Commentary
The potential of human regulatory T cells generated *ex vivo* as a treatment for lupus and other chronic inflammatory diseases

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Abstract

Regulatory T cells prevent autoimmunity by suppressing the reactivity of potentially aggressive self-reactive T cells. Contact-dependent CD4+ CD25+ 'professional' suppressor cells and other cytokine-producing CD4+ and CD8+ T-cell subsets mediate this protective function. Evidence will be reviewed that T cells primed with transforming growth factor (TGF)-β expand rapidly following restimulation. Certain CD4+ T cells become contact-dependent suppressor cells and other CD4+ and CD8+ cells become cytokine-producing regulatory cells. This effect is dependent upon a sufficient amount of IL-2 in the microenvironment to overcome the suppressive effects of TGF-β. The adoptive transfer of these suppressor cells generated *ex vivo* can protect mice from developing chronic graft versus host disease with a lupus-like syndrome and alter the course of established disease. These data suggest that autologous T cells primed and expanded with TGF-β have the potential to be used as a therapy for patients with systemic lupus erythematosus and other chronic inflammatory diseases. This novel adoptive immunotherapy also has the potential to prevent the rejection of allogeneic transplants.

Keywords: autoimmunity, IL-2, regulatory T cells, systemic lupus erythematosus, transforming growth factor-β

Introduction
It has become evident that self-reactive T cells with the potential to cause autoimmune disease comprise a part of the normal T-cell repertoire, but their activation is prevented by suppressor cells [1–3]. Although originally described in the 1970s [4], significant progress in characterizing suppressor T-cell subsets has been made only recently, where they have been renamed 'regulatory' T cells.

A subset of thymus-derived CD4+ cells that constitutively expresses CD25, the α-chain of the IL-2 receptor, protect their host from spontaneous organ-specific autoimmune diseases. These CD4+ CD25+ cells have been called 'professional' suppressor cells and have a contact-dependent mechanism of action, at least *in vitro* [5]. Other subsets of CD4+ and CD8+ cells, natural killer T cells, and cells displaying γδ TCRs also have downregulatory (suppressor) activity. In the periphery, suppressor T cells generated in response to environmental antigens protect their

hosts from immune-mediated tissue injury by producing immunosuppressive cytokines.

The mechanisms responsible for the generation of suppressor T cells were poorly understood until recently. Our group has accumulated evidence that the multifunctional cytokine transforming growth factor-β (TGF-β) plays an essential role in the expansion of thymus-derived, professional, CD4+ CD25+ precursors that circulate in the blood. TGF-β also plays a key role in the generation of peripherally induced CD4+ and CD8+ cytokine-producing suppressor cell subsets.

This article will briefly review the evidence for contact-mediated and cytokine-producing suppressor cells, especially in humans, and the role of TGF-β in the generation of these cells. This knowledge can be used to generate suppressor T cells *ex vivo* in large numbers, and raises the possibility that the transfer of these cells back to the donor

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CTLA-4 = cytotoxic T-lymphocyte-associated antigen-4; TGF-β = transforming growth factor-β; IL = interleukin; MHC = major histocompatibility complex; SLE = systemic lupus erythematosus; TCR = T-cell receptor; Th = T helper cells; Tr1 = Treg 1 regulatory CD4+ cells.

can serve as a therapy for autoimmune diseases such as systemic lupus erythematosus (SLE). This T-cell-based therapy could also be used to prevent graft rejection.

Thymus-dependent, 'professional', contact-dependent, regulatory T cells

The existence of thymus-derived suppressor cells was suggested by studies in mice where neonatal thymectomy on day 3 led to the development of a multiorgan autoimmune disease [6]. This disease is due to the loss of CD4+ CD25+ suppressor cells that do not appear until the first week after birth [7,8]. Mature CD4+ CD25+ cells are found in the CD45RBlow activated/memory fraction mouse T cells. Because potentially aggressive, self-reactive T cells are found in the CD45RBhi naive fraction of mouse T cells, the injection of CD45RBhi cells from nonautoimmune, normal mice into immunodeficient mice results in generalized, multiorgan inflammatory disease. Similar to neonatal thymectomy, this disease is prevented by supplementing the injected cells with purified CD4+ CD25+ cells [9,10]. Because these thymus-derived CD4+ CD25+ T cells appear to be crucial for the prevention of spontaneous autoimmune diseases, they have been called 'professional' suppressor cells [5,8].

In general, the properties of rodent and human CD4+ CD25+ T cells appear to be very similar. In humans, 6–18% of CD4+ T cells constitutively express CD25 [11–17]. Purified CD4+ CD25+ cells do not proliferate in response to cross-linking of their TCRs. They inhibit the activation of other T cells by a contact-dependent mechanism [5–17]. A large percentage constitutively express intracellular cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4 or CD152), the IL-2 receptor β-chain (CD122), transferrin receptors (CD71) and class II MHC markers [17].

Almost all the CD4+ CD25+ are in the 'activated' state (CD45RBlow in the mouse, CD45RA− RO+ in the human). This suggests they may be continuously stimulated by their internal environment. Although activation of CD4+ CD25+ cells is antigen specific, once these cells are activated they not only suppress T cells stimulated by the same antigen, but they also inhibit T cells stimulated by other antigens; so-called bystander effects [18]. Although CD4+ CD25+ cells are nonresponsive to cross-linking their TCRs, they do proliferate when costimulated with IL-2 or anti-CD28.

Cytokine production by CD4+ CD25+ cells is controversial. While some groups claim that these cells do not produce cytokines [8,17], other groups have found that they can produce IL-10 [12,13,19], TGF-β [15,19], IL-4 [12] and low amounts of interferon-γ [15]. All groups agree that these cells do not produce IL-2 and that they have a contact-dependent mechanism of action. Their suppressive activities are not abolished by neutralizing antibodies to IL-10, and all groups agree that suppression is not abolished by anti-TGF-β, but for one exception [19]. Nakamura *et al.* reported that immunosuppression by CD4+ CD25+ regulatory T cells is mediated by cell surface-bound TGF-β [19]. Many of these differences can possibly be explained by the heterogeneity of CD4+ CD25+ T cells. One group separated human CD4+ CD25+ cells into high- and low-intensity fractions by cell sorting, and they found that the suppressive effects were only displayed by the high-intensity fraction [17]. This subset did not produce cytokines.

Cytokine-dependent regulatory T cells

CD8+ and CD4+ T cells that produce immunosuppressive cytokines have been described. Those that produce predominantly TGF-β and variable amounts of IL-10 and IL-4 have been called Th3-type cells, and they have been generated *in vivo* by immunization through an oral or other mucosal route [2,20]. This route of antigen administration, however, does not only result in Th3 cells. Both Th2 cells and CD4+ CD25+ cells can also be generated by this procedure [20–22]. The conditions needed for the generation of Th3 cells are poorly understood.

Other workers have produced regulatory CD4+ cells by repeatedly stimulating with the antigen in the presence of IL-10 [23–26], or using immature antigen-presenting cells that lack potent costimulatory activity [27]. These regulatory CD4+ cells have been called Treg 1 (Tr1) cells and they produce significant quantities of IL-10. They do not proliferate in response to antigen and do not produce IL-2. Therefore, they are anergic.

Th3 and Tr1-like cells have been described in humans. One group has reported the appearance of Th3 cells in patients with multiple sclerosis following oral administration of myelin basic protein [28]. Human Tr1 cells suppressed an alloantigen-induced proliferative response [29]. Th3 or Tr1 cells mediate antigen-specific cellular hyporesponsiveness in patients with chronic helminth infections [30].

The fact that some regulatory T cells produce predominantly TGF-β and others IL-10 is not fortuitous. The combination of TGF-β and IL-10 is more immunosuppressive than either of the cytokines by themselves [31]. Significantly, shortly after antigen activation, T cells downregulate their signal transducing type II receptor (TGF-βRII) and become refractory to the effects of TGF-β [32]. These cells then become mature effector cells. IL-10 appears as a feedback regulator later in the response and induces the re-expression of TGF-βRII. The synergistic inhibitory effects of TGF-β and IL-10 then terminate the response.

Whether Th3 cells and Tr1 cells come from similar precursors or comprise different subsets of regulatory T cells is not known. Many variables determine the differentiation

pathway that a naive T cell will take following activation. These include the antigen concentration and route of administration, the cytokine milieu, and the pattern of co-stimulatory signals. Self-MHC-reactive T cells in humans can either provide B-cell helper function or suppress antibody production, depending on how they are activated. In each case, regulatory function depends on the cytokines produced [33]. In determining the T-cell response to myelin basic protein, another group found that TCR usage was similar whether the T cells became Th1 encephalitogenic cells or regulatory Th3 cells [34]. These studies suggest common precursors for T cells that take different differentiation pathways.

TGF-β induces CD4+ and CD8+ T cells to become suppressor cells

While TGF-β has well-known inhibitory effects on lymphocyte cytokine production and functional properties [35], our laboratory has accumulated data that these effects can be overcome by IL-2 and can be superceded by co-stimulatory activities. The net effect is that TGF-β induces IL-2-activated CD8+ and CD4+ T cells to develop potent suppressive activities. In parallel, other groups have observed that TGF-β inhibits the differentiation of T cells to Th1 or Th2 subsets [36,37].

The initial observation that TGF-β is an IL-2-dependent differentiation factor for regulatory T cells was made in a study designed to determine the conditions required for human CD8+ T cells to become suppressors of antibody production. Using a model where we could induce T-cell-dependent antibody production without accessory cells, we found that CD4+ T cells, by themselves, lacked suppressor-inducing activity. The CD4+ cells produced IL-2 but, notwithstanding previous reports [38,39], this cytokine could not induce suppressor cells by itself. We learned that the interaction of IL-2-activated natural killer cells with CD8+ cells leads to the production of active TGF-β, and that the presence of this cytokine was critical for CD8+ cells to suppress antibody production (Figure 1) [40,41]. Moreover, the suppression was cytokine dependent and was abolished by a neutralizing anti-TGF-β monoclonal antibody (JD Gray and DA Horwitz, unpublished observation, 2000). Both IL-2 and TGF-β were thus critical for CD8+ cells to become Th3-like regulatory cells.

We have also induced CD4+ T cells to become Th3 cells. We used the superantigen, staphylococcal enterotoxin B, as the T-cell activating agent. Low-dose staphylococcal enterotoxin B can induce T-cell-dependent antibody production without additional accessory cells [42]. Briefly exposing CD4+ cells to TGF-β downregulated B-cell helper activity and induced certain CD4+ cells to develop suppressive activity that was neutralized by anti-TGF-β. Activating both CD4+ and CD8+ cells in the presence of TGF-β thus induced them to develop cytokine-dependent

Figure 1
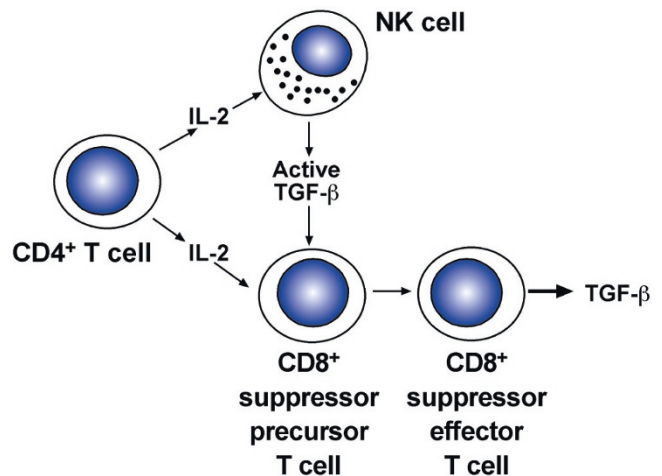
The role of transforming growth factor-β (TGF-β) in the differentiation pathway of CD8+ regulatory T cells. In response to antigen stimulation, the combination of IL-2 produced by CD4+ cells and the active form of TGF-β produced by natural killer (NK) cells or macrophages (not shown) induce CD8+ cells to lose their cytotoxic potential and become regulatory, TGF-β-producing, Th3-like cells. IL-2 also enhances the extracellular conversion of TGF-β from the latent to the biologically active form.

suppressive activity [43]. Other workers have also reported similar effects of TGF-β on CD8+ T cells [44]. One group found that IL-4 and TGF-β are involved in the differentiation of naive CD4+ cells to cytokine-producing Th3-type cells [45]. Another group reported that *in vitro* differentiation of Th3-type cells from Th0 precursors from TCR transgenic mice is enhanced by culture with TGF-β [20].

We next focused our attention on the induction of naive (CD45RA+ RO−) CD4+ T cells to become suppressor cells. Using the alloantigens as the T-cell activating agent, we found that TGF-β induced naive CD4+ T cells to develop extremely potent suppressive activity. These CD4+ cells had the phenotype and functional characteristics of 'professional' regulatory T cells. Using the generation cytotoxic T-cell activity and T-cell proliferation to assess suppressive activity, we learned that the suppressor cells were CD25+, and that a large percentage expressed CTLA-4. Their suppressive effects were contact dependent and were not neutralized by anti-TGF-β or IL-10. Adding less than 1% of these cells to T cells strongly inhibited the generation of cytotoxic T-lymphocyte activity by preventing the activation of CD8+ cells [14]. Other workers have also reported that CD4+ CD25+ cells have potent suppressive effects on CD8+ cells. Rodent CD4+ CD25+ regulatory cells cause CD8+ cells to enter cycle arrest [46].

The precursors of the human CD4+ CD25+ T cells induced by IL-2 and TGF-β appear to be the small number of CD25+

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cells in the naive fraction. Although <1% of these cells express CD25, depletion of these cells abrogated the generation of suppressive activity in some experiments [14]. The principal difference between the cytokine-induced CD4+ CD25+ cells and the murine and human positively selected CD4+ CD25+ cells that are predominantly found in the CD45RO+ 'memory' fraction is their capacity for expansion. The positively selected cells are anergic while the CD4+ CD25+ cells generated from naive cells can be expanded in IL-2 and retain their suppressive activity [14].

Studies on the mechanism of action of TGF-β have revealed that this cytokine has potent costimulatory effects on IL-2-activated T cells. These effects include upregulation of CD25, CTLA-4 and CD40 ligand expression on CD4+ cells [14,47], and increased tumor necrosis factor-α production by both CD4+ and CD8+ cells [47]. The TGF-β costimulated human CD4+ T cells are resistant to activation-induced apoptosis. They took up less annexin and expanded fivefold greater in primary cultures than control, alloactivated CD4+ T cells [14] (SG Zheng and DA Horwitz, unpublished observations, 2001). Some workers have reported that TGF-β can accelerate activation-induced cell death of some T cells [48,49], while others observed that this cytokine protected T cells from apoptosis [50,51]. We favor the hypothesis that TGF-β promotes the death of mature Th1 and Th2 cells while protecting newly generated regulatory T cells from undergoing apoptosis. This view is consistent with a report indicating positive effects of TGF-β on naive T cells [52].

In summary, using several different stimuli to activate T cells, we have found that the combination of IL-2 and TGF-β can induce CD4+ and CD8+ T cells to become either cytokine-producing Th3-like or contact-dependent professional suppressor cells. In our studies with CD8+ cells, the cultures were always supplemented with IL-2. When human CD4+ cells are activated in the presence of TGF-β by irradiated allogeneic stimulator cells or with superantigens, however, sufficient IL-2 is produced for the costimulatory effects of TGF-β and suppressor cell differentiation. By contrast, cultures with mouse lymphocytes must generally be supplemented with IL-2.

As shown in Figure 2, we propose that TGF-β induces thymic-derived CD25 precursors in the naive fraction of CD4+ cells to expand and to become contact-dependent 'professional' regulatory T cells. TGF-β also induces CD4+ and CD8+ cells that are CD25− to become Th3-like cells. Although almost all naive CD4+ cells are CD25−, why the predominant TGF-β effect on T cells in this fraction is the generation of 'professional' regulatory T cells remains to be determined. Our finding that both IL-2 and TGF-β are critical in the generation of regulatory T cells is of particular importance in patients with SLE since production of IL-2 and the active form of TGF-β is decreased [53].

Figure 2
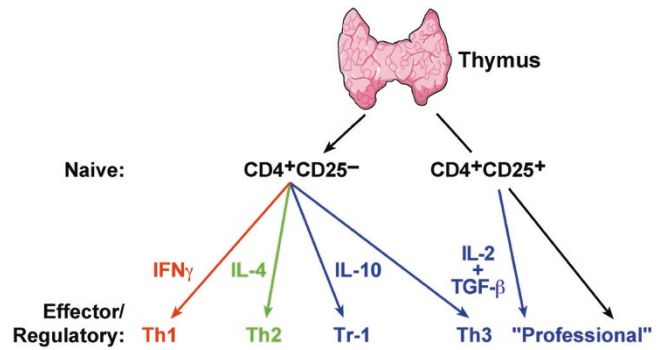
The role of transforming growth factor-β (TGF-β) in the differentiation pathway of CD4+ regulatory T cells. Following T-cell activation where a sufficient amount of IL-2 is produced to overcome the inhibitory effects of TGF-β, the costimulatory effects of this cytokine induce the precursors of CD4+ CD25+ T cells to become contact-dependent 'professional' suppressor cells or induces CD4+ CD25− cells to produce immunosuppressive quantities of TGF-β. IFN, interferon; Tr-1, Treg 1 regulatory CD4+ cells.

In vivo effects of Treg

Cloned Th3 cells protect mice from several autoimmune diseases that include experimental allergic encephalitis, diabetes mellitus, colitis, and uveitis [20,29,54–56]. Cytokine-producing CD8+ cells were described initially [55], but reports of CD4+ cells with this characteristic have become predominant. Cloned Tr1 cells protect rodents from an experimental colitis [29]. Small numbers of adoptively transferred noncloned CD4+ CD25+ cells protect lymphopenic mice from developing spontaneous organ-specific autoimmune diseases and also protect animals from developing graft-versus-host disease [8–10,57].

We have begun to learn whether regulatory T cells generated *ex vivo* with TGF-β can have protective effects *in vivo*. For this purpose, we selected a mouse model of SLE that has a rapid onset. The transfer of parental T cells to F1 mice can result in acute or chronic graft-versus-host disease depending on the precursor frequency of CD8+ parental cells reactive against the allogeneic MHC antigens [58,59]. The transfer of DBA/2 T cells into DBA/2 x C57BL/6 F1 mice results in a lupus-like syndrome with high titers of anti-DNA antibodies and an immune complex glomerulonephritis. While alloactivated DBA/2 T cells accelerated the disease, alloactivation of splenic T cells or CD4+ cells in the presence of TGF-β markedly suppressed and even prevented the development of the lupus-like syndrome. Both anti-DNA antibody production and proteinuria were significantly suppressed [60]. Recent studies have revealed that these suppressor T cells can also alter the course of established disease. A single transfer of 5 million T cells conditioned with TGF-β markedly improved survival of these mice (SG Zheng and DA Horwitz, unpublished observations, 2001).

Since it has been possible to significantly expand regulatory T cells generated with TGF-β, it should be possible to generate sufficient numbers in humans for clinical trials. Although this will be carried out initially with mitogens as the T-cell activating agent, the ultimate goal is to induce autoantigen-specific regulatory T cells. This should be possible based on the progress being made in characterizing the pathogenic peptides that trigger autoimmune diseases. It may even be possible to induce potentially aggressive naive self-reactive cells to become protective suppressor cells by activating them with TGF-β. An adoptive immunotherapy using the patients own T cells that have regained a protective function they had lost should lack the serious toxic effects associated with the agents now in use. This treatment is especially promising in autoimmune diseases characterized by a relapsing and remitting course such as SLE, inflammatory bowel disease or certain forms of multiple sclerosis. The adoptive transfer of regulatory T cells generated *ex vivo* also has the potential to prevent the rejection of allogeneic organ transplants.

Acknowledgements

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