Please cite this article in press as: Khare et al., An NLRP7-Containing Inflammasome Mediates Recognition of Microbial Lipopeptides in Human Macrophages, Immunity (2012), doi:10.1016/j.immuni.2012.02.001

Immunity
Article

An NLRP7-Containing Inflammasome Mediates Recognition of Microbial Lipopeptides in Human Macrophages

Sonal Khare,[1] Andrea Dorfleutner,[1] Nicole B. Bryan,[1,4] Chawon Yun,[1] Alexander D. Radian,[1,3] Lucia de Almeida,[1] Yon Rojanasakul,[5] and Christian Stehlik[1,2,*]
[1]Division of Rheumatology, Department of Medicine
[2]Robert H. Lurie Comprehensive Cancer Center, Interdepartmental Immunobiology Center and Skin Disease Research Center
[3]Driskill Graduate Program
Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA
[4]Program in Cancer Cell Biology, Health Sciences Center
[5]Department of Pharmaceutical Sciences, School of Pharmacy
West Virginia University, Morgantown, WV 26506, USA
*Correspondence: c-stehlik@northwestern.edu
DOI 10.1016/j.immuni.2012.02.001

SUMMARY

Cytosolic pathogen- and damage-associated molecular patterns are sensed by pattern recognition receptors, including members of the nucleotide-binding domain and leucine-rich repeat-containing gene family (NLR), which cause inflammasome assembly and caspase-1 activation to promote maturation and release of the inflammatory cytokines interleukin-1β (IL-1β) and IL-18 and induction of pyroptosis. However, the contribution of most of the NLRs to innate immunity, host defense, and inflammasome activation and their specific agonists are still unknown. Here we describe identification and characterization of an NLRP7 inflammasome in human macrophages, which is induced in response to microbial acylated lipopeptides. Activation of NLRP7 promoted ASC-dependent caspase-1 activation, IL-1β and IL-18 maturation, and restriction of intracellular bacterial replication, but not caspase-1-independent secretion of the proinflammatory cytokines IL-6 and tumor necrosis factor-α. Our study therefore increases our currently limited understanding of NLR activation, inflammasome assembly, and maturation of IL-1β and IL-18 in human macrophages.

INTRODUCTION

Pathogen infection triggers a host defense program utilizing distinct germline-encoded pattern recognition receptors (PRRs), which collectively mount an inflammatory host response via production of proinflammatory cytokines and induction of pyroptosis to eliminate invading pathogens. PRRs are not limited to specifically recognizing conserved molecules on pathogens referred to as pathogen-associated molecular patterns (PAMPs) but also sense host-derived damage-associated molecular patterns (DAMPs). Although engagement of some PRRs, such as TLRs and some nucleotide-binding domain and leucine-rich repeat-containing gene family members (NLRs) containing a caspase recruitment domain (NLRCs), lead to a transcriptional response, activation of other NLRCs and NLRPs (NLRs containing a PYRIN domain; PYD) promote the maturation of the proinflammatory cytokines interleukin-1β (IL-1β) and IL-18 in inflammasomes and the induction of pyroptosis in macrophages (Khare et al., 2010; Schroder and Tschopp, 2010). The human NLR family consists of 22 intracellular PRRs with a tripartite domain architecture featuring a C-terminal leucine-rich region (LRR), a central nucleotide binding NACHT domain, and an N-terminal effector domain crucial for downstream signaling. Inflammasomes are protein scaffolds linking PAMP and DAMP recognition by PRRs to the activation of caspase-1-dependent processing and release of IL-1β and IL-18 (Martinon et al., 2002). PAMP and DAMP sensing occurs by the LRRs and results in receptor unfolding, oligomerization, and PYD-mediated adaptor protein binding (Faustin et al., 2007). ASC is the essential adaptor for bridging NLRPs with caspase-1 (Srinivasula et al., 2002; Stehlik et al., 2003), and macrophages deficient in *Asc* are impaired in caspase-1 activation and maturation of IL-1β and IL-18 (Mariathasan et al., 2004; Yamamoto et al., 2004).

Little is known about the nature of NLRP agonists. NLRP1 recognizes muramyl-dipeptide (MDP) and *B. anthraces* lethal toxin (Boyden and Dietrich, 2006; Faustin et al., 2007). NLRP3 senses a variety of infectious and stress conditions by multiple mechanisms that include potassium efflux, lysosomal damage, and generation of reactive oxygen species (ROS) (Schroder and Tschopp, 2010). Although the signals that activate NLRP6 are unknown, it assembles an inflammasome to control the gut microflora (Chen et al., 2011; Elinav et al., 2011; Grenier et al., 2002). However, the physiological function of most NLRPs and their agonists is currently unknown.

Bacterial acylated (ac) lipopeptides (LP) signal through TLR2 and promote IL-1β maturation and release from macrophages and cause septic shock in mice (Aliprantis et al., 1999; Guan et al., 2010; Takeuchi et al., 2000; Zhang et al., 1997). However, the mechanism of acLP-induced IL-1β release is still elusive. In the present study we identified an NLRP7-containing inflammasome that senses microbial acLP and promotes caspase-1-dependent

Please cite this article in press as: Khare et al., An NLRP7-Containing Inflammasome Mediates Recognition of Microbial Lipopeptides in Human Macrophages, Immunity (2012), doi:10.1016/j.immuni.2012.02.001

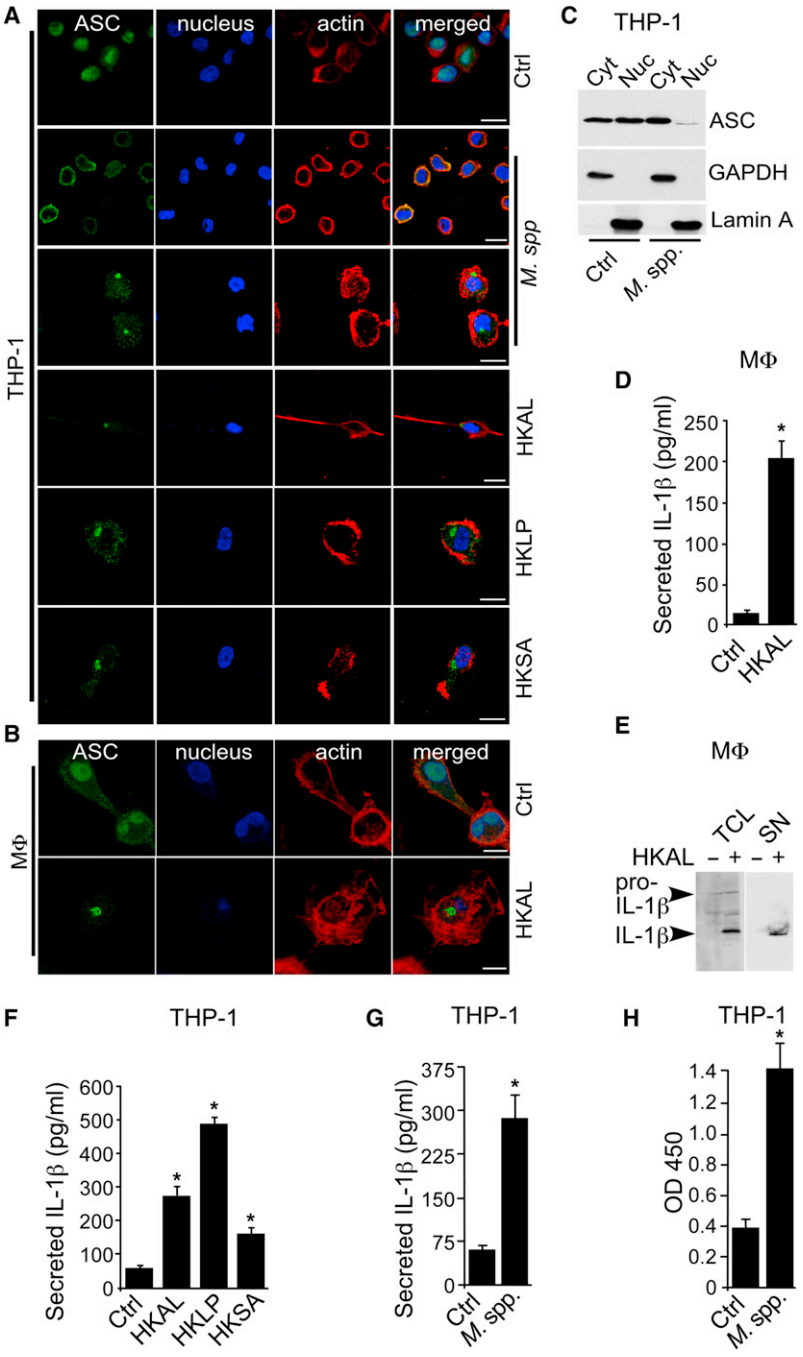

Figure 1. MP Causes ASC Translocation from the Nucleus to the Cytosol and Secretion of IL-1β from MΦ

(A) THP-1 cells were treated with culture SN from HEK293 cells either negative (Ctrl, row 1) or positive for MP for 30 min (row 2) or for 16 hr (row 3), and treated with HKAL (row 4), HKLP (row 5), or HKSA (row 6) for 6 hr.

(B) MΦ were either mock treated (upper row) or treated with HKAL (lower row) for 6 hr and immunostained for ASC, DNA, and actin.

Scale bars represent 20 μm.

(C) Mock and MP-infected THP-1 cells were separated into nuclear (Nuc) and cytosolic (Cyt) fractions and analyzed by protein immunoblot.

(D and E) MΦ were treated with vehicle Ctrl or HKAL for 16 hr and (D) SN were analyzed for IL-1β (n = 3 ± SD) or (E) TCL and concentrated SN were analyzed by protein immunoblot.

(F–H) THP-1 cells were treated with vehicle Ctrl or HK bacteria for 16 hr (F) or with Ctrl or MP-positive culture SN for 24 hr (G). SN were analyzed for IL-1β as above or analyzed for the presence of MP (H) ($OD_{600} \geq 1.0$ indicates the presence of MP); n = 3 ± SD.

*$p \leq 0.05$.

IL-1β and IL-18 maturation in human macrophages to restrict bacterial replication. Therefore, our study is an important contribution toward a better understanding of the pathogen-derived agonists that trigger NLR activation and inflammasome assembly in human macrophages.

RESULTS

Mycoplasma spp. Activate Macrophage Inflammasomes

Mycoplasma spp. (MP) are intracellular pathogens that cause macrophage activation (Sacht et al., 1998), so we tested whether MP are sensed by inflammasomes. We previously described NLRP3 agonist-induced redistribution of ASC from the nucleus to the cytosol and inflammasome formation (Bryan et al., 2009). We noticed that MP infection of human THP-1 monocytic cells (Figure 1A) and treatment of THP-1 cells (Figure 1A) and human primary macrophages (MΦ) (Figure 1B) with heat-killed (HK) *Acholeplasma laidlawii* (HKAL) also caused redistribution of ASC. Inflammasome formation was also induced by HK Gram-negative *Legionella pneumophilia* (HKLP) and Gram-positive *Staphylococcus aureus* (HKSA) (Figure 1A), which are sensed by NLRP3 and NLRC4 or NLRP3, respectively, and require ASC (Mariathasan et al., 2006; Vinzing et al., 2008). Subcellular fractionation also revealed nuclear and cytosolic ASC in mock-infected cells, but only cytosolic ASC in MP-infected cells (Figure 1C). To directly test MP for inflammasome activation, we treated MΦ with HKAL, which caused a significant increase in IL-1β release into culture supernatants (SN) (Figure 1D), indicating that live and HK MP are sufficient to prime MΦ and promote inflammasome activation. HKAL promoted secretion of the mature IL-1β, as shown by the fact that total cell lysates (TCLs) contained the 32 kDa precursor and the mature 17 kDa IL-1β, but only the mature IL-1β was detected in the culture SN (Figure 1E). Treatment of THP-1 cells with HKAL, HKLP, and HKSA not only induced redistribution and aggregation of ASC but also caused release of comparable IL-1β (Figure 1F). Furthermore, infection of THP-1 cells with MP promoted IL-1β release (Figure 1G). Infection was confirmed with the MycoAlert assay (Figure 1H).

Please cite this article in press as: Khare et al., An NLRP7-Containing Inflammasome Mediates Recognition of Microbial Lipopeptides in Human Macrophages, Immunity (2012), doi:10.1016/j.immuni.2012.02.001

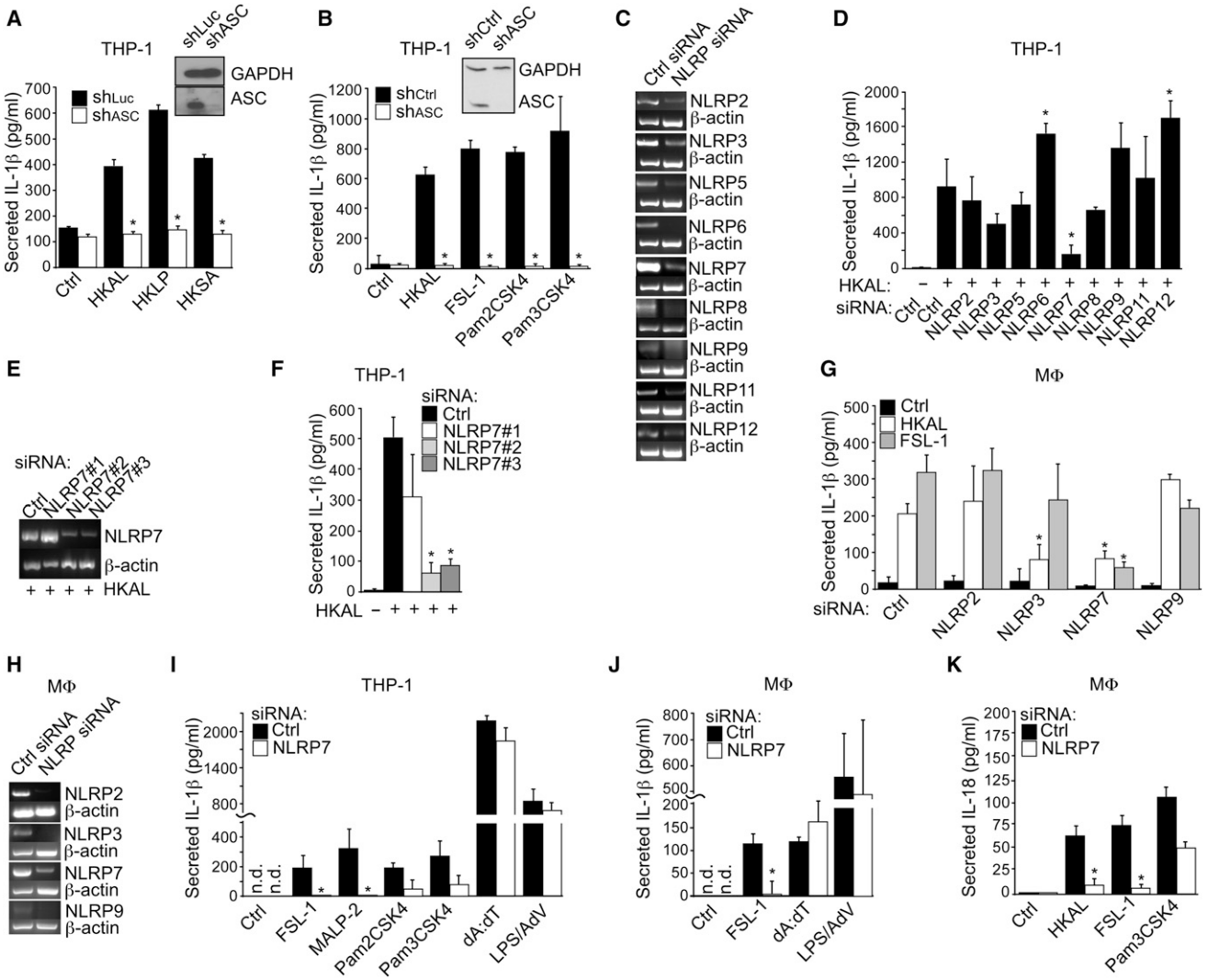

Figure 2. ASC and NLRP7 Are Required for HKAL- and acLP-Induced IL-1β Secretion

(A and B) THP-1^{shLuc}, THP-1^{shCtr}, and THP-1^{shASC} cells were treated with vehicle Ctrl or HK bacteria (A) or with vehicle Ctrl, HKAL, or acLP (B) for 16 hr and SN were analyzed for IL-1β (n = 3 ± SD). TCL were analyzed by protein immunoblot.

(C–F) THP-1 cells were transfected with pooled (C, D) or individual nontargeting (E, F) siRNAs (Ctrl) and siRNAs targeting NLRPs, as indicated, and treated with HKAL for 16 hr. mRNA expression of NLRPs was analyzed by RT-PCR and β-actin control (C, E) and SN were analyzed for IL-1β as above (D, F). n = 3 ± SD.

(G and H) MΦ were transfected with Ctrl or NLRP-specific siRNAs, treated with HKAL or FSL-1 for 16 hr. IL-1β in SN was determined (n = 3 ± SD) (G) and silencing of NLRP expression was confirmed by RT-PCR (H).

(I–K) THP-1 cells (I) or MΦ (J, K) were transfected with Ctrl or NLRP7 siRNAs, mock or acLP treated, transfected with dA:dT or primed with ultrapure LPS for 6 hr, and infected with adenovirus (AdV) for 16 hr, as indicated, and analyzed for IL-1β (I, J) and IL-18 (K) release as above. n = 3 ± SD.

*p ≤ 0.05.

MP and MP-Derived PAMPs Promote ASC- and NLRP7-Dependent IL-1β Release

Maturation and release of IL-1β in BMDM is ASC dependent (Mariathasan et al., 2004; Yamamoto et al., 2004). We therefore investigated whether MP-induced IL-1β maturation also requires ASC, by using THP-1 cells with stably silenced ASC (THP-1^{shASC}), scrambled (THP-1^{shCtrl}), or luciferase-targeted control shRNA (THP-1^{shLuc}) (Bryan et al., 2009; Kung et al., 2012). IL-1β release into culture SN of THP-1^{shASC} cells treated with HKAL, HKLP, or HKSA was impaired compared to THP-1^{shLuc} cells (Figure 2A). MP-induced TLR2 activation occurs via diacylated LP, such as FSL-1 (Garcia et al., 1998; Sacht et al., 1998; Takeuchi et al., 2000). We therefore investigated the contribution of acLP to this ASC-dependent response. Although treatment of THP-1^{shCtrl} cells with HKAL, FSL-1, diacylated Pam2CSK4, and triacylated Pam3CSK4 caused IL-1β release, it was impaired in THP-1^{shASC} cells (Figure 2B), indicating that microbial acLP are sufficient to cause ASC-dependent IL-1β release, as shown for *Asc*-deficient BMDM (Ozören et al., 2006).

The requirement of ASC for MP- and acLP-induced IL-1β release suggests the involvement of inflammasomes and we hypothesized that NLRs are involved in this response in addition

Please cite this article in press as: Khare et al., An NLRP7-Containing Inflammasome Mediates Recognition of Microbial Lipopeptides in Human Macrophages, Immunity (2012), doi:10.1016/j.immunol.2012.02.001

to TLRs. To identify this NLR, we performed an RNAi screen focusing on the 14 human NLRPs resulting from the ASC requirement. We tested expression of all NLRPs in resting and HKAL-treated THP-1 cells by reverse transcriptase-polymerase chain reaction (RT-PCR) and confirmed expression of 11 NLRPs (NLRP1–NLRP3, NLRP5–NLRP9, NLRP11–NLRP13) in THP-1 cells, with no change in expression after HKAL treatment (data not shown). We then silenced NLRP expression with pooled siRNAs and silencing was achieved, as confirmed by RT-PCR and qPCR, for all NLRPs except NLRP1 and NLRP13, which we excluded from further experiments (Figure 2C, Figure S1A available online, and data not shown). Only siRNAs targeting NLRP7 consistently prevented HKAL-induced IL-1β release (Figure 2D). We also noticed reduced IL-1β release in cells with silenced NLRP3, although it was not as potent as NLRP7 silencing. The increased inflammasome activity observed in response to HKAL in NLRP12-silenced cells agrees with a previous report showing an inhibitory role of NLRP12 on NF-κB activation and IL-1β release in response to TLR agonists, including acLP (Williams et al., 2005). We also validated individual siRNAs targeting NLRP7. Only the two siRNAs causing efficient silencing, as determined by RT-PCR (Figure 2E) and quantitative PCR (qPCR) (Figure S1B), blocked HKAL-induced IL-1β release (Figure 2F), further suggesting that NLRP7 is required for MP-mediated IL-1β release.

Microbial acLP Are Specifically Sensed by NLRP7

FSL-1 was sufficient to cause ASC-dependent IL-1β release (Figure 2B) and we therefore hypothesized that NLRP7 should also be required. We silenced NLRP7, NLRP3, and randomly chosen NLRP2 and NLRP9 as controls in MΦ and determined HKAL- or FSL-1-induced IL-1β release. Silencing of NLRP7, but not NLRP2 or NLRP9, impaired HKAL- and FSL-1-induced IL-1β release in MΦ (Figure 2G). Silencing of NLRP3 affected HKAL- but not FSL-1-induced IL-1β release. NLR silencing was confirmed by RT-PCR (Figure 2H) and qPCR (Figure S1C). These results support a specific role of NLRP7 in the cytosolic MP response through FSL-1 recognition. Other acLP, such as MALP-2, Pam2CSK4, and Pam3CSK4, mediate a TLR2-mediated response similar to FSL-1 (Alexopoulou et al., 2002; Guan et al., 2010; Takeuchi et al., 2000, 2001, 2002). We therefore investigated whether NLRP7 is selective for MP-derived LP. Silencing of NLRP7 prevented IL-1β release in response to MP-derived diacylated FSL-1 and MALP-2 and reduced the response to the synthetic diacylated Pam2CSK4 and triacylated Pam3CSK4, but did not impair IL-1β release induced by dsDNA (dA:dT), which is recognized by absent in melanoma 2 (AIM2) (Fernandes-Alnemri et al., 2010; Rathinam et al., 2010) and to adenovirus (AdV), which elicits an NLRP3-mediated response (Muruve et al., 2008) in THP-1 cells (Figure 2I) and MΦ (Figure 2J). Activation of inflammasomes promotes release of IL-1β and IL-18 and accordingly, silencing of NLRP7 also impaired acLP-induced IL-18 release in MΦ (Figure 2K).

NLRP7 and NLRP3 Sense Bacteria, but Only NLRP7 Senses acLP

Activation of BMDM with bacterial RNA or HK bacteria requires costimulation with exogenous ATP (Kanneganti et al., 2007; Mariathasan et al., 2006), but we observed that exogenous ATP is not necessary for NLRP3 inflammasome activation in MΦ. Silencing of NLRP3 in MΦ was sufficient to block IL-1β release in response to *E. coli* total RNA, a known NLRP3 agonist (Kanneganti et al., 2006), and NLRP3 silencing was confirmed by RT-PCR (Figure S2A). In addition, we observed that NLRP7 also sensed HKAL and acLP in the absence of exogenous ATP. We therefore tested the inflammasome response to HK Gram-negative (HKLP and *Porphyromonas gingivalis*, HKPG) and Gram-positive (HKSA and *Listeria monocytogenes*, HKLM) bacteria, which was NLRP3 and NLRP7 dependent in MΦ (Figures 3A and 3B) and THP-1 cells (Figures S2B and S2C). Because the NLRP3-ASC complex senses various PAMPs, we confirmed that silencing of NLRP7 did not affect NLRP3 or ASC expression in MΦ by RT-PCR (Figure 3C) and qPCR (Figure S2D). To further examine NLR specificity, we silenced NLRP3 and NLRP7 and treated cells with FSL-1. We had used NLRP2 as control before and used NLRP12 because of its inhibitory effect. Although both NLRP3 and NLRP7 are required for HK bacteria-induced IL-1β release, only silencing of NLRP7, but not of NLRP3 or NLRP2, impaired FSL-1-induced IL-1β release in MΦ (Figure 3D) and THP-1 cells (Figure S2E), whereas NLRP12 silencing enhanced FSL-1-induced IL-1β release.

Nlrp3-deficient BMDM show impaired caspase-1 activation and IL-1β release in response to infection with Gram-positive *S. aureus* and *L. monocytogenes* (Mariathasan et al., 2006), but *L. monocytogenes* is also sensed by NLRC4 and AIM2 (Wu et al., 2010). *S. aureus*- and *L. monocytogenes*-induced IL-1β release was blunted in cells with silenced NLRP3 and also in cells with silenced NLRP7 (Figures 3E and 3G), indicating that NLRP7 is another cytosolic PRR recognizing both bacteria by sensing bacterial acLP. Consistent with impaired inflammasome activation, we observed an increase in intracellular bacteria in NLRP3- and NLRP7-silenced cells at 345 min postinfection (Figures 3F and 3H). Because bacterial counts were identical at 75 min postinfection, NLRP3 and NLRP7 silencing did not affect bacterial uptake. Thus, reminiscent of NLRP3, NLRP7 inflammasome activation contributes to the restriction of intracellular growth of *S. aureus* and *L. monocytogenes.*

Activation of inflammasomes is sometimes linked to pyroptosis and silencing of NLRP3-impaired early time points of pyroptosis (McCoy et al., 2010) of up to 2 hr postinfection of THP-1 cells with *S. aureus*, as determined by lactate dehydrogenase (LDH) release and annexin V staining, but not later time points (Figures 3I and S2F). In contrast, NLRP7 silencing did not affect pyroptosis at any time point tested (Figures 3I and S2F), suggesting that NLRP7 is involved only in cytokine release.

NLRP7 Is Required for acLP-Induced Caspase-1 Activation

HKAL- and acLP-induced IL-1β release was caspase-1 dependent, as shown by the fact that zYVAD-fmk, a specific cell permeable caspase-1 inhibitor, completely abolished it, whereas the caspase-9 inhibitor zLEHD-fmk had no effect (Figures 4A and S3A). In addition, NLRP7 silencing prevented FSL-1-induced caspase-1 activation, as demonstrated by impaired processing of caspase-1 p45 into p35 (Figure 4B). Crude lipopolysaccharide (cLPS) was used as a positive control. Furthermore, NLRP7 silencing prevented release of active caspase-1

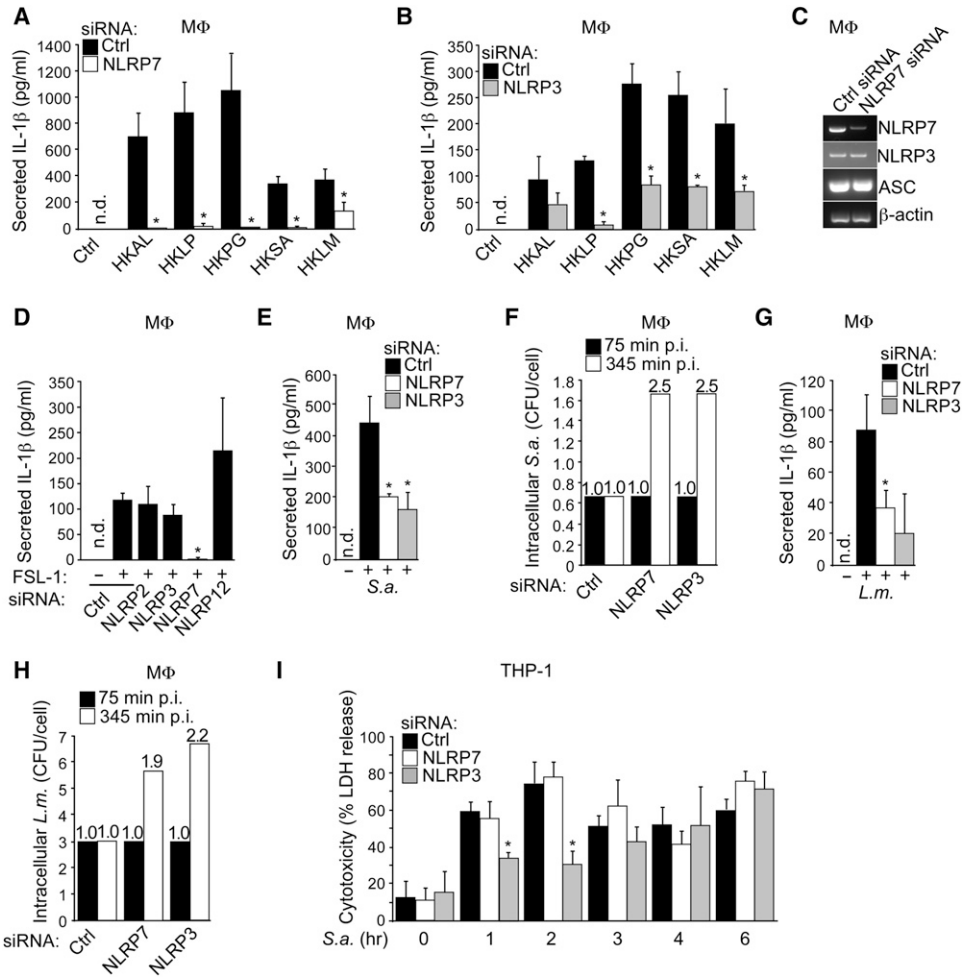

Figure 3. NLRP3 and NLRP7 Recognize Intracellular Bacteria and Restrict Bacterial Replication

(A–D) MΦ were transfected with Ctrl and NLRP siRNAs, mock treated or treated with HK bacteria or FSL-1 for 16 hr, as indicated, and analyzed for IL-1β (A, B, D) (n = 3 ± SD, *p ≤ 0.05) or analyzed by RT-PCR (C).

(E–H) MΦ were transfected with Ctrl, NLRP3, or NLRP7 siRNAs and left uninfected or were infected with *S. aureus* (S.a.) (E, F) or *L. monocytogenes* (L.m.) (G, H) and analyzed for IL-1β 345 min p.i. (E, G) (n = 3 ± SD) or were lysed at 75 and 345 min p.i. and intracellular colony forming units (CFU) were determined (F, H). Results from a representative experiment are presented as CFU/cell and the fold increase compared to control siRNA-transfected cells at 75 min p.i. is indicated.

(I) THP-1 cells were transfected with siRNAs as indicated and kept uninfected or infected with S.a. as above. SN were analyzed at the indicated times for released LDH and presented as percent cytotoxicity (n = 3 ± SD; *p ≤ 0.05); n.d., not detectable.

p20 into culture SN (Figure 4C), directly demonstrating that NLRP7-mediated caspase-1 activation is required for acLP-induced IL-1β release.

NLRP7 Is a Bona Fide Inflammasome Activator and Does Not Affect NF-κB

acLP, LPS, and tumor necrosis factor-α (TNF-α) activate TLR and cytokine receptor-mediated NF-κB-dependent transcription of inflammatory cytokines, such as TNF-α and IL-6, which is sufficient for secretion. However, release of IL-1β requires in addition a caspase-1-dependent maturation step. To rule out that NLRP7 silencing had a general effect on the expression of proinflammatory cytokines, we analyzed FSL-1-induced secretion of IL-6 and TNF-α, with cLPS as control, which activates TLR2 and TLR4. To eliminate any potential autocrine IL-1β effects, we pretreated cells with the IL-1 receptor (IL-1R) antagonist anakinra (Taxman et al., 2006). However, contrary to the release of IL-1β, NLRP7 silencing did not affect the release of IL-6 and TNF-α in MΦ (Figures 4D and S3C) and THP-1 cells (Figure 4E and S3B), indicating that NLRP7 is not involved in the transcriptional regulation of cytokines. Furthermore, NLRP7 silencing did not affect FSL-1-, cLPS-, or TNF-α-induced IκBα phosphorylation and degradation in MΦ and THP-1 cells (Figure 4F), further ruling out NLRP7 effects on NF-κB activation.

NLRP7 and TLR2 Are Required for acLP-Induced IL-1β Release

FSL-1 upregulates IL-1β and IL-18 transcription through a TLR- and NF-κB-dependent mechanism. Although we achieved efficient silencing of NLRP7, this did not affect IL-1β and IL-18 transcription, as determined by qPCR (Figure 5A) and expression of pro-IL-1β in MΦ (Figure 5B), although we achieved efficient

Please cite this article in press as: Khare et al., An NLRP7-Containing Inflammasome Mediates Recognition of Microbial Lipopeptides in Human Macrophages, Immunity (2012), doi:10.1016/j.immunol.2012.02.001

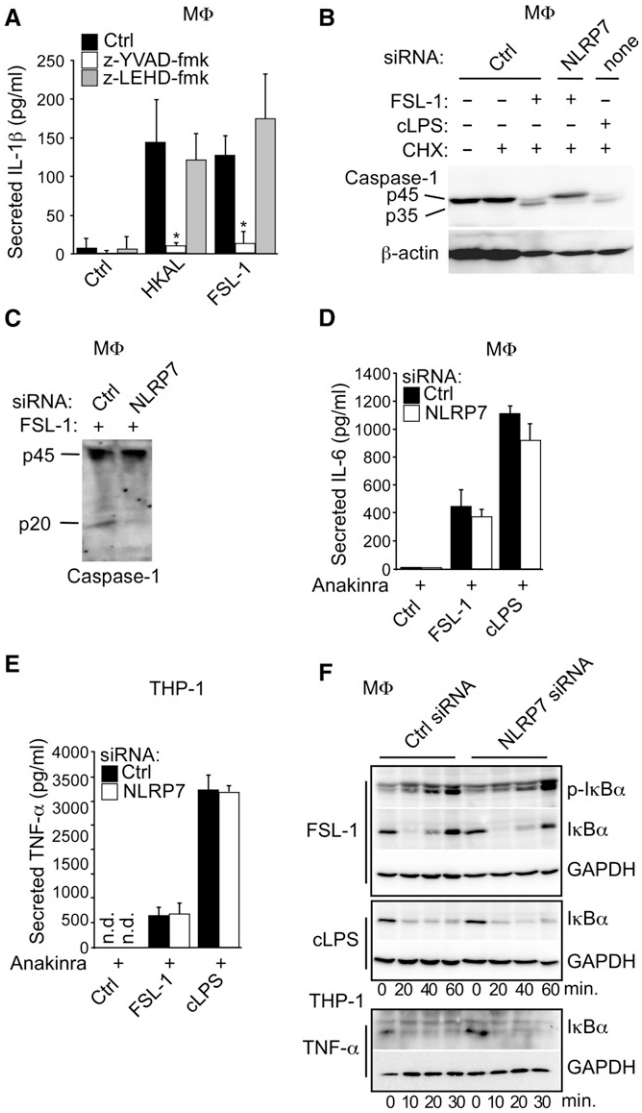

Figure 4. NLRP7 Mediates acLP-Induced IL-1β Release through Caspase-1 Activation

(A) MΦ were pretreated with vehicle Ctrl (DMSO), zYVAD-fmk, or zLEHD-fmk for 1 hr, treated with vehicle Ctrl, HKAL, or FSL-1 for 16 hr, and analyzed for IL-1β release (n = 3 ± SD); *p ≤ 0.05.

(B and C) MΦ were not transfected (none) or transfected with Ctrl or NLRP7 siRNAs. Samples were mock or cycloheximide (CHX) treated (to prevent resynthesis of pro-caspase-1) and FSL-1 or cLPS activated as indicated (B) or FSL-1 activated (C) for 6 hr. TCL (B) and SN (C) were analyzed by protein immunoblot.

(D and E) MΦ (D) or THP-1 cells (E) were transfected with Ctrl or NLRP7 siRNAs and activated with FSL-1 or cLPS for 16 hr as indicated and SN were analyzed for IL-6 (D) and TNF-α (E) by ELISA (n = 3 ± SD). Cells were pretreated for 1 hr with anakinra to prevent autocrine IL-1β signaling. n.d., not detectable.

(F) MΦ (upper panel) and THP-1 cells (lower panel) were transfected as indicated and activated with FSL-1, cLPS, or TNF-α for the indicated times, and TCL were analyzed by protein immunoblot.

silencing of NLRP7 protein (Figure 5C). In contrast, TLR2 silencing abolished FSL-1-induced transcription of IL-1β and IL-18 (Figure 5D) and silencing was confirmed by immunoblot (Figures 5E and S4A). As expected, silencing of either TLR2 or NLRP7 prevented FSL-1-induced IL-1β release in MΦ (Figure 5F) and in THP-1 cells (Figure S4B). Because NLRP7 silencing did not affect IL-1β transcription, we conclude that NLRP7 is not involved in TLR2 signaling. *Tlr2* and *Asc* are required for host defense against *L. monocytogenes* through transcription factor and caspase-1 activation, respectively (Ozören et al., 2006). Accordingly, silencing of NLRP7 and TLR2 impaired *L. monocytogenes*-induced IL-1β release in MΦ (Figure 5G) and caused increased intracellular *L. monocytogenes* counts (Figure 5H). Because TLR2 is also required for efficient phagocytosis of *L. monocytogenes* (Shen et al., 2010), intracellular bacterial counts were lower upon TLR2 silencing compared to NLRP7 silencing, but TLR2 also controls *L. monocytogenes* infection (Torres et al., 2004). Collectively, these results demonstrate that TLR2 and NLRP7 are required for acLP-induced IL-1β release through distinct molecular mechanisms.

NLRP7, ASC, and Caspase-1 Assemble into a High-Molecular-Weight Complex in Response to Bacterial Infection

ASC, caspase-1, and NLRP7 are required for acLP-induced maturation and release of IL-1β. Therefore, we expected ASC to be recruited to NLRP7, similar to its recruitment to NLRP3. Coexpression of NLRP7 with ASC caused their colocalization in aggregates, similar to NLRP3, although additional factors might influence this interaction, because it was not as efficient as NLRP3 (Figure 6A). NLRP7 alone localized diffusely throughout the cytosol (data not shown), but aggregates appeared in cells with higher NLRP7 expression (Figure 6B). Distribution of NLRP7 was not affected in the presence or absence of caspase-1 expression (Figure 6B, compare lower and upper panels). However, coexpression of NLRP7 with ASC and caspase-1 caused colocalization of all three proteins in aggregates (Figure 6C), suggesting that ASC is required to bridge NLRP7 to caspase-1. To demonstrate that NLRP7 and ASC physically interact, we transiently transfected GFP, GFP-NLRP7, or GFP-NLRP3 into stably ASC-expressing HEK293 cells (HEK293^{ASC}) and immunoprecipitated ASC. ASC copurified NLRP3 and NLRP7, but not GFP, indicating that NLRP7 can interact with ASC (Figure 6D). As predicted, the interaction required the PYD of NLRP7, because NLRP7$^{\Delta LRR}$ but not NLRP7$^{\Delta PYD}$ was copurified with ASC (Figure 6E). HEK293 cells lack endogenous inflammasome proteins, but restoring defined concentrations of pro-caspase-1, pro-IL-1β, ASC, and NLRP3 is sufficient to release IL-1β, even in the absence of agonists (Bryan et al., 2009). To demonstrate that the NLRP7 complex contains inflammasome activity, we first purified NLRP7 from stable inflammasome-reconstituted cells expressing pro-caspase-1 and ASC, transfected with NLRP7, by using immobilized NLRP7 antibodies and control IgG. NLRP7 specifically copurified ASC and caspase-1, further indicating that all three proteins existed in a complex (Figure 6F). We then subjected the purified NLRP7 protein complexes, which were isolated from these cells and from inflammasome-reconstituted cells lacking NLRP7, to an in vitro caspase-1 activity assay. Caspase-1 activity was copurified only from cells expressing NLRP7, indicating that an active caspase-1 is present in the NLRP7 protein complex (Figure 6G). To further demonstrate that it has IL-1β converting enzyme (ICE) activity, we incubated the purified NLRP7 protein

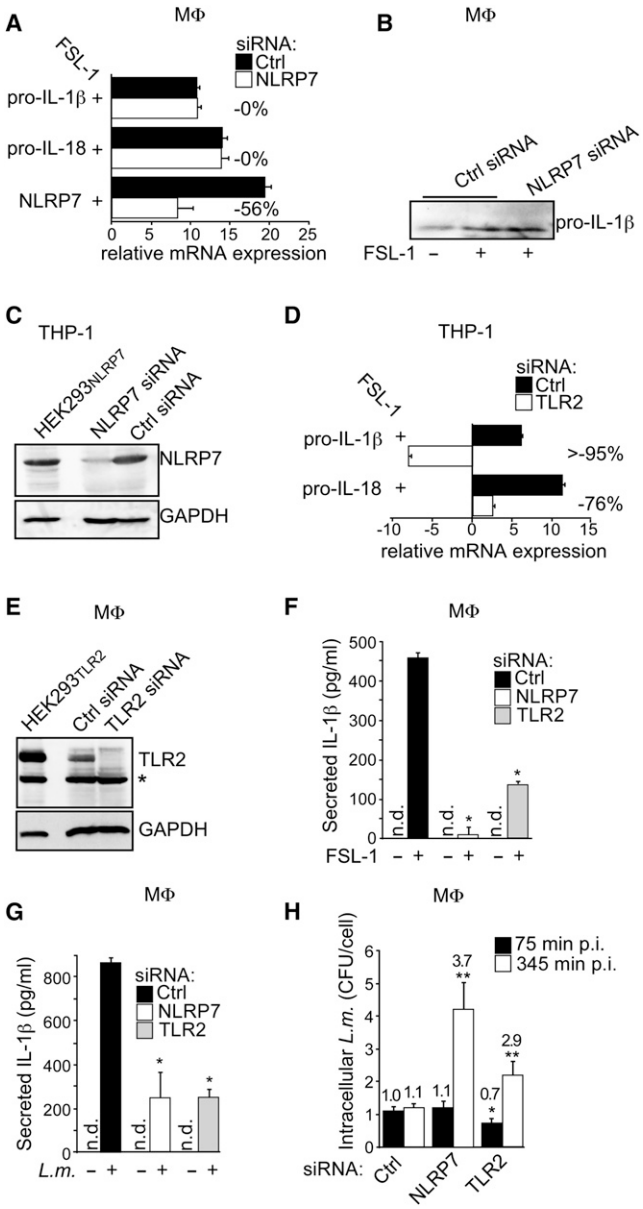

Figure 5. NLRP7 and TLR2 Are Required for acLP-Induced IL-1β Release

(A) MΦ were transfected with Ctrl or NLRP7 siRNAs, activated with FSL-1 for 16 hr, and analyzed by qPCR (relative expression compared to β-actin, n = 3 ± SD). Silencing efficiency is indicated in percentage compared to control siRNA.

(B) MΦ were transfected with Ctrl or NLRP7 siRNAs and activated with FSL-1 for 16 hr in the presence of zYVAD-fmk to prevent maturation of IL-1β, and TCL were analyzed for pro-IL-1β by protein immunoblot.

(C) THP-1 cells were transfected with Ctrl or NLRP7 siRNAs and analyzed by protein immunoblot, with NLRP7-transfected HEK293^{NLRP7} cells as control.

(D) THP-1 cells were transfected with Ctrl or TLR2 siRNAs, treated with FSL-1 for 16 hr, and analyzed by qPCR for IL-1β and IL-18 mRNA, as described above. n = 3 ± SD.

(E and F) MΦ were transfected with Ctrl, NLRP7, or TLR2 siRNAs, mock treated or treated with FSL-1 for 16 hr. TCL were analyzed by protein immunoblot with TLR2-transfected HEK293^{TLR2} cells as control (E). Asterisk indicates cross-reactive protein. TCL were analyzed for IL-1β release (F) (n = 3 ± SD).

complexes with a protein lysate containing pro-IL-1β as a substrate. Only the NLRP7 protein complex purified from cells expressing NLRP7 contained ICE activity, as determined by its capability to mature pro-IL-1β (Figure 6H).

We then investigated endogenous NLRP7 inflammasome formation by size exclusion chromatography (SEC) of TCL (Figure S5). Although NLRP7 was present in fractions corresponding to its molecular weight in resting cells, it was efficiently shifted into a high-molecular-weight (HMW) complex after *S. aureus* infection of MΦ (Figure 6I). This complex was less than 1.5 MDa, as shown by the fact that NLRP7 was not present in the void volume (first fraction). Also, ASC and caspase-1 shifted into the NLRP7-containing HMW fractions in *S. aureus*-infected MΦ. Caspase-1 copurified ASC and NLRP7 from the HMW (fractions 2–6) but copurified only ASC from the low-molecular-weight (LMW) (fractions 19–24) after *S. aureus* infection (Figure 6J). We also performed the reciprocal experiment and purified NLRP7 from both fractions, and caspase-1 activity was purified only from the HMW fraction (Figure 6K), which also contained ICE activity (Figure 6L), providing biochemical support for NLRP7-containing inflammasome formation.

NLRP7, ASC, and Caspase-1 Are Sufficient for the Inflammasome Response to MP and acLP

We used the inflammasome reconstitution system to dissect NLRP7 signaling. NLRP7 expression alone was not sufficient to cause IL-1β release in the presence of pro-caspase-1 but required coexpression of ASC (Figure 7A), further emphasizing the essential role of ASC in the NLRP7 inflammasome. To directly demonstrate that NLRP7 is sufficient for HKAL and FSL-1 recognition, we transfected cells with suboptimal concentrations of NLRP7, followed by a second transfection to deliver HKAL and FSL-1 to the cytosol. Although transfection of HKAL and FSL-1 did not promote IL-1β release in control cells, it enhanced IL-1β release in NLRP7-transfected cells (Figure 7B). Addition of HKAL or FSL-1 to the culture medium also caused inflammasome activity, albeit less than the activity obtained by cytosolic delivery of HKAL or FSL-1 (Figure S6A), because agonists need to reach the cytosol for NLR activation. To demonstrate the specificity of acLP for NLRP7, we reconstituted NLRP7 and NLRP3 inflammasomes and used Nod1, which can directly interact with caspase-1 (Yoo et al., 2002), and the inflammasome-unrelated AFAP1 as controls. FSL-1 caused significantly increased IL-1β release only in reconstituted NLRP7 inflammasomes (Figure 7C). As expected, AFAP1 did not promote IL-1β maturation. It is generally believed that the LRR is required for ligand sensing and specificity, and deletion of the LRR renders NLRP3 unresponsive in vivo (Hoffman et al., 2010). However, previous studies demonstrate that deletion of the LRR in several NLRs renders the protein hyperactive in vitro. NLRP7$^{\Delta LRR}$ similarly displayed enhanced activity in the inflammasome

(G and H) MΦ were transfected with Ctrl, NLRP7, or TLR2 siRNAs and either left uninfected or were infected with *L. monocytogenes* (L.m.) and were analyzed for IL-1β release 345 min p.i. (G), or were lysed at 75 and 345 min p.i. (H) and CFU were determined. Results are presented as CFU/cell and the fold increase compared to Ctrl siRNA-transfected cells at 75 min p.i. is indicated (n = 3 ± SD).

n.d., not detectable; *p ≤ 0.05.

Please cite this article in press as: Khare et al., An NLRP7-Containing Inflammasome Mediates Recognition of Microbial Lipopeptides in Human Macrophages, Immunity (2012), doi:10.1016/j.immuni.2012.02.001

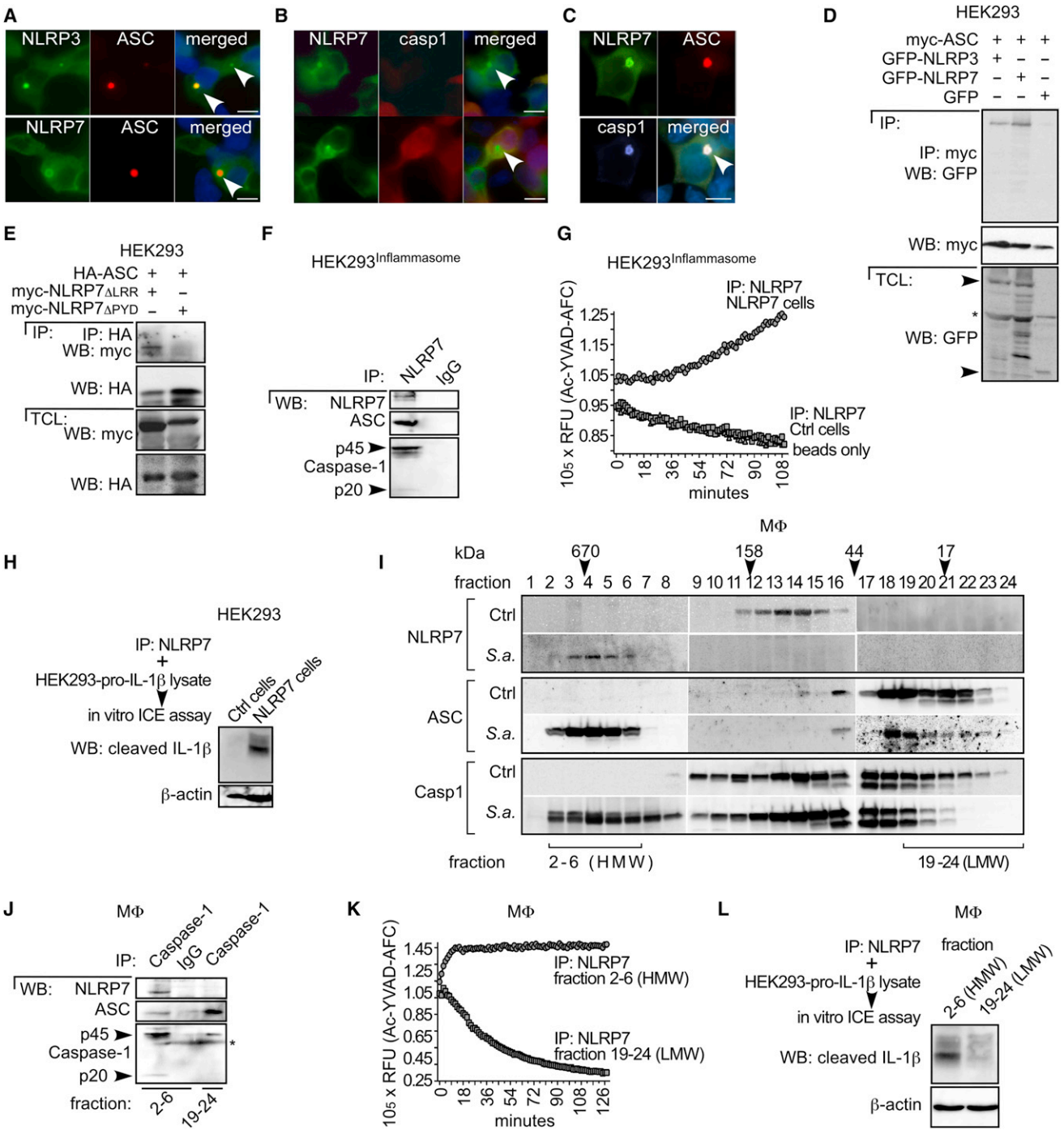

Figure 6. Activation of NLRP7 Causes the Formation of a High-Molecular-Weight Inflammasome in MΦ

(A–C) Immunofluorescence staining of HEK293 cells transiently transfected with ASC and NLRP3 (upper row), ASC and NLRP7 (bottom panel) (A), NLRP7 and pro-caspase-1 (upper row shows a cell expressing only NLRP7; lower row shows cells expressing NLRP7 and caspase-1) (B), or NLRP7, ASC, and pro-caspase-1 (C). NLR-containing aggregates are marked by arrowheads. Scale bars represent 20 μm.

(D) HEK293^{ASC} cells were transfected with GFP, GFP-NLRP3, or GFP-NLRP7 as indicated and TCL were used for IP with immobilized myc antibodies and protein immunoblot as indicated.

(E) HEK293 cells were transfected with HA-ASC, myc-NLRP7$^{\Delta LRR}$, or myc-NLRP7$^{\Delta PYD}$, as indicated, and TCL were used for IP with immobilized HA antibodies and protein immunoblot as indicated.

(F–H) Stable HEK293 inflammasome-reconstituted cells (HEK293^{Inflammasome}) were transfected with control or NLRP7 as indicated, and TCL were used for IP with immobilized NLRP7 antibodies. Immune complexes were analyzed by protein immunoblot as indicated (F) or equilibrated in caspase-1 assay buffer and subjected to in vitro caspase-1 activity assay (G) or for ICE activity incubated with a TCL isolated from HEK293 cells transfected with pro-IL-1β and analyzed by protein immunoblot for mature IL-1β (H).

Please cite this article in press as: Khare et al., An NLRP7-Containing Inflammasome Mediates Recognition of Microbial Lipopeptides in Human Macrophages, Immunity (2012), doi:10.1016/j.immuni.2012.02.001

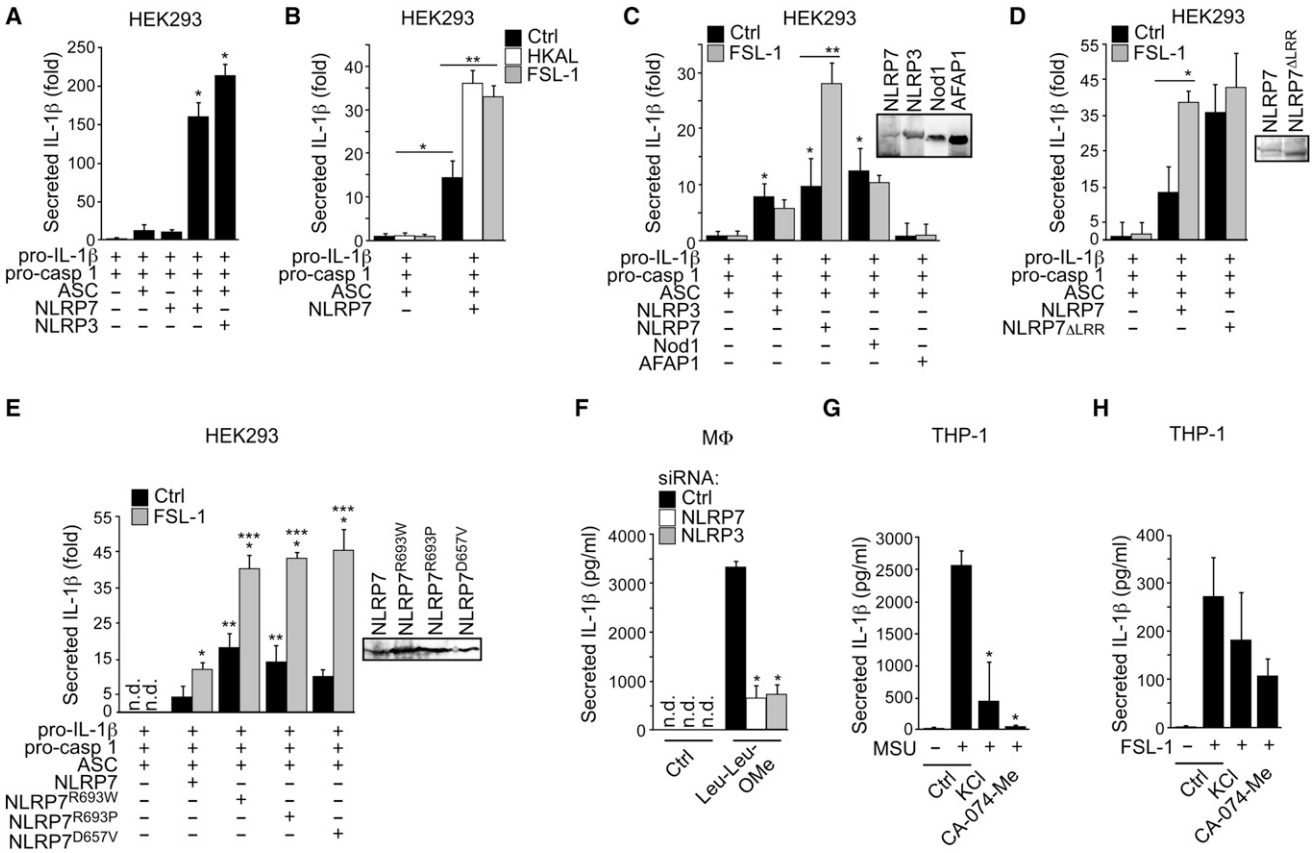

Figure 7. Reconstituted NLRP7 Inflammasomes Are Sufficient to Respond to HKAL and acLP

(A) HEK293 cells were transiently transfected as indicated and release of IL-1β was determined in SN. Results are presented as fold increase compared to cells transfected with pro-IL-1β and pro-caspase-1 (n = 3 ± SD); *p ≤ 0.05 compared to cells not transfected with NLRPs.

(B) HEK293 cells were transfected as above with suboptimal concentrations of NLRP7, followed by a second transfection after 24 hr with transfection reagent alone (Ctrl), HKAL (8 × 10^5 cfu), or FSL-1 (0.5 μg). Secreted IL-1β is presented as fold increase compared to control transfected cells (n = 3 ± SD); *p ≤ 0.05 compared to cells lacking NLRP7; **p ≤ 0.05 compared to the second round of mock transfected cells.

(C) HEK293 cells were transfected as above with suboptimal concentrations of NLRP7, NLRP3, Nod1, or AFAP1, followed by FSL-1 transfection. Secreted IL-1β is presented as fold increase compared to Ctrl transfected cells (n = 3 ± SD); *p ≤ 0.05 compared to cells lacking NLRs; **p ≤ 0.05 compared to the second round of mock transfected cells. Expression of NLRP7 (118 kDa), NLRP3 (118 kDa), Nod1 (107 kDa), and AFAP1 (110 kDa) in the inflammasome reconstitution assay was verified by protein immunoblot.

(D) HEK293 cells were transfected and analyzed for IL-1β secretion as above, but with NLRP7 and NLRP7$^{\Delta LRR}$ (n = 3 ± SD); *p ≤ 0.05 compared to the second round of mock transfected cells.

(E) HEK293 cells were transfected with NLRP7, NLRP7^{R693W}, NLRP7^{R693P}, or NLRP7^{D657V}, followed by FSL-1 transfection as above and secreted IL-1β is presented as fold increase compared to cells transfected with pro-IL-1β and pro-caspase-1 (n = 3 ± SD); *p ≤ 0.05 compared to cells not transfected with FSL-1; **p ≤ 0.05 compared to cells transfected with NLRP7; ***p ≤ 0.05 compared to cells transfected with NLRP7 and FSL-1. Expression of NLRP7 was verified by protein immunoblot.

(F) MΦ were transfected with Ctrl, NLRP3, or NLRP7 siRNAs, primed with ultrapure LPS for 4 hr, and either left untreated or lysosomal damage was inflicted with Leu-Leu-OMe for 16 hr and analyzed for IL-1β release (n = 6 ± SD).

(G and H) THP-1 cells were pretreated with either medium containing 130 mM KCl (KCL) or a cathepsin B inhibitor (CA-074-Me), mock treated or treated with MSU (G) or FSL-1 (H) for 8 hr and secreted IL-1β was determined (n = 3 ± SD); *p ≤ 0.05.

reconstitution assay but failed to respond to FSL-1 (Figure 7D). Collectively, these experiments demonstrate that NLRP7 is sufficient to promote FSL-1-induced inflammasome activation and that the LRR is necessary to sense acLP.

We next started to investigate the mechanism of NLRP7 activation. Hereditary mutations within the NACHT-LRR region of NLRP3 cause excessive release of IL-1β through constitutive inflammasome activation. Several hereditary mutations have also been identified in NLRP7 and are linked to recurrent hydatidiform moles (HM), but a link to IL-1β or IL-18 is not known (Murdoch et al., 2006). Comparison of WT and three HM-linked NLRP7 mutations (R693W, R693P, and D657V) in the inflammasome

(I–L) Ctrl or S.a.-infected MΦ were fractionated by SEC and pooled fractions were TCA precipitated and analyzed by protein immunoblot as indicated (I); caspase-1 was immunoprecipitated from fractions 2–6 and 19–24 of S.a.-infected MΦ with IgG as control and analyzed by protein immunoblot as indicated (J); NLRP7 was immunoprecipitated from the same fractions (K, L) and assayed for caspase-1 activity assay (activity purified from fractions 2–6 saturated the assay) (K) or assayed for ICE activity as above (L). Asterisk indicates cross-reactive protein.

Please cite this article in press as: Khare et al., An NLRP7-Containing Inflammasome Mediates Recognition of Microbial Lipopeptides in Human Macrophages, Immunity (2012), doi:10.1016/j.immuni.2012.02.001

reconstitution assay provided evidence that HM-linked mutations are more potent in activating inflammasomes than NLRP7 (Figure 7E).

Direct interaction of any ligand with an NLRP has not been demonstrated, but several indirect mechanism have been proposed for NLRP3 activation, including lysosomal damage, cathepsin B release, ROS generation, and K^+ efflux (Schroder and Tschopp, 2010). We could not detect colocalization of fluorescently labeled FSL-1 with NLRP7, nor could we copurify biotinylated FSL-1 with NLRP7 (data not shown), suggesting an indirect mechanism of NLRP7 activation, as proposed for NLRP3. Lysosomal destabilization with the dipeptide Leu-Leu-OMe resulted in IL-1β release in MΦ, which was significantly blocked upon silencing of NLRP3 and NLRP7 (Figure 7F), suggesting that both function downstream of lysosomal damage. Lysosomal rupture releases cathepsin B, which promotes NLRP3 activation in response to particulates (Hornung et al., 2008). Although MSU crystal-induced NLRP3 activation was blocked in cells treated with the cathepsin B inhibitor CA-074-Me (Figure 7G), FSL-1-induced IL-1β release was only partially prevented, suggesting mechanistic differences of NLRP3 and NLRP7 activation (Figure 7H). A distinct activation mechanism is also supported by the observation that MSU-induced NLRP3 activation was impaired when cells were cultured in 130 mM KCl-containing medium (Figure 7G), which blocks K^+ efflux (Pétrilli et al., 2007), but FSL-1-induced IL-1β release was only slightly affected (Figure 7H). The ROS scavenger NAC and the NADPH oxidase inhibitor APDC prevent MSU-induced IL-1β release (Dostert et al., 2008), but we did not observe any effect (data not shown), which is not surprising considering that NLRP7 activation is independent of K^+ efflux, which is frequently linked to ROS generation.

DISCUSSION

Our study in MΦ identified the NLRP7 inflammasome, which sensed microbial infection through recognition of acLP. An earlier study failed to detect IL-1β release by immunoblot in response to the acLP Pam3Cys after only 3 hr of activation (Martinon et al., 2004). The use of a more sensitive ELISA assay and increased activation time allowed us to detect acLP-induced IL-1β release, in agreement with *Nlrp3*-independent acLP-induced IL-1β secretion (Kanneganti et al., 2006). NLRP3 is activated by diverse stimuli, including phagocytosis of bacteria, and silencing of NLRP3 or NLRP7 inhibited the response to HK and live bacteria, but only silencing of NLRP7 impaired the acLP-induced response. We propose that NLRP7 specifically recognizes bacterial acLP. Our results indicate that NLRP7 is an inflammasome activator and not a signaling component downstream of TLR2. Nevertheless, an initial extracellular acLP-induced transcriptional response through TLR2 is required for NF-κB-dependent IL-1β and IL-18 transcription (Aliprantis et al., 1999; Guan et al., 2010; Takeuchi et al., 2000). Accordingly, silencing of TLR2 impaired IL-1β transcription and release, but caspase-1 activation is not impaired in MΦ of *Tlr2*-, *Tlr4*-, *MyD88*-, and *Trif*-deficient mice, clearly separating it from TLR signaling (Kanneganti et al., 2007), and we ruled out effects on cytokine transcription. Furthermore, in our inflammasome reconstitution assay, synthesis of IL-1β was TLR2 and NF-κB independent, as shown by the fact that HEK293 cells lack TLR2. Collectively, our results indicate that TLR2 signaling is necessary for IL-1β and IL-18 transcription, whereas NLRP7 is essential for IL-1β and IL-18 maturation and release in response to acLP. In contrast, a previous in vitro study suggested that NLRP7 is an inhibitor of caspase-1, based on the stable expression of a truncated NLRP7 in THP-1 cells (Kinoshita et al., 2005). A HEK293 cell overexpression study suggested that NLRP7 negatively regulates expression of cotransfected pro-IL-1β (Messaed et al., 2011a), whereas another study in HEK293 cells did not observe effects on IL-1β secretion (Grenier et al., 2002). In contrast, we showed that endogenous NLRP7 forms an ASC-dependent inflammasome in response to acLP in MΦ. Insufficient sensitivity may prevent detecting the NLRP7 inflammasome, as indicated by our observation that overexpressed NLRP7 is unstable and proteasomally degraded, resulting in lower expression compared to other NLRs. In addition, NLRs are spliced, resulting in altered activities. NLRP7 in our study contained nine LRRs (118 kDa), but we also cloned a shorter transcript (NLRP7-S; 109 kDa) with five LRRs and reduced activity (Figure S6B).

Differences exist in the repertoire of human and mouse NLRs and *Nlrp7* is lacking from mice, but nevertheless, one can expect the existence of an acLP-sensing functional NLRP7 mouse analog. Another difference is that BMDM require exogenous ATP for IL-1β release, whereas MΦ release IL-1β in response to acLP alone. Future studies will need to identify the mechanism of NLRP7 activation and address whether the agonist repertoire of NLRP7 is similar to that of TLR2 (i.e., restricted to acLP) or whether it responds to non-TLR2 agonists also. acLP are used as vaccine adjuvant and immune-activating serum acLP have been identified (Thacker et al., 2009). Thus, NLRP7-activating DAMPs might exist, similar to the ones recognized by NLRP3.

Hereditary mutations in NLRP7 are linked to HM, which predisposes women toward molar pregnancy and may develop into a choriocarcinoma, but only 60% of HM patients harbor NLRP7 mutations (Murdoch et al., 2006). It is not known whether IL-1β or IL-18 contribute to HM (Slim and Mehio, 2007). Monocytes from some HM patients with NLRP7 mutations show reduced IL-1β secretion (Messaed et al., 2011a), whereas two out of four HM patients showed increased IL-1β secretion in another study (Messaed et al., 2011b), but even the general population show varying IL-1β secretion (Gattorno et al., 2007). Our preliminary results from three common HM mutations showed increased activity in the inflammasome reconstitution system, but future studies need to investigate the contribution of IL-1β and IL-18 to HM. In addition, a connection among latent MP infection, NLRP7 activation, and chronic inflammatory disease will need to be elucidated. Inhibition of bacterial replication is independently achieved by autocrine IL-1β signaling and pyroptosis (Broz et al., 2010), but we did not observe NLRP7-mediated pyroptosis, indicating that NLRP7 acts through cytokine release to limit bacterial replication, supported by the critical role of IL-1β in the host defense against *S. aureus* (Bernthal et al., 2011). Thus, our finding of NLRP7 as an activator of caspase-1, which restricts intracellular bacterial replication, has broad implications for understanding host defense and inflammatory and autoimmune disease and expands our limited understanding of NLRs and their respective agonists.

EXPERIMENTAL PROCEDURES

Materials and Cell Culture

THP-1 cells and MΦ were treated with HK *Acholeplasma laidlawii* (HKAL), *Legionella pneumophila* (HKLP), *Staphylococcus aureus* (HKSA), *Porphyromonas gingivalis* (HKPG), *Listeria monocytogenes* (HKLM) (2×10^5 cfu/ml), FSL-1 (0.1 μg/ml), Pam2CSK4 (2 μg/ml), Pam3CSK4 (2 μg/ml), ultrapure LPS (10 ng/ml, Invivogen), crude *E. coli* LPS (cLPS, 0111:B4, 600 ng/ml, Sigma), MALP-2 (0.2 μg/ml, Imgenex), TNF-α (20 ng/ml, Biosource), or Leu-Leu-OMe (1 mM; Bachem) or cells were transfected with dA:dT (2 ng/ml, Sigma) with lipofectamine LTX+ (Invitrogen) or infected with adenovirus (AdV, serotype 5). Where indicated, cells were pretreated for 1 hr with Anakinra (10 μg/ml, Amgen), zYVAD-fmk or zLEHD-fmk (100 μM, Calbiochem), CA-074-Me (50 μM), or with medium containing 130 mM KCl. Stable THP-1^{shASC}, THP-1^{shLuc}, and THP-1^{shCtrl} cells were described previously (Bryan et al., 2009; Kung et al., 2012). Blood from healthy donors was drawn after obtaining informed consent under a protocol approved by NU Institutional Review Board.

Bacterial Infection

THP-1 cells were treated with culture SN from HEK293 cells that tested negative or positive for MP, as determined by morphology, DAPI staining, and MP test (MycoAlert, Lonza). Control HEK293 cells were grown in the presence of Plasmocin and Plasmocure (Invivogen) to prevent MP infection. At 67 hr posttransfection, cells were infected with *Listeria monocytogenes* (MOI = 12) or *Staphylococcus aureus* (MOI = 3) for 45 min. Extracellular bacteria were eliminated with gentamycin (50 μg/ml) for 30 min, followed by collection of culture SN for ELISA as indicated and lysed in 0.02% Triton X-100 for CFU determination.

Immunofluorescence Microscopy

Cells were fixed, permeabilized, and immunostained as described (Bryan et al., 2009). For colocalization studies, HEK293 cells were transfected with NLRP7 or cotransfected with ASC and/or pro-caspase-1. Images were acquired by confocal laser scanning and epifluorescence microscopy on a Zeiss LSM 510 and a Nikon TE2000E2-PFS with a 100× oil objective and image deconvolution.

Subcellular Fractionation

10^6 cells were fractionated by hypotonic lysis and centrifugation into cytosolic and nuclear fractions as previously described (Bryan et al., 2009).

Cytokine and Caspase-1 Measurement

Cytokine secretion was quantified from clarified culture SN by ELISA (BD Biosciences, Invitrogen) from 3×10^5 MΦ after treatment for 5 hr (live bacteria) or 16 hr (all other treatments), or 24 hr posttransfection of 4×10^5 HEK293 cells. Samples were analyzed in triplicate and repeated at least three times, showing a representative result. TCL (3×10^5 MΦ) and SN (4×10^6 MΦ) were analyzed for caspase-1 by protein immunoblot. Cycloheximide (10 μg/ml) was added to prevent resynthesis of pro-caspase-1 when analyzing TCL. In vitro caspase-1 activity was determined by kinetic fluorescence assay with NLRP7 immune complexes isolated from 5–12 × 10^7 *S. aureus*-infected and size fractionated MΦ, or from HEK293^{Inflammasome} cells transfected with control or NLRP7 in assay buffer (50 mM HEPES [pH 7.4], 50 mM NaCl, 1 mM EDTA, 0.1% Chaps, 10% sucrose, 10 mM DTT, 50 μM AcYVAD-AFC), and fluorescence was determined at 37°C at 400/505 nm. Caspase-1 proteolytic activity was determined by incubating NLRP7 complexes in assay buffer, supplemented with TCL from HEK293 cells transfected with pro-IL-1β, and analysis of mature IL-1β by protein immunoblot.

Pyroptosis

Lactate dehydrogenase (LDH) activity was measured with a kit (Clontech). Cells were lysed with 1% Triton X-100 for maximal LDH release.

Inflammasome Reconstitution System

HEK293 cells were transfected with expression constructs for mouse pro-IL-1β and human pro-caspase-1, ASC, and NLRs by Lipofectamine 2000 (Invitrogen). A stably transfected HEK293 reconstitution system (HEK293^{Inflammasome}) was used in some experiments. Cells were transfected a second time with transfection mixture, HKAL (8×10^5 cfu), or FSL-1 (500 ng) with Lipofectamine 2000.

Coimmunoprecipitation

Stable myc-ASC HEK293 cells (HEK293^{ASC}) were transfected with GFP, GFP-NLRP3, or GFP-NLRP7, adjusted to yield comparable expression. Alternatively, HEK293 cells were transfected with HA-ASC and myc-NLRP7$^{\Delta LRR}$ or NLRP7$^{\Delta PYD}$, or HEK293^{Inflammasome} cells were transfected with control or NLRP7. Cells were lysed (120 mM NaCl, 20 mM Tris [pH 7.4], 10% glycerol, 0.2% Triton X-100, and protease inhibitors) 36 hr posttransfection. Cleared TCL or SEC fractions were subjected to IP with immobilized antibodies as indicated for 12 hr at 4°C and washed in lysis buffer, and bound proteins were analyzed by protein immunoblot with HRP-conjugated secondary native IgG-recognizing antibodies (eBioscience), ECL detection (Pierce), and image acquisition (Ultralum). TCL (10% volume) were analyzed where indicated.

Size Exclusion Chromatography

TCL were prepared from 5–12 × 10^7 Ctrl and 90 min *S. aureus*-infected MΦ by lysis in SEC buffer (10 mM $Na_4P_2O_7$, 10 mM NaF, 5 mM Tris [pH 7.4], 150 mM NaCl, 1% octylglucoside, and protease inhibitors) for 5 min on ice and Dounce homogenization. TCL were cleared by centrifugation (12,000 × g at 4°C for 10 min) and 0.45 μm filtration. SEC was performed on a 16 × 600 mm HiPrep Sephacryl S300HR column (GE Healthcare) in 50 mM Tris (pH 7.4), 150 mM NaCl at 4°C at a flow rate of 0.5 ml/min. Collected fractions were either TCA precipitated for protein immunoblot analysis or used to purify protein complexes.

siRNA Transfection

3×10^5 THP-1 cells were electroporated (Invitrogen) with 60 nM single or pooled siRNA duplexes and MΦ were transfected with 120 nM siRNA (F2/virofect; Targeting Systems) and analyzed 72 hr posttransfection.

RNA Isolation and RT-PCR

Total RNA was isolated (Trizol, Invitrogen), DNase I digested, reverse transcribed (Superscript III, Invitrogen), and analyzed by exon-spanning RT-PCR (Table S2) or qPCR (Applied Biosystems) and displayed as relative expression compared to β-actin. PCR products were sequence verified.

Statistics

A standard two-tailed t test was used for statistical analysis and values of $p \leq 0.05$ were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at doi:10.1016/j.immuni.2012.02.001.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (GM071723, GM071723-S1, AI082406, AI082406-S1/S2, and AR057216 to C.S.). S.K. is an Arthritis Foundation fellow (AF161715) and L.deA. is supported by the American Heart Association (11POST585000). This work was supported by the Northwestern University Monoclonal Antibody Facility, Flow Cytometry Facility, a Cancer Center Support Grant (CA060553), and the Skin Disease Research Center (P30AR057216). We thank S. Kramer for isolation of PBMCs and C. Cuda and H. Perlman for help with flow cytometry.

Received: January 14, 2011
Revised: December 21, 2011
Accepted: February 2, 2012
Published online: February 23, 2012

REFERENCES

Alexopoulou, L., Thomas, V., Schnare, M., Lobet, Y., Anguita, J., Schoen, R.T., Medzhitov, R., Fikrig, E., and Flavell, R.A. (2002). Hyporesponsiveness to

vaccination with *Borrelia burgdorferi* OspA in humans and in TLR1- and TLR2-deficient mice. Nat. Med. *8*, 878–884.

Aliprantis, A.O., Yang, R.B., Mark, M.R., Suggett, S., Devaux, B., Radolf, J.D., Klimpel, G.R., Godowski, P., and Zychlinsky, A. (1999). Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science *285*, 736–739.

Bernthal, N.M., Pribaz, J.R., Stavrakis, A.I., Billi, F., Cho, J.S., Ramos, R.I., Francis, K.P., Iwakura, Y., and Miller, L.S. (2011). Protective role of IL-1β against post-arthroplasty *Staphylococcus aureus* infection. J. Orthop. Res. *29*, 1621–1626.

Boyden, E.D., and Dietrich, W.F. (2006). Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. Nat. Genet. *38*, 240–244.

Broz, P., von Moltke, J., Jones, J.W., Vance, R.E., and Monack, D.M. (2010). Differential requirement for Caspase-1 autoproteolysis in pathogen-induced cell death and cytokine processing. Cell Host Microbe *8*, 471–483.

Bryan, N.B., Dorfleutner, A., Rojanasakul, Y., and Stehlik, C. (2009). Activation of inflammasomes requires intracellular redistribution of the apoptotic speck-like protein containing a caspase recruitment domain. J. Immunol. *182*, 3173–3182.

Chen, G.Y., Liu, M., Wang, F., Bertin, J., and Núñez, G. (2011). A functional role for Nlrp6 in intestinal inflammation and tumorigenesis. J. Immunol. *186*, 7187–7194.

Dostert, C., Pétrilli, V., Van Bruggen, R., Steele, C., Mossman, B.T., and Tschopp, J. (2008). Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. Science *320*, 674–677.

Elinav, E., Strowig, T., Kau, A.L., Henao-Mejia, J., Thaiss, C.A., Booth, C.J., Peaper, D.R., Bertin, J., Eisenbarth, S.C., Gordon, J.I., and Flavell, R.A. (2011). NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. Cell *145*, 745–757.

Faustin, B., Lartigue, L., Bruey, J.M., Luciano, F., Sergienko, E., Bailly-Maitre, B., Volkmann, N., Hanein, D., Rouiller, I., and Reed, J.C. (2007). Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation. Mol. Cell *25*, 713–724.

Fernandes-Alnemri, T., Yu, J.W., Juliana, C., Solorzano, L., Kang, S., Wu, J., Datta, P., McCormick, M., Huang, L., McDermott, E., et al. (2010). The AIM2 inflammasome is critical for innate immunity to *Francisella tularensis*. Nat. Immunol. *11*, 385–393.

Garcia, J., Lemercier, B., Roman-Roman, S., and Rawadi, G. (1998). A *Mycoplasma fermentans*-derived synthetic lipopeptide induces AP-1 and NF-kappaB activity and cytokine secretion in macrophages via the activation of mitogen-activated protein kinase pathways. J. Biol. Chem. *273*, 34391–34398.

Gattorno, M., Tassi, S., Carta, S., Delfino, L., Ferlito, F., Pelagatti, M.A., D'Osualdo, A., Buoncompagni, A., Alpigiani, M.G., Alessio, M., et al. (2007). Pattern of interleukin-1β secretion in response to lipopolysaccharide and ATP before and after interleukin-1 blockade in patients with CIAS1 mutations. Arthritis Rheum. *56*, 3138–3148.

Grenier, J.M., Wang, L., Manji, G.A., Huang, W.J., Al-Garawi, A., Kelly, R., Carlson, A., Merriam, S., Lora, J.M., Briskin, M., et al. (2002). Functional screening of five PYPAF family members identifies PYPAF5 as a novel regulator of NF-kappaB and caspase-1. FEBS Lett. *530*, 73–78.

Guan, Y., Ranoa, D.R., Jiang, S., Mutha, S.K., Li, X., Baudry, J., and Tapping, R.I. (2010). Human TLRs 10 and 1 share common mechanisms of innate immune sensing but not signaling. J. Immunol. *184*, 5094–5103.

Hoffman, H.M., Scott, P., Mueller, J.L., Misaghi, A., Stevens, S., Yancopoulos, G.D., Murphy, A., Valenzuela, D.M., and Liu-Bryan, R. (2010). Role of the leucine-rich repeat domain of cryopyrin/NALP3 in monosodium urate crystal-induced inflammation in mice. Arthritis Rheum. *62*, 2170–2179.

Hornung, V., Bauernfeind, F., Halle, A., Samstad, E.O., Kono, H., Rock, K.L., Fitzgerald, K.A., and Latz, E. (2008). Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat. Immunol. *9*, 847–856.

Kanneganti, T.D., Ozören, N., Body-Malapel, M., Amer, A., Park, J.H., Franchi, L., Whitfield, J., Barchet, W., Colonna, M., Vandenabeele, P., et al. (2006). Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. Nature *440*, 233–236.

Kanneganti, T.D., Lamkanfi, M., Kim, Y.G., Chen, G., Park, J.H., Franchi, L., Vandenabeele, P., and Núñez, G. (2007). Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. Immunity *26*, 433–443.

Khare, S., Luc, N., Dorfleutner, A., and Stehlik, C. (2010). Inflammasomes and their activation. Crit. Rev. Immunol. *30*, 463–487.

Kinoshita, T., Wang, Y., Hasegawa, M., Imamura, R., and Suda, T. (2005). PYPAF3, a PYRIN-containing APAF-1-like protein, is a feedback regulator of caspase-1-dependent interleukin-1β secretion. J. Biol. Chem. *280*, 21720–21725.

Kung, V.L., Khare, S., Stehlik, C., Bacon, E.M., Hughes, A.J., and Hauser, A.R. (2012). An rhs gene of *Pseudomonas aeruginosa* encodes a virulence protein that activates the inflammasome. Proc. Natl. Acad. Sci. USA *109*, 1275–1280.

Mariathasan, S., Newton, K., Monack, D.M., Vucic, D., French, D.M., Lee, W.P., Roose-Girma, M., Erickson, S., and Dixit, V.M. (2004). Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. Nature *430*, 213–218.

Mariathasan, S., Weiss, D.S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., Lee, W.P., Weinrauch, Y., Monack, D.M., and Dixit, V.M. (2006). Cryopyrin activates the inflammasome in response to toxins and ATP. Nature *440*, 228–232.

Martinon, F., Burns, K., and Tschopp, J. (2002). The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-β. Mol. Cell *10*, 417–426.

Martinon, F., Agostini, L., Meylan, E., and Tschopp, J. (2004). Identification of bacterial muramyl dipeptide as activator of the NALP3/cryopyrin inflammasome. Curr. Biol. *14*, 1929–1934.

McCoy, A.J., Koizumi, Y., Higa, N., and Suzuki, T. (2010). Differential regulation of caspase-1 activation via NLRP3/NLRC4 inflammasomes mediated by aerolysin and type III secretion system during *Aeromonas veronii* infection. J. Immunol. *185*, 7077–7084.

Messaed, C., Akoury, E., Djuric, U., Zeng, J., Saleh, M., Gilbert, L., Seoud, M., Qureshi, S., and Slim, R. (2011a). NLRP7, a nucleotide oligomerization domain-like receptor protein, is required for normal cytokine secretion and co-localizes with Golgi and the microtubule organizing center. J. Biol. Chem. *286*, 43313–43323.

Messaed, C., Chebaro, W., Di Roberto, R.B., Rittore, C., Cheung, A., Arseneau, J., Schneider, A., Chen, M.F., Bernishke, K., Surti, U., et al; H M Collaborative Group. (2011b). NLRP7 in the spectrum of reproductive wastage: rare non-synonymous variants confer genetic susceptibility to recurrent reproductive wastage. J. Med. Genet. *48*, 540–548.

Murdoch, S., Djuric, U., Mazhar, B., Seoud, M., Khan, R., Kuick, R., Bagga, R., Kircheisen, R., Ao, A., Ratti, B., et al. (2006). Mutations in NALP7 cause recurrent hydatidiform moles and reproductive wastage in humans. Nat. Genet. *38*, 300–302.

Muruve, D.A., Pétrilli, V., Zaiss, A.K., White, L.R., Clark, S.A., Ross, P.J., Parks, R.J., and Tschopp, J. (2008). The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. Nature *452*, 103–107.

Ozören, N., Masumoto, J., Franchi, L., Kanneganti, T.D., Body-Malapel, M., Ertürk, I., Jagirdar, R., Zhu, L., Inohara, N., Bertin, J., et al. (2006). Distinct roles of TLR2 and the adaptor ASC in IL-1β/IL-18 secretion in response to *Listeria monocytogenes*. J. Immunol. *176*, 4337–4342.

Pétrilli, V., Papin, S., Dostert, C., Mayor, A., Martinon, F., and Tschopp, J. (2007). Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. Cell Death Differ. *14*, 1583–1589.

Rathinam, V.A., Jiang, Z., Waggoner, S.N., Sharma, S., Cole, L.E., Waggoner, L., Vanaja, S.K., Monks, B.G., Ganesan, S., Latz, E., et al. (2010). The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. Nat. Immunol. *11*, 395–402.

Sacht, G., Märten, A., Deiters, U., Süssmuth, R., Jung, G., Wingender, E., and Mühlradt, P.F. (1998). Activation of nuclear factor-kappaB in macrophages by mycoplasmal lipopeptides. Eur. J. Immunol. *28*, 4207–4212.

Schroder, K., and Tschopp, J. (2010). The inflammasomes. Cell *140*, 821–832.

Shen, Y., Kawamura, I., Nomura, T., Tsuchiya, K., Hara, H., Dewamitta, S.R., Sakai, S., Qu, H., Daim, S., Yamamoto, T., and Mitsuyama, M. (2010). Toll-like receptor 2- and MyD88-dependent phosphatidylinositol 3-kinase and Rac1 activation facilitates the phagocytosis of Listeria monocytogenes by murine macrophages. Infect. Immun. *78*, 2857–2867.

Slim, R., and Mehio, A. (2007). The genetics of hydatidiform moles: new lights on an ancient disease. Clin. Genet. *71*, 25–34.

Srinivasula, S.M., Poyet, J.-L., Razmara, M., Datta, P., Zhang, Z., and Alnemri, E.S. (2002). The PYRIN-CARD protein ASC is an activating adaptor for caspase-1. J. Biol. Chem. *277*, 21119–21122.

Stehlik, C., Lee, S.H., Dorfleutner, A., Stassinopoulos, A., Sagara, J., and Reed, J.C. (2003). Apoptosis-associated speck-like protein containing a caspase recruitment domain is a regulator of procaspase-1 activation. J. Immunol. *171*, 6154–6163.

Takeuchi, O., Kaufmann, A., Grote, K., Kawai, T., Hoshino, K., Morr, M., Mühlradt, P.F., and Akira, S. (2000). Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway. J. Immunol. *164*, 554–557.

Takeuchi, O., Kawai, T., Mühlradt, P.F., Morr, M., Radolf, J.D., Zychlinsky, A., Takeda, K., and Akira, S. (2001). Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int. Immunol. *13*, 933–940.

Takeuchi, O., Sato, S., Horiuchi, T., Hoshino, K., Takeda, K., Dong, Z., Modlin, R.L., and Akira, S. (2002). Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J. Immunol. *169*, 10–14.

Taxman, D.J., Zhang, J., Champagne, C., Bergstralh, D.T., Iocca, H.A., Lich, J.D., and Ting, J.P. (2006). Cutting edge: ASC mediates the induction of multiple cytokines by Porphyromonas gingivalis via caspase-1-dependent and -independent pathways. J. Immunol. *177*, 4252–4256.

Thacker, J.D., Brown, M.A., Rest, R.F., Purohit, M., Sassi-Gaha, S., and Artlett, C.M. (2009). 1-Peptidyl-2-arachidonoyl-3-stearoyl-sn-glyceride: an immunologically active lipopeptide from goat serum (Capra hircus) is an endogenous damage-associated molecular pattern. J. Nat. Prod. *72*, 1993–1999.

Torres, D., Barrier, M., Bihl, F., Quesniaux, V.J., Maillet, I., Akira, S., Ryffel, B., and Erard, F. (2004). Toll-like receptor 2 is required for optimal control of Listeria monocytogenes infection. Infect. Immun. *72*, 2131–2139.

Vinzing, M., Eitel, J., Lippmann, J., Hocke, A.C., Zahlten, J., Slevogt, H., N'guessan, P.D., Günther, S., Schmeck, B., Hippenstiel, S., et al. (2008). NAIP and Ipaf control *Legionella pneumophila* replication in human cells. J. Immunol. *180*, 6808–6815.

Williams, K.L., Lich, J.D., Duncan, J.A., Reed, W., Rallabhandi, P., Moore, C., Kurtz, S., Coffield, V.M., Accavitti-Loper, M.A., Su, L., et al. (2005). The CATERPILLER protein monarch-1 is an antagonist of toll-like receptor-, tumor necrosis factor α-, and *Mycobacterium tuberculosis*-induced pro-inflammatory signals. J. Biol. Chem. *280*, 39914–39924.

Wu, J., Fernandes-Alnemri, T., and Alnemri, E.S. (2010). Involvement of the AIM2, NLRC4, and NLRP3 inflammasomes in caspase-1 activation by *Listeria monocytogenes*. J. Clin. Immunol. *30*, 693–702.

Yamamoto, M., Yaginuma, K., Tsutsui, H., Sagara, J., Guan, X., Seki, E., Yasuda, K., Yamamoto, M., Akira, S., Nakanishi, K., et al. (2004). ASC is essential for LPS-induced activation of procaspase-1 independently of TLR-associated signal adaptor molecules. Genes Cells *9*, 1055–1067.

Yoo, N.J., Park, W.S., Kim, S.Y., Reed, J.C., Son, S.G., Lee, J.Y., and Lee, S.H. (2002). Nod1, a CARD protein, enhances pro-interleukin-1β processing through the interaction with pro-caspase-1. Biochem. Biophys. Res. Commun. *299*, 652–658.

Zhang, H., Peterson, J.W., Niesel, D.W., and Klimpel, G.R. (1997). Bacterial lipoprotein and lipopolysaccharide act synergistically to induce lethal shock and proinflammatory cytokine production. J. Immunol. *159*, 4868–4878.

Immunity, Volume *36*

Supplemental Information

An NLRP7-Containing Inflammasome Mediates Recognition of Microbial Lipopeptides in Human Macrophages

Sonal Khare, Andrea Dorfleutner, Nicole B. Bryan, Chawon Yun, Alexander D. Radian, Lucia de Almeida, Yon Rojanasakul, and Christian Stehlik

Inventory of Supplemental Information (7 figures, 2 tables, experimental procedure)

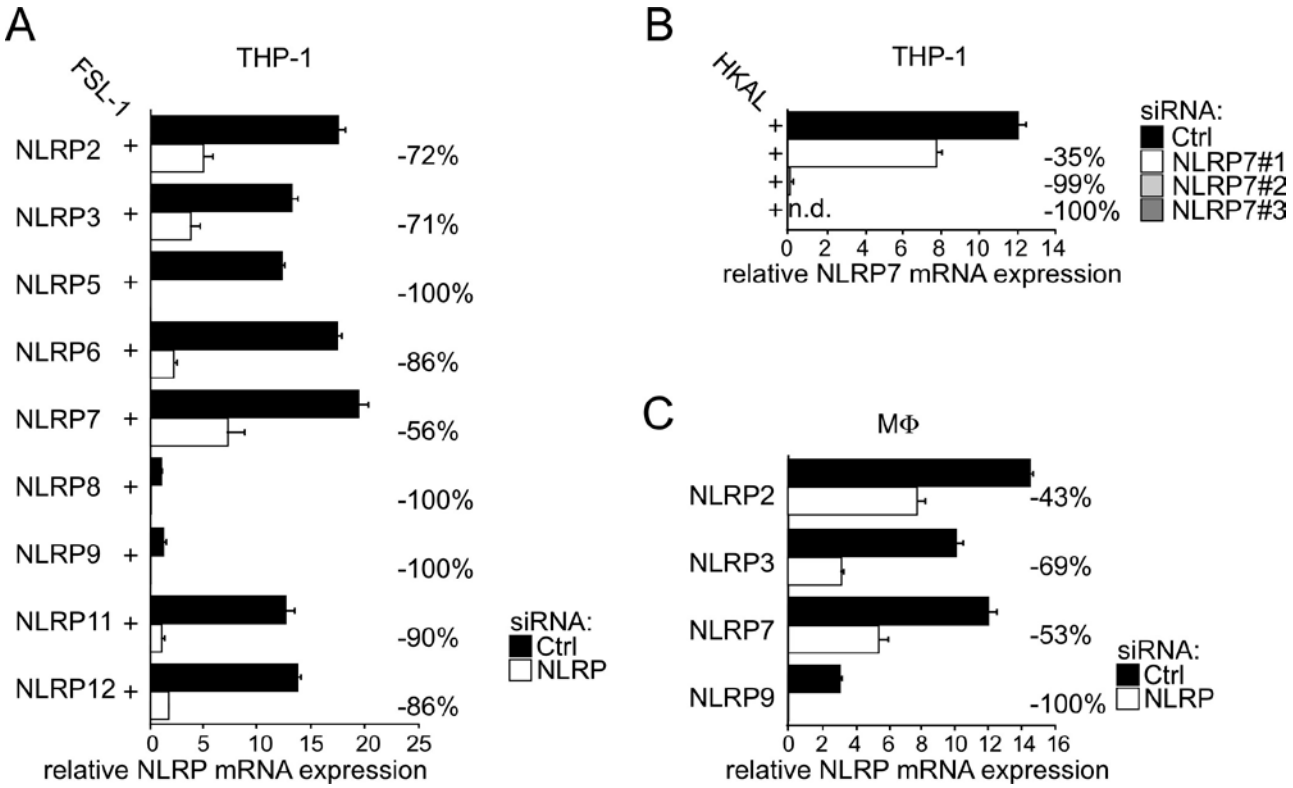

Figure S1. Validation of siRNA-mediated knock-down of NLRPs in THP-1 cells and MΦ by real time PCR. THP-1 cells were electroporated with non-targeting siRNA pools and **(A)** siRNA pools targeting NLRPs or **(B)** three individual siRNAs targeting NLRP7 as indicated, and total RNA was isolated 72 hrs post transfection, transcribed into cDNA for real time PCR. **(C)** MΦ were transfected with non-targeting siRNA pools and siRNA pools targeting NLRPs as indicated, and analyzed as above. Results are presented as relative expression compared to βactin (n=3, ±SD); n.d.: not detectable; Silencing efficiency is indicated in % compared to control siRNA.

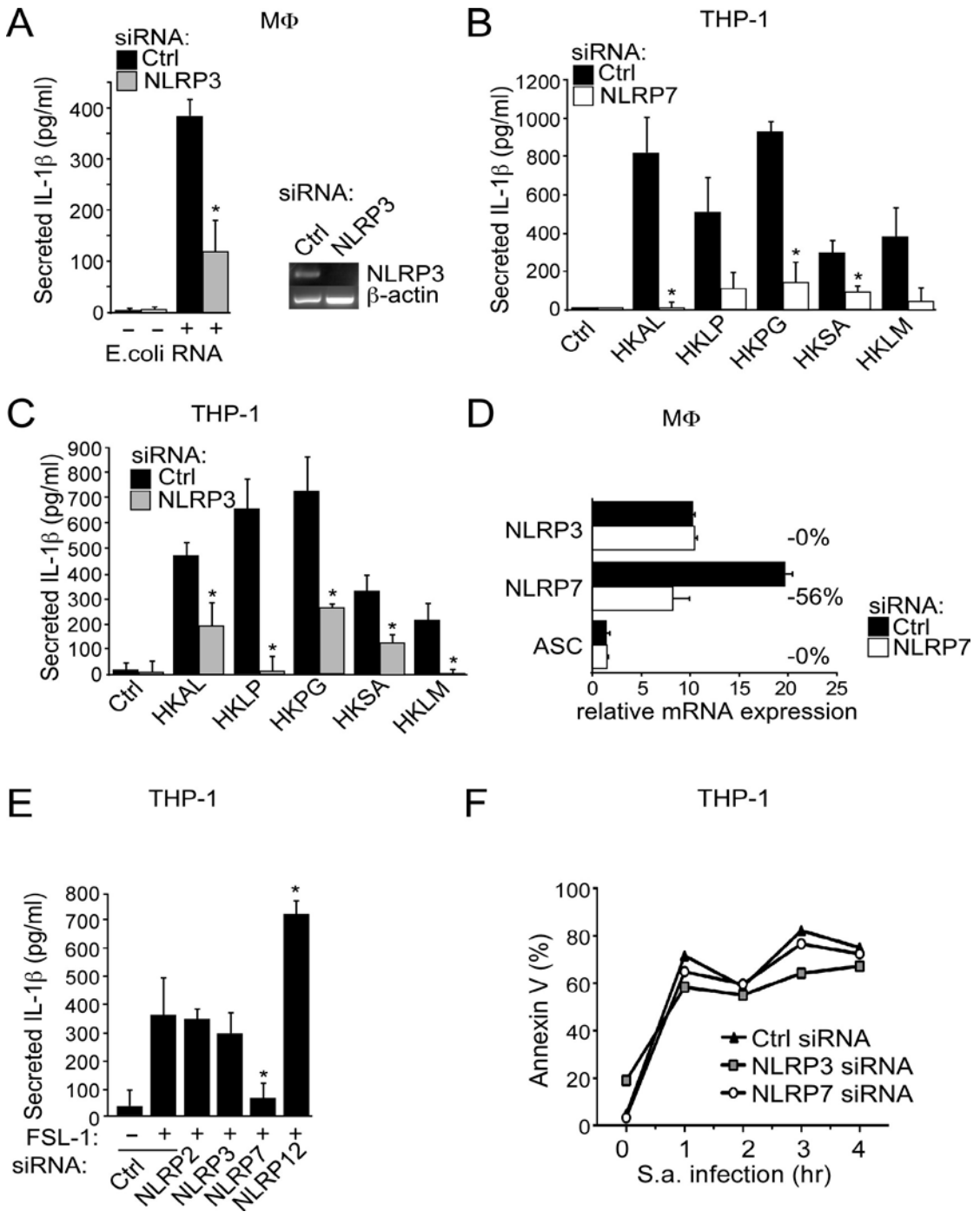

Figure S2. NLRP3 and NLRP7 recognize HK bacteria. (A) MΦ were transfected with Ctrl or pooled NLRP3 siRNAs and mock treated or treated with *E. coli* total RNA (1 μg/ml) and analyzed for IL-1β by ELISA (pg IL-1β/ml/10^6 cells, n=3, ±SD). Silencing of NLRP3 expression in MΦ was confirmed by RT-PCR, using βactin as a control. **(B)** THP-1 cells were transfected with Ctrl or NLRP7 siRNAs or **(C)** NLRP3 siRNAs and mock treated or treated with HKAL, HKLP, HKPG, HKSA or HKLM ($2x10^5$ cfu/ml) and analyzed for mature IL-1β as above. **(D)** MΦ were transfected with non-targeting siRNAs and siRNAs targeting NLRP7, and total RNA was isolated 72 hrs post transfection, transcribed into cDNA for real time PCR and analyzed for NLRP3, NLRP7 and ASC. Results are presented as relative expression compared to βactin (n=3, ±SD). Silencing efficiency is indicated in % compared to control siRNA. **(E)** THP-1 cells were transfected with Ctrl or NLRP2, NLRP3, NLRP7, and NLRP12-targeting siRNAs and were either mock treated or treated with FSL-1 (0.1 μg/ml) as indicated for 16 hrs and analyzed for IL-1β as above. **(F)** THP-1 cells were transfected with Ctrl, NLRP3 and NLRP7 siRNAs as indicated and kept uninfected or were infected with *S.a.,* and were stained for annexin V, analyzed by flow cytometry and presented as % compared to control cells. *p≤0.05.

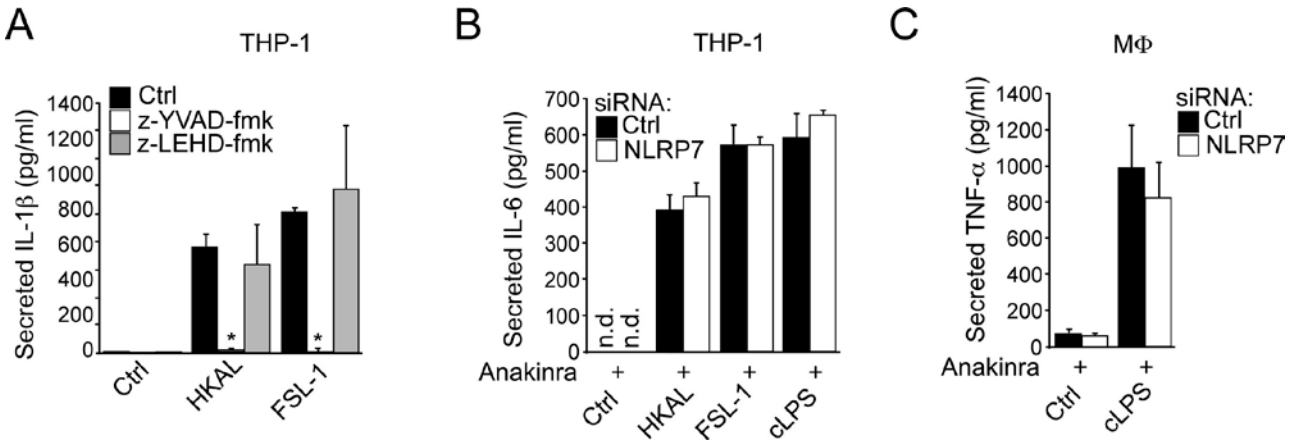

Figure S3. NLRP7 activation affects IL-1β processing and release, but does not influence IL-6 and TNF-α production. (A, B) THP-1 cells or **(C)** h MΦ were transfected with pooled Ctrl or NLRP7 siRNAs and activated with HKAL ($2x10^5$ cfu/ml), FSL1 (0.1 µg/ml), or cLPS (600 ng/ml) as indicated for 16 hrs, and cell free culture supernatants were analyzed for secreted IL-1β, L-6 or TNF-α by ELISA (pg/ml/10^6 cells, n=3, ±SD). Cells were either pretreated with vehicle control (DMSO), z-YVAD-fmk (a specific caspase-1 inhibitor, 100 µM) or z-LEHD-fmk (a specific caspase-9 inhibitor, 100 µM) for 1 hr before further treatment **(A)**. Cells were pre-treated with the IL-1R antagonist anakinra to prevent autocrine IL-1β-mediated IL-6 and TNF-α secretion before further treatment **(B, C)**. n.d.: not detectable; *p≤0.05.

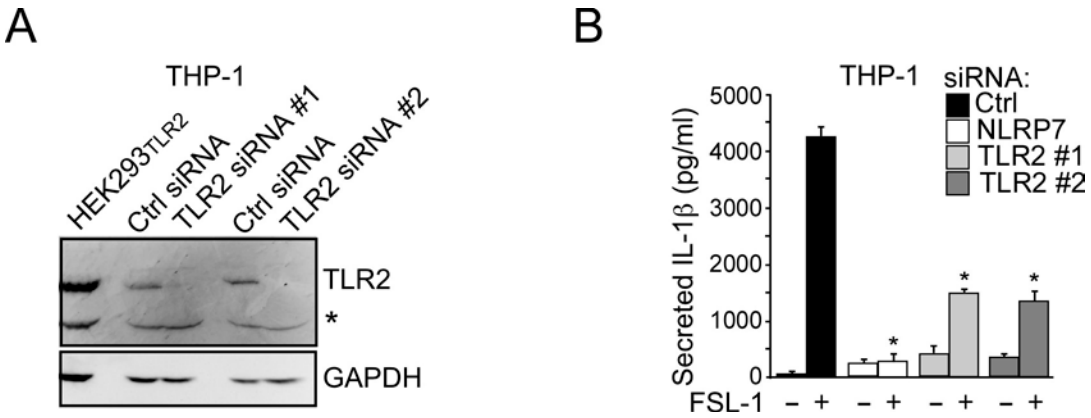

Figure S4. Silencing of TLR2 impairs IL-1β release. THP-1 cells were transfected with Ctrl, NLRP7 or two TLR2 siRNAs, mock treated or treated with FSL-1 for 16 hrs. **(A)** Cells were lysed and analyzed by immunoblot for expression of TLR2 and GAPDH as loading control, using HEK293 cells stably transfected with TLR2 ($HEK293^{TLR2}$) as controls. An "*" marks a cross-reactive protein recognized by the TLR2 antibody. **(B)** Culture supernatants were analyzed for mature IL-1β by ELISA (pg IL-1β/ml/10^6 cells, n=3, ±SD); *p≤0.05.

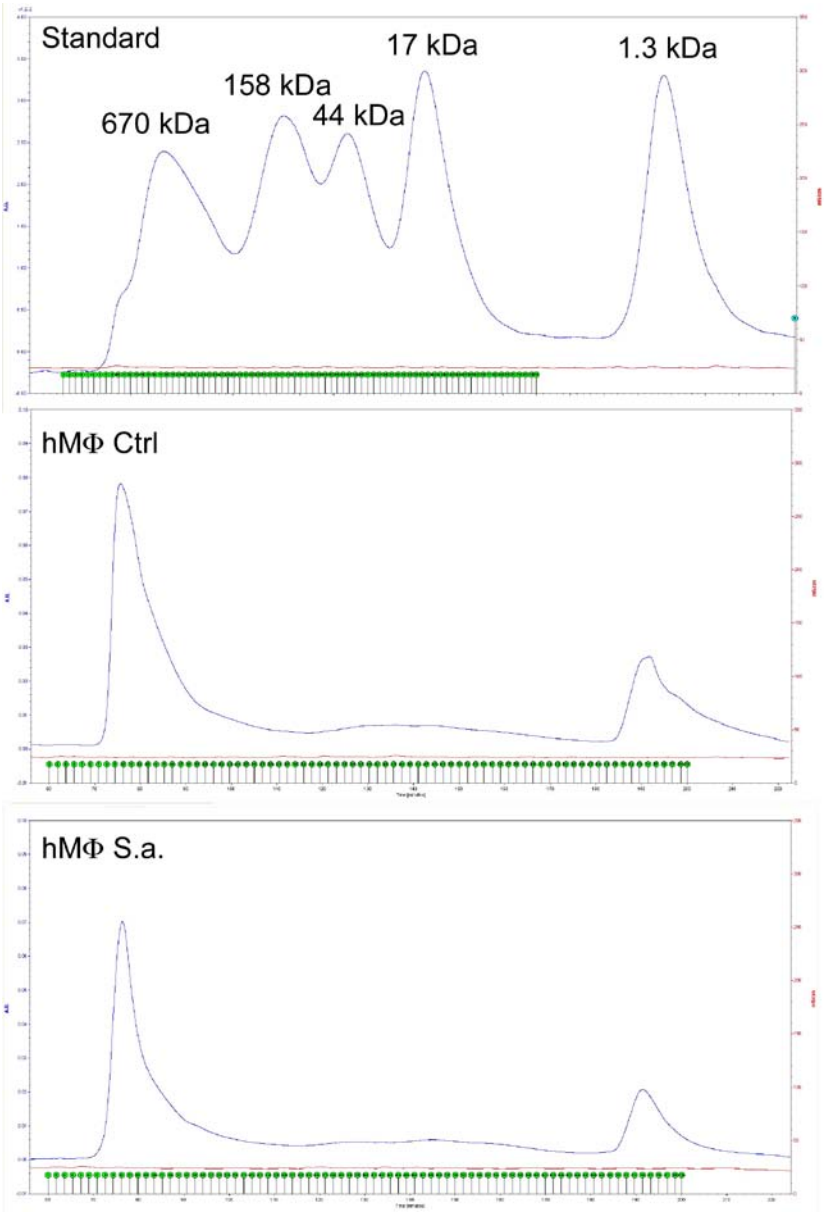

Figure S5. UV spectra of fractions eluted from the SEC column. Total cell lysates (TCL) from 5-12x10^7 control MΦ and MΦ infected with *S.a.* were subjected to size exclusion chromatography (SEC). The UV spectra for eluted proteins are shown for molecular weight standard **(upper panel)** control MΦ **(middle panel)**, *S.a.*-infected MΦ **(lower panel)**. The fractions are indicated at the bottom. One representative fractionation is shown.

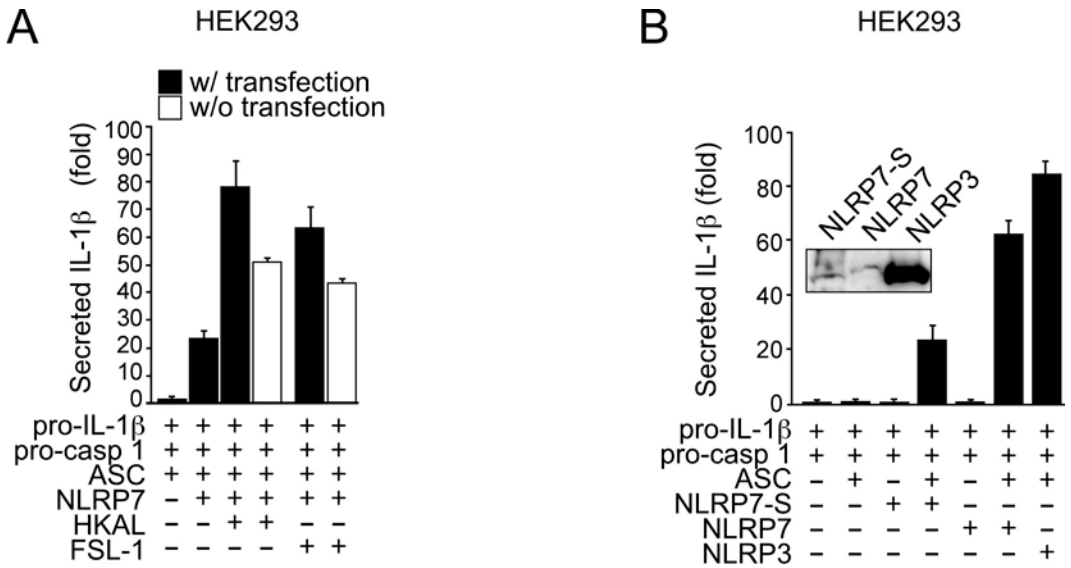

Figure S6. Inflammasome reconstitution system. (A) HEK293 cells were transiently transfected with pro-IL-1β, pro-caspase-1, ASC and suboptimal concentrations of NLRP7. Cells were transfected a second time with transfection mixture only or transfection mixture combined with HKAL or FSL1 (black bars). Alternatively, the medium was supplemented with HKAL or FSL-1 without transfection mixture (white bars). **(B)** Cells were transfected as above with either NLRP7 (7 LRRs; 118 kDa), NLRP7-S (5 LRRs; 109 kDa), or NLRP3 as indicated. Expression of NLRP7-S, NLRP7, and NLRP3 was determined by immunoblot of total lysates, showing equal expression of NLRP7 and NLRP7-S. IL-1β was determined in cell free culture supernatants by ELISA. Results are presented as fold increase compared to cells transfected with pro-IL-1β, pro-caspase-1 and ASC (n=3, ±SD).

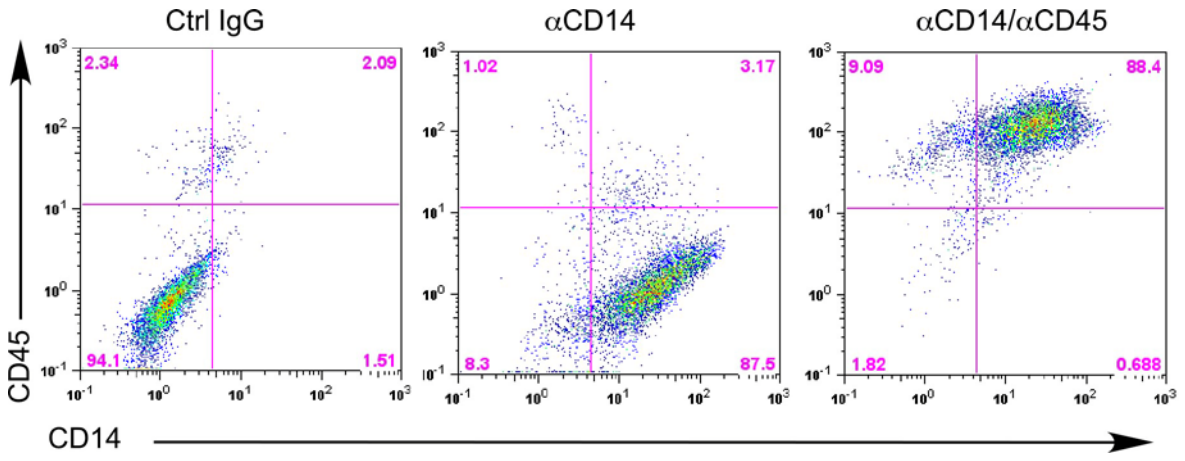

Figure S7. Human macrophage phenotyping. Differentiated human peripheral blood derived MΦ were immunostained at day 7 post isolation with anti-CD45-PE and anti-CD14-FITC antibodies and analyzed by flow cytometry. Non-differentiated monocytes die at day 3 post isolation.

NLR	duplex 1	duplex 2	duplex 3
NLRP2	S: CUCUAGACGUGGACGAAAUtt AS: AUUUCGUCCACGUCUAGAGtt	S: GCAAAGAGGUCCAGGUUAUtt AS: AUAACCUGGACCUCUUUGCtt	S: CGAUGCGACAUAAGUUGUAtt AS: UACAACUUAUGUCGCAUCGtt
NLRP3	S: CGCUAAUGAUCGACUUCAAtt AS: UUGAAGUCGAUCAUUAGCGtt	S: GAGUACCUUUCGAGAAUCUtt AS: AGAUUCUCGAAAGGUACUCtt	S: CCAAGAUUGAGAUCAAUCUtt AS: AGAUUGAUCUCAAUCUUGGtt
NLRP5	S: GGCUUCCGAUUAACCAGAAtt AS: UUCUGGUUAAUCGGAAGCCtt	S: GAACGAAGGUGUAAAUCUAtt AS: UAGAUUUACACCUUCGUUCtt	S: GUUGGUUACUGGAUUUGAAtt AS: UUCAAAUCCAGUAACCAACtt
NLRP6	S: GUGUCCGAGUACAAGAAGAtt AS: UCUUCUUGUACUCGGACACtt	S: CAAGGACAAGAAGAAGUAUtt AS: AUACUUCUUCUUGUCCUUGtt	S: ACGUCAGUGUACCUGCUUUtt AS: AAAGCAGGUACACUGACGUtt
NLRP7	S: GGAAUUGCUGCAAUGCAAAtt AS: UUUGCAUUGCAGCAAUUCCtt	S: GUGUUCCUGGAGAAUUACAtt AS: UGUAAUUCUCCAGGAACACtt	S: GUCAGAGGGUCACAUGUUAtt AS: UAACAUGUGACCCUCUGACtt
NLRP8	S: GUAGUCCGCAGAGAGAUAAtt AS: UUAUCUCUCUGCGGACUACtt	S: GAAGACCACUAUGUCUUUAtt AS: UAAAGACAUAGUGGUCUUCtt	S: CAAGGUAACGGGCAUCUAAtt AS: UUAGAUGCCCGUUACCUUGtt
NLRP9	S: GCAUCUCAGAUUACAAUGAtt AS: UCAUUGUAAUCUGAGAUGCtt	S: GGAUUGACGAGGAAUACAAtt AS: UUGUAUUCCUCGUCAAUCCtt	S: CCUAAGUGUUCUAGCAAUAtt AS: UAUUGCUAGAACACUUAGGtt
NLRP11	S: CCAACUGCUAGUCAAAUGAtt AS: UCAUUUGACUAGCAGUUGGtt	S: CAGAAAGGAUUCUGUCUAAtt AS: UUAGACAGAAUCCUUUCUGtt	S: CUAGAAGAGUGCAUGUUAAtt AS: UUAACAUGCACUCUUCUAGtt
NLRP12	S: CUCUCAUAGCCAAUAAGAAtt AS: UUCUUAUUGGCUAUGAGAGtt	S: CCUCAACUCUCAGUGUGAAtt AS: UUCACACUGAGAGUUGAGGtt	S: GAGACAGACUCAUGCAGAUtt AS: AUCUGCAUGAGUCUGUCUCtt
TLR2	S: GGUUCCUUGUUUACUUUCAtt AS: UGAAAGUAAACAAGGAACCtt	S: GGAAUGCAAUAACUACGUUtt AS: AACGUAGUUAUUGCAUUCCtt	S: GCAAGUGGAUCAUUGACAAtt AS: UUGUCAAUGAUCCACUUGCtt

Table S1. NLRP and TLR2 siRNA sequences.

NLR	Fwd primer	Rev primer
NLRP1	5’-cacctcagcccagcctagcac-3’	5’-tcaggccagcttctcttgaccag-3’
NLRP2	5’-ataacacggaaagaacgacca-3’	5’-tagaacagggcagtgagaaac-3’
NLRP3	5’-tgtgatatgccaggaagacag-3’	5’-cgaggatggtccagcaa-3’
NLRP4	5’-caggagttctgtgccgccttgttctat-3’	5’-ctgaggtgcccgctggtggtga-3’
NLRP5	5’-ggaaaggcacgggatgacatgaa-3’	5’-tctccttcttccgctgcatctctctaac-3’
NLRP6	5’-cgcggagcagctggcccagttcta-3’	5’-ggaagaggcggttgaaagtgtgcgtgtc-3’
NLRP7	5’-aggacggacaggtgcaagaaata-3’	5’-ccctgggtaacatcttcctct-3’
NLRP8	5’-gaaccagaggacttgaatgtggg-3’	5’-agaagttggtcgggtttg-3’
NLRP9	5’-ggcctccaaagaagatgtagcaa-3’	5’-tttccctctgcccagtcca-3’
NLRP10	5’-gggccttgagtgaccttgaggagaacga-3’	5’-cgagagttgtctttccagtgccagccga-3’
NLRP11	5’-agacgaaaccgcaatcaggaggcat-3’	5’-gtcaacgcatcagcaagaaagtgggcat-
NLRP12	5’-tggtggcccgtcctcacttg-3’	5’-cttcccgtccgcccagtc-3’
NLRP13	5’-atccaaaccaagaagaaccagag-3’	5’-ggccaatccaaagaaatc-3’
NLRP14	5’-gactataggaccagatgatgccaagg-3’	5’-ctcaggttcttcaaaggcaaagttcagttc-3’
ASC	5’- gctgtccatggacgccttgg-3’	5’- gcttggctgccgactgaggag-3’

Table S2. RT-PCR primer pairs.

Supplemental Experimental Procedures:

Materials and Cell Culture

THP-1 monocytes were obtained from ATCC and cultured as suggested, used only at low passage numbers, and screened routinely for mycoplasma infections (MycoAlert, Lonza). For immunofluorescence staining THP-1 cells were differentiated into adherent macrophages (Bryan et al., 2009). Human PBMCs were isolated by Ficoll-Hypaque centrifugation (Sigma) from healthy donor buffy coats and countercurrent centrifugal elutriation in the presence of 10 µg/ml polymyxin B using a JE-6B rotor (Beckman Coulter). To ensure the purity of PBMCs, cells were washed in Hank's Buffered Salt Solution, resuspended in serum-free RPMI for 1 hr, followed by culturing in complete medium supplemented with 20% FBS for 7 days to differentiate peripheral blood macrophages, which were then cultured in medium supplemented with 10% FBS. Isolated and differentiated peripheral blood macrophages were routinely phenotyped to ensure >85% purity, as determined by flow cytometry for CD45 and CD14 (Figure S7). Cells were scraped from dishes, washed in flow cytometry buffer (1xPBS, 1% FBS, 0.1% sodium azide), incubated with PE-conjugated anti-CD45 (Immunotech, Immu 19.2) and FITC-conjugated anti-CD14 (BD Biosciences, M5E2) at 4°C for 30 min. Samples were washed in flow cytometry buffer and analyzed on a Beckman Coulter CyAn using FloJo (Tree Star, Inc.).

Subcellular fractionation

10^6 cells were resuspended in hypotonic lysis buffer (10 mM Tris-HCL pH 7.4, 10 mM NaCl, 3 mM MgCl2, 1 mM EDTA, and 1mM EGTA, supplemented with protease inhibitors), incubated on ice, adjusted to 250 mM sucrose, and lysed in a Dounce homogenizer. Samples were initially centrifuged at 4°C at 1,000 xg for 3 min to remove any intact cells and then centrifuged at 4°C at 2,000 xg for 10 minutes to pellet the nuclei. The cytosolic supernatant was removed, and the nuclear pellet was washed three times in hypotonic lysis buffer with the addition of 250 mM sucrose and 0.1% NP-40 and incubated for 20 min on ice. Both fractions were adjusted to 50 mM Tris-HCl pH 7.4, 20 mM NaCl, 3 mM MgCl2, 250 mM sucrose, 0.5% deoxycholate, 0.1% SDS, 0.2% NP-40, and protease and phosphatase inhibitors, and fully solubilized by brief sonication. 50 µg of protein lysates were separated by SDS-PAGE, transferred to a PVDF membrane, and probed with anti-ASC antibodies specific to the linker (CS3, 1:15,000) and HRP-conjugated secondary antibodies (Amersham Pharmacia) in conjunction with an ECL detection system (Pierce). Membranes were stripped and re-probed with anti-GAPDH (Sigma, 1:1000) and anti-Lamin A (Santa Cruz Biotechnology, 1:500) antibodies as control for cytosolic and nuclear fractions, respectively.

Immunofluorescence microscopy

Cells were fixed in 3.7% paraformaldehyde, incubated in 50 mM glycine for 5 min, permeabilized and blocked with 0.5% saponin, 1.5% BSA, 1.5% normal goat serum for 30 min. ASC was immunostained with affinity purified polyclonal antibodies directed to the CARD from Chemicon (1:200) or custom raised directed to the linker (CS3; 1: 2,500). Secondary Alexa Fluor 488-conjugated antibodies, DAPI, and Alexa Fluor 546-conjugated phalloidin were from Molecular Probes. Cells were washed with PBS containing 0.5% saponin, and cover slips were mounted using Fluoromount-G (Southern Biotech). Suspension cells were fixed and stained as above and adhered to poly-L-lysine-coated slides using a cytocentrifuge (StatSpin). For co-localization studies, HEK293 cells were transiently transfected with NLRP7 alone or co-transfected with ASC and/or pro-caspase-1 and immunostained with mouse monoclonal anti-myc (Northwestern University), rat monoclonal anti-HA (Roche) and rabbit polyclonal anti-ASC (CS3) and secondary Alexa Fluor 488-, 546- and 647-conjugated antibodies and DAPI. Images were acquired by confocal laser scanning and epifluorescence microscopy on a Zeiss LSM 510 and a Nikon TE2000E2-PFS with a 100x oil immersion objective and image deconvolution.

AnnexinV Pyroptosis assay

THP-1 cells were also harvested and re-suspended in annexin-binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$), incubated for 15 min with annexin V (5 µg/ml, Invitrogen) and analyzed by flow cytometry (LSRII, BD Biosciences) and FlowJo (Tree Star, Inc.).

Real time PCR
Real time PCR was performed on an ABI 7300 Real time PCR machine (Applied Biosystems) using the TaqMan Gene expression system (Applied Biosystems) and predesigned FAM labeled primer/probes (NLRP2: Hs00215284_m1, NLRP3: Hs00918082_m1, NLRP5: Hs00411266_m1, NLRP6: Hs00373246_m1, NRP7: Hs00373683_m1, NLRP8: Hs00603419_m1, NLRP9: Hs00603423_m1, NLRP11: Hs00935472_m1, NLRP12: Hs00536435_m1, ASC: Hs00203118_m1, βActin: 4352935E, IL-1β Hs01555410_m1, IL-18: Hs01038788_m1). Results are presented as relative expression compared to βactin.

Inflammasome reconstitution system
Stable myc-ASC HEK293 cells ($HEK293^{ASC}$) were selected by limited dilution to yield low ASC expression, which prevents spontaneous ASC aggregation. The stable HEK293 reconstitution system ($HEK293^{Inflammasome}$) was based on $HEK293^{ASC}$ cells stably transfected with pro-IL-1β-Flag and pro-caspase-1 from a dual expression vector and selected to only cause Caspase-1 activation and IL-1β release upon transient transfection of NLRP3 or NLRP7.

siRNA transfection
$3x10^5$ THP-1 cells were electroporated (Neon, Invitrogen; setting: 1400-20-2 using the 10 µl tip) with 60 nM single or pooled siRNA duplexes, and analyzed 72 hrs post electroporation. hMΦ were transfected in 24-well plates (F2 in combination with virofect; Targeting Systems) using 120 nM siRNA and analyzed 72 hrs post transfection.