The PYRIN Domain-only Protein POP1 Inhibits Inflammasome Assembly and Ameliorates Inflammatory Disease

**Highlights**
- POP1 inhibits inflammasome-mediated responses to PAMPs and DAMPs
- POP1 prevents IL-1β and IL-18 release and pyroptosis
- POP1 prevents ASC danger particle-mediated response propagation to bystander cells
- Transgenic POP1 expression protects mice from systemic inflammation

**Authors**
Lucia de Almeida, Sonal Khare, Alexander V. Misharin, ..., Hal M. Hoffman, Andrea Dorfleutner, Christian Stehlik

**Correspondence**
a-dorfleutner@northwestern.edu (A.D.), c-stehlik@northwestern.edu (C.S.)

**In Brief**
Inflammatory responses need to be tightly controlled to maintain homeostasis. Stehlik and colleagues demonstrate that the PYRIN domain-only protein POP1 inhibits ASC-containing inflammasome assembly and consequently caspase-1 activation, IL-1β and IL-18 release, pyroptosis, and the release of ASC particles in macrophages. Importantly, transgenic POP1 expression protects mice from systemic inflammation.

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The PYRIN Domain-only Protein POP1 Inhibits Inflammasome Assembly and Ameliorates Inflammatory Disease


Correspondence: a-dorfleutner@northwestern.edu (A.D.), c-stehlik@northwestern.edu (C.S.)

School of Medicine, Northwestern University, Chicago, IL 60611, USA
(CS) and San Diego Branch, Ludwig Institute of Cancer Research, La Jolla, CA 92093, USA
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SUMMARY

In response to infections and tissue damage, ASC-containing inflammasome protein complexes are assembled that promote caspase-1 activation, IL-1β and IL-18 processing and release, pyroptosis, and the release of ASC particles. However, excessive or persistent activation of the inflammasome causes inflammatory diseases. Therefore, a well-balanced inflammasome response is crucial for the maintenance of homeostasis. We show that the PYD-only protein POP1 inhibited ASC-dependent inflammasome assembly by preventing inflammasome nucleation, and consequently interfered with caspase-1 activation, IL-1β and IL-18 release, pyroptosis, and the release of ASC particles. There is no mouse ortholog for POP1, but transgenic expression of human POP1 in monocytes, macrophages, and dendritic cells protected mice from systemic inflammation triggered by molecular PAMPs, inflammasome component NLRP3 mutation, and ASC danger particles. POP1 expression was regulated by TLR and IL-1R signaling, and we propose that POP1 provides a regulatory feedback loop that shuts down excessive inflammatory responses and thereby prevents systemic inflammation.

INTRODUCTION

Inflammation is an essential and tightly controlled process initiated by the innate immune system in response to infection and tissue damage and is responsible for pathogen clearance, wound healing, and restoring homeostasis. It is triggered by the sensing of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs or danger signals) by germline-encoded pattern recognition receptors (PRRs). Particularly, cytosolic PRRs of the AIM2-like receptor (ALR) and Nod-like receptor (NLR) families facilitate the activation of the pro-inflammatory caspase-1 within large macromolecular protein complexes in macrophages, referred to as inflammasomes. Inflammasomes promote the proteolytic maturation and release of the leaderless pro-inflammatory cytokines interleukin (IL)-1β and IL-18 and the induction of pyroptotic cell death, which causes the release of IL-1α and HMGB1 (Martinon et al., 2002; Khare et al., 2010; Wen et al., 2013). Inflammasomes are composed of a sensory PRR, which is linked to caspase-1 via the adaptor protein ASC (Srinivasula et al., 2002; Stehlik et al., 2003a; Martinon et al., 2002). This complex exhibits specific protein-protein interactions, which are mediated by homotypic PYRIN domain (PYD) interactions between PRRs and ASC and by homotypic caspase recruitment domain (CARD) interactions between ASC and pro-caspase-1. Within this protein complex, pro-caspase-1 is activated by induced proximity-mediated oligomerization (Martinon et al., 2002; Lu et al., 2014). The underlying assembly mechanism of this ternary complex has recently been delineated and indicates that PRR activation triggers prion-like, self-propagating ASC polymerization into filaments, which is initialized by PRR-induced ASCPYD nucleation (Cai et al., 2014; Lu et al., 2014; Franklin et al., 2014; Dorfleutner et al., 2015). PRRs localize to the end of the hollow ASC filaments and allow efficient self-propagation of the ASCPYD, whereby the ASCCARD is flexibly linked to the outside of the filaments, allowing recruitment and clustering of pro-caspase-1 (Lu et al., 2014). Eventually, these filaments can assemble into a spherical structure, where caspase-1 is placed in the hollow core (Man et al., 2014). Thus, the PYD is essential for inflammasome assembly after activation of NLRP3 and other PYD-containing PRRs, and disassembly of this ternary signaling complex is required for its termination. NLRP3 is one of the best-studied inflammasomes—activating PRRs and the NLRP3 inflammasome is regulated by a two-step mechanism, referred to as priming and activation (Khare et al., 2010). After priming with LPS or other NF-κB-inducing signals, NLRP3 is activated in a second step by a wide variety of PAMPs that cause potassium (K⁺) efflux, including exogenous ATP, nigericin, and uric acid crystals, as well as environmental and endogenous mediators released in response to stress and tissue damage (Khare et al., 2010; Khare et al., 2009; Dorfleutner et al., 2015).
Muñoz-Planillo et al., 2013). Besides inflammasome-linked cytokines, oligomeric ASC particles are also released and phagocytized by neighboring cells to perpetuate inflammasome responses (Franklin et al., 2014; Baroja-Mazo et al., 2014). Overall, inflammasomes play an essential role in host defense. However, impaired inflammasome activation results in failure to restrict colitogenic microbiota species and subsequently promotes metabolic dysfunction. In contrast, constitutive inflammasome activation through disease-associated mutations in NLRP3 promotes excessive IL-1β release and causes inflammatory diseases, including Cryopyrinopathies (or Cryopyrin-associated periodic syndromes; CAPS) (Henao-Mejia et al., 2012; Hoffman and Brydges, 2011). Therefore, a controlled and well-balanced inflammasome response is essential for maintaining homeostasis. However, the molecular mechanisms regulating assembly and disassembly of inflammasomes are largely unknown. We discovered a family of PYD-only proteins (POPs) that are encoded in humans but not in mice (Stehlik and Dorfleutner, 2007; Khare et al., 2014; Stehlik et al., 2003b; Dorfleutner et al., 2007a, 2007b; Bedoya et al., 2007; Johnston et al., 2005). We recently demonstrated that POP3 functions as a specific inhibitor for ALR inflammasomes (Khare et al., 2014). POP3 binds to the PYD of ALRs, but not to ASC or NLRs, and thereby prevents ALR interactions with the inflammasome adaptor ASC. However, the function of endogenous POP1 (PYD-containing 1, PYDCl) has not yet been established. POP1 is highly similar to the PYD of ASC and in vitro overexpression experiments showed that POP1 interacts with the PYD of ASC; however, its role in vivo is unknown.

Here we report that POP1 functions in an IL-1β-induced regulatory loop to inhibit the assembly and consequently the activity of ASC-containing inflammasomes by preventing nucleation of ASC. POP1 also prevented the release of oligomeric ASC danger particles, and incorporation of POP1 into ASC particles rendered them inactive, thereby preventing self-perpetuation of inflammasome responses in neighboring cells. To demonstrate the importance of inflammasomes particularly in monocytes and macrophages, we engineered mice that usually lack POP1 to specifically express transgenic POP1 in monocytes, macrophages, and DCs. We used the NLRP3 inflammasome as an example to demonstrate that transgenic POP1 expression in the monocyte-macrophage-DC lineage was sufficient to blunt excessive systemic inflammation in response to PAMPs, ASC danger particles, and CAPS-associated mutations. Interestingly, CAPS patients exhibited reduced POP1 expression, suggesting that impaired POP1 expression might contribute to excessive inflammasome-driven inflammation. Our data reveal a mechanism by which human inflammasome assembly is regulated and detail how healthy tissue puts the brakes on inflammasome-induced systemic inflammation. In addition, our results emphasize the crucial role of the monocyte-macrophage-DC lineage in this response.

RESULTS

POP1 Inhibits Inflammasome-Mediated Cytokine Release in Human Macrophages

To investigate the role of POP1 in inflammasome signaling, we established stable shRNA-mediated POP1 silencing in human monocytic THP-1 cells (shPOP1 THP-1). POP1 knockdown (via two different shRNAs causing elevated IL-1β release in response to LPS and POP1 silencing) was confirmed by qPCR (Figure 1A). Similarly, siRNA-mediated silencing of POP1 in primary human macrophages resulted in elevated IL-1β and IL-18, but not IL-6, release in response to LPS. POP1 silencing was again confirmed by qPCR (Figure 1B). Conversely, THP-1 cells stably expressing GFP-POP1 displayed diminished IL-1β secretion in response to LPS and also in response to non-canonical inflammasome activation with LPS transfection or cytosolic delivery of LPS with cholera toxin subunit B (CTB) (Figure 1C). Cytokine release after non-canonical inflammasome activation requires NLRP3. Consistently, POP1 blocked IL-1β release after NLRP3 inflammasome activation with nigericin, calcium pyrophosphate dehydrate (CPPD) crystals, or K+ depletion. Furthermore, POP1 blocked IL-1β release after AIM2 and NLRC4 activation with poly(dA:dT) or flagellin transfection, respectively (Figure 1D), and POP1 expression was confirmed by qPCR (Figure 1E). Hence, POP1 inhibits inflammasome-dependent cytokine release. Comparable results were also obtained for myc-tagged POP1, thus establishing that the GFP tag does not affect the function of POP1 (Figure S1A).

POP1 Impairs ASCPYD Nucleation and Caspase-1 Activation in Human Macrophages

Caspase-1 activation in canonical inflammasomes of macrophages is required for IL-1β and IL-18 release and for pyroptosis. Therefore, we determined caspase-1 activity in the presence and absence of POP1. Treatment of Pam3CSK4-primed shPOP1 knockdown cells with nigericin resulted in elevated active caspase-1 p10 and mature IL-1β in culture supernatants when compared to control THP-1 cells (Figure 2A). Further, intracellular caspase-1 activation was also augmented in shPOP1 THP-1 compared to control THP-2, as shown by caspase-1 FLICA assay (Figure 2B). Conversely, expression of GFP-POP1 in primed THP-1 cells showed drastically reduced caspase-1 activity in response to nigericin compared to GFP THP-1 cells (Figure 2C). Consequently, POP1 expression also caused reduced pyroptosis, as determined by LDH release (Figure 2D). TLR-mediated priming is necessary for NLRP3 inflammasome activation (Bauerfeind et al., 2009; Juliana et al., 2012; Schroder et al., 2012; Lin et al., 2014). However, stable POP1 expression in THP-1 cells did not affect transcription of IL1B (Figure 1B) and resulted in mild increased transcription of NLRP3 or PYCARD (ASC) in response to LPS (Figure S1C), and contrary to transient overexpression in epithelial cell lines (Stehlik et al., 2003b), stable POP1 expression also did not affect phosphorylation of IκBα (Figure S1D). Furthermore, silencing of POP1 in THP-1 cells also did not affect transcription of PYCARD and NLRP3 (Figure S1E). Thus, POP1 seems to directly regulate inflammasome assembly or activation in macrophages. Recruitment of ASC to upstream sensors is essential for inflammasome activation and we hypothesized that POP1 interferes with this interaction. POP1 specifically bound to endogenous ASC, but not to the PYD-containing PRRs NLRP3 and AIM2 in LPS-primed THP-1 cells (Figure 2E). Binding of ASC to NLRP3 induces ASCPYD nucleation, which provides the oligomeric platform essential for caspase-1 activation (Cai et al., 2014; Lu et al., 2014). Therefore, we hypothesized that POP1 prevents ASCPYD-NLRP3PYD interactions and thus ASCPYD nucleation (Chu et al., 2015).
Indeed, the nigericin-induced interaction of NLRP3 and ASC was abolished in LPS-primed GFP-POP1 THP-1 cells, indicating that POP1 might prevent NLRP3-mediated ASC nucleation (Figure 2F). Upon ASC nucleation, the ASC-PYD polymerizes in a prion-like self-perpetuating manner that further promotes caspase-1 activation (Lu et al., 2014). Because ASC-ASC, ASC-POP1, and ASC-NLRP3 interactions utilize the same key residues (Vajjhala et al., 2012), POP1 binding to the ASCPYD could also directly prevent ASCPYD self-polymerization. Surprisingly, POP1 expression in HEK293 cells stably expressing GFP or GFP-POP1 were analyzed for IL-1β release by ELISA in untransfected cells (Ctrl) or in response to LPS treatment, LPS transfection, or incubation with LPS complexed with CTB (C) and nigericin or CPPD treatment or K+ depletion in LPS-primed cells or transfection of poly(dA:dT) or flagellin (D). (E) Real-time PCR of POP1 transcripts in above cells. Data are representative of three (A) or three (B–E) replicates; error bars represent SEM. *p = 0.0009, **p = 0.0128, ***p < 0.0001, ****p < 0.0001 in (A); *p = 0.0284, **p = 0.0315, ***p = 0.0001 in (B); *p < 0.0001, **p < 0.0001, ***p < 0.0001 in left panel of (D); *p < 0.0001, **p < 0.0001 in right panel of (D); and *p = 0.0024, **p = 0.0022, ***p = 0.0139 in (E); all two-tailed unpaired t test. See also Figure S1A.

**POP1 Prevents Caspase-1 Activation and Cytokine Release in Mouse Macrophages**

Because the genes for all POPs, including POP1, are absent in mice (Stehlik and Dorfleutner, 2007; Khare et al., 2014), we generated GFP-POP1 transgenic (TG) mice. Caspase-1 is essential for IL-1β and IL-18 release in monocytes and macrophages and we detected POP1 expression in CD68+ bone-marrow-derived macrophages (BMDMs) in inflamed lung tissue (Figure S2A). Therefore, to limit POP1 expression to macrophages, we used the hCD68/IVS-1 promoter/enhancer (Iqbal et al., 2014; Khare et al., 2014; Gough et al., 2001) for GFP-POP1 expression in TG mice. We confirmed POP1 expression specifically in CD68+ BMDMs and not in wild-type (WT) mice by qPCR analysis of whole blood cell RNA (Figure S2B). Accordingly, we also detected POP1 expression in transgenic POP1 bone-marrow-derived macrophages (BMDMs) by immunoblot (Figure 3A). Human and mouse ASCPYD have a high degree of homology (Figure S2C) and therefore it was not surprising that, similar to human ASC in THP-1 cells, POP1 also interacted with mouse ASC, but not NLRP3 or AIM2 in BMDMs (Figure 3B). ASC polymerization can be captured by non-reversible cross-linking and functions as a read-out for inflammasome activation (Fernandes-Alnemri et al., 2007), which was markedly reduced in LPS-primed and ATP-treated POP1 BMDMs compared to WT BMDMs (Figure 3C). Consequently, POP1 BMDMs lacked active caspase-1 p10 and mature IL-1β in culture supernatants.
of LPS/ATP-treated cells to a similar extent as the caspase-1 inhibitor zYVAD-fmk (Figure 3D). Reduced caspase-1 activity was also detected by flow cytometry in intact cells (Figure 3E), indicating that POP1 also inhibits caspase-1 activation in mouse macrophages.

As expected from impaired caspase-1 activation, LPS/ATP-treated POP1 BMDMs also displayed significantly reduced levels of IL-1β, IL-1α, and IL-18 in culture supernatants by ELISA. However, secretion of TNF-α, which occurs independently of caspase-1, was not affected (Figure 3E). Significantly, reduced IL-1β release in POP1 BMDMs was comparable to Pycard−/− BMDMs and Nlrp3−/− BMDMs (Figure 3G), K⁺ efflux is the unifying mechanism of NLRP3 activation in BMDMs (Muñoz-Planillo et al., 2013), and culturing POP1 BMDMs in K⁺-free medium showed impaired IL-1β release compared to WT BMDMs (Figure 3H).

Similarly, peritoneal POP1 macrophages (POP1 PMs) showed impaired IL-1β release in response to activation of ASC-dependent inflammasomes containing NLRP3 with ATP, AIM2 with poly(dA:dT), and NLRC4 with flagellin (Figure 3I). POP1 BMDMs also showed reduced LDH release, and thus pyroptosis, when compared to WT BMDMs in response to NLRP3 activation (Figure 3J), but did not reveal any altered LPS-induced activation of NF-κB, p38, JNK, or ERK (Figure S2D) or altered transcription of Il1b, Il18, Pycard, and Nlrp3 (Figure S2E), ruling out POP1 effects on inflammasome priming in mouse macrophages. Collectively, these data indicate that POP1 impairs assembly of the NLRP3 inflammasome in human and mouse macrophages by impairing the PRR-mediated nucleation of ASC and consequently the release of inflammasome-dependent cytokines.

Monocyte-Macrophage-DC-Specific Expression of POP1 Ameliorates LPS-Induced Peritonitis

NLRP3 senses endogenous danger signals and PAMPs and promotes inflammatory responses that can be detrimental to the host. We therefore used the NLRP3 inflammasome to investigate the role of POP1 in vivo. To initially characterize the CD68-POP1 TG mice, we analyzed peripheral blood, which revealed POP1 expression selectively in monocytes (Figures 4A and S3), with equal expression in classical Ly6C⁺CD43⁺, intermediate Ly6C⁺CD43⁺, and non-classical Ly6C⁻CD43⁻ monocytes (Figure S4A). POP1 was also expressed in myeloid...
precursors (MPs), macrophage and DC precursors (MDPs), and common DC precursors (CDPs) in the bone marrow (Figures S4B and S4C), large peritoneal macrophages (LPMs), small peritoneal macrophages (SPMs), and peritoneal DCs (Figures S4D and S4E), as well as in splenic red pulp macrophages (RPMs), monocytes, and CD11b+ DCs, but not plasma-cytoid DCs (pDCs) (Figures S5A and S5B). Monocyte-macrophage-DC-specific POP1 expression was also observed in other tissues, with no detectable expression in CD45- cells (Figure S5C and data not shown). Collectively, these results demonstrate POP1 expression in the monocyte-macrophage-DC lineage.

Caspase-11 is responsible for LPS- and Gram-negative-bacteria-induced lethal shock, but ASC and NLRP3 are both

Figure 3. POP1 Inhibits the NLRP3 Inflammasome in Mouse Macrophages
(A) Immunoblot of POP1 expression in BMDMs using a GFP antibody.
(B) Interaction of GST-POP1 with endogenous ASC from LPS-primed BMDM total cell lysates (TCL) using GST as negative control and showing 10% TCL as input. Asterisk (*) marks a degradation product of GST-POP1.
(C) Immunoblot analysis of ASC polymerization (oligomerization) in untreated or LPS/ATP-treated wild-type (WT) and POP1 BMDMs after cross linking of pellets (P) and in TCL.
(D) Immunoblot analysis of caspase-1 and IL-1β in culture SN of LPS/ATP-treated WT and POP1 BMDMs. Pro-caspase-1 expression in TCL confirms equal loading.
(E) Flow cytometric quantification of active caspase-1 in WT and POP1 BMDMs in response to LPS/ATP.
(F) Analysis of culture supernatants (SN) for IL-1β, IL-18, IL-1α, and TNF-α by ELISA in LPS/ATP-treated WT and POP1 BMDMs.
(G) Analysis of culture SN for IL-1β by ELISA in LPS-primed and ATP-treated WT, POP1, Pycard−/−, and Nlrp3−/− BMDMs.
(H) WT and POP1 BMDMs cultured in K+ depleted medium.
(I) WT and POP1 PMs treated with LPS/ATP or transfected with flagellin or poly(dA:dT).
(J) LPS-primed WT and POP1 BMDMs were treated with nigericin or CPPD crystals and released LDH in culture SN was quantified.

Data are representative of four (A, H, I) or two (B–G, J) replicates; error bars represent SEM. *p = 0.0027, **p = 0.003, ***p = 0.0403 in (F); *p = 0.0214, **p = 0.0043, ***p = 0.0052 in (G); *p = 0.01 in (H); *p = 0.0009, **p < 0.0001 in (I); and *p < 0.0001 in (J); all two-tailed unpaired t test. See also Figure S2.
necessary for amplifying this response to LPS in vivo (Kayagaki et al., 2011). Accordingly, Pycard−/− and Nlrp3−/− mice are protected from LPS-induced lethality in response to moderate LPS doses (Mariathasan et al., 2004, 2006; Kayagaki et al., 2011). NLRP3 inflammasome-released IL-1β is essential for neutrophil recruitment during sterile inflammation (McDonald et al., 2010). Therefore, we injected WT and CD68-POP1 TG mice i.p. with a low dose of LPS and determined neutrophil infiltration 3 hr after LPS challenge by quantifying myeloperoxidase (MPO) activity in vivo. Contrary to PBS, injection of LPS recruited a substantial number of neutrophils into the peritoneal cavity, which was completely abolished in CD68-POP1 TG mice (Figure 4B). Consequently, CD68-POP1 TG mice experienced significantly less hypothermia (Figure 4C) and were significantly more protected from a lethal LPS dose (Figure 4D). Compared to 100% lethality in WT mice, only 30% of CD68-POP1 TG mice died within 96 hr, which is similar to Pycard−/− mice (Mariathasan et al., 2004). Consistent with reduced neutrophil infiltration and increased survival, serum IL-1β was reduced, but TNF-α levels remained unchanged (Figure 4E), indicating that expression of POP1 in the monocyte-macrophage-DC lineage is sufficient to impair inflammasome activation in response to PAMPs in vivo, thereby blocking the secretion of IL-1β and ameliorating an excessive host response.

Monocyte-Macrophage-DC-Specific Expression of POP1 Ameliorates CAPS

Because sepsis is a rather complex disease, we investigated a disease model, for which the pathology is absolutely dependent on the NLRP3 inflammasome. Cryopyrinopathies (or Cryopyrin-associated periodic syndromes; CAPS) are caused by mutations in NLRP3 (Hoffman et al., 2001), are therefore directly linked to the NLRP3 inflammasome, and can be recapitulated in mice by knocking-in CAPS-associated NLRP3 mutations (Brydges et al., 2009, 2013; Meng et al., 2009). We employed a mouse model for Muckle Wells syndrome (MWS), where floxed Nlrp3A350V, corresponding to human NLRP3A352V, is expressed exclusively in myeloid cells in the presence of lysozyme M-Cre (CreL) (Brydges et al., 2009, 2013). Nlrp3A350V/CreL mice develop systemic inflammation that affects multiple organs, display characteristic skin inflammation, and die within 2 weeks of birth. This phenotype is caused by excessive IL-1β and IL-18 release as well as pyroptosis (Brydges et al., 2009, 2013). Nlrp3A350V/CreL mice had inflammatory skin abscesses and lesions shortly after birth, which developed into scaling erythema (Brydges et al., 2009), but Nlrp3A350V/CreL CD68-POP1 mice did not display this phenotype (Figure S6A). Histological analysis revealed that POP1 expression prevented leukocytic infiltrates in multiple organs, including the liver and the skin, and also restored skin architecture (Figure 5A). Also, the systemic IL-1β
levels were reduced (Figure 5B). Significantly, POP1 expression rescued the severe growth delay (Figure 5C) and prevented mortality of Nlrp3<sup>A350V/+</sup> CreL mice from multi-system organ failure (Figure 5D). Because expression of POP1 efficiently ameliorated CAPS, we analyzed POP1 expression in a previously published patient cohort (Boisson et al., 2012) and found that CAPS patients displayed significantly lower POP1 expression compared to healthy controls (Figure 5E). We also observed the same trend in leukocytes from two large, independent septic patient cohorts, when compared to healthy controls (Figure S6B; Tang et al., 2008; Wong et al., 2009), although the reduced POP1 expression in patients was less pronounced. These findings demonstrate that POP1 inhibits excessive NLRP3 inflammasome activity in vivo and thereby ameliorates auto-inflammatory disease. We also identified reduced POP1 expression in CAPS patients, which might circumvent appropriate inflammasome control.

**POP1 Prevents ASC Particle Release and Ameliorates ASC Particle-Induced Inflammation**

Recently, polymerized ASC particles were detected in the serum of active CAPS patients (Baroja-Mazo et al., 2014), which are released from macrophages through inflammasome-dependent pyroptosis and act as danger signals on neighboring cells (Baroja-Mazo et al., 2014; Franklin et al., 2014). POP1 prevented ASC nucleation and the subsequent ASC polymerization, caspase-1 activation, and pyroptosis, which are all required for the ASC particle response. Hence, culture supernatants from LPS-primed and nigericin- or ATP-treated GFP THP-1 cells and WT BMDMs contained ASC, but supernatants from GFP-POP1 THP-1 cells (Figure 6A) or POP1 BMDMs (Figure 6B) did not contain any ASC. Particulare, the release of polymeric ASC was inhibited by POP1 (Figure 6C). Extracellular ASC particles are phagocytized by macrophage and activate caspase-1 in an NLRP3- and ASC-dependent process (Baroja-Mazo et al., 2014; Franklin et al., 2014). FACS-purified ASC-GFP particles (Figure S6C) induced IL-1β release in LPS-primed GFP THP-1 cells, but not in GFP-POP1 THP-1 cells (Figure 6D), suggesting that POP1 prevents ASC nucleation downstream of NLRP3 activation. POP1 is an ASC binding protein, which can be incorporated into ASC<sub>CARD</sub> filaments (Figure S1F). This can result in reduced ASC<sub>CARD</sub> density and subsequently prevent caspase-1 nucleation and
To directly prove that POP1 incorporation into ASC particles renders them inactive, we generated mixed ASC-GFP/RFP-POP1 particles (Figure S6C), which, in contrast to ASC-GFP particles, failed to cause IL-1β release in THP-1 cells (Figure 6E). Importantly, i.p. injection of ASC-GFP particles into WT mice resulted in neutrophil recruitment (Figure 6F) and IL-1β release (Figure 6G), which was substantially reduced in CD68-POP1 TG mice.

Based on the reduced POP1 expression in CAPS patients and its inflammasome inhibitory function in macrophages, we designed a proof-of-concept treatment approach targeting ASC inflammasomes. Cell-penetrating peptides are frequently employed for the delivery of molecules targeting intracellular signaling pathways (Schwarze et al., 1999), and so we produced recombinant POP1 and GFP as a control fused to the cell-penetrating HIV TAT sequence (TAT-POP1 and TAT-GFP) (Figure S6D). TAT-GFP was efficiently taken up by PMs after i.p. injection in vivo (Figure S6E), and injection of TAT-POP1, but not TAT-GFP, ameliorated LPS-induced peritonitis (Figure 6H), reminiscent to transgenic POP1 expression. Collectively, these data show that POP1 also blocks the release of ASC danger particles and consequently propagation of secondary inflammasome responses in neighboring cells and that delivery of POP1 might be used as the basis for future therapeutic approaches in patients with CAPS and other inflammasomopathies.
POP1 Functions in an Inducible Inflammasome Regulatory Loop

Overwhelming evidence supports the necessity for a balanced inflammasome response to maintain tissue homeostasis (Hernao-Mejia et al., 2012). Therefore, we hypothesized that POP1 expression needs to be tightly regulated. We observed LPS-induced late response gene expression of POP1 in human macrophages (Figure 7A) and THP-1 cells (Figure S7A), but in contrast to POP3, POP1 was not upregulated in response to IFN-β (Khare et al., 2014), which emphasizes the distinct function of individual POPs. Notably, POP1 expression peaked right before the inducible expression of HMGB1, which is released through pyroptosis (Lamkanfi et al., 2010; Willingham et al., 2009) and contributes to inflammatory disease (Harris et al., 2012). Thus, the late response expression of POP1 potentially enables inflammasome functions in early host defense and might provide a mechanism to counter excessive release of late mediators that perpetuate systemic inflammation, such as HMGB1. Importantly, this LPS-inducible expression of POP1 was also observed in leukocytes isolated from human subjects after in vivo LPS infusion (Figure 7B; Calvano et al., 2005). TLR4 is required for inducible POP1 expression by LPS, as indicated by the fact that a TLR4 inhibitor reduced POP1 expression (Figure S7B). TLR signaling leads to NF-κB activation and blocking NF-κB also reduced POP1 transcription (Figure S7B). Inducible POP1 transcription was caused not only by TLR4, but also by TLR2 activation by Pam3CSK4 (Figure S7C). IL-1R and IL-18R share signaling components with TLRs and accordingly POP1 expression was also elevated in human macrophages after IL-1β (Figure 7C) and IL-18 (Figure S7D) treatment. Thus, TLR, IL-1R, and IL-18R engagement contributes to NF-κB-dependent inducible transcription of POP1. At the transcriptional level, we observed ~4-fold increased POP1 expression in human macrophages after TLR or IL-1R stimulation and our stable GFP-POP1 THP-1 and Myc-POP1 THP-1 cells showed ~3-fold and ~7-fold more POP1 compared to baseline expression levels, respectively (Figures 1E, S1A, and S1E). Hence, our stable cell lines closely mimicked the induced POP1 expression levels, which were therefore also sufficient to impair inflammasome activity. At the protein level, POP1 PM expressed less POP1 than GFP-POP1 THP-1, as determined by flow cytometry and immunoblot (Figure S7E), indicating that POP1 expression in our TG mice was decreased relative to human macrophages. Still, we found that POP1 expression levels in CD68-POP1 TG mice varied slightly and correlated inversely with inflammasome activity, as determined by measuring IL-18 levels 4 hr after i.p. LPS injection (R² = 0.9316) (Figure S7F). Overall, our results demonstrate that POP1 regulates the inflammasome-mediated IL-1β and IL-18 release, which in turn regulates POP1 expression.

DISCUSSION

ASC-containing inflammasomes are responsible for cytokine release through canonical and non-canonical inflammasomes (Marianthasan et al., 2004; Kayagaki et al., 2011, 2013) and consequently play a central role in facilitating the beneficial inflammatory responses to clear pathogen infections and initiate wound healing after tissue damage. However, uncontrolled inflammasome responses cause inflammatory disease through excessive cytokine release (Strowig et al., 2012; Hoffman and Brydges, 2011). Thus, a well-balanced inflammasome response is crucial for maintaining homeostasis. Therefore, inflammasome regulatory proteins probably exist to maintain an appropriate level of activity and in particular to limit inflammasome activity during the resolution phase of these responses. We proposed that POP1 is one of these proteins. Assembly of the inflammasome platform is initiated by nucleation of ASC through PYD interactions with PYD-containing PRRs followed by prion-like ASC self-polymerization (Cai et al., 2014; Franklin et al., 2014; Lu et al., 2014). Moreover, POP1 might also interact with PYD-containing proteins (Cai et al., 2014; Franklin et al., 2014; Lu et al., 2014). Furthermore, POP1 enhances IL-1β release in vitro (Stehlik et al., 2003c). However, based on our results, this is probably a consequence of overexpression in HEK293 cells.
because we also observed a weak nucleation of ASC polymerization by POP1 in these cells. Other PYDs, including PYD from NLRP3, can also induce weak nucleation of ASC polymerization in this cell type (Lu et al., 2014), and the NLRP3PYD also binds to the same ASC region as POP1 (Vajjhala et al., 2012).

Overexpression studies of several PYD proteins in HEK293 cells have implicated PYDs in NF-κB regulation, but these results were not observed in macrophages and in vivo. Similarly, we previously observed an inhibitory effect of POP1 on NF-κB activity in HEK293 cells (Stehlik et al., 2003b) but did not observe such activity in THP-1 cells, human macrophages, or BMDMs in response to all tested stimuli. However, POP1-expressing THP-1 cells showed a slightly reduced iNOS phosphorylation in response to LPS, but this cannot be responsible for the potent inhibition of caspase-1 and we did not observe significant altered transcription of inflammasome components or release of IL-6 and TNF-α. Filament formation of prion activity containing signaling components is one of the mechanisms of innate immune pathway activation. Although ASCPYD, AIM2PYD, and NLRP3PYD contain such intrinsic prion activity (Lu et al., 2014; Cai et al., 2014), POP1 does not appear to have this activity, because it does not form these characteristic filaments (Stehlik et al., 2003b), but POP1 expression levels probably are crucial for its inflammasome inhibitory function. Inflammasome activity increased upon POP1 silencing in human macrophages and THP-1 cells but decreased upon stable expression of POP1 at levels that are comparable to its induced endogenous expression levels. POP1 transgene expression in mice was lower than in our stable cells and by extension also probably lower than in human macrophages, and therefore closely mimicked physiologically relevant POP1 levels. Despite this lower expression, POP1 was still able to inhibit the inflammasome, and variable POP1 expression levels in individual TG mice even correlated with its inflammasome inhibitory activity. An earlier study mapping the interaction of ASC with NLRP3 did not observe reduced NLRP3-ASC interaction in the presence of POP1 when using in vitro binding assays with recombinant proteins (Vajjhala et al., 2012). However, protein folding and posttranslational modifications might not be recapitulated in vitro and might yield different results compared to our studies of endogenous inflammasome assembly in human and mouse macrophages. Posttranslational modifications, such as ubiquitination (Py et al., 2013) and phosphorylation (Lin et al., 2015; Har et al., 2013), have been implicated in NLRP3 inflammasome activity, and POP1 phosphorylation (Stehlik et al., 2003b) might also be involved in the binding mechanism.

Excessive and uncontrolled release of inflammasome mediators contributes to auto-inflammatory and auto-immune diseases (Strowig et al., 2012). Hence, blocking IL-1β has proved beneficial in various inflammatory diseases in human and mice (Dinarello, 2011). Furthermore, oligomeric ASC particles have been identified in CAPS and pulmonary disease (Franklin et al., 2014; Baroja-Mazo et al., 2014). Despite the tight regulation of inflammasome responses, even a single point mutation in NLRP3 can drive excessive systemic inflammation (Hoffman and Brydges, 2011). Thus, inflammasome regulatory mechanisms might also be impaired. In line with this possibility, we observed reduced POP1 expression in CAPS patients. This observation suggested that in addition to uncontrolled activation of NLRP3, recruitment and oligomerization of ASC and extracellular release of ASC danger particles proceed uncontrolled, due to reduced POP1 expression. The presence of POP1 might also increase the required threshold for inflammasome assembly. At low expression levels, as observed in resting macrophages, POP1 would probably not interfere with acute host defense and maintenance of metabolic health. However, upon inducible expression as a late response gene, POP1 might become involved in the resolution phase of inflammasome responses (Figure S7G), which is still poorly understood. In particular, POP1 expression before the onset of HMGBl expression might be important, because inflammasome-dependent release of HMGBl is directly linked to inflammatory disease (Yang et al., 2013). Accordingly, in mice that normally lack POP1 and other POP members (Stehlik and Dorfleutner, 2007; Khare et al., 2014), POP1 expression potently ameliorated systemic inflammasome-driven inflammation. Interestingly, CAPS is caused not only by excessive IL-1β secretion, but also by IL-18 secretion and pyroptosis (Brydges et al., 2009, 2013). Furthermore, ASC inflammasomes are also responsible for cytokine release by the non-canonical inflammasome (Kayagaki et al., 2011). By blocking all inflammasome-dependent mediators, POP1 exhibited a potent anti-inflammatory function in peritonitis, sepsis, and CAPS, and this function can probably be extended to other ASC-dependent inflammatory diseases. Employing a cell-permeable recombinant POP1 provided proof of concept that a POP1-based therapy could be effective in CAPS and other inflammasomopathy patients with impaired POP1 expression, where it might function as a novel broad-spectrum anti-inflammatory treatment strategy targeting all inflammasome effectors.

Assembly of inflammasomes is not limited to macrophages, but the cell types that are responsible for systemic inflammation have not been elucidated yet. We demonstrated that monocyte-macrophage-DC-lineage-specific expression of POP1 was sufficient to prevent systemic inflammation in three different inflammatory disease models, which strongly implicates that inflammasome activation and defects in inflammasome control in this lineage are crucial for promoting systemic inflammation. Many studies highlight the importance of inflammasomes for homeostasis and disease pathology and suggest that understanding the mechanism by which healthy tissues put the brakes on inflammasome-induced systemic inflammation will be the next important step for designing future therapies. With the results from our study, we started to provide some answers to this important question. POP1 provides a unique mechanism that evolved in humans to possibly allow tighter control of an essential host defense system by guarding against excessive and out-of-control responses that cause inflammatory disease.

**EXPERIMENTAL PROCEDURES**

**Animals**

B6.Tg(ND468-Pop1) TG mice were generated as described with GFP-Pop1 (Khare et al., 2014; Iqbal et al., 2014). C57BL/6 wild-type (WT) and Lysozyme M-Cre knock-in mice (CreL) were obtained from the Jackson Laboratories and Nlrp3−/−, Pycard−/−, and floxed Nlrp3Asp365G knock-in mice were described previously (MARIATHASAN et al., 2004, 2006; Brydges et al., 2009). Mice were housed in a specific-pathogen-free animal facility and all experiments were performed on age- and gender-matched, randomly assigned 8- to 14-week-old mice conducted according to procedures approved by the Northwestern
University Committee on Use and Care of Animals. Floxed Nlrp3<sup>flxed</sup> mice (Brydges et al., 2009, 2013) were crossed with CreL and CD68-POP1 TG mice and male and female offspring were analyzed for body weight and survival. Histological analysis was performed at day 8 after birth.

**Macrophage Isolation, Culture, and Transfection**
Peripheral blood-derived human macrophages, BMDMs, and peritoneal macrophages (PMs) were isolated as described (Khare et al., 2012, 2014).

**Quantitative Real-Time PCR**
Total RNA was isolated and analyzed as described (Khare et al., 2012, 2014).

**LPS-Induced Peritonitis**
8- to 12-week-old female WT and CD68-POP1 TG mice had their abdomen shaved under anesthesia and were randomly selected for i.p. injection with PBS or LPS (2.5 mg/kg, E. coli 0111:B4, Sigma). After 3 hr, mice were i.p. injected with XenoLight Rediject Inflammation probe (200 mg/kg, PerkinElmer) (Crosst et al., 2009) and in vivo bioluminescence was captured by imaging (NIS Spectrum, PerkinElmer) 10 min after injection with a 5 min exposure on anesthetized mice (Khare et al., 2014). Images were quantified with Living Image software (PerkinElmer). Endotoxic shock was induced by i.p. injection of a lethal dose of 20 mg/kg LPS (E. coli 0111:B4) and mice were monitored four times daily for survival. Body temperature was measured with an animal rectal probe. Blood was collected 3 hr after LPS injection by mandibular bleed, and serum cytokine levels were quantified by ELISA.

**ASC-Particle-Induced Peritonitis**
ASC-GFP-containing particles were FACS purified and verified by microscopy. 14-week-old male WT and CD68-POP1 TG mouse's abdomen shaved under anesthesia and were randomly selected for i.p. injection with PBS or FACS-purified ASC-GFP particles (1 x 10<sup>10</sup> particles/mouse). After 4 hr, MPO activity was determined as above. Peritoneal lavage fluids were collected and assayed for IL-1β by ELISA.

**Plasmids**
pcDNAs and pGEX-based expression constructs for ASC, POP1, NLRP3, ASC<sup>ΔN</sup>, and NLRP3<sup>ΔN</sup> were described earlier (Khare et al., 2014; Stehlik et al., 2003a, 2003b).

**Antibody-Based Detection**
Co-immunoprecipitations (IP), GST pull down, ASC cross-linking, immunohistochemistry, ELISA, flow cytometry, and caspase-1 activity were performed as previously described (Khare et al., 2014; Fernandes-Alnemri and Alnemri, 2008).

**Cell-Penetrating Recombinant Proteins**
6xHIS-POP1 and a 6xHIS-GFP cDNAs were fused with the HIV TAT sequence and purified from E. coli. 12-week-old male WT mice had their abdomen shaved under anesthesia and were randomly selected for i.p. injection with TAT-GFP or TAT-POP1 (40 μg/kg) for 30 min prior LPS i.p. injection (2.5 mg/kg, E. coli 0111:B4, Sigma) and were quantified for MPO activity in vivo 1 hr later, as described above.

**Statistics**
Graphs represent the mean ± SEM. A standard two-tailed unpaired t test was used for statistical analysis of two groups with all data points showing a normal distribution and Kaplan-Meier survival curves were used to investigate differences in survival. Values of p < 0.05 were considered significant and listed in the figure legends (Prism 5, GraphPad). The investigators were not blinded to the genotype of the mice/cells. Sample sizes were selected on the basis of preliminary results to ensure a power of 80% with 95% confidence between populations.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes seven figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.07.018.

**AUTHOR CONTRIBUTIONS**

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