

# Roles of caspase-1 in *Listeria* infection in mice

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## Abstract

**Caspase-1 [IL-1 $\beta$ -converting enzyme (ICE)] processes substrate precursor molecules to yield the biologically active form of IL-1 $\beta$  and IL-18, both of which are considered to play important roles in the host defense by activation of both innate and adaptive immunity. We evaluated the immune response of caspase-1<sup>-/-</sup> mice to *Listeria monocytogenes* (LM) infection. LM eradication in the early phase of infection was impaired in the mutant mice with a prominent decrease in IL-18 and IFN- $\gamma$  production, but not in IL-12. Caspase-1<sup>-/-</sup> spleen cells including dendritic cells and NK cells produced less IFN- $\gamma$  in response to heat-killed LM than wild-type cells *in vitro*. IFN- $\gamma$  production and bactericidal activity in LM-infected caspase-1<sup>-/-</sup> mice was reconstituted to normal levels by adding back IL-18 at the initial phase of infection, suggesting that the lack of this cytokine is primarily responsible for the susceptibility of caspase-1<sup>-/-</sup> mice against LM infection. Moreover, IFN- $\gamma$  injection of caspase-1<sup>-/-</sup> mice corrected the deficiency in pathogen clearance. In contrast, LM-specific acquired immunity in caspase-1<sup>-/-</sup> mice was normal and they successfully cleared the pathogen following secondary infection, in spite of a moderate skewing of cytokine profile to T<sub>H</sub>2 when compared to wild-type mice. These data shed light on the importance of caspase-1-mediated IL-18 processing in innate immunity against facultative intracellular pathogens.**

## Introduction

*Listeria monocytogenes* (LM), a Gram-positive bacterium and a facultative intracellular pathogen, invades and proliferates in the liver and spleen of hosts, and sometimes causes lethal infection in immunocompromised hosts. Immunity to LM is regulated by pro-inflammatory cytokines secreted by activated macrophages and lymphocytes (1–3). In the early infectious phase, innate immune responses, associated with IFN- $\gamma$  production, play a central role in host defense against LM. IFN- $\gamma$  activates macrophages to produce the bactericidal elements such as NO and tumor necrosis factor (TNF)- $\alpha$ , which stimulate neutrophils to recruit and kill LM (4–7). Recent

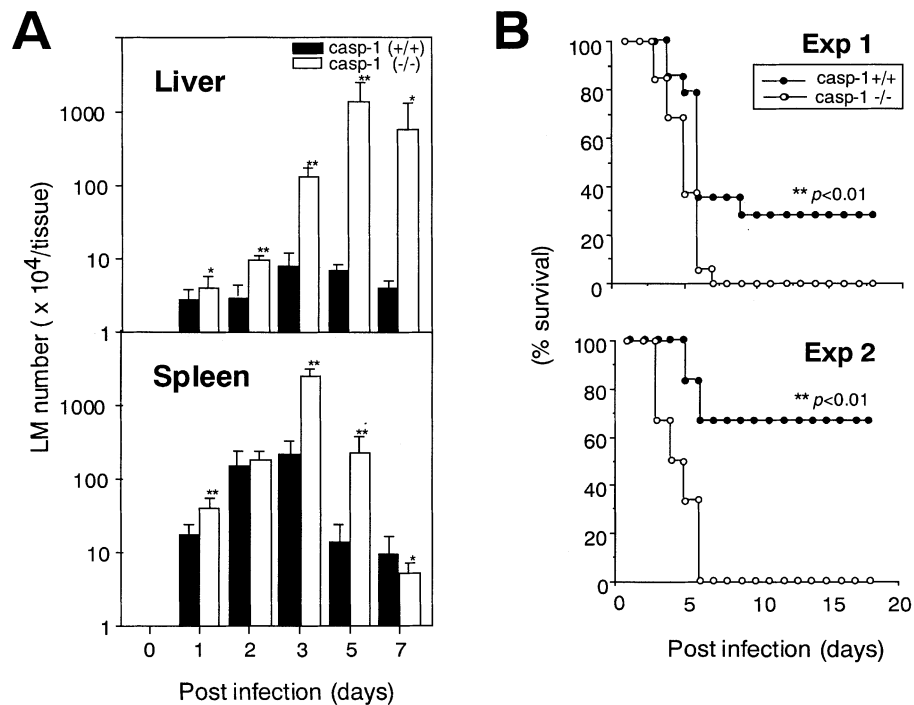
studies by us and others revealed that LM products directly activate macrophages and dendritic cells (DC) via Toll-like receptors, which are potential innate immune signaling receptors, to secrete TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IFN- $\gamma$ -inducing cytokines, such as IL-12 and IL-18 (8–10). IL-18 together with IL-12 induces production of large amounts of IFN- $\gamma$  by NK cells and DC (11–16). Very recently, our study revealed the hierarchy of pro-inflammatory cytokines for the early eradication of LM in a LM-resistant mouse strain (9). Based on the comparison of LM eradication among IL-18<sup>-/-</sup>, IL-12<sup>-/-</sup>, IFN- $\gamma$ <sup>-/-</sup> and wild-type mice, we found that IFN- $\gamma$  is the most important,

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**Fig. 1.** Acute resistance to LM infection is impaired in caspase-1<sup>-/-</sup> mice. (A) A high dose ( $5 \times 10^5$ /head) of LM was injected i.v. At 1, 2, 3, 5, and 7 days after infection, spleens and livers were harvested from caspase-1<sup>-/-</sup> and control mice, and their homogenates were plated onto agar plates of trypticated casein. Colony numbers were counted after overnight culture at 37°C. The data represent the average of five mice  $\pm$  SD of values from individual mice. Difference of data between caspase-1<sup>-/-</sup> and control mice was statistically significant except day 2 (spleen). Representative data from two independent experiments are shown (\*\* $P < 0.01$ , \* $P < 0.05$ ). (B) A high dose ( $1 \times 10^6$ /head) of LM was injected i.v. After infection, mice were observed every day up to day 18. The number of mice used in each group  $n = 14$ . Representative data from two independent experiments are shown.  $P < 0.01$  in both experiments.

followed by IL-12 and then IL-18, whereas the hierarchy in susceptible mouse strains is quite distinct (17). In contrast, LM-specific adaptive immunity is essential for its eradication in late-phase infection and upon re-infection. In particular, IFN- $\gamma$  produced by LM-specific cytotoxic T lymphocytes is critical for LM eradication (18–21).

Caspase-1 [IL-1 $\beta$ -converting enzyme (ICE)], an intracellular cysteine protease, is an essential processing enzyme for release of biologically active IL-1 $\beta$  and IL-18, the precursors of which are intracellularly produced as biologically inactive molecules (22–26). Caspase-1<sup>-/-</sup> macrophages do not secrete IL-18 or IL-1 $\beta$  after stimulation with lipopolysaccharide (LPS) (27). Moreover, caspase-1<sup>-/-</sup> mice show impaired elevation of serum levels of IL-18 upon LM infection, indicating an essential role of caspase-1 for IL-18 secretion after infection with some types of microbes (28). In this study, we investigated whether caspase-1 is required for LM clearance in a LM-resistant mouse strain, particularly focusing on its role in the induction of IFN- $\gamma$ . Caspase-1<sup>-/-</sup> mice showed a decrease in serum levels of IL-18 and IFN- $\gamma$ , but not IL-12, and also impairment in LM eradication at the early phase of infection. However, caspase-1<sup>-/-</sup> mice showed normal LM expulsion in the late phase of its initial infection and also upon re-infection. These results indicate the importance of caspase-1 for innate immunity against LM, but not in adaptive immunity. In addition, these results provide us with a novel regimen targeting caspase-1 to

protect hosts from the pathological inflammatory responses following microbial infection without affecting their development of adaptive immunity required for successful microbe eradication.

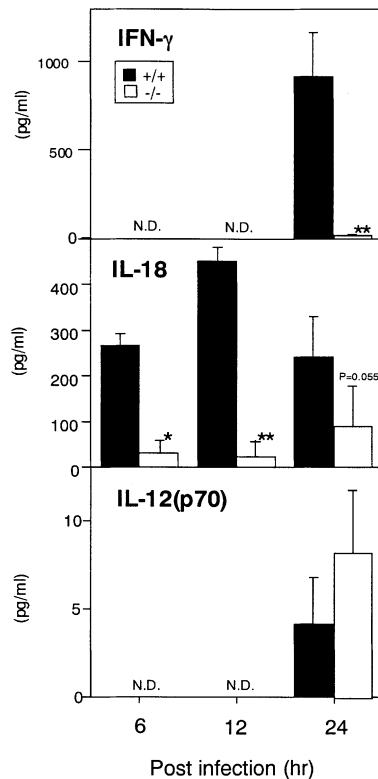
## Methods

### Mice

Caspase-1 gene-deleted mice, caspase-1<sup>-/-</sup>, and control wild-type mice, caspase-1<sup>+/+</sup>, were generated on C57BL/6J  $\times$  129SV/J (H-2<sup>b</sup>) mice as previously described (27). They were backcrossed with C57BL/6J mice for eight generations to eliminate gene effects of the 129SV/J background. Wild-type mice of comparable background were used for each experiment as control mice. C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) or Japan Clea (Tokyo, Japan) at 5 weeks of age. All experiments were initiated with 6- to 8-week-old mice, and all animals received humane care as outlined in the Guide for the Care and Use of Experimental Animals (Yale Animal Care Committee, Hyogo Medical College Animal Care Committee and National Institute of Agrobiological Sciences Animal Care Committee).

### Antibodies and reagents

Commercial kits for IL-6, IL-12, TNF- $\alpha$  and IFN- $\gamma$  (R & D, Minneapolis, MN or Genzyme, Cambridge, MA), and antibody



**Fig. 2.** The serum levels of IL-18 and IFN- $\gamma$  after LM infection are attenuated in caspase-1<sup>-/-</sup> mice, while IL-12 levels are normal. Sera were taken from caspase-1<sup>-/-</sup> and control mice at 6, 12 and 24 h after a high-dose LM infection, and subjected to cytokine ELISA assay for IFN- $\gamma$ , IL-12 and IL-18. The data represent the average of five mice  $\pm$  SD of values from individual mice. ND, not detected. Representative data from three independent experiments with similar results are shown, \* $P < 0.05$ , \*\* $P < 0.01$ .

sets for IL-2, IL-4, and IFN- $\gamma$  (PharMingen, San Diego, CA) were used to detect the cytokines in the culture supernatants or sample serum by two-sided sandwich ELISA. Recombinant cytokines for standards were either provided in ELISA kits or purchased from PharMingen. Pairs of antibodies for the analysis of IL-18 by ELISA (74 and #93-10C-HRPO) and recombinant murine IL-18 standard were kind gifts from Hayashibara Biochemical Laboratories (Okayama, Japan). Antibodies used for purification of T cells were anti-mouse NK-1.1 (PharMingen) and anti-I-A (M5/114; ATCC, Rockville, MD). Culture medium generally used in this study was RPMI 1640 supplemented with 10% FCS, 100 U/ml of penicillin, 100  $\mu$ g/ml of streptomycin, 50  $\mu$ M 2-mercaptoethanol and 2 mM L-glutamine.

#### LM infection and protection assay

LM 43251 was cultured in Trypsinase Soy Broth (Becton Dickinson, MD) till absorbance at 600 nm reached 0.1 of optical density. In some experiments, heat-killed LM (HKLM) (60°C for 4 h) were used. Caspase-1<sup>+/+</sup> and caspase-1<sup>-/-</sup> mice were infected i.v. with 5000 as a low dose, or  $5 \times 10^5$  or  $1 \times 10^6$  as a high dose of live LM. The protocol of low-dose infection

was used for the primary infection to examine the immunity of mice against secondary infection. A high dose of  $1 \times 10^6$  or  $2 \times 10^6$  LM was used for the evaluation of survival rate, in which mice were observed every day post-infection. C57BL/6J mice were generally used except for the protection assay shown in Fig. 1, in which mice with the genetic background of C57BL/6J  $\times$  129SV/J were used for. A high dose of  $5 \times 10^5$  LM was used for all other experiments. At 1, 2, 3, 5 or 7 days after infection, spleens and livers were harvested into 0.1% Triton in PBS, and homogenized with a stainless mesh and a plunger. An aliquot of 10 ml of the desired dilution of the homogenate was plated onto agar plates of trypticated casein. Plates were cultured overnight at 37°C and colony numbers were counted. Injection of IL-18 (1  $\mu$ g/animal) or IFN- $\gamma$  ( $4 \times 10^5$  U/animal) was performed i.v. 30 min prior to infection.

#### Cytokine analysis

Sera were collected from caspase-1<sup>+/+</sup> and caspase-1<sup>-/-</sup> mice at the indicated time points after LM infection, and kept at -20°C until assayed. DC-containing spleen cells ( $1 \times 10^6$ /ml) were isolated by the collagenase digestion method from uninfected mice (29) and were incubated with HKLM at 1:10 cells:HKLM ratio. In some experiments, the spleen cells were incubated with anti-CD11c or anti-DX5 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and depleted of DC and NK cells respectively by AutoMACS (Miltenyi Biotec), and the residual cells were cultured ( $1 \times 10^6$ /ml) with  $1 \times 10^6$ /ml of HKLM. Supernatants after 24 h of incubation were collected and kept at -20°C until assayed. To investigate the responsiveness of caspase-1<sup>-/-</sup> mice to IL-12 and/or IL-18, spleen cells ( $1 \times 10^6$ /ml) were incubated with 2 ng/ml of rIL-12, 20 ng/ml of IL-18 or both for 24 h, and IFN- $\gamma$  levels in each supernatant were measured. For the analysis of DC-mediated cytokines, bone marrow-derived DC (BMDC) were generated generally as shown previously (30). Briefly, bone marrow cells of caspase-1<sup>-/-</sup> or wild-type mice were incubated in the culture medium supplemented with 10% supernatant of granulocyte macrophage colony stimulating factor (GM-CSF)-secreting cell line (plasmacytoma line X63-Ag8 transfected with GM-CSF gene; a kind gift from Dr D. Gray) for 10 days. Non-adherent cells were collected and used as BMDC. BMDC were cultured in the culture medium in the presence or absence of HKLM (BMDC:HKLM 1:10). For the analysis of T cell-mediated cytokines after secondary infection, mice that had been infected with low-dose LM ( $5 \times 10^3$  LM/animal) 14 days before were re-infected with high-dose LM ( $5 \times 10^5$  LM/animal). At 2 days after re-infection, spleen cells were harvested and incubated on plastic dishes for 50 min to remove macrophages. B cells and NK cells were subsequently depleted using BioMag (Perseptive Biosystems, Framingham, MA), and specific antibodies against I-A and NK1.1. The resulted cells were suspended in BRUFFS medium supplemented with 5% FCS, glutamine and streptomycin (modified BRUFFS medium). Supernatants after 24 h of incubation were collected and kept at -20°C until assayed. IL-2, IL-4, IL-6, IL-12, IL-18, TNF- $\alpha$  and IFN- $\gamma$  levels were measured by ELISA.

### Statistics

Statistical evaluation between control and experimental samples was performed using the two-tailed Student's *t*-test. Survival curves were plotted using Kaplan–Meier estimates and the Mantel–Cox log-rank test was used for the statistical analysis. *P* values <0.05 were considered to indicate significance.

## Results

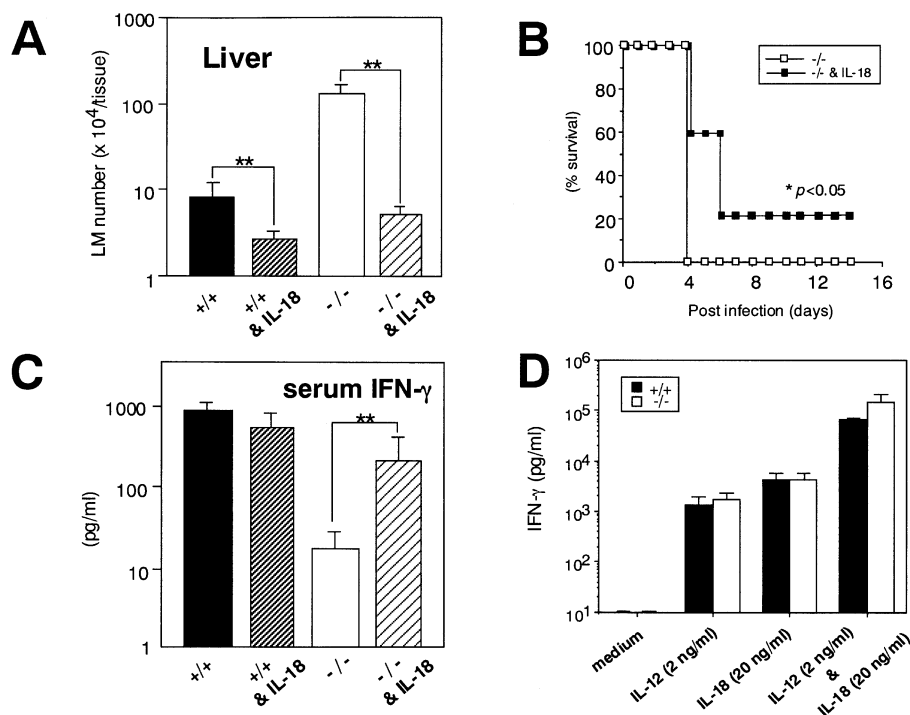
### Partial impairment of caspase-1<sup>-/-</sup> mice in LM eradication in early-phase infection

To investigate whether caspase-1 is involved in LM eradication in the early infectious phase, we inoculated caspase-1<sup>-/-</sup> mice with  $5 \times 10^5$  LM, and counted the LM burden in their livers and spleens at days 1, 2, 3, 5 and 7 post-infection. As shown in Fig. 1(A), the bacterial numbers peaked at day 3 post-infection in both organs of wild-type mice. Caspase-1<sup>-/-</sup> mice showed higher LM titers throughout up to 7 days after infection when compared to wild-type mice (Fig. 1A). In particular, the liver of the mutant mice exhibited severer impairment in LM eradication than spleen (Fig. 1A).

Next, we investigated the susceptibility of caspase-1<sup>-/-</sup> mice to LM. We examined the mortality of the mutant mice after inoculation with the LD<sub>50</sub> of LM for wild-type mice. As shown in Fig. 1(B), all the mutant mice succumbed within 7 days after infection, while 40–70% of wild-type mice survived up to 18 days (*P* < 0.01). These results indicate that caspase-1<sup>-/-</sup> mice exhibit impairment in LM eradication, particularly in the early phase of infection.

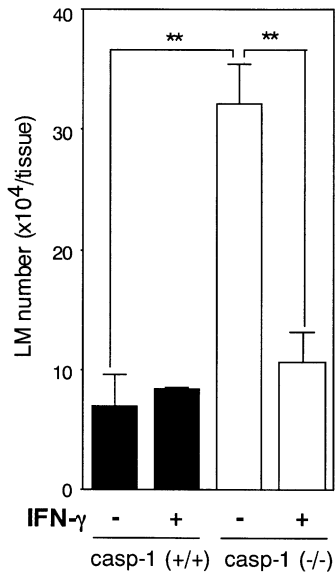
### Impairment of IFN- $\gamma$ induction in LM-infected caspase-1<sup>-/-</sup> mice

As IFN- $\gamma$  is essential for LM eradication (31), we measured serum levels of IFN- $\gamma$  in caspase-1<sup>-/-</sup> mice after LM infection. As shown in Fig. 2, caspase-1<sup>-/-</sup> mice showed only a small increase in serum levels of IFN- $\gamma$  upon LM infection at 24 h post-infection, while wild-type mice exhibited a marked increase in IFN- $\gamma$  levels of their sera, indicating a requirement of caspase-1 for IFN- $\gamma$  production induced by LM infection (Fig. 2). We measured the serum concentration of IFN- $\gamma$  in both wild-type and the mutant mice at 48 or 72 h post-infection, but these levels were below the detection limit (data not shown). As both IL-18 and IL-12 are important for IFN- $\gamma$  production (9), we compared serum levels of IL-12p70 and IL-18 between the



**Fig. 3.** Exogenous IL-18 corrects LM clearance in caspase-1<sup>-/-</sup> mice. (A) Injection of IL-18 (1  $\mu$ g/animal) was performed i.v. 30 min prior to high-dose ( $5 \times 10^5$ /animal) infection. Three days after infection, livers were harvested from caspase-1<sup>-/-</sup> and control mice, and their homogenates were plated onto agar plates of trypticated casein. Colony numbers were counted after overnight culture at 37°C. The data represent the average of five mice  $\pm$  SD of values from individual mice. Representative data from three independent experiments are shown. (B) IL-18 (1  $\mu$ g/animal) was administered i.v. 30 min prior to infection with  $2 \times 10^6$  LM. After infection, caspase-1<sup>-/-</sup> mice were observed every day up to day 14. The number of mice used in each group *n* = 5. Similar results were obtained in three independent experiments. *P* < 0.05. (C) Injection of IL-18 (1  $\mu$ g/animal) was performed i.v. 30 min prior to high-dose LM ( $5 \times 10^5$ /animal) infection. At 24 h after infection, mice were bled and sera were subjected to cytokine ELISA analysis. Representative data from three independent experiments are shown. (D) Splenocytes from uninfected caspase-1<sup>-/-</sup> or wild-type mice were cultured in the presence of IL-12 (2 ng/ml) and/or IL-18 (20 ng/ml) for 24 h. IFN- $\gamma$  levels in each culture supernatant were analyzed by specific ELISA. The data represent the average of cultures  $\pm$  SD of values from individual mice. The number of mice used per group *n* = 3. The data represent the average of five mice  $\pm$  SD of values from individual mice. Representative data from three different experiments with similar results are shown.

mutant and wild-type mice. Serum levels of IL-12 in caspase-1<sup>-/-</sup> mice were comparable with those in wild-type mice,



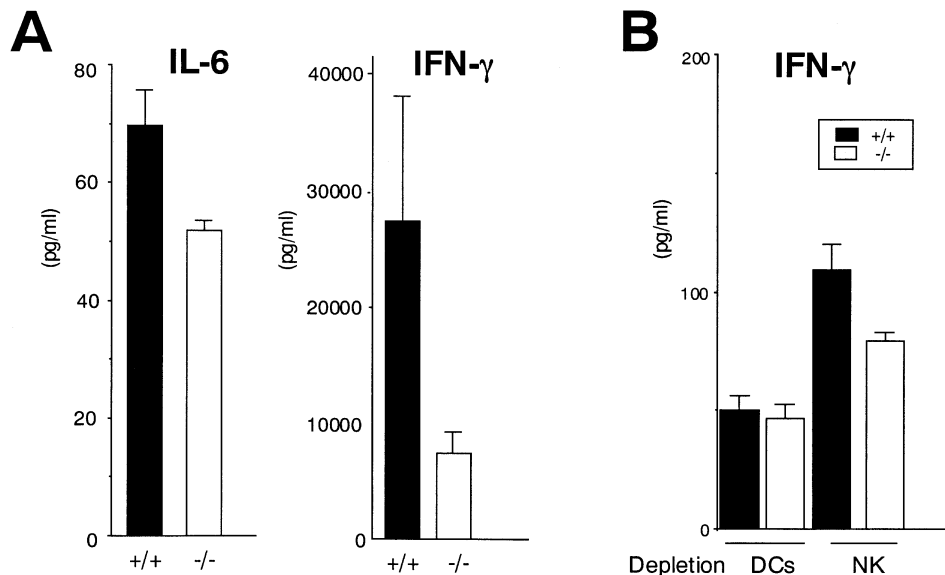
**Fig. 4.** Effects of recombinant IFN- $\gamma$  on early clearance of LM in caspase-1<sup>-/-</sup> mice. Injection of IFN- $\gamma$  ( $4 \times 10^5$  U/animal) was performed i.v. 30 min prior to high-dose ( $5 \times 10^8$ /animal) infection. Three days after infection, livers were harvested from caspase-1<sup>-/-</sup> and control mice, and their homogenates were plated onto agar plates of trypticated casein. Colony numbers were counted after overnight culture at 37°C. The data represent the average of five mice  $\pm$  SD of values from individual mice. Representative data from two independent experiments with similar results are shown. \*\* $P < 0.01$ .

indicating a dispensable role of caspase-1 for production of IL-12. In contrast, IL-18 serum levels of the mutant mice were lower than wild-type mice, indicating a requirement of caspase-1 for the release of IL-18. IL-12p70 was undetectable in the serum of caspase-1<sup>-/-</sup> and wild-type mice at 48 and 72 h post-LM infection (data not shown). These results indicate that caspase-1 is required for IL-18 release and IFN- $\gamma$  induction after LM infection.

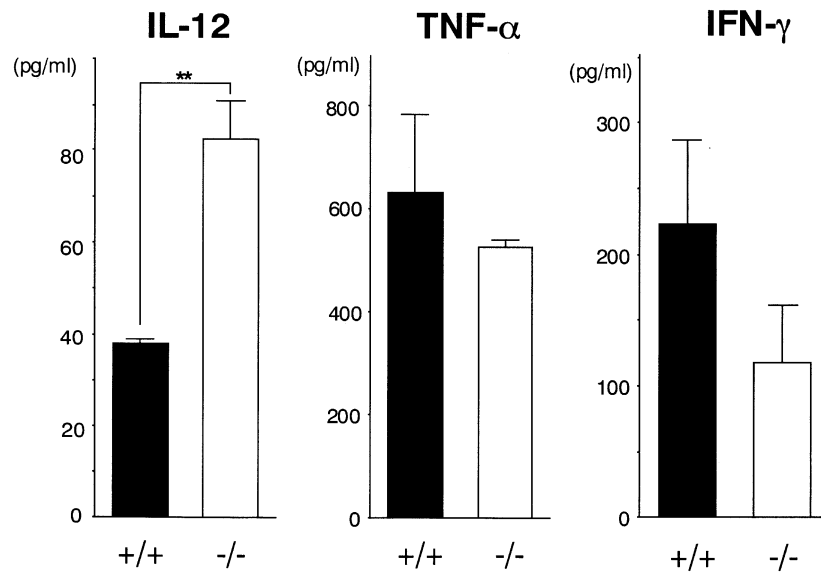
#### Requirement of IL-18-dependent IFN- $\gamma$ for LM eradication

To test whether the impairment in innate phase LM eradication and/or IFN- $\gamma$  induction and the high LM susceptibility of caspase-1<sup>-/-</sup> mice is due to their failure of IL-18 release, we investigated whether exogenous IL-18 corrects these deficiencies. As shown in Fig. 3(A), the exogenous IL-18 obviously decreased the LM titer in the liver of caspase-1<sup>-/-</sup> mice to comparable levels as in normal wild-type mice. Survival was also restored by the administration with IL-18 in the mutant mice (Fig. 3B). However, the same treatment did not significantly increase survival of wild-type mice (data not shown), although the LM burden of IL-18-treated wild-type mice was significantly reduced (Fig. 3A). These results suggest that the dose of IL-18 administered is sufficient to restore both LM eradication and survival of the mutant mice, but insufficient to increase the survival of wild-type mice.

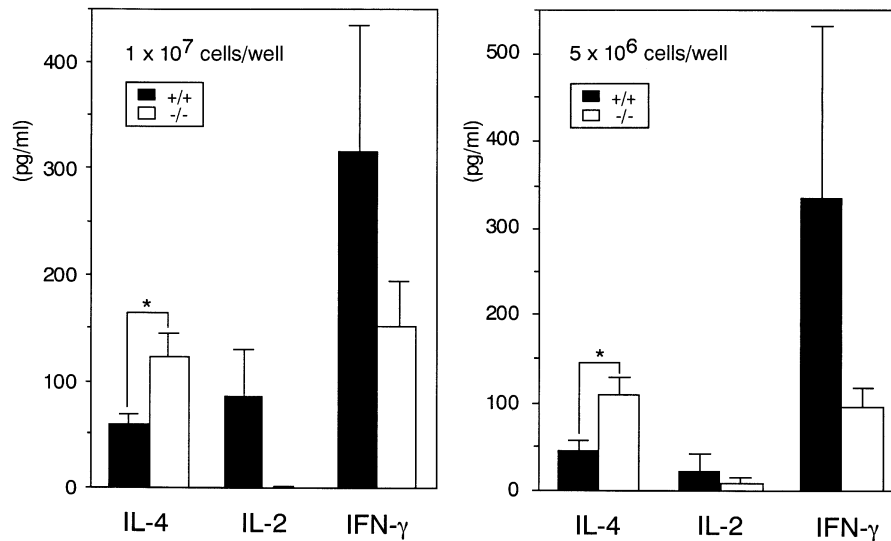
Next, we investigated whether IFN- $\gamma$  is involved in the correction of LM eradication in caspase-1<sup>-/-</sup> mice by IL-18. As expected, serum levels of IFN- $\gamma$  in caspase-1<sup>-/-</sup> mice were corrected by exogenous IL-18, while those in wild-type mice were not significantly affected (Fig. 3C). We confirmed that caspase-1<sup>-/-</sup> cells have no impairment in IFN- $\gamma$  production in response to IL-12 and/or IL-18. Caspase-1<sup>-/-</sup> spleen cells were incubated with IL-12 and/or IL-18, and IFN- $\gamma$  concentration in



**Fig. 5.** Production of cytokines in spleen cells in response to HKLM. (A) Supernatants from unfractionated spleen cell cultures ( $1 \times 10^6$ /ml) were collected 24 h after stimulation with HKLM ( $1 \times 10^7$ /ml), and analyzed for IL-6 (left panel) and IFN- $\gamma$  (right panel) by ELISA. (B) Antibodies against CD11c or DX5 were used to deplete DC or NK cells from wild-type and caspase-1<sup>-/-</sup> splenocytes shown in (A) respectively. The DC-depleted and NK-depleted cells ( $1 \times 10^6$ /ml) were incubated with HKLM ( $1 \times 10^7$ /ml) for 24 h, and IFN- $\gamma$  in each supernatant was measured by ELISA. The data represent the average of three cultures  $\pm$  SD of values. Representative data from three different experiments with similar results are shown.



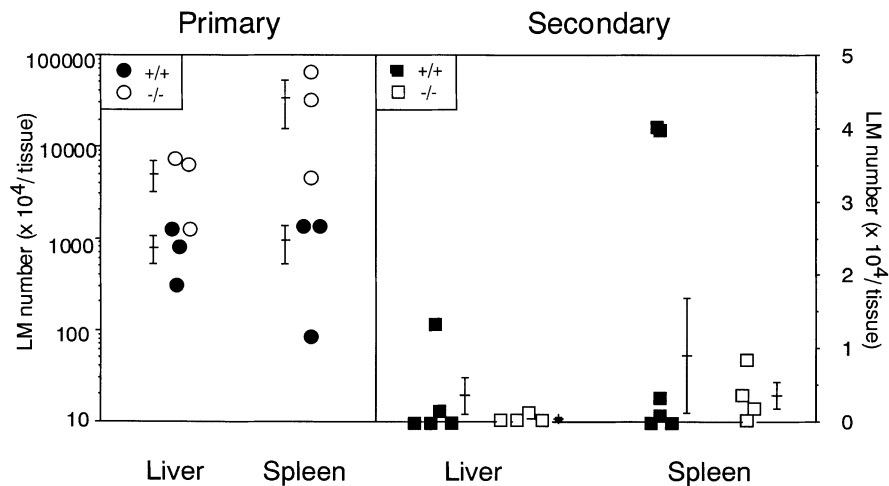
**Fig. 6.** Production of cytokines in BMDC in response to HKLM is not overly impaired in caspase-1<sup>-/-</sup> mice. Supernatants from BMDC cultures ( $1 \times 10^6$ /ml) were collected at 24 h after stimulation with HKLM ( $1 \times 10^7$ /ml) and analyzed for cytokines by ELISA. The data represent the average of three cultures  $\pm$  SD of values. Representative data from three different experiments with similar results are shown. \*\* $P < 0.01$ .



**Fig. 7.** Cytokines in culture supernatant of LM-re-infected spleen cells. Caspase-1<sup>-/-</sup> and control mice had been infected with low-dose LM via i.v. On day 14 after primary infection, mice were re-infected with high-dose LM. Spleen T cells were prepared at 24 h after LM re-infection and cultured for an additional 24 h. Supernatants were subjected to ELISA analysis. The data represent the average of five mice  $\pm$  SD of values from individual mice. Representative data from three independent experiments with similar results are shown.

each supernatant was determined. As shown in Fig. 3(D), spleen cells from the mutant mice produced comparable amounts of IFN- $\gamma$  upon stimulation with IL-12 and/or IL-18 as in wild-type cells, indicating normal responsiveness of the mutant cells to exogenous IL-12 and/or IL-18. Again, IFN- $\gamma$  serum levels paralleled with LM elimination and survival, confirming an important role of IL-18-mediated IFN- $\gamma$  induction in LM eradication in mice.

We next tested whether IFN- $\gamma$  is responsible for the restoration of LM eradication by exogenous IL-18 in the mutant mice. As shown in Fig. 4, exogenous IFN- $\gamma$  corrected the LM burden in the liver of the mutant mice, while the same amount of IFN- $\gamma$  did not affect the LM titer in wild-type mice. Collectively, these results indicate that the partial impairment in LM eradication in caspase-1<sup>-/-</sup> mice is attributable to the failure of IL-18-mediated IFN- $\gamma$  induction.



**Fig. 8.** Normal LM clearance in caspase-1<sup>-/-</sup> mice upon LM re-infection. Mice had been injected with (squares; right panel as experimental group) or without (circles; left panel as control group) low-dose (5000/animal) LM i.v. and re-infected with high-dose ( $5 \times 10^5$ /animal) LM after 14 days. Two days later, livers and spleens were harvested, and colony numbers of LM were counted. The data for individual mice and their mean  $\pm$  SD are shown. The number of mice per experimental group  $n = 4$  or 5, while for the control group  $n = 3$ . Representative data from two independent experiments with similar results are shown.

#### Involvement of NK cells and DC in IFN- $\gamma$ production *in vitro*

As both NK cells and DC are essential for LM eradication in the early infectious phase (7,29,32), we investigated whether NK cells and/or DC are important for IFN- $\gamma$  production in response to HKLM *in vitro*. Collagenase-dispersed wild-type spleen cells, including lymphocytes, macrophages and DC, produced IFN- $\gamma$  and IL-6 in response to HKLM (Fig. 5A). In contrast, wild-type spleen cells depleted of NK cells or DC showed little or only poor ability to produce IFN- $\gamma$  upon HKLM stimulation (Fig. 5B). Thus, both NK cells and DC play an important role in IFN- $\gamma$  production upon stimulation with HKLM. As DC have the potential to produce both IFN- $\gamma$ -inducing factors (i.e. IL-12 and IL-18) and IFN- $\gamma$  (29), the depletion of DC might result in a more severe decrease of IFN- $\gamma$  production than the NK cell-depleted cells (Fig. 5B). In parallel, we performed the same experiments on caspase-1<sup>-/-</sup> cells. The unfractionated spleen cells prepared from caspase-1<sup>-/-</sup> mice produced similar amounts of IL-6, but much less IFN- $\gamma$  than those from wild-type mice (Fig. 5A), indicating again the importance of caspase-1 for IFN- $\gamma$  production. Depletion of DC and NK cells resulted in the reduction of IFN- $\gamma$  to the equivalent levels as compared to wild-type cells (Fig. 5B).

Next, we substantiated further whether caspase-1<sup>-/-</sup> DC are impaired in the production of IFN- $\gamma$ -inducing cytokines and/or IFN- $\gamma$  itself in response to HKLM. Although caspase-1<sup>-/-</sup> BMDC produced higher levels of IL-12 than wild-type cells, the mutant cells produced smaller amounts of IFN- $\gamma$ , suggesting the importance of caspase-1 in the release of other IFN- $\gamma$ -inducing factors, most likely IL-18 (Fig. 6). Indeed, both mutant and wild-type BMDC produced comparable amounts of IFN- $\gamma$  in response to exogenous IL-12 and IL-18 (data not shown). BMDC prepared from caspase-1<sup>-/-</sup> mice produced similar amounts of TNF- $\alpha$  to those from wild-type cells (Fig. 6), indicating that the mutant DC can respond to LM. Collectively, all the results indicated that caspase-1<sup>-/-</sup> mice have partial

defects in production of IFN- $\gamma$ , but not IL-12 or TNF- $\alpha$ , in response to HKLM.

#### Normal LM eradication after re-infection

Finally, we investigated the possible involvement of caspase-1 in memory responses upon LM re-infection. First, we analyzed whether caspase-1<sup>-/-</sup> CD4<sup>+</sup> T cells have impairment in T<sub>h</sub>1 cell development after LM infection, because IL-18 is important for *in vivo* T<sub>h</sub>1 cell development (33). To test this, we prepared CD4<sup>+</sup> T cells from re-infected caspase-1<sup>-/-</sup> mice and analyzed their production of T<sub>h</sub>1- and T<sub>h</sub>2-type cytokines without extra stimulation *ex vivo*. As shown in Fig. 7, caspase-1<sup>-/-</sup> CD4<sup>+</sup> T cells produced moderately higher amounts of IL-4, but less IFN- $\gamma$ /IL-2, than wild-type cells, indicating the importance of caspase-1 for T<sub>h</sub>1 cell development in LM infection. Despite their partial defects in T<sub>h</sub>1 cell development, caspase-1<sup>-/-</sup> mice showed a normal response to LM re-infection as compared to wild-type mice (Fig. 8). These results indicate only a minor role of caspase-1 in development of the memory responses required for the prompt eradication of LM after re-infection.

#### Discussion

This study clearly showed that partial impairment of LM eradication in caspase-1<sup>-/-</sup> mice at the early infectious phase is caused by defects in IFN- $\gamma$  production due to failure of IL-18 release. In contrast, caspase-1<sup>-/-</sup> mice showed normal adaptive immunity against LM because they have retained the ability to eliminate LM after re-infection, based on their normal production of IL-12 that is essential for T<sub>h</sub>1 cell development.

The normal production of IL-12 in caspase-1<sup>-/-</sup> mice should be necessary to mount the subsequent acquired cellular immune response. In fact, these mice successfully cleared the pathogen after secondary infection and LM-

specific CD4<sup>+</sup> T cell activity, evaluated by T cell proliferative response and delayed-type hypersensitivity responses, was normal (data not shown), although a moderate skewing towards T<sub>H</sub>2 response was observed (Fig. 8). Consistent with our previous work (33), endogenous IL-18 seems to be important for efficient development of T<sub>H</sub>1 cells after LM infection, although IL-18 alone cannot cause the development of T<sub>H</sub>1 cells (34).

LM clearance after secondary infection was normal in caspase-1<sup>-/-</sup> mice despite the impaired production of IFN- $\gamma$  from LM-specific T<sub>H</sub>1 cells (Figs 7 and 8). This is plausible because IFN- $\gamma$  is not crucial for the induction of LM-specific CD8<sup>+</sup> T cells and for acquired resistance to LM during secondary infection (35,36). Since acquired immunity of LM infection was demonstrated to be largely dependent on CD8<sup>+</sup> T lymphocytes (19), successful clearance of LM during secondary infection in caspase-1<sup>-/-</sup> mice implies the normal development of the anti-LM CD8<sup>+</sup> T cell response. The fact that caspase-1<sup>-/-</sup> mice can comparably control this pathogen by day 7 in spleen upon initial infection as in wild-type mice (Fig. 1A) also suggests the normal development of LM-specific cellular immunity in these mice. Caspase-1<sup>-/-</sup> mice are also able to induce CD8<sup>+</sup> cytotoxic T lymphocytes against lymphocytic choriomeningitis virus (LCMV) with normal kinetics (Borrow and Oldstone, pers. commun.). Moreover, antibody titers against LM or LCMV were normal in caspase-1<sup>-/-</sup> mice as compared to wild-type mice (data not shown, and Borrow and Oldstone, pers. commun.). Thus, acquired immunity following infection is relatively intact in caspase-1<sup>-/-</sup> mice, suggesting a dependable way of regulating inflammation via innate machinery, without affecting acquired cellular immunity.

Many investigators have demonstrated the significance of IL-1 in listeriosis. The blockade of IL-1 receptor exacerbates the disease (37). IL-1 receptor antagonist (IL-1Ra)-deficient mice and IL-1Ra-overproducing mice are less and more susceptible respectively to listeriosis (38). However, IL-1 $\beta$  by itself seems not to be important for LM immunity because IL-1 $\beta$ -deficient mice show a normal immune response to LM (39). Moreover, the level of IFN- $\gamma$  was normal in mice treated with anti-IL-1 $\beta$  antibody after LM infection (40) and caspase-1<sup>-/-</sup> mice expressed normal IL-1 $\alpha$  serum levels after LM infection (data not shown). It is therefore possible that the normal IL-1 $\alpha$  production overcomes the deficiency of IL-1 $\beta$  release in these mutant mice.

Interestingly, LM clearance in caspase-1<sup>-/-</sup> mice seems to be largely delayed at the early phase of the infection (Fig. 1A). It has been reported that the activity of neutrophils is an important mechanism to combat this microbe in the liver (2), suggesting the importance of caspase-1 in neutrophil activation. This should be further addressed, since we observed fewer activated neutrophils in LM-infected caspase-1<sup>-/-</sup> mice (Tsuji *et al.*, manuscript in preparation).

As adaptive immunity is not overly attenuated in caspase-1<sup>-/-</sup> mice, the inhibition of caspase-1 may be useful to reduce acute inflammation without inhibiting the induction of an antigen-specific T cell response. This approach may offer the potential to control organ failure in patients who suffer from such infections.

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## Abbreviations

BM	bone marrow
DC	dendritic cell
GM-CSF	granulocyte macrophage colony stimulating factor
HKLM	heat-killed LM
IL-1Ra	IL-1 receptor antagonist
LCMV	lymphocytic choriomeningitis virus
LM	<i>Listeria monocytogenes</i>
TNF	tumor necrosis factor

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