Interaction of TNF with TNF Receptor Type 2 Promotes Expansion and Function of Mouse CD4+CD25+ T Regulatory Cells[1]

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Although TNF is a major proinflammatory cytokine, increasing evidence indicates that TNF also has immunosuppressive feedback effects. We have demonstrated in this study that, in both resting and activated states, mouse peripheral CD4+CD25+ T regulatory cells (Tregs) expressed remarkably higher surface levels of TNFR2 than CD4+CD25− T effector cells (Teffs). In cocultures of Tregs and Teffs, inhibition of proliferation of Teffs by Tregs was initially transiently abrogated by exposure to TNF, but longer exposure to TNF restored suppressive effects. Cytokine production by Teffs remained continually suppressed by Tregs. The profound anergy of Tregs in response to TCR stimulation was overcome by TNF, which expanded the Treg population. Furthermore, in synergy with IL-2, TNF expanded Tregs even more markedly up-regulated expression of CD25 and FoxP3 and phosphorylation of STAT5, and enhanced the suppressive activity of Tregs. Unlike TNF, IL-1β and IL-6 did not up-regulate FoxP3-expressing Tregs. Furthermore, the number of Tregs increased in wild-type mice, but not in TNFR2−/− mice following sublethal cecal ligation and puncture. Depletion of Tregs significantly decreased mortality following cecal ligation and puncture. Thus, the stimulatory effect of TNF on Tregs resembles the reported costimulatory effects of TNF on Teffs, but is even more pronounced because of the higher expression of TNFR2 by Tregs. Moreover, our study suggests that the slower response of Tregs than Teffs to TNF results in delayed immunosuppressive feedback effects. *The Journal of Immunology*, 2007, 179: 154–161.

T he pleiotropic cytokine TNF is a major participant in the initiation and orchestration of inflammation and immunity (1). TNF has been shown to have proinflammatory effects; nevertheless, increasing evidence reveals TNF to also have unexpected immunosuppressive effects. For example, TNF has been considered as a major proinflammatory mediator of sepsis (1). This cytokine is markedly elevated in human infectious diseases (2) and is reported to be required for survival following cecal ligation and puncture (CLP)[3] (3), which represents a mouse model of polymicrobial peritonitis simulating human sepsis (4). However, both TNF- and TNFR-deficient mice fail to develop the usual sepsis-induced immunosuppression after CLP (5). In addition in chronic inflammatory states TNF-deficient mice also exhibit more

severe inflammation (6–8). We have therefore tested the hypothesis that the suppressive effects of TNF on the inflammatory response may involve CD4+CD25+ T regulatory cells (Tregs).

Tregs actively restrain the inflammatory response, suppress development of autoimmune diseases and dampen a wide spectrum of immune responses (9, 10). Tregs express TNFR2 (11), suggesting that TNF may either overcome or promote the activity of Tregs directly. Indeed, it has been reported that neutralization of TNF restored Treg function and reduced the spontaneous apoptosis of Tregs in rheumatoid arthritis patients (12, 13). In neonatal NOD mice, administration of TNF also promoted the development of diabetes accompanied with reduced number and impaired function of Tregs (14). Paradoxically, injection of young adult NOD mice with TNF suppressed the development of diabetes and expanded Tregs (14). In one report TNF failed to have any effect on in vitro cocultures of human Tregs and CD4+CD25− T effector cells (Teffs) (12), whereas in another study TNF blocked inhibition of Teffs by Tregs (15). These studies suggest TNF may play an important role in either the down- or up-regulation of Treg activity, but the precise effect of TNF on Tregs remains elusive.

Activation of TNFR2 has direct costimulatory effects on TCR-mediated mouse T cell responses and consequently reduces the activation threshold of Ag-driven CD4 T cell responses (16). We found initially that the signal mediated by TNFR2 effectively stimulated Teffs when cocultured with Tregs, which may enable TNF-stimulated Teffs to escape from Treg-mediated inhibition and consequently mount a TNF-dependent inflammatory response. Indeed, it was recently reported that TNF also overcomes the immunosuppressive effect of mesenchymal stem cells on T cell proliferation (17). Furthermore, anti-TNF is a well known therapeutic for a number of inflammatory autoimmune conditions. Thus, liberating Teffs from Treg-mediated inhibition may account for proinflammatory effects of TNF. However, there are paradoxical reports

[3] Abbreviations used in this paper: CLP, cecal ligation and puncture; Teff, CD4+CD25− T effector cell; Treg, CD4+CD25+ T regulatory cell.

that prolonged exposure to TNF actually is protective in some autoimmune states (7, 8, 18), suggesting that duration and dose of TNF may modify its effects on Teffs and Tregs. Therefore we investigated the effects of different times and doses of TNF exposure on cocultures of Tregs and Teffs, and the response of Tregs in TNFR2-deficient mice to a septic challenge to determine whether and when TNF stimulated Tregs to reassert their suppressive capacity.

Materials and Methods

Mice and reagents

Female C57BL/6 (CD45.2) and C57BL/6 Ly5.2 congenic mice (CD45.1) and BALB/c mice, 8- to 12-wk-old, were provided by Animal Production Area of the National Cancer Institute (NCI, Frederick, MD). NCI is accredited by the American Association for the Accreditation of Laboratory Animal Care International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the Guide for Care and Use of Laboratory Animals (National Research Council, National Academy Press). Mice deficient in TNFR2 (19) were kept and bred in the animal facility of the University of Regensburg (Regensburg, Germany). Abs purchased from BD Pharmingen consisted of FITC anti-CD4 (GK1.5), PE anti-CD25 (PC61), PE anti-phospho-STAT5, purified anti-CD3 (145-2C11), purified anti-CD16/CD32 (2.4G2), and anti-CD28 (35.71). PE anti-mouse/rat FoxP3 staining set (FJK-16s) was purchased from eBioscience. PE anti-TNFR1 and PE anti-TNFR2 were obtained from Serotec. Purified anti-CD25 (PC61) was a gift of W. Falk (University of Regensburg, Regensburg, Germany). Recombinant mouse cytokines (IL-2, TNF, IL-1β, and IL-6) were purchased from PeproTech.

Purification of cells

CD4$^+$ cells were purified with mouse CD4 (L3T4) microbeads and LS column (Miltenyi Biotec). CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ cells were purified from lymph node (inguinal, axillary, and mesenteric regions) cells using Cytomation MoFlo cytometer, yielding a purity of ~98% for both subsets. T-depleted spleen cells were used as APCs and were prepared by depletion of CD90$^+$ cells with anti-mouse CD90 MicroBead and LD column (Miltenyi Biotec). APCs were irradiated with 3000 rad.

Flow cytometry

After treatment with anti-CD16/CD32 Ab, cells were incubated with appropriately diluted Abs. For CFSE labeling assay, cells were labeled with 2 μM CFSE for 8 min at room temperature. FACS analysis was performed on a FACScan (BD Biosciences) using CellQuest software.

In vitro cell culture and proliferation assay

CD4$^+$CD25$^-$ T cells (5 × 10^4 cells/well) were seeded in a U-bottom 96-well plate in the medium with 2 × 10^5 cells/well of APCs plus 0.5 μg/ml soluble anti-CD3 Ab. CD4$^+$CD25$^+$ T cells were added to the wells at desired ratio to CD4$^+$CD25$^-$ cells. Cells were pulsed with 1 μCi [^{3}H]thymidine (Amersham Biosciences) per well for the last 6 h of culture period. In some settings, cells were stimulated with 2 μg/ml soluble anti-CD3 Ab (without APCs). In a parallel experiment, the supernatants were collected and cytokine measurement was performed by analysis of supernatants with SearchLight Mouse Cytokine Array (Pierce). In some experiments, IL-2 (10 ng/ml) was supplemented to the medium, with or without TNF (10 ng/ml). In some experiments, CD4$^+$CD25$^-$ T cells from C57BL/6 Ly5.2 (CD45.1) mice were labeled with CFSE and then cocultured with CD4$^+$CD25$^+$ T cells from C57BL/6 (CD45.2) mice. The cocultures were stimulated with APC from C57BL/6 (CD45.2) mice and 0.5 μg/ml soluble anti-CD3 Ab. The proliferation of CD4$^+$CD25$^-$ T cells was assessed by CFSE dilution using flow cytometry, gating on CD45.1$^+$ cells. In some experiments, CFSE-labeled Tregs or CFSE-labeled Teffs were cultured alone or CFSE-labeled Teffs were cocultured with unlabeled Tregs, or vice versa unlabeled Teffs were cocultured with CFSE-labeled Tregs at a "physiological" ratio (ratio of Teff to Treg was 10:1) with IL-2 or IL-2 with TNF. After incubation for 72 h, the proliferation of CFSE-labeled cells was analyzed by flow cytometry. In some experiments, purified Tregs were cultured with medium alone or with TNF (10 ng/ml) for 16 h. The resultant intracellular expression of FoxP3 was analyzed by FACS. In some settings, purified Tregs and Teffs were cultured with IL-2 (10 ng/ml) or IL-2 with TNF (10 ng/ml, each) for 24–72 h, after washing, the intracellular expression of FoxP3 was analyzed by FACS, and the immunosuppressive activity of cultured Tregs was determined by coculturing
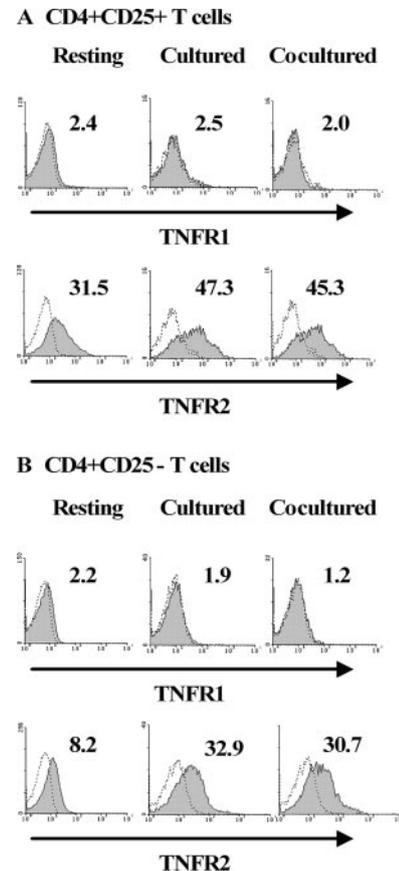

FIGURE 1. Surface expression of TNFRs on Teffs and Tregs. CD45.1$^+$ Tregs (5 × 10^4 cells/well) (*A*) and CD45.2$^+$ Teffs (5 × 10^4 cells/well) (*B*) were cultured alone (cultured) or cocultured together (cocultured) at a ratio for Teff to Treg of 2:1. The cells were stimulated with APC and anti-CD3. After 48 h, the surface expression of TNFR1 and TNFR2 was analyzed by gating on CD4$^+$ and CD45.1$^+$ (for Tregs) or CD4$^+$ and CD45.2$^+$ (for Teffs) population. Freshly isolated cells (resting) were used for comparison. Values shown are the percentage of TNFR-positive cells. Data shown are representative of two separate experiments.

with freshly isolated Teffs, either using the [^{3}H]thymidine incorporation assay or CFSE-labeling assay.

Sublethal CLP

C57BL/6 mice were anesthetized by i.p. injection of 75 mg/kg Ketanest (Parke-Davis) and 16 mg/kg Rompun (Bayer). The cecum was exteriorized and the distal end (<30%) was ligated and punctured once with a needle (0.4 mm diameter) to achieve a sublethal CLP as previously described (2). For Treg depletion mice received 200 μg of anti-CD25 (PC61) or rat IgG, respectively, in 500 μl of PBS 3 days before CLP. Splenocytes were isolated on day 1 or 2 after CLP.

Statistical analysis

Comparisons of data were analyzed by two-tailed Student's *t* test using GraphPad Prism 4.0 or using the Mann-Whitney *U* test. The log-rank test was used to compare Kaplan-Meier survival curves.

Results

Expression of TNFRs on resting and activated Tregs and Teffs

TNF mediates its biological functions through its receptors TNFR1 and TNFR2, the latter which is largely confined to cells of the immune system (20). Unlike TNFR1, which contains a death domain in its cytoplasmic tail, the primary function of TNFR2 is to promote cellular proliferation and survival (1). We were able to confirm the data of Kim et al. (21) showing that mouse splenic
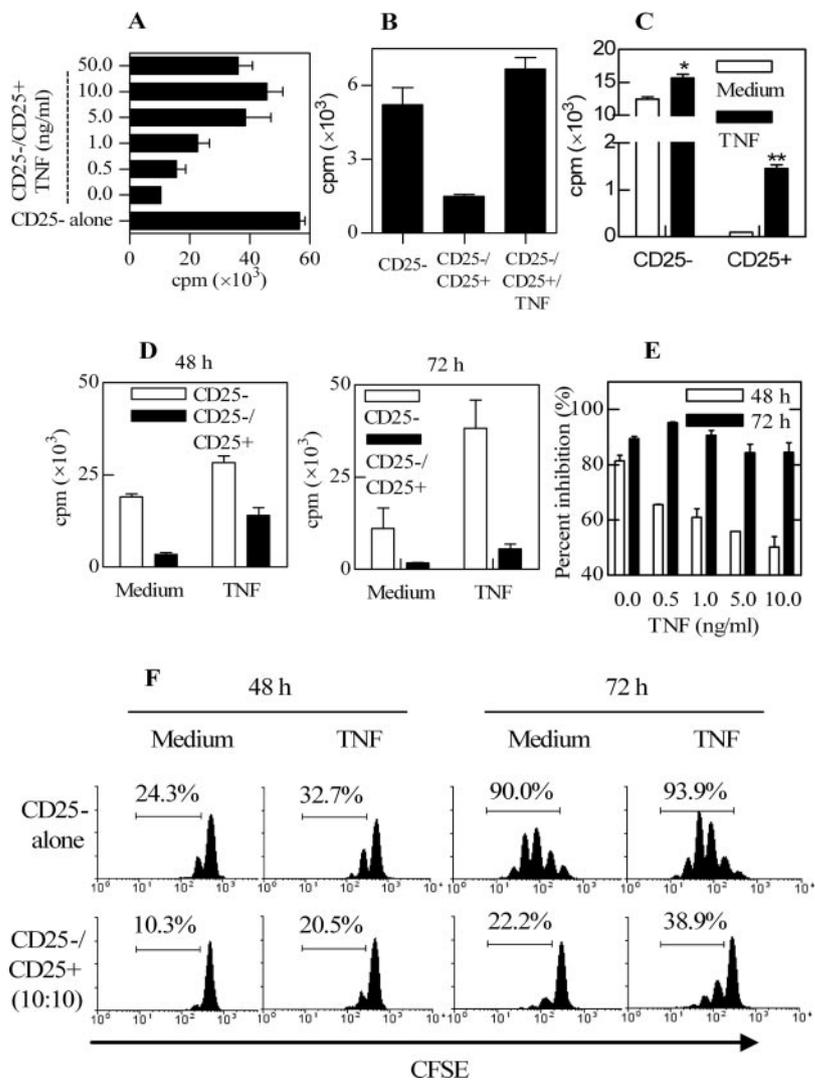
FIGURE 2. Effect of exogenous TNF on the lymphocyte proliferation in cocultures. *A*, Teffs (5×10^4 cells/well) cultured alone or cocultured with Tregs at a ratio for Teff to Treg of 2:1 were stimulated with APC and anti-CD3 in the presence of increasing levels of TNF. *B*, Cocultures of Teffs (5×10^4 cells/well) and Tregs (2.5×10^4 cells/well) were stimulated with soluble anti-CD3 alone in the presence or absence of 10 ng/ml TNF. *C*, Teffs or Tregs (5×10^4 cells/well) were cultured alone and stimulated with APC and anti-CD3 with or without 10 ng/ml TNF. The proliferative response was determined after 48 h culture (*A–C*). *D*, Cells were cultured and stimulated as in *A* for 48 or 72 h in the presence or absence of 10 ng/ml TNF. *E*, Cells were cultured and stimulated as in *A* for 48 or 72 h in the presence of an increasing concentration of TNF. Data shown represent the percentage of inhibition. *F*, FACS-sorted Teffs from C57BL/6 Ly5.2 (CD45.1) mice were labeled with CFSE and were cultured alone or cocultured with Tregs from C57BL/6 (CD45.2) mice in the presence or absence of 10 ng/ml TNF. The cells were stimulated with CD45.2$^+$ APC and anti-CD3. After incubation for 48 and 72 h, proliferation by Teffs was analyzed by gating on CD45.1$^+$ cells. Percentages show the proportion of replicating Teffs. Results represent at least three separate experiments with similar results.

CD4$^+$ as well as CD8$^+$ T cell subpopulations express moderately high levels of TNFR2. The expression of TNFR2 was further increased by T cell activation (data not shown). CD4$^+$ splenocytes from TNFR2-deficient mice were less able than splenocytes from wild-type mice to proliferate in response to anti-CD3 and anti-CD28 (data not shown). It has been reported that human thymic Tregs expressed high levels of surface TNFR2 (11). Consistent with this report, we found considerably higher constitutive expression of surface TNFR2 on mouse peripheral Tregs (32%) than on Teffs (8%) (Fig. 1). Upon TCR stimulation, expression of this receptor on Tregs was further increased to 47% and to 33% on activated Teffs. Furthermore, the activation-dependent elevation of TNFR2 expression by Teffs was not reduced by the presence of Tregs at a ratio of Teffs to Tregs of 2:1. Unlike TNFR2, the surface expression of TNFR1 was barely detectable by FACS on either resting or activated Teffs or Tregs (Fig. 1).

Effect of TNF on cocultures of Teffs and Tregs

Next we evaluated the effect of relative short-term (48 h) exposure of TNF on the outcome of in vitro cocultures of Tregs and Teffs. Exogenous TNF markedly increased cell proliferation in the cocultures (Fig. 2*A*). This effect of TNF was observed over various ratios of Tregs to Teffs (data not shown). To exclude potential interference by APC, cocultures of Teffs and Tregs were stimulated with soluble anti-CD3 alone. TNF also increased the prolif-

eration by these APC-free cocultures (Fig. 2*B*). Thus, the proliferative effect of TNF is due to direct action on CD4 subsets. TNF not only enhanced proliferation of Teffs, but it also overcame the nonresponsiveness of Tregs to TCR stimulation (Fig. 2*C*), presumably by costimulatory signaling through TNFR2, which reduces the threshold of both Teffs and Tregs to TCR-dependent activation. At 48 h, TNF-stimulated proliferation of Teffs and reduced the inhibition of Teffs by Tregs by [^{3}H]thymidine incorporation assay in a dose-dependent manner (Fig. 2, *D* and *E*). In contrast, more prolonged exposure of cocultures to TNF (72 h) restored the inhibition of proliferation of Teffs by Tregs (Fig. 2, *D* and *E*).

Because both Teffs and Tregs incorporated thymidine by 48 and 72 h of incubation in the presence of TNF, we further examined the impact of TNF on the proliferation of cocultured congenic CD45.1$^+$ Teffs by CFSE dilution assay. As shown in Fig. 2*F*, CFSE dilution assay was more delayed compared with the [3H]thymidine incorporation assay because DNA synthesis occurs before cell replication. The CFSE assay showed considerable proliferation by 72 h of incubation because 90% of Teffs have by this time replicated. This growth was reduced to 22.2% by the addition of Tregs. When Teffs alone were cultured with TNF, the proportion of replicating Teffs similarly was 93.9%, but the number of cells resulting from multiple replications by CFSE assay was greater than the number of cells in the absence of TNF. In the presence of Tregs and TNF, replicating Teffs decreased to 38.9%.

FIGURE 3. Effect of exogenous TNF on cytokine production by cocultures. Teffs and Tregs (5×10^4 cells/well) were cultured alone or cocultured at a ratio of 1:1. The cells were stimulated with APC and anti-CD3 in the presence or absence of 10 ng/ml TNF. After incubation for 48 h, IL-4, IFN-γ, and IL-2 in the supernatant were determined by multiplex mouse cytokine measurement. Data shown are representative of at least three separate experiments with similar results.
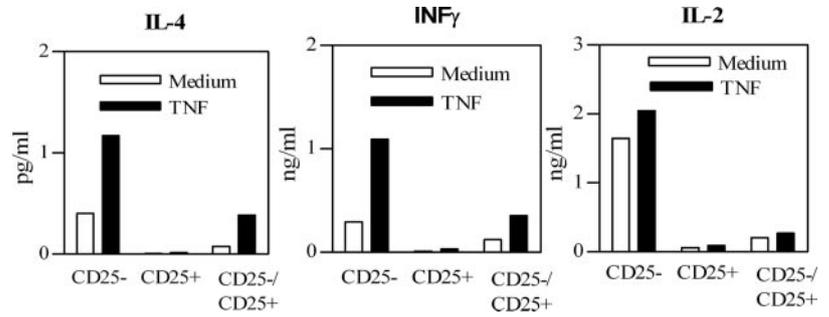
Based on the proportion of replicating Teffs, the percentage of inhibition by Tregs at 48 h was 57.6% and this was reduced to 37.3% in the presence of TNF. At 72 h, the percentage of inhibition by Tregs was 75.3% and 58.6% in the absence and presence of TNF, respectively. The reduction in inhibition by TNF was therefore 35.2% and 20.8% at 48 h and 72 h, respectively. Because TNF stimulated multiple rounds of division by Teffs at 72 h, the precise percentage of inhibition by Tregs in the presence of TNF
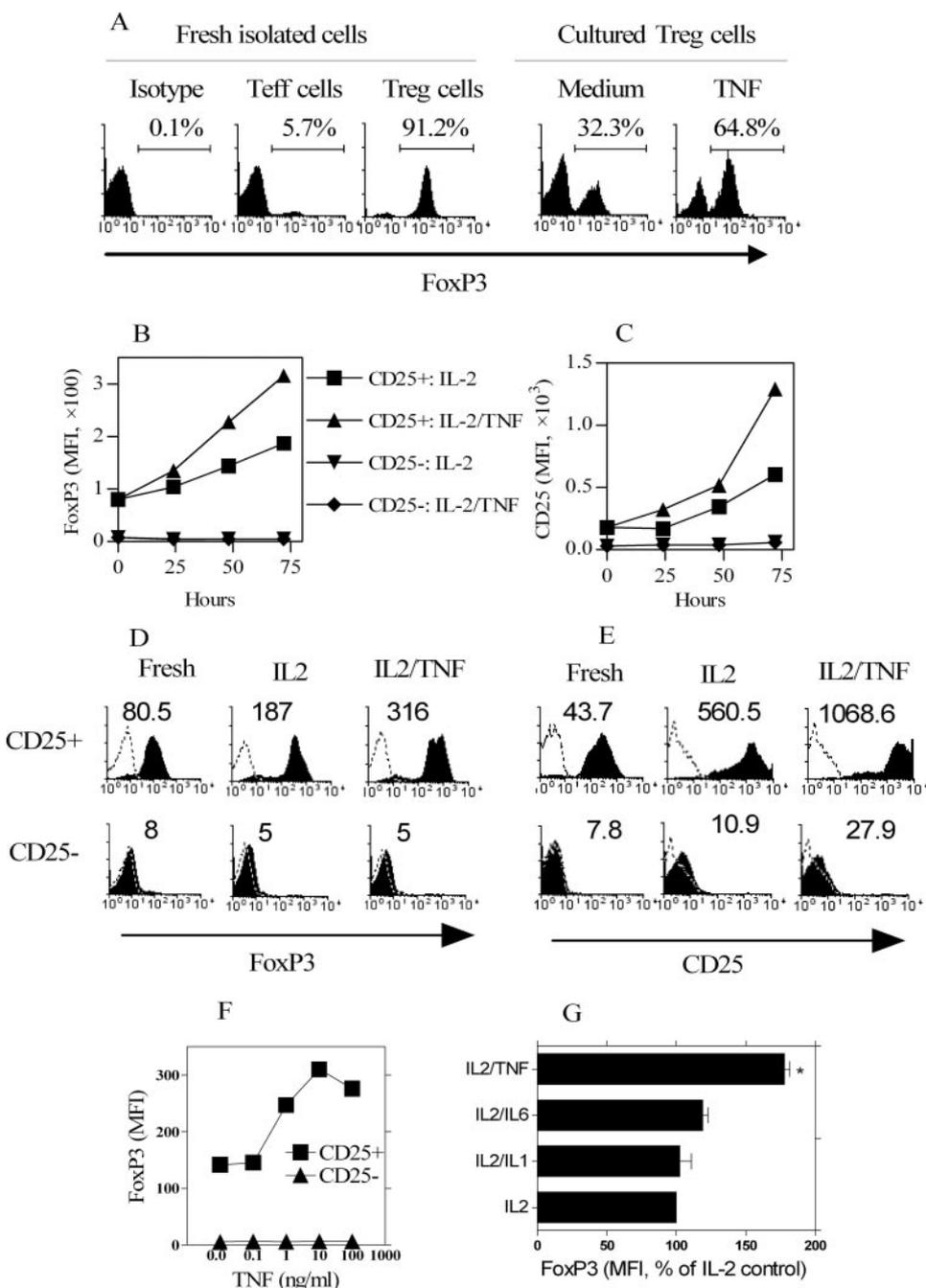

FIGURE 4. TNF, synergizing with IL-2, selectively activates Tregs. *A*, Tregs were cultured with RPMI 10 alone (medium) or 10 ng/ml TNF. At 16 h later cells were harvested, and expression of FoxP3 was analyzed by FACS, compared with freshly isolated cells. *B–E*, Tregs or Teffs were cultured in medium supplemented with 10 ng/ml IL-2 with or without 10 ng/ml TNF for 24, 48, and 72 h. Kinetic expression of FoxP3 (*B*) and CD25 (*C*) by Tregs and Teffs cultured in IL-2 with or without TNF was determined by FACS. FoxP3 (*D*) and CD25 (*E*) expression by 72-h cultured Treg or Teffs, compared with freshly isolated cells, are shown. Isotype control (open histogram) and FoxP3 (*D*) or CD25 (*E*) Ab staining (filled histogram) are shown. The value shown in each histogram is mean fluorescence intensity (MFI). *F*, Dose response of TNF (0.1–100 ng/ml) on FoxP3 expression (MFI) by Tregs and Teffs cultured with medium containing IL-2 (10 ng/ml) for 72 h. Data are representative of at least three separate experiments with similar results. *G*, Effect of proinflammatory cytokines on FoxP3 expression by IL-2 cultured Tregs. IL-1β (10 ng/ml), IL-6, or TNF on FoxP3 expression (MFI) by Tregs cultured with IL-2 for 72 h. Data are mean $\pm$ SEM and shown as a percentage of IL-2 culture alone, summarized from three separate experiments. *, $p < 0.05$ compared with IL-2 culture alone.

at this time point was actually greater than 58.6%, and consequently, the reduction in inhibition by TNF was actually <20.8%. Similar results obtained in three separate experiments. Altogether these results lead us to conclude that the shorter duration of treatment with TNF (48 h) showed partial abrogation of inhibition of Teffs by Tregs, whereas more prolonged exposure to TNF (72 h) restored Treg-mediated inhibition.

The Treg-mediated suppression of cytokine production by Teffs was detected early on. At 48 h of incubation, in the absence of TNF, Tregs produced very low cytokine levels in response to TCR stimulation and markedly inhibited the capacity of Teffs to produce IL-4, IFN-γ, and IL-2 by 81%, 67%, and 87% (Fig. 3). Addition of TNF led to a 3-fold increase in IL-4 and IFN-γ production by purified Teffs and partially restored the production of IL-4 and IFN-γ (but not IL-2) in cocultures. However, in the presence of Tregs plus TNF, the percentage of inhibition of IL-4, IFN-γ, and IL-2 production by Teffs was still as high as 77%, 67%, and 86%, respectively, which is comparable to the inhibitory effect of Tregs in the absence of TNF (Fig. 3). Therefore, short-term exposure to TNF for up to 48 h reversed inhibition of Teffs proliferation by Tregs, but the capacity of Tregs to inhibit cytokine production by Teffs occurred even at the earlier time point.

TNF synergizes with IL-2 in activating Tregs

To determine whether TNF acted directly on Tregs, TNF was added to the purified Tregs cultured without APC or anti-CD3. Both FoxP3 expression and immunosuppressive activity of unstimulated Tregs rapidly decreased after 16 h incubation in medium, but addition of TNF partially prevented this decrease in FoxP3 expression (Fig. 4*A*) and partially maintained the immunosuppressive function of Tregs (data not shown). However, we could not evaluate the effect of longer incubation periods under such condition because TNF was not able to support prolonged survival of Tregs.

It has been amply demonstrated that IL-2 is crucial for survival of in vitro cultured Tregs (22). We therefore used IL-2 to enable us to observe a more prolonged in vitro effect of TNF on Tregs. As predicted, IL-2 alone increased the expression of FoxP3 and CD25 by Tregs with time. This effect of IL-2 was markedly enhanced by the addition of TNF, in a time-dependent (Fig. 4, *B–E*) and dose-dependent manner (Fig. 4*F*). The up-regulation of FoxP3 by TNF was unique because other inflammatory cytokines such as IL-1β and IL-6 did not share this property (Fig. 4*G*). These data indicated that TNF, in combination with IL-2, markedly increased Tregs FoxP3 and CD25 expression. In contrast, Teffs did not show changes in FoxP3 or CD25 expression in response to IL-2/TNF (Fig. 4, *B–D*), suggesting TNF cannot support de novo differentiation of Tregs from the non-Treg population.

We further determined the effect of TNF on the expansion of the physiological level of ~10% Tregs present in purified CD4 cells. After 72 h of culture with IL-2 alone, the proportion of FoxP3^{+} cells increased by 2-fold above the level in freshly isolated CD4 cells. The expression level of FoxP3 (mean fluorescence intensity) was also dramatically enhanced by TNF (Fig. 5*A*). Addition of IL-2 plus TNF increased the proportion of FoxP3-expressing cells by 4-fold. The proportion of FoxP3^{+} cells did not increase further after 5–7 days culture, but remained high at a 30–40% level (data not shown). CFSE-labeling assays indicated that IL-2 induced the proliferation of a small population (~10%) of Tregs. Supplementation of IL-2 with TNF further increased the proportion of proliferating cells by 4-fold over IL-2 alone in cultures of Tregs and in cocultures of Teffs and Tregs at a 10:1 ratio (Fig. 5*B*). In contrast, IL-2 or IL-2 plus TNF failed to stimulate the proliferation of Teffs (Fig. 5*B*).
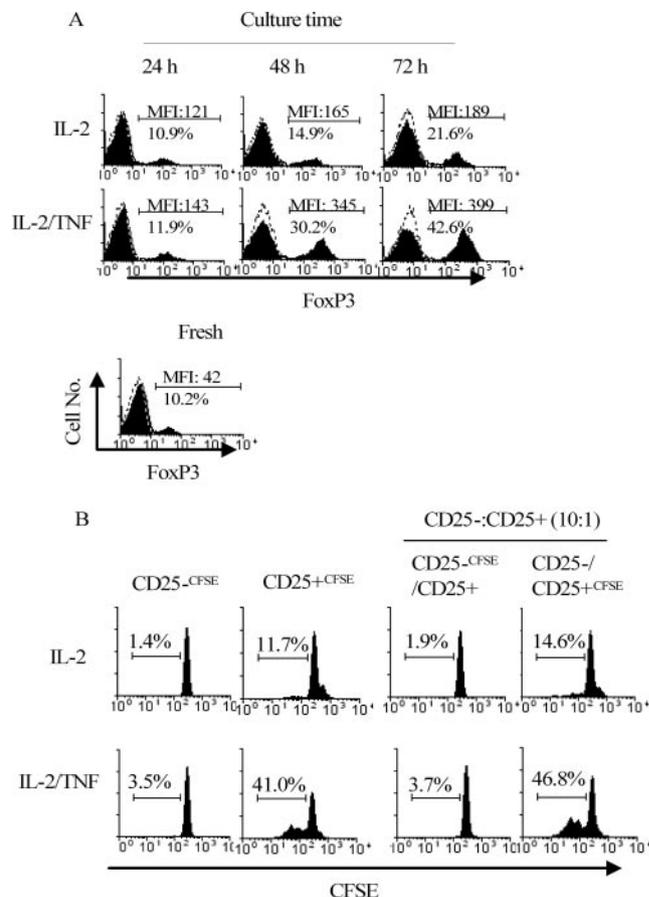

FIGURE 5. In combination with IL-2, TNF preferentially expands Tregs in vitro. *A*, Purified CD4 cells (pooled from lymph nodes and spleen) were cultured with 10 ng/ml IL-2 with or without 10 ng/ml TNF for 24, 48, or 72 h. The expression of FoxP3 was analyzed by FACS. Mean fluorescence intensity (MFI) and the percentage of positive cells are indicated in the histograms. *B*, CFSE-labeled Tregs or CFSE-labeled Teffs were cultured by themselves or CFSE-labeled Teffs were cocultured with unlabeled Tregs, or vice versa unlabeled Teffs were cocultured with CFSE-labeled Tregs at a "physiological" ratio (Teff to Treg at 10:1) with IL-2 or IL-2 plus TNF. After incubation for 72 h, the proliferation of CFSE-labeled cells was analyzed by FACS. The proportion of proliferating cells is indicated in the histogram. The data shown are representative of three separate experiments.

TNF increased IL-2-induced phosphorylation of STAT5 and immunosuppressive activity of Tregs

It was recently reported that up-regulation of FoxP3 expression and expansion of Tregs by IL-2 involved activation of STAT5 (23). We found that IL-2-stimulated phosphorylation of STAT5 in Tregs was further enhanced by TNF (Fig. 6*A*). This finding may be based on TNF up-regulation of CD25 expression, which therefore promotes an IL-2 response by Tregs.

The effect of IL-2 or IL-2 plus TNF on the inhibitory activity of Tregs was examined in cocultures (72 h) with freshly isolated Teffs. Pretreatment with IL-2 significantly enhanced the immunosuppressive activity of Tregs (data not shown). Tregs pretreated with IL-2 plus TNF exhibited greater inhibitory activity than Tregs cultured with IL-2 alone ($p < 0.01$ to 0.05) (Fig. 6*B*). Because Tregs themselves may proliferate in response to 3 days of incubation with IL-2 and TNF, we further examined the inhibitory potential of the washed Tregs by coculturing with CFSE-labeled congenic CD45.1[+] Teffs. As shown in Fig. 6*D*, at ratios for Teff to Treg of 10:1, 10:5, and 10:10, the percentage of replicating Teffs was
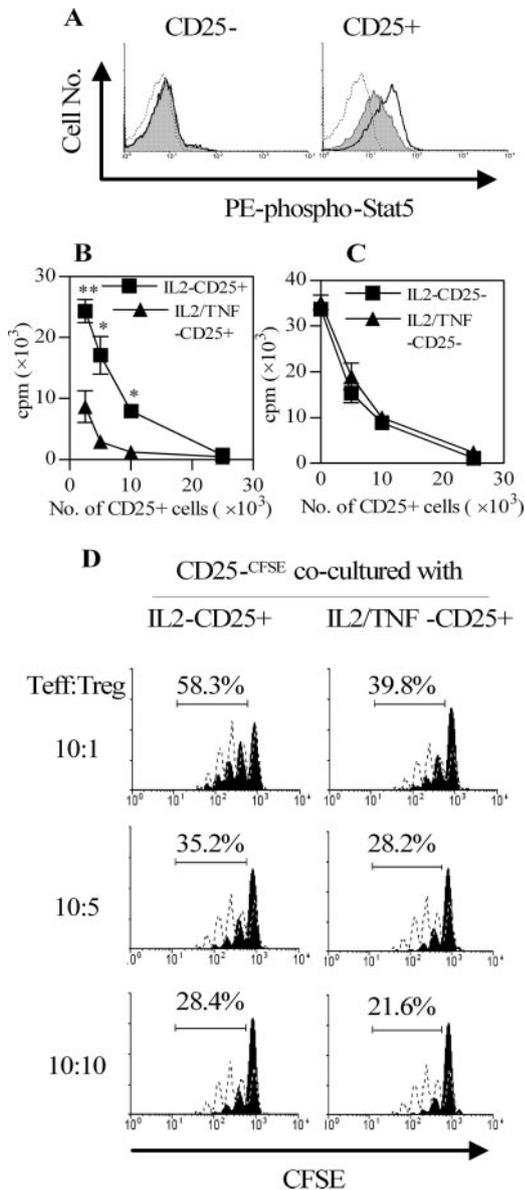
FIGURE 6. TNF increases IL-2-mediated phosphorylation of STAT5 and enhances function of Tregs. Tregs or Teffs were cultured in the medium supplemented with 10 ng/ml IL-2 with or without 10 ng/ml TNF for 72 h. *A*, Intracellular expression of phosphorylated STAT5 was determined by FACS. Isotype control (dotted line histogram), cells cultured with IL-2 (gray-filled histogram), and cells cultured with IL-2 plus TNF (thick line histogram) are shown. *B*, Inhibitory activity of IL-2-cultured (IL2-CD25$^+$) or IL-2 plus TNF-pretreated (IL2/TNF-CD25$^+$) Tregs (2.5×10^3 to 2.5×10^4 cells/well) was determined by coculturing with freshly isolated Teffs (5×10^4 cells/well). The mean value of Teffs alone was 66,280 cpm. *C*, The susceptibility of IL-2 cultured (IL2-CD25$^-$) or IL-2 plus TNF-pretreated (IL2/TNF-CD25$^-$) Teffs (5×10^4 cells/well) was examined by coculture with an increasing number of freshly isolated Tregs (5×10^3 to 2.5×10^4 cells/well). The proliferation was determined after 72 h incubation. Compared with proliferation of IL-2 cultured cells. *, $p < 0.05$; **, $p < 0.01$. *D*, CD45.1$^+$ Teffs were labeled with CFSE and cocultured with IL-2-pretreated or IL-2 plus TNF-pretreated CD45.2$^+$ Treg at a ratio of 10:1, 10:5, and 10:10. The cells were stimulated with CD45.2$^+$ APC and anti-CD3 for 72 h. The proliferation of cocultured Teffs was analyzed by FACS gating on CD45.1$^+$ cells (filled histogram). Proliferation profile of Teffs when they were cultured alone is shown by an open histogram. Percentage shown is of replicating Teffs. Results shown are representative of at least three separate experiments.
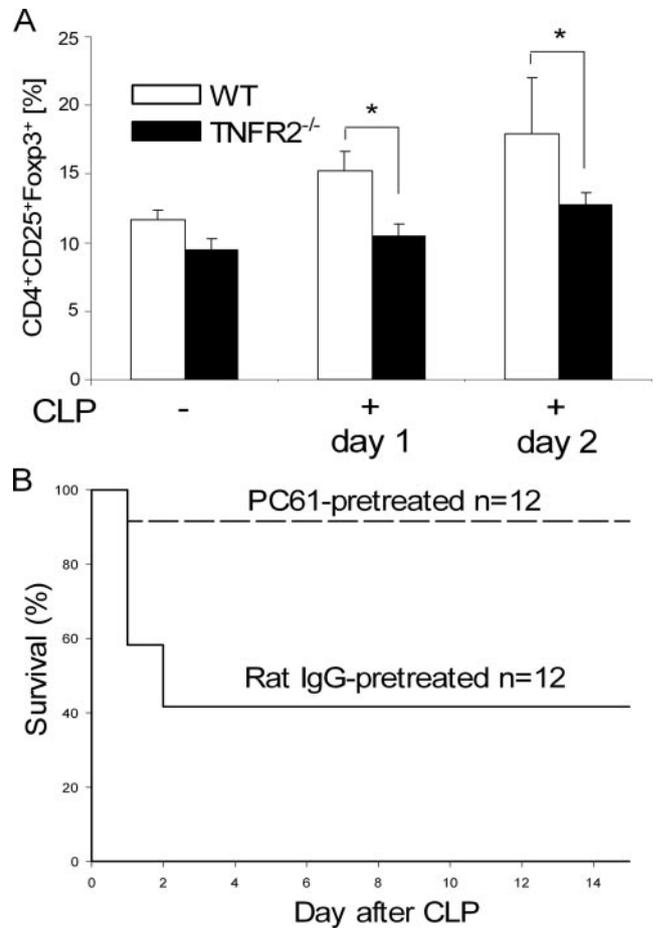

FIGURE 7. In TNFR2$^{-/-}$ mice, the proportion of splenic Tregs failed to increase after CLP. *A*, Wild-type and TNFR2$^{-/-}$ mice were subjected to sublethal CLP ($n = 3$ without CLP, $n = 6$ with CLP for each group). Splenocytes were isolated and the proportion of CD4$^+$CD25$^+$ FoxP3$^+$ Tregs was determined by FACS from mice without CLP or on day 1 or 2 after CLP. *, $p < 0.01$ compared with wild-type mice. Data shown are representative of two separate experiments. *B*, Mice ($n = 12$ per group) were treated with anti-CD25 (PC61) or rat IgG, respectively, 3 days before sublethal CLP operation. Survival was monitored over 15 days and compared. $p = 0.03$. Data shown are representative of three separate experiments.

58.3%, 35.2%, and 28.4%, when cells were cocultured with IL-2-pretreated Tregs, and was reduced to 39.8%, 28.2%, and 21.6%, respectively when Teffs were cocultured with IL-2/TNF-pretreated Tregs. Thus, the CFSE dilution assay was consistent with [^{3}H]thymidine incorporation assay, and showed that pretreatment with IL-2 plus TNF enhanced the suppressive activity of Tregs. The effect of these cytokines on the susceptibility of Teffs to inhibition by Tregs was also examined. As shown in Fig. 6*C*, the inhibitory effect of freshly isolated Tregs on Teffs pretreated with IL-2 or IL-2 plus TNF was similar in potency ($p > 0.05$), indicating that after pretreatment with IL-2/TNF, Teffs were still fully susceptible to Treg-mediated suppression. Thus, TNF in combination with IL-2 selectively activated an increased in number of fully functional Tregs.

TNFR2$^{-/-}$ mice subjected to CLP failed to expand Tregs

To further evaluate the in vivo relevance of our findings, we investigated the role of Tregs in TNFR2-deficient mice. Because the number and function of Tregs was normal in such mice (data not shown), we studied the effect of challenging mice with CLP, which

revealed a rapid elevation in TNF level (3, 4, 24). In septic patients, the percentage of circulating Tregs is markedly increased, which presumably contributed to the postseptic immunosuppression (25, 26). Consistent with those observations, we found that the number of splenic Tregs significantly increased in wild-type mice at 1 and 2 days after CLP. In contrast, Tregs failed to increase in the TNFR2-deficient mice that were subjected to CLP (Fig. 7*A*). Furthermore, treatment of wild-type mice with PC61 Abs directed against CD25 to deplete Tregs resulted in a remarkable reduction in the early lethality following the CLP procedure (Fig. 7*B*). Our data indicate that in vivo expansion of Tregs by interaction of TNF with TNFR2 may play an important role in the induction of postseptic immunosuppression, which results in the failure of bacterial clearance and lethality.

Discussion

It was recently reported that ligands for TLR2, but not for TLR4 or TLR9, in conjunction with TCR stimulation induce proliferation of Tregs in vitro and in vivo (27). This response is associated with a transient loss of suppressive activity by the Tregs, which is subsequently restored in an amplified manner. Another group reported TLR2 activation to result in a transient suppression of FoxP3 mRNA expression by mouse Tregs in association with resistance of Teffs to suppression by Tregs by 8–15 h (28). Similarly, our study suggests that short-term effects of TNF, as seen in the early phases of inflammatory response, may enable Teffs to expand despite the presence of Tregs, whereas more prolonged exposure to TNF may favor expansion and activation of Tregs. These recent results concerning the effect of TLR2 expression and signaling on the expansion and function of Tregs suggest that during acute infection, pathogen-induced TLR2 activation promote IL-2-dependent Treg proliferation in parallel with temporarily abrogating the suppression of the ongoing immune response by Tregs (27, 28). Because activated T cells are a good source of TNF, it is likely that TNF and TNFR2 participate in these TLR2 responses. The initial transient loss of suppressive activity of Tregs presumably favors elimination of pathogens. Tregs subsequently regain their suppressive capabilities and/or Teffs become more susceptible, thus down-regulate inflammation and contributing to the balance between tolerance and immunity.

The capacity of TNF to stimulate Tregs may underlie the temporal role of TNF in the inflammatory response and account for the opposing effects of TNF in inflammation (29) and autoimmune diseases (30). For example, wild-type mice responded promptly with granuloma formation and hepatosplenomegaly to a microbial agent followed by subsequent resolution of inflammation, whereas similarly treated TNF$^{-/-}$ mice showed little or no initial response, but subsequently developed a vigorous, disorganized and lethal inflammatory response (29). In experimental autoimmune encephalomyelitis, TNF$^{-/-}$ mice had a delayed onset of disease; however, once developed, the disease was more severe than in wild-type controls (30). It is possible that in the absence of TNF, Teffs are unable to initiate an effective immune response. However, in the later phase of inflammatory response, deficiency of TNF may result in failure to expand and activate Tregs and cause excessive self-destructive inflammation.

TNF has been implicated in the pathogenesis of sepsis for decades as a major mediator of inflammation (1, 2). It is well established that sepsis deeply perturbs immune homeostasis by inducing an initial tremendous systemic inflammatory response that is rapidly followed by immunosuppression (31) and correlated with a fatal outcome (32). Tregs suppress a broad spectrum of immune responses, including dampening host defense against microbial infection (9, 10). Recently, it has been reported that the percentage

of circulating Tregs in sepsis-induced immunosuppression is markedly increased, which correlates with the progression of sepsis (25, 26). Consistent with these human sepsis studies, we found that the number of Tregs significantly increased in wild-type mice with CLP, but not in TNFR2-deficient mice with CLP. Furthermore, we observed that depletion of Tregs prevented CLP-induced mortality. Our data suggest that elevated levels of TNF, by interacting with TNFR2 predominantly expressed by Tregs, together with the sepsis-induced expansion of Tregs contribute in the postseptic immunosuppression and fatal consequences. In contrast with our observation, a recent report observed that adoptive transfer of in vitro-stimulated Tregs (but not unstimulated Tregs) improved the survival in the mouse CLP model (33). However, as indicated in the study, the protective effect of adoptive transferred Tregs was not attributable to the classic immunosuppressive effects of Tregs because rather than inducing local or systemic immunosuppression, Treg transfer resulted in greater peritoneal cytokine production and mast cells accumulation (33). Their findings may be due to contamination by Teffs because MACS is subefficient purification approach.

Our findings that TNF up-regulated FoxP3 expression and immunosuppressive activity of Tregs contrasts with another study of human Tregs, which reports that TNF down-regulates FoxP3 expression and blocks suppressive function of Tregs (15). This discrepancy is unlikely due to the species difference of Treg response to TNF because we observed that TNF in combination with IL-2 also enhanced FoxP3 expression by human CD4$^+$CD25$^+$ T cells (data not shown). Valencia et al. (15) found that circulating CD4$^+$CD25^{high} Tregs incubated with relatively high concentrations of TNF (50 ng/ml) and IL-2 (100 U/ml) lost immunosuppressive activity, probably reflecting only the early transient immunostimulatory phase of TNF.

Our in vitro and in vivo data are compatible with the observation of Wu and McDevitt (14) that administration of TNF to adult NOD mice resulted in expansion of Tregs in vivo and inhibited diabetes. Thus, TNF not only up-regulated FoxP3 and CD25 expression, but also contributed to expanding the Treg pool in mice subjected to an autoimmune or infectious challenge.

In conclusion, TNFR2 preferentially expressed by Tregs endows Tregs with greater capability to use TNF for cellular activation and expansion. Although TNFR2$^{-/-}$ mice have normal numbers of functional Tregs, these fail to expand when challenged by sepsis. Activated T cells are major sources of TNF as well as IL-2 and these two cytokines synergize in promoting the capacity of Tregs to temper both innate and adaptive immune-mediated inflammatory reactions. It is surprising that of the proinflammatory cytokine TNF, but not IL-1β or IL-6, up-regulate Tregs. Perhaps this is because TNF is the "master regulator" and is the upstream inducer of the other cytokines (34). The stimulatory effect of TNF mediated by TNFR2 on Treg activity resembles the costimulatory effect of TNF on Teffs (16), but is even greater because Tregs express higher levels of TNFR2.

Disclosures

The authors have no financial conflict of interest.

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