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ASC Directs NF-κB Activation by Regulating Receptor Interacting Protein-2 (RIP2) Caspase-1 Interactions

Anasuya Sarkar,* Michelle Duncan,* Judy Hart,* Erin Hertlein,† Denis C. Guttridge,† and Mark D. Wewers2*

Receptor interacting protein-2 (RIP2) is a caspase recruitment domain (CARD)-containing kinase that interacts with caspase-1 and plays an important role in NF-κB activation. Apoptosis-associated speck-like protein containing a CARD (ASC) is a PYRIN and CARD-containing molecule, important in the induction of apoptosis and caspase-1 activation. Although RIP2 has also been linked to caspase-1 activation, RIP2 knockout animals fail to show a defect in caspase-1-mediated processing of proIL-1β to its active form. Therefore, RIP2 function in binding to caspase-1 remains poorly understood. We hypothesized that caspase-1 may serve as a scaffolding molecule that promotes RIP2 interaction with 1b kinase-γ thus inducing NF-κB activation. We further hypothesized that ASC, which also interacts with caspase-1 via its CARD, may interfere with the caspase-1 RIP2 interaction. In HEK293 cells, ASC induced prominent activation of caspase-1 and proIL-1β processing. RIP2 transient transfection induced transcription of an NF-κB reporter gene. This RIP2-induced NF-κB activity and caspase-1 binding was inhibited in a dose-dependent fashion by ASC. Consistent with a role for caspase-1 as a scaffold for RIP2, caspase-1 knockout macrophages were suppressed in their ability to activate NF-κB, and septic caspase-1 knockout animals produced less IL-6, a functional marker of NF-κB activity. Lastly, THP-1 cells treated with small interfering RNA for ASC decreased their caspase-1 activity while enhancing their NF-κB signal. These data suggest that ASC may direct caspase-1 away from RIP2-mediated NF-κB activation, toward caspase-1-mediated processing of proIL-1β by interfering with the RIP2 caspase-1 interaction. The Journal of Immunology, 2006, 176: 4979–4986.

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regulation of NF-κB and caspase activation, it seems logical that imbalances in these pathways result in disease conditions.

ASC (apoptosis-associated speck-like protein containing a CARD) is one of the few proteins in the human genome that contains both an N-terminal PYD and a C-terminal CARD. ASC derives its name from its reported ability to trigger apoptosis upon overexpression in tumor cell lines as well as from its unique property to condense to cytosolic speck structures (33). ASC, also known as target of methylation induced silencing (TMS1), is silenced by methylation in certain breast cancer cells and lung cancers (34). ASC is predominantly expressed in monocytes and mucosal epithelial cells (35). ASC interacts with the CARD of procaspase-1 and induces aggregation of a protein complex (recently termed the inflammasome), thereby regulating activation of caspase-1 and secretion of IL-1β (36, 37). In addition, it has also been shown that ASC can up-regulate NF-κB activation upon co-expression with other PYD family proteins like cryopyrin and PYPAF7 (38, 39). However, the mechanism of the ASC effect on the NF-κB pathway remains poorly understood. In the present report, ASC uses its CARD interaction not only to induce caspase-1 activation and IL-1β processing, but also to down-regulate NF-κB signaling by modifying RIP2 interaction with procaspase-1 upon LPS activation. Moreover, this inhibitory property of ASC depends on the stoichiometry of its expression compared with other adaptor proteins and also whether certain other PYD family proteins are expressed upon activation. Thus ASC may serve to regulate the RIP2 connection between the inflammasome and the signalosome.

Materials and Methods

Expression plasmids

Caspase-1 plasmid was a gift of Merck Research Laboratory (Rahway, NJ). RIP2 plasmid was also the gift of J. Tschopp (University of Lausanne, Lausanne, Switzerland). ASC and pro-IL-1β were cloned from human monocyte cDNA and confirmed by DNA sequencing. An ASC mutant (lacking the functional CARD) and catalytically active and catalytically inactive caspase-1 (mutated at cysteine 284) were created and confirmed by DNA sequencing. GFP fusion vectors were generated by subcloning into pEGFP expression vectors (BD Clontech). Caspase-1 inhibitor Ac-Tyr-Val-Ala-Asp-chloromethyl ketone (YVAD-cmk) was purchased from Calbiochem.

Cell culture, transfection, and luciferase reporter assay

HEK293 cells were cultured in DMEM, whereas THP-1 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS. HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen Life Technologies), whereas THP-1 cells were transfected using a Nucleofector kit (Amaxa) according to the manufacturer’s recommendation. When indicated, cells were stimulated with LPS (1 μg/ml) for various amounts of time. For small interfering RNA (siRNA) experiments, THP-1 cells were transfected with either siRNA ASC or scrambled control (both from Ambion).

For NF-κB reporter gene assays, HEK293 cells were transfected with NF-κB luciferase plasmid (Stratagene) along with other plasmids as per experimental design, whereas THP-1 cells were nucleofected with the NF-κB luciferase plasmid using the Nucleofector kit from Amaxa, according to the manufacturer’s protocol. After transfection, cells were lysed in 100 μl of cell lysis reagent (Promega). Luciferase activity was measured using Promega Luciferase assay reagent. Data are represented as fold induction of NF-κB activity in cells.

Cytokine measurements

Sandwich ELISA analyses were developed in our laboratory to detect human proIL-1β and mature IL-1β as previously described (40). Briefly, mouse monoclonal anti-human IL-1β Ab (clone 8516; R&D Systems) was used as the coating Ab, and rabbit polyclonal anti-human proIL-1β-specific peptide Ab generated against aa 3–21 was used to sandwich the Ag for the ELISA system using rat monoclonal anti-mouse IL-1β Ab (clone 30331; MAB401; R&D Systems) and goat biotinylated anti-mouse IL-1β Ab (BAF401; R&D Systems) as coating and sandwich Abs, respectively. Streptavidin-conjugated HRP (Amersham Biosciences) and TMB Microwell Peroxidase Substrate System (Kirkgegaard & Perry Laboratories) were used for detection. Western blotting of mouse peritoneal macrophage released IL-1β used a goat anti-mouse IL-1β Ab (AF-401-NA; R&D Systems) and rabbit anti-goat Ab (Bio-Rad) as primary and secondary Abs, respectively.

Caspase activity assay

Caspase-1 activity was measured using the WEHD-AMC assay (40). Briefly, 106 cells were lysed in 60 μl of a lysis buffer (50 mM HEPEs (pH 7.4), 100 mM NaCl, 0.1% CHAPS, 0.1 mM EDTA and protease inhibitors) for 20 min on ice, followed by centrifugation at 14,000 × g for 10 min at 4°C. Fifty microliters of the cell extract was mixed with 50 μl of assay buffer (50 mM HEPEs (pH 7.4), 100 mM NaCl, 0.1% CHAPS, 20% glycercol, 10 mM DTT and 0.1 mM EDTA) and 5 μl of 1 mM Ac-WEHD-AMC (41). The mixture was placed in Costar 96-well flat-bottom plate (Corning Glass) and immediately subjected to a kinetic fluorometric assay at room temperature using a Cytofluor 4000 fluorometer (PerSeptive Biosystems) with filters of 360 nm excitation and 460 nm emission. The linear change of fluorescence of hydrolyzed free AMC per time and protein concentrations of the assayed samples were used for calculating unit activity with a conversion factor obtained from the assay for constructing an AMC standard curve.

Common pathway

For immunoprecipitation, cells were lysed in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 0.2% Nonidet P-40, 1 mM PMSF and 1× protease inhibitor mix (Roche). Lysates were clarified and then subjected to immunoprecipitation using protein A-conjugated anti-caspase-1 Ab (goat anti-human caspase-1), a gift of D. Miller, Merck Research Laboratory (Rahway, NJ), anti-RIP2 Ab, and anti-ASC Ab (eBioscience). After incubation overnight at 4°C, immune complexes were washed three times in lysis buffer, separated by SDS-PAGE, and analyzed by immunoblotting using various Abs as specifically indicated, in conjunction with ECL detection system (Amersham Biosciences). Alternatively, cell lysates were directly analyzed by immunoblotting after normalization for total protein content.

Murine macrophage isolation

Caspase-1 knockout (caspase-1−/−) mice were a gift of J. Mudgett, Merck Research Laboratory (Rahway, NJ). Caspase-1−/− animals were originally derived from 129ES male chimeras bred to C57B6 females. F1/F2 offspring were backcrossed to B10.RIII mice (the Jackson Laboratory) to the N3 level. Heterozygotes from the provided strains were then backcrossed in our laboratory to B10.RIII mouse strain for an additional three generations to obtain a strain with a similar genetic background. Genotyping was performed using primers specific for wild-type caspase-1 and the neomycin cassette, respectively, as following: ICE (forward) CGCGGAAGCCTTATCTTTCGTTG, reverse) CAGGAAATCAACCCAAAAACAC and neomycin (forward) AGA CAATGCGTCTGCTTCTAT, reverse) CTGCTTCCGACTTCACTTCA. All animal experiments were performed according to animal protocols approved by the Animal Care Use Committee of the Ohio State University College of Medicine.

Peritoneal macrophages were isolated 7 days after sterile i.p. thioglycolate injection by peritoneal lavage using sterile saline and then resuspended with RPMI 1640 medium with 10% FBS and antibiotics (penicillin and streptomycin; Invitrogen Life Technologies). Cells were plated at 104/ml and stimulated with LPS (1 μg/ml) for the indicated period of time in 5% CO2 at 37°C.

NF-κB DNA-binding activity assay

EMSA was used to measure NF-κB DNA-binding activity as described (42). Briefly, nuclear extracts of LPS stimulated and unstimulated peritoneal macrophages from caspase-1 wild-type and knockout mice were pre-pared as described (43), except that 0.25% Nonidet P-40 was used to extract the nuclei. A 5-μg portion of extract was preincubated with 1 mM PMSF and 1 μg of poly(dI:poly(dC)-oligodeoxycytidylic acid in a volume of 12 μl for 10 min. This mixture was subsequently incubated in a total volume of 20 μl at room temperature for 20 min with 2 × 108 cpm of a 32P-labeled oligonucleotide probe containing a κB site (underlined) from the class I MHC promoter (5′-CAG GCC TGG GGA TTC CCC ATC TCC-3′) and stained with DNA binding reagents in 5% agarose gel and then visualized under UV light.
ASC induces IL-1β processing by caspase-1 activation

To determine whether ASC is capable of inducing IL-1β processing, HEK293 cells were cotransfected with plasmids encoding procaspase-1, proIL-1β, and RIP2. IL-1β processing was measured 10 h after the additional transfection of ASC or plasmid control. That overexpression of ASC did not affect the expression levels of caspase-1, RIP2, and proIL-1β was confirmed by immunoblot (Fig. 1A). The supernatants were measured for the mature form of IL-1β using ELISA and immunoblot. Enhanced expression of mature IL-1β in the supernatants suggested that ASC significantly increased IL-1β processing in these experiments (Fig. 1B). To determine whether this processing of IL-1β by ASC was accompanied by the activation of caspase-1, caspase-1 activity was measured using the standard WEHD-AMC assay system (40). ASC transfection resulted in increased caspase-1 activity in a dose-dependent manner that correlated with the IL-1β processing induced by ASC (Fig. 1C).

ASC down-regulates RIP2-mediated NF-κB activation in 293 cells

Although ASC has been reported to have an inhibitory effect on the NF-κB pathway, the mechanism of its inhibitory effect still remains unclear. To understand this unique role of ASC, we first measured the effect of ASC on RIP2-induced NF-κB activation in HEK293 cells. Briefly, the cells were first cotransfected with procaspase-1 and proIL-1β along with RIP2 or plasmid control. The samples were then analyzed for NF-κB activation using the luciferase reporter assay system. RIP2 induced NF-κB activation in a dose-dependent manner (Fig. 2A). To determine the effect of ASC on this RIP2-induced NF-κB activation, we then transfected the cells with RIP2, procaspase-1, and proIL-1β along with increasing doses of ASC. Total plasmid DNA was kept constant in all experiments. ASC down-regulated RIP2-mediated NF-κB by ~80% (Fig. 2B). Increasing RIP2 expression in the presence of ASC increased NF-κB, suggesting that increasing amounts of RIP2 could overcome the down-regulatory effect of ASC on NF-κB (Fig. 2C). To show that the ASC inhibitory effect on NF-κB activation was not an artifact of transfection, we determined the effect of ASC when coexpressed with pyrin. Pyrin, in a dose-response manner, resulted in a significant increase of NF-κB activation (Fig. 2D). IL-1β processing was also measured for each of these samples. As expected, no IL-1β was detected in the supernatants of the cells lacking ASC (Fig. 2A), whereas increasing amounts of IL-1β were detected in the supernatants of the cells transfected with increasing amounts of ASC (Fig. 2B). However, despite an augmentation in NF-κB when RIP2 expression was increased in the presence of ASC and caspase-1, RIP2 had minimal inhibitory effect on ASC-induced IL-1β processing (Fig. 2C), as was also the case with pyrin (Fig. 2D).

Because earlier reports showed that ASC can also induce NF-κB activation (38, 39, 44), we also determined the effect of lower doses of ASC on RIP2-induced NF-κB activation. HEK293 cells were transfected with very low to high concentrations of ASC along with procaspase-1, RIP2, and proIL-1β. Both IL-1β and NF-κB activation were measured. ASC led to increased processing of IL-1β in a dose-dependent manner (as observed in our earlier experiments), which reached saturation at the highest doses. This pattern of IL-1β processing also correlated with caspase-1 activation (data not shown). Although at very low doses ASC did induce NF-κB activation; at higher doses it led to the inhibition of RIP2-induced NF-κB activation (Fig. 3A).

To determine the relative roles of the PYD and CARD of ASC, we compared wild-type ASC to an ASC construct that lacked a functional CARD. Unlike the wild-type, CARD-deficient ASC was unable to form specks, as shown in Fig. 3B. Both wild-type and mutant ASC were analyzed for their effects on NF-κB and IL-1β processing in HEK293 cells. Upon overexpression, mutant ASC only minimally inhibited NF-κB compared with the wild-type ASC (Fig. 3C). IL-1β processing was also impaired in the mutant expressed cells compared with the wild-type ASC (Fig. 3D).

ASC modifies RIP2/procaspase-1 interaction

Previous work of others (36, 37), as well as our own experiments as described earlier, have shown that ASC-mediated caspase-1 activation leads to IL-1β processing. Also previous reports (27–29, 45) and unpublished work from our own laboratory also suggested the interaction of RIP2 with procaspase-1. These data led us to hypothesize that the ability of ASC to inhibit RIP2-induced NF-κB activation was due to the fact that ASC interferes with the interaction of RIP2 and procaspase-1 by competing with RIP2 binding to procaspase-1. To understand this idea, we again cotransfected HEK293 cells with RIP2, procaspase-1, and proIL-1β with or without ASC. The cell lysates of these samples were then subjected to immunoprecipitation using caspase-1 Ab. Immunoprecipitation experiments provided evidence that ASC competes with RIP2 to bind to procaspase-1 (Fig. 4A).
To extend these observations to a monocyte cell, we turned to an in vivo system, the THP-1 monocytic cell line. THP-1 cells were stimulated with LPS, and the interaction of ASC and RIP2 with procaspase-1 was determined by immunoprecipitation. As previously observed in the HEK293 cells, ASC binding to procaspase-1 increased with time, whereas the RIP2-procaspase-1 interaction correlated with the decrease in NF-κB activity seen in untreated THP-1 cells and not further induced by LPS stimulation (Fig. 4B). This increased binding of ASC to procaspase-1 after LPS induction suggested the association of ASC and caspase-1 into the inflammasome complex. The decreased interaction between RIP2 and caspase-1 was also associated with a decreased interaction between IκB kinase (IKK)γ and RIP2. This decreased interaction correlated with the decrease in NF-κB activity seen in Figs. 2 and 3.

Caspase-1 knockout macrophages have decreased NF-κB activity

RIP2 has been reported to be an upstream activator of NF-κB (13, 19–21, 45–47). Because we also observed that ASC not only down-regulates RIP2-induced NF-κB activation, but also competes with RIP2 for binding to caspase-1 (bringing caspase-1 to the inflammasome complex), we asked whether caspase-1 itself can affect the NF-κB pathway. For this purpose, we stimulated thioglycolate-induced peritoneal macrophages from caspase-1 wild-type and knockout mice with LPS and analyzed NF-κB DNA-binding activity (Fig. 5A). As a second measure of NF-κB activation in caspase-1 wild-type and knockout mice, plasma IL-6 cytokine levels were measured at different time points after i.p. challenge with live *Escherichia coli* in caspase-1 wild-type mice and caspase-1 knockout mice. Plasma IL-6 levels were significantly suppressed in the caspase-1 knockout mice (data not shown). To further demonstrate the direct involvement of caspase-1 in NF-κB pathway, peritoneal macrophages from the caspase-1 knockout and wild-type mice were nucleofected with a luciferase NF-κB reporter plasmid along with a cysteine mutant caspase-1 that is unable to process IL-1β, to determine whether the effect on NF-κB after LPS stimulation was independent of caspase-1 catalytic activity. The caspase-1 knockout exhibited lower luciferase activity than the wild type. However, addition of catalytically inactive caspase-1 corrected the depressed NF-κB activity of the caspase-1 knockout mice (Fig. 5B). To compare the effect of wild-type and catalytically inactive caspase-1 on the
NF-κB activation we also transfected THP-1 cells with the luciferase reporter plasmid and either wild-type or catalytically inactive human caspase-1 gene and analyzed the NF-κB response to LPS. As shown, catalytically inactive caspase-1 was at least as functional as wild-type caspase-1 at inducing NF-κB. Interestingly, the wild-type caspase-1 effect on NF-κB was further augmented by inhibition of caspase-1 activity with YVAD-cmk (suggesting the possibility that active caspase-1 may also induce apoptosis that is prevented by the YVAD-cmk).

**Suppression of endogenous ASC reduces IL-1β processing but enhances NF-κB activation**

To further explore the role of ASC as both an inducer of IL-1β processing and an inhibitor of NF-κB activation, we reduced the expression of ASC in THP-1 cells using siRNA to ASC. Briefly, THP-1 monocyte cells were nucleofected with either control oligonucleotides or small interfering ASC and luciferase reporter plasmids at equal concentrations and stimulated with LPS (1 µg/ml) for different time periods. IL-1β processing was measured by ELISA and NF-κB activity was analyzed both by EMSA and luciferase reporter assay. Densitometry analysis of immunoblots of whole cell lysates from these experiments revealed ~50% reduction of endogenous ASC protein levels (Fig. 6A). Equal expression of other proteins of interest like caspase-1 and RIP2 confirmed the specificity of small interfering ASC. NF-κB measurements between the two groups demonstrated that suppressing ASC expression increased NF-κB activity (Fig. 6, B and C), further supporting the hypothesis that ASC plays an inhibitory role in the NF-κB pathway. As expected, ASC suppression significantly reduced IL-1β processing in the THP-1 cells (Fig. 6D) as compared with control siRNA.

**Discussion**

ASC is one of only two genes in the human genome that contain both PYD and CARD. Such modular protein-protein interaction domains are known to play an important role in many intracellular signal transduction pathways. ASC has been recently reported to collaborate with certain PYD family members like PYPAF1 and PYPAF7 and thereby affect NF-κB activity or procaspase-1 activation (38, 39). In this report, we have extended existing data regarding the role of ASC in both caspase-1 activation and NF-κB pathway. Our data confirm that ASC induces processing of proIL-1β to its mature form and documents that such processing of IL-1β is associated with caspase-1 activation. We extend this observation to the in vivo situation, in which suppression of endogenous THP-1 cell ASC, using siRNA, significantly reduced IL-1β processing upon LPS stimulation.

Recently ASC has also been reported to bind to pyrin and cryopyrin via its PYD. These CATERPILLER (CARD, transcription enhancer, R(purine)-binding, pyrin, lots of leucine repeats) proteins are thought to be causative genes involved in familial Mediterranean fever, familial cold autoinflammatory syndrome, Muckle-Wells syndrome, and chronic infantile neurological cutaneous and articular syndrome (38, 48). ASC has also been reported to collaborate with cryopyrin and PYPAF7 in inducing NF-κB activity in an overexpression system (38, 39). However, reports also exist showing ASC as a suppressor of NF-κB activity (44), suggesting that the ASC role may be more complex. RIP2, another CARD-containing serine/threonine kinase molecule, is also known to be an inducer of NF-κB signaling. RIP2-deficient mice showed severely reduced NF-κB activation (45, 47). Moreover, cytokine production in RIP2-deficient cells is also reduced on stimulation of

![Figure 3](http://www.jimmunol.org/)
TLRs with LPS, peptidoglycans, and dsRNA, but not by bacterial DNA, indicating RIP2 to be downstream of TLR2/3/4 but not TLR9 (47). In this context, we sought to analyze the role of ASC in NF-κB signaling. We first demonstrate that overexpression of RIP2 and caspase-1 in HEK293 cells leads to the induction of NF-κB activity; thereby confirming that RIP2 is an activator of NF-κB. For reasons that are not entirely clear, low levels of ASC actually promote NF-κB activity. However, overexpression of ASC in the presence of RIP2 and caspase-1 results in a decrease in NF-κB activity, demonstrating an inhibitory role of ASC in NF-κB signaling. This observation was confirmed in an in vivo THP-1 system in which NF-κB activity was up-regulated upon ASC suppression using siRNA.

Thus, the ASC effect on NF-κB appears to have a biphasic dose response. Such dual function for a single molecule is not uncommon. Proteins such as c-FLIPL have been reported to function either as a procaspase-8 activator or inhibitor, depending on cell context (49). As mentioned, transient transfection with very low doses of ASC enhanced NF-κB activity. Although we do not have direct evidence to explain this effect, we speculate that the dual role of ASC in regulating NF-κB may depend upon the stoichiometry of the CARDs among the binding proteins (Fig. 7). Low ratios of ASC to RIP2 and caspase-1 may enhance RIP2 association with caspase-1, thus promoting RIP2 association with IKKγ and hence subsequent NF-κB activation. In contrast, high ratios of ASC to RIP2 and caspase-1 may serve to disrupt the RIP2-caspase-1 interaction. Our overexpression studies aimed to address the stoichiometry issue to support this hypothesis. We hypothesize that it is RIP2 association with caspase-1 that is critical to RIP2-IKKγ interaction. This concept is supported by the caspase-1 knockout experiments. That is, in the absence of caspase-1, RIP2 may be unable to activate the IKK complex.

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FIGURE 4. ASC interferes with RIP2-procaspase-1 interaction. A, HEK293 cells were transiently transfected with increasing doses of ASC along with RIP2 (0.1 μg), caspase-1 (0.1 μg), and proIL-1β (0.1 μg). Total DNA amount was kept constant during transfection using empty vector. Interaction of ASC and RIP2 with caspase-1 was analyzed by immunoprecipitation of caspase-1 with a capturing Ab as compared with control Ab. B, THP-1 monocyctic cells were stimulated with LPS (1 μg/ml) for indicated period of time. Expression pattern of ASC, RIP2, caspase-1, and IKKγ was analyzed by immunoblotting. C, Immunoprecipitation using caspase-1, RIP2, and ASC Abs were performed on the cell extracts to analyze the interaction pattern of ASC, RIP2, and caspase-1.

![Image](http://www.jimmunol.org/)

FIGURE 5. Caspase-1 promotes NF-κB activation. A, Time course of LPS (1 μg/ml) induced NF-κB activation was compared between wild-type (+/+), and caspase-1 knockout (−/−) peritoneal macrophages using gel EMSA of radiolabeled NF-κB promoter. B, Peritoneal macrophages were isolated from wild-type (C57BL/6) and caspase-1 knockout (C3H/HeN) mice and nucleofected with functionally inactive cysteine mutant caspase-1 or empty vector and a luciferase reporter plasmid, following manufacturer’s protocol. Cells were stimulated with or without LPS (1 μg/ml) and analyzed for NF-κB activity as determined by relative light units and expressed as fold induction over the wild-type with vector controls in the absence of LPS. Effect of wild-type and mutant caspase-1 on NF-κB activity was measured using luciferase reporter assay. Caspase-1 knockout mice showed decreased NF-κB activity compared with the wild-type mice (*, p < 0.023 caspase-1 wild-type vs knockout). C, THP-1 cells were nucleofected with luciferase plasmid along with functionally active and inactive caspase-1 or empty vector. Cells were then stimulated with LPS (1 μg/ml) in the presence or absence of caspase inhibitor YVAD-cmk (100 μM) and analyzed for NF-κB activity as determined by relative light units and expressed as fold induction over cells with vector control in the absence of LPS and caspase inhibitor.
mimic the effect of the caspase-1 knockout by preventing RIP2 interaction with caspase-1 and hence with IKKγ. Furthermore our report and other reports have already demonstrated that ASC leads to the activation of caspase-1 by its CARD interactions (36, 37, 44). Supporting this concept is our over expression studies using wild type and ASC mutated in the CARD (Fig. 3). Also it has been shown by others, and by our own yeast 2 hybrid experiments (data not shown), that caspase-1 binds to RIP2 via its CARD. We therefore propose that ASC competes with RIP2 binding to caspase-1. Because caspase-1 knockout macrophages have diminished NF-κB activity and because the catalytically inactive mutant of caspase-1 is able to correct this defect, we hypothesize that caspase-1 serves as a scaffold for RIP2 to interact with IKK and NF-κB activation. In this context, prior work has provided evidence that ASC down-regulates IKK complex phosphorylation (44). The present findings provide evidence that the inhibitory role of ASC in NF-κB pathway involves RIP2 and caspase-1. They further suggest that the ratio of ASC molecules to caspase-1 molecules may be critical to determining the final effect on NF-κB.

It should be mentioned that our hypothesis also implies that increasing RIP2 might compete with ASC binding to and activation of caspase-1. However, increasing RIP2 had only a modest effect on ASC-dependent proIL-1β processing and release (Fig. 2C). Thus, the RIP2 effect is not completely reciprocal in its ability to interfere with the ASC activation of caspase-1. We do not have a complete explanation for this phenomenon but attribute this to the transient nature of the RIP2-caspase-1 interaction. As we have shown in Fig. 4C, RIP2 interacts only briefly with caspase-1 after LPS challenge. Thus, it may be that the RIP2 ability to inhibit the ASC caspase-1 interaction is dependent upon a transient postranslational event that we have not controlled for in our model systems.

Although ASC was originally described by its ability to induce apoptosis upon overexpression in certain tumors (33, 50), we did not observe apoptosis at the doses of ASC-encoding plasmid used in our transfection system. ASC is silenced by methylation of the ASC CpG rich promoter in breast cancer cells and lung cancer cells (34). We propose that in situations in which ASC overexpression causes apoptosis, NF-κB is highly down-regulated. In this context, coexpression of pyrin with ASC reduces the number of apoptotic cells (38). We show in this report that pyrin can block the inhibitory effect of ASC on NF-κB activation. Recent work of Dixit and colleagues (51) demonstrates that macrophages from Lipa and ASC knockout mice, respectively, are markedly resistant to Salmonella typhimurium induced cell death. It is highly possible that cross-talk between this novel caspase-1-mediated NF-κB pathway and other NF-κB activation pathways does exist. It is evident from our study that ASC plays a critical role in communications between the inflammasome and the signalosome. We show that RIP2 is one component of the molecular bridge that is regulated by ASC. However, much still remains to be learned regarding the complex role of ASC in regulating apoptosis and inflammation. Future studies will require gene depletion of ASC, RIP2, and caspase-1 to understand the upstream signaling pathways and the cellular and physiological context in which the dual function of ASC is operating.

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**Disclosures**

The authors have no financial conflict of interest.

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**FIGURE 6.** Effect of suppression of ASC on IL-1β processing and NF-κB activation. LPS (1 μg/ml) stimulated THP-1 cells were nucleofected with ASC siRNA (●) or control siRNA ( ○) and luciferase reporter vector (keeping total concentrations constant). A. Efficiency of ASC suppression was confirmed by immunoblot analysis. B and C. Effect of ASC suppression on NF-κB activation was measured by EMSA (B) and luciferase reporter assay system (C). D. IL-1β processing was measured by ELISA in the supernatants.

**FIGURE 7.** Proposed model of NF-κB regulation by ASC, RIP2, and caspase-1. A. Early after stimulation, RIP2 and caspase-1 interact leading to RIP2 binding to IKKγ, which results in NF-κB activation. B. At later time points, ASC prevents caspase-1-RIP2 interactions, thereby down-regulating NF-κB and activating IL-1β processing.