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Human CD4⁺CD25⁺ Regulatory T Lymphocytes Inhibit Lipopolysaccharide-Induced Monocyte Survival through a Fas/Fas Ligand-Dependent Mechanism¹

Fabienne Venet,* Alexandre Pachot, ‡ Anne-Lise Debard, ‡ Julien Bohe, ‡ Jacques Bienvenu, ‡ Alain Lepape, § William S. Powell, † and Guillaume Monneret²*

Although it is known that septic shock induces immunosuppression, the mechanism for this phenomenon is not well understood. Monocytes play a central role in septic shock pathophysiology, which is also characterized by an increased proportion of natural regulatory T (Treg) cells. We therefore investigated whether Treg could be involved in the decreased monocyte expression of CD14 and HLA-DR observed during septic shock. We demonstrated that human Treg inhibit LPS-induced retention of monocyte CD14. Because loss of CD14 is a hallmark of monocyte apoptosis, this suggests that Treg inhibit monocyte survival. This effect was largely mediated through the release of a soluble mediator that was not identical with either IL-10 or IL-4. The Fas/FasL pathway participated in the effect as it was blocked by anti-FasL Abs and reproduced by Fas agonist and recombinant soluble FasL. Furthermore, expression of FasL was much higher on Treg than on their CD25⁻ counterparts. Collectively, these results indicate that Treg act on monocytes by inhibiting their LPS-induced survival through a proapoptotic mechanism involving the Fas/FasL pathway. This may be an important mechanism for septic shock-induced immunosuppression and may offer new perspectives for the treatment of this deadly disease. The Journal of Immunology, 2006, 177: 6540–6547.

Despite the great advances in modern medicine, septic shock remains a major cause of death in intensive care units, with a mortality rate that is regularly reported as high as 40–50% (1, 2). It is now agreed that septic shock deeply perturbs immune homeostasis by inducing an initial intense systemic inflammatory response that is rapidly followed by an anti-inflammatory process, acting in a negative feedback manner (1, 3). These inhibitory mechanisms may become deleterious as nearly all immune functions are compromised. Therefore, they may account for the majority of septic shock related death. Indeed, most nonsurviving patients die after initial resuscitation in a delayed immunosuppressive state (3–5).

The mechanistic bases for septic shock-induced immunosuppression have not yet been clearly established. It is known that this condition is characterized by profound immunological dysfunction partly due to cell anergy and increased apoptosis of various immune cells (1, 3). Monocytes play a central role in septic shock pathophysiology given that they participate in both the initial inflammatory response and the secondary immunodepression (4). Initially, as components of the innate immune system, they sense microbial products and in response release inflammatory cytokines and initiate adaptive T cell responses due to their capacity of Ag presentation. Subsequently, they exhibit decreased Ag presentation (likely due to a decreased HLA-DR expression), decreased proinflammatory cytokine production, and increased apoptosis (1, 2, 4, 6).

We recently found that the percentage of CD4⁺CD25⁺ regulatory T lymphocytes (Treg)³ is increased in septic shock patients (7, 8). These cells possess potent regulatory properties that are directed at different arms of the immune system (9–11). Although they have been shown to modulate the innate immune response in various murine models of infectious diseases, their role in human diseases has not yet been established (12). In particular, their potential role in septic shock has never been addressed. As their immunosuppressive properties are not limited to effects on other T cell responses, but also include inhibition of immune pathology mediated by cells of the innate immune system (13), we hypothesized a link between Treg and monocytes. The goal of the current study was thus to examine whether monocyte deactivation might be due to a suppressive effect of Treg during septic shock. We found that CD14 expression is down-regulated in monocytes from septic shock patients and that this effect can be mimicked by incubation of LPS-treated monocytes with conditioned medium from Treg. Treg produce a soluble factor that prevents LPS-induced monocyte survival by a mechanism involving the Fas/FasL pathway, which has previously been linked to sepsis-induced immunosuppression (14). Our findings suggest that Treg may play an important role in the increased level of monocyte apoptosis observed in immunodepressed septic patients.

Patients, Materials and Methods

Patients

The study group consisted of 33 consecutive patients with septic shock according to the diagnostic criteria of the American College of Chest Physicians/Society of Critical Care Medicine (age, 58 ± 3 years; 9 female, 24

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3 Abbreviations used in this paper: Treg, regulatory T cell; DIOC-6, 3,3′-dihexyloxacarbocyanine iodide.

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male; mortality, 39%. mean Simplified Acute Physiology Score II on admission, 47, with a range of 15–94. These patients belong to a larger group examined for their percentage of HLA-DR-positive monocytes (results reported elsewhere) (5). Septic shock was defined by an identifiable site of infection, hypotension persisting despite fluid resuscitation, and requiring vasopressor therapy, and evidence of a systemic inflammatory response manifested by at least two of the following criteria: 1) temperature >38°C or <36°C; 2) heart rate >90 beats/min; 3) respiratory rate >20 breaths/min; 4) white blood cell count >12,000/mm³ or <0.5 × 10⁹/mm³. Severity was assessed by the Simplified Acute Physiology Score II. Mortality was defined as death occurring within 28 days after the onset of shock. Cell phenotyping was performed at days 1 and 2 and until day 15 after the onset of shock on residual blood after completing routine follow-up performed in our intensive care unit, in accordance with the human experimentation guidelines set for clinical research of our institute. To provide a panel values from healthy donors, we also included 36 individuals from the laboratory staff of our hospital (ages 23–59 years, 27 female, 9 male, without comorbidity) after informed consent was given.

Cell isolation

Heparinized blood was taken from healthy individuals (n = 40) after informed consent was given (age 34 ± 2 years, 21 female, 19 male). Mononuclear cells were isolated from freshly drawn human blood by Ficoll-Paque Plus gradient centrifugation (Amersham). Monocytes were first

...CD25+/CD45RO staining. T cell subpopulations of CD4+ T cells (CD25+ or CD25−) were cultured immediately after isolation.

Cells and culture conditions

Cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich) in 24-well ultralow attachment plates (Corning-Costar). The number of monocytes per well was adjusted to the number of CD4+CD25− T lymphocytes (ratio 1:1). LPS was added at a concentration of 10 μg/ml (15). Cells were incubated 16 h at 37°C in a humidified 5% CO₂ atmosphere.

Reagents and flow cytometry

The following Abs were used: FITC- or energy-coupled dye-labeled anti-CD4; PC5-labeled anti-CD4; FITC-labeled anti-CD25; anti-CD45RO (BD Pharmingen); PE- or PE-Cy5-labeled anti-CD25; and PE-labeled anti-HLA-DR (BD Biosciences); PE-labeled anti-TLR4 (eBiosciences); and PE-labeled anti-FasL (BD Biosciences). The lower limit of detection was 3 pg/ml, according to the manufacturer’s instructions. Soluble Fasl measurements were performed using a commercial kit from R&D Systems. The lower limit of detection was 3 pg/ml according to the supplier’s instructions. ELISAs were quantified by OD₄₅₀ on a microplate reader (Dynatech).

Statistics

Data are presented as mean ± SEM. The results are considered as significant at p < 0.05, as determined by the Wilcoxon nonparametric paired test. Due to the sample size, comparisons between groups of patients were made with the nonparametric Mann-Whitney U test without correction on the number of test performed.

Results

Loss of CD14 expression on monocytes from septic shock patients

During septic shock, monocytes present with decreased response to endotoxin stimulation (1, 6, 19). Surprisingly, little work has been specifically devoted to the study of LPS coreceptors on monocytes. We therefore retrospectively investigated whether the expression of CD14 and TLR4 was altered on monocytes from septic patients during the course of this condition. We monitored CD14 in 33 consecutive patients (between day 1 and day 15 after the onset of shock) in comparison with 36 healthy individuals. In agreement with our hypothesis, in addition with a decreased HLA-DR expression, septic patients displayed during the whole monitoring, a decrease in CD14 expression on monocytes in comparison with healthy individuals (Fig. 1A). When survivors (n = 20) were separated from nonsurvivors (n = 13), the latter had...
performed (H11569) significance between groups without correction on the number of test CD4 shown). mRNA microarray analysis demonstrated that purified each purification (based on CD4/CD25/CD45RO staining, data not performed (H11001). Because of the reduced expression of CD14 (Fig. 1) and HLA-DR We investigated the effects of Treg on LPS-treated monocytes. CD4 T cells display the characteristics usually described for Treg (9, 5, 20) that we observed on monocytes from septic patients, we were especially interested to determine whether Treg could block the effect of LPS on the expression of these molecules by monocytes. Monocytes were cultured in the presence or absence of LPS (10 μg/ml) with or without an equal number of either Treg or CD4+CD25− T cells. After culture for 16 h, we did not detect any modification of HLA-DR expression in response to either LPS or Treg (data not shown). In the absence of LPS, few monocytes retained CD14 after 16 h (13 ± 3%; Fig. 3, Aa and Ab), whereas in its presence, a distinct population of these cells (44 ± 4%) exhibited near normal CD14 expression (Fig. 3, Ad). Overall, CD14 expression was '~2.5 times higher in LPS-treated monocytes than in control cells (p < 0.001), and this effect was completely blocked by Treg but not by CD4+CD25− T cells (Fig. 3, B).

**Characterization of purified CD4+CD25+ Treg**

To determine whether Treg could have effects on monocytes similar to what we observed in septic shock patients we purified these cells from peripheral blood of healthy individuals. The purity of CD4+CD25+ lymphocytes was monitored by flow cytometry after each purification (based on CD4/CD25/CD45RO staining, data not shown). mRNA microarray analysis demonstrated that purified CD4+CD25+ lymphocytes overexpress FOXP3, CD25, and CTLA4 but exhibit decreased expression of CD69 in comparison with their CD25− counterpart (Fig. 2A). The suppressive capacity of CD4+CD25+ T lymphocytes on CFSE-stained CD4+CD25− T cell proliferation was also assessed. The addition of an equal number of non-stained CD4+CD25− T lymphocytes induced a substantial decrease in CD4+CD25− T cell proliferation in response to PHA (Fig. 2B), whereas the addition of an equal number of nonstained CD4+CD25− T cells had no effect (data not shown).

Collectively, these data indicate that the purified CD4+CD25+ T cells display the characteristics usually described for Treg (9, 11), including suppression of T cell proliferation, and can thus be considered to be CD4+CD25+ Treg.

**Treg inhibit LPS-induced retention of CD14 on monocytes**

We investigated the effects of Treg on LPS-treated monocytes. Because of the reduced expression of CD14 (Fig. 1) and HLA-DR (5, 20) that we observed on monocytes from septic patients, we significantly lower CD14 expression (Fig. 1B). CD14 down-regulation was thus present during septic shock and was correlated with severity. In contrast, TLR4 expression by monocytes was the same in septic patients as in healthy control subjects and did not change during the course of this condition (data not shown).

Because of the reduced expression of CD14 (Fig. 1) and HLA-DR (5, 20) that we observed on monocytes from septic patients, we were especially interested to determine whether Treg could block the effect of LPS on the expression of these molecules by monocytes. Monocytes were cultured in the presence or absence of LPS (10 μg/ml) with or without an equal number of either Treg or CD4+CD25− T cells. After culture for 16 h, we did not detect any modification of HLA-DR expression in response to either LPS or Treg (data not shown). In the absence of LPS, few monocytes retained CD14 after 16 h (13 ± 3%; Fig. 3, Aa and Ab), whereas in its presence, a distinct population of these cells (44 ± 4%) exhibited near normal CD14 expression (Fig. 3, Ad). Overall, CD14 expression was ~2.5 times higher in LPS-treated monocytes than in control cells (p < 0.001), and this effect was completely blocked by Treg but not by CD4+CD25− T cells (Fig. 3, B).

**The inhibitory effect of Treg is largely mediated by a soluble factor**

In vitro, Treg usually exert their effect by direct cellular contact with responder cells (9). We thus tested the necessity of cell-cell contact for their effect on CD14 expression. Monocytes and Treg were incubated overnight in 24-well Transwell plates, which permit soluble factors to diffuse from one chamber to another but...
prevent any cell-cell contact. The physical separation of Treg and monocytes did not prevent the ability of Treg to suppress the effect of LPS on CD14 expression (Fig. 4A).

To provide further support for the involvement of a soluble factor, conditioned medium from Treg obtained after 4 h in culture was incubated for 16 h with monocytes in the presence of LPS. These supernatants reproduced the regulatory effect of Treg on LPS-induced CD14 expression (Fig. 4B). These results clearly indicate that the effect of Treg on monocyte CD14 expression is largely mediated by the production of a soluble factor.

The soluble factor released by Treg is not identical with IL-4 or IL-10

Human Treg may produce IL-10 (9, 11). Both this cytokine and IL-4 have been implicated in sepsis-induced immunosuppression (2) and might be able to regulate CD14 expression on monocytes (21, 22). These cytokines were measured in conditioned medium from Treg and in coculture supernatants. Under the conditions used, the levels of IL-4 were below the detection limits of our assay. Moreover, anti-IL-4-blocking Abs did not suppress the effect of Treg on LPS-induced CD14 expression (Fig. 5A).

In contrast to IL-4, little amounts of IL-10 were present in the media from monocytes cultured with Treg and LPS (15 ± 4 pg/ml vs 10 ± 5 pg/ml in the media of monocytes cultured with LPS, p ≤ 0.05), whereas we were unable to detect this cytokine in medium from either monocytes or Treg when cultured alone (data not shown). The possible involvement of IL-10 (as a secondary induced mediator) in the effect of Treg on CD14 expression was further tested by the addition of anti-IL-10-blocking Abs to monocyte/Treg cocultures. However, these Abs were unable to suppress the effect of Treg on LPS-induced CD14 expression (Fig. 5A). Furthermore, addition of recombinant human IL-10 to monocytes failed to reproduce the effect of Treg on CD14 expression (Fig. 5B). We thus conclude that neither IL-4 nor IL-10 can explain the effect of Treg on monocytes.

Treg inhibit LPS-induced monocyte survival

There is evidence that monocytes undergoing apoptosis display reduced surface expression of CD14 (23, 24). We therefore hypothesized that Treg act by preventing LPS-induced monocyte survival. To test this hypothesis, we monitored, in addition to CD14, the apoptotic markers annexin V and DIOC-6 in purified monocytes (24). Monocytes cultured for 16 h in the absence of LPS exhibited a profound reduction in CD14 expression coupled to a marked increase in annexin V staining and a decrease in DIOC-6 staining, indicative of increased spontaneous apoptosis (Fig. 6A).

We next observed that the population of CD14high cells present in LPS-treated monocytes express lower levels of annexin V than CD14 low monocytes in the same sample (Fig. 6B). This CD14 high/annexin V population was modulated in the presence of Treg. It represented 22% of total monocytes in the presence of LPS but only 12% when monocytes were cultured alone or with Treg and LPS (Fig. 6C). These results suggest that the inhibitory effect of Treg on LPS-induced CD14 expression is due to the blockade of the antiapoptotic properties of LPS due to the release of a soluble factor with apoptotic properties from Treg (Fig. 4).
The proapoptotic effect of Treg on monocytes involves the Fas/ FasL pathway

To investigate the nature of the soluble proapoptotic factors involved in the effect of Treg, we added blocking Abs directed against FasL, TNF-α, and TRAIL to LPS-treated monocyte-Treg cocultures. Neither anti-TNF nor anti-TRAIL Abs had an effect whereas anti-FasL Abs strongly suppressed the blockade of LPS-induced CD14 expression by Treg (Fig. 7A). A similar tendency was observed in coculture experiments in which anti-FasL, added to the bottom chamber with monocytes, reduced the inhibitory effect of Treg, added to the top chamber. However, this effect was less pronounced than in coculture experiments (data not shown).

Finally, to determine whether activation of Fas on monocytes could reproduce the effect of Treg, we incubated monocytes in the presence of LPS and increasing concentrations of agonistic anti-FasL Abs. As with Treg conditioned medium, these Abs completely blocked LPS-induced CD14 expression in a dose-dependent manner (Fig. 7, D and E). Identical results were obtained using recombinant human soluble FasL (Fig. 7F).

Collectively, these data support a role for the Fas/FasL proapoptotic pathway in the inhibitory effect of Treg on LPS-induced monocyte survival.

Discussion

Septic syndromes are still the leading cause of death in intensive care units. In the United States, they develop in 750,000 people annually, of whom >210,000 die (1). Among septic syndromes, septic shock is the most severe, with a mortality ranging from 40 to 50% despite adequate initial treatments. In fact, there is growing recognition that a large number of septic patients rapidly manifest immunosuppression, which may be the principal cause of death, because most of them survive the initial proinflammatory state (1, 2, 4).

An increased percentage of CD4+CD25+ Treg has been observed in septic patients. Because these cells possess potent regulatory properties on cellular activation (9, 11), they may participate...
in sepsis-induced immunosuppression. The role of Treg during infectious processes has been studied in mouse models but is still far from being understood in humans (12). Treg can be activated and expand against bacterial, viral, and parasite Ags in vivo. Particularly, Caramalho et al. (15) demonstrated that Treg express various TLRs and that LPS directly activates survival and markedly increases the suppressive activity of Treg through TLR4. Similar results were recently demonstrated using flagellin as a TLR5 ligand (25). Such activated Treg can thus prevent infection-induced immunopathology but may also increase the load of infection and prolong pathogen persistence by suppressing protective immune responses (9, 26). Therefore, their beneficial or deleterious effect is dependent on their relative proportion during infection.

Because the immunosuppressive properties of Treg are not limited to inhibition of T cell responses but also include inhibition of immune pathology mediated by cells of the innate immune system (13), we were prompted to investigate the role of the increased proportion of Treg present during septic shock. We therefore studied the effect of Treg on activation of monocytes, usually considered as key players in septic shock pathophysiology. In a model of LPS-stimulated purified human cells, we describe here two important results: 1) Treg may act on monocytes by inhibiting their LPS-induced survival through a proapoptotic mechanism; 2) this effect is largely mediated by a soluble factor and involves the Fas/FasL pathway.

Relatively little information is available on the interaction of Treg with monocytes. A direct suppressive effect of Treg on APC has been proposed. It has been demonstrated that murine Treg down-regulate the expression of CD80 and CD86 on bone marrow-derived dendritic cells in a cell contact-dependent manner (27). These results were then confirmed in humans (28). Secondly, a recent study by Taams et al. (29) demonstrated that human monocytes incubated with Treg (cell-cell contact) were severely limited in their capacity to induce Ag-specific response and to produce proinflammatory cytokines in response to LPS. However, the mechanisms responsible for these effects were not identified in these models. Thus, Treg may modulate APC functions and thereby make them unable to activate effector T cells.
The present study constitutes the first description of an effect of human Treg on monocyte CD14 expression, which may explain the decreased expression of this molecule on monocytes in septic shock patients. The regulation of CD14 expression on purified human monocytes by cytokines and other factors has received considerable attention (21, 22, 30). CD14 is also regulated on monocytes during their apoptosis (23). In low serum supplementation milieu, it has been shown that monocytes display decreased CD14 expression due to their spontaneous apoptosis (21, 24, 31–33). This decrease represents an early event during monocyte apoptosis because it precedes annexin V binding, in contrast to HLA expression, which is unchanged in apoptotic monocytes (23). In this context, LPS constitutes a well-known stimulatory factor able to rescue monocytes from apoptosis through c-Flip up-regulation (22–24, 34).

The major pathway involved in monocyte apoptosis appears to involve Fas/FasL (34–37). Kiener et al. (37) demonstrated that human monocytes express both Fas and FasL and that the autocrine and paracrine interactions of these two molecules are largely responsible for the spontaneous induction of apoptosis that occurs on culture of peripheral monocytes. They also observed that human monocytes contain high levels of intracellular preformed FasL that can be rapidly released (within 30 min) in an active soluble form (36). Finally, Fas may also be required in vivo for regulating circulating monocyte numbers, as mice deficient in the Fas pathway (lpr/lpr) display increased circulating monocytes (35). Taken together, these data are consistent with the proposed role for Fas/FasL in the current study.

Our present data demonstrate that human Treg may participate in Fas/FasL-induced apoptosis by releasing a soluble factor. We observed that Treg suppression of LPS-induced monocyte survival was blocked by anti-FasL Abs and was reproduced by Fas agonist. Moreover, Treg overexpressed FasL in comparison with their CD25− counterparts. Very few studies have investigated the role of Treg in the induction of apoptosis. It has been recently demonstrated that Treg may use the perforin pathway to kill target cells both in humans (38) and mice (39, 40). Grossman et al. (38) demonstrated that human Treg express granzyme A and display perforin-dependent cytotoxicity. All of these effects necessitated adhesion immunological synapses and were independent of Fas-FasL interactions. In a murine cell line model, Treg were able to lyse Ag-presenting B cells through Fas-FasL interactions in a cell-cell contact-dependent manner. Treg up-regulate FasL expression through which they transduce a death signal, inducing target APC cell apoptosis (41). Our study reports thus on the involvement of human Treg in apoptosis through the release of a soluble factor. The precise mechanisms involved in this effect remain to be more specifically elucidated. Regarding the sensitivity of Treg themselves to apoptosis, contrasting results have been published. Some studies proposed a high sensitivity of Treg to Fas-induced apoptosis (42), whereas others demonstrated the resistance of Treg to apoptosis induced by either Fas (43) or dexamethasone (44). Interestingly, in the context of septic shock, we previously observed that the increased percentage of Treg was due to a reduction in the numbers of CD4+CD25+ T cells, whereas the absolute numbers of Treg remained in the normal range (8). To explain this, we proposed that Treg might be resistant to the apoptotic processes occurring after shock. This seems consistent with our current experiments.

In septic shock, apoptotic pathways are largely activated (14, 45). Apoptosis of circulating monocytes has been described in humans (6, 14, 46–47). Among different mechanisms, the involvement of the Fas/FasL pathway has been well established (48, 49) and seems to be of primary importance given that several studies associated Fas and FasL expression with severity and even mortality in sepsis (50, 51). Furthermore, recent studies using a variety of strategies to inhibit apoptotic processes suggest that blocking programmed cell death is beneficial to sepsis, especially blocking the Fas/FasL pathway (49, 52). Treatments using bcl-2 overexpression (53, 54) and caspase inhibitors have also shown good results in terms of survival (52, 55, 56).

In light of the present study, it seems relevant to propose a link among the increased percentage of Treg, the activated Fas/FasL pathway, and the monocyte apoptosis observed during septic shock. Besides, recent studies observed a participation of Treg in the modulation of the innate immune system during severe inflammatory processes: murine models of septic shock (57) and severe injury (58). It has been also very recently demonstrated that Treg contribute to the development of immune suppression in a mouse burn injury model (59). In summary, we observed that human CD4+CD25+ Treg inhibit LPS-induced monocyte survival partly through a Fas/FasL mechanism. This constitutes the first description of the involvement of human Treg in an apoptotic process that is largely mediated by the release of a soluble mediator. The identification of the precise mechanism by which Treg induce monocyte apoptosis warrants further investigation. This should give a better understanding of septic shock pathophysiology and may offer new perspectives for the treatment of this deadly disease.

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Disclosures
The authors have no financial conflict of interest.

References


