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E. D. Murphey

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Cecal Ligation and Puncture-Induced Impairment of Innate Immune Function Does Not Occur in the Absence of Caspase-1

E. D. Murphey

Mice that have been subjected to cecal ligation and puncture (CLP) have an impaired ability to clear a subsequent *Pseudomonas aeruginosa* challenge compared with that of sham CLP controls. We hypothesized that this outcome is dependent upon a caspase-1 mechanism and tested this hypothesis by measuring caspase-1 after CLP and by measuring clearance of a bacterial challenge in caspase-1–deficient mice after CLP. Wild-type mice subjected to CLP had increased caspase-1 activity as well as increased IL-1β and increased IL-18 production in splenocytes stimulated with heat-killed *Pseudomonas* and had increased plasma concentrations of IL-1β and IL-18 and impaired clearance of a *P. aeruginosa* challenge compared with sham controls. Healthy, uninjured caspase-$1^{-\backslash -}$ mice did not differ from wild-type mice in their ability to clear a *Pseudomonas* challenge. However, unlike wild-type mice, caspase-$1^{-/-}$ mice subjected to CLP had no impairment of bacterial clearance of the *Pseudomonas* challenge, suggesting that caspase-1 induction after CLP played a role in impairment of bacterial clearance. This was further substantiated by the use of a specific caspase-1 inhibitor, Ac-YVAD-CMK. Wild-type mice treated with Ac-YVAD-CMK (10 mg/kg s.c. twice daily, initiated at time of CLP) did not have impaired clearance of a *Pseudomonas* challenge compared with that of sham mice and had significantly improved bacterial clearance compared with that of untreated CLP mice. Increased caspase-1 expression and activity after CLP injury appears to contribute to diminished innate immune function. ***The Journal of Immunology*, 2011, 187: 905–910.**

Sepsis occurs most commonly as an adverse complication in patients who have suffered major trauma or illness, and it is commonly believed that these patients are predisposed to sepsis because they have impaired immune function secondary to their primary lesion. We have tried to mimic this clinical scenario in our investigations of postinjury immunosuppression by assessing clearance of a bacterial challenge in mice 5 d after cecal ligation and puncture (CLP). Mice subjected to CLP have impaired ability to clear the subsequent bacterial challenge as efficiently as sham control mice, suggesting that the innate immune response was adversely affected by CLP (1, 2). CLP induces activation and mobilization of immune cells to the abdomen and results in local and systemic inflammatory responses. CLP also induces an increase in cellular apoptosis and death, particularly in cells of the lymphocytic lineage (3). Some of these responses have a beneficial role in the immediate response to CLP but are not inducible to the same degree upon secondary immune challenge. Other host responses do not appear necessary for an effective defense against CLP but, in combination with attenuation of the necessary responses, may play a role in diminished innate immune function against secondary immune challenges.

Caspases (cysteinyl aspartate-specific proteinases) are a group of 15 enzymes that are involved in processing proteins critical to major changes in cell state, including nuclear proteins, transcription factors, cell cycle regulators, kinases, and cytoskeletal proteins (4). Caspases have been recognized to play an important role in inflammation and cell death. Caspase-1, also known as IL-1β converting enzyme, is a class I cysteine protease that lies in quiescence in a complex of molecules termed the "inflammasome." Cellular activation by microbial ligands of TLRs leads to a chain reaction resulting in release of caspase-1 from the inflammasome complex. Caspase-1 activity results when the released caspase-1 molecules form dimers that cleave the proforms of IL-1β and IL-18 (IFN-γ–inducing factor) into their mature, active forms (5, 6), and also cleave other proteins involved in glycolysis and chaperone activities (7). Because of this activity, caspase-1 has been categorized as an inflammatory caspase, although activation of caspase-1 was also reported to induce apoptosis in macrophages and fibroblasts (8, 9). Further, although mice deficient in caspase-1 do not have obvious defects in apoptosis, overexpression of caspase-1 can induce cell death (8). Caspase-1–deficient mice are markedly resistant to endotoxic shock (10), presumably due to deficits in secretion of mature IL-1β and IL-18.

The purpose of this study was to determine if caspase-1 was activated in response to CLP and, if so, whether caspase-1 activity played a role in the development of CLP-induced immune suppression.

Materials and Methods

Animal model

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1996) and with the approval of the Institutional Animal Care and Use Committee at the University of Texas Medical Branch. Male mice (6–8 wk) were purchased from The Jackson Laboratory (Bar Harbor, ME) and included C57BL/6J, caspase-1 knockout mice [NOD.129S2(B6)-Casp1^{tm1Sesh}/LtJ], and their corresponding wild-type controls (NOD/ShiLtJ). The animals were allowed to acclimatize for at least 7 d after delivery and were maintained on 12-h light–dark cycles with ad libitum food and water at all times. Cecal ligation was performed as previously described (11) with some modifications. Isoflurane anesthesia (2.5% in 100% O_2) was initiated in an induction chamber and

Department of Anesthesiology, University of Texas Medical Branch, Galveston, TX 77555; and Shriners Burns Institute, University of Texas Medical Branch, Galveston, TX 77555

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Address correspondence and reprint requests to Dr. E. D. Murphey, University of Texas Medical Branch, 301 University Avenue, Galveston, TX 77555-0591. E-mail address: edmurphe@utmb.edu

Abbreviations used in this article: CLP, cecal ligation and puncture; FLICA, fluorochrome inhibitor of caspase-1.

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maintained by delivery through a face mask. After shaving and cleaning the ventral abdominal wall with alcohol, a midline incision was made, and the cecum was exteriorized. Cecal contents were massaged out of the tip and toward the base of the cecum, and the distal 0.5 cm of the tip was ligated with 3-0 silk suture. A 25-gauge needle was used to perforate the ligated portion of the cecum once in a through-and-through manner. Some groups of mice were studied for survival after a more severe model of CLP in which cecal contents were massaged into the cecal apex, 1 cm of the cecal apex was ligated, and a 23-gauge needle was used to perforate the cecum. The cecum was returned to the abdominal cavity, the abdominal wall was closed with 4-0 Vicryl (Ethicon, Somerville, NJ) suture, and the skin was reapposed with cyanoacrylate tissue adhesive. Mice were allowed to recover for 5 d before bacterial challenge. Some groups of mice were treated with the caspase-1 inhibitor Ac-YVAD-CMK (10 mg/kg s.c. twice daily; Bachem, Torrance, CA) starting immediately after CLP and continuing until the *Pseudomonas* challenge. Treatment controls were treated with vehicle (1:1 v/v saline/polyethylene glycol 300) in the same manner.

Bacterial challenge and clearance

Pseudomonas aeruginosa (strain 19660; American Type Culture Collection, Rockville, MD) was inoculated into tryptic soy broth and allowed to replicate overnight in a shaking incubator at 37˚C. The resulting bacterial culture was washed with 10 ml sterile 0.9% saline. Viable numbers of CFUs were determined by plating serial dilutions overnight on tryptic soy agar. Bacteria were suspended in sterile 0.9% saline at a final concentration of 1×10^9 CFU/ml. Mice were challenged with 0.1 ml of this suspension (1×10^8 CFU; i.v.) 5 d after the CLP (or sham) procedure.

The mice were sacrificed under isoflurane 6 h after i.v. injection of *P. aeruginosa*. Lungs, spleens, and liver tissue samples were aseptically excised, weighed, and homogenized in sterile saline (1:10 w/v) using sterile tissue grinders. Serial dilutions of tissue homogenates were plated on tryptic soy agar and incubated overnight at 37˚C. *Pseudomonas* bacterial CFUs were identified by colony color and morphology and were counted to assess bacterial clearance. *Pseudomonas* colony identification was confirmed as necessary by the use of Taxo N Discs (BD, Franklin Lakes, NJ).

Measurement of plasma and cell culture supernatant cytokine concentrations

Commercially available ELISA kits were used to measure plasma concentrations of IFN-γ and IL-10, as well as plasma and cell culture supernatant concentrations of IL-1β (eBioscience, San Diego, CA) and IL-18 (MBL International, Woburn, MA) in cell culture supernatant following the manufacturers' recommended procedures.

Measurement of cell-associated caspase-1 activity by flow cytometry

Caspase-1 activity was measured in splenocytes using the cell-permeable fluorochrome inhibitor of caspase-1 (FLICA) FAM-YVAD-FMK (Immunochemistry Technologies, Bloomington, MN). Spleens were aseptically excised 24, 48, or 120 h after CLP (or sham) and transferred to 6-well culture plates containing RPMI 1640. The tissue was minced and passed through sterile mesh. Erythrocytes were lysed (erythrocyte lysis kit; R&D Systems). The remaining splenocytes were resuspended to a concentration of 1×10^6 cells/ml in PBS and incubated with the FLICA reagent (1:30 dilution) for 1 h at 37˚C. The cells were washed, resuspended, and incubated for 30 min at 4˚C with fluorescence-labeled Abs against cell-surface Ags including anti-F4/80, anti-CD3, anti-CD4, and anti-CD8 (Invitrogen Laboratories, Carlsbad, CA). Isotype-matched Abs were used as controls. Cells were washed with PBS and resuspended in 1% paraformaldehyde before analysis by a FACScan flow cytometer (BD Biosciences, San Jose, CA). Alternatively, some groups of cells were counterstained with propidium iodide after the FLICA incubation and were analyzed on the flow cytometer immediately without fixation. Data were analyzed and graphed by FlowJo software (Tree Star, Ashland, OR).

Splenocyte production of IL-1β and IL-18

Spleens were excised from mice 5 d after CLP and transferred to 6-well culture plates containing RPMI 1640. The tissue was minced and passed through sterile mesh. Erythrocytes were lysed (erythrocyte lysis kit; R&D Systems). The remaining splenocytes were resuspended to a concentration of 1×10^6 cells/ml in RPMI 1640 and incubated with heat-killed *Pseudomonas* (1×10^8 CFU/ml) overnight in 5% CO_2 at 37˚C. Cell supernatants were collected and assayed for IL-1β and IL-18 by ELISA.

Data analysis

Statistical analyses were performed using GraphPad Prism 4 software (GraphPad, La Jolla, CA). All data are presented as mean ± SEM. Multiple group data were analyzed by ANOVA and post hoc Tukey test, and comparisons between two groups were performed by an unpaired *t* test. A p value <0.05 was considered statistically significant.

Results

CLP was associated with a diminished ability to clear a subsequent bacterial challenge

CLP or sham CLP was performed, and the mice were allowed to recover for 5 d. Mice subjected to CLP had overt signs of injury, including piloerection and lethargy for the first 1–2 d, but continued to eat, drink, and maintain near-normal levels of activity afterward. There were no deaths in any of the groups after CLP or sham procedures. On the 5th day after CLP or sham CLP, the mice were anesthetized briefly and subjected to an i.v. challenge of live *P. aeruginosa* (or saline control). The mice were sacrificed 6 h later for collection of tissues. Five days after CLP, the site of the cecal puncture was typically walled off by abscess formation, and there was little to no bacterial growth on culture of the spleens from mice that were not subjected to further bacterial challenge. The small amount of other bacteria cultured from the spleens of mice subjected to CLP were mostly Gram-negative rods and occasional Gram-positive cocci. No *Pseudomonas* colonies were identified in spleens of mice that were not challenged with *Pseudomonas*. Mice that had been subjected to CLP and were subsequently challenged with *Pseudomonas* had an increased number of *Pseudomonas* CFUs in spleen tissue samples compared with that of sham control mice (Fig. 1).

CLP was associated with increased caspase-1 activity in splenocytes in both macrophage and granulocyte cell populations

Caspase-1 activity was determined in intact cells from spleen tissue using a cell-permeable caspase-1 inhibitor fluorochrome, and these results were further extended by examining specific cell populations for higher levels of caspase-1 activity. Caspase-1 activity was found to be higher in splenocytes of mice 24 h after CLP compared with that in splenocytes from sham mice (Fig. 2). When concurrently stained with cell surface markers, it was apparent that both macrophages ($F4/80^+$) and granulocytes ($Ly6\text{-}G^+$) had

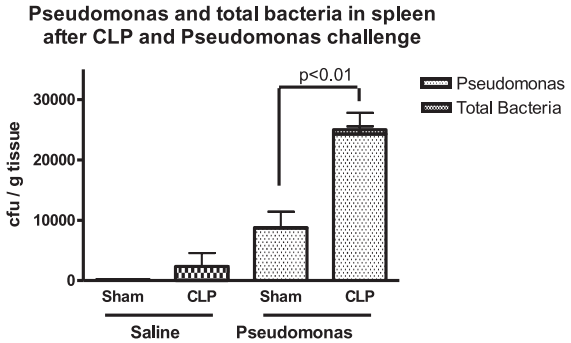

FIGURE 1. CLP was associated with a diminished ability to clear bacterial challenge. Mice challenged with live *P. aeruginosa* (or saline control) 5 d after CLP were sacrificed 6 h later, and spleen homogenates were plated and cultured overnight to measure bacterial growth. CLP was associated with a diminished ability to clear the *Pseudomonas* challenge compared with that of sham controls. Mice subjected to CLP or sham procedures and sacrificed 5 d later with no *Pseudomonas* challenge had little to no bacterial growth on culture of their spleen. n = 7–9/group.

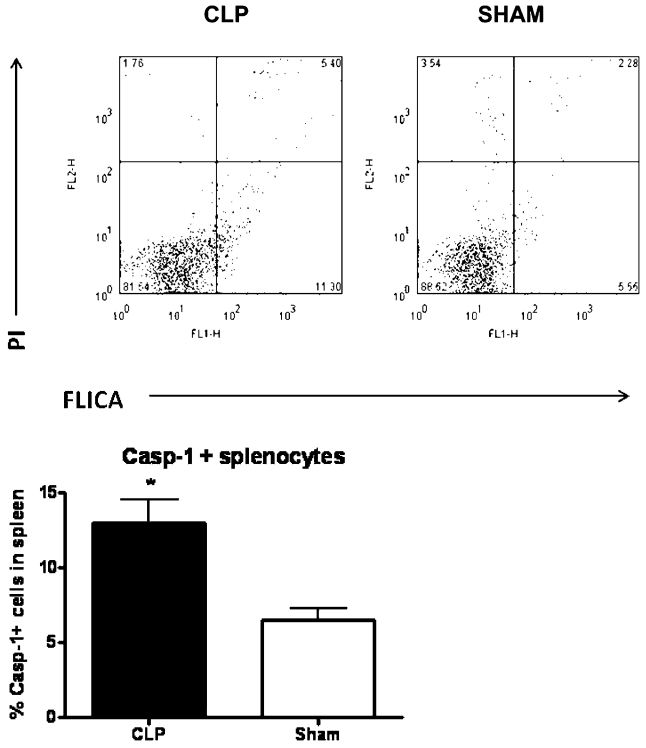

FIGURE 2. Caspase-1 activity was increased in splenocytes 24 h after CLP. FLICA was used to detect caspase-1 activity in splenocytes harvested from mice 24 h after CLP (*left*) or sham (*right*) procedures. The *top panels* shows propidium iodide leakage into dead cells (*upper quadrants*) and FLICA activation (*right quadrants*) by caspase-1 in the total splenocyte population. The *bottom panel* shows caspase-1 activity in splenocytes derived from mice after CLP or sham surgery. n = 7/group. *$p < 0.05$.

a higher level of activity of caspase-1 in mice that had been subjected to CLP (Fig. 3).

CLP primed for increased production of IL-1β and IL-18 responses to a secondary stimulus

Transcription and translation of proforms of IL-1β and IL-18 occur in response to TLR stimulation, but caspase-1 activity is necessary to cleave the proforms resulting in the secretion of mature, active IL-1β and IL-18. Therefore, caspase-1 activity may also be indirectly reflected by measurement of IL-1β and IL-18. The *top panels* of Fig. 4 show that plasma concentrations of IL-1β and IL-18 were elevated in post-CLP mice 6 h after *Pseudomonas* challenge compared with those of shams. The *bottom panels* of Fig. 4 show that CLP mice had increased induction of splenocyte production of IL-1β and IL-18 compared with that in sham mice. Splenocytes were collected 24 h after CLP or sham and incubated in culture media overnight in the presence of heat-killed *Pseudomonas*. IL-1β and IL-18 were measured to be significantly higher in cultures of cells collected from post-CLP mice compared with those from sham CLP control mice.

CLP-induced impairment of Pseudomonas *clearance did not occur in the absence of caspase-1*

To test directly whether induction of caspase-1 activity was involved in the development of post-CLP immunosuppression, caspase-1 knockout mice and their respective wild-type controls were subjected to CLP (or sham CLP) and challenged 5 d later with *P. aeruginosa.* As in the C57BL6 mice used in the aforementioned experiments, the wild-type control mice strain (NOD/ShiLtJ) had diminished ability to clear the bacterial challenge after being subjected to CLP compared with that of sham CLP mice. In contrast, there did not appear to be any adverse effect of CLP on subsequent bacterial clearance in the absence of caspase-1 activity, as caspase-1 knockout mice cleared the bacterial challenge as readily as mice subjected to sham CLP (Fig. 5, *top panel*). To test whether the difference in bacterial clearance may have been due to an attenuation of the CLP-induced injury in the absence of caspase-1, wild-type and caspase-1 knockout mice were subjected to a highly lethal model of CLP and monitored for 5 d without further challenge. There was no significant difference in survival in caspase-1–deficient mice after CLP alone (7 of 13 versus 5 of 14 in wild-type mice, $p = 0.44$). To confirm further the role that caspase-1 induction by CLP had in our investigations and to test the possibility of targeting capsase-1 therapeutically, mice were subjected to CLP and treated with a specific caspase-1 inhibitor (Ac-YVAD-CMK; N-1330) prior to *Pseudomonas* challenge. The results (Fig. 5, *bottom panel*) show that mice treated with the caspase-1 inhibitor had an attenuation of CLP-induced impairment of bacterial clearance, with treated CLP mice having significantly less *Pseudomonas* bacterial growth than that in nontreated CLP mice and equivalent to that seen in sham control mice challenged with *Pseudomonas.*

Depletion of caspase-1 activity was associated with smaller but similar changes in plasma IFN-γ and IL-10 as that seen in wild-type mice after CLP

To determine whether induction of caspase-1 activity had any effect on the inflammatory cytokine profile in post-CLP mice challenged with *Pseudomonas*, blood samples were collected 6 h after the *Pseudomonas* challenge for measurement of plasma cytokine concentrations. Wild-type mice subjected to CLP had an attenuation of IFN-γ and an exaggeration of IL-10 responses to the *Pseudomonas* challenge compared with those of the sham control mice. Caspase-1 knockout mice subjected to CLP had a similar change in the pattern of cytokine response (i.e., lower IFN-γ and higher IL-10) compared with that of sham control caspase-1

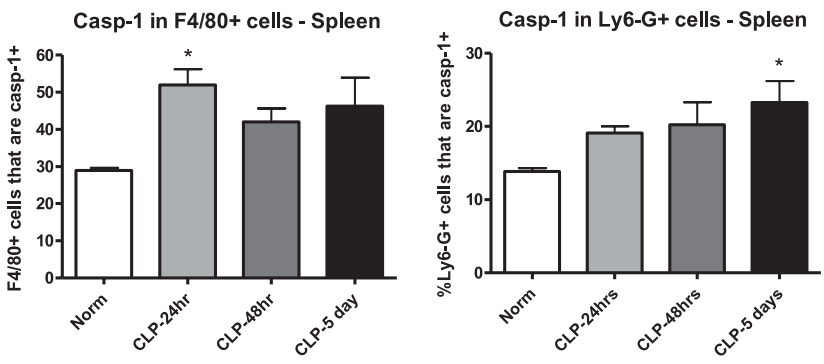

FIGURE 3. Caspase-1 activity was increased in both macrophages and granulocytes after CLP. Spleen tissues were collected at 24 h, 48 h, or 5 d after CLP. Caspase-1 activity was measured by the FLICA assay in both macrophages ($F4/80^+$ cells) and granulocytes ($Ly6\text{-}G^+$ cells). n = 4–5/group. *$p < 0.05$.

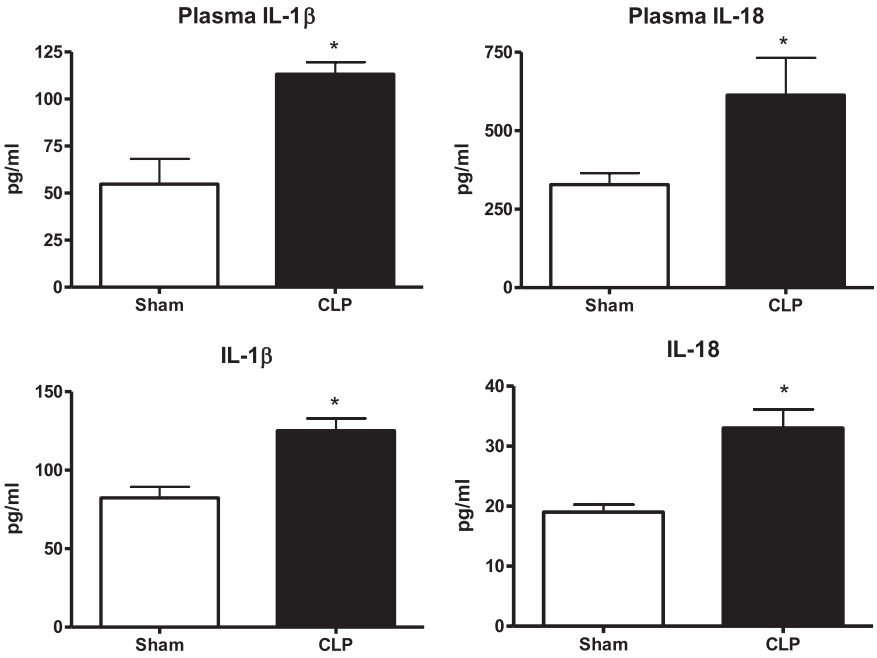

FIGURE 4. Post-CLP mice had increased IL-1β and IL-18 responses to the subsequent *Pseudomonas* challenge. Mice were subjected to CLP and allowed to recover for 5 d. The *top panels* demonstrate the higher plasma concentrations of IL-1β and IL-18 in post-CLP mice 6 h after challenge with live *P. aeruginosa*. The *bottom panels* show that splenocytes derived from mice 5 d after CLP and cultured in the presence of heat-killed *Pseudomonas* had higher production of IL-1β and IL-18 than that of splenocytes from sham mice. n = 11/group for IL-1β, n = 4/group for IL-18. $*p < 0.05$.

knockout mice, although the concentrations of those cytokines were ~4 times less than that seen in the wild-type mice. IL-1β and IL-18, which were higher in post-CLP wild-type mice compared with those in sham wild-type mice, were not detectable in mice deficient in caspase-1 (Fig. 6).

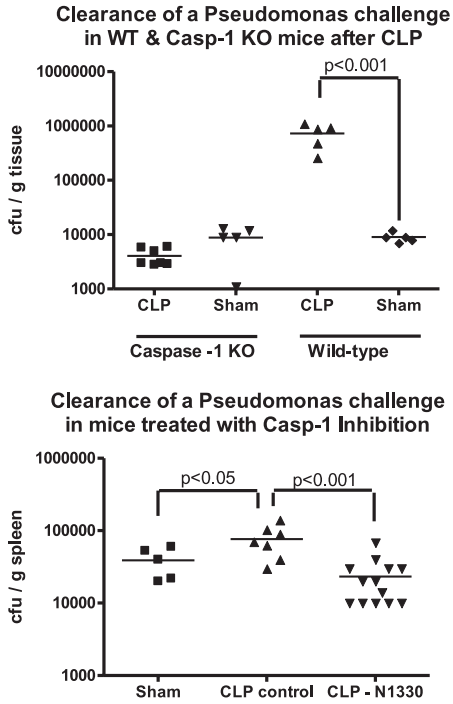

FIGURE 5. Mice deficient in caspase-1 activity did not demonstrate CLP-induced impairment of clearance of a subsequent *Pseudomonas* challenge. Wild-type mice subjected to CLP had higher numbers of *Pseudomonas* CFUs in spleen tissue 6 h after a *Pseudomonas* challenge than those of mice subjected to sham procedures. However, caspase-1 knockout mice subjected to CLP did not differ from sham mice in the amount of *Pseudomonas* isolated from spleen tissue after a *Pseudomonas* challenge (*top panel*). Wild-type mice treated with a specific caspase-1 inhibitor after CLP had less *Pseudomonas* bacterial growth in spleen tissue compared with that of nontreated CLP mice and did not differ significantly from that of the sham control mice (*bottom panel*). n = 5–13/group.

Discussion

Sepsis occurs most commonly as an adverse complication in patients who have suffered major trauma or major illness, and it is commonly believed that these patients are predisposed to sepsis because they have impaired immune function secondary to their primary lesion. That belief is supported by the fact that many of the bacteria isolated from septic patients are not typically virulent in people with competent immune function. Sepsis is thought to develop when an initial systemic proinflammatory response is followed by a sustained anti-inflammatory state concurrent with immunoparalysis (12–19). Mortality can occur during the proinflammatory phase, such as in patients with meningococcemia or toxic-shock syndrome (16, 20); however, most septic patients survive the initial inflammatory response and enter a prolonged immunosuppressive state in which they are unable to resolve their primary infection or continue to develop new secondary infections.

We have modeled that paradigm in mice by using CLP to induce a major injury and initial systemic proinflammatory response. When the mice are challenged with *Pseudomonas* 5 d later, compromise of innate immune function is revealed by diminished ability of the mice to clear the bacterial challenge compared with that of uninjured (sham) controls. Caspase-1 propagates inflammation in that it does not appear to be part of an apoptotic mechanism but contributes to the inflammatory cytokine response by cleaving proforms of IL-1β and IL-18 to their mature, active forms. In this study, we showed that caspase-1 activity was higher in wild-type mice that had been subjected to CLP compared with that of tissues from sham control mice. We also demonstrated that CLP primed for increased production of IL-1β and IL-18 in response to subsequent challenges, both of which require caspase-1 activity for cleavage and release from precursor forms. These findings are consistent with a recent article reporting that circulating concentrations of caspase-1 and IL-18 were higher in septic patients (21).

We further demonstrated that bacterial clearance was adversely affected in wild-type mice subjected to CLP but not in caspase-1–deficient mice subjected to CLP, suggesting that caspase-1

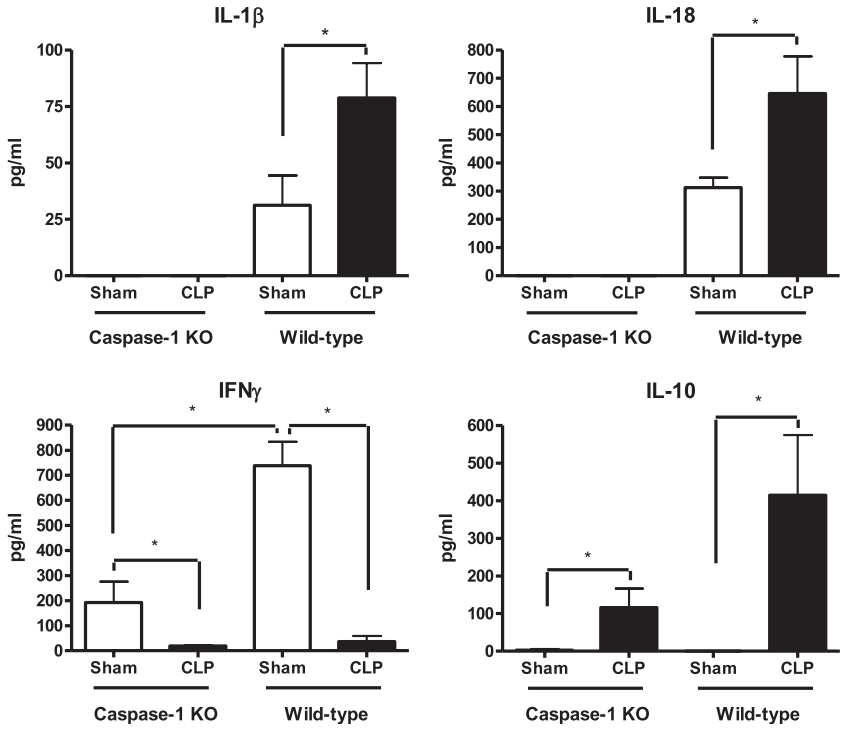

FIGURE 6. Mice deficient in caspase-1 activity had no detectable IL-1β or IL-18 but had similar changes in the serum IFN-γ and IL-10 cytokine response to a *Pseudomonas* challenge as those seen in wild-type mice after CLP. Caspase-1 knockout mice subjected to CLP had no detectable circulating concentrations of caspase-1–dependent IL-1β or IL-18 in response to a subsequent *Pseudomonas* challenge. Both caspase-1 knockout mice and wild-type mice that were subjected to CLP had similar suppression of the IFN-γ response and exacerbation of the IL-10 response to a subsequent *Pseudomonas* challenge compared with those responses in sham mice. n = 5–7/group. *$p < 0.05$.

activation in response to injury plays a mechanistic role in the impairment of subsequent innate immune function. This was not due to a baseline difference in innate immune function, as the absence of caspase-1 activity did not affect the ability of healthy, uninjured caspase-1 knockout mice to clear the *Pseudomonas* challenge. Nor does it seem likely to be due to a more severe injury after CLP, as there was no difference in mortality of capase-1–deficient mice compared with that of wild-type mice after each group was subjected to a more severe CLP model. The use of a caspase-1–specific inhibitor further confirmed those results and also suggests the therapeutic potential that caspase-1 inhibition might have for the prevention of postinjury immunosuppression. Other recent studies have confirmed the role of caspase-1 in the proinflammatory process and hinted at the adverse effect caspase-1 activity might have in the immune process. Caspase-1–deficient mice are markedly resistant to endotoxic shock (10), presumably due to deficits in maturation of IL-1β and IL-18. When a caspase-1 inhibitor was nebulized into the lungs of rats subjected to i.v. infusion of LPS, the rats had a decreased IL-1β and IL-18 cytokine response both in the lungs and systemically as well as decreased expression of inducible NO synthase and cyclooxygenase-2 in the lung tissue (22). Caspase-1–deficient mice were resistant to *Escherichia coli*-induced peritonitis in another study compared with both wild-type and IL-1β knockout mice (23).

Recent investigations have reported an improvement in survival after CLP in animals treated with broad caspase inhibitors and associated those results with diminished inflammation and apoptosis (24, 25). It is not clear how the specific depletion of caspase-1 activity prevented the decline in innate immune function in mice subjected to CLP. We have not been able to demonstrate any difference in cell death in post-CLP mice compared with that in sham controls, most likely because our model of CLP is less severe than that used by other investigators (24, 25). Therefore, this seems to rule out attenuation of cell death in the absence of caspase-1 as a mechanism for our findings. Nor did it appear that the altered ability to clear the bacterial challenge could be explained by an attenuation of the severity of the CLP-induced injury in caspase-1–deficient mice, as caspase-$1^{-/-}$ mice had a similar level of mortality as that of wild-type mice when subjected to a more severe model of CLP. Caspase-1 knockout mice did have a subdued IFN-γ response to the *Pseudomonas* challenge, which is likely due to the impaired release of IL-18, a cytokine that contributes to induction of IFN-γ (26). Although circulating concentrations of IL-10 were elevated in caspase-1 knockout mice subjected to CLP compared with those in sham caspase-1 knockout mice, the serum concentrations were lower than those measured in wild-type mice subjected to CLP. Thus, the anti-inflammatory cytokine balance was not as pronounced in the absence of caspase-1, and perhaps this contributed to the attenuation of impaired immune function in these mice. We also showed that CLP was followed by increased levels of caspase-1 activity. Caspase-1 levels were elevated in macrophages and neutrophils, two cell populations that would be expected to play a primary role in the innate immune response to the *Pseudomonas* challenge.

Although administration of several TLR agonists such as LPS can induce caspase-1 activation (27, 28), TLR signaling does not seem to be required for the activation of caspase-1. Neither TLR2 nor TLR4 were necessary for caspase-1 activation in response to heat-killed Gram-negative or Gram-positive bacteria (29). *P. aeruginosa* seems to induce caspase-1 independently of TLR5 by introduction of flagellin into the cytosol through a type III secretion system (30, 31). However, there are reports that *Pseudomonas* may also activate caspase-1 by a flagellin-independent pathway (32). Consistent with that, infection of macrophages with flagellin-deficient *Pseudomonas* induced activation of caspase-1, suggesting an alternate pathway of *Pseudomonas*-induced caspase-1 activation (30).

The current findings suggest that caspase-1 may play a role in the development of sepsis secondary to a major injury. Selective depletion of caspase-1 did not seem to adversely affect immune competence in uninjured mice and attenuated a decline in immune competence in injured mice. Although caspase-1 may provide a potential therapeutic target in patients at high risk for

development of sepsis, further work is necessary to determine if treatments against caspase-1 would be effective in patients in which sepsis is already established.

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Disclosures

The author has no financial conflicts of interest.

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