TNF downmodulates the function of human CD4⁺CD25hi T-regulatory cells

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CD4⁺CD25⁺ T-regulatory cells (Tregs) play an essential role in maintaining immunologic homeostasis and preventing autoimmunity. However, little is known about the exogenous factors that regulate their differentiation and function. Here, we report that TNF inhibits the suppressive function of both naturally occurring CD4⁺CD25⁺ Tregs and TGFβ1–induced CD4⁺CD25⁺ T-regulatory cells. The mechanism of this inhibition involves signaling through TNFRII that is constitutively expressed selectively on unstimulated Tregs and that is up-regulated by TNF. TNF-mediated inhibition of suppressive function is related to a decrease in Foxp3 mRNA and protein expression by the Tregs. Notably, CD4⁺CD25⁺ Tregs isolated from patients with active rheumatoid arthritis (RA) expressed reduced levels of Foxp3 mRNA and protein and poorly suppressed the proliferation and cytokine secretion of CD4⁺ effector T cells in vitro. Treatment with anti-TNF antibody (infliximab) increased Foxp3 mRNA and protein expression by CD4⁺CD25⁺ Tregs and restored their suppressive function. Thus, TNF has a novel action in modulating autoimmunity, by inhibiting CD4⁺CD25⁺ Treg activity. (Blood. 2006; 108:253-261)

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Introduction

Immunologic self-tolerance is critical for the prevention of autoimmunity and maintenance of immune homeostasis. The ability of the immune system to discriminate between self and nonself is controlled by central and peripheral tolerance mechanisms. The former involves deletion of self-reactive T cells in the thymus at an early stage of development, whereas peripheral tolerance involves several mechanisms, including T-cell anergy and ignorance. Since these mechanisms are not completely effective and potentially autoantigen-reactive lymphocytes escape into the periphery, additional mechanisms are involved in the maintenance of self-tolerance. A number of subsets of regulatory T cells play an important role in preventing activation of autoantigen-reactive T cells. Among these are naturally occurring “professional” regulatory T cells (Tregs). In this regard, studies carried out during the past decade provided strong evidence for the existence of a unique CD4⁺CD25⁺ population of naturally occurring regulatory/suppressor T cells that actively prevent both the activation and the effector function of autoreactive T cells that have escaped other mechanisms of tolerance. Removal of this population from normal rodents leads to the spontaneous development of various autoimmune diseases, such as destructive arthritis, experimental allergic enteritis, and diabetes.

It has been proposed that during the initiation of an adaptive immune response, dendritic cells can induce effector CD4⁺ T cells to become resistant to the suppressive effects of Tregs by secreting IL-6, thus allowing a productive immune response to take place. Similarly, glucocorticoid-induced tumor necrosis factor–like receptor (GITR) engagement on effector T cells by its ligand (GITRL) expressed on antigen-presenting cells (APCs) has been claimed to render them resistant to suppression by CD4⁺CD25⁺ Tregs, and may also have an effect on the function of Tregs.

Recent studies have revealed the presence of CD4⁺CD25⁺ Tregs in human peripheral blood, where they constitute up to 5% of the CD4⁺ T cells. These cells are similar to those described in the mouse in that they require cell-to-cell contact to exert their suppressive effect. Whether a soluble factor is involved depends on the experimental system used.

Tumor necrosis factor (TNF) is a pleiotropic cytokine critical for cell trafficking, inflammation, maintenance of secondary lymphoid organ structure, and host defense against various pathogens. Because of this panoply of effects, TNF plays a critical role in bridging innate and adaptive immunity. However, its role in regulating the function of Tregs or their impact on effector cells is presently unknown. Rheumatoid arthritis (RA) is one of the most common human autoimmune diseases, with a prevalence of nearly 1%. The pivotal role that inflammatory cytokines such as TNF, IL-1β, and IL-6 play in the induction and maintenance of rheumatoid synovitis is well established. Treatment of RA patients with TNF blockers results in significant clinical benefit, although the possibility that anti-TNF restores immunologic homeostasis has not been fully explored. Recent studies in RA patients have suggested that the function of CD4⁺CD25⁺ Tregs may be impaired as they may suppress the proliferation of autologous CD4⁺CD25⁻...
T cells, but not the production of proinflammatory cytokines, such as interferon γ. Notably, treatment of RA patients with anti-TNF antibodies appeared to result in an increased frequency of CD4<sup>+</sup>CD25<sup>+</sup> Tregs and reversed their defect in inhibition of cytokine secretion, although a direct effect of TNF on CD4<sup>+</sup>CD25<sup>+</sup> Treg function in vitro could not be demonstrated.

Although these studies suggested that TNF might play some direct or indirect role in modulating the suppressive effects of Tregs, the cellular targets and the molecular mechanism of TNF action on those cells have not been delineated. Here, we demonstrate that human Tregs express TNFRII and that the percentage of Tregs that express TNFRII can be enhanced by exposure to TNF. The addition of TNF or agonistic antibody to TNFRII reversed their suppressive activity by downmodulating the expression of FOXP3. The beneficial effect of TNF blockade in autoimmune/inflammatory diseases could involve the restoration of immune homeostasis by permitting the full expression of CD4<sup>+</sup>CD25<sup>+</sup> Treg function.

**Patients, materials, and methods**

**Subjects**

Forty healthy donors between the ages of 23 and 69 years with no history of autoimmune disease were examined. Subjects with a history of infection within 3 weeks and comorbidities, such as diabetes mellitus, were excluded. In addition, 15 patients with active RA were studied (disease activity score [DAS] > 5), each of whom met the American College of Rheumatology (ACR) criteria. RA patients were evaluated before and after anti-TNF therapy. Infliximab was given at a dose of 5 mg/kg intravenously at weeks 0, 2, and 6, and every 6 weeks in combination with stable doses of methotrexate (7.5 to 15 mg/wk orally). Patients were re-examined after the 3-month infusion. Informed consent was provided in accordance with the Declaration of Helsinki. The study was approved by the institutional review board of the National Institute of Arthritis and Musculoskeletal and Skin Diseases/National Institute of Diabetes and Digestive and Kidney Diseases, NIH.

**Cell culture reagents**

RPMI 1640 (Gibco-BRL, Grand Island, NY) supplemented with 1% heat-inactivated autologous plasma, 20 μg/mL gentamicin (Gibco-BRL), and 2 mM glutamine (Gibco-BRL) was used for the generation of dendritic cells (DCs). X-VIVO 20 (Bio Whitaker, Walkersville, MD) supplemented with 1% heat-inactivated normal human serum (Bio Whitaker), 20 μg/mL gentamicin, amphotericin B 1 μg/mL, and 2 mM glutamine (all from Gibco-BRL) was used for T-cell culture. Fetal bovine serum was obtained from HyClone (Logan, UT).

**Cytokines**

All cytokines used in this study were recombinant human proteins. Final concentrations were as follows: 100 ng/mL granulocyte-macrophage–colony-stimulating factor (GM-CSF) and IL-4; 2 ng/mL TGFβ1 (R&D Systems, Minneapolis, MN); and 100 U/mL IL-2 (NCI preclinical). For final DC maturation, 500 ng/mL TNF-R&D Systems was added.

**Monoclonal Abs**

For immunostaining, mouse PE-, FITC-, and Cyochrome-conjugated mAbs against CD3 human (UCHT 1), CD4 (RPA-T4), CD8 (RPA-T8), CD14 (M5E2), CD25 (M-A251), CD45RA (HI 100), CD45RO (UCHL 1), CD62L (DREG-56), CD80 (L307.4), CD83 (HB15e), CD86 (FUN-1), CD122 (MIK-β2), CD152 (BN13.1), HLA-DR (G46-6), CCR4 (1G1), and corresponding mouse isotype controls (all from BD-Pharmingen); GITR-FITC (110416), TNFR1-FITC (16803), and TNFRIII (22221.311; all from R&D Systems); and CD25-PE (Beckman-Coulter, Hialeah, FL) were used. Unconjugated mouse anti-human IL-10 (JES3-19F1) and anti-IL-10R (3F9; both from BD-Pharmingen); and anti-TGFβ1 (9016.2), anti-GITR (110416), anti-TNFRII (16803), and anti-TNFRIII (22221.311; all from R&D Systems) were used for neutralization experiments, and anti-CD3 (64.12) was used for polyclonal activation of T cells.

**Cytokine assays**

T cells were stimulated with plate-bound anti-CD3 (1 μg/well). Cytokine analysis was carried out at 72 hours by analysis of supernatants with commercially available enzyme-linked immunosorbent assay (ELISA) kits for human IL-10, IFNγ, IL-4, and TGFβ1 (BD-Pharmingen) according to the manufacturer’s instructions or by the cytometric bead array kit (CBA; BD Biosciences, San Jose, CA). A direct comparison of capture ELISA and CBA demonstrated that the 2 methods were comparable in terms of the amount of cytokine detected.

**Cell isolation and DC generation**

DCs were generated from elutriated monocytes (obtained from the Department of Transfusion Medicine, Clinical Center, NIH) from healthy donors. In brief, monocytes were cultured in RPMI 1640 supplemented with 10% FBS, IL-4, and GM-CSF. At day 6, TNF was added to mature the cells fully. At day 7, nonadherent cells were harvested and considered to be mature DCs. More than 90% were double positive for costimulatory molecules (CD80, CD86) and CD83.

CD4<sup>+</sup> T cells were enriched from peripheral-blood mononuclear cells (PBMCs) by negative selection using the AutoMACS (Miltenyi Biotec, Auburn, CA). Enriched T cells were stained with anti-CD4-Cyochrome and PE-conjugated anti-CD25 (15 μg/10<sup>6</sup> cells) for 20 minutes at 4°C. CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>–</sup> Tregs were purified using a MoFlo high-speed cell sorter (Dako Cytomation, Carpinteria, CA) to a purity of more than 98%. In some experiments, CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> Tregs were stimulated in vitro before analysis. This was accomplished by culturing them for 3 days in microtiter plates coated with anti-CD3 mAb (1 μg/well).

**TNF preincubation experiments**

Purified CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells were incubated overnight with TNF at 50 ng/mL in medium supplemented with 1% NHS and 100 U/mL IL-2. Afterward, cells were washed extensively and used in the assays of Treg function.

**Flow cytometric analysis**

Single-cell suspensions were prepared and stained for 20 minutes at 4°C with optimal dilutions of each mAb. Cells were stained with FoxP3-APC (PCH101; eBioscience, San Diego, CA), according to the manufacturer’s instructions for fixation, permeabilization, and staining, after the cells were stained for surface expression of CD4 and CD25 with CD25-PE and CD4-Cyochrome. An isotype-matched control mAb was used to determine nonspecific staining. Expression of cell-surface or intracellular markers was assessed using the flow cytometer (FACSCalibur; Becton Dickinson, Ashland, OR).

**Proliferation assays**

To assess proliferation, 5 × 10<sup>4</sup> sorted cells were incubated in X-VIVO-20 medium with 10% fetal bovine serum in 96-well U-bottom plates coated with anti-CD3 at 1 μg/well. For assessment of regulatory properties, 5 × 10<sup>4</sup> CD4<sup>+</sup>CD25<sup>+</sup> T cells were cultured with plate-bound anti-CD3 in 96-well U-bottom plates. Purified autologous CD4<sup>+</sup>CD25<sup>+</sup> T cells were added, usually at a 1:1 ratio if not indicated differently. After 3 to 4 days of culture, 100 μL supernatant was removed from each well and used for cytokine detection, and 1 μCi/well (37 KBq/well) [H]Tdr was added for an additional 16 hours to each well. [H]Tdr incorporation was measured using a liquid scintillation counter.
Real-time polymerase chain reaction (PCR)

Total RNA was isolated from sorted cells using RNAasy Mini kit (QUIGEN, Valencia, CA), according to the manufacturer’s instructions. RNA samples were treated with DNase I to remove contaminating genomic DNA and reverse transcribed with Superscript II (Invitrogen, Carlsbad, CA). FOXP3 expression was tested using Assays on Demand reagents (Hs00203958_m1) from Applied BioSystems (Foster City, CA). All reported mRNA levels were normalized to the GAPDH mRNA level, where GAPDH = 1.

Statistical analysis

The mean ± SEM thymidine uptake and mean ± SEM cytokine secretion of triplicate cultures were calculated for each experimental condition. The Mann-Whitney test was used to evaluate possible differences in the CD4+CD25hi function following TNF stimulation. Percent suppression was determined as 1 – (cpm incorporated in the coculture/cpm of responder population alone) × 100%.

Results

CD4+CD25hi and CD4+CD25− T cells exhibit distinct phenotypic and functional differences

Previous work has suggested that the subset of CD4+CD25hi cells is enriched in Tregs, and, therefore, this population was isolated. CD4+CD25hi cells were defined as the 2% of CD25+ cells with the highest density of this molecule, and represented approximately 0.5% to 2% (mean ± SEM: 1.1 ± 1.8 [n = 40]) of total CD4+ T cells. By flow cytometry, CD4+CD25hi Tregs uniformly expressed high levels of FoxP3 protein, whereas CD4+CD25− cells did not express this transcription factor that governs Treg function. CD4+CD25hi cells expressed minimal levels (Figure 1B). By scatter characteristics, CD4+CD25hi Tregs were not larger or more complex than CD4+CD25− effector T cells (data not shown). Fifteen percent (mean ± SEM: 15% ± 5% [n = 40]) of the CD4+CD25hi subset expressed the GITR, which is a marker of CD4+CD25+ Tregs in the murine system. We also observed that 18% (mean ± SEM: 18% ± 6% [n = 40]) of freshly isolated CD4+CD25hi cells expressed TNFRII (CD120b) compared with 2.5% ± 1.5% (mean ± SEM [n = 40]) in CD4+CD25− cells. In contrast, TNFRI (CD120a) was practically undetectable (mean ± SEM: 1.3% ± 0.6% [n = 10]; Figure 1C). TNFRII expression increased and was expressed by 100% of both cell subsets after in vitro anti-CD3 stimulation (Figure 1D). In contrast, after overnight incubation with TNF and IL-2, we noticed a specific up-regulation of the expression of TNFRII by the CD4+CD25hi T-cell subset, such that 59% became positive (Figure 1D). In contrast, no up-regulation of TNFRI was observed after overnight stimulation (data not shown).

Analysis of CD69 expression on the Treg subset (mean ± SEM: 10% ± 0.1% [n = 40]) confirmed that these cells were not simply contaminated with recently activated CD4+ T cells as these would be mainly CD69+. Of importance, the majority of CD4+CD25hi Tregs were CD45ROhi and less than 25% were HLA-DR+ as has been reported previously, thus confirming that the isolated CD4+CD25hi Tregs were not contaminated simply with recently activated T cells. Our data also confirmed previous findings of selective expression of CCR4 on CD4+CD25hi cells (mean ± SEM: 90% ± 7% [n = 40]) compared with CD4+CD25− (mean ± SEM: 30% ± 10%).

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[n = 40]. These phenotypic characteristics indicated that the cells analyzed were typical CD4+CD25hi Tregs.

CD4+CD25hi T cells proliferated poorly to immobilized anti-CD3 (Figure 2A), and produced no detectable amounts of IL-2, IL-10, IL-4, TGFβ, IFNγ, or IL-6 (data not shown). At a ratio of 1:1, CD4+CD25hi Tregs inhibited the proliferation of CD4+CD25− T cells stimulated by plate-bound anti-CD3 by a mean (± SEM) of 85% ± 5% (n = 20; Figure 2B). These data indicate that CD4+CD25hi Tregs have a direct suppressive effect on T cells that is independent of APCs. In addition, CD4+CD25hi Tregs suppressed the production of IFNγ by CD4+CD25− T cells activated with anti-CD3 mAb (Figure 2C). The suppressive effects of the CD4+CD25hi Tregs were not reversed by the addition of neutralizing mAb to TGFβ1, IL-10, IL-10R, and GITR (Figure 2D). These results are all consistent with the conclusion that the CD4+CD25hi cells isolated and assessed were bona fide Tregs with the characteristics previously described for these cells.

TNF blocks suppressive activity of CD4+CD25hi Tregs by signaling through TNFRII

As Tregs appeared to express TNFRII, we next determined whether signaling through TNFRII influenced the regulatory function of CD4+CD25hi Tregs. The addition of soluble TNF to the culture of CD4+CD25− responders and CD4+CD25hi Tregs completely reversed the suppression of the proliferation of CD4+CD25− T cells without influencing the anergic phenotype of CD4+CD25hi Tregs (Figure 3A). Furthermore, an agonistic mAb to TNFRII also reversed the suppressive activity of CD4+CD25hi Tregs (Figure 3B). Since a previous study20 failed to demonstrate an effect of low concentrations of TNF on the suppressor function of CD4+CD25+ Tregs isolated with magnetic beads, we carried out dose-response curves of both TNF and the mAb to TNFRII. As can be seen in Figure S1A (available on the Blood website; see the Supplemental Figures link at the top of the online article), a low concentration of either TNF (<20 ng/mL) or the anti-TNFRII mAb (0.05 μg/well) had minimal effects on the function of Tregs, whereas higher concentrations of TNF (50 ng/mL) or the anti-TNFRII mAb (≥0.1 μg/well) suppressed Treg function in a concentration-dependent manner. Therefore 50 ng TNF or 0.2 μg/well anti-TNFRII mAb was used for the remainder of the studies. Notably, the addition of the antibody did not reverse the anergic phenotype of the CD4+CD25hi Tregs and did not cause increased proliferation of CD4+CD25− T cells. Anti-TNFRII mAb also reversed the suppression of IFNγ secretion mediated by CD4+CD25hi Tregs (Figure 3C). Similar results were observed when the anti-TNFRII mAb was added to cultures of CSFE-labeled cells (Figure 3D). CD4+CD25hi Tregs diluted CFSE minimally, whereas CD4+CD25− T cells underwent extensive CFSE dilution. The anti-TNFRII mAb did not reverse the anergy of the Tregs and did not augment the proliferation of the CD4+CD25− effectors. In contrast, CD4+CD25hi Tregs suppressed the dilution of CFSE by CD4+CD25− T cells and the suppressive effect was blocked by the anti-TNFRII mAb.

To determine whether the effect of TNF was to alter the function of the CD4+CD25hi Tregs or increase the resistance of the effector cells to suppression, a series of preincubation studies were performed. Preincubation with TNF had little effect on the capacity of CD4+CD25− T cells to proliferate in response to anti-CD3 stimulation (Figure 4). Similarly, preincubation of CD4+CD25hi Tregs did not increase their subsequent proliferative capacity and actually appeared to make them more anergic. In contrast, preincubation of Tregs with TNF inhibited their capacity to suppress control of TNF-preincubated effector cells, whereas preincubation of CD4+CD25− T cells did not make them resistant to suppression by CD4+CD25hi Tregs. These studies strongly support the conclusion that TNF can directly modulate Treg function.

To determine whether the lack of in vitro suppressive function we observed when TNFRII was engaged could be attributed to down-regulation of FoxP3, flow cytometric evaluation and quantitative PCR was performed on freshly isolated CD4+CD25− effectors and CD4+CD25hi Tregs. Notably, TNF caused a marked decrease in the FOXP3 mRNA as well a decrease in protein expression by CD4+CD25hi Tregs (Figure 4B-C). Similar results were noted when TNFRII was cross-linked with mAb.

TNF also blocked the suppressive activity and down-regulated FOXP3 expression of TGFβ1-induced regulatory T cells, as shown in Figure S2.

CD4+CD25hi Tregs from active RA patients are both phenotypically and functionally abnormal

The studies presented in the preceding section strongly suggest that TNF/TNFRII interactions play a major role in modulation of Treg function. Patients with active RA have high levels of serum TNF,27 and some studies have suggested that Treg function in these patients is compromised.20 The phenotype of Tregs from patients with active RA was therefore examined. As can be seen in Figure 5, CD4+CD25hi Tregs from patients with active RA contained a
greater percentage of TNFRII+ cells (mean ± SEM: 44% ± 10% [n = 15]) and GITR+ cells (mean ± SEM: 55% ± 15% [n = 15]) compared with healthy controls (depicted in Figure 1). It is unlikely that the cells expressing these receptors were activated conventional T cells as the levels of expression of the activation antigen, CD69, were not elevated (mean ± SEM: 1% ± 0.7%). To examine the function of regulatory T cells in active RA patients, we isolated highly pure CD4+CD25hi Tregs and the CD4+CD25− effector-cell population by flow cytometry. As shown in Figure 6, CD4+CD25hi Tregs isolated from 15 RA patients were anergic to stimulation by immobilized anti-CD3. Although CD4+CD25hi Tregs isolated from active RA were anergic, they did not suppress the proliferation of CD4+CD25− T cells (Figure 6) and suppressed the production of IFNγ from CD4+CD25− T cells less effectively than CD4+CD25hi Tregs from healthy controls (Figure 7A). To determine whether the loss of regulatory function in active RA was explained by a decrease in the intrinsic function of CD4+CD25hi Tregs or an increase in the resistance of CD4+CD25− effector T cells to inhibition, we carried out mixing experiments with cells from RA patients and healthy controls. Tregs from patients with active RA failed to suppress the proliferation and exhibited diminished regulation of IFNγ production by autologous CD4+CD25− effector T cells, whereas CD4+CD25hi Tregs from healthy controls readily suppressed the proliferative response of CD4+CD25− effectors from RA patients (Figure 7B) as well as their IFNγ secretion (Figure 7A). Notably, CD4+CD25hi Tregs from active RA patients also failed to suppress the proliferation of CD4+ effector T cells from healthy donors (Figure S3). These data clearly indicate that the primary regulatory defect is in the function of CD4+CD25hi Tregs isolated from the circulation of patients with active RA. As our in vitro studies demonstrated that the levels of expression of the transcription factor FOXP3 in Tregs were modulated by TNF, we compared the levels of FOXP3 expression on Tregs from healthy controls and from patients with active RA. Freshly isolated CD4+CD25hi Tregs from active RA expressed significantly less FOXP3 mRNA and protein (Figure 8A-B) compared with that expressed by normal CD4+CD25hi Tregs.

Finally, we compared the phenotype and function of Tregs in 15 patients with RA before and after treatment with anti-TNF. After treatment with infliximab, there was a significant decrease in expression of TNFRII (12% ± 5% vs 44% ± 10%, n = 15) and GITR (35% ± 10% vs 55% ± 15%, n = 15) by CD4+CD25hi Tregs (Figure 5B). Moreover, 3 months after anti-TNF therapy was begun, CD4+CD25hi Tregs regained their ability to inhibit the proliferation of autologous CD4+CD25− effectors, whereas CD4+CD25hi Tregs from these same RA patients were unable to suppress the proliferation of CD4+CD25− effectors before therapy was begun (Figure 8C). In addition, following treatment with infliximab, expression of FOXP3 mRNA (Figure 8A) and protein (Figure 8B) by CD4+CD25hi Tregs significantly increased compared with that noted before treatment and was not significantly different from that noted in healthy control subjects.

Discussion

The current studies have revealed a novel and potentially important immunoregulatory action of TNF. This proinflammatory cytokine was found to inhibit the suppressive function of CD4+CD25hi Tregs. This effect was mediated through TNFRII and was
isolated from all CD25 expression. However, when similar criteria are followed to isolate high levels of CD25.

The fluorescence intensity of the stained cells indicates the percentage of positive cells and those in parentheses, the mean fluorescence intensity of the 24-hour incubation. Data are representative of 3 different experiments. Numbers in each box indicate the percentage of positive cells and those in parentheses, the mean fluorescence intensity of the stained cells.

Two-way ANOVA was used to compare mean 

associated with downmodulation of FOXP3. These results suggest a potential novel interaction between the innate and adaptive immune systems, in which TNF, a product of the former, could foster immune reactivity by limiting the action of CD4+CD25hi Tregs. Our data presented here provide new insight into the biology of CD4+CD25hi Tregs within the context of a human autoimmune disease.

Previous investigations identified human constitutive CD4 regulatory function only within CD4+CD25+ cells that expressed high levels of CD25.

In contrast, the mouse CD4+CD25+ regulatory subset is isolated from all CD25+ T cells regardless of their levels of CD25 expression. However, when similar criteria are followed to isolate these cells from human blood, the resulting CD25+ cells (high and low together) did not exhibit an anergic phenotype or significant suppressive function, likely because the population is a mixture of effector cells and Tregs.24 To avoid this problem, we analyzed the suppressive function of isolated CD4+CD25hi T cells. These cells exhibited all of the expected properties of human Tregs, including functional anergy, high expression of FOXP3, and the capacity to suppress the responses of CD4+CD25+ effector cells in a cytokine-independent manner. Of importance, the function of human Tregs was tested in cultures stimulated by immobilized anti-CD3 mAb in the absence of APCs. Therefore, the Tregs analyzed suppressed both proliferative and cytokine production of CD4+CD25+ effector cells by a direct interaction and not indirectly through an action on APCs as has been shown previously.12

Figure 4. Preincubation with TNF suppresses the subsequent ability of CD4+CD25hi Tregs to inhibit the proliferation of CD4+CD25+ T cells and decreases FOXP3 expression. (A) Freshly sorted CD4+CD25+ T cells and CD4+CD25hi Tregs were incubated with TNF at 50 ng/mL in medium supplemented with IL-2 at 100 U/mL. After overnight incubation, cells were washed and examined for the capacity of CD4+CD25hi Tregs to suppress proliferation of CD4+CD25+ T cells. Data are mean ± SEM from 6 different experiments. (B) FoxP3 expression is reduced by TNF incubation of CD4+CD25hi Tregs. CD4+CD25+ and CD4+CD25hi T cells were sorted as described and cultured overnight with IL-2 and with or without TNF (50 ng/mL). Real-time PCR was carried out in triplicate for FOXP3 in CD4+CD25hi Tregs after overnight incubation compared with that found in fresh cells (P < .01). (C) Intracellular FoxP3 staining was carried out with freshly isolated cells and the same cells after a 48-hour incubation with IL-2 alone or the combination of IL-2 and TNF (50 ng/mL). Similar results were noted after a 24-hour incubation. Data are representative of 3 different experiments. Numbers indicate the percentage of positive cells and those in parentheses, the mean fluorescence intensity of the stained cells.

Figure 5. Phenotype of CD4+CD25hi Tregs from RA patients. (A) Purified CD4+ T cells were stained with anti-CD4 Cyochrome and anti-CD25 PE and the population was sorted into CD4+CD25+ and CD4+CD25hi subsets as indicated in “Patients, materials, and methods.” The resultant purity of CD4+CD25+ and CD4+CD25hi T cells is shown. Purified populations were stained with anti–TNFRI-FITC–, anti–TNFRII–APC–, anti–GITR–FITC–, anti–CD69–FITC–, and anti–CCR4–APC–conjugated mAb as indicated. (B) Anti-TNF therapy down-regulates expression of TNFRII and GITR on CD4+CD25+ T cells. Freshly sorted CD4+CD25+ T-regulatory cells and CD4+CD25+ effector cells were isolated from 15 RA patients treated with infliximab and their phenotype was characterized. Numbers in each box indicate the percentages of positive cells and those in parentheses, the mean fluorescence intensity of the stained cells. These changes in phenotype were compared with the phenotype of healthy donors, shown in Figure 1.

Figure 6. CD4+CD25hi T cells from active RA patients fail to suppress proliferation. CD4+CD25+ donors (5 × 10^4/well) and CD4+CD25hi Tregs (5 × 10^4/well) were cultured with plate-bound anti-CD3 (1 μg/well) either alone or at a 1:1 ratio. After 72 hours, [3H] thymidine incorporation was determined. Results are the mean ± SEM of 15 separate experiments using patient samples compared with healthy donors. Also shown is the percent inhibition of proliferation of these 15 experiments.
We found that the suppressive function of Tregs can be modulated by TNF through its receptor TNFRII constitutively expressed on human Tregs. Expression of TNFRII is largely confined to cells of the immune system, but this is the first report that TNFRII is constitutively expressed by Tregs and not by circulating CD4+CD25+ effector cells. TNFRII was expressed by neither Tregs nor effector cells. As expected from the distribution of TNFRs, the effect of TNF was mediated through TNFRII, and the impact of TNF or the agonistic mAb to TNFRII was directed at the function of Tregs and not the effector T cells. This was clearly documented functionally in preincubation experiments. Because expression of FOXP3 appears to play a necessary role in governing Treg action, we directly tested whether engagement of TNFRII modulates FOXP3 expression. Brief pretreatment of Tregs with TNF resulted in a marked down-regulation of FOXP3 mRNA and protein expression of Tregs.

Extrinsic factors that regulate the impact of Tregs have been described in the mouse. Thus, IL-6 has been shown to increase the resistance of effector T cells to the suppressive action of Tregs, whereas engagement of GITR has been claimed to interfere with the action of Tregs' and/or the sensitivity of effector T cells to the suppressive effect of Tregs. In contrast to murine Tregs, we found that freshly isolated human CD4+CD25hi Tregs expressed considerably less GITR; only 10% of Tregs expressed GITR. Moreover, cross-linking of GITR failed to reverse the suppressive activity of Tregs, although it is not certain that this mAb was a fully agonistic mAb. Confirming that GITR is not a lineage-specific marker in the human, we detected a marked up-regulation on both CD4+CD25+ and CD4+CD25hi T cells upon in vitro anti-CD3 activation (data not shown). Since we could not find an effect of GITR on the suppressive function of human CD4+CD25hi Tregs, we sought to study other molecules that might modulate Treg function.

There is considerable data that the function of Tregs is governed by the effects of a transcriptional repressor, FOXP3. In animals and humans lacking FOXP3 expression, Treg function is absent, whereas overexpression of FOXP3 by in vitro transfection induces T cells both to become anergic and to exert a complete or partial suppressive activity. Thus, expression of FOXP3 appears to play a necessary role in governing Treg action. The finding that TNF by signaling through TNFRII down-regulates FOXP3 expression suggests a mechanism by which this inflammatory cytokine can influence Treg function. It is notable that TNF altered the suppressive function of Tregs but not their proliferative unresponsiveness or inability to produce cytokines. These results suggest that different mechanisms may govern anergy and suppression in Tregs and that, of importance, only the latter is regulated by TNF.

Figure 7. CD4+CD25hi Tregs from active RA patients are defective suppressors. (A) CD4+CD25hi responders (5 × 10⁴/well) and CD4+CD25hi Tregs (5 × 10⁴/well) were cultured with plate-bound anti-CD3 (1 μg/well) either alone or at a 1:1 ratio. After 72 hours, ³H-thymidine incorporation was determined. CD4+CD25hi effectors from active RA patients were also cocultured with CD4+CD25hi Tregs from healthy individuals. Results are the mean ± SEM of 3 separate experiments. (B) Culture supernatants were diluted and analyzed to determine the amount of interferon-γ. Data represent the mean ± SE of 5 different experiments.

Figure 8. CD4+CD25hi Tregs from RA patients recover their suppressive function after infliximab therapy. (A) FOXP3 mRNA expression is reduced in CD4+CD25hi Tregs of patients with active RA and recovers following treatment with infliximab. CD4+CD25hi and CD4+CD25hi T cells were sorted as described from RA patients before (RA pre) and after (RA post) treatment with infliximab. Real-time PCR was carried out in triplicate for FOXP3 mRNA and relative fold changes were normalized to GAPDH. Data represent the mean ± SEM of 5 different experiments. The same RA patients were examined following 3 months of therapy with infliximab. Following infliximab therapy, FOXP3 mRNA levels in CD4+CD25hi Tregs of RA patients were significantly increased (P < .05) and not significantly different from that found in healthy controls. (B) FoxP3 protein expression in CD4+CD25hi Tregs recovers after anti-TNF treatment of RA patients. CD4+CD25hi and CD4+CD25hi T cells were sorted as described. Intracellular FoxP3 staining was carried out in CD4+CD25hi and CD4+CD25hi T cells sorted from an RA patient before and after infliximab therapy. (C) CD4+CD25hi responders (5 × 10⁴/well) and CD4+CD25hi Tregs (5 × 10⁴/well) were isolated from active RA patients before infliximab therapy and after 3 months of infliximab treatment. Cells were cultured with plate-bound anti-CD3 (1 μg/well) either alone or at a 1:1 ratio. After 72 hours, ³H-thymidine incorporation was determined. Data represent the mean ± SE of 15 RA patients before and after infliximab treatment.
protein expression. The finding that TNF by signaling through TNFRII regulates FOXP3 expression suggests a mechanism by which this inflammatory cytokine can influence Treg function. Further studies are needed to determine the biochemical basis for TNFRII-mediated down-regulation of FOXP3. It is known that TNF by engaging TNFRII can alter the function of T cells, but no previous studies have examined the impact of TNF signaling through TNFRII on the function of Tregs.

A previous paper reported the lack of a direct effect of TNF on Tregs. However, there are methodological differences that may explain their results. Of most importance, a lower concentration of TNF was used than was used in our study. This is an important consideration because the effect of TNF on Tregs was mediated via TNFRII. It is believed that TNFRII predominantly recognizes membrane-bound TNF during cell-to-cell contacts owing to its significantly lower avidity for soluble TNF. This may provide a mechanism for highly localized regulation of Tregs only in the region of cells expressing membrane-bound TNF. Moreover, higher concentrations of soluble TNF would be expected to be required to observe an effect on Treg function. In this regard, we found minimal effects on Treg function with lower concentrations of TNF. It is notable that high levels of circulating TNF similar to that used in our studies and sufficient to bind and activate TNFRII can be found in serum of healthy volunteers after infection with S. aureus and higher levels have been reported in sepsis. Moreover, analysis of TNF bioactivity in the serum of patients with RA resulted in the detection of levels that were comparable with those used in the current study, although bioactivity of TNF did not correlate with levels measured by ELISA. These results indicate that sufficiently high levels of TNF may occur in infections or chronic inflammation to inhibit Treg function more globally.

Treatment of patients with rheumatoid arthritis with a mAb to TNF has been reported to enhance some actions of circulating Tregs. CD4+CD25+ Tregs isolated from patients with active RA, although anergic, showed compromised function as demonstrated by their inability to regulate proliferation and their reduced capacity to regulate cytokine secretion by effector CD4+CD25− T cells. Our results differ from a previous report that found preserved suppression of proliferation but lack of suppression of cytokine production by CD4+CD25+ Tregs from patients with active RA. One potential explanation for the difference between the 2 studies is that we analyzed highly purified CD4+CD25+ Tregs prepared by flow cytometry, whereas the previous report used largely CD4+CD25+ Tregs isolated with magnetic beads that likely included both CD25hi and CD25lo subpopulations. Of most importance, we demonstrated that CD4+CD25+ Tregs from patients with active RA express high levels of TNFRII, markedly decreased levels of FOXP3 mRNA and protein, and a diminished capacity to suppress both proliferation and IFNγ production by CD4+CD25− effector T cells. TNF has previously been shown to up-regulate TNFRII expression in human malignant epithelial cell lines, but no previous studies have examined this in Tregs. In the context of in vitro experiments demonstrating that TNF could up-regulate TNFRII expression on Tregs, as well as drive the changes in FOXP3 expression and regulatory function, it is likely that all of these abnormalities in RA patients reflect the effect of in vivo exposure to TNF. Of importance, mixing experiments clearly demonstrated that the decrease in Treg function was caused by a defect in the CD4+CD25hi T-cell subset rather than the possibility that the responder CD4+CD25− cells were refractory to suppression. All of the abnormalities in Treg phenotype and function were reversed after infliximab treatment. Although the results are most consistent with the conclusion that the effect of in vivo treatment with infliximab reflected blockade of the action of TNF directly on Tregs, it is formally possible that other activities of TNF were involved, including a potential alteration in the distribution of Tregs because of changes in cell trafficking.

Supporting our data on the role of TNF in Treg function is recent evidence that TNF impaired the ability of regulatory T cells to suppress disease in the NOD mouse model of diabetes. These authors suggested that endogenous levels of TNF might act centrally in the thymus to mediate these effects on regulatory T cells. Very recently, up-regulation of TNFRII in Tregs in the inflammatory site in the NOD mouse was also demonstrated, and is consistent with the possibility that these cells might have increased sensitivity to the modulatory effects of TNF. Also pertinent to our data is the recent observation that CD4+CD25+ T cells derived from the thymus of healthy donors have an increased expression of TNFRII compared with CD4+CD25− T cells, which might result in an increased susceptibility of regulatory T cells to the actions of TNF.

In summary, we have demonstrated a novel function of TNF as a critical factor downmodulating Treg function. We also uncovered the mechanism of this effect, a reduction in FoxP3 expression in CD25+CD4+ Tregs that has been correlated with the suppressive function of CD4+CD25+ Tregs. It is intriguing to consider the physiologic role of TNF in regulating immune responses to invading microorganisms. During initial tissue invasion, exuberant TNF production may limit the activity of Tregs and foster induction of immune reactivity and the effector phase of lymphocyte responses. As the insult is resolved, a decrease in TNF production may result in enhanced Treg function that limits immune reactivity so as to avoid triggering immune responses to autologous tissue antigens revealed during inflammation. In this way, TNF may play an important instructive role in controlling adaptive immunity. Strategies designed to manipulate TNF signaling in Tregs may result in novel therapeutic approaches to augment the limited and/or inadequate function of these regulatory T cells in inflammatory or autoimmune diseases.

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TNF downmodulates the function of human CD4+CD25hi T-regulatory cells

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