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ASC is an activating adaptor for NF-κB and caspase-8-dependent apoptosis☆,☆☆

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Abstract

ASC is a pro-apoptotic protein containing a pyrin domain (PD) and a caspase-recruitment domain (CARD). A previous study suggests that ASC interacts with Ipaf, a member of the Apaf-1/Nod1 protein family. However, the functional relevance of the interaction has not been determined. Here, we report that co-expression of ASC with Ipaf or oligomerization of ASC induces both apoptosis and NF-κB activation. Apoptosis induced through ASC was inhibited by a mutant form of Caspase-8 but not by that of Caspase-1. The PD of ASC physically interacted with Caspase-8 as well as with pyrin, the familial Mediterranean fever gene product. Caspase-8 deficiency rescued mouse fibroblasts from apoptosis induced by ASC oligomerization. Pyrin disrupted the interaction between ASC and Caspase-8, and inhibited both apoptosis and NF-κB activation induced by ASC. These findings suggest that ASC is a mediator of NF-κB activation and Caspase-8-dependent apoptosis in an Ipaf signaling pathway.

Keywords: ASC; Nod protein family; Caspase-recruitment domain; NF-κB; Ipaf; Caspase-8; Apoptosis

ASC is a pro-apoptotic protein originally found as a component of a "speck" in apoptotic cells and contains an N-terminal pyrin domain (PD) and a C-terminal caspase-recruitment domain (CARD) (Fig. 1A) [1]. Both CARD and PD are known as protein–protein interaction domains and are structurally related to the death domain (DD) and death effector domain (DED) [2,3]. Signaling pathways induced through ASC are poorly characterized, although ASC has been described to interact with several proteins including the Apaf-1/Nod1-like proteins called Nods [4].

ASC physically interacts with Ipaf (CARD12/CLAN), a CARD-containing Nod protein (Fig. 1A). However, the functional relevance of the interactions between ASC and Ipaf has not been characterized yet, although Ipaf is suggested to be involved in Caspase-1 activation [5,6]. ASC also interacts with other members of the Nod protein family which contain CARD and/or PD, including cryopyrin (PYPAF1), NALP1 (NAC/CARD7/DEFCAP), PYPAF5, and PYPAF7 [7–10]. Previous studies suggested that ASC might be an

☆ *Abbreviations:* Ab, antibody; CARD, caspase-recruitment domain; IKK, IκB-kinase; MEF, mouse embryonic fibroblast; NF-κB, nuclear factor-κB; PD, pyrin domain; WT, wild-type.

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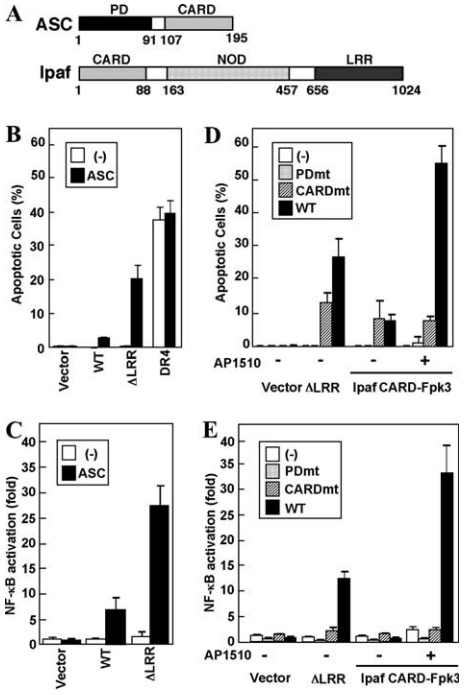

Fig. 1. Apoptosis and NF-κB activation induced by co-expression of Ipaf and ASC. (A) Schematic representation of ASC and Ipaf. (B) Ectopic expression of ASC and Ipaf synergistically induces apoptosis. HEK293T cells (5×10^4) were co-transfected with pcDNA3 (Vector), pcDNA3-Ipaf-Myc (WT), pcDNA3-Ipaf(1-647)-Myc (ΔLRR) or pcDNA3-DR4-FLAG (DR4, positive control) together with pcDNA3 (−) or pcDNA3-ASC-Myc (ASC) in the presence of pcDNA3-β-gal. Twenty-four hours post-transfection, the percentage of apoptotic cells was determined as a function of total β-galactosidase-positive cells from triplicate cultures. The results are given as means ± standard deviation. (C) Ectopic expression of ASC and Ipaf synergistically induces NF-κB activation. HEK293T cells were co-transfected with pcDNA3 (Vector), pcDNA3-Ipaf-Myc (WT), pcDNA3-Ipaf(1-647)-Myc (ΔLRR) together with pcDNA3 (−) or pcDNA3-ASC-Myc (ASC) in the presence of pBVIx-Luc and pEF1-BOS-β-gal. Twenty-four hours post-transfection, NF-κB-dependent transcription was determined. Values are normalized to β-galactosidase from triplicate cultures. (D) Both PD and CARD mutants of ASC failed to induce apoptosis with activated Ipaf. HEK293T cells were co-transfected with vector control (Vector), pcDNA3-Ipaf(1-647)-Myc (IpafΔLRR), or pcDNA3-Ipaf-(1-97)-Fpk3-Myc (IpafCARD-Fpk3) together with vector control (−), pcDNA3-ASC-Myc (WT), pcDNA3-ASC-L12Q-Myc (PDmt), or pcDNA3-ASC(1-166)-Myc (CARDmt) in the presence of pcDNA3-β-gal. Eight hours post-transfection, cells were treated with 200 nM AP1510 or left untreated. Twenty-four hours post-transfection, the percentage of apoptotic cells was determined. (E) Both PD and CARD mutants of ASC failed to induce NF-κB activation with activated Ipaf. HEK293T cells were co-transfected with vector control (Vector), pcDNA3-Ipaf(1-647)-Myc (ΔLRR), or pcDNA3-Ipaf(1-97)-Fpk3-Myc (IpafCARD-Fpk3) together with vector control (−), pcDNA3-ASC-Myc (WT), pcDNA3-ASC-L12Q-Myc (PDmt), or pcDNA3-ASC(1-166)-Myc (CARDmt) in the presence of the reporter plasmids. Eight hours post-transfection, cells were treated with 200 nM AP1510 or left untreated. Twenty-four hours post-transfection, NF-κB-dependent transcription was determined.

adaptor molecule which mediates the activation of NF-κB and caspases in Nod signaling pathways [6–11]. Pyrin, a putative immunosuppressor encoded by the gene mutated in familial Mediterranean fever, interacts and co-localizes with ASC, suggesting that pyrin might be involved in the same signaling pathway [3,12].

Here we show that co-expression of Ipaf with ASC induces NF-κB activation and apoptosis mediated by IκB kinase (IKK) and Caspase-8, respectively. The PD of ASC physically interacted with Caspase-8 and pyrin in a competitive manner. Our findings provide novel insights into the mechanism by which NF-κB activation and apoptosis are induced through the ASC-mediated signaling pathway and inflammatory diseases.

Materials and methods

Plasmid constructions. pcDNA3-Myc, pcDNA3-Fpk3-Myc, pcDNA3-ASC, pcDNA3-Nod1(1-648)-FLAG, pcDNA3-p35, pcDNA3-CrmA, pcDNA3-Caspase-8-C377S-HA, pcDNA3-Caspase-9-C287S-HA, pcDNA3-pro-Caspase-1-C285S-FLAG, pcDNA3-FADD-DN-AU1, pcDNA3-DR4-FLAG, pcDNA3-HA-pyrin, pCMV-Myc-pyrin, pCMV-Myc-pyrin-E1 (for PD), pCMV-Myc-pyrin-E2-10 (for ΔPD), pcDNA3-Nod2-Myc, pcDNA3-Nod2(1-301)-Fpk3-Myc, pRK7-FLAG-IKKβ-K44A, pcDNA3-IKKγ(134-419)-HA, pcDNA3-MyD88-DN, pcDNA3-β-gal, pBVIx-Luc, and pEF1-BOS-β-gal have been described previously [1–3,13–15]. The DNA fragments of ASC were constructed from pcDNA3-ASC using polymerase chain reaction. NALP2 and Ipaf were amplified from cDNA derived from a mixture of multiple human tissues. Fragments were cloned into the vectors pcDNA3-Myc and pcDNA3-Fpk3 to generate pcDNA3-Ipaf-Myc, pcDNA3-Ipaf(1-647)-Myc, pcDNA3-Ipaf(1-97)-Fpk3-Myc, pcDNA3-ASC-Myc, pcDNA3-ASC-Fpk3-Myc, pcDNA3-ASC-L12Q-Myc, pcDNA3-ASC-L12Q-Fpk3-Myc, pcDNA3-Fpk3-ASC(95-195)-Myc, pcDNA3-ASC(1-94)-Fpk3-Myc, pcDNA3-ASC(1-166)-Myc, pcDNA3-ASC(1-166)-Fpk3-Myc, and pcDNA3-NALP2(1-104)-Myc. The authenticity of all constructs was confirmed by sequencing.

Transfection, expression, immunoprecipitation, immunodetection of tagged proteins, cell death assay, and NF-κB activation reporter assay. HEK293T cells were transfected with expression plasmids using the calcium phosphate method as described previously [13]. Mouse embryonic fibroblasts (MEFs) derived from Caspase-8 deficient and control mice were generously provided by Dr. David Wallach (the Weizmann Institute of Science). MEFs were transfected using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. Immunoprecipitation and immunodetection of tagged proteins were performed by using anti-Myc (Santa Cruz), anti-HA (Roche), anti-FLAG-M2 (Sigma), and anti-ASC Abs as described [1,13,16]. Cell death assays with HEK293T cells and MEFs, and NF-κB reporter assay were performed as described [13].

Results and discussion

Cooperation between Ipaf and ASC induces apoptosis and NF-κB activation

To assess the functional relevance of the interaction between ASC and Ipaf, we first tested the ability of Ipaf to cooperate with ASC to induce apoptosis and NF-κB activation in HEK293T cells. In the absence of ASC, expression of wild-type (WT) Ipaf or a constitutively active form of Ipaf (IpafΔLRR) [6] induced neither apoptosis nor NF-κB activation (Figs. 1B and C). In contrast,

co-expression of ASC with WT Ipaf induced significant but low level apoptosis, that was enhanced when IpafΔLRR was co-expressed with ASC (Fig. 1B). Unexpectedly, co-expression of WT Ipaf with ASC also induced NF-κB activation that was enhanced by IpafΔLRR with ASC (Fig. 1C). This suggests that Ipaf is involved in signaling pathways which induce or enhance both apoptosis and NF-κB activation like Nod1 or Nod2 (13, 15).

The enforced oligomerization of Ipaf and ASC induces apoptosis and NF-κB activation

The Nod family members Apaf-1 and Nod1 require self-association of nucleotide-binding oligomerization domain (NOD) to promote the proximity and activity of downstream effectors that bind through CARD-CARD interactions [4,14]. Moreover, recent studies have shown that zebrafish ASC activates Caspy, a PD-containing caspase, by an induced proximity mechanism through a PD homophilic interaction [17]. Therefore, we tested whether the enforced oligomerization of the CARD of Ipaf could enhance ASC-dependent apoptosis. To perform these studies, we constructed plasmids to express chimeric proteins in which the CARD of Ipaf was fused to the Fpk3 domain (Fig. 2A), which can be oligomerized by the ligand AP1510 [14]. Expression of Ipaf-CARD-Fpk3-Myc with ASC in HEK293T cells induced both apoptosis and NF-κB activation in an AP1510-dependent manner as did the expression of IpafΔLRR with ASC (Figs. 1D and E). Thus, the oligomerization of the CARD of Ipaf alone can mimic signaling induced through Ipaf. The mutant forms in which a highly conserved leucine at position 12 in the PD was mutated to glutamine (PDmt) or the CARD of ASC partially deleted (CARDmt) did

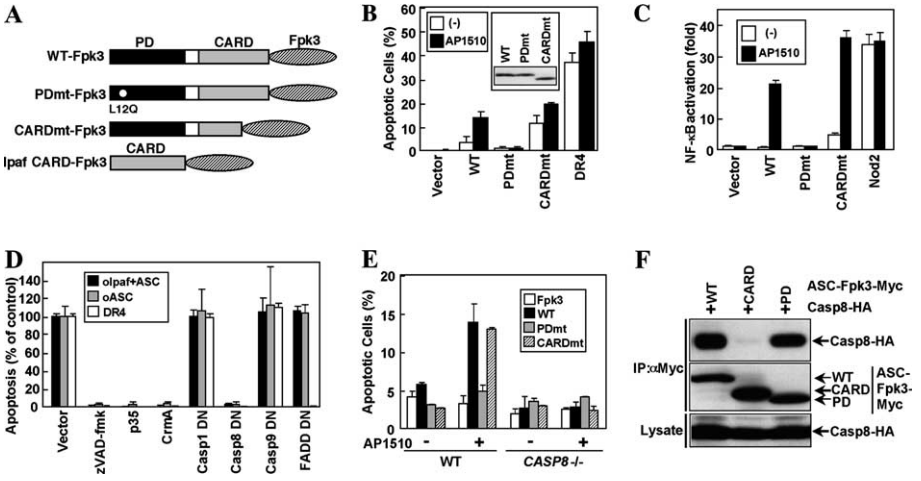

Fig. 2. Caspase-8-dependent apoptosis mediated by oligomerization of ASC and Ipaf. (A) Schematic representation of Fpk3-fusion proteins. (B) The enforced oligomerization of WT and CARDmt but not that of PDmt of ASC induces apoptosis. HEK293T cells were transfected with pcDNA3-Fpk3-Myc (Vector), pcDNA3-ASC-Fpk3-Myc (WT), pcDNA3-ASC-L12Q-Fpk3-Myc (PDmt), pcDNA3-ASC-(1-166)-Fpk3-Myc (CARDmt), or pcDNA3-DR4-FLAG (DR4) in the presence of pcDNA3-β-gal. Eight hours post-transfection, cells were treated with 200 nM AP1510 (black bars) or left untreated (white bars). Twenty-four hours post-transfection, the percentage of apoptotic cells was determined. As a control, Myc-tagged proteins in lysates from transfected cells were detected by anti-Myc Ab (inset). Statistical analysis showed that the enhancement of apoptosis by AP1510 is significant (the *p*-values are <0.0001 and 0.0310 for WT and CARDmt, respectively). (C) The enforced oligomerization of WT and CARDmt but not that of PDmt of ASC induced NF-κB activation. HEK293T cells were co-transfected with vector control (Vector), pcDNA3-ASC-Fpk3-Myc (WT), pcDNA3-ASC-L12Q-Fpk3-Myc (PDmt), pcDNA3-ASC-(1-166)-Fpk3-Myc (CARDmt), or pcDNA3-Nod2-Myc (Nod2, positive control) in the presence of the reporter plasmids. Eight hours post-transfection, cells were treated with 200 nM AP1510 (black bars) or left untreated (white bars). Twenty-four hours post-transfection, NF-κB-dependent transcription was determined as described above. (D) Ipaf and ASC oligomerization-induced apoptosis was specifically inhibited by the Caspase-8 mutant. HEK293T cells was co-transfected with vector control (Vector and zVAD-fmk), pcDNA3-p35 (p35), pcDNA3-crmA (CrmA), pcDNA3-pro-Caspase-1-C285S-FLAG (Casp1 DN), pcDNA3-Caspase-8-C377S-HA (Casp8 DN), pcDNA3-Caspase-9-C287S-HA (Casp9 DN), or pcDNA3-FADD-DN-AU1 (FADD DN) together with pcDNA3-Ipaf-CARD-Fpk3-Myc plus pcDNA3-ASC-Myc (oIpaf + ASC) or pcDNA3-ASC-Fpk3-Myc (oASC) or pcDNA3-DR4-FLAG (DR4) in the presence of pcDNA3-β-gal. Eight hours post-transfection, cells transfected by the Fpk3 constructs were treated with 200 nM AP1510 to oligomerize and indicated cells (zVAD-fmk) were treated with 20 μM zVAD-fmk. Twenty-four hours post-transfection, the percentage of apoptotic cells after stimulation with oligomerized Ipaf-CARD plus ASC (oIpaf + ASC), oligomerized ASC (oASC), and DR4 was 40%, 16%, and 70%, respectively, in the absence of apoptosis inhibitors and these values were considered as 100%. (E) ASC-mediated apoptosis was defective in Caspase-8 deficient MEFs. *CASP8*+/+ (WT) or *CASP8*−/− MEFs were transfected with Fpk3 vector control (Fpk3), pcDNA3-ASC-Fpk3-Myc (WT), pcDNA3-ASC-L12Q-Fpk3-Myc (PDmt), or pcDNA3-ASC-(1-166)-Fpk3-Myc (CARDmt) in the presence of pcDNA3-β-gal. Eight hours post-transfection, cells were treated with 200 nM AP1510 (+) or left untreated (−). Twenty-four hours post-transfection, the percentage of apoptotic cells was determined. (F) Pyrin domain of ASC but not CARD of ASC is required for the interaction between ASC and Caspase-8. HEK293T cells (5×10^5) were co-transfected with 3 μg pcDNA3-ASC-Fpk3-Myc (WT), pcDNA3-Fpk3-ASC-CARD-Myc (CARD), or pcDNA3-ASC-PD-Fpk3-Myc (PD) with 1.0 μg pcDNA3-Caspase-8-DN-HA (Casp8mt-HA). Twenty-four hours post-transfection, anti-Myc immunoprecipitates (upper and middle panels) and total lysates (lower panel) from the cells were immunoblotted with anti-HA (upper and lower panels) or anti-Myc Abs (middle panel).

not enhance the apoptosis or NF-κB activation by activated Ipaf, indicating that both PD and CARD of ASC are necessary for Ipaf signaling (Figs. 1D and E).

The CARD of ASC binds to Ipaf [5], suggesting that the PD might be the effector domain of ASC. To test this hypothesis, we constructed plasmids to express WT and mutant ASC proteins fused with Fpk3 (Fig. 2A). Oligomerization of WT and the CARD mutant of ASC (CARDmt) induced both apoptosis and NF-κB activation (Figs. 2B and C). In contrast, oligomerization of the L12Q mutant of ASC (PDmt) did not (Figs. 2B and C), indicating that the PD of ASC is required for ASC-mediated apoptosis and NF-κB activation. Immunoblotting analysis revealed that WT and ASC mutants were expressed at the same levels (Fig. 2B inset), indicating that the lack of activity was not due to reduced protein levels of the mutant. These results indicate that the PD of ASC functions as an effector domain to mediate apoptosis and NF-κB activation and its oligomerization can short-circuit signaling induced by Ipaf.

Asc mediates Caspase-8-dependent apoptosis

We determined next as to which caspases are required for Ipaf and ASC-mediated apoptosis. The pro-apoptotic activity induced by oligomerized Ipaf plus ASC or oligomerized ASC was completely inhibited by a catalytically inactive form of Caspase-8 as well as by the caspase inhibitors zVAD-fmk, viral p35, and CrmA (Fig. 2D). In contrast, catalytically inactive mutants of Caspase-1, Caspase-9 or a dominant negative mutant of FADD did not inhibit ASC-mediated apoptosis (Fig. 2D). These results suggest that the pro-apoptotic activity of Ipaf and ASC interaction is mediated through Caspase-8. To verify these results, we tested the ability of oligomerized ASC to induce apoptosis in MEFs deficient in Caspase-8 and control. Oligomerization of WT and a CARD mutant of ASC (CARDmt) induced significant apoptosis in control MEFs but not in those deficient in Caspase-8 (Fig. 2E). Using an immunoprecipitation assay, we found that catalytically inactive Caspase-8 was co-immunoprecipitated with ASC (Fig. 2F). Moreover, the PD of ASC but not the CARD of ASC associated with Caspase-8 (Fig. 2F). This result is consistent with the observation that the PD of ASC acts as an effector domain to mediate apoptosis.

Ipaf, ASC, and Caspase-8 form a signaling protein complex that is disrupted by pyrin

A previous study demonstrated a physical interaction between pyrin, the causative gene product of familial Mediterranean fever, and ASC through their PDs [3]. This prompted us to test the ability of pyrin to regulate

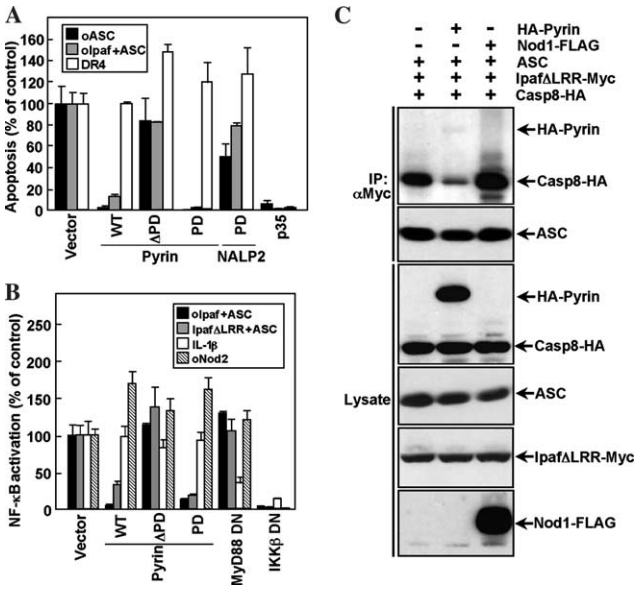

Fig. 3. Inhibition of Ipaf/ASC/Caspase-8 ternary complex by pyrin. (A) ASC-mediated apoptosis is inhibited by pyrin. HEK293T cells were co-transfected with pcDNA3-ASC-Fpk3-Myc (oASC), pcDNA3-Ipaf-(1-97)-Fpk3-Myc plus pcDNA3-ASC-Myc (oIpaf + ASC), or pcDNA3-DR4-FLAG (DR4) together with vector control (Vector), pCMV-Myc-pyrin (Pyrin-WT), pCMV-Myc-pyrin-E2-10 (Pyrin-ΔPD), pCMV-Myc-pyrin-E1 (Pyrin-PD), pcDNA3-NALP2-(1-104)-Myc (NALP2-PD), or pcDNA3-p35 (p35) in the presence of pcDNA3-β-gal. Eight hours post-transfection, cells transfected with the Fpk3 constructs were treated with 200 nM AP1510. Twenty-four hours post-transfection, the percentage of apoptotic cells after stimulation with oligomerized ASC, oligomerized Ipaf plus ASC (oIpaf + ASC) and DR4 (DR4) was 9.3%, 43%, and 25%, respectively, in the absence of apoptosis inhibitors and these values were considered as 100%. (B) ASC-mediated NF-κB activation is inhibited by pyrin. HEK293T cells were co-transfected with pcDNA3-Ipaf-(1-97)-Fpk3-Myc plus pcDNA3-ASC-Myc (oIpaf + ASC), pcDNA3-Ipaf(1-647)ΔLRR plus pcDNA3-ASC-Myc (IpafΔLRR + ASC) and pcDNA3-Nod2-Fpk3-Myc (oNod2) together with vector control (Vector), pCMV-Myc-pyrin (Pyrin-WT), pCMV-Myc-pyrin-E2-10 (Pyrin-ΔPD), pCMV-Myc-pyrin-E1 (Pyrin-PD) pcDNA3-MyD88DN (MyD88DN), or pRK7-FLAG-IKKβ-K44A (IKKβDN) in the presence of the reporter plasmids. Eight hours post-transfection, cells transfected by the Fpk3 constructs were treated with 200 nM AP1510. Twenty-two hours post-transfection, indicated cells were treated with 10 ng/ml interleukin-1β. Twenty-four hours post-transfection, NF-κB-dependent transcription was determined. 80-, 13-, 70-, and 41-fold induction of NF-κB activation was observed in cells with oligomerized Ipaf plus ASC, IpafΔLRR plus ASC, interleukin-1β treatment, and oligomerized Nod2, respectively, without the NF-κB inhibitors and these values were considered as 100%. (C) The complex comprising of Ipaf, ASC, and Caspase-8 was disrupted by pyrin. HEK293T cells were co-transfected with vector control (Vector), pcDNA3-HA-pyrin (HA-Pyrin), or pcDNA3-Nod1(1-648)-FLAG (Nod1-FLAG) in the presence of pcDNA3-ASC (ASC), pcDNA3-Caspase-8-DN-HA (Casp8mt-HA), and pcDNA3-Ipaf(1-647)-Myc (IpafΔLRR). Twenty-four hours post-transfection, proteins in anti-Myc immunoprecipitates (top and second panels) and total lysates (third to bottom panels) from the cells were detected with anti-HA (top and third panels), anti-ASC (second and fourth panels), anti-Myc (fifth panel), and anti-FLAG Abs (bottom panel).

apoptosis and NF-κB activation mediated by ASC. As shown in Figs. 3A and B, pyrin inhibited Ipaf-induced ASC-mediated apoptosis and NF-κB activation. These results indicate that Ipaf/ASC-mediated apoptosis and NF-κB activation are negatively regulated by pyrin. We also found that the NF-κB activation was dependent on IKKβ (Fig. 3B) and IKKγ (data not shown) but independent of MyD88 (Fig. 3B). To find out the mechanism, by which pyrin inhibits the ASC-signaling pathway, we tested the effect of pyrin on the ternary complex of Ipaf, ASC, and Caspase-8. ASC and Caspase-8mt-HA were co-immunoprecipitated with IpafΔL RR-Myc, indicating that Ipaf, ASC, and Caspase-8 form a ternary signaling complex (Fig. 3C). Notably, pyrin, but not control Nod1, inhibited the interaction between the Ipaf/ASC complex and Caspase-8 (Fig. 3C), suggesting that pyrin and Caspase-8 interact with ASC in a competitive manner.

The findings in this study suggest that Ipaf and ASC mediate NF-κB activation and apoptosis in an IKK- and Caspase-8-dependent manner. This dual cellular function is similar to that observed for death receptors including DR4. However, the mechanism by which ASC/Ipaf and DR4 induce apoptosis appears to be different because a FADD dominant negative mutant blocked apoptosis induced by DR4 but not that induced by ASC/Ipaf. Both ASC and FADD physically interact with Caspase-8, suggesting that they may have function in a similar manner to promote apoptosis. Human ASC was also reported to interact via its CARD with pro-Caspase-1, and with cryopyrin and NALP1, two members of the Nod protein family, through the PD [7–9,11]. ASC, unlike FADD, might act as a bi-directional adaptor for different caspases. Notably, we also show that pyrin, the anti-inflammatory protein mutated in familial Mediterranean fever, inhibits Ipaf/ASC-mediated signaling. Therefore our findings might provide an insight into the mechanism whereby pyrin mutations may cause inflammatory disease.

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