Deficiency of $\gamma\delta$ T lymphocytes contributes to mortality and immunosuppression in sepsis

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Chung, Chun-Shiang, Lara Watkins, Antonio Funches, Joanne Lomas-Neira, William G. Cioffi, and Alfred Ayala. Deficiency of $\gamma\delta$ T lymphocytes contributes to mortality and immunosuppression in sepsis. Am J Physiol Regul Integr Comp Physiol 291: R1338–R1343, 2006. First published June 22, 2006; doi:10.1152/ajpregu.00283.2006.—Studies have indicated that $\gamma\delta$ T lymphocytes play an important role in the regulation of immune function and the clearance of intracellular pathogens. We have recently reported that intraepithelial lymphocytes (IEL), which are rich in $\gamma\delta$ T cells, within the small intestine illustrated a significant increase in apoptosis and immune dysfunction in mice subjected to sepsis. However, the contribution of $\gamma\delta$ T cells to the host response to polymicrobial sepsis remains unclear. In this study, we initially observed that after sepsis induced by cecal ligation and puncture (CLP), there was an increase in small intestinal IEL CD8$^+$ $\gamma\delta$ T cells in control $\gamma\delta^{+/+}$ mice. Importantly, we subsequently found an increased early mortality in mice lacking $\gamma\delta$ T cells ($\gamma\delta^{--/-}$ mice) after sepsis. This was associated with decreases in plasma TNF-α, IL-6, and IL-12 levels in $\gamma\delta^{--/-}$ mice compared with $\gamma\delta^{+/+}$ mice after sepsis. In addition, even though in vitro LPS-stimulated peritoneal macrophages showed a reduction in IL-6 and IL-12 release after CLP, these cytokines were less suppressed in macrophages isolated from $\gamma\delta^{--/-}$ mice. Alternatively, IL-10 release was not different between septic $\gamma\delta^{+/+}$ and $\gamma\delta^{--/-}$ mice. Whereas T helper (Th)1 cytokine release by anti-CD3-stimulated splenocytes was significantly depressed in septic $\gamma\delta^{+/+}$ mice, there was no such depression in $\gamma\delta^{--/-}$ mice. However, $\gamma\delta$ T cell deficiency had no effect on Th2 cytokine release. These findings suggest that $\gamma\delta$ T cells may play a critical role in regulating the host immune response and survival to sepsis, in part by alteration of the level of IEL CD8$^+$ $\gamma\delta$ T cells and through the development of the Th1 response.

T helper 1 cytokines; intraepithelial lymphocytes; mice

Although there have been major technological advances in the treatment of traumatic/surgical injury, postoperative complications (such as sepsis) remain one of the most common causes of morbidity and mortality in the intensive care unit (8). Studies have indicated that there is a marked suppression of immune function during sepsis (25, 29), which may contribute to the development of subsequent multiple organ failure. The mechanism behind the induction of immunosuppression in sepsis is only beginning to be understood. In this respect, accumulative evidence has consistently suggested the existence of active suppressor/regulator lymphoid populations such as CD4$^+$ T helper (Th)2, T regulatory, Th3, natural killer T, and CD8$^+$ cells as well as $\gamma\delta$ T cells, which might be responsible for mediating aspects of this immune dysfunction (30). Our and other laboratories (3, 24) have previously demonstrated that the development of Th2 cells, which exhibit an immune suppressive phenotype, occurs in response to septic challenge. However, studies revealing the role of $\gamma\delta$ T lymphocytes in trauma/sepsis are still limited.

Murine $\gamma\delta$ T lymphocytes comprise ~5–10% of the total T cell population; however, they are especially predominant in mucosal surfaces such as epithelial layers of the gut, tongue, lung, and female reproductive tract (20). It is becoming clear that unlike $\alpha\beta$ T cells, which recognize processed peptide antigen-major histocompatibility complexes, $\gamma\delta$ T cells appear to recognize a variety of nascent proteins without antigen processing (11). Therefore, pathogens, damaged tissue, and/or T and B cells can be recognized directly and cellular immunity can be initiated without antigen-presenting cells or prior antigen degradation (28). Along with their unique features in antigen recognition and localization in the mucosa, $\gamma\delta$ T cells have been shown to produce a variety of cytokines upon stimulation. However, whereas the important roles of $\alpha\beta$ T cells in immune function are well studied, our comprehension of the biological functions of $\gamma\delta$ T cells remains incomplete. Allison and Havran (1) proposed that $\gamma\delta$ T cells may be responsible for the first line of defense. This concept is supported by the increase/accumulation of $\gamma\delta$ T cells earlier before $\alpha\beta$ T cell responses in bacterial and viral infections (18, 35). An increasing number of studies has documented and suggested that $\gamma\delta$ T cells may play a role in regulation of immune function (13, 28) and may also downregulate the immune response during bacterial infection by producing cytokines (26). Several studies have shown that $\gamma\delta$ T cells are critical for mouse survival after burn injury (38), Klebsiella pneumonia (33), and proinflammatory cytokine development (33, 38). Furthermore, recent clinical studies have shown that the circulating $\gamma\delta$ T cell count was significantly lower in septic patients compared with normal healthy donors (31, 45), which suggests that $\gamma\delta$ T cells may play an important role in the inflammatory response after sepsis. Nonetheless, the precise role of $\gamma\delta$ T cells in sepsis-induced immune depression remains unclear.

A previous study (12) in our laboratory using cecal ligation and puncture (CLP) to produce a polymicrobial septic challenge that is thought to more closely approximate clinical sepsis (14) has indicated that there is dysfunction in the intraepithelial lymphoid (IEL) compartment of the small intestine, which is a site rich in $\gamma\delta$ T cells. However, the contribution of $\gamma\delta$ T cells was not directly examined in that study (12). We, therefore, tested the hypothesis that $\gamma\delta$ T cells play a role...
in the regulation of the immune response in the polymicrobial septic mouse. In the present study, we investigated the effect of γδ T cell deficiency on mouse overall survival and possible suppression/regulation in immune function during the course of polymicrobial sepsis.

MATERIALS AND METHODS

CLP. Polymicrobial sepsis was induced in mice using the model of CLP described by our laboratory (4). Male inbred C57BL/6J (background control, γδTγδ−/−) and C57BL/6γδTγδ+/+ (γδTγδ+/−) mice (Jackson Laboratory, Bar Harbor, ME), 7–10 wk of age, were lightly anesthetized with Metofane (methoxyflurane, Pitman-Moore, Mundelein, IL), shaved at the abdomen, and scrubbed with betadine. A midline incision (1.5–2.0 cm) was made below the diaphragm to expose the cecum. The cecum was ligated, punctured twice with a 22-gauge needle, and gently compressed to extrude a small amount of fecal contents through the punctured holes. The cecum was returned to the abdomen, and the incision was sutured in layers. Animals were resuscitated with 0.8 ml of lactated Ringer solution by a subcutaneous bath. Cell suspensions from three incubations were pooled, centrifuged (800 g at 4°C for 15 min), resuspended in fresh DMEM at 1 × 10^6 cells/ml, and plated onto plastic tissue culture plates, and then incubated at 37°C for 2 h. Nonadherent cells were removed by repeated washing for three times with fresh DMEM. This protocol provided adherent cells that were >95% positive by nonspecific esterase staining and exhibited typical macrophage morphology.

Stimulation and assessment of cytokine release. Splenocytes were stimulated in vitro with or without monoclonal rat anti-mouse CD3 antibody (15 μg/ml in PBS and coated overnight at 4°C in tissue culture plates) for 48 h at 37°C (5% CO2 and 95% humidity). Adherent macrophage monolayers were stimulated with or without 10 μg LPS/ml DMEM supplemented with 10% fetal bovine serum for 24 h (37°C and 5% CO2). At the end of the incubation period, cultured supernatants were collected and stored at −70°C until analysis.

Th1 (IL-2 and IFN-γ) or Th2 (IL-10) cytokines produced from splenocytes upon plate-bound anti-CD3 stimulation and IL-6, IL-12, and IL-10 produced from macrophages upon LPS stimulation were measured in culture supernatants by the sandwich ELISA technique as previously described (3) with monoclonal antibody pairs and appropriate mouse cytokine standards (obtained from BD Pharmingen).

Results

Presentation of data and statistical analysis. Data are presented as means ± SE for each group. Differences in percent data (i.e., percentage of apoptosis positive and phenotypic positive) and cytokine levels were considered to be significant if P < 0.05, as determined by the Mann-Whitney U-test. Survival data were compared using the Fisher’s exact test and Kaplan-Meier log rank test and considered as significant at P < 0.05.

RESULTS

Sepsis induced a concomitant increase in the percentage of CD8γδ+ cells and a decrease in CD8αβ+ cells in the small intestinal IEL compartment of the gut. IEL phenotypic distribution was characterized by staining cells with anti-αβ or γδ T cell receptors and anti-CD8. In control γδTγδ-/- mice, CD8αβ+ and CD8γδ+ were about equally distributed in the small intestine of sham animals. Figure 1 shows percentages of CD8αβ+ and CD8γδ+ cells of total CD8+ cells in IELs isolated from sham or CLP mice. Whereas total CD8+ (data not shown) and CD8αβ+ IELs decreased in CLP mice, there...
was a significant increase in \( \text{CD}8^+ \gamma \delta^+ \) cells at 4 h after the onset of sepsis. Although not statistically significant, the percentage of \( \text{CD}8^+ \gamma \delta^+ \) cells at 24 h was still higher in septic mice.

Mice lacking \( \gamma \delta \) T cells succumbed more rapidly after the initial onset of polymicrobial sepsis. To determine whether \( \gamma \delta \) T cell deficiency could affect the animals’ ability to survive septic challenge, C57BL/6J (background control, \( \gamma \delta^{+/+} \)) and C57BL/6JcrdMom (\( \gamma \delta^{-/-} \)) mice were subjected to CLP, and survival was monitored for 10 days. \( \gamma \delta^{-/-} \) mice showed a significantly increased mortality rate early after CLP (day 1, \( P < 0.05 \) by Fisher’s exact test; Fig. 2). However, whereas a difference of \( \sim20\% \) persisted over time (>2 days), this was no longer statistically different based on the log rank Kaplan-Meier-type comparison, which assesses cumulative change over time typically using a much larger number of animals per group. Nevertheless, this did not reduce the significance of changes seen early after CLP.

\( \gamma \delta \) T cell deficiency suppressed systemic levels of proinflammatory cytokine release after CLP. At both 4 and 24 h after CLP, plasma levels of proinflammatory cytokines such as TNF-\( \alpha \), IL-6, and IL-12 (Fig. 3, A–C) were significantly elevated compared with the sham control \( \gamma \delta^{+/+} \) mice. Alternatively, \( \gamma \delta^{-/-} \) mice did not respond to septic challenge as vigorously as \( \gamma \delta^{+/+} \) mice. At 4 h, although cytokine levels in \( \gamma \delta^{-/-} \) septic mice were significantly elevated compared with their shams, they were markedly lower than \( \gamma \delta^{+/+} \) septic mice. By 24 h after CLP, the production of these cytokines in \( \gamma \delta^{-/-} \) septic mice was essentially absent.

\( \gamma \delta \) T cell deficiency ablated the suppression of Th1 but not Th2 cytokine release in anti-CD3-stimulated splenocytes after CLP. Whereas Th1 cytokine (IL-2 and IFN-\( \gamma \)) release by splenocytes in response to anti-CD3 stimulation in \( \gamma \delta^{+/+} \) mice was decreased compared with their shams 24 h after sepsis, no such suppression was seen in \( \gamma \delta^{-/-} \) mice (Fig. 4, A and B). However, although an increase in Th2 cytokine (such as IL-10) release after CLP was observed, this was not attenuated in \( \gamma \delta^{-/-} \) mouse cells, there were no changes between the two mouse strains (Fig. 4C). No cytokines were detected in the absence of stimulation (data not shown).

**Effect of \( \gamma \delta \) T cell deficiency on IL-6, IL-12, and IL-10 release in LPS-stimulated peritoneal macrophages after CLP.** Figure 5 illustrates that peritoneal macrophages harvested from septic \( \gamma \delta^{+/+} \) mice showed a significant decrease in IL-6 (A) and IL-12 (B) release in response to LPS compared with their shams. Deficiency of \( \gamma \delta \) T cells also suppressed IL-6 release by peritoneal macrophages from septic \( \gamma \delta^{-/-} \) mice; however, the degree of suppression was significantly less than that seen in control mice. IL-12 release in peritoneal macrophages was not suppressed in \( \gamma \delta^{-/-} \) mice after CLP. Alternatively, \( \gamma \delta \) T cells had no effect on the increased IL-10 release in peritoneal macrophages after sepsis (Fig. 5C). It should be noted that without LPS stimulation, a significant increase in IL-6 and IL-12 release was observed in control \( \gamma \delta^{+/+} \) mouse peritoneal macrophages after sepsis; however, there was no or low cytokine release in septic \( \gamma \delta^{-/-} \) mice. In addition, IL-10 release was not detectable without LPS stimulation in peritoneal macrophages from both mouse strains.
DISCUSSION

The fact that T cell deficiency contributes to the high mortality rate seen in many different models of bacterial infection suggests that T cells are critical to the overall protection of the host against pathogenic insults (22, 39, 41). Studies (2, 10, 25) have shown that loss of immune (T and B cells and monocytes) and/or nonimmune cells (epithelial cells, myocytes, endothelial cells, and hepatocytes) through the apoptotic process may contribute to the immunosuppression seen in sepsis, which may lead to the subsequent multiple organ failure and mortality. In this study, we reported that T cell-deficient mice succumb more rapidly earlier compared with background control mice after the induction of sepsis by CLP. One possible explanation for the higher mortality seen in mice during the early stage of polymicrobial sepsis could be related to the deficit of T cells in the small intestinal IEL compartment, because we observed an increase in IEL T cells taken from control mice. Increased numbers of T cells have been shown to be associated with a variety of infectious conditions (9). Studies have indicated that an accumulation of T cells was found in the lungs of mice with sepsis (23) and pulmonary bacterial infection (44). Nonetheless, T cells seem to have a complex role in host defense because the depletion of these cells has been shown to lead to not only changes in Th1 and Th2-type responses but also can be either detrimental or protective to host survival/infection depending on the specific pathogen nature and stage of infection (28).

Another explanation for the high mortality in septic mice could be related to the apparent inability of these mice to mount an adequate proinflammatory (innate) response. In this respect, one of the most striking differences between T and T mice was that little or no increase in systemic proinflammatory cytokine levels (TNF-α, IL-6, and IL-12) were detected in T mice in response to sepsis. The current paradigm for the pathophysiology of shock/sepsis is that organ injury results from an uncontrolled proinflammatory response (19). Proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 have been suggested to be responsible for the initiation of cell and organ dysfunctions associated with sepsis and multiple organ failure (7). This was based primarily on the observation that shock and death were the most common results when high bacterial toxin and/or monospecific microbe infusions or high...
concentrations of the given cytokine (e.g., TNF, IL-1β, etc.) were given in various animal models. However, when the dosage of these proinflammatory agents was titrated back toward levels actually encountered in septic animals/patients or where the model used produced a somewhat more comparable state to that of the septic patient (e.g., the CLP used here) (14), the extent of proinflammation observed was substantially lower than that seen in models of toxic shock. Furthermore, a number of investigators (6, 17, 21) have demonstrated that ablating key members of this proinflammatory response to septic challenge can actual result in significant harm to the animal, suggesting that γδ T cells may be essential in controlling the inflammatory response. Thus, the depletion of these cells may lead to dysregulation and/or mortality. The failure of antiinflammatory therapeutic trials for sepsis also suggests that the process by which septic patients develop multiple organ failure is far more complex than that produced in simple models of lethal toxic/bacterial shock. Therefore, the early proinflammatory mediator response may reflect the animals’ attempt to contain/respond to the developing infection in CLP and hence may be an important component for survival.

The contribution of αβ T cell receptor+ T cells, let alone γδ T cell receptor+ T cells, to the development of the innate/proinflammatory response is poorly understood, and αβ T cells are, for the most part, thought to play a critical role in the acquired/adaptive response to infection and not innate immunity (27, 43). The impact of γδ T cells on innate and/or adaptive immunity during infection is even less well understood. However, a number of recent studies have appeared to suggest that γδ T cells may play a critical role in the cross-talk between T cells in the development of an adaptive response and macrophages during the induction of innate immunity. Macrophages from γδ−/− mice are defective in TNF-α production, suggesting that a mechanism exists for γδ T cells where cellular components contribute to the regulation of the innate responsiveness (34). Moore et al. (15) reported that γδ T cell deficiency impaired early expression of TNF-α and IFN-γ mRNA, which was associated with increasing susceptibility to Klebsiella pneumoniae infection. γδ T cell depletion has been shown to downregulate chemokine and chemokine receptor expression in a model of experimental autoimmune encephalomyelitis (36). Studies have also suggested that γδ T cells can function as a potent source of cytokines themselves that may stimulate or attract other cells into a site of inflammation (37). In this regard, our results further support the suggestion that γδ T cells may play a critical regulatory role in the development of a competent innate/proinflammatory response. However, the precise nature of the interaction between these cells is still unclear.

Previous studies from many laboratories including our own have indicated that Th1 cytokine release in anti-CD3-stimulated splenocytes is suppressed with enhancement of Th2 cytokine release after the onset of sepsis (2). However, little is known about the mechanism responsible for these changes. Here, we have observed that Th1 cytokine release in splenocytes from septic γδ−/− mice was not suppressed as is typically seen in γδ+/- mice. This suggests at least two things about the immune response seen in γδ−/− animals. First, the restoration of the splenocyte IL-2/IFN-γ response suggests γδ T cells may contribute directly or indirectly to the developing Th1 cell dysfunction. Second, the development of this late immune dysfunction may not be a critical component contributing to the mortality in γδ−/− animals. In this respect, we and others have previously reported that antiinflammatory agents such as IL-10, which can modulate the induction of suppression of the cell-mediated lymphoid immune response, can also have marked effects on the innate/proinflammatory responsiveness (16, 40). We found that when mice were administered a neutralizing antibody to IL-10 immediately after the induction of CLP, a time point at the beginning of the proinflammatory phase in this sepsis model, survival of these animals was markedly reduced early after CLP (42). However, when the antibody was given at 12 h post-CLP, a period just outside the majority of the proinflammatory response but before the marked splenic immune suppression, survival was not only improved but Th1 responsiveness was preserved. This demonstrates that the susceptibility to septic morbidity and mortality can be differentially affected by the nature of the phase of the host response that may predominate at the time of treatment, i.e., early defects in the innate response versus the late development of cell-mediated changes. Thus, we suggest that alterations in the innate response more likely may be affected by this deficiency in γδ T cells, whereas the change in cell-mediated (Th1) responsiveness is a secondary aspect. However, further studies are needed to delineate the effect of these T cells on innate as well as cell-mediated immune responsiveness.

In summary, our findings indicated that a deficiency in γδ T cells may induce early mortality in sepsis. An increase in γδ T cells in small intestinal IELs was observed after sepsis. The extensive decrease in systemic levels of the proinflammatory cytokine release along with the lack of changes in Th1 cytokine release observed in splenic T cells and the attenuation of proinflammatory cytokine release by peritoneal macrophages in septic γδ−/− mice appear to implicate the γδ T cells’ role in communication between adaptive (cell mediated) responsiveness and the innate response mediated by macrophages, granulocytes, epithelial cells, etc. Thus, while accounting for only a small fraction of all mature T cells, γδ T cells appear to play a critical role in maintaining the innate response, which is sufficient to ward off the lethal effects of polymicrobial septic challenge.

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