Increased Natural CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cells and Their Suppressor Activity Do Not Contribute to Mortality in Murine Polymicrobial Sepsis

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Increased Natural CD4\(^+\)CD25\(^+\) Regulatory T Cells and Their Suppressor Activity Do Not Contribute to Mortality in Murine Polymicrobial Sepsis\(^1\)

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Regulatory T cells (Tregs), including natural CD4\(^+\)CD25\(^+\) Tregs and inducible IL-10 producing T regulatory type 1 (TR1) cells, maintain tolerance and inhibit autoimmunity. Recently, increased percentages of Tregs have been observed in the blood of septic patients, and ex vivo-activated Tregs were shown to prevent polymicrobial sepsis mortality. Whether endogenous Tregs contribute to sepsis outcome remains unclear. Polymicrobial sepsis, induced by cecal ligation and puncture, caused an increased number of splenic Tregs compared with sham-treated mice. Splenic CD4\(^+\)CD25\(^+\) T-cells from septic mice expressed higher levels of Foxp3 mRNA and were more efficient suppressors of CD4\(^+\)CD25\(^-\) T effector cell proliferation. Isolated CD4\(^+\) T-cells from septic mice displayed increased intracellular IL-10 staining following stimulation, indicating that Treg cells may also be elevated in sepsis. Surprisingly, Ab depletion of total CD4\(^+\) or CD4\(^+\)CD25\(^+\) populations did not affect mortality. Furthermore, no difference in survival outcome was found between CD25 or IL-10 null mice and wild-type littermates, indicating that Treg or TR1-generated IL-10 are not required for survival. These results demonstrate that, although sepsis causes a relative increase in Treg number and increases their suppressive function, their presence does not contribute significantly to overall survival in this model. The Journal of Immunology, 2006, 177: 7943–7949.

Despite progress in antibiotic and other supportive therapies over the past 20 years, sepsis remains the leading cause of death in the intensive care unit affecting over 750,000 patients in the United States and causing >200,000 deaths annually (1). Significant advancements in our understanding of sepsis pathophysiology and immune dysfunction have occurred during this time period, predominantly focusing on the roles of inflammation and activation of the innate immune system. Unfortunately, mortality from severe sepsis has only declined modestly (1, 2), and several anti-inflammatory approaches have failed in clinical trials. There is a growing recognition that defects in the acquired immune response may also contribute to this adverse clinical outcome and may represent an appropriate target for therapeutic intervention.

New insights into specific immunomodulatory cells that contribute to immune tolerance, such as T regulatory cells (Tregs),\(^4\) may yield valuable insights into immune perturbations in sepsis syndromes. Despite their initial description over 30 years ago by Gershon and Kondo (3), “suppressor T cell” research was largely abandoned until Sakaguchi and colleagues (4) suggested that a small population of CD4\(^+\)CD25\(^+\) T-cells, referred to as Tregs, was responsible for the prevention of organ-specific autoimmunity. Since their original description, a growing body of studies have suggested that naturally occurring Tregs play a major role in suppressing immune reactivity, ranging from autoimmunity to infectious disease (5) and to injury (6). Other studies have discovered inducible Treg subpopulations possessing immunoregulatory functions, including TGF-\(\beta\)-producing Th3 cells having a role in oral tolerance to ingested Ags (7), and IL-10-producing T regulatory type 1 (T\(_R\)1) cells (8, 9). Many cell surface molecules serve as markers coexpressed on Tregs, including glucocorticoid-induced TNF receptor (GITR) (10) and intracellular CTLA-4 (11, 12), while still other factors contribute to the development and activity of regulatory T-cells, such as the forkhead box transcription factor, Foxp3 (13, 14), and TLRs (15–17), which recognize pathogen associated molecular patterns or can augment Treg function or proliferation.

Monneret et al. (18) first observed that sepsis increases CD4\(^+\)CD25\(^+\) T-cells in the peripheral blood of septic patients. This was subsequently found to be a relative increase in Tregs due to a decrease in the CD4\(^+\)CD25\(^-\) T effector cell populations (19). However, whether these Tregs suppress T effector cell proliferation was not examined. Interestingly, expanding the endogenous population of activated Tregs by adoptive transfer before or following the initiation of polymicrobial sepsis improved outcome by enhancing peritoneal and mast cell TNF-\(\alpha\) production and bacterial clearance (20). At the same time, whether endogenous Tregs modulate outcomes in sepsis is unknown.

In this study, we used a murine cecal ligation and puncture (CLP) model to examine the effects of polymicrobial sepsis on endogenous Treg number, phenotype, and suppressor activity. We...
also ascertained whether Ab-mediated depletion of Treg, genetic deficiency of CD25, or the Tg1-produced cytokine IL-10 would affect outcome to sepsis. Understanding the immunomodulatory effects of endogenous Tregs may lead to novel therapeutic approaches to ameliorate the mortality observed in severe sepsis.

Materials and Methods

Mice

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Florida College of Medicine (Gainesville, FL) before their initiation. Specific pathogen-free 6-wk-old C57BL/6 female mice were purchased from The Jackson Laboratory and were used between 8 and 12 wk of age. Heterozygous CD25-null (B6.129S4-Il2ra−/−) mice backcrossed to a C57BL/6 background, and C57BL/6 IL-10-null mice (B6.IL-10−/−) breeding pairs were also purchased from The Jackson Laboratory and were maintained in a breeding colony at the University of Florida College of Medicine. Homozygous recessive mice (referred to as CD25 null) from the B6.129S4-Il2ra−/− colony, and wild-type littermates were genotyped from tail DNA using primer sequences provided by The Jackson Laboratory. Experiments using the CD25-null and age- and sex-matched littermates were performed at 5 wk of age since the mice readily developed symptoms of inflammatory bowel disease shortly after 6 wk of age. Similarly, IL-10-null mice were used before 6 wk of age as they also developed symptoms of colitis at ~8 wk of age.

Cecal ligation and puncture

For induction of polymicrobial sepsis, mice were subjected to a CLP as described previously (21, 22). In brief, a laparotomy was performed, and the cecum was isolated, ligated below the ileocecal valve, and punctured through and through with a 23-gauge needle. Sham operation was performed by isolating the cecum without ligation or puncture. When indicated, animals were given either an i.p. injection of 500 μg of affinity-purified CD4 (GK1.5 hybridoma) or CD25 (PC61 hybridoma)-depleting Ab (BioExpress) or an identical volume (200–250 μL) of sterile normal saline 72 h before the CLP. Administration of the Ab (250 μg) was repeated 1 h before the procedure. Depletion of cell populations was confirmed by flow cytometry and lasted up to 8 days following the injection. Depending on the experiment, mice were either euthanized at 24 h after surgery to harvest splenocytes, or animals were observed for up to 7 days to determine survival.

Flow cytometry

Spleens were harvested 24 h after CLP or sham-treatment, and processing and flow cytometry was performed as described previously (21). Total cell counts were obtained using a hemacytometer. All Abs were purchased from BD Pharmingen, except the anti-GITR Ab and the anti-Foxp3 staining set, which were purchased from eBioscience. Abs used include anti-CD4 (RM4.4 conjugated to FITC, or GK1.5 conjugated to allophycocyanin), anti-CD25 (3C7 conjugated to PE or PC61 conjugated to allophycocyanin), anti-Foxp3 (FJK-16s) conjugated to FITC or allophycocyanin, and anti-CD25 (90D3) conjugated to PE. The purity of CD3−CD4+CD25high cells (Tregs) was >95% and the purity of CD3−CD4+CD25low cells (T effector cells) was >99%.

Foxp3 real-time RT-PCR

Quantitative real-time RT-PCR was performed as previously described, with some modifications (23). To measure quantities of Foxp3 mRNA, total RNA was extracted from an identical number (2.5 × 10^7) of cells using TriReagent (Molecular Research Center). Samples were treated with DNase (RNase-free DNase; Invitrogen Life Technologies), and total RNA was reverse transcribed into complementary DNA using Superscript II cDNA kit (Invitrogen Life Technologies). Foxp3 mRNA was quantified using quantitative RT-PCR and SYBR Green real-time PCR kit (Applied

FIGURE 1. Sepsis increases the percentage of Tregs. Sepsis was induced by CLP. Twenty-four hours later, splenocytes were stained with fluorescein-labeled Abs. A, Representative examples of CD25-PE vs CD4 APC on flow plots from one septic (top left panel) or one sham-treated (top right panel) spleen gated on 2 × 10^7 nondebris, living (7-AAD negative) CD4+ T cells. The gate represents cells that were considered CD4+CD25+ in the calculations for the bottom panel (n = 3 in each group). These results are consistent with results obtained from four different experiments. B, Representative examples of CD25 PE vs CD69 FITC from one septic (top left panel) or one sham-treated spleen gated on 2 × 10^7 nondebris, living (7-aminoactinomycin D negative) CD4+ T cells. The upper left quadrant represents CD4+CD25+CD69−nonactivated Tregs, whereas the upper right panel represents CD4+CD25+CD69−activated T cells. The average percentage of CD4+CD25+CD69−T cells (n = 3/group) was increased in septic mice (lower panel). C, Representative examples of CD25 APC vs GITR PE from one septic (upper left panel) vs one sham-treated (upper right panel) mice gated on 1 × 10^7 nondebris, live-gated CD4+ T cells. The average proportion (upper panel) of CD4+CD25+GITR−T cells (n = 4/group) was increased in septic mice (lower panel). *, p < 0.05 vs sham treated.
Quantitative RT-PCR for Foxp3 mRNA was performed in triplicate for each sample. Experiments were repeated a total of three times. Shown is a representative example, and error bars represent the difference between triplicate runs for each sample. Differences among groups in flow cytometric analyses were evaluated by Student’s t test. Differences in suppressor activity of septic vs sham-treated Tregs were determined by comparing differences between septic and sham-treated Treg-mediated suppression from separate experiments by paired t test. Significance was set at p = 0.05.

Results

Increased proportion of splenic CD4⁺CD25⁺ T cells expressing Treg markers in murine sepsis

We first examined whether Treg numbers were increased following polymicrobial sepsis. Because no differences in Treg proportion or total number (by any marker used) were found between normal control mice and sham-operated mice at the time intervals examined, we included only the sham vs CLP comparisons in this study. The percentage of CD25-expressing CD4⁺ T cells in the spleens of sham or control mice differed from experiment to experiment but was consistent with the literature as it ranged from 4 to 10% of the total CD4⁺ T cell population. We observed a 2- to 2.5-fold increase in the percentage of CD4⁺CD25⁺ T cells in the spleen 24 h following induction of generalized peritonitis from a CLP (Fig. 1A). Since CD25 is also a marker of activated T cells, we examined a more specific marker of T cell activation, CD69, and more specific Treg-associated markers, such as GITR, intracellular CTLA-4, and Foxp3. We found that there was an approximate 75% increase in the percentage of CD4⁺CD25⁺CD69⁺ T cells, indicating that a nonactivated CD4⁺CD25⁺ T cell subset was increasing following sepsis (Fig. 1B; p < 0.001). However,
we did not find an increase in the absolute number of these cells in the spleen. Interestingly, there was also an approximate 4-fold elevation in the percentage of CD4<sup>+</sup>CD69<sup>+</sup>CD25<sup>+</sup> cells, indicating a dramatic increase in the activation of nonregulatory or effector splenic T cells 24 h after a CLP as well (data not shown). We subsequently examined whether the CD4<sup>+</sup>CD25<sup>+</sup> T cells expressed other known markers of endogenous Tregs. To this question, we saw a similar increase in the percentage of CD4<sup>+</sup>CD25<sup>+</sup>GITR<sup>+</sup> T cells but not the absolute number of these cells in the spleen of septic compared with sham-treated mice (Fig. 1C; p < 0.03).

Because GITR may also be expressed on activated T cells, we examined the expression of the transcription factor Foxp3 as a more definitive marker of Tregs. The relative level of Foxp3 mRNA was assessed by quantitative real-time RT-PCR from 2.5 × 10<sup>7</sup> CD4<sup>+</sup>CD25<sup>+</sup> or CD4<sup>+</sup>CD25<sup>-</sup> T cells isolated by cell sorting of splenocytes from septic or sham-treated mice. Consistent with previous reports, Foxp3 mRNA expression was limited to the CD4<sup>+</sup>CD25<sup>+</sup> population in control (sham) mice (Fig. 2A). CD4<sup>+</sup>CD25<sup>+</sup> cells from sham-treated mice expressed ~30-fold the amount of Foxp3 mRNA over control splenocytes, whereas CD4<sup>+</sup>CD25<sup>-</sup> cells from sham-treated mice expressed near baseline values. CD4<sup>+</sup>CD25<sup>+</sup> T cells from septic mice, on the other hand, expressed 140-fold the amount of Foxp3 mRNA over control splenocytes, and 3.5-fold greater expression of Foxp3 mRNA than the CD4<sup>+</sup>CD25<sup>-</sup> population from sham-treated mice. Interestingly, there was about a 3-fold increase in Foxp3 mRNA levels over baseline (and overexpression from CD4<sup>+</sup>CD25<sup>-</sup> T cells from sham-treated animals) in the CD4<sup>+</sup>CD25<sup>-</sup> T cells from septic animals (Fig. 2A). Similar data were obtained from three independent experiments, and one representative example is shown. These data confirm that there is an increase in the expression of Foxp3 per CD4<sup>+</sup>CD25<sup>+</sup> T cell, indicating perhaps these cells may possess increased suppressor cell activity.

Since the expression of Foxp3 mRNA may not necessarily translate to the expression of Foxp3 protein, we further examined whether the augmented CD4<sup>+</sup>CD25<sup>+</sup> T cell population from septic mice expressed Foxp3 protein. We found that spleens from septic mice had almost a 2-fold increase in the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells (Fig. 2, B and C; p < 0.001) by 24 h, but again, there was no significant increase in the absolute number of this cell population at 24 h. We also observed that the relative increase in CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells was maintained up to 3 days following the septic challenge, but levels soon diminished to baseline in surviving animals thereafter (Fig. 2C, left panel) and remained that way for up to 10 days after CLP. Interestingly, there was an increase in the absolute number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells at day 3 when the number of total splenocytes rebounded following the septic insult (Fig. 2C, right panel).

When examined, over 75–85% of the CD4<sup>+</sup>Foxp3<sup>+</sup> population from septic or control mice also expressed intracellular CTLA-4 (data not shown), indicating that the phenotype of these cells (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> GITR<sup>+</sup> CTLA-4<sup>+</sup> CD69<sup>+</sup>) was more likely to be Tregs than activated T cells. Also, these data demonstrate that a relative increase in the percentages of Tregs during sepsis is probably due to persistence of these cells (possibly because they are more resistance to apoptosis) instead of proliferation during sepsis, whereas a loss of many cells such as T cells, B cells, and dendritic cells (most likely by apoptosis) is known to occur (24, 25).

CD4<sup>+</sup>CD25<sup>+</sup> Tregs from septic mice are better suppressors of T effector cell proliferation

Tregs are best characterized as being poor responders to mitogenic stimuli and suppressors of T effector cell proliferation. Since the CD4<sup>+</sup>CD25<sup>+</sup> T cells from septic mice expressed more Foxp3 than CD4<sup>+</sup>CD25<sup>+</sup> T cells from sham-treated mice, we examined whether they possessed increased suppressor cell activity. Indeed, we found that the CD4<sup>+</sup>CD25<sup>+</sup> T cells from both septic and sham-treated mice were hyporesponsive to CD3/CD28 stimulation and suppressed syngeneic T effector cell proliferation (Fig. 3A). Interestingly, the CD4<sup>+</sup>CD25<sup>+</sup> T cells from septic mice displayed increased suppressive properties over CD4<sup>+</sup>CD25<sup>-</sup> T cells from sham-treated animals (Fig. 3B; p = 0.032). Also of note, Tregs from either septic or sham-treated mice suppressed T effector cell proliferation regardless of whether the T effector cells were from septic or sham-treated animals (data not shown). However, T effector cells from septic mice only proliferated to approximately one-half the magnitude of cells from sham-treated mice, indicating some dysfunction exists with regards to T effector cell proliferation during the early sepsis period (Fig. 4A).

Ab-mediated depletion of Tregs does not alter sepsis-induced mortality

Elimination of CD4<sup>+</sup>CD25<sup>+</sup> Tregs by administration of a mAb to CD25 is one of the most used methods to determine the role of Tregs in immunological reactions or various disease processes. In this study, we used mAbs against CD4 and CD25 to deplete both of these cell types before sepsis. First, we confirmed that injection of the Ab depleted the cell of interest. We found that our treatment regimen depleted >90% of the cell population of interest for at least 8 days following the second dose with >95% depletion on the day of CLP (Fig. 4, A and B). Surprisingly, depletion of CD4<sup>+</sup> or CD4<sup>+</sup>CD25<sup>+</sup> cells did not alter mortality to sepsis (Fig. 4C; p > 0.05). Furthermore, when we varied the baseline mortality produced by the CLP by altering the size of the enterotomy or the...
ligation, mortality rates between the Ab-treated and nontreated mice remained similar (data not shown).

To confirm that CD4\(^+\)/CD25\(^+\) cells do not influence survival outcome in this model of sepsis, we performed CLP in CD25-null mice. Again, we found no survival difference in mice deficient in Tregs vs control mice (Fig. 5D; \(p < 0.05\)). Taken together, with the Ab-mediated depletion experiments, we conclude that CD4\(^+\)/CD25\(^+\) Tregs do not influence survival in sepsis.

CLP increases IL-10 expressing T cells but IL-10-null mice do not have increased susceptibility to CLP

Although CD4\(^+\)/CD25\(^+\) Tregs did not influence survival in sepsis, other Treg populations exist, including the inducible T\(_{reg}\)-1-type Tregs. These T\(_{reg}\)-1 Tregs are known to produce copious amounts of IL-10, and the generation of T\(_{reg}\)-1 cells by contact with natural Treg may actually mediate some of the in vivo-suppressive effects of natural Tregs. We therefore examined whether IL-10-expressing T cells were increased in response to sepsis. We found that stimulation of CD4\(^+\) T cells from the spleens of septic mice with CD3/CD28 stimulation in the presence of IL-2 caused a 4-fold increase in the number of cells producing IL-10 when compared with cells from sham-treated mice (Fig. 5, A and B; \(p < 0.001\)). Since IL-10 is important in the suppressive activity of T\(_{reg}\)-1 cells, we next wished to determine whether IL-10-null mice displayed increased mortality to sepsis and whether this was due to a lack of T\(_{reg}\)-1 cells.

In contrast to previous reports demonstrating that mortality progressed more rapidly in IL-10-null mice (26), we did not identify any role for IL-10 in sepsis mortality using this strain of mice (Fig. 5C). Therefore, the production of this cytokine by a subset of cells that produce it (T\(_{reg}\)-1 cells) most likely did not have an effect on sepsis mortality.

Discussion

Studies ongoing for more than a decade have provided firm evidence for the existence of a unique CD4\(^+\)/CD25\(^+\) population of regulatory/suppressor T cells that prevent the activation and effector function of autoreactive T cells that have escaped other mechanisms of tolerance (4). Subsequently, these Tregs have been found to play major roles in a wide assortment of biological processes including transplantation tolerance (28), infection (29), inflammation/injury (6, 30), and tumor persistence/progression (31). CD4\(^+\)/CD25\(^+\) T cells have unique immunologic characteristics...
compared with other immune cells. For example, they do not proliferate in response to antigenic stimulation in vitro (naturally anergic) and can potentially suppress the activation and proliferation of CD4⁺/CD25⁺ T cells through either an Ag-nonspecific (32) or Ag-specific (32, 33) manner through cell-to-cell contact.

In this study, we found that polymicrobial sepsis increases the percentage of splenic CD4⁺/CD25⁺ Tregs 1 day after sepsis and the percentage and the absolute number 3 days after a septic insult, and these changes are transient, lasting only a few days. The findings are consistent with those of Venet et al. (19), who observed that sepsis in humans results in the relative increase of Tregs in peripheral blood. In contrast to our findings, they found no increase in Foxp3 mRNA expression in the blood of septic patients. They collected their blood in PAXgene tubes, however, in which lymphocyte RNA is significantly diluted with RNA from erythroid precursor cells as well as other leukocyte populations, such as neutrophils, which make up the majority of leukocytes in the blood of septic patients. When examining highly purified T cell subpopulations, we not only found that sepsis increases Foxp3 mRNA expression in CD4⁺/CD25⁺ cells but also causes a similar increase in CD4⁺/CD25⁺ cells (albeit at a much lower absolute expression level). Similarly, we observed a low number of Foxp3⁺ cells in the CD4⁺/CD25⁺ fraction of sham-treated mice that was increased in septic mice (data not shown). However, these cells were not included in our analysis as our suppressor assay was performed using the CD4⁺/CD25⁺ fraction as Tregs.

Interestingly, we found that Tregs from septic mice had increased suppressor cell activity ex vivo when compared with Tregs from sham-treated mice. It is known that activation of Tregs by anti-CD3 engagement can increase their suppressive properties (20). Recently, engagement of TLRs by pathogen-associated molecular patterns on Tregs has also been shown to induce their proliferation and activation, while causing an increase in their suppressor cell function (15, 16). This is a likely explanation for the increase in the suppressive function of Tregs from septic mice in our experiments, as bacteremia and organ colonization in the spleen by enteric microorganisms is a natural component of the sepsis response to a CLP.

Despite their increased proportion and suppressor cell activity ex vivo, however, genetic depletion of endogenous Tregs or Ab-mediated elimination of Tregs before the onset of sepsis did not alter survival to the septic challenge. Furthermore, elimination of the total Th cell population with anti-CD4 Abs also did not affect survival to sepsis. These data are not fully consistent with reports by Hotchkiss et al. (25, 33) that apoptotic loss of Th cells is detrimental to sepsis outcome. To be certain that the Ab-mediated depletion of a cell type can actually alter survival to sepsis, we pretreated mice exposed to sepsis with an anti-Gr-1 Ab (targeted to deplete certain populations of cells, including macrophages, neutrophils, and activated T cells). We found a dramatic increase in sepsis mortality with that Ab treatment (targeted to deplete certain populations of cells). Furthermore, elimination of the total Th cell population with anti-CD4 Abs also did not affect survival to the septic challenge. However, when sepsis was induced in IL-10–null mice, we found no appreciable difference in survival. Since IL-10 deficiency yielded no difference in outcome, we could not fully ascertain the role of IL-10-producing Th cells in vivo. When sepsis was induced in IL-10–null mice, we found no appreciable difference in survival. Since IL-10 deficiency yielded no difference in outcome, we could not fully ascertain the role of IL-10-producing Th cells in vivo. These data suggest that compensatory mechanisms in addition to IL-10 or IL-10-producing Th cells may exist in the IL-10–deficient mice to limit the inflammatory sequelae in sepsis.

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Disclosures

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