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Caspase-1 Activation of IL-1β and IL-18 Are Essential for *Shigella flexneri*–Induced Inflammation

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Summary

Caspases are intracellular proteases that mediate mammalian cell apoptosis. Caspase-1 (Casp-1) is a unique caspase because it activates the proinflammatory cytokines interleukin (IL)-1β and IL-18. *Shigella flexneri*, the etiological agent of bacillary dysentery, induces macrophage apoptosis, which requires Casp-1 and results in the release of mature IL-1β and IL-18. Here we show that *casp-1*$^{-/-}$ mice infected with *S. flexneri* do not develop the acute inflammation characteristic of shigellosis and are unable to resolve the bacterial infection. Using *casp-1*$^{-/-}$ mice supplemented with recombinant cytokines and experiments with *IL-1β*$^{-/-}$ and *IL-18*$^{-/-}$ mice, we show that IL-1β and IL-18 are both required to mediate inflammation in *S. flexneri* infections. Together, these data demonstrate the importance of Casp-1 in acute inflammation and show the different roles of its substrates, IL-1β and IL-18, in this response.

Introduction

Caspase-1 (Casp-1), like all caspases, is a cysteine protease that induces apoptosis when overexpressed in cultured cells (Miura et al., 1993). However, unlike mice with targeted deletions in most other caspase genes, *casp-1*$^{-/-}$ mice do not have any developmental defects, and cells isolated from these animals respond normally to "classical" apoptotic stimuli (Kuida et al., 1995; Li et al., 1995), suggesting that Casp-1 causes apoptosis only on dysregulated activation. In addition, the cleavage products of Casp-1, IL-1β, and IL-18 are proinflammatory cytokines (Dinarello, 1998), although apoptosis is often immunologically silent and does not cause inflammation (Savill, 1997).

Bacillary dysentery is a severe bloody diarrhea caused by the gram-negative bacteria *Shigella*e. *Shigella flexneri* accounts for the worldwide endemic form of the disease, which is particularly prevalent in young children living in developing areas in which poor hygiene facilitates transmission (Kotloff et al., 1999). After ingestion, *S. flexneri* invades and remains localized to the colonic and rectal mucosa, where it causes an acute inflammation characterized by a massive influx of polymorphonuclear cells (PMN) (Mathan and Mathan, 1991). Inflammation in classical shigellosis is of striking severity, resembling the acute phase of inflammatory bowel diseases (IBD), such as ulcerative colitis (MacDermott, 1994; Sartor, 1995). The inflammatory response causes significant tissue destruction and facilitates further tissue invasion by the bacteria (Perdomo et al., 1994). Eventually, however, the inflammation eradicates *Shigella* (Maurelli and Sansonetti, 1988; Hale, 1991; Lindberg and Pál, 1993).

Shigella invades host cells by macropinocytosis (Adam et al., 1995) and quickly escapes from phagosomes into the cytoplasm (Sansonetti et al., 1986). In contrast to epithelial cells, which allow *Shigella* to multiply in their cytoplasm, macrophages undergo apoptosis after bacterial uptake (Zychlinsky et al., 1992). The upregulation of apoptosis was demonstrated in animal models of *Shigella* infection (Zychlinsky et al., 1996) and in intestinal biopsies from dysenteric patients (Islam et al., 1997). The invasion plasmid antigen (Ipa) B is a protein encoded in the *Shigella* virulence plasmid that is secreted from bacteria through a type III secretion apparatus. IpaB alone is sufficient to induce apoptosis (Chen et al., 1996). IpaB binds to Casp-1 in the macrophage cytosol and activates an apoptotic cascade (Chen et al., 1996; Thirumalai et al., 1997). Interestingly, macrophages isolated from mice with a targeted deletion in *casp-1* but not from *casp-3* or *casp-11* null mice are resistant to *Shigella*-induced apoptosis (Hilbi et al., 1998). Thus, unlike most other apoptotic stimuli, *Shigella*-induced apoptosis in macrophages requires Casp-1.

Here we demonstrate that Casp-1 is essential for the inflammation provoked by *Shigella. Shigella* causes an inflammatory response of the lung tissue of mice that parallels the acute inflammation in the intestinal mucosa of dysenteric patients, when administered intranasally (Voino-Yasenetsky and Voino-Yasenetska, 1961; Phalipon et al., 1995). We use this model of infection because *Shigella* cannot infect mice orally. Wild-type mice develop a severe inflammatory response within 6 hr of infection. The bacterial load diminishes over time and the inflammation starts to resolve 48 hr later. In contrast, *casp-1*$^{-/-}$ mice do not generate an inflammation in response to a *Shigella* infection until much later. Moreover, the inflammation that eventually develops in *casp-1*$^{-/-}$ mice is more severe than the acute inflammation in wild-type mice. Even after extended periods of infection,

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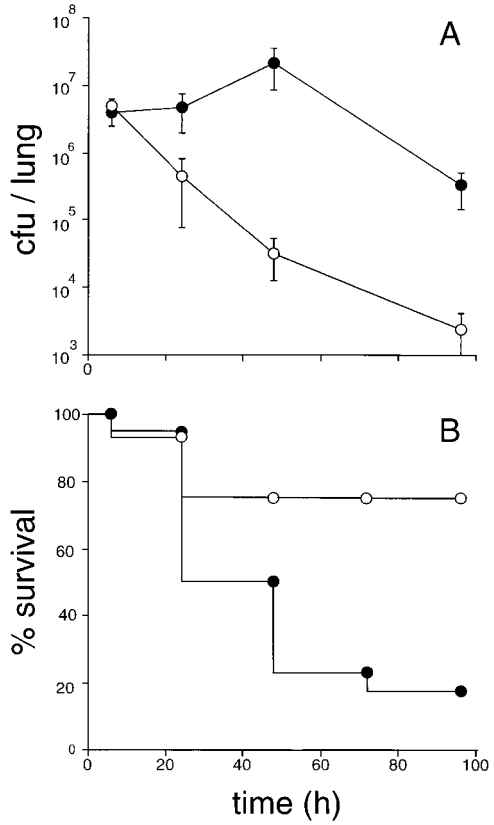

Figure 1. *casp-1*$^{-/-}$ Mice Cannot Control *Shigella* Infections

(A) The number of bacteria in the lungs of wild-type (open circles) and *casp-1*$^{-/-}$ (closed circles) mice are presented as the mean and standard error of the number of colony-forming units. Six hours after infection, there were similar numbers of bacteria in wild type and *casp-1*$^{-/-}$ mice. The number of bacteria significantly decreased in wild-type animals starting as early as 24 hr and continuing up to 96 hr. In *casp-1*$^{-/-}$ mice, the bacterial load increased slightly by 24 hr and remained unresolved even at 96 hr postinfection.

(B) Wild-type (open circles) and *casp-1*$^{-/-}$ (closed circles) mice were infected with *Shigella* and their survival recorded for 96 hr. In contrast to wild-type mice, *casp-1* null mice were susceptible to *Shigella* especially 48 hr after infection.

casp-1$^{-/-}$ mice are unable to control the infection and the inflammation continues to worsen. We demonstrate that IL-1β is the Casp-1 substrate responsible for the intensity of the acute inflammation observed in *Shigella* infections, while the other substrate, IL-18, is important in eliciting an effective anti-bacterial response. Therefore, in vivo activation of Casp-1 by *Shigella* is proinflammatory.

Results

Bacterial Load and Mortality in *Shigella* Infections of *casp-1*$^{-/-}$ and Wild-Type Mice

To evaluate the in vivo role of Casp-1 in *Shigella* infections, we infected *casp-1*$^{-/-}$ and wild-type mice with a sublethal dose of bacteria intranasally. The number of bacteria in the lungs of infected wild-type and *casp-1*$^{-/-}$ mice after 6 hr of infection was not statistically different ($p = 0.59$) (Figure 1). However, a difference was manifest at later time points. In wild-type mice, there was a significant drop in the number of bacteria recovered 24 hr ($p = 0.0039$) after infection, and by 96 hr bacterial recovery was reduced by three orders of magnitude. In contrast, 24 and 48 hr after infection, *casp-1*$^{-/-}$ mouse lungs had 10- and 670-fold more bacteria than wild-type mice, respectively ($p = 0.0011$). *casp-1*$^{-/-}$ mice still harbored a large number of bacteria even at 96 hr postinfection.

Mice with a targeted deletion in *casp-1* succumbed to the *Shigella* infection. As shown in Figure 1B, there were a few wild-type mice that died early on after infection. In contrast, the mortality of *casp-1*$^{-/-}$ mice increased during the experiment. Together, these data show that wild-type but not *casp-1* null mice are able to efficiently control the microbial infection.

Shigella Does Not Upregulate Apoptosis in *casp-1*$^{-/-}$ Mice

To determine whether *Shigella* induces apoptosis in this infection model, we labeled sections of infected lung tissue by the terminal d-transferase dUTP nick end labeling (TUNEL) technique. In histopathological sections taken at 6 hr after infection, we observed apoptotic cells that had a macrophage morphology in wild type (Figures 2A–2C), but we did not see apoptosis in *casp-1*$^{-/-}$ mice (Figures 2D–2F).

Reconstruction of complete sections of lungs from wild-type (Figures 2I and 2J) and *casp-1*$^{-/-}$ (Figures 2G and 2H) mice demonstrated a striking difference in the number of TUNEL-positive cells. Many apoptotic cells were present in the middle portion of the wild-type lung with greatest density around the bronchi, mirroring the distribution of bacteria. Very few apoptotic cells were detected in the lungs of *casp-1*$^{-/-}$ mice. These apoptotic cells localized to the periphery of the tissue. A similar distribution of apoptotic cells was observed in uninfected lungs or lungs infected with *S. flexneri* strain with a targeted deletion in *ipaB* (Δ*ipaB*, data not shown), suggesting that this was a phenomenon unrelated to shigellosis. Thus, in vivo, *Shigella* requires Casp-1 to induce apoptosis.

Inflammation in *Shigella* Infections of *casp-1*$^{-/-}$ Mice

To determine the role of Casp-1 in inducing an inflammatory response, we evaluated the extent of tissue inflammation in *casp-1*$^{-/-}$ mice by histopathological analysis. A qualitative analysis of a selection of typical histopathological aspects is shown in Figure 3. In wild-type mice, extensive inflammation is observed at 6 hr in the peribronchial/bronchiolar area and in the pulmonary tissue, causing acute diffuse alveolitis (Figure 3A). At 24 hr, inflammation is less extensive; alveolar walls remain thickened but the alveolitis is already resolving (Figures 3B and 3C). By 48 hr, the overall alveolar structure has almost returned to normal, with a few remaining macrophages and PMN (Figure 3D). Although a persistent edema still thickens the alveolar walls, the tissue has almost recovered. The disappearance of inflammatory cells at 96 hr (Figure 3E) shows the complete regression of the inflammation.

The pathology is strikingly different in *casp-1*$^{-/-}$ animals. Very few inflammatory foci are observed at 6 hr

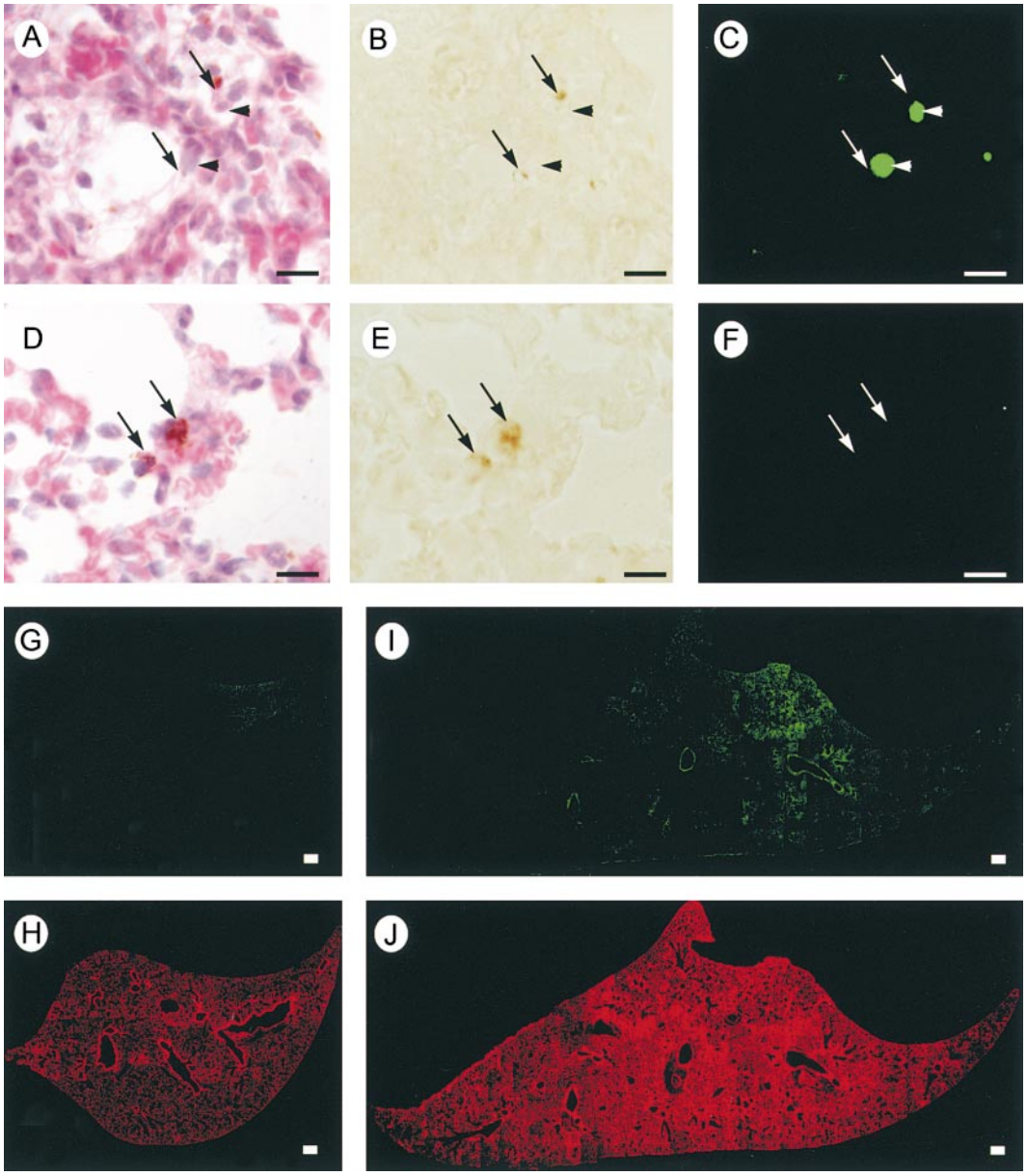

Figure 2. *Shigella* Induces Macrophage Apoptosis in Wild-Type but Not in *casp-1*$^{-/-}$ Infected Mice

(A) Two cells with macrophage morphology (arrowheads) were identified in wild-type mice in a section stained with hematoxilin and eosin (H&E) stain.

(B) The same cells were labeled with an anti-LPS antiserum (arrows) indicating that they are infected with *S. flexneri.*

(C) The same two cells were positively stained by fluorescent TUNEL (arrows). Thus, we were able to identify infected macrophages undergoing apoptosis in these animals.

(D and E) H&E-stained macrophages (arrowheads) of *casp-1* null mice were also labeled with anti-LPS serum (E), indicating that they were infected.

(F) These same cells were not stained by TUNEL, showing that in vivo *casp-1*$^{-/-}$ macrophages do not undergo apoptosis with Shigella.

(G) Reconstruction of a section from a lung from a *casp-1*$^{-/-}$ mouse infected with *S. flexneri* and stained with fluorescent TUNEL. Few cells toward the edge of the lung were labeled, serving as a positive control.

(H) Reconstruction of the same section shown in (G) stained with the DNA stain propidium iodide that allows the visualization of the complete lung section.

(I) Reconstruction of a lung from a wild-type mouse infected with *S. flexneri* and stained with fluorescent TUNEL. Large numbers of TUNEL-positive cells are evident in the middle portion of the lung, with a greater concentration in close proximity to bronchi, a distribution similar to that of bacteria.

(J) Reconstruction of the same section shown in (I) and stained with propidium iodide. Scale bars in (A–F) are 10 μm. Scale bars in (G–J) are 1 mm.

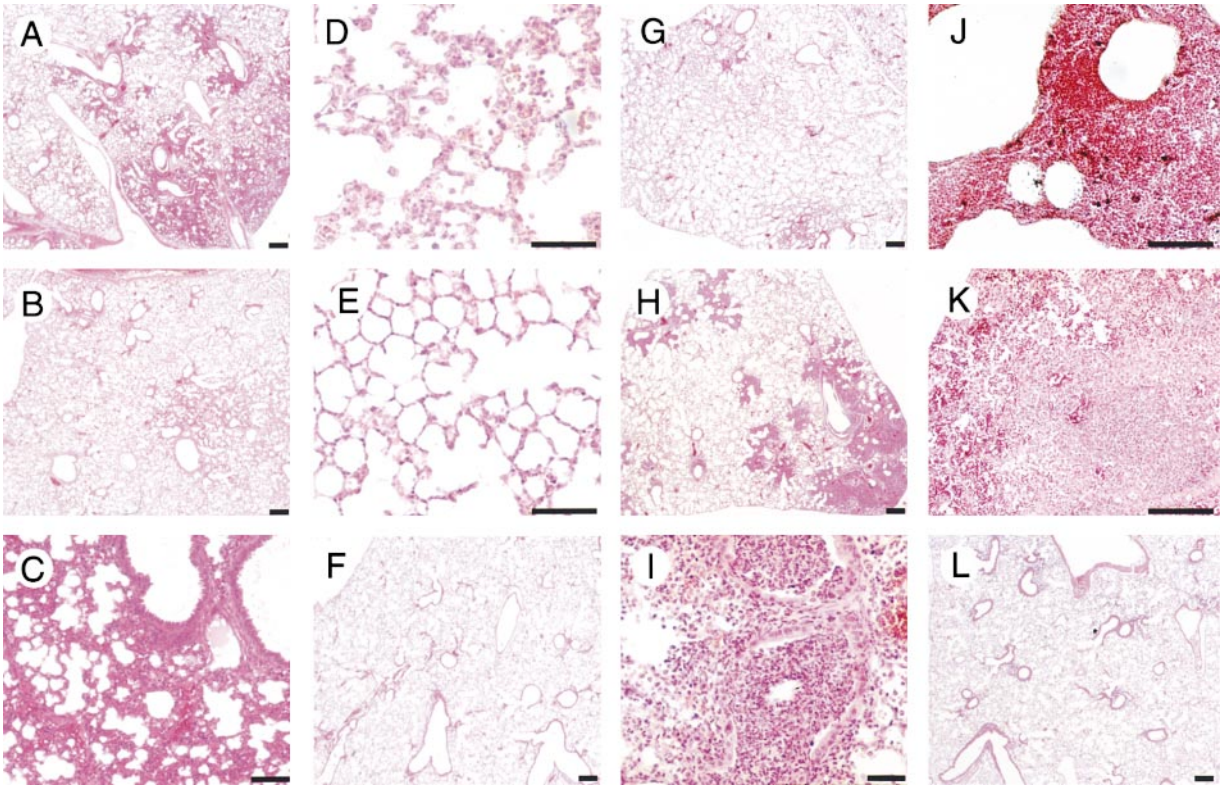

Figure 3. Inflammation in the Lungs of Wild-Type and *casp-1*$^{-/-}$ Mice Infected with *S. flexneri*

Lungs of infected mice were processed for histopathological analysis and stained with H&E.

(A) Wild-type mice 6 hr after infection show extensive inflammation, starting in the peribronchial region and spreading through the lung, causing acute diffuse alveolitis.

(B) In wild-type mice 24 hr after infection, the inflammation is starting to resolve.

(C) High magnification of (B) showing some thickened alveolar walls.

(D) Forty-eight hr after infection some mononuclear cells are still observed in thickened alveoli in wild-type mice.

(E) Ninety-six hours postinfection the lung has almost normal morphology.

(F) Wild-type mice 24 hr after infection with Δ*ipaB* do not show any signs of inflammation.

(G) *casp-1*$^{-/-}$ mice 6 hr after infection present very few inflammatory sites concentrated in the peribronchial and bronchiolar areas, without significant acute condensing alveolitis.

(H) At 24 hr postinfection there is a strong diffuse inflammation with formation of multiple foci of acute condensing alveolitis.

(I) Higher magnification of (H) showing intense inflammation and many bronchi and bronchioli occluded by inflammatory exudates in *casp-1* null mice.

(J) Forty-eight hours after infection *casp-1*$^{-/-}$ mice still show condensing inflammation with edema and emphysema.

(K) The inflammation, edema, and emphysema are unabated 96 hr postinfection. Strong hemorrhagic necrosis is also evident at this late time point.

(L) *casp-1* null mice 24 hr after infection with Δ*ipaB* without any sign of inflammation. Scale bars, 30 μm.

(Figure 3G). However, 24 hr after infection, there is a strong diffuse inflammation with extensive areas of alveolitis. Many bronchi and bronchioli are occluded by a massive inflammatory exudate (Figure 3H and 3I). By 48 hr postinfection (Figure 3J), the tissue damage worsens as condensing lesions predominate with tissue necrosis and hemorrhages. Ninety six hours after inoculation (Figure 3K), the histopathology remains the same: large areas of condensing inflammation, often centering around vessels occluded with mononuclear cells, and areas with hemorrhages and necrotic tissue. The inflammation in *casp-1*$^{-/-}$ mice was richer in macrophages than in wild-type mice.

There were no lesions observed in either wild type or *casp-1*$^{-/-}$ mice infected with Δ*ipaB* either at 6 hr (data not shown) or 24 hr (Figures 3F and 3L).

In order to quantify the severity of inflammation, lung sections stained by hematoxilin and eosin (H&E) were scanned at high resolution onto a computer. Using image analysis, we measured the area of the tissue sections which had a high color density and granularity. Because of its structure, a healthy lung has very few areas with high color density, while an inflamed lung, where the alveoli are infiltrated with inflammatory cells, has large areas with high color density. The ratio of the high color density surface to the entire surface of the tissue section, excluding the anatomic "holes" (i.e., the lumen of large blood vessels and bronchi) yields an "inflammatory index" (IF). This measure underestimates the inflammation, since it is biased toward well-delineated and condensed foci, omitting diffuse noncondensing processes. The analysis showed that by 6 hr, wild-type mice developed an IF of 15.0, whereas the *casp-1*$^{-/-}$ mice had an IF of 0.80, indicating a lack of an inflammatory response in the latter. The inflammation decreased in wild-type animals at 24 and 48 hr and reached an IF of

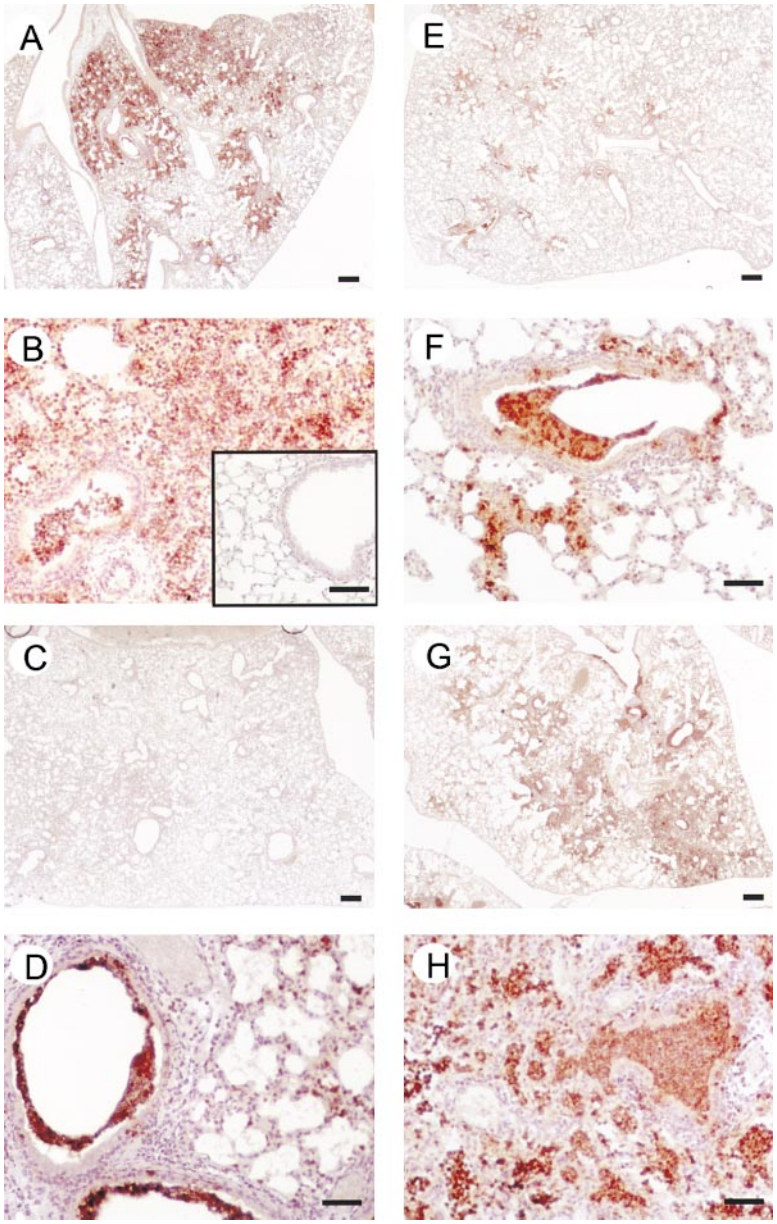

Figure 4. Distribution of LPS in Lungs of Wild-Type and *casp-1*$^{-/-}$ Mice Infected with Virulent *S. flexneri*

Lungs of infected mice were processed for histopathological analysis using standard techniques and immunostained with an anti-*S. flexneri* serotype 5 LPS-specific antiserum. (A and B) Wild-type mice 6 hr after infection present large amounts of LPS throughout the lung, particularly in association with areas of acute alveolitis. Inset shows a section stained with an isotype control mAb (B) and demonstrates the specificity of the immunostaining. (C and D) Wild-type mice 24 hr after infection display diffuse localization of LPS, which is more abundant in bronchial/bronchiolar walls. (E and F) In *casp-1*$^{-/-}$ mouse 6 hr after infection LPS is diffusely distributed in the lung and is more prominent in the lumen and walls of bronchi and bronchioli.

(G and H) *casp-1* null mice 24 hr after infection show massive amounts of LPS that fill luminal spaces and are associated with inflammatory cells. Scale bars, 300 μm.

only 3.6 by 96 hr. In contrast, *casp-1*$^{-/-}$ mice experienced a striking increase in the intensity of their lesions by 24 hr when they achieved an IF of 12.5, which continued to increase to almost 40 by 48 hr and 96 hr. The inflammation seen in the wild-type lungs was significantly different from *casp-1*$^{-/-}$ at each time point ($p = <0.001$).

We used a serotype-specific anti-LPS monoclonal antibody to determine the distribution of bacteria in the infected tissue. As shown in Figure 4, *Shigella* were present throughout the middle portion of the lung section 6 hr postinfection. The LPS immunostaining was specific, since a control with an isotype-matched monoclonal did not stain the tissue (Figure 4B, inset). The bacteria were found in more defined foci at 24 hr in the lungs of wild-type mice. Although there was almost no inflammation in the lungs of *casp-1*$^{-/-}$ mice at 6 hr postinfection, there were areas in the lungs that were occupied by bacteria. The distribution and amount of bacteria in *casp-1*$^{-/-}$ mice 24 hr after infection were unchanged from 6 hr. These results are in agreement with the data on the number of colony-forming units in the lungs of wild-type and *casp-1*$^{-/-}$ mice presented in Figure 1.

Recombinant IL-1β Exacerbates the Infection While IL-18 Restores the Wild-Type Phenotype in *casp-1* Null Mice

Casp-1 activates the proinflammatory cytokines IL-1β and IL-18 (Dinarello, 1998). To test the roles of IL-1β and IL-18 individually in inflammation during *Shigella* infection, we injected *casp-1*$^{-/-}$ mice intraperitoneally with either recombinant IL-1β or IL-18 and sacrificed the mice 48 hr postinfection. *Shigella*-infected *casp-1*$^{-/-}$ mice presented the previously described phenotype of condensing inflammation with defined areas of infection (Figures 5A and 5B). Treatment with recombinant IL-1β resulted in a more diffuse and severe inflammatory response and a more dispersed distribution of bacteria

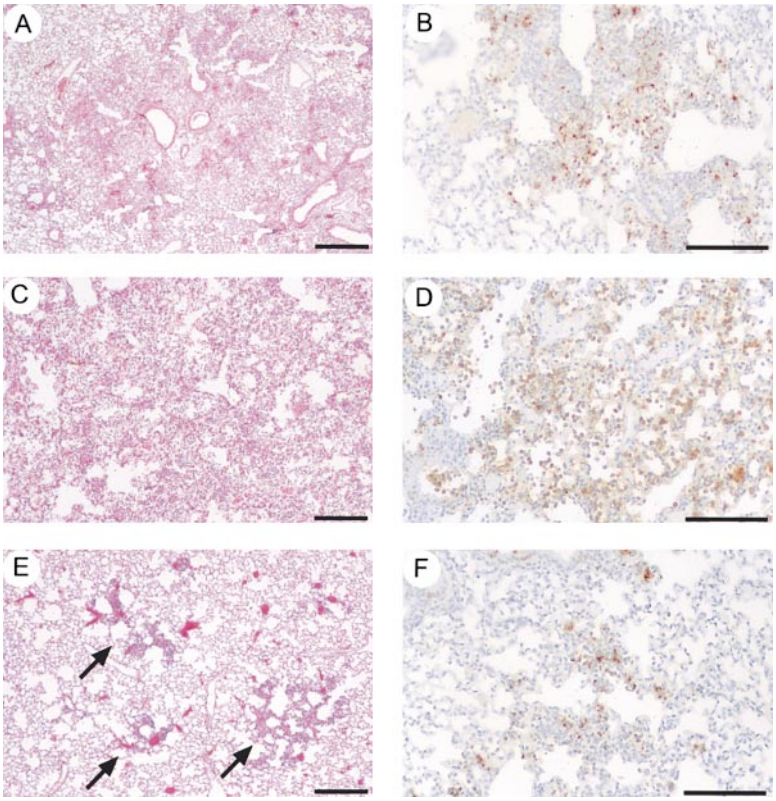

Figure 5. Recombinant IL-1β and IL-18 Have Different Functions in *Shigella* Infections

casp-1 null mice were either mock treated or treated with recombinant IL-1β or IL-18 as described in Experimental Procedures and infected with *Shigella* for 24 hr. The tissue was processed either for H&E staining or immunostaining with an anti-*Shigella* serotype 5 mAb.

(A and B) Control *casp-1* null mice show a strong diffuse inflammation with formation of multiple foci of acute condensing alveolitis and (B) large amounts of LPS.

(C and D) *casp-1*$^{-/-}$ mice treated with IL-1β show a similar, if slightly increased, inflammation and (D) abundant LPS staining.

(E and F) In contrast, *casp-1* null mice injected with recombinant IL-18 show a decreased nondiffuse inflammation (arrows) (F) and the corresponding localization of LPS.

in the lungs of these mice (Figures 5C and 5D). In contrast, treating *casp-1*$^{-/-}$ mice with recombinant IL-18 decreased the inflammation and restricted the distribution of the bacteria to a similar degree to that observed in wild-type mice (Figures 5E and 5F).

The enumeration of bacteria in the lungs of these mice corroborated the histological observations. At 48 hr wild-type mice were resolving the infection ($7.2 \times 10^3 \pm$ 4.5 SD cfu/lung) while *casp-1* null mice were still heavily infected ($110 \times 10^3 \pm 4.5$ SD, $p = 0.01$), as observed earlier. Surprisingly, at the same time point, *casp-1* null mice injected with recombinant IL-1β presented an even higher number of bacteria ($966 \times 10^3 \pm 526$), that was significantly greater ($p = 0.01$) than seen in untreated *casp-1*$^{-/-}$ mice. In contrast, administration of recombinant IL-18 resulted in a bacterial load ($18 \times 10^3 \pm 16.3$) that was not different from wild-type mice ($p = 0.7$).

Infection of *IL-1*β and *IL-18* Null Mice

Macrophages from both *IL-1*β null and *IL-18* null mice are susceptible to *Shigella*-induced apoptosis (data not shown). Thus, neither of these cytokines is necessary in the Casp-1-dependent apoptotic pathway. To determine downstream effectors of Casp-1-mediated inflammation in response to *Shigella*, we infected *IL-1*β$^{-/-}$ and *IL-18*$^{-/-}$ mice with these bacteria. The number of bacteria in wild-type and *IL-1*β$^{-/-}$ mice was not significantly different at 6, 24, or 48 hr postinfection (Figure 6A). In contrast, although the level of infection was similar at 6 hr between *IL-18* null and wild-type mice, *IL-18* null mice did not control the infection at later time points (Figure 6B). Twenty four and 48 hr after infection, the lungs of *IL-18*$^{-/-}$ mice had 61 ($p = 0.02$) and 1150 ($p = 0.04$) fold more bacteria than wild-type mice, respectively. Thus, similar to *casp-1*$^{-/-}$ mice, *IL-18*$^{-/-}$ mice could not control the *Shigella* infection.

Figure 7 shows the LPS immunostaining of wild-type, *IL-1*β null, and *IL-18* null mice at 24 and 48 hr postinfection. Lungs from wild-type mice showed a restricted

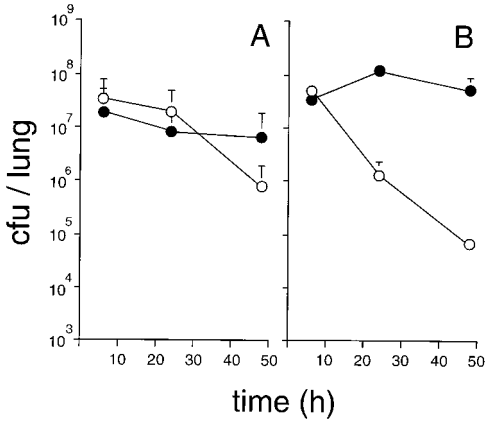

Figure 6. IL-18 but Not IL-1β Is Required to Control *Shigella* Infection

The number of bacteria in the lungs of wild-type (open circles) and *IL-1*β$^{-/-}$ (A) or *IL-18*$^{-/-}$ (B) (closed circles) mice are presented as the mean and standard error of the number of colony-forming units. Six hours after infection, there were similar numbers of bacteria in wild-type and either *IL-1*β null or *IL-18* null mice infected with *Shigella*. The number of bacteria was similar in wild-type and *IL-1*β$^{-/-}$ mice at later times after infection. In contrast, the bacterial load increased slightly by 24 hr and was higher than wild type by 48 hr in *IL-18* null mice.

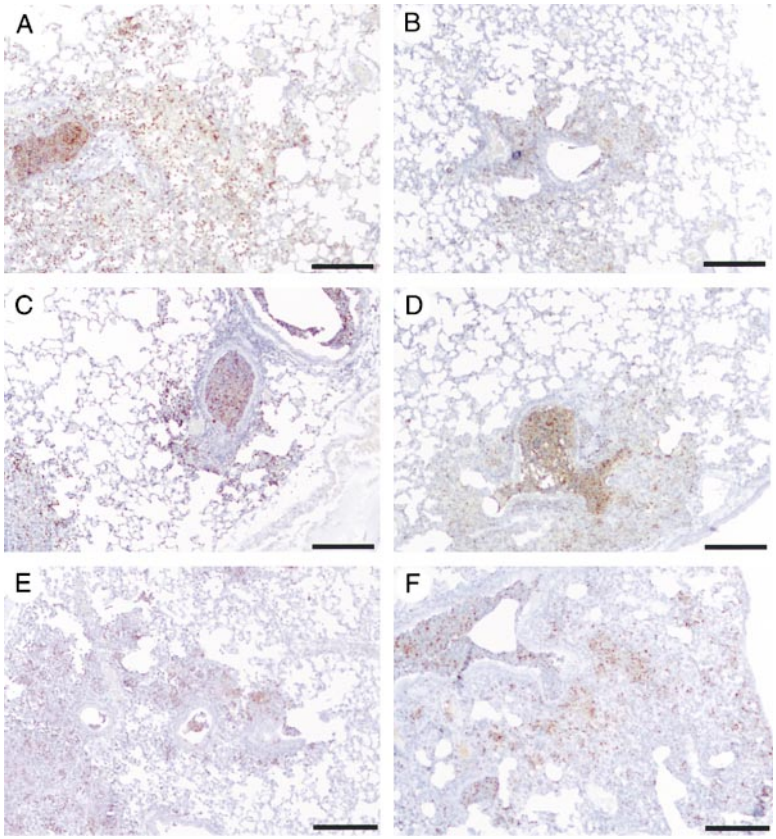

Figure 7. IL-1β and IL-18 Have Distinct Roles in the Acute Inflammation Characteristic of Shigellosis

The distribution of LPS and inflammation in lungs of wild-type, *IL-1*β null, and *IL-18* null mice infected with virulent *S. flexneri* is visualized using standard histopathological techniques and immunostaining with an anti-*S. flexneri* serotype 5 LPS-specific mAb.

(A) In wild-type mice 24 hr after infection, the inflammation is starting to resolve and there is a diffuse localization of LPS, which is more abundant in bronchial/bronchiolar walls.

(B) Forty-eight hours after infection, wild-type mice show a clear resolution of the inflammation and a decrease in LPS staining.

(C) Mice with a targeted deletion in *IL-1*β show a diffuse inflammation at 24 hr.

(D) This inflammatory response increases and LPS is abundant at 48 hr.

(E) In contrast, *IL-18* null mice show a very intense inflammation with abundant LPS staining at 24 hr.

(F) Forty eight hours after infection, the *IL-18*$^{-/-}$ mice show an unabated inflammation rich in LPS staining, reminiscent of the phenotype of *casp-1* null mice.

bacterial distribution with occlusion of a few bronchi at 24 hr postinfection and a diminution in both inflammation and the amount and distribution of LPS. This progression was similar to the one already described in Figures 3 and 4. Mice with a targeted deletion in *IL-1*β showed a more localized inflammation than wild-type mice at 24 hr. This inflammatory response did not appear to progress toward resolution at 48 hr. Consistent with the results obtained from infection of *casp-1* null mice treated with recombinant IL-18, mice with a targeted deletion in *IL-18* showed a very intense inflammation at 24 hr, with diffuse LPS staining, reflecting the spread of *Shigella*. At 48 hr postinfection, the inflammation was more severe, with several bronchi occluded and a continued diffuse distribution of LPS. Thus, the patterns of infection and inflammation in *casp-1* and *IL-18* null mice were similar.

Discussion

Severe intestinal inflammation is a hallmark of bacillary dysentery. Therefore, to understand the pathogenesis of dysentery, it is crucial to determine how the bacteria activate this inflammatory response. In vivo, *Shigella* induces apoptosis in a rabbit ileal loop model (Zychlinsky et al., 1996), in dysenteric patients (Islam et al., 1997), and in the murine lung infection model (Figure 2). *Shigella*-induced apoptosis appears to initiate a unique cell death pathway, since, in contrast to other apoptotic stimuli (Li et al., 1995), *Shigella* does not induce apoptosis in macrophages from *casp-1*$^{-/-}$ mice (Hilbi et al., 1998). These data probably reflect the fact that IpaB binds to Casp-1 and not to other caspases (Hilbi et al., 1998). The requirement for Casp-1 in *Shigella*-induced apoptosis allowed us to study the proinflammatory functions of this caspase in *Shigella* infection.

Shigella is an invasive bacteria that triggers an acute inflammation that eliminates the infection in humans. The murine lung model of infection mimics the invasive and proinflammatory properties of *S. flexneri* in the human intestinal mucosa. When mice are inoculated intranasally, shigellae invade the tracheobronchial and alveolar epithelia. An acute tracheobronchitis and alveolitis characterized by a massive influx of PMN results from the infection (Voino-Yasenetsky and Voino-Yasenetska, 1961; Phalipon et al., 1995). Furthermore, mice, like humans, are able to control the *Shigella* infection. Noninvasive mutants of *Shigella* such as the Δ*ipaB* strain, used as a control in this study, do not cause pulmonary pathology (Phalipon et al., 1995), confirming the validity of the model.

Mice with a targeted deletion in *casp-1* were unable to generate an early inflammation (6 hr) or resolve the infection (Figures 1A and 4) and eventually succumbed to the infection significantly more frequently (Figure 1B) than wild-type mice. Interestingly, at later time points, the lungs of *casp-1*$^{-/-}$ mice develop more severe inflammation than that in wild-type mice. Monocytes, rather than PMN, dominated the inflammatory response in *casp-1*$^{-/-}$ mice. In order to test the induction of a proinflammatory cytokine not dependent in Casp-1 activation, we tested the concentration of IL-6 in the serum

of infected animals. At 6 hr postinfection, the levels of IL-6 were similar in *casp-1*$^{-/-}$ and wild-type mice (data not shown), indicating that Casp-1-independent proinflammatory mechanisms were active in *casp-1* null mice. Furthermore, the distribution of *Shigella* seems to be more restricted in *casp-1*$^{-/-}$ than in wild-type mice (Figure 4). *Shigella* is a nonmotile organism that uses the host cell cytoskeleton to move from cell to cell (Bernardini et al., 1989). Therefore, it is possible that in wild-type mice, but not in *casp-1*$^{-/-}$ nulls, *Shigella* spreads more efficiently after tissue disruption by the inflammatory response. *casp-1*$^{-/-}$ mice could not eradicate the *Shigella* infection (Figures 1 and 4), suggesting that only Casp-1-mediated inflammation could effectively clear the bacteria. Interestingly, there were 2-fold higher levels of serum IL-6 in *casp-1*$^{-/-}$ than in wild-type mice, probably reflecting the persistence of the infection (data not shown). These data indicate that the host requires Casp-1 to mount the inflammatory response characteristic of shigellosis. Future investigation will determine whether the timing or the type of cell recruited in the inflammation is important for the eradication of *Shigella*.

Both IL-1β and IL-18 are synthesized as inactive precursors that lack a secretion signal sequence and are proteolytically activated by Casp-1 (Thornberry et al., 1992; Ghayur et al., 1997; Gu et al., 1997). We used two independent approaches to test the roles of IL-1β and IL-18 in *Shigella* infections; *casp-1*$^{-/-}$ mice reconstituted with recombinant IL-1β or IL-18 as well as *IL-1*β null and *IL-18* null mice. Recombinant IL-1β enhanced both the inflammatory response and, more importantly, the bacterial infection load in *casp-1* null mice challenged with *Shigella* (Figure 5). Consistent with this notion, infection of *IL-1*β$^{-/-}$ mice resulted in an inflammatory process that was more localized than in wild-type mice and that restricted the distribution of bacteria to the sites of entry more effectively than wild-type mice (Figure 7). These mice, however, progressed toward the resolution of the infection (Figure 6). It has been proposed that the destruction of the tissue by the initial inflammation in response to *Shigella* allows further bacterial infection (Perdomo et al., 1994). These data would be consistent with the hypothesis that IL-1β is involved in the initial inflammatory response to *Shigella* and invasion into the lungs, and that in the absence of this cytokine, the infectious foci are more restricted than in wild-type mice. Indeed, IL-1β is induced and activated very early after *Shigella* infection (Arondel et al., 1999) and blocking IL-1 with IL-1 receptor antagonist (IL-1ra) decreases the inflammation in the rabbit ileal loop model (Sansonetti et al., 1995).

Recombinant IL-18 reconstituted a wild type inflammatory response in *casp-1*$^{-/-}$ mice (Figure 5). The role of IL-18 in *Shigella* infections was corroborated by the *IL-18*$^{-/-}$ mice infected with *Shigella*, which did not regulate the inflammation nor control the bacterial growth (Figures 6 and 7), a phenotype similar to that observed in *casp-1* null mice. Taken together, these data show that IL-18 is required to generate an inflammatory response capable of eradicating *Shigella* effectively. IL-18 induces IFNγ and activates a T_H1 response (reviewed in Dinarello, 1998). Interestingly, Way et al. showed that IFNγ-deficient mice were significantly more susceptible to *Shigella* infection at later time points (Way et al., 1998). Given the rapid inflammatory response to *Shigella*, it would be surprising if IL-18 was acting solely as an IFNγ-inducing factor. In fact, IL-18 seems to have IFNγ-independent proinflammatory functions (Kohka et al., 1998; Shapiro et al., 1998). More detailed studies on the early events of infection using IFNγ null mice will further delineate the functions of IL-18 in *Shigella* infections.

IL-1β is essential in the innate immune response to many different microbial pathogens (Dinarello, 1998), and recently IL-18 was also shown to be crucial in the immune response to *Mycobacterium tuberculosis* (Sugawara et al., 1999), *Salmonella typhimurium* (Mastroeni et al., 1999), *Yersinia enterocolitica* (Bohn et al., 1998), *Leishmania major*, and in sequelae to *Staphylococcus aureus* (Wei et al., 1999). The mechanisms of IL-1β and IL-18 activation in these infections, except in *Salmonella*, are not yet clear. *Salmonella* encodes SipB, a homolog of IpaB that activates Casp-1 (Hersh et al., 1999). Recently Casp-1 was shown to be essential for the progression of salmonellosis in vivo (Monack et al., unpublished data). It remains to be determined whether Casp-1-mediated inflammation through IL-1β and IL-18 is a common pathway in other microbial infections.

The data presented here directly link the bacterial virulence factor IpaB with the initiation of inflammation through Casp-1. We propose that Casp-1 is a component of the innate immune response. Invasion of macrophages by *Shigella* directly activates Casp-1, which triggers an acute inflammation by proteolytically activating IL-1β and IL-18. It remains to be determined whether induction of apoptosis is necessary to allow the rapid release of these two cytokines. Thus, Casp-1 might have coevolved with the virulence factors of acute pathogens such as *Shigella*. In this model, Casp-1 emerges as a protease that coordinates the use of an apoptotic pathway to release mature cytokines.

Experimental Procedures

Bacteria and Growth Conditions

M90T, an invasive isolate of *S. flexneri* serotype 5, was our virulent strain of reference (Sansonetti et al., 1981). An IpaB deletion mutant of M90T referred to as Δ*ipaB* was used as a negative control (Ménard et al., 1993). Bacteria were routinely grown in Luria Broth at 37°C with aeration. The bacteria were washed and resuspended in RPMI for macrophage infection and in PBS for mouse infections.

Mice

casp-1$^{-/-}$ mice were obtained from the Animal Resource Facility at BASF Bioresearch Corporation (Li et al., 1995). *IL-1*β$^{-/-}$ mice were obtained from Merck (Zheng et al., 1995), and *IL-18*$^{-/-}$ mice (Takeda et al., 1998) were obtained from Dr. Kiyoshi Takeda at Osaka University. Animals were housed at the Institut Pasteur or NYU Medical Center animal facility. *casp-1* and *IL-18* null mice are C57BL/6, and *IL-1*β$^{-/-}$ mice are in the I29 Sv background. All three null strains were backcrossed at least eight times and were controlled with the appropriate isogenic mice. The course of the *Shigella* infection is indistinguishable in mice with different genetic background (P. J. S. et al., unpublished data). Control mice were obtained from the Unité de Génétique Murine and Central Animal Facility at the Institut Pasteur.

Treatment with Recombinant Cytokines

Mice were injected intraperitoneally with 0.5 μg/mouse mature murine recombinant IL-1β (Cirulli et al., 1998; Neveu et al., 1998) and 10 μg/mouse IL-18 (both cytokines from Biosource International, CA) at the infection time and every 24 hr thereafter. These protocols

have been shown to be effective in other systems (Kawakami et al., 1997; Cirulli et al., 1998; Neveu et al., 1998).

Mice Infection and Bacterial Counts

Mice were inoculated intranasally with 2×10^8 bacteria in 20 μl as described (Phalipon et al., 1995). For bacterial enumeration, the mice were sacrificed at the indicated time points and the lungs were rapidly removed "en bloc" and ground in 10 ml sterile PBS (Ultra Turrax T25 apparatus, Janke and Kunkel IKA Labortechnik GmbH, Staufen, Germany). Dilutions were then plated on Trypticase Soy Broth plates for cfu enumeration. Each data point is the mean of at least 10 (range 10–12) infected mice per group from two independent experiments. Susceptibility to *Shigella* infections was done in groups of at least 16 animals.

TUNEL

Terminal d-transferase dUTP nick end labeling (TUNEL) was done using the Apoptosis Detection System, Fluoresceine kit (Promega, Madison, WI). For double-labeling experiments, the sections were first labeled with ant-LPS antiserum as described (Sansonetti et al., 1996), then labeled with TUNEL and mounted in 50% glycerol in PBS. After photographing the LPS label by light microscopy and the TUNEL label with epifluorescence, the sections were washed, stained with H&E, dehydrated, and mounted in Permount. The same field that was photographed for LPS and TUNEL labeling was localized and photographed again. To reconstitute the TUNEL and propidium iodide label in a whole section, each field was scanned with a confocal microscope (Molecular Dynamics, Sunnyvale, CA) in both channels. The sections were reconstructed using Adobe Photoshop.

Histopathological Studies

At the indicated time points, the mice were anesthetized, their trachea catheterized, and 4% formalin injected in order to fill the broncheoalveolar space. Lungs were then removed and fixed in 4% formalin before being processed for histopathological studies. Ten-micrometer paraffin sections were stained with H&E, and observed with a BX50 Olympus microscope (Olympus Optical, Europa, GmbH, Hamburg, Germany). To quantify the inflammatory response, the microscopic images were scanned into a computer and analyzed with NIH image software. The ratio of the surface of these areas to the entire surface of the tissue section minus the anatomic "holes", i.e., the lumen of large blood vessels and bronchi, was computed as the "inflammatory index." The resulting inflammatory index was the median of at least 17 mice (range 17–24) in each group.

Statistics

All two-way comparisons were done using the Mann Whitney non-parametric test.

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