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# Critical Role for NALP3/CIAS1/Cryopyrin in Innate and Adaptive Immunity through Its Regulation of Caspase-1

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## Summary

**Mutations in the *NALP3/CIAS1/cryopyrin* gene are linked to three autoinflammatory disorders: Muckle-Wells syndrome, familial cold autoinflammatory syndrome, and chronic infantile neurologic cutaneous and articular syndrome. NALP3, with the adaptor molecule ASC, has been proposed to form a caspase-1-activating "inflammasome," a complex with pro-IL1β-processing activity. Here, we demonstrate the effect of NALP3 deficiency on caspase-1 function. NALP3 was essential for the ATP-driven activation of caspase-1 in lipopolysaccharide-stimulated macrophages and for the efficient secretion of the caspase-1-dependent cytokines IL-1α, IL-1β, and IL-18. IL-1β has been shown to play a key role in contact hypersensitivity; we show that ASC- and NALP3-deficient mice also demonstrate an impaired contact hypersensitivity response to the hapten trinitrophenylchloride. NALP3, however, was not required for caspase-1 activation by *Salmonella typhimurium*, and NALP3 deficiency only partially protects mice from the lethal effects of endotoxin. These data suggest that NALP3 plays a specific role in the caspase-1 activation pathway.**

## Introduction

Interleukin (IL)-1β and IL-18 are both proinflammatory cytokines involved in host response to infection and injury. Excessive production of these cytokines is associated with septic shock and autoimmune disorders. Both IL-1β and IL-18 are produced as inactive cytoplasmic precursors (pro-IL-1β and pro-IL-18), which are cleaved by caspase-1 (IL-1β-converting enzyme [ICE]) to their mature active forms (Dinarello, 1998). Recently, IL-33 has also been described to be cleaved by caspase-1 (Schmitz et al., 2005). Recent studies have suggested that caspase-1 is activated in a complex termed the "inflammasome" (Agostini et al., 2004; Martinon et al., 2002). Overexpression studies have reported that ASC (apoptosis-associated speck-like protein containing a CARD [caspase-recruitment domain]; also known as Pycard or TMS1) and NALP3 can activate caspase-1 (Agostini et al., 2004). ASC contains a C-terminal CARD as well as an N-terminal Pyrin domain (PYD, PAAD, DAPIN) (Conway et al., 2000; Martinon et al., 2002; Masumoto et al., 1999). The CARD domain within ASC binds to the caspase-1 CARD domain (Srinivasula et al., 2002; Stehlik et al., 2003; Wang et al., 2002), and recent studies utilizing ASC-deficient mice have shown that ASC plays a key role in caspase-1 activation (Mariathasan et al., 2004; Yamamoto et al., 2004). The PYD of ASC interacts with the PYD of NALP3. NALP3, also known as cryopyrin/CIAS1/PYPAF1, is a member of the NACHT-LRR (NLR) family (Inohara et al., 2005; Martinon and Tschopp, 2005, Ting and Davis, 2005). Mutations within the *NALP3* gene are responsible for three autoinflammatory disorders, Muckle-Wells syndrome (MWS), familial cold autoinflammatory syndrome (FCAS), and chronic infantile neurological cutaneous and articular syndrome (CINCA), which compose a subset of the periodic fever syndromes (Hoffman et al., 2001). The mutations within NALP3 seen in MWS, FCAS, and CINCA are thought to result in a constitutively active form of NALP3 that causes the spontaneous activation of caspase-1 and the hypersecretion of IL-1β. However, the physiological importance of NALP3 in caspase-1 activation and IL-1β production remains unclear.

We report here a mouse strain deficient in NALP3, and we demonstrate that NALP3 is required for the ATP-driven activation of caspase-1 in response to multiple TLR ligands. We also demonstrate that NALP3 is involved in a specific caspase-1 activation pathway for the processing of proinflammatory cytokines, but not Salmonella-induced cell death. Taken together, these data help to delineate a critical role for NALP3 in caspase-1 activation.

## Results

### Expression of NALP3

The tissue distribution of mouse NALP3 mRNA was examined by using RT-PCR analysis (Figure 1A). NALP3 mRNA was detected in all tissues examined, with the exception of thymus. This is in contrast to Northern blot analysis of human tissues where NALP3 expression was found to be restricted to peripheral blood leukocytes (Manji et al., 2002). Anderson et al. (2004) recently examined the expression of NALP3 in mouse tissues by quantitative real-time PCR, and they also noted NALP3 expression in a wide variety of tissues, with particularly

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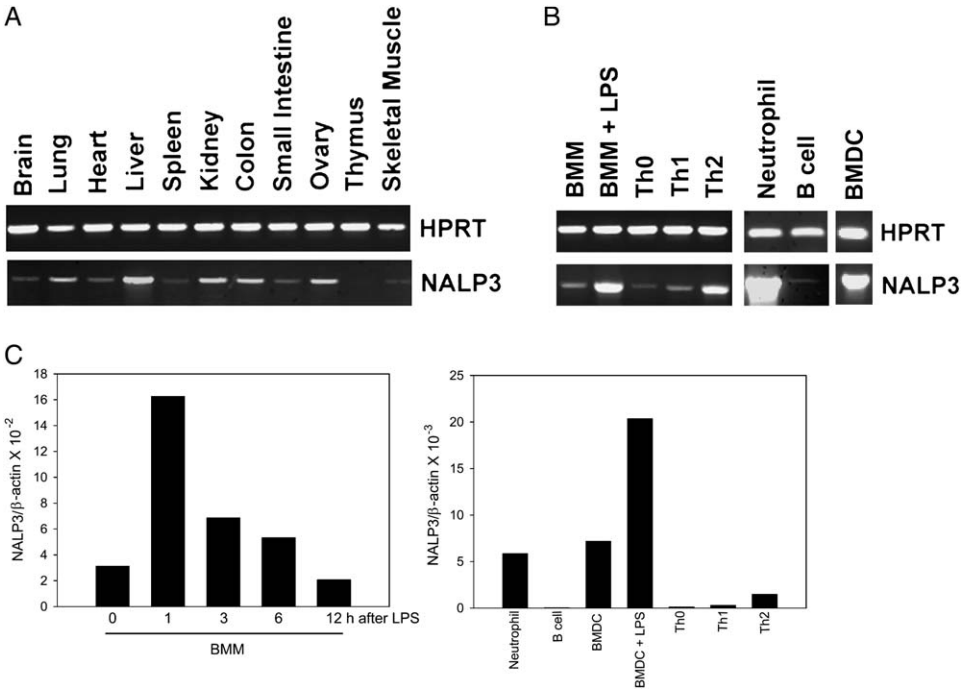


Figure 1. Expression of Mouse NALP3 mRNA

(A) NALP3 expression in C57BL/6 tissue was determined by RT-PCR.

(B) NALP3 expression in bone marrow-derived macrophages (BMM), BMM stimulated with 50 ng/ml LPS for 6 hr, T helper cell 0 (Th0), Th1, Th2, $GR1^+$ neutrophils isolated from bone marrow by using positive selection beads (Miltenyi Biotec), $CD19^+$ B cells isolated from spleens by using positive selection beads, and bone marrow-derived dendritic cells (BMDC) was determined by RT-PCR.

(C) BMM and BMDC were stimulated with 50 ng/ml LPS for 3 hr or the indicated times, and RNA was isolated and used to generate cDNA. Neutrophils, B cells, and T cells were isolated as described in (B). NALP3 expression was determined by quantitative RT-PCR analysis.

high expression in the skin and eye. We also detected NALP3 mRNA by RT-PCR and SYBR green quantitative RT-PCR analysis in various primary immune cells (Figures 1B and 1C). NALP3 is expressed in a variety of resting immune cells, including macrophages, dendritic cells, and neutrophils, and it is seen at low levels in B cells (Figures 1B and 1C). The expression of NALP3 mRNA was upregulated in Th2 cells compared to Th1 and Th0 cells (Figures 1B and 1C). There does not, however, appear to be a defect in the ability of NALP3-deficient $CD4^+$ cells to be differentiated into Th1 or Th2 cells in vitro (data not shown). The expression of NALP3 in macrophages was increased by lipopolysaccharide (LPS) stimulation (Figures 1B and 1C). Real-time PCR analysis carried out on cDNA isolated from LPS-stimulated macrophages demonstrated that the NALP3 message was maximally upregulated 1 hr after LPS stimulation (Figure 1C), which correlates well with the expression of human NALP3, which is also inducible by TLR agonists (O'Connor et al., 2003).

### NALP3 Is Required for Caspase-1 Activation in TLR-Stimulated Macrophages

We investigated the role of NALP3 in the caspase-1-dependent secretion of IL-1β in vitro by using NALP3-deficient bone marrow-derived macrophages. The synthesis, processing, and release of IL-1β by macrophages require two stimuli. An inflammatory stimulus, such as LPS, induces large intracellular stores of the 31 kDa pro-IL-1β, and a second stimulus results in the release of the active mature 17 kDa IL-1β. The second stimulus can be a variety of agents, such as ATP, nigericin, and bacterial toxins (Cheneval et al.,1998; Monack et al., 2001; Perregaux and Gabel, 1994). These stimuli also probably cause the autocatalytic processing of the 45 kDa caspase-1 to generate the two subunits p20 and p10. Macrophages from $NALP3^{+/+}$ and $NALP3^{-/-}$ mice were assessed for caspase-1 activation and IL-1β release after treatment with LPS and ATP. ATP induces the caspase-1-dependent maturation of IL-1β by activating $P2X_7$ receptors, which results in the rapid efflux of intracellular $K^+$ (Solle et al., 2001). Unlike $NALP3^{+/+}$ macrophages, $NALP3^{-/-}$ macrophages did not process caspase-1 in response to LPS and ATP, as measured by the detection of the p10 subunit of caspase-1 (Figure 2A). We examined if the defect in caspase-1 activation had any effect on the production and secretion of IL-1β. Western blot analysis revealed comparable amounts of pro-IL-1β induced in LPS-treated $NALP3^{+/+}$ and $NALP3^{-/-}$ macrophages (Figure 2B). There was, however, a decrease in intracellular pro-IL-1β detected in $NALP3^{+/+}$ macrophages treated with LPS plus ATP in comparison to macrophages treated with LPS alone; this decrease likely reflects the processing and secretion of IL-1β. This decrease in pro-IL-1β after LPS plus ATP treatment was not detected in $NALP3^{-/-}$ macrophages. Secreted IL-1β levels in the supernatants of stimulated macrophages was determined by ELISA (Figure 2C). $NALP3^{+/+}$ macrophages expectedly secreted IL-1β in response to LPS plus ATP, but not to

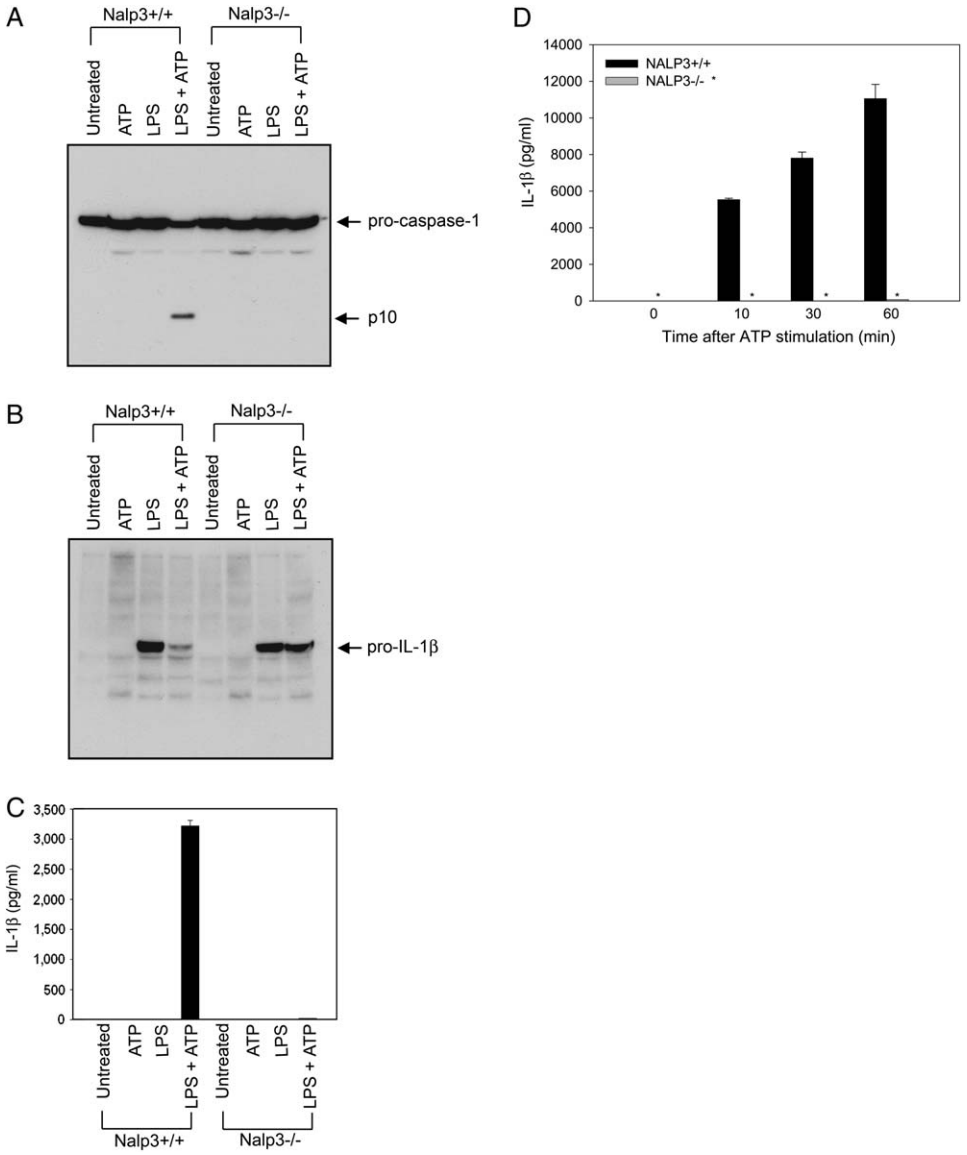


Figure 2. NALP3 Is Required for Caspase-1 Activation and Secretion of IL-1β

(A and B) Bone marrow-derived macrophages were stimulated with or without LPS (10 ng/ml) for 6 hr and then treated with 1 mM ATP for 30 min. Cell lysates were immunoblotted with antibodies against the p10 subunit of (A) caspase-1 and (B) IL-1β. Results in (A) are representative of two separate experiments.

(C and D) (C) IL-1β release into culture supernatants was measured by ELISA. (D) Bone marrow-derived macrophages were stimulated with LPS (10 ng/ml) for 6 hr, followed by stimulation with 1 mM ATP for the indicated times, and IL-1β released into culture supernatants was measured by ELISA. (C and D) Determinations were performed in triplicate and were expressed as the mean ± SD. Results in (C) are representative of four separate experiments.

either stimulus alone, consistent with the decrease seen in intracellular pro-IL-1β. Release of IL-1β after LPS priming and ATP treatment was markedly impaired in NALP3$^{-/-}$ macrophages (Figure 2C), indicating a specific requirement for NALP3 in the caspase-1-dependent secretion of IL-1β. LPS-stimulated NALP3-deficient macrophages treated with ATP for up to 60 min still did not secrete IL-1β (Figure 2D), suggesting that NALP3 is required for caspase-1-mediated secretion of IL-1β in response to LPS plus ATP and does not merely delay this process. Recent studies have demonstrated similar defects in caspase-1 activation and IL-1β secretion in macrophages from ASC-deficient mice (Mariathasan et al., 2004). Our results demonstrate that like ASC, NALP3 is indispensable for caspase-1 activation in response to LPS plus ATP.

The roles of ASC and NALP3 in caspase-1 activation and IL-1β secretion led us to evaluate if other caspase-1-dependent cytokines were affected by ASC and NALP3 deficiency. IL-18 is a proinflammatory cytokine produced as a 24 kDa precursor and processed to an 18 kDa mature form by caspase-1. Although pro-IL-1α is not a substrate for caspase-1, caspase-1 deficiency has been shown to also affect the secretion of IL-1α (Kuida et al., 1995; Li et al., 1995). Release of IL-1α and IL-1β from TLR-primed and ATP-treated ASC$^{-/-}$

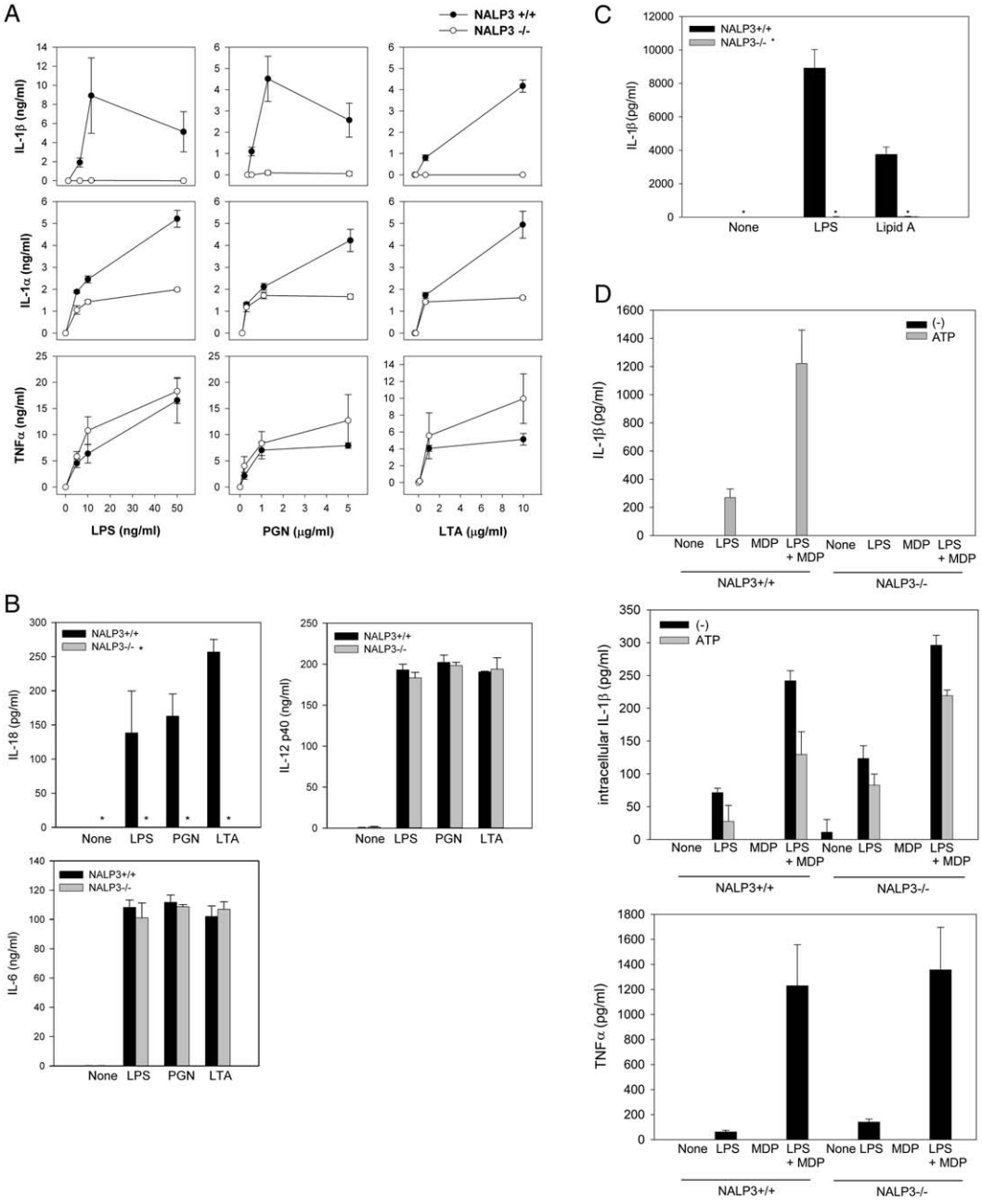


Figure 3. NALP3 Is Required for Secretion of IL-1α, IL-1β, and IL-18 in Response to TLR Agonists Plus ATP

(A and B) Thioglycollate-elicited peritoneal macrophages were stimulated with LPS (50 ng/ml), PGN (5 μg/ml), LTA (10 μg/ml), or at the concentration indicated, for 16 hr, pulsed with 5 mM ATP for 20 min, and then further incubated in fresh medium for 3 hr. (A) IL-1β (upper panel), (A) IL-1α (middle panel), and (B) IL-18 release into culture supernatants was measured by ELISA. (A) TNFα (lower panel), (B) IL-12 p40, and (B) IL-6 secreted during the initial 16 hr incubation were measured by ELISA. In (A), results are representative of two separate experiments.

(C) Bone marrow-derived macrophages were stimulated with or without LPS (10 ng/ml) or lipid A (1 μg/ml) for 6 hr, followed by stimulation with 1 mM ATP for 30 min. IL-1β release into culture supernatants was measured by ELISA.

(D) Bone marrow-derived macrophages were stimulated with LPS (1 ng/ml) or MDP (10 μg/ml) or both LPS plus MDP for 6 hr, followed by incubation with or without 1 mM ATP for 30 min. IL-1β and TNFα release into culture supernatants was measured by ELISA (panels 1 and 3). Intracellular pro-IL-1β and IL-1β were detected by lysing macrophage monolayers with 1% Nonidet P40 and measuring IL-1β in cell lysates by ELISA (panel 2).

In (A)–(D), determinations were performed in triplicate and are expressed as the mean ± SD.

macrophages was reduced when compared to ASC$^{+/+}$ macrophages (see Figure S1 in the Supplemental Data available with this article online), confirming the findings of others that used independently derived ASC-deficient mouse strains (Mariathasan et al., 2004). The release of IL-1α and IL-1β from TLR-primed and ATP-treated NALP3$^{-/-}$ macrophages was also markedly reduced compared to NALP3$^{+/+}$ macrophages (Figure 3A). This

defect was not only seen by using LPS as a priming agent, but also in response to peptidoglycan (PGN), lipoteichoic acid (LTA), and synthetic lipid A (Figures 3A and 3C). NALP3$^{+/+}$ and NALP3$^{-/-}$ macrophages secreted comparable amounts of the proinflammatory cytokine TNF$\alpha$ (Figure 3A). The release of IL-18 from TLR-primed and ATP-treated NALP3$^{-/-}$ macrophages was also decreased compared to NALP3$^{+/+}$ macrophages (Figure 3B). In contrast, no significant difference in IL-6 and IL-12 p40 production was seen in NALP3$^{-/-}$ macrophages compared to NALP3$^{+/+}$ macrophages (Figure 3B), further suggesting that NALP3 is specifically required for caspase-1-dependent secretion of IL-1$\alpha$, IL-1$\beta$, and IL-18. Bacterial muramyl dipeptide (MDP) has been recently proposed to activate NALP3 (Martinon et al., 2004). MDP is also the ligand for NOD2, which is also an NLR family member (Girardin et al., 2003; Inohara et al., 2003). Macrophages stimulated with MDP alone did not secrete detectable amounts of IL-1$\beta$ or TNF$\alpha$ (Figure 3D). MDP enhanced LPS-induced secretion of TNF$\alpha$ in both NALP3$^{+/+}$ and NALP3$^{-/-}$ macrophages (Figure 3D). Intracellular pro-IL-1$\beta$ was enhanced by MDP plus LPS in both NALP3$^{+/+}$ and NALP3$^{-/-}$ macrophages (Figure 3D). MDP also enhanced caspase-1-dependent IL-1$\beta$ secretion from NALP3$^{+/+}$ macrophages, but no secretion of IL-1$\beta$ was detected in NALP3$^{-/-}$ macrophages (Figure 3D). Because NALP3 was required for IL-1$\beta$ secretion in response to LPS plus ATP, we could not evaluate if the enhancement in IL-1$\beta$ secretion seen in LPS plus MDP-treated wild-type macrophages was due to the interaction of NALP3 with MDP. Our data do demonstrate a crucial role for NALP3 in the ATP-driven activation of caspase-1.

### Role of NALP3 in Endotoxic Shock

Wild-type mice treated with high doses of endotoxin produce multiple proinflammatory cytokines, which are thought to mediate the lethal effects of endotoxin. Caspase-1-deficient mice have been shown to have increased resistance to the lethal effect of a high dose of LPS, demonstrating a central role for caspase-1 in mediating inflammatory responses in vivo (Kuida et al., 1995; Li et al., 1995). To determine if ASC and NALP3 are involved in the lethal effects seen with caspase-1 activation in response to LPS, the sensitivity of ASC- and NALP3-deficient mice to endotoxic shock was determined. ASC$^{+/+}$ and ASC$^{-/-}$ mice were injected intraperitoneally with a lethal dose of 37.5 mg/kg LPS. By 24 hr, 90% of ASC$^{+/+}$ mice had died; however, only 15% of ASC$^{-/-}$ mice had died by 24 hr, with a maximum of 30% mortality at day 7 (Figure 4A). ASC$^{-/-}$ mice challenged with LPS also demonstrated significantly decreased serum IL-1$\beta$ and IL-18 levels compared to ASC$^{+/+}$ mice (Figure 4E). These data are consistent with those of others who also noted increased resistance and decreased serum IL-1$\beta$ and IL-18 levels in ASC$^{-/-}$ mice in response to LPS compared to wild-type mice (Mariathasan et al., 2004). In contrast to the increased resistance to endotoxic shock seen in ASC$^{-/-}$ mice, death occurred with a similar time course in NALP3$^{+/+}$ and NALP3$^{-/-}$ mice when challenged with a lethal dose of 37.5 mg/kg LPS (Figure 4B). No enhanced survival to endotoxic shock was observed in NALP3$^{-/-}$ mice despite significantly reduced amounts of serum IL-1$\beta$ and IL-18 in response LPS (Figure 4E). Serum concentrations of TNF$\alpha$ in response to LPS were similar in both NALP3$^{+/+}$ and NALP3$^{-/-}$ mice (Figure 4E). These data suggest that the protective effect of ASC and caspase-1 deficiency seen in endotoxic shock is not due to defects in IL-1$\beta$ and IL-18 production. These findings also demonstrate a divergence in the roles of NALP3 with those of ASC and caspase-1. However, when NALP3$^{-/-}$ mice were challenged with lower doses of LPS (18.75 and 9.38 mg/kg), some degree of protection was observed (Figures 4C and 4D). NALP3$^{-/-}$ mice challenged with 18.75 mg/kg LPS showed a delay in mortality compared to NALP3$^{+/+}$ mice (Figure 4C). In mice challenged with 9.38 mg/kg LPS (Figure 4D), 87.5% of NALP3$^{-/-}$ mice survived, compared to only 25% of NALP3$^{+/+}$ mice by day 7. These data suggest that NALP3 plays a partial role in protection from endotoxic shock, although to a far lesser degree than observed with ASC.

### *Salmonella typhimurium* Can Activate Caspase-1 Independently of NALP3

Caspase-1 has been shown to play an important role in host resistance to Salmonella infections, and macrophages infected with *S. typhimurium* undergo a caspase-1-dependent cell death (Hueffer and Galán, 2004). The Salmonella pathogenicity island (SPI)-1-encoded protein SipB has been postulated to bind to and activate caspase-1, thereby causing macrophage cell death (Hersh et al., 1999). To test whether ASC and NALP3 are also involved in *S. typhimurium*-induced cell death, wild-type ASC$^{-/-}$ and NALP3$^{-/-}$ macrophages were infected with *S. typhimurium*, and cell death was measured by lactate dehydrogenase (LDH) release. ASC has previously been implicated in *S. typhimurium*-induced cell death (Mariathasan et al., 2004), and we were able to confirm that ASC$^{-/-}$ macrophages display a delayed cell death in response to *S. typhimurium* infection (data not shown); however, by 60 min, ASC$^{+/+}$ and ASC$^{-/-}$ macrophages displayed similar cell death in response to *S. typhimurium*. The delay in *S. typhimurium*-induced cell death seen in ASC$^{-/-}$ macrophages was not observed in NALP3$^{-/-}$ macrophages (Figure 5A). Both NALP3$^{+/+}$ and NALP3$^{-/-}$ macrophages displayed a similar level of cell death in response to infection with *S. typhimurium* at all of the time points examined. As expected, the *invG* mutant strain of *S. typhimurium*, defective in the type III secretion system, did not kill NALP3$^{+/+}$ or NALP3$^{-/-}$ macrophages (Figure 5A). We next examined the effect of *S. typhimurium* on caspase-1 activation. LPS plus ATP treatment resulted in the activation of caspase-1 in wild-type macrophages, but not in NALP3-deficient macrophages (Figure 5B). Wild-type macrophages challenged with *S. typhimurium* also rapidly activated caspase-1 (Figure 5B). ASC-deficient macrophages challenged with *S. typhimurium* failed to produce detectable amounts of the p10 subunit of caspase-1 (data not shown; Mariathasan et al., 2004), consistent with the findings of a delayed Salmonella-induced cell death in ASC$^{-/-}$ macrophages. However, NALP3$^{-/-}$ macrophages infected with *S. typhimurium* were capable of activating caspase-1, as measured by the detection of the p10 subunit of caspase-1

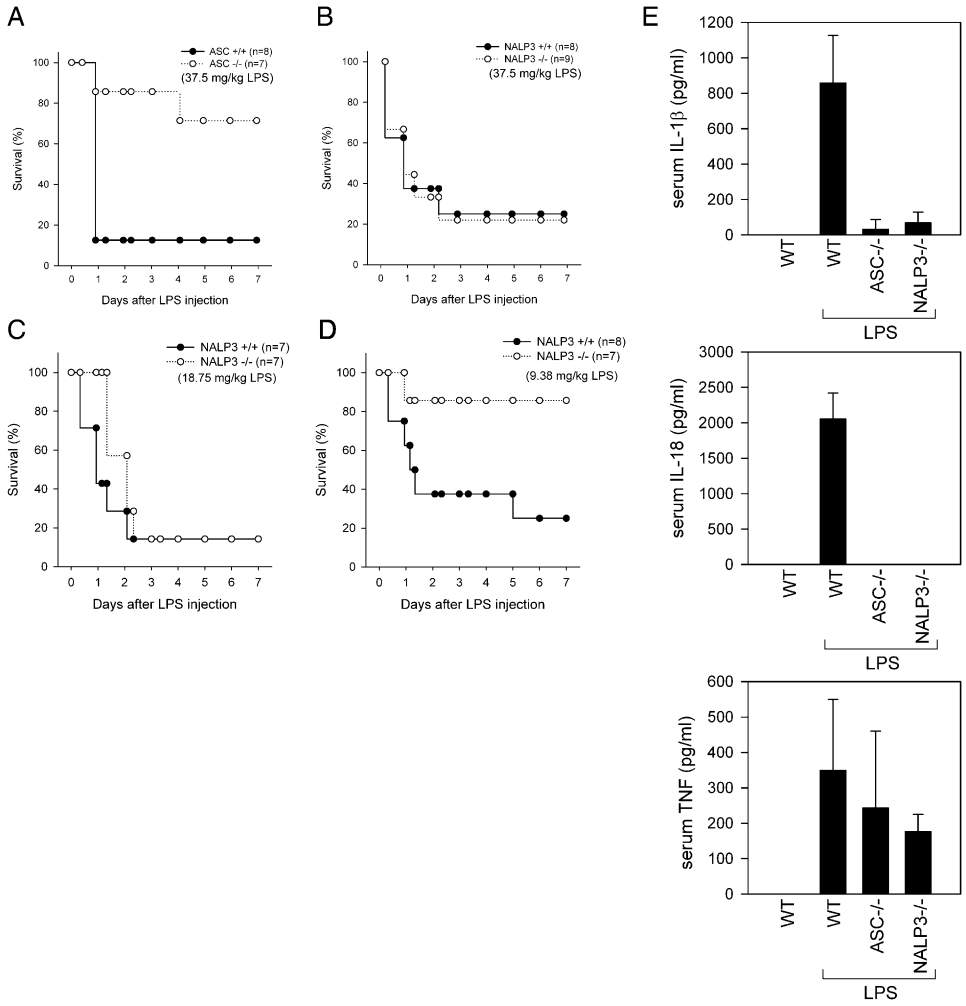


Figure 4. Susceptibility of ASC- and NALP3-Deficient Mice to Endotoxic Shock

(A–D) Mice were injected intraperitoneally with the indicated dose of LPS (37.5, 18.75, or 9.38 mg/kg), and survival was followed for 7 days. In (B), data are representative of two independent experiments.

(E) Wild-type, ASC$^{-/-}$, and NALP3$^{-/-}$ mice were injected intraperitonaelly with LPS (37.5 mg/kg); 5 hr after LPS injection, mice were euthanized and blood was collected by cardiac puncture. Serum IL-1β, IL-18, and TNFα concentrations were measured by ELISA. Serum from untreated wild-type mice was used as a control. Data represent the mean ± SD for 3–4 mice of each genotype.

(Figure 5B). NALP3$^{-/-}$ macrophages infected with *S. typhimurium* were also capable of producing IL-1β, as detected by ELISA (Figure 5C). These findings again are consistent with a distinct role for NALP3 and ASC in caspase-1 function, since NALP3$^{-/-}$ macrophages did not show the delay in *S. typhimurium*-induced cell death observed in ASC$^{-/-}$ macrophages. Furthermore, although ASC appears to be is involved in *S. typhimurium*-mediated caspase-1 activation, *S. typhimurium* can activate caspase-1 independently of NALP3.

### ASC- and NALP3-Deficient Mice Display Impaired Contact Hypersensitivity

Contact hypersensitivity is a T cell-mediated cellular immune response to repeated epicutaneous exposure to contact allergens. Contact hypersensitivity can be divided into two phases: sensitization and elicitation. IL-1β has been implicated to play a central role in contact hypersensitivity to trinitrophenylchloride (TNP-Cl), primarily at the sensitization phase. Mice treated with neutralizing antibodies to IL-1β or mice deficient in IL-1β have impaired contact hypersensitivity to TNP-Cl (Enk et al., 1993; Shornick et al., 1996). In order to determine if ASC and NALP3 are involved in contact hypersensitivity, we examined the responsiveness of wild-type, ASC$^{-/-}$, and NALP3$^{-/-}$ mice after sensitization with TNP-Cl. Wild-type, ASC$^{-/-}$, and NALP3$^{-/-}$ mice were sensitized with 150 μl 5% TNP-Cl on 2 consecutive days, and they were then challenged by painting 10 μl 0.4% TNP-Cl onto both ears 4 days later. Significant ear swelling was detected in the ears of ASC$^{+/+}$ and NALP3$^{+/+}$ mice at the site of challenge at 24 hr (Figures 6A and 6B). In contrast, ASC$^{-/-}$ and NALP3$^{-/-}$ mice showed no increase in 24 hr ear swelling compared to nonsensitized control mice. To determine at which stage of the TNP-Cl response NALP3 is involved, we transferred splenocytes and lymph node cells from wild-type TNP-Cl-sensitized mice into naïve NALP3$^{+/+}$ versus NALP3$^{-/-}$ recipients (Figure 6C). The recipient mice were challenged with 0.4% TNP-Cl, and ear swelling

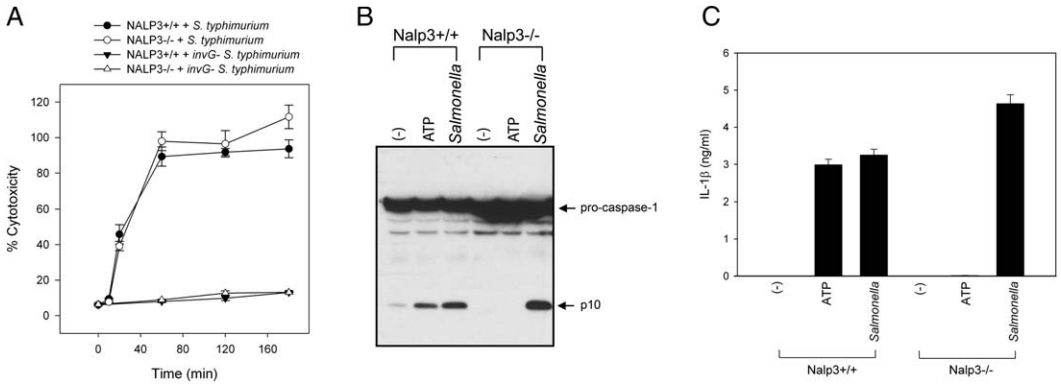


Figure 5. Unaltered *S. typhimurium*-Induced Cell Death in NALP3-Deficient Macrophages

(A) LPS-stimulated bone marrow-derived macrophages from NALP3$^{+/+}$ and NALP3$^{-/-}$ mice were infected with either wild-type *S. typhimurium* or *invG* mutant *S. typhimurium* at an MOI of 20 bacteria per macrophage, and cytotoxicity was measured by LDH release. Cytotoxicity is expressed as a percentage of LDH release by Triton X-100 detergent. Determinations were performed in triplicate and are expressed as the mean ± SD. Results are representative of three separate experiments.

(B and C) LPS-stimulated bone marrow-derived macrophages from NALP3$^{+/+}$ and NALP3$^{-/-}$ mice were either stimulated with 1 mM ATP for 30 min or infected with wild-type *S. typhimurium* at an MOI of 10 bacteria per macrophage for 2 hr. Caspase-1 cleavage in cell lysates was then detected by Western blotting with an (B) anti-caspase-1 p10 antibody, and (C) IL-1β release into the culture supernatants was measured by ELISA.

was measured at 24 hr. As expected, unsensitized NALP3$^{+/+}$ mice that received cells from sensitized wild-type mice developed ear swelling comparable to actively TNP-Cl-sensitized NALP3$^{+/+}$ mice upon TNP-Cl challenge. Unsensitized NALP3$^{-/-}$ mice that received cells from sensitized wild-type mice also developed ear swelling comparable to that seen in NALP3$^{+/+}$ mice (Figure 6C). This suggests that the defect seen in TNP-Cl contact hypersensitivity in NALP3-deficient mice is occurring at the sensitization step, as an appropriate elicitation response occurs in NALP3$^{-/-}$ mice that have received effector cells from sensitized wild-type mice. Together, these data demonstrate a significant defect in the ability of ASC- and NALP3-deficient mice to manifest a contact hypersensitivity response to TNP-Cl.

## Discussion

Our results demonstrate that both NALP3 and ASC are required for caspase-1 activation in macrophages in response to TLR stimulation plus ATP. This defect in caspase-1 activation seen in NALP3-deficient macrophages affects the subsequent production of the caspase-1-dependent cytokines IL-1α, IL-1β, and IL-18 (Figures 3A–3C). Although NALP3 has been reported to attenuate NF-κB activity (O'Connor et al., 2003), we did not observe any consistent differences in the production of TNFα, IL-6, or IL-12 p40 from NALP3$^{-/-}$ macrophages (Figures 3A and 3C), suggesting that NALP3 does not influence macrophage NF-κB activity, but specifically affects caspase-1. NALP3 along with ASC have been proposed to form a caspase-1-activating inflammasome (Agostini et al., 2004). The existence of the inflammasome under physiologic conditions in primary cells remains to be shown. We postulate, however, that in this model NALP3 would be activated by an upstream signal induced by ATP plus TLR stimulation, and would then go on to activate ASC and caspase-1. Although it is known that caspase-1 activation by ATP is mediated through the P2X$_7$ receptor and subsequent K$^+$ efflux (Solle et al., 2001), further studies will be required to elucidate the upstream events linking ATP plus TLR stimulation to NALP3. Recent work has suggested that human NALP3 may function as an intracellular pattern recognition receptor, specifically for bacterial MDP (Martinon et al., 2004). Our studies suggest that NALP3 plays a far more central role in the activation of caspase-1 and is required for the ATP-driven activation of caspase-1 in response to multiple TLR ligands. Unlike human monocytes, mouse macrophages do not secrete IL-1β in response to MDP alone (Figure 3D) and will secrete IL-1β in response to TLR stimulation plus ATP in the absence of MDP. As the possibility of MDP contamination of TLR agonists such as LPS and LTA could not be completely ruled out, due to the method of preparation of these agonists from bacterial sources, we looked at macrophage responses to synthetic lipid A. NALP3$^{-/-}$ macrophages were also unable to secrete IL-1β in response to stimulation with synthetic lipid A plus ATP (Figure 3C), confirming that the results seen with TLR agonists are not a result of MDP contamination.

The autoinflammatory disorders MWS, FCAS, and CINCA are characterized by bouts of fever, arthralgias, and rash. These disorders are associated with over 20 distinct mutations in the NACHT domain of NALP3, resulting in the spontaneous activation of caspase-1 and the secretion of IL-1β (Agostini et al., 2004; Dode et al., 2002; Feldmann et al., 2002; Hoffman et al., 2001). This has been demonstrated with monocytes from patients with Muckle-Wells syndrome, who spontaneously secrete active IL-1β (Agostini et al., 2004). This hypersecretion of IL-1β has provided a rationale for therapy; notably, the administration of the IL-1β antagonist IL-1Ra has proven to be highly successful in abrogating disease severity (Hawkins et al., 2003; Hoffman et al., 2004). Our data help support the hypothesis that the mutations within NALP3 seen in MWS, FCAS, and CINCA result in a constitutively active form of NALP3 that leads to the spontaneous activation of caspase-1 (Dowds

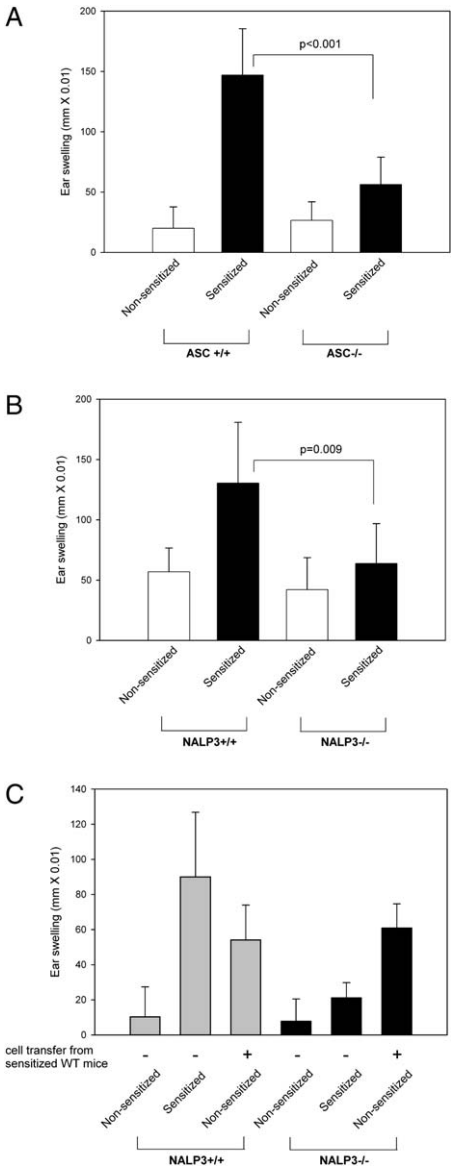


Figure 6. Reduced Contact Hypersensitivity Response in ASC- and NALP3-Deficient Mice

(A and B) (A) ASC$^{+/+}$ and ASC$^{-/-}$ mice or (B) NALP3$^{+/+}$ and NALP3$^{-/-}$ mice were sensitized on the abdomen and chest by topical application of 0.15 ml 5% TNP-Cl on 2 consecutive days (closed bars) or were left unsensitized (open bars). A total of 4 days later, zero time ear thickness was measured before challenge via topical application to both sides of both ears of 10 μl 0.4% TNP-Cl. The subsequent increase in ear thickness was measured at 24 hr. Data in (A) represent the mean ± SD for four (nonsensitized) and five (sensitized) mice per group. Data in (B) represent the mean ± SD for three (nonsensitized) and four (sensitized) mice per group and are representative of three separate experiments.

(C) C57BL/6 mice were sensitized on the abdomen and chest by topical application of 0.15 ml 5% TNP-Cl on 2 consecutive days. A total of 4 days later, spleens and axillary and inguinal lymph nodes were isolated from sensitized mice, made into a single cell suspension, and injected intravenously into unsensitized NALP3$^{+/+}$ and NALP3$^{-/-}$ mice at a 1:1 ratio. Mice were then challenged via topical application to both sides of both ears of 10 μl 0.4% TNP-Cl. Increase in ear thickness was measured at 24 hr. Data represent the mean ± SD for two nonsensitized mice with no cell transfer, two sensitized mice with no cell transfer, and five nonsensitized mice with cell transfer per group.

et al., 2004). MWS and FCAS both have significant skin manifestations that abate after treatment with IL-1Ra (Hawkins et al., 2003; Hoffman et al., 2004). This led us to examine the role of ASC and NALP3 in contact hypersensitivity responses. Both ASC- and NALP3-deficient mice displayed an impaired contact hypersensitivity response to the hapten TNP-Cl (Figures 6A and 6B). This defect in contact hypersensitivity seen in ASC- and NALP3-deficient mice is likely due to their impaired IL-1β secretion, which is consistent with studies that have shown IL-1β-deficient mice to have similar impairment in their contact hypersensitivity response to TNP-Cl (Shornick et al., 1996).

NALP3-deficient mice remained susceptible to endotoxic shock (Figure 4B), which is in marked contrast to the protection from endotoxic shock-mediated mortality seen in ASC-deficient mice challenged with the same dose of LPS. Some degree of protection from the lethal effects of endotoxin was observed in NALP3$^{-/-}$ mice challenged with lower doses of LPS (Figure 4D). These data demonstrate that, although NALP3 is important for ATP-driven caspase-1 activation in response to TLR stimulation, it is only partially responsible for the lethal effects of endotoxin. It remains unclear from these studies how caspase-1 is involved in causing injury in response to endotoxin, but it appears to be only partially NALP3 dependent. The distinct roles of ASC and NALP3 in caspase-1 activation could again be demonstrated in response to *S. typhimurium* infection of macrophages. Macrophages infected with *S. typhimurium* undergo a caspase-1-dependent cell death (Hueffer and Galán, 2004). We show that *S. typhimurium*-induced cell death is, however, not NALP3 dependent, and that *S. typhimurium* can activate caspase-1 independently of NALP3 (Figures 5A and 5B). It is interesting that *S. typhimurium* activates caspase-1 via another NLR family member, namely, IPAF. Recent studies have shown that IPAF-deficient macrophages were resistant to *S. typhimurium*-induced cell death (Mariathasan et al., 2004). ASC also plays a role in this process, as caspase-1 activation is not observed in ASC-deficient macrophages infected with *S. typhimurium*, and there is a delay in *S. typhimurium*-induced cell death in ASC-deficient macrophages (Mariathasan et al., 2004). Thus, ASC seems to play a role as a common downstream factor for both NALP3 and IPAF. The activation of caspase-1 within a macrophage can lead to two distinct endpoints, i.e., the processing of the proinflammatory cytokines IL-1β and IL-18, and caspase-1-dependent cell death induced by pathogens such as Salmonella, Shigella, and Yersinia. It remains unclear as to how caspase-1 can be specifically activated to process IL-1β and IL-18 but not lead to cell death. We observed that NALP3 is involved specifically in the activation of caspase-1 for processing of proinflammatory cytokines, but not Salmonella-induced cell death, while IPAF is known to be required for Salmonella-induced cell death, but not for cytokine processing by ATP plus TLR ligands (Mariathasan et al., 2004). Thus, NALP3 and IPAF seem to be key players in assigning the direction of caspase-1 activity toward cytokine processing and/or cell death. In addition to NALP3 and IPAF, other NALP subfamily proteins, which carry Pyrin and/or CARD domains, may also be responsible for regulating caspase-1 activity

by converging signals from various stimuli to ASC. Analysis of mice lacking these molecules will help reveal their roles in caspase-1 function.

## Experimental Procedures

### Generation of ASC- and NALP3-Deficient Mice

ASC (Figure S2A) and NALP3 (Figure S3A) targeting vectors were electroporated into 129SvEvBrd Lex-1 embryonic stem (ES) cells. Homologous recombinant ES cells were identified by Southern blot analysis and were microinjected into C57BL/6 blastocysts. Chimeric offspring were backcrossed to C57BL/6 mice, and germline transmission was confirmed by PCR of tail genomic DNA (Figures S2B and S3B). Screening of ASC mice with the primers 5′-GTGGACGGAGTGCTGGATG-3′ and 5′-GTCCATCACCAAGTAGGGATG-3′ amplifies a 185 bp product from the wild-type allele; primers 5′-GTCCATCACCAAGTAGGGATG-3′ and 5′-GCTGACCGCTTCCTCGTGCTTTAC-3′ amplify a 275 bp product from the targeted allele. PCR genotyping from NALP3 tail genomic DNA with primers 5′-GCTGTGCCCAGGTCCTAGC-3′ and 5′-CAGCAGCAGCCCTTTCGAG-3′ amplify a 431 bp wild-type band; primers 5′-CGGTGGTTGCTAGGAGATGG-3′ and 5′-ATCGCCTTCTTGACGAGTTC-3′ amplify a 317 bp targeted band. RT-PCR analysis of cDNA isolated from $ASC^{+/+}$, $ASC^{+/-}$, and $ASC^{-/-}$ (Figure S2C) and $NALP3^{+/+}$, $NALP3^{+/-}$, and $NALP3^{-/-}$ (Figure S3C) splenocytes confirmed the absence of ASC and NALP3 mRNA in $ASC^{-/-}$ and $NALP3^{-/-}$ mice, respectively. Primers used for RT-PCR analysis were as follows: NALP3, 5′-CTGAAGATGACGAGTGTCCGTT-3′, 5′-CTCGGGCTCAAACAGCAGCTCCAGCTTAA-3′; ASC, 5′-GCGAGCAGCAGCAAGAGTAAAA-3′, 5′-TACTTCAGCTCTGCTCCAGGT-3′.

ASC- and NALP3-deficient mice were backcrossed onto the C57BL/6 genetic background for at least four generations. $ASC^{+/-}$ and $NALP3^{+/-}$ mice were each intercrossed to generate mutant and control mice. All procedures used in this study complied with federal guidelines and institutional policies by the Yale animal care and use committee.

### Macrophages and $CD4^+$ T Cells

Bone marrow-derived macrophages were generated as previously described (Sutterwala et al., 1997). Thioglycollate-elicited peritoneal macrophages were generated by injecting 1 ml 3% thioglycollate solution into the peritoneal cavity of mice. A total of 4–5 days later, macrophages were collected by peritoneal lavage and plated in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin. $CD4^+$ T cells were prepared from lymph node and spleen cells by immunomagnetic negative selection as described previously (Kamogawa et al., 1993). Cells were cultured in Bruff's medium supplemented with 10% FCS, L-glutamine, penicillin, and streptomycin. $CD4^+$ T cells were stimulated with plate bound antibodies to CD3 and CD28 and 20 U/ml recombinant mouse IL-2 and (Th0) anti-IFNγ and anti-IL-4 or (Th1) 3.5 ng/ml rIL-12 and anti-IL-4 or (Th2) 500 U/ml rIL-4 and anti-IFNγ. Effectors were collected after 4 days of culture and were used to generate cDNA.

### RT-PCR and SYBR Green Quantitative RT-PCR Analysis

RNA was isolated from various C57BL/6 mouse tissues by using Trizol (Invitrogen, Carlsbad, CA). cDNA was synthesized by using SuperScript II reverse transcriptase (Invitrogen). The primers used for RT-PCR amplification were as follows: HPRT, 5′-GTTGGATACAGGCCAGACTTTGTTG-3′, 5′-GAGGGTAGGCTGGCCTATAGGCT-3′; NALP3, 5′-CTGAAGATGACGAGTGTCCGTT-3′, 5′-ATGCTGCAGTTTCTCCAAGGCT-3′. Quantitative RT-PCR analysis was performed by using iQ SYBR Green I Supermix in the iCycler thermal cycler (Bio-Rad, Hercules, CA) with the following primers: NALP3, 5′-CGAGACCTCTGGGAAAAAGCT-3′ and 5′-GCATACCATAGAGGAATGTGATGTACA-3′.

### Western Blotting

Electrophoresis of proteins was performed by using the NuPAGE system (Invitrogen) according to the manufacturer's protocol. Briefly, bone marrow-derived macrophages stimulated with or without LPS and/or ATP were collected and lysed in NuPAGE sample loading buffer (Invitrogen). Proteins were separated on a NuPAGE gel and were transferred to PVDF membrane by electroblotting. To detect caspase-1 and IL-1β, rabbit polyclonal anti-mouse casapse-1 p10 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and goat polyclonal anti-mouse IL-1β antibody (R&D Systems, Minneapolis, MN) were used, respectively.

### Cytokine ELISA Measurements and TLR Agonists

Lipoteichoic acid from *Staphylococcus aureus*, peptidoglycan from *Escherichia coli* serotype 0111:B4, and LPS from *E. coli* serotype 0111:B4 were purchased from Invivogen (San Diego, CA). Synthetic lipid A, LA-15-PP, was purchased from Peptide International (Louisville, KY). MDP was purchased from Sigma (St. Louis, MO). Thioglycollate-elicited peritoneal macrophages were stimulated with the indicated TLR agonist for 16 hr, and culture supernatants were assayed for TNFα, IL-6, and IL-12 p40. The TLR-stimulated macrophages were cultured for an additional 20 min in the presence of 5 mM ATP (Sigma), washed, and then incubated for an additional 3 hr in fresh culture medium. Culture supernatants were then assayed for IL-1α, IL-1β, and IL-18. Antibody pairs for TNFα and IL-1α ELISAs were from eBiosciences (San Diego, CA). Antibody pairs for IL-6 and IL-12 p40 ELISAs were from BD Pharmingen (San Diego, CA). The IL-1β ELISA kit was from R&D Systems. Antibody pairs for the IL-18 ELISA were from MBL (Woburn, MA).

### Endotoxic Shock

Mice (8–12 weeks old) were injected intraperitoneally with 37.5, 18.75, or 9.38 mg/kg *E. coli* LPS (serotype 0111:B4; Sigma). Mice were monitored for lethality twice daily for a total of 7 days. For serum cytokine measurements, a separate cohort of mice received 37.5 mg/kg LPS; 5 hr later, mice were euthanized and blood was collected by cardiac puncture.

### *S. typhimurium* Infection of Macrophages

The *S. typhimurium* strain SB161, which carries a nonpolar mutation in the *invG* gene, has been previously described (Kaniga et al., 1994). In vitro infection of macrophages with *S. typhimurium* was performed as described (Chen et al., 1996). Briefly, bone marrow-derived macrophages were seeded in 24-well dishes at $2 \times 10^5$ cells/well. A total of 18 hr later, nonadherent cells were removed, and macrophages were further incubated for 4 hr in DMEM supplemented with 10 % FCS, 2 mM L-glutamine, and 50 ng/ml LPS. Before infection, the medium was replaced with serum-free HBSS. Macrophages were then infected with wild-type *S. typhimurium* or the *invG* mutant *S. typhimurium* strain SB161 at an MOI of 10 or 20 bacteria per macrophage as indicated. After 25 min, culture supernatants were supplemented with 100 μg/ml gentamicin, added to kill any extracellular bacteria. Macrophage cell death was determined at the indicated time points by LDH release by using a cytotoxicity (LDH) detection kit (Roche, Indianapolis, IN).

### Contact Hypersensitivity

Mice (8–12 weeks old) were sensitized by topical application of 0.15 ml 5% TNP-Cl (Chemica Alta, Edmonton, Canada) in a 1:3 acetone-ethanol mixture to the shaved abdomen and chest on 2 consecutive days. A total of 4 days later, ear thickness was measured with an engineer's micrometer (Mitutoyo Mfg. Co., Aurora, IL) before local ear skin challenge via topical application to both sides of both ears of 10 μl 0.4% TNP-Cl in a 1:1 olive oil-acetone mixture. The subsequent increase in ear thickness was measured at 24 hr. In all experiments, a group of nonsensitized control animals was also challenged on the ears with 0.4% TNP-Cl, and the resulting background increase in ear thickness was measured. For adoptive transfer experiments, C57BL/6 mice were sensitized with 5% TNP-Cl as described above. A total of 4 days later, spleens and axillary and inguinal lymph nodes were isolated from sensitized mice, made into a single cell suspension, and injected intravenously into unsensitized $NALP3^{+/+}$ and $NALP3^{-/-}$ mice at a 1:1 ratio. Mice that received transferred cells were challenged with 0.4% TNP-Cl, and changes in ear thickness were measured at 24 hr.

### Statistical Analysis

We performed statistical analysis by using an unpaired Student's t test. We considered values of $p < 0.05$ to be statistically significant.

## Supplemental Data

Supplemental Data including three figures are available at http://www.immunity.com/cgi/content/full/24/3/317/DC1/.

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