Caspase-1 Regulates *Escherichia coli* Sepsis and Splenic B Cell Apoptosis Independently of Interleukin-1 β and Interleukin-18

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Rationale: Caspase-1 processes interleukin 1 β (IL-1 β) and IL-18 but may also contribute to apoptosis. In this context, caspase-1 knockout mice have been shown to be protected from endotoxin-induced mortality, whereas IL-1 β knockout mice are not protected.

Objectives: We therefore sought to delineate the mechanisms responsible for the differential responses between caspase-1 and IL-1 β knockout mice.

Methods: Caspase-1 knockout, IL-1 β knockout, and IL-1 β /IL-18 double knockout mice were compared with wild-type mice for survival after intraperitoneal challenge with live *Escherichia coli*.

Measurements and Main Results: Caspase-1 knockout animals were protected from bacterial challenge, whereas wild-type, IL-1 β knockout, and IL-1 β /IL-18 double knockout animals were not. Wild-type animals and both IL-1 β knockout and IL-1 β /IL-18 double knockout mice demonstrated significant splenic B lymphocyte apoptosis, which was absent in the caspase-1 knockout mice. Importantly, IL-1 β /IL-18 double knockout mice were protected from splenic cell apoptosis and sepsis-induced mortality by the caspase inhibitor zVAD-fmk. Furthermore, wild-type but not caspase-1 knockout splenic B lymphocytes induced peritoneal macrophages to assume an inhibitory phenotype.

Conclusion: Taken together, these findings suggest that caspase-1 is important in the host response to sepsis at least in part via its ability to regulate sepsis-induced splenic cell apoptosis.

Keywords: apoptosis; caspase inhibition; septic shock; spleen

More than 500,000 people develop sepsis annually and 175,000 of them die in the United States alone (1). Septic shock activates numerous proinflammatory mediators, which can result in multiple organ injury (2, 3). In addition, executioner cysteine-aspartate proteases (caspases) play a key role in the disassembly of cells during septic shock via various proapoptotic stimuli. Pharmacologic blockade of caspase activation improves organ function and survival in animal models of sepsis and ischemia reperfusion injury (4). Interleukin 1 β (IL-1 β) is one of the major proinflammatory cytokines known to be produced in sepsis (5–8). It is synthesized as an inactive 31-kD precursor that requires a unique cysteine protease, IL-1 β -converting enzyme (caspase-1), to generate biologically active 17-kD IL-1 β (9, 10).

Although caspase-1 plays no part in the spontaneous apoptosis of monocytes and macrophages (11), its activation via intracellular pathogens can induce macrophage apoptosis (12, 13) and its deletion has been linked to survival in animal models of endotoxin shock (14). This protective effect could logically be attributed to caspase-1's role in activating the precursor, pro-IL-1 β . Unexpectedly, however, active IL-1 β does not regulate survival from endotoxin shock, because IL-1 β knockout animals are not protected from endotoxin-induced death (15). This difference may hold an important key to understanding the role of caspase-1 in host responses. Importantly, prior caspase-1 knockout experiments have not analyzed the apoptotic role of caspase-1 in sepsis. Furthermore, it is important to expand the model to a live bacterial challenge because IL-1 β may be critical to coordinating the more complex host eradication of pathogens (16, 17).

The present study was designed to determine the mechanisms responsible for the caspase-1 knockout protection from the sepsis response to live intraperitoneal *Escherichia coli* injections. Mice were genetically deficient in caspase-1, IL-1 β , or IL-1 β and IL-18 or pharmacologically deficient in functional caspase-1. Our studies confirm for the first time that the reported differences in survival between caspase-1 and IL-1 β knockout animals are translatable to complex live-infection models of sepsis. We show that the protection is unique to caspase-1 and not IL-1 β or IL-18. Furthermore, we show that, although the IL-1 β /IL-18 double knockout mice are not protected from the *E. coli* challenge, these mice are protected by a synthetic caspase inhibitor. We also show that the caspase-1 knockout state or the use of a synthetic caspase inhibitor prevents splenic B cell apoptosis. Finally, we demonstrate that apoptotic splenic B lymphocytes induce macrophages to assume an inhibitory phenotype. These results are particularly relevant because they support the hypothesis that inhibition of apoptosis can promote sepsis survival (18) and that caspase-1 may be a critical determinant of the apoptosis response in sepsis. Some of the results of these studies have been previously reported in the form of an abstract (19).

METHODS

Mice

All animal experiments performed were done according to animal protocols approved by the Animal Care Use Committee of the Ohio State University College of Medicine. Caspase-1 knockout (caspase-1 $^{-/-}$) and IL-1 β knockout (IL-1 β $^{-/-}$) mice of B10.RIII background were generated by Merck Research Laboratory (Rahway, NJ) (20). IL-1 β /IL-18 double knockout mice of C57Bl6 background were obtained from Dr. A. Zychlinsky, Max Planck Institute, Berlin (authorized by Dr. S. Akira, Japan). Age-matched control mice were purchased from Jackson Laboratory (Bar Harbor, ME).

Genotyping of Mice

Based on the maps of the wild-type and caspase-1, IL-1 β , and IL-1 β /IL-18 knockout mice, primers were designed for genotyping. Before

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each experiment, polymerase chain reaction of tail snip DNA was performed to identify the specific genotypes. Details of primer designs are provided in the online supplement.

Mouse Sepsis Survival

Mice weighing 17 to 20 g were injected intraperitoneally with live *E. coli* BL21DE3 strain at a dose of 5×10^8 cfu/kg (based on our prior finding that the LD₅₀ for wild-type mice was 10^8 cfu/kg) or saline as control. Viability and accuracy of bacterial dosing were confirmed by repeat colony counts at the time of injection. In experiments involving caspase inhibitors, mice were injected first with bacteria or saline and then the caspase inhibitor was injected intraperitoneally 90 min after bacterial injection and then every 12 h for 96 h. The survival rates of the mice were monitored every 6 h for 7 d.

Histology and Apoptosis Quantification

Spleen and lung were collected from experimental mice and subjected to hematoxylin and eosin staining. Apoptotic bodies in the spleens were quantified both by light microscopy and read by a blinded observer with randomized samples and by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining. B and T cells from spleen sections were also immunostained using CD79 and CD3 antibodies, respectively (BD Bioscience, San Jose, CA). Details of histopathology, immunostaining, and apoptosis quantification are provided in the online supplement.

Caspase-3 and Caspase-1 Assay

Caspase-3 was measured as a marker for apoptotic death in the spleens, as previously described (11, 21). Details of assay method are provided in the online supplement.

Murine Macrophage Isolation and IL-1 β Release

Peritoneal macrophages cells were plated at 10^6 /ml and stimulated with either LPS (1 μ g/ml) alone or LPS (1 μ g/ml) for the indicated period followed by a 30-min pulse with ATP (sodium salt; 5mM) in 5% CO₂ at 37°C and analyzed for IL-1 β release. Details are provided in the online supplement.

Lymphocyte Isolation

B and T cells were isolated from spleens using positive selection by CD19 and CD90 from Miltenyi Biotec (Auburn, CA), following the manufacturer's protocol. The isolated B and T cells were then confirmed by flow cytometry analysis.

Murine Cytokine Measurement

Cytokine levels (IL-1 β , IL-18, IL-6, tumor necrosis factor α [TNF- α], and transforming growth factor β [TGF- β]) were measured by both ELISA and immunoblot as detailed in the online supplement.

Statistical Analysis

Data are represented as the mean $\pm$ SEM from at least three independent experiments. Differences in group survival were analyzed using a log rank test (Prism4 Graphical Software, Inc., San Diego, CA). All other simple comparisons were performed with Student's *t* test, with *p* < 0.05 considered to represent statistical significance.

RESULTS

Caspase-1 Knockout Mice Are Resistant to Live Bacterial Challenge

To investigate the effect of septic shock by live bacteria in our murine model, caspase-1 wild-type (+/+), heterozygote (+/-), and knockout littermates (-/-) (*n* = 9–10/group) were injected intraperitoneally with 5×10^8 cfu/kg live *E. coli* or saline and

monitored for survival (Figure 1A). In the live model after 3 d of sepsis, only 2 of 9 caspase-1^{+/+} mice survived compared with 7 of 9 caspase-1^{+/-} and 10 of 10 caspase-1^{-/-} mice (< 0.001 for caspase^{-/-} vs. wild types). After 7 d, none of the wild-type animals but all the knockout mice survived. All the saline control animals survived (data not shown). The caspase-1 heterozygotes showed an intermediate trend for survival. All the mice demonstrated signs of illness after bacterial injection, including marked lethargy, hair ruffling, and piloerection between 12 and 48 h.

Knockout mice had been previously confirmed to have a functional deficit in caspase-1 function as demonstrated by *in vitro* activation of peritoneal macrophages (Figure 1B). Peritoneal macrophages from untreated wild-type animals released IL-1 β abundantly in response to *in vitro* priming with endotoxin followed by induction of IL-1 β processing and release by exogenous ATP. In contrast, caspase-1 knockout macrophages were markedly impaired in this response, providing further confirmation of the functional deficiency of caspase-1 in our knockout animals.

To further address the issue that this sepsis model truly reflected live bacterial challenge and not simply endotoxin, spleen, lung, and liver were collected from both wild-type and knockout mice challenged with live *E. coli* (5×10^8 cfu/kg) or saline as control, and bacterial loads were counted at 4, 12 and 24 h after injection. Live bacteria were detected in all three organs at approximately $1\text{--}5 \times 10^6$ cfu/g tissue in livers and spleens and $3\text{--}9 \times 10^5$ cfu/g lung at both 4 and 12 h postinjection from both wild-type and knockout mice. Bacterial numbers decreased minimally at 24 h in the wild-type animals but fell approximately a log-fold in the caspase-1 knockout animals (data not shown).

Plasma cytokine analyses of wild-type and caspase-1^{-/-} animals documented the release of IL-6 and TNF- α in septic animals but the absence of IL-1 β and IL-18 release with the knockout of caspase-1 (Table 1).

Splenocyte Apoptosis Is Diminished in Caspase-1 Knockout Mice

To investigate the histopathologic difference between the wild-type and knockout animals, sepsis experiments were repeated, harvesting organs at selected time points after intraperitoneal injection of *E. coli*. Wild-type animals demonstrated increased alveolar wall swelling and inflammatory cell accumulation in the lungs but liver and kidneys were not histologically different from knockout animals. However, notably, wild-type animals demonstrated marked apoptosis in their splenic germinal centers, with destruction of normal splenic architecture visible on low power and numerous apoptotic bodies visible on higher power light microscopy. Apoptosis was confirmed by TUNEL assay (Figure 1C). Spleens from caspase-1^{-/-} mice showed significantly less apoptosis than the caspase-1^{+/+} mice (Figure 1D). Splenic cell apoptosis was also confirmed through the measurement of caspase-3 activation, with caspase-1^{+/+} animals demonstrating a 16-fold increase in caspase-3 activity and only a 2-fold increase in the caspase-1^{-/-} spleens.

IL-1 β Knockout and IL-1 β / IL-18 Double Knockout Mice Are Sensitive to Bacterial Challenge

Because a principal function of caspase-1 is to activate pro-IL-1 β to functional IL-1 β , we first tested whether IL-1 β knockout mice are similarly protected from live bacteria-induced sepsis. IL-1 β wild-type (+/+; *n* = 7), heterozygote (+/-; *n* = 5), and knockout (-/-; *n* = 7) mice were injected intraperitoneally with live *E. coli* (5×10^8 cfu/kg) or saline (as control), and survival was monitored. In contrast to the effect seen with caspase-1 knockout animals, there was no significant difference between the survival rates of IL-1 β knockout animals or wild-type littermates (Figure 2A). Splenic histology from the

Figure 1. Effect of caspase-1 knockout on response to intraperitoneal *Escherichia coli*. (A) Survival curves. Caspase-1-deficient ($-/-$; $n = 9$), heterozygote ($+/-$; $n = 9$), and wild-type ($+/+$; $n = 10$) littermates were injected intraperitoneally with 5×10^8 cfu/kg live *E. coli* or saline and monitored for survival ($p < 0.001$, $-/-$ vs. $+/+$). (B) Interleukin (IL)-1 β processing and release. Thioglycolate-induced macrophages obtained from caspase-1 wild-type ($n = 3$) and caspase-1 knockout ($n = 3$) mice were left unstimulated or stimulated with LPS ($1 \mu\text{g/ml}$) for total of 3.5 h. In one group, ATP (5 mM) was added for the final 30 min. Supernatants were analyzed for mature IL-1 β by ELISA and immunoblot (pro-IL-1 β , 31 kD ; mature IL-1 β , 17 kD ; inset). (C) Splenic cell apoptosis. Live *E. coli* (5×10^8 cfu/kg) or saline was injected intraperitoneally into caspase-1 $^{+/+}$ and caspase-1 $^{-/-}$ mice, and animals were killed at 0, 4, 12 and 24 h, and spleens harvested. Histology of hematoxylin and eosin (H&E)-stained spleens by light microscopy at $10\times$ and $100\times$ (data shown for 24 h). Sections were further stained by terminal deoxynucleotidyl transferase nick end labeling (TUNEL) staining. Representative $40\times$ micrographs are shown in lower panel. (D) Apoptosis quantification. Splenic apoptotic bodies are expressed as numbers of apoptotic bodies per high power field (hpf) counted by H&E (data = mean $\pm$ SEM). * $p < 0.001$ for wild type at 12 h versus all knockout time points; $^{\psi}p < 0.001$ for caspase-1 $^{+/+}$ at 24 h versus caspase-1 $^{-/-}$ all time points *E. coli* group, *t* test.

bacteria-challenged wild-type and the IL-1 β knockout mice showed similar numbers of apoptotic bodies (Figures 2B and 2C).

Because the other product of caspase-1 activation is functional IL-18, we performed similar experiments with animals that contained the knockouts of both known substrates of caspase-1, IL-1 β , and IL-18. IL-1 β /IL-18 double knockout mice ($n = 10$) and age-matched control animals ($n = 9$) were injected intraperitoneally with *E. coli* (5×10^8 cfu/kg) or saline (as control), and survival was monitored. IL-1 β /IL-18 double knockout mice showed no difference in survival compared with wild-type animals (Figure 2D). Histopathologic studies on the spleens of the wild-type and IL-1 β /IL-18 double knockout mice also showed no differences in apoptosis (Figures 2E and 2F). The proinflammatory cytokines IL-1 β , IL-18, IL-6, and TNF- α were measured from the plasma of both bacterially challenged and saline-injected mice. IL-1 β and IL-18 were abundant in the wild-type plasma but undetectable in the double knockouts, at 12 h after bacterial challenge (Table 1). There was no significant difference in the amounts of IL-6 and TNF- α in the plasma of wild-type or double knockout mice.

IL-1 β / IL-18 Double Knockout Mice Are Protected by Synthetic Caspase Inhibition

Because caspase-1 $^{-/-}$ mice are protected from *E. coli* sepsis-induced death but IL-1 β $^{-/-}$ and IL-1 β /IL-18 double knockout mice are not, we hypothesized that the protective effect of the

caspase-1 deficiency was due to its ability to prevent apoptosis independent of the IL-1 and IL-18 processing. We therefore asked whether the IL-1 β /IL-18 double knockout mice would be protected from septic death by pharmacologically inhibiting caspase-1 function with the pancaspase inhibitor zVAD. As demonstrated in Figure 3, zVAD-fmk but not the zFA-fmk inhibitor control, simulated the effect of the caspase-1 $^{-/-}$ state. Not only did zVAD protect against mortality but it also prevented splenic cell apoptosis without affecting TNF- α release. Inhibition of caspase-1 by zVAD-fmk was confirmed by measuring caspase-1 activity in spleen cell lysates (data not shown). Thus, in summary, the protection provided by caspase-1 $^{-/-}$ cannot be duplicated by deletions of the two key substrates of caspase-1, IL-1 β and IL-18 but can be replicated by inhibiting caspase-1 function with a synthetic pancaspase inhibitor.

Apoptotic B Cells Induce Suppressive Macrophage Phenotype

To address the significance of the caspase-1-mediated splenic cell apoptosis, spleens from wild-type and caspase-1 knockout animals were further characterized. Using immunohistochemistry, the apoptotic foci that developed in response to the *E. coli* sepsis were demonstrated to be B lymphocytes (Figure 4A). In an effort

TABLE 1. PLASMA CYTOKINE LEVELS AFTER LIVE *Escherichia coli* PERITONEAL SEPSIS

Cytokine	Time (h)	Gene Deletion Model*					
		Caspase-1			IL-1 β /IL-18		
		+/+	-/-	p Value	+/+	-/-	p Value
IL-1 β	12	1.4 $\pm$ 0.17	0.13 $\pm$ 0.02	0.001	0.9 $\pm$ 0.14	0.095	†
IL-18	12	0.8 $\pm$ 0.08	0.06 $\pm$ 0.01	0.002	0.64	0.056	†
TNF- α	1.5	1.4 $\pm$ 0.15	1.2 $\pm$ 0.11	0.06	1.7 $\pm$ 0.01	1.5 $\pm$ 0.01	0.05
	3	0.49 $\pm$ 0.10	0.39 $\pm$ 0.11	0.55	0.45 $\pm$ 0.01	0.6	†
	12	ND	ND	†	0.02	0.02	†
IL-6	1.5	0.24 $\pm$ 0.02	0.08 $\pm$ 0.02	0.002	0.3 $\pm$ 0.05	0.3 $\pm$ 0.06	0.06
	3	0.42 $\pm$ 0.09	0.2 $\pm$ 0.10	0.003	0.70	0.67	†
	12	0.2 $\pm$ 0.07	0.05 $\pm$ 0.03	0.0008	0.22	0.24	†

Definition of abbreviations: IL = interleukin; ND = not detectable; TNF = tumor necrosis factor.

* Caspase-1 wild-type (+/+), caspase-1-deficient (-/-), and IL-1 β /IL-18 wild-type (+/+) and knockout (-/-) mice were injected intraperitoneally with live *E. coli* (5×10^8 cfu/kg), and plasma was collected at 1.5, 3, and 12 h after injection and measured for demonstrated cytokines by ELISA and expressed as ng/ml.

† Samples were done in duplicate.

to understand the significance of the apoptotic B lymphocytes, splenic cells were fractionated into B and T lymphocytes from wild-type and caspase-1 knockout spleens harvested 12 h after *E. coli* peritonitis. These lymphocyte subsets were confirmed to be more than 98% pure by flow cytometry using CD3 and CD19 markers (Figure 4B). Of note, the percentage of annexin V-positive cells from CD19- and CD3-purified splenocytes of septic wild-type mice was 57 vs. 2%, respectively. When B lymphocytes from septic animals were added back to wild-type peritoneal macrophages from control animals, only the B lymphocytes (i.e., apoptotic B cells) from wild-type animals induced increases in TGF- β production (Figure 4C). These findings suggest that apoptotic B lymphocytes may change the function of macrophages. Clearance of apoptotic B lymphocytes may suppress the host's ability to clear infections during sepsis, thus providing a potential connection between caspase-1 deficiency and survival in this model.

DISCUSSION

Although it has been previously shown that the caspase-1^{-/-} state protects animals from endotoxin-induced mortality (14), it has been controversial as to whether caspase inhibition can be translated to live bacteria-induced sepsis because there is support for the notion that IL-1 β and IL-18 may be necessary for bacterial clearance (16, 22). This current work extends our understanding of the role of caspase-1 in sepsis by comparing caspase-1^{-/-} animals with IL-1 β ^{-/-} and IL-1 β /IL-18 double knockout animals for survival to an intraperitoneal challenge with live *E. coli*. We demonstrated that caspase-1 is critical to surviving live *E. coli*-induced septic shock and that this effect is independent of caspase-1's function in processing and releasing IL-1 β and IL-18. Although only 22% of caspase-1^{+/+} mice survived the first 72 h of bacterial injection, all caspase-1^{-/-} mice survived. In contrast, IL-1 β -deficient animals and IL-1 β /IL-18 double knockout animals (i.e., lacking the key substrates for caspase-1) demonstrated no protective advantage to live bacterial challenge. We believe that our comparison of IL-1 β and caspase-1 deficiency is unique and raises important new questions about the role of caspase-1 in sepsis. Our experiments confirm that caspase-1 deficiency provides protection against live bacterial challenge, whereas knockouts of the classical substrates of caspase-1, IL-1 β , and IL-18 do not. Importantly, caspase-1^{-/-} mice are protected from splenic cell apoptosis. Furthermore, the synthetic caspase inhibitor zVAD also protects from septic

shock and from splenic cell apoptosis. The zVAD experiment supports the concept that caspase-1 is a component of sepsis-induced apoptosis, although it must be considered that zVAD is a pancaspase inhibitor and may also inhibit other endogenous enzymes such as cathepsins (23). Thus, caspase-1 regulates sepsis responses in a fashion that is independent from pro-IL-1 β and pro-IL-18 activation, suggesting a novel function of caspase-1 in host innate defenses.

The mechanisms responsible for the prominent role of caspase-1 in sepsis survival remain unknown. However, our data provide strong support for the role of apoptosis as a critical component of the sepsis connection to caspase-1. Sepsis is known to accelerate lymphocyte apoptosis in both animals and patients (24, 25) and prevention of apoptosis in mice improves survival (18). In this context, the present study showed remarkable contrasts in the amount of splenic apoptosis between caspase-1-deficient animals and wild-type littermates and between caspase-1-deficient mice and IL-1 β -deficient or IL-1 β /IL-18-deficient animals. Furthermore, we demonstrated that it is predominantly B lymphocytes that undergo apoptosis in the spleens from our sepsis model. Ayala and colleagues have shown that it is predominantly B lymphocytes that undergo apoptosis in the lamina propria with sepsis (26). Although we did not observe T lymphocyte apoptosis in our spleens, it should be noted that T lymphocyte apoptosis in human sepsis is well accepted (25). In this context, it should be noted that there may be technical problems in the accurate recognition of T and B cells in the advanced stages of apoptosis. For example, we found that the flow cytometry quantitation of apoptotic cells in our spleens vastly underestimated the numbers that were seen by histochemistry (data not shown). Nevertheless, regardless of the lymphocyte types undergoing apoptosis, it is reasonable to hypothesize that the reduction in apoptosis of splenic germinal centers plays a major role in the enhanced survival induced by caspase-1 deficiency.

How apoptosis might regulate survival in our model remains unknown. One consideration of how loss of splenic B lymphocytes may affect sepsis survival is the connection between B1 lymphocytes from the spleen and the presence of natural antibodies (27). A significant component of the initial defense to bacterial challenge rests with natural antibodies. Hence, a loss of natural antibody levels may occur as a result of the B cell apoptosis seen in sepsis. This could predispose to rapid bacterial proliferation early and hence increased mortality.

However, we favor the concept that the apoptotic cells are inducing a hypimmune state due to their own clearance.

Figure 2. Effect of IL-1 β and IL-18 gene knockout on survival after intra-peritoneal bacterial challenge. (A) Live *E. coli* (5×10^8 cfu/kg) was injected intraperitoneally into IL-1 β wild-type (+/+), IL-1 β heterozygote (+/-), and IL-1 β -deficient (-/-) mouse littermates. Survival was monitored for a period of 7 d ($n = 5-7$ mice/group). This represents one of two independent experiments ($p = 0.99$). (B) *E. coli* or saline was injected intraperitoneally into IL-1 β wild-type and knockout mice. Animals were killed 24 h later and spleens were harvested. H&E-stained sections at 10 $\times$ and 40 $\times$ (inset, 100 $\times$) from 24-h samples. (C) Splenic apoptotic bodies as counted from H&E-stained specimens (data = mean $\pm$ SEM). * $p < 0.0001$ *E. coli* versus saline control, *t* test; $\psi p = 0.79$ IL-1 β ^{+/+} *E. coli* versus IL-1 β ^{-/-} *E. coli* group, *t* test. (D) Live *E. coli* (5×10^8 cfu/kg) were intraperitoneally injected into wild-type (+/+) and IL-1 β /IL-18-deficient (-/-) mice. Survival was monitored for a period of 7 d ($n = 9-10$ mice/group). This represents one of two independent experiments ($p = 0.51$, log rank test). (E) *E. coli* or saline was injected intraperitoneally into wild-type and IL-1 β /IL-18 double knockout mice. Animals were killed 24 h later and harvested spleens were H&E stained (sections at 10 $\times$ and 40 $\times$ [inset, 100 $\times$] for spleen of the *E. coli*-injected animals are shown). (F) Splenic apoptotic bodies (data = mean $\pm$ SEM). * $p < 0.0001$ versus saline control, *t* test; $\psi p = 0.12$ wild-type versus IL-1 β /IL-18^{-/-} *E. coli* group, *t* test.

Hotchkiss and colleagues have generated a significant body of work that supports the notion that lymphocyte apoptosis is a central event in sepsis (4, 28). For example, prevention of lymphocyte apoptosis significantly improves the survival of mice subjected to a cecal ligation and puncture model of sepsis (18). There is ample support for the hypothesis that macrophages involved in the clearance of apoptotic cells experience a profound suppression of their inflammatory responses as a consequence of this phagocytosis (29). In support of this concept, we have purified B lymphocytes from the spleens of septic wild-type and caspase-1 knockout animals to test their relative ability to influence macrophage function. Wild-type (apoptotic) but not caspase-1 knockout (nonapoptotic) B lymphocytes induced a suppressive phenotype in peritoneal macrophages. Thus, caspase-1

plays an important functional role in sepsis that is independent of classical cytokine regulation.

Members of the caspase-related protease family have been shown to play an important role in apoptosis (30, 31). However, the specific role of caspase-1 in apoptosis is controversial. Caspase-1 knockout animals are born healthy without detectable morphologic abnormalities, whereas caspase-3-deficient animals have major birth defects, particularly neurologic defects, which implies a role for caspase-3 in developmental apoptosis (32-34). Furthermore, we have previously documented that spontaneous monocyte apoptosis is not dependent on caspase-1 but on caspase-3 activity (11). On the other hand, overexpression of caspase-1, in a rat fibroblast cell line, induces apoptosis, which is blocked by crmA, a cow pox virus protein that inhibits caspase-1 (30). The involvement of caspase-1 in neuronal cell

Figure 3. IL-1 β /IL-18 double knockout mice are protected from bacterial sepsis by synthetic caspase inhibition. IL-1 β /IL-18-deficient ($-/-$) were injected intraperitoneally with either saline or live *E. coli* (5×10^8 cfu/kg) in the presence or absence of the caspase inhibitor zVAD-fmk or the inactive caspase inhibitor zFA-fmk (each at 10 mg/kg). Inhibitors were injected intraperitoneally 90 min after bacterial injection and then every 12 h for 96 h. (A) Survival was monitored for a period of 7 d ($n = 7$ mice/group; $p < 0.001$, zVAD vs. zFA). (B) In a separate set of experiments, animals were killed 24 h later and spleens were harvested. H&E-stained sections for spleen at 40 $\times$ (insets, 100 $\times$) of the animals are shown. Sections were also stained by TUNEL staining to confirm apoptotic bodies. Representative 40 $\times$ micrographs are shown. (C) Splenic apoptotic bodies (data = mean $\pm$ SEM). * $p < 0.001$ zFA versus zVAD-treated IL-1 β /IL-18 $-/-$ *E. coli* group, *t* test.

apoptosis is well established (35). Gagliardini and colleagues observed the ability of a caspase-1 inhibitor to prevent apoptosis induced by nerve growth factor deprivation (36). Furthermore, caspase-1 has been implicated in the death of *Salmonella*-

infected dendritic cells and monocyte-derived macrophages (37, 38). Thus, the present work lends support to the notion that caspase-1 is important in at least some forms of programmed cell death. Our findings suggest that caspase-1 either directly or indirectly regulates apoptosis in the spleen.

Other potential mechanisms to explain the protection afforded by the caspase-1 knockout state are the changes in the resulting cytokine profiles. Caspase-1 is known to be principally responsible for the production of mature IL-1 β from its precursor form. Cells not expressing caspase-1 were unable to process pro-IL-1 β unless cotransfected with caspase-1 cDNA constructs (39, 40). This characteristic feature of caspase-1-deficient mice was used to confirm genotyping results. In agreement with existing literature (34), we demonstrated that the peritoneal macrophages of caspase-1-deficient mice are incapable of processing pro-IL-1 β to its mature form after stimulation with endotoxin or with ATP augmentation of endotoxin. Macrophages from wild-type mice, on the other hand, readily release mature IL-1 β with similar treatments. This was further confirmed by *in vivo* measurements of IL-1 β production in the plasma of our *E. coli*-infected animals.

It is conceivable that a relative decrease in inflammatory cytokine production is responsible for the protection afforded by caspase-1 deficiency. Inflammatory cytokines have been linked to organ injury and failure (2, 3, 8). Thus, the caspase-1-deficient state may have a more profound effect on inflammatory cytokines than the IL-1 β - or IL-1 β /IL-18-deficient state. However, in this context, we found no statistical difference between caspase-1 $-/-$ and caspase-1 $+/+$ animals for TNF- α , an inflammatory cytokine that has had a strong link to outcome, in response to a septic challenge.

Last, and perhaps linked to the apoptosis concept, is the possibility that caspase-1 deficiency has important effects on the host intracellular defense system. This idea is supported by the growing body of evidence that places caspase-1 at the center of complex of regulatory proteins termed by some the inflammasome (41, 42). Caspase-1 is known to interact with intracellular defense molecules (variously termed NLR, NALP [NACHT-, LRR-, and PYD-containing], NOD [nucleotide-binding oligomerization domain], NACHT [nucleotide binding domain], CATERPILLER [CARD, transcription enhancer, R(purine)-binding, pyrin, lots of leucine repeats], PYPAF [PYRIN-containing Apaf-1-like protein], PAAD [Pyrin, AIM, ASC, and death domain-like], or CARD [caspase recruitment domain]) that have striking structural homology to plant disease resistance proteins (43–45). It is believed that caspase-1 interacts with various members of these novel molecules via its caspase recruitment domain, or CARD. It is likely that caspase-1 may play a central role in the structural integrity, organization, and/or regulation of these intracellular protein complexes. For example, caspase-1 is known to interact with RIP2, a kinase that is important in upstream activation of nuclear factor (NF)- κ B, via CARDs on both molecules (46, 47). We have recently demonstrated that caspase-1 may also function as a scaffolding molecule that promotes RIP2-mediated NF- κ B activation (47). In keeping with this hypothesis, we noted that IL-6 production (often used as a readout for NF- κ B activity) was significantly suppressed in the caspase-1 $-/-$ animals ($p < 0.0002$) but not in the IL-1 β or IL-1 β /IL-18 knockout animals.

Finally, it is important to comment about the role of the inflammasome in host defense against live pathogens. The inflammasome, as currently conceived, is linked via CARD domains to intracellular pathogen sensors, such as NOD2 in monocytes and NOD1 in epithelial cells. NOD2 can detect bacterial products, such as muramyl dipeptide, and NOD1 detects diaminopimelic acid (45). In this context, it has been demonstrated that certain pathogens,

Figure 4. Apoptotic B cells induce suppressive macrophage phenotype. (A) Caspase-1 wild-type ($+/+$) and caspase-1-deficient ($-/-$) mice were injected intraperitoneally with live *E. coli* (5×10^8 cfu/kg), and spleens were harvested at 24 h. Sections were then immunostained with CD79 (B cells) and CD3 (T cells). (B) B and T cells were also isolated from the spleens of caspase-1 $+/+$ and caspase-1 $-/-$ mice after intraperitoneal *E. coli* injection. Isolated cells were identified by flow cytometry using CD19 and CD3 antibody and respective isotypes. (C) Isolated macrophages (Mac) and B and T cells from septic wild-type and caspase-1 $-/-$ mice were cultured alone, or B and T cells from either wild-type or knockout mice were cocultured in the presence of freshly isolated macrophages (isolated from unstimulated control [$+/+$] mice), and supernatants were measured for transforming growth factor (TGF)- β . The results are from two separate experiments done in duplicate.

In summary, our results demonstrate that caspase-1 plays a central role in the regulation of the response to live bacterial challenge that is closely tied to splenic cell apoptosis and which cannot totally be explained by its established function as a convertase for pro-IL-1 β or pro-IL-18. These findings emphasize the likely role that caspase-1 plays in regulating the organization and function of intracellular defense molecules.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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such as *Salmonella*, *Shigella*, and *Francisella*, can activate caspase-1 and induce dendritic cell and macrophage apoptosis (12, 37, 38, 48-50). Thus, caspase-1 may profoundly modify host responses independently from its ability to function as a convertase.

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