with ECL (GE Healthcare) and Kodak Biomax films.

Quantitative real-time PCR. CD4⁺ T cells were isolated from untreated or peptide-treated mice by positive selection, and total RNA was extracted from the cell pellets with TRIzol according to the manufacturer's instructions (Invitrogen). Extracted RNA was reverse transcribed with Superscript III RNase H⁻ Reverse transcription (20 U/µl) and random primer oligonucleotides (15 ng/µl; Invitrogen). A negative control for subsequent PCR reactions was provided by omitting Superscript III RTase from reactions. The sequences of primers (Sigma-Aldrich) were as follows: *t-bet*, 5'-CTA-AGCAAGGACGCGAATG-3' (forward) and 5'-CACCAAGACCA-CATCCACAAA-3' (reverse); *egr-2*, 5'-GGCGGGAGATGGCATGAT-3' (forward) and 5'-CCCATGTAGGTGAAGGTCTGGT-3' (reverse); *icos*, 5'-TGCAGGTGTGACCTCATAAGC-3' (forward) and 5'-GCCCTGTGGTCCAGAAAATA-3' (reverse); *gata-3*, 5'-ACCGGGTTCGGATG-TAAGTC-3' (forward) and 5'-GTTACACACTCCCTGCCTTCT-3' (reverse); and the housekeeping gene *β₂-microglobulin*, 5'-GCTATC-CAGAAAACCCCTCAA-3' (forward) and 5'-CGGGTGAACCTGTGT-TACGT-3' (reverse). For transcript quantitation, PCR reactions were performed in a final volume of 20 µl containing cDNA template, Platinum SYBR Green qPCR Supermix-UDG (Invitrogen), and forward/reverse primers (*t-bet*, *egr-2*, *icos*, and *β₂-microglobulin* concentrations 0.2 µM and *gata-3* 0.1 µM). Fluorescent amplicons were derived after an initial denaturation step at 95°C for 2 min, 35 cycles of amplification of 30 s at 94°C, 30 s annealing at 58°C (*t-bet*, *egr-2*, *icos*, and *β₂-microglobulin*) or 64°C (*gata-3*), 30 s extension at 72°C, and finally detected on an Opticon 2 DNA Engine (Bio-Rad Laboratories).

Online supplemental material. Fig. S1 shows changes in the splenocyte and splenic CD4⁺ T cell numbers during the course of tolerance induction in the Tg4 *Rag1*^{-/-} mouse. Fig. S2 shows changes in the serum cytokine profile of Tg4 *IL-10*^{-/-} mice assessed at 2 h after successive i.n. peptide

administration. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20082118/DC1>.

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SUPPLEMENTAL MATERIAL

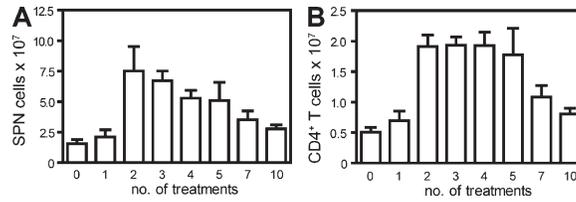
Gabryšová et al., <http://www.jem.org/cgi/content/full/jem.20082118/DC1>

Figure S1. Changes in splenocyte and CD4⁺ T cell numbers during the course of tolerance induction. Tg4 *Rag1*^{-/-} mice were treated with successive i.n. doses of MBP Ac1-9[4Y]. Total splenocyte (A) and CD4⁺ T cell (B) counts at 2 h after each peptide administration. Results are depicted as the mean \pm SEM for six mice per treatment group on pooled data from two separate experiments.

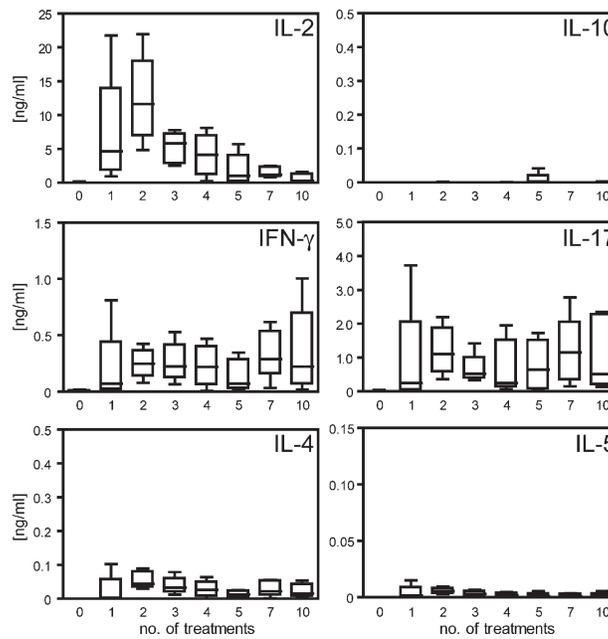


Figure S2. Changes in serum cytokine profile over the course of i.n. peptide treatment in Tg4 *IL-10*^{-/-} mice. Tg4 *IL-10*^{-/-} mice were bled before treatment and after each successive i.n. MBP Ac1-9[4Y] administration. Serum concentrations of IL-2, IL-10, IFN- γ , IL-17, IL-4, and IL-5 were measured by cytometric bead assay. Results show the mean serum cytokine concentration of six mice per treatment group on pooled data from two separate experiments.