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Interplay between TNF and Regulatory T Cells in a TNF-Driven Murine Model of Arthritis

Jérôme Biton,* Luca Semerano,**† Laure Delavallée,* Delphine Lemeiter,* Marion Laborie,‡ Géraldine Groud-Vogel,‡ Marie-Christophe Boissier,*,‡ and Natacha Bessis*

CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Treg) are involved in several autoimmune diseases, including rheumatoid arthritis. TNF-α blockers induce therapeutic benefits in rheumatoid arthritis via a variety of mechanisms. We aimed to characterize the impact on Treg of TNF-α overexpression in vivo and of TNF-α inhibiting treatments. We used human TNF-α transgenic mice as a model of strictly TNF-α-dependent arthritis. Our study showed that initial Treg frequency was lower in TNF-α transgenic mice than in wild-type mice. However, the course of arthritis was marked by elevation of Treg frequency and a dramatic increase in expression of TNFR2. Antagonizing TNF-α with either the anti-human TNF-α Ab (infliximab) or active immunotherapy (TNF-kinoid) increased the Treg frequency and upregulated CTLA-4, leading to enhancement of suppressor activity. Moreover, both anti–TNF-α strategies promoted the differentiation of a CD62L<sup>+</sup> Treg population. In conclusion, in an in vivo model of TNF-α–driven arthritis, Treg frequency increased with inflammation but failed to control the inflammatory process. Both passive and active TNF-α–inhibiting strategies restored the suppressor activity of Treg and induced the differentiation of a CD62L<sup>+</sup> Treg population.  The Journal of Immunology, 2011, 186: 000–000.

Rheumatoid arthritis is a chronic autoimmune disease involving T lymphocytes and whose hallmark is hyperplastic synovitis responsible for cartilage and bone destruction. Factors involved early in the disease process include proinflammatory cytokines such as TNF-α, IL-1, and chemokines. Among all of the cytokines involved in the disease process, TNF-α has a particularly important role in the cascade of pathogenic events in rheumatoid arthritis (RA). TNF-α acts within a complex network of cells and mediators of inflammation, as shown by the ability of IL-1β or IL-17 to induce TNF-α. The hypothesis that TNF-α drives much of the pathophysiology of the rheumatoid joint is supported by studies of TNF-α overexpression or TNF-α neutralization in animal models of RA (1, 2). The experimental results in animal models reflect findings from studies of TNF-α antagonism in patients with RA. Whereas previous medications used in RA were developed primarily based on serendipitous observations, TNF-α antagonists (mainly mAbs such as infliximab and soluble receptors such as etanercept) were the first rationally designed drugs and the first U.S. Food and Drug Administration–approved recombinant proteins ("biologics") for the treatment of RA. Although TNF-α antagonists provide substantial therapeutic benefits in most patients, primary unresponsiveness and secondary escape phenomena are not uncommon, indicating a need for alternative treatments (3). We recently developed a novel concept consisting of active immunotherapy to cytokines (4, 5). In this anticytokine immunotherapy strategy, the immunogen (TNF-kinoid [TNF-K]) induces the production of Abs that block the effects of the targeted cytokine. This strategy protected human TNF-α transgenic (TTg) mice against clinical and histological arthritis in short- and long-term experiments involving preventive (4, 5) or curative (6) immunotherapy.

TNF-α antagonists may act through mechanisms involving regulatory T cells (Treg), which exhibit the CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> phenotype. Treg are essential for maintaining immune homeostasis, preventing autoimmunity, and limiting chronic inflammatory diseases. Treg act by preventing both the activation and the effector function of T cells that have escaped other mechanisms of tolerance (7, 8). Their central place in the maintenance of peripheral tolerance is underlined by the fact that Treg deficiency results in spontaneous autoimmunity in both mice and humans (9, 10). Furthermore, Treg play a pivotal role in preventing autoimmune diseases such as type 1 diabetes (11) and in limiting chronic inflammatory diseases such as asthma and inflammatory bowel disease (12, 13).

In patients with RA, Treg functions, including suppression of proinflammatory cytokine secretion by activated T cells and monocytes, are diminished compared with healthy individuals (14, 15). The regulatory role for Treg in experimental models of RA has been demonstrated in a few studies (16, 17). The link between TNF-α antagonists (infliximab and adalimumab) and Treg is that TNF-α antagonists normalize immune homeostasis by restoring the capacity of Treg to inhibit cytokine production and by conveying a suppressive phenotype to conventional T cells (14, 18). However, direct interactions between TNF-α and Treg have been...
documented chiefly in in vitro studies, which have produced conflicting results. In a first study, TNF-α had no direct effect on Treg (14), but subsequent work showed that TNF-α inhibited the suppressive capacity of Treg via a TNFR2-dependent mechanism leading to downregulation of Foxp3 expression (19). More recently, TNF-α interaction with TNFR2 was shown to promote Treg expansion and function (20, 21).

Our aim in this work was to study T cell differentiation to the Treg phenotype in an in vivo model of TNF-α–driven disease, namely the hTNF-αTg model. This model is relevant for investigating the consequences of TNF-α overexpression on arthritides and the effect of TNF-α blockade. We show that Treg differentiation may constitute a critical mode of action of TNF-α blocking treatments such as infliximab and TNF-K immunotherapy.

Materials and Methods

Mice

Male transgenic mice (1006-T) aged 6–9 wk were purchased from Taconic Farms (Germantown, NY). These mice were produced using the microinjection construct previously used to generate the Tg197 strain (5). They were hemizygous for the hTNF-α transgene and maintained on a C57BL/6 background. All pups were genotyped by PCR, which established the presence of the hTNF-α transgene in all the animals. C57BL/6 mice aged 7–31 wk were purchased from Janvier (Le Genest-Saint-Isle, France) and used as controls. All procedures were approved by the Animal Care Use Committee of Sorbonne Paris Cité-Université Paris 13 (Bobigny, France).

Clinical and histological assessments

1006-T transgenic mice develop spontaneous arthritis, similar to the Tg197 mice first described by Keffler et al. (1). Clinical arthritis begins to develop in 1006-T mice at ∼8–10 wk of age (22). A blinded procedure was used to monitor body weight and arthritis in all four limbs. Clinical severity of arthritis in each limb was scored from 0 (normal) to 3 (severe inflammation with deformities) (23). For incidence determination, arthritis was defined as a score ≥1. The mean arthritis score on each clinical observation day was calculated in each treatment group. In each mouse, we recorded the maximal arthritis score as the highest score reached during the observation period. For histological analysis, the hind paws were dissected and processed as described elsewhere (24). Numerous sections were cut from each paw, and at least four sections per paw were examined. Slides were then stained with H&E before microscopic observation (optical microscopy). A blinded procedure was used to evaluate the lesions in each joint as described elsewhere (6), using a 4-point scale for synovitis (0–3, where 0 is normal and 3 indicates severe synovial proliferation or inflammatory cell infiltration) and a 4-point joint-destruction scale (cartilage, irregularities, and bone erosions). For prevalence determinations, histological inflammation or destruction was defined as an inflammation or destruction score ≥0.5.

Treatments

We obtained hTNF-α kinoid from Neovacs (Paris, France). Briefly hTNF-α (Boehringer, Austria) (1 mg/ml) in 0.5 EDTA/0.1 M phosphate buffer (pH 7.8) was treated with 1% DMSO for 30 min. Keyhole limpet hemocyanin, purchased from Intracel (Frederick, MD), was added, followed by glutaraldehyde. After 45 min incubation at 4°C, the preparation was dialyzed against the working buffer and then treated with formaldehyde. After

FIGURE 1. Evolution of arthritis in untreated mice and effect of hTNF-α blockade on established disease. TTg mice received three primary injections of TNF-K at 15, 16, and 19 wk of age (n = 20, gray squares) or infliximab weekly from week 15 to euthanasia (n = 16, black triangles). Control mice received no treatment (n = 20, open triangles). Because half the mice were sacrificed at 24 wk of age and the other half at 31 wk of age, A shows the change in clinical scores only until 24 wk of age. Data are expressed as means ± SEM for each group. p < 0.0001 for TNF-K versus untreated TTg. Examples are shown of histological evaluation of 24-wk-old TTg mice immunized with TNF-K (B), treated with infliximab (C), and untreated (D). Histological sections (original magnification ×40) of the knee were prepared (see Materials and Methods) and stained with H&E. Date represent one experiment representative of three similar experiments.
quenching with glycine (0.1 M) and subsequent dialysis against Dulbecco’s PBS, the preparation was stored at 4°C (4).

Immunization involved i.m. injections of TNF-K emulsified in ISA51 (Seppic, Paris, France). Three injections were given (4 μg TNF-K) at 15, 16, and 19 wk of age. In parallel, other mice were given weekly i.p. injections of infliximab (10 mg/kg; Schering-Plough, Levallois Perret, France) from week 15 to week 24 or 31, when they were sacrificed.

**Ab assays**

Sera obtained from blood samples collected at different time points during the experiment were tested for anti-hTNF-α Ab titers and for anti-hTNF-α Ab-neutralizing capacity. Specific anti-hTNF-α titers were determined using a direct ELISA (4). The neutralizing capacity of sera was assessed using the L929 cytotoxicity assay reflecting neutralizing Abs (4).

**Real-time quantitative RT-PCR**

Total RNA was extracted from synovial tissue using the TRI-Reagent kit (Euromedex, Mundolsheim, France). Then, 5 μg RNA was primed with oligo(dT) and reverse transcribed into a 20 μl volume using SuperScript III RNase H- reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative PCR reactions were performed using PCR LightCycler FastStart DNA Master SYBR Green I (Roche Molecular Biochemicals, Indianapolis, IN). Foxp3 expression was measured by real-time PCR. The Foxp3 primers were 5’-CAGGCTGCCCTGACAGAA-3’ (forward) and 5’-GGGGTTCAAGGAAAGAGCG-3’ (reverse). Each reaction mix contained 5 μl cDNA, 0.2 μM each primer, and LightCycler FastStart DNA Master SYBR Green I mix (Roche Molecular Biochemicals) to a final volume of 15 μl. For Foxp3 amplification, initial 8-min holds at 95°C were followed by 45 cycles of 10 s at 95°C, 5 s at 62°C, and 8 s at 72°C. Amplification was analyzed using LightCycler software (RealQuant, version 1.0; Roche, Indianapolis, IN).

**Cell and tissue preparation**

Leukocytes from the spleen were prepared using a homogenizer, and RBCs were lysed in hemolysis buffer (NH4Cl, KHCO3, and EDTA). Afferent and popliteal lymph nodes were dissected out of the hind limbs, and leukocytes were prepared using a homogenizer. Blood was collected by heart puncture. Finally, the knees were dissected and opened and synovial tissue was removed from both knees and pooled for further processing and analysis.

**Flow cytometry**

For FACS, surface cells were stained with FITC-labeled anti-CD25 (clone 3C7), PE-labeled anti-TNF-2R (clone TR75-89) or PE-labeled anti–CTLA-4 Ig mix (Roche Molecular Biochemicals) to a final volume of 15 μl. For Foxp3 amplification, initial 8-min holds at 95°C were followed by 45 cycles of 10 s at 95°C, 5 s at 62°C, and 8 s at 72°C. Amplification was analyzed using LightCycler software (RealQuant, version 1.0; Roche, Indianapolis, IN).

**Lymphocyte purification**

CD4+CD25+ and CD4+CD25− T cells from the spleen were purified using a Treg isolation kit according to the manufacturer’s protocol (Miltenyi Biotec, Bergisch-Gladbach, Germany). In brief, CD4+CD25+ T cells were isolated using a two-step procedure. First, CD4+ T cells were isolated by

<p>| Table I. Clinical and histological parameters of arthritis in TNF-K− or infliximab-treated TTg mice |</p>
<table>
<thead>
<tr>
<th>Clinical and Histological Parameters</th>
<th>Clinical Parameters</th>
<th>Histological Parameters</th>
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<tbody>
<tr>
<td></td>
<td>Omit (wk)</td>
<td>Clinical Score</td>
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<td>--------------------------------------</td>
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<tr>
<td>Untreated</td>
<td>9.6 ± 0.5</td>
<td>100 (10/10)</td>
</tr>
<tr>
<td>Infliximab</td>
<td>10 ± 1.2</td>
<td>100 (10/10)</td>
</tr>
<tr>
<td></td>
<td>18 ± 0.3</td>
<td>100 (10/10)</td>
</tr>
<tr>
<td></td>
<td>24 ± 0.2</td>
<td>100 (10/10)</td>
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<tr>
<td></td>
<td>31 ± 0.1</td>
<td>100 (10/10)</td>
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<td></td>
<td>38 ± 0.1</td>
<td>100 (10/10)</td>
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<tr>
<td></td>
<td>45 ± 0.1</td>
<td>100 (10/10)</td>
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<tr>
<td></td>
<td>52 ± 0.1</td>
<td>100 (10/10)</td>
</tr>
<tr>
<td></td>
<td>59 ± 0.1</td>
<td>100 (10/10)</td>
</tr>
<tr>
<td></td>
<td>66 ± 0.1</td>
<td>100 (10/10)</td>
</tr>
<tr>
<td></td>
<td>73 ± 0.1</td>
<td>100 (10/10)</td>
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<tr>
<td></td>
<td>80 ± 0.1</td>
<td>100 (10/10)</td>
</tr>
<tr>
<td></td>
<td>87 ± 0.1</td>
<td>100 (10/10)</td>
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<tr>
<td></td>
<td>94 ± 0.1</td>
<td>100 (10/10)</td>
</tr>
<tr>
<td></td>
<td>101 ± 0.1</td>
<td>100 (10/10)</td>
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</table>
| The mice are the same as in Fig. 1. Time at arthritis onset, mean maximum arthritis score (% of mice), clinical scores at 24 wk, and clinical scores at 31 wk of age, and inflammation and destruction scores are expressed as means ± SEM. (1) p < 0.05; (2) p < 0.01; (3) p < 0.001; (4) p < 0.001 versus untreated 24 wk TTg; (5) p < 0.001 versus untreated 31 wk TTg. The incidence of arthritis, inflammation, and destruction are expressed as percentages; (6) p < 0.001 versus untreated 24 wk TTg.
negative selection using a mixture of biotin-conjugated Abs, anti-biotin microbeads, an LD column, and QuadroMACS (all from Miltenyi Biotec). Then, CD4+ T cells were directly labeled with a PE-conjugated anti-CD25 Ab and anti-PE microbeads. The cell suspension was loaded onto an MS column, which was placed in the magnetic field of a MACS separator (OctoMACS; Miltenyi Biotec). The flow-through cells were collected and used as CD4+CD25− cells, whereas the retained cells were eluted from the column and used as CD4+CD25+ Treg. To increase purity, two consecutive column runs were performed. Flow cytometry analysis showed that purity of the CD4+CD25− and CD4+CD25+ cell-enriched fractions was 90–95% (data not shown).

**Measurement of CD4+CD25− effector T cell IFN-γ secretion**

Spleen CD4+CD25− (2.5 × 10^4) effector T cells (Teff) were cultured in RPMI 1640 with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, 1 M HEPES, and 5 μg/ml of soluble anti-CD3 (clone 2C11) (BD Biosciences) in U-bottom 96-well plates. APCs (2.5 × 10^4) treated with mitomycin were added to the culture medium. The cells were then incubated at 37°C in a 5% CO2 atmosphere. After 4 d culture, IFN-γ levels in culture supernatants were measured using commercially available ELISA kits (Quantikine; R&D Systems, Abingdon, U.K.) according to the manufacturer’s instructions. The sensitivity of the cytokine assays was 2 pg/ml.

**Assessment of Treg suppressive effect on CD4+CD25− Teff**

Spleen CD4+CD25− Teff were prelabeled with 5 μM CFSE (Invitrogen) for 10 min. Then, CFSE-labeled Teff (2.5 × 10^4) were cocultured in RPMI 1640 with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, 1 M HEPES, and 5 μg/ml soluble anti-CD3 (clone 2C11) (BD Biosciences) in U-bottom 96-well plates with Treg (2.5 × 10^4 or 1.25 × 10^4) to produce Teff/Treg ratios of 1:1 and 2:1, respectively. Controls were performed using non–CFSE-labeled Teff instead of Treg (CD4+CD25−; 2.5 × 10^4 or 1.25 × 10^4). APCs (2.5 × 10^4) treated with mitomycin were added to the culture medium. The cells were then incubated at 37°C in a 5% CO2 atmosphere. After 4 d culture, the cells were stained with allophycocyanin-labeled anti-CD4 (clone RM4-5, BD Biosciences), and Teff proliferation was then determined for each Teff/Treg ratio using flow cytometry to measure the CFSE dilution. The values were compared with the control, in which Teff cells were cultured without Treg. The percentage of suppression was calculated as follows: % suppression = [(Teff proliferation − Treg proliferation) / Teff proliferation] × 100.

**TABLE II. Frequency of Teff and Treg in the spleen during the course of arthritis in hTNF-α transgenic mice**

<table>
<thead>
<tr>
<th></th>
<th>% Teff (%)</th>
<th>% Treg (%)</th>
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<tbody>
<tr>
<td>7 wk wild-type</td>
<td>20.40 ± 0.39</td>
<td>2.04 ± 0.09(2)</td>
</tr>
<tr>
<td>7 wk TTg</td>
<td>20.81 ± 0.72</td>
<td>1.36 ± 0.16</td>
</tr>
<tr>
<td>12 wk TTg</td>
<td>19.19 ± 0.93</td>
<td>1.73 ± 0.08</td>
</tr>
<tr>
<td>17 wk TTg</td>
<td>17.80 ± 0.89</td>
<td>1.59 ± 0.17</td>
</tr>
<tr>
<td>24 wk TTg</td>
<td>18.55 ± 0.52(1)</td>
<td>1.90 ± 0.15(2)</td>
</tr>
<tr>
<td>24 wk wild-type</td>
<td>15.07 ± 1.05</td>
<td>1.83 ± 0.15</td>
</tr>
</tbody>
</table>

Splenocytes from TTg mice were stained with fluorochrome-conjugated anti-CD4, anti-CD25, and anti-Foxp3. The mice are the same as in Fig. 2. Teff and Treg in the spleen were monitored using flow cytometry. Percentages of Teff and Treg among splenocytes are given as mean values ± SEM.

(1) p < 0.05 versus 24 wk wild-type, (2) p < 0.05 versus 7 wk TTg.
eration without Treg-Teff proliferation with Treg)/Teff proliferation without Treg]. IFN-γ levels were measured in the supernatants after 4 d culture using commercially available ELISA kits (Quantikine; R&D Systems) according to the manufacturer’s instructions. The sensitivity of the cytokine assays was 2 pg/ml.

Statistical analysis

According to data distribution, a parametric test (ANOVA, Student test) or a nonparametric test (Kruskal–Wallis, Mann–Whitney U) with appropriate post hoc comparisons was used to compare data across the different groups. Clinical scores curves were compared with ANOVA. Categorical data were compared by a χ² test. All statistical analyses were performed using StatView version 5.0 software (Abacus Concepts, Berkeley, CA).

Results

Active and passive anti–hTNF-α immunotherapy improves established arthritis

As expected, clinical arthritis in untreated TTg mice was detected at 8 wk of age and arthritis severity increased over time (Fig. 1A). Fifteen-week-old TTg mice were given either classical hTNF-α–neutralizing mAb (infliximab) or active immunotherapy (TNF-K). At baseline, all mice had clinical scores >3. In mice immunized with TNF-K, anti–hTNF-α Ab was detected 5 wk after the first injection (Supplemental Fig. 1A). These anti–hTNF-α Abs inhibited hTNF-α bioactivity in vitro (Supplemental Fig. 1B). Infliximab-treated mice and TNF-K–immunized mice experienced...
dramatic improvements in the disease compared with untreated mice (Fig. 1A). Although infliximab exerted its therapeutic effect more rapidly than did TNF-K, both treatments were effective. As expected, disease severity increased in untreated mice between 24 and 31 wk of age (Table I). Histological evaluation also indicated decreased joint inflammation and destruction in TNF-K− (Fig. 1B) and infliximab-treated (Fig. 1C) groups compared with untreated mice (Fig. 1D) (Table I).

**hTNF-α overexpression modifies Treg frequency**

We investigated whether in untreated TTg mice, in vivo systemic hTNF-α overexpression influenced the numbers and percentages of Treg (defined as CD4+CD25+Foxp3+ cells) and Teff (defined as CD4+Foxp3− cells) (Fig. 2A) in secondary lymphoid organs from untreated TTg mice during the course of arthritis. The total leukocyte count increased progressively from week 7 to week 24 in the lymph nodes (24.3 $\times$ 10^3 ± 5.2 $\times$ 10^3 versus 38.1 $\times$ 10^3 ± 3.8 $\times$ 10^3, p < 0.05). In lymph node, Teff counts did not change significantly over time (data not shown) but their percentage decreased from week 7 to week 24 (Fig. 2B). However, a similar decrease was found in the wild-type mice (Fig. 2B), indicating that the cause was not hTNF-α overexpression. Compared to wild-type mice at the same age, 7-wk-old TTg mice had a lower percentage of Treg (Fig. 2C). Subsequently, Treg count (7 wk wild-type, 7.9 $\times$ 10^3 ± 1.7 $\times$ 10^3; 7 wk TTg, 6.1 $\times$ 10^3 ± 1.2 $\times$ 10^3; 24 wk wild-type, 8.9 $\times$ 10^3 ± 1.4 $\times$ 10^3; 24 wk TTg, 13.5 $\times$ 10^3 ± 1.0 $\times$ 10^3) p < 0.05 versus 24 wk TTg) and percentage (Fig. 2C) increased in the lymph nodes of TTg mice but not in those of wild-type mice. In the spleen, Treg and Teff populations in TTg mice showed similar changes to those seen in the lymph nodes (Table II). Given that the percentage and the number of Tregs in the lymph nodes increased progressively during the course of arthritis, we assessed Treg in the synovium by measuring the level of Foxp3 mRNA expression. An increase, although nonsignificant, in Foxp3 mRNA expression between weeks 7 and 24 was detected (Supplemental Fig. 2). Taken together, our results in TTg mice show that hTNF-α overexpression leading to arthritis is accompanied by an initial Treg deficiency followed by an increase in Treg proportions.

**Treg phenotype, but not suppressive activity, is modified during arthritis in hTNF-α transgenic mice**

Because hTNF-α overexpression in untreated TTg mice led to an initial Treg deficiency followed by an increase in the lymph nodes and spleen, we characterized the Treg phenotype throughout arthritis development by evaluating the expressions of CTLA-4, CD62L, and TNFR2. As shown in Fig. 3A and 3B, CTLA-4 expression and mean fluorescence intensity (MFI) increased in Treg from the lymph nodes between week 7 and week 24, but it was also the case in wild-type mice. In contrast, percentage of Treg-expressing CD62L decreased slightly over time (Fig. 3C); however, the same was true in wild-type mice. As shown in Fig. 3E and 3F, lymph node Treg exhibited gradual and marked increases in TNFR2 expression (65.8 and 88.6% at weeks 7 and 24, respectively; p < 0.05) and in MFI during the course of arthritis. Similar results were observed in the spleen, except for TNFR2 MFI, which was similar in all groups (Table III).

To better characterize Treg activity during arthritis development in TTg mice, we evaluated the ability of Treg to suppress Teff proliferation and IFN-γ production. We found no significant difference in Treg suppressive activity across age groups at each of the Teff/Treg ratios studied either for proliferation (Fig. 4) or for IFN-γ secretion (data not shown).

**hTNF-α blockade increases the Treg/Teff ratio in 24-wk-old TTg mice**

We investigated whether the mechanism of action of passive and active anti–hTNF-α immunotherapy involved Treg in the hTNF-α−dependent arthritis model.

Table III. Treg phenotype in spleen during arthritis in TTg mice

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>TNFR2 (%)</th>
<th>MFI</th>
<th>CTLA-4 (%)</th>
<th>MFI</th>
<th>CD62L (%)</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 TTg</td>
<td>59.32 ± 5.35</td>
<td>33.06 ± 1.33</td>
<td>91.54 ± 1.99</td>
<td>51.46 ± 3.74</td>
<td>89.95 ± 1.60</td>
<td>85.07 ± 18.66</td>
</tr>
<tr>
<td>12 TTg</td>
<td>68.99 ± 1.86</td>
<td>30.33 ± 0.44</td>
<td>95.00 ± 0.95</td>
<td>54.80 ± 2.76</td>
<td>84.05 ± 2.43</td>
<td>66.36 ± 8.11</td>
</tr>
<tr>
<td>17 TTg</td>
<td>70.12 ± 2.30</td>
<td>29.46 ± 1.07</td>
<td>94.91 ± 1.51</td>
<td>53.96 ± 9.09</td>
<td>79.10 ± 2.76</td>
<td>60.51 ± 9.21</td>
</tr>
<tr>
<td>24 TTg</td>
<td>82.25 ± 1.94</td>
<td>34.35 ± 1.92</td>
<td>98.47 ± 0.46</td>
<td>74.97 ± 6.03</td>
<td>81.86 ± 1.91</td>
<td>78.61 ± 9.25</td>
</tr>
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</table>

The mice are the same as in Fig. 2. Spleen cells were labeled with fluorochrome-conjugated anti-CD25, anti-CD4, anti-Foxp3, and anti–TNFR2 or anti–CTLA-4 or anti–CD62L. Then, TNFR2, CTLA-4, and CD62L expression was studied by gated CD4+CD25+Foxp3+ cells using flow cytometry. Percentage of Treg expressing TNFR2, TNFR2 MFI on TNFR2− Treg, percentage of Treg expressing CTLA-4, CTLA-4 MFI on CTLA-4− Treg, percentage of Treg expressing CD62L, and CD62L MFI on CD62L− Treg are given as means ± SEM.

(1) p < 0.05 versus 7 wk TTg, (2) p < 0.05 versus 24 wk TTg.

**FIGURE 4.** Treg suppressive activity during arthritis in TTg mice. TTg mice were sacrificed at the age of 7 (n = 6), 12 (n = 3), 17 (n = 4), or 24 wk (n = 5), CD4+CD25+ (Treg) and CD4+CD25− (Teff) were isolated from the spleens of all mice. CD4+CD25− CFSE-labeled T cells were cocultured with CD4+CD25+ Treg at ratios of 1:1 and 2:1 for 96 h, with 5 μg/ml soluble anti-CD3 and mitomycin-treated APCs. Teff proliferation was determined by flow cytometry measurement of CFSE dilution and was compared with Teff proliferation in the absence of Treg. The percentage of suppression was calculated as described in Materials and Methods. Results are expressed as mean ± SEM for each group.
At 24 and 31 wk of age, the total leukocyte count in lymph nodes was markedly decreased in TNF-K− or infliximab-treated mice (infliximab 31 wk, 16.3 × 10^5 ± 2.5 × 10^4/1; TNF-K 31 wk, 17.2 × 10^5 ± 2.5 × 10^4/1; untreated 31 wk, 67.2 × 10^5 ± 8.9 × 10^4/1; (1)p < 0.05 versus untreated 31 wk). This decrease in total cell number leads to a diminished lymph node Treg count (infliximab 31 wk, 5.3 × 10^5 ± 0.5 × 10^5/1; TNF-K 31 wk, 5.4 × 10^5 ± 0.5 × 10^5/1; untreated 31 wk, 12.5 × 10^5 ± 1.2 × 10^5/1; (1)p < 0.05 versus untreated 31 wk) and Treg count (infliximab 31 wk, 5.3 × 10^5 ± 0.8 × 10^5/1; TNF-K 31 wk, 5.3 × 10^5 ± 0.5 × 10^5/1; untreated 31 wk, 16.5 × 10^5 ± 1.6 × 10^5/1; (1)p < 0.05 versus untreated 31 wk) in these treated mice at 31 wk. However, the percentages of lymph node Treg and Treg were higher in the TNF-K− and infliximab-treated mice than in the untreated mice (Fig. 5A, 5B), and these differences were more pronounced at 31 wk than at 24 wk. We determined whether the increases in both Treg and Treg percentages in lymph nodes seen in the TNF-K− or infliximab-treated mice modified the Treg/Teff ratio. The most interesting finding was that both of the anti–hTNF-α treatments increased the Treg/Teff ratio at 24 wk compared with untreated TTg mice (Fig. 5C). However, a decreased Treg/Teff ratio was observed at 31 wk in anti–TNF-α-treated mice, but percentages of Treg increase in those mice (Fig. 5B) and percentages of Treg dramatically increase in anti–TNF-α-treated mice at 31 wk (Fig. 5A). Collectively, these data suggest that the decreased Treg/Teff ratio is due to the more pronounced Treg percentage increase and not to a decreased Treg frequency. Overall, results in the spleen were similar to those in the lymph nodes for both Treg and Teff populations (Table IV).

**hTNF-α blockade modifies Treg phenotype and enhances Treg suppressive activity**

Because the improvement in arthritis produced by hTNF-α blockade was accompanied by an increase in Treg percentages, we investigated whether hTNF-α blockade also modified the Treg phenotype. As shown in Fig. 6A, TNFR2 expression (percentage and MFI) at 24 wk was higher in mice treated with infliximab or TNF-K compared with untreated mice. CTLA-4 expression (percentage and MFI) on Treg from the lymph nodes and spleen was also higher in 24- and 31-wk-old mice after both anti–hTNF-α treatments compared with untreated mice (Fig. 6B, Table V). Interestingly, the percentage of CD62L− cells within the Treg population was significantly increased in 31-wk-old mice treated with infliximab or TNF-K (Fig. 6C). Moreover, CD62Llow Treg frequency among CD62L+ Treg increased in infliximab and TNF-K−treated mice at 31 wk in the lymph node (Fig. 6D). Taken together, our results show that hTNF-α blockade by passive (infliximab) or active (TNF-K) immunotherapy induced CTLA-4 upregulation on Tregs and, most importantly, expansion of CD62L− Treg.

Because the improvement in established arthritis by hTNF-α blockade was accompanied by modifications in the Treg phenotype and an increase in the Treg/Teff ratio in 24-wk-old mice, we...
characterized the biological suppressive activity of Treg on Teff after hTNF-α blockade. We found that Treg from 24-wk-old mice treated with infliximab or TNF-K induced greater suppression of Teff proliferation than did Treg from untreated mice (Fig. 7, B, C), whereas no difference was seen for suppression of IFN-γ secretion (data not shown).

Table IV. Effect of hTNF-α blockade on splenic Teff and Treg frequencies

<table>
<thead>
<tr>
<th>Group</th>
<th>% Teff</th>
<th>% Treg</th>
<th>% Treg/% Teff Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>15.07 ± 1.05(1)</td>
<td>1.83 ± 0.15(1)</td>
<td>0.122 ± 0.006</td>
</tr>
<tr>
<td>Untreated</td>
<td>15.99 ± 0.55</td>
<td>1.41 ± 0.14(1)</td>
<td>0.091 ± 0.009</td>
</tr>
<tr>
<td>Infliximab</td>
<td>19.84 ± 1.27(2)</td>
<td>2.41 ± 0.13</td>
<td>0.124 ± 0.012</td>
</tr>
<tr>
<td>TNF-K</td>
<td>18.64 ± 1.26</td>
<td>2.02 ± 0.10(1)(2)</td>
<td>0.111 ± 0.006(2)</td>
</tr>
<tr>
<td>31 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>14.37 ± 1.15</td>
<td>1.56 ± 0.19(3)</td>
<td>0.110 ± 0.0007(3)</td>
</tr>
<tr>
<td>Untreated</td>
<td>16.43 ± 0.68</td>
<td>2.09 ± 0.06</td>
<td>0.129 ± 0.004</td>
</tr>
<tr>
<td>Infliximab</td>
<td>16.92 ± 1.07</td>
<td>1.68 ± 0.20</td>
<td>0.118 ± 0.009</td>
</tr>
<tr>
<td>TNF-K</td>
<td>17.80 ± 0.95</td>
<td>1.80 ± 0.10(3)</td>
<td>0.107 ± 0.005(3)</td>
</tr>
</tbody>
</table>

Splenic mononuclear cells from the mice used in Fig. 1 were stained with fluorochrome-conjugated anti-CD4, anti-CD25, and anti-Foxp3. Splenic Teff (CD4+Foxp3− cells) and Treg (CD4+CD25+Foxp3+ cells) were monitored using flow cytometry. Percentages of Teff and Treg among splenocytes and the % Treg/% Teff ratio are given as means ± SEM.

(1) p < 0.05 versus infliximab 24 wk TTg, (2) p < 0.05 versus 24 wk TTg, (3) p < 0.05 versus untreated 31 wk TTg.

FIGURE 6. Effect of hTNF-α blockade on Treg phenotype in lymph nodes. Lymph node leukocytes from the mice used in Table I were labeled with fluorochrome-conjugated anti-CD25, anti-CD4, anti-Foxp3, and anti-TNF-R2 or anti–CTLA-4 or anti-CD62L. Then, TNFR2, CTLA-4, and CD62L expression was studied by gated CD4+CD25+Foxp3+ cells using flow cytometry. A, Dot plots are shown for one representative mouse in each group and the numbers are the percentages of Treg expressing TNFR2 and the MFI of TNFR2 on TNFR2+ Treg, both given as means ± SEM for each group. B, Dot plots are shown for one representative mouse in each group and the numbers are the percentages of Teff expressing CTLA-4 and MFI of CTLA-4 on CTLA-4+ Treg, both given as means ± SEM for each group. A histogram from a representative mouse in each group is shown; the shaded histogram represents the untreated 31-wk-old group, the gray line the TNF-K–treated 31-wk-old group, and the black dotted line the infliximab-treated 31-wk-old group. C, Dot plots are shown for one representative mouse in each group and the numbers indicate the percentage of Treg expressing CD62L and the MFI of CD62L on CD62L+ Treg, both given as means ± SEM for each group. A histogram from a representative mouse in each group is shown; the shaded histogram represents the untreated 31-wk-old group, the gray line the TNF-K–treated 31-wk-old group, and the black dotted line the infliximab-treated 31-wk-old group. (1) p < 0.05 versus untreated 24 wk, (2) p < 0.05 versus infliximab 24 wk, (3) p < 0.05 versus untreated 31 wk. D, Frequency of Treg CD4+CD25+ Foxp3+CD62Llow among CD4+CD25+Foxp3+CD62L+ cells. Data are expressed as mean ± SEM for each group.
**Table V. Effect of hTNF-α blockade on splenic Treg phenotype**

<table>
<thead>
<tr>
<th>Group</th>
<th>% TNFR2 (MFI)</th>
<th>% CTLA-4 (MFI)</th>
<th>% CD62L (MFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>74.84 ± 2.90</td>
<td>30.42 ± 1.17</td>
<td>82.08 ± 1.93</td>
</tr>
<tr>
<td>Infliximab</td>
<td>81.25 ± 1.21(2)</td>
<td>33.58 ± 0.96(2)</td>
<td>97.05 ± 0.46(2)</td>
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<tr>
<td>TNF-K</td>
<td>66.97 ± 2.99(1)</td>
<td>29.47 ± 0.92(1)</td>
<td>89 ± 2.84(1)(2)</td>
</tr>
<tr>
<td>31 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>80.78 ± 1.80</td>
<td>35.96 ± 1.22</td>
<td>92.65 ± 0.74</td>
</tr>
<tr>
<td>Infliximab</td>
<td>86.05 ± 1.12</td>
<td>42.35 ± 1.76(3)</td>
<td>95.30 ± 0.90(3)</td>
</tr>
<tr>
<td>TNF-K</td>
<td>82.91 ± 0.75</td>
<td>40.31 ± 1.58(3)</td>
<td>94.94 ± 0.36(3)</td>
</tr>
</tbody>
</table>

TNFR2, CTLA-4, and CD62L expression by gated CD4+CD25+Foxp3+ from the spleen was studied using flow cytometry. The mice are the same as in Fig. 1. Percentage of Treg expressing TNFR2, TNFR2 MFI among TNFR2+ Treg, percentage of Treg expressing CTLA-4, CTLA-4 MFI among CTLA-4+ Treg, percentage of Treg expressing CD62L, and CD62L MFI among CD62L+ Treg are given as means ± SEM.

(1) p < 0.05 versus infliximab 24 wk TTg, (2) p < 0.05 versus untreated 24 wk TTg, (3) p < 0.05 versus untreated 31 wk TTg.

**hTNF-α blockade influences Th1 cells but not Th17 cells**

RA is characterized not only by a Treg deficiency but also by an imbalance between proinflammatory Th1 and Th17 cells (25). To determine whether hTNF-α blockade acted via a mechanism involving Th1 or Th17 cells, we monitored the proportions of these cells in the lymph nodes and spleen. No modification was observed for Th17 (Fig. 8D), but we found a decrease in the percentage of Th1 cells among CD4+ cells from lymph nodes at 31 wk of age in TNF-K−treated (nonsignificant) and infliximab-treated mice compared with untreated mice (Fig. 8C). To further investigate the involvement of Th1 cells in the effects of TNF-α antagonist therapy in TTg mice, we also assessed INF-γ production. Cultured Teff cells from TNF-K− or infliximab-treated 24-wk-old mice produced less IFN-γ than did those from untreated mice (Fig. 8E).

**Discussion**

A link exists between TNF-α and Treg, but its nature remains controversial and has been chiefly studied in vitro. In the current study, we used hTNF-α transgenic mice to characterize the Treg/...
TNF-α link in vivo. TTg mice constitute a relevant model of severe RA and, moreover, a suitable system for studying the effects of human TNF-α antagonists including infliximab and new agents such as active immunotherapy to hTNF-α (4, 6) that are being tested in clinical trials (NCT00808262 and NCT01040715). A major finding from our study is that in vivo in animals exhibiting a proinflammatory state characterized by hTNF-α overexpression, Treg frequency is initially decreased compared with wild-type mice. Then, chronic inflammation development is accompanied by an increased frequency and a phenotype modification of Treg. In TTg mice, hTNF-α blockade by the conventional TNF-α antagonist infliximab or by anti–hTNF-α immunization improved the clinical manifestations of arthritis and induced modification in the amount and activation phenotype of Treg and, more importantly, enhanced their biological suppressive activity on Teff proliferation.

Overexpression of hTNF-α in TTg mice creates a proinflammatory environment that leads mainly to severe joint inflammation and destruction. We found that in TTg mice, although Treg percentages were consistently lower than in wild-type mice, they increased slightly over time. In keeping with this finding, administration of TNF-α to young adult NOD mice increased the number of CD4+CD25+ Treg in the spleen and thymus (26). In the RA synovium, most studies showed increased Treg counts. However, Treg counts in peripheral blood varied across studies (14, 27–30). These discrepancies can probably be ascribed to differences in RA patient selection and in the definition of Tregs, which hinder comparisons of results across studies. Interestingly, in patients with RA, a smaller percentage of Treg in peripheral blood was found compared with healthy controls only in a patient subgroup with early active RA (31).

Previous studies produced conflicting results regarding the effect of TNF-α on Treg phenotype and function in vitro and in vivo. In RA, TNF-α had no direct effect on Treg in one study (14), but in another study TNF-α inhibited the Treg suppressive effect via a TNFR2-dependent mechanism leading to downregulation of Foxp3 expression (19). Furthermore, in mice, TNF-α interaction with TNFR2 promoted Treg expansion and enhanced Treg function (20). Besides that, Treg are able to shed TNFR2, resulting in TNF-α inhibition (32). More recently, TNFR2+ Treg from human peripheral blood were found to exhibit a stronger suppressive effect than TNFR2− Treg, suggesting that CD25 and TNFR2 co-expression might identify a Treg population characterized by greater potency compared with the CD4+CD25high Treg population (21). In our study, arthritis development was accompanied by progressive and marked TNFR2 upregulation on Treg. We can hypothesize that this increased expression leads to TNFR2 shedding by Treg (32), leading to TNF-α neutralization, but insufficiently to inhibit inflammation. Nevertheless, Treg suppressive activity showed no change during the course of arthritis in TTg mice. Taken together, these data indicate that hTNF-α overexpression does not diminish the suppressive effect of Treg.

This study confirms our previous work showing that immunization against TNF-α is effective in treating established chronic inflammatory disease in TTg mice (4, 6), and hTNF-α blockade with infliximab or anti–TNF-α immunotherapy increased the frequency of Treg. In agreement with this finding, a study in RA patients showed that the percentage of CD4+CD25+ T cells in...
peripheral blood was higher in responders to anti–TNF-α therapy than in patients with active RA (14). Moreover, the proportion of CD4+Foxp3+ cells among PBMC was increased in patients treated with infliximab (18). Regulation of the Treg/Teff balance is crucial to the control of immunity, and in our study hTNF-α blockade increased the Treg/Teff ratio in 24-wk-old mice. Furthermore, hTNF-α blockade had little effect on Th17 cells, whereas another study showed expansion of pathogenic Th17 cells in the lymph nodes and inhibited accumulation of these cells in the synovium (33). We found smaller proportions of Th1 cells among CD4+ T cells, suggesting that Treg might not have been the only T cell subpopulation involved in the effect of TNF-α blockade. Importantly, in addition to Foxp3 Treg, other types of Treg can be induced from naive CD4+ T cells in the periphery, so that TGF-β and IL-10–producing Tr1 cells and TGF-β–producing Th3 cells. These various Treg types probably cooperate to regulate the immune response.

It is now well established that two distinct populations of Treg coexist, that is, natural Treg (CD44lowCD62LhighCD103+ T cells) and induced from naive CD4+ T cells in the periphery, so that TGF-β and IL-10–producing Tr1 cells and TGF-β–producing Th3 cells. These various Treg types probably cooperate to regulate the immune response.

**References**


Disclosures

The authors have no financial conflicts of interest.

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Supplemental material

Figure S1. Evaluation of anti-hTNF-α antibody production in TTg mice immunized with TNF-K

TTg mice (n=20) were immunized with TNF-K at the age of 15, 16, or 19 weeks. Mice are the same as in Table I. (A) Serum anti-hTNF-α antibody titers. (B) The neutralizing capacity (NC) of the anti-hTNF-α antibody was evaluated on L929 cells. The NC is expressed as the mean of the reciprocal of the serum dilution that neutralized 50% of hTNF-α activity. Each point represents an individual mouse. Values are means±SEM. Data represent one experiment representative of 3 similar experiments.

Figure S2. FoxP3 mRNA expression in synovium during the course of arthritis in hTNF-α transgenic mice

Expression of FoxP3 mRNA was evaluated using real-time quantitative reverse-transcription PCR in synovial membrane cells in TTg mice aged of 7 (n=2), 12 (n=5), 17 (n=5), and 24 weeks (n=5). Results are given as mean±SEM.
Fig. S1

Graph A: Anti-hTNF antibody levels (UI/ul) vs. weeks post-immunization. The data shows a significant increase over time. P < 0.001.

Graph B: Reciprocal of serum dilution corresponding to one NC50 (UI/ul) vs. weeks post-immunization. The data indicates a consistent level of dilution. P > 0.30.
Fig. S2