Dual Role of Apoptosis-Associated Speck-Like Protein Containing a CARD (ASC) in Tumorigenesis of Human Melanoma

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Apoptosis-associated Speck-like protein containing a CARD (caspase recruitment domain) (ASC) was originally named because it triggered apoptosis in certain tumors. More recently, however, ASC was found to be a central adaptor protein of inflammasome, which mediates the secretion of protumorigenic inflammation. Here we examined the role of ASC in tumorigenesis of human melanoma. Compared with primary melanoma, ASC protein expression was generally downregulated in metastatic melanoma. Although overexpressing ASC in metastatic melanoma showed no effects on cell viability, silencing ASC with short hairpin RNA induced G1 cell cycle arrest, reduced cell viability, and suppressed tumorigenesis in metastatic melanoma. On the other hand, silencing ASC in primary melanoma reduced cell death, increased cell viability, and enhanced tumorigenesis. In primary and metastatic melanoma cells, ASC knockdown inhibited inflammasome-mediated caspase-1 activity and IL-1β secretion. However, phosphorylated IkB kinase (IKK)α/β expression and NF-κB activity were suppressed in metastatic melanoma and enhanced in primary melanoma after ASC knockdown. These findings suggest stage-dependent dual roles of ASC in tumorigenesis. ASC expression in primary melanoma inhibits tumorigenesis by reducing IKKβ phosphorylation and inhibiting NF-κB activity. In metastatic melanoma, on the other hand, this inhibitory effect is diminished, and ASC induces tumorigenic pathways through enhanced NF-κB activity and inflammasome-mediated IL-1β secretion.

INTRODUCTION

ASC (Apoptosis-associated Speck-like protein containing a CARD, caspase recruitment domain) was originally named because it induced apoptosis when overexpressed in HL-60 human leukemia cells (Masumoto et al., 1999). ASC facilitates translocation of Bax to mitochondria, and increases mitochondrial permeability, cytochrome c release, and activation of caspase-9 and caspase-3 (McConnell and Vertino, 2004; Ohtsuka et al., 2004). ASC is also called TMS1 (Target of Methylation-mediated Silencing) because ASC expression is suppressed in many human tumors by methylation of CpG islands in the ASC gene, which likely prevents cancer cells from undergoing apoptosis (Conway et al., 2000; Stone et al., 2004; Das et al., 2006). These data suggest a tumor suppressor role of ASC.

Structurally, ASC contains an N-terminal pyrin domain (PYD) and a C-terminal CARD (Martinon et al., 2002). ASC is highly expressed in immune cells, particularly in neutrophils and monocytes. As a central adaptor protein of inflammasome, ASC was recently found to mediate inflammatory signals by recruiting CARD-containing pro-caspase-1 to several PYD-containing NOD-like receptors (NLRs), including NLR family containing PYD (NLRP)3. ASC thus has a pivotal role in the caspase-1-dependent processing of proinflammatory cytokines such as IL-1β. Because IL-1β is a pleiotropic proinflammatory cytokine involved in cell growth, differentiation, tissue repair, and regulation of immune response (Dinarello, 2009), tumor cells secreting IL-1β have a greater propensity for invasion, angiogenesis, antitumor suppression, and metastasis (Song et al., 2003, 2005), and IL-1β is strongly implicated in tumor progression (Dinarello, 2010; Okamoto et al., 2010). We have previously shown that the NLRP3 inflammasome (composed of NLRP3, ASC, and pro-caspase-1) is constitutively assembled and activated in human melanoma cells, resulting in spontaneous IL-1β secretion in metastatic melanoma cells (Okamoto et al., 2010). These results suggest a tumor-promoting role of ASC in human melanoma.

In this study, we examined the role of ASC in melanoma-genesis using primary and metastatic human melanoma cell...
lines. We provide evidence that ASC has stage-dependent dual roles in tumorigenesis by differentially regulating NF-kB activity and IL-1β secretion in human melanoma cells.

RESULTS

ASC expression is downregulated in metastatic melanoma

We analyzed 12 human melanoma tumor specimens (six primary and six metastatic melanoma tumors) for ASC expression. Consistent with a published report (Guan et al., 2003), ASC was highly expressed in normal melanocytes (Supplementary Figure 1 online) and suppressed in human melanoma tissues. There was a trend of ASC to a further reduction in metastatic melanoma compared with primary melanoma (Figure 1a and Supplementary Table 1). We then analyzed ASC expression in 10 human melanoma cell lines (five primary and five metastatic lines). Western blot analysis showed reduced ASC expression in most metastatic melanoma cell lines (Figure 1b). To investigate expression-dependent and/or stage-dependent roles of ASC in human melanoma, we used two primary melanoma cells, WM35 and WM115, expressing relatively higher (WM35) and lower (WM115) ASC among primary cells, and two metastatic melanoma cells, HS294T and 1205Lu, expressing higher (HS294T) and lower (1205Lu) ASC among metastatic cells, in the subsequent experiments.

ASC overexpression in metastatic melanoma has little effect on cell viability despite its killing effects on primary melanoma

Because ASC expression was higher in primary melanoma than in metastatic melanoma, we hypothesized that ASC overexpression may induce growth suppression in human melanoma. 1205Lu and HS294T cells were transfected with an ASC expression plasmid. Successful transfection was confirmed by an increase in ASC mRNA (11.3-fold increase in 1205Lu cells and 3.7-fold increase in HS294T cells; Figure 1c) and protein (Figure 1d) expression. Overexpression of ASC induced no significant change in cell viability in 1205Lu and HS294T cells (Figure 1e); however, it reduced cell viability in primary melanoma cells (Figure 1f).

ASC knockdown reduces cell viability and inhibits tumor growth in metastatic melanoma

To investigate the role of ASC on tumor growth, we knocked down ASC expression by transducing ASC short hairpin RNA (shRNA) into HS294T and 1205Lu cells. Successful transduction was confirmed by a decrease in ASC mRNA (84% reduction in 1205Lu cells and 95% reduction in HS924T cells) and protein expression in 1205Lu and HS294T cells compared with their control cells (Figure 2a). Silencing ASC reduced cell viability in 1205Lu cells (32% reduction) and HS294T cells (38% reduction) (Figure 2b). G1 cell cycle arrest was induced in both cells (Figure 2c) and increased cell death was observed in 1205Lu cells after ASC knockdown (Figure 2d). We then determined whether the same effect can be observed in vivo. Nude mice were injected with 1205Lu-control-shRNA cells or 1205Lu-ASC-shRNA cells, and tumor growth was monitored. Mice injected with 1205Lu-ASC-shRNA cells showed slower tumor growth and decreased tumor volume (60% reduction on day 25) compared with the mice injected with 1205Lu-control-shRNA cells (Figure 2e). Tumor tissues from mice injected with 1205Lu-ASC-shRNA cells exhibited decreased mitosis (arrows), proliferation (Ki-67-positive cells), and angiogenesis (CD31-positive cells) compared with control tumors (Figure 2f). These results indicate that ASC promotes tumor growth in metastatic melanoma.

ASC knockdown increases cell viability and enhances tumor growth in primary melanoma

To investigate the role of ASC in primary melanoma, we knocked down ASC expression in WM35 and WM115 cells. Successful transduction was confirmed by a decrease in ASC mRNA (87% reduction in WM35 and 60% reduction in WM115) and protein expression (Figure 3a). In contrast with metastatic melanoma cells, however, silencing ASC in primary melanoma cells enhanced cell viability (1.7-fold in WM35 and 1.6-fold in WM115; Figure 3b). Cell proliferation was enhanced in WM35 cells (Figure 3c) and cell death was decreased in both WM35 and WM115 cells after ASC knockdown (Figure 3d). Tumors from WM35-ASC-shRNA cells grew in mice after day 30, whereas those from WM35-control-shRNA cells remained small in mice (Figure 3e). Histological analysis revealed enhanced proliferation (Ki-67-positive cells) and angiogenesis (CD31-positive cells) in tumor tissues from WM35-ASC-shRNA cells compared with those from WM35-control-shRNA cells (Figure 3f). Taken together, these results suggest that ASC has differential effects on primary and metastatic melanoma cells: ASC inhibits tumor growth in primary melanoma cells, whereas it promotes tumorigenesis in metastatic melanoma cells.

ASC knockdown impairs inflammasome-mediated caspase-1 activation in primary and metastatic melanoma

As an integral component of inflammasome, ASC has an important role in caspase-1 activity and IL-1β secretion. To examine inflammasome function, we measured both IL-1β synthesis and secretion in primary (WM35 and WM115) and metastatic (HS294T and 1205Lu) melanoma cells after ASC knockdown (Figure 4a). Consistent with a previous report (Okamoto et al., 2010), the amount of IL-1β synthesized in WM35 and WM115 was very low (just above the limit of sensitivity) and IL-1β secretion was not detected in these primary melanoma cells (<3 pg ml⁻¹). In all four cells transduced with ASC shRNA, IL-1β synthesis was not changed relative to control cells (Figure 4a). However, IL-1β secretion was significantly decreased in metastatic melanoma cells transduced with ASC shRNA (62% reduction in HS924T-ASC-shRNA cells and 46% reduction in 1205Lu-ASC-shRNA cells; Figure 4a). Consistent with these results, activated caspase-1 (20-kDa fragments) was decreased in all cells transduced with ASC shRNA (Figure 4b). Caspase-1 activity was also decreased in these cells (78, 73, 85, and 88% reduction in WM35-ASC-shRNA, WM115-ASC-shRNA, HS294T-ASC-shRNA, and 1205Lu-ASC-shRNA cells, respectively; Figure 4c), indicating that ASC knockdown impairs inflammasome-mediated caspase-1 activation in both primary and metastatic melanoma cells.
ASC knockdown decreases NF-κB activity in metastatic melanoma but increases the activity in primary melanoma

Through its PYD or CARD, ASC can associate with PYD- or CARD-containing proteins. In particular, ASC has been shown to mediate signals by upregulating or downregulating NF-κB (Stehlik et al., 2002; Hasegawa et al., 2005; Sarkar et al., 2006). To assess the association of ASC and NF-κB, we first measured NF-κB activity after ASC knockdown by luciferase assay. Although NF-κB activity was increased in primary melanoma cells (10.9-fold in WM35 and 7.3-fold in WM115), it was decreased in metastatic melanoma cells (72% reduction in HS294T and 64% reduction in 1205Lu) after ASC knockdown (Figure 5a). To further decipher the role of ASC on NF-κB activity in melanoma cells, we examined
proteins involved in the NF-κB activation pathway. Immunoprecipitation revealed the interaction of ASC with IκB kinase (IKK)α/β in both primary (WM35) and metastatic (1205Lu) cells (Figure 5b, 1st and 3rd panels immunoblotted with ASC). Whereas interaction of ASC and IKKα/β was reduced after ASC knockdown (Figure 5b, 1st and 3rd panels), the expression of IKKα/β was not changed in the melanoma cells transduced with ASC shRNA (Figure 5b, 2nd and 4th panels; Figure 5c, 2nd panels). Phosphorylated IKKα/β expression, on the other hand, was increased in WM35 cells and decreased in 1205Lu cells after ASC knockdown (Figure 5c, 3rd panels). Consistent with these results, IκBα expression was decreased in WM35 cells and increased in 1205Lu cells after ASC knockdown (Figure 5c, 4th panels). The findings in IκBα expression were further confirmed in xenografted tumor tissues derived from these cells (Figure 5d). The results indicate that ASC inhibits NF-κB activity in primary melanoma cells by suppressing IKKα/β phosphorylation and inhibiting IκB degradation. On the contrary, ASC increases IKKα/β phosphorylation and IκB degradation and enhances NF-κB activity in metastatic melanoma cells.

Figure 2. Effects of Apoptosis-associated Speck-like protein containing a CARD (caspase recruitment domain) (ASC) short hairpin RNA (shRNA) on metastatic melanoma. (a) Quantitative RT-PCR (qRT-PCR) (left panel) and western blot (right panel) of ASC in cells transduced with control or ASC shRNA. mRNA expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). β-Actin served as a loading control. (b) Cell viability in cells transduced with shRNA. (c) Cell cycle analysis in cells transduced with control and ASC shRNA. (d) Annexin-V/propidium iodide (PI) staining in cells transduced with control and ASC shRNA. (e) Effects of ASC knockdown in vivo. Nude mice injected with 1205Lu-control-shRNA or 1205Lu-ASC-shRNA cells (upper panel). Tumor growth curve (lower panel). (f) Representative tumor sections stained with hematoxylin and eosin (H&E) (upper panel), Ki-67 (middle panel), and CD31 (lower panel). Bar = 100μm. Data represent mean ± SE (n = 3 except in e, and n = 8 in e). **P<0.01; ***P<0.001. OD, optical density.
ASC knockdown decreases IL-1 receptor signaling–dependent activation of NF-κB in metastatic melanoma

Previously, we have shown a positive feedback loop of IL-1 receptor (IL-1R) signaling through secreted IL-1β in metastatic melanoma cells (Okamoto et al., 2010). IL-1R signaling activates the NF-κB pathway. Therefore, to examine the role of ASC on NF-κB in human melanoma cells, we investigated IL-1R signaling. IL-1α was used to stimulate IL-1R signaling, whereas an IL-1 receptor antagonist was used to inhibit a positive feedback loop. Primary melanoma cells (WM35) did not respond to the stimulation by IL-1α unless ASC is silenced (Figure 5e, 1st vs. 2nd bars and 4th vs. 5th bars), indicating that IL-1R is functional, but IL-1R signaling is impaired by ASC in primary melanoma. In contrast, in metastatic melanoma cells (1205Lu) with ASC knockdown, IL-1α treatment rescued the suppressed NF-κB activity to the same level as that in 1205Lu-control-shRNA cells (Figure 5f, 2nd vs. 5th bars), suggesting that IL-1R signaling in metastatic melanoma is functional despite the ASC knockdown. However, treatment with IL-1 receptor antagonist did not reduce NF-κB activity in 1205Lu-ASC-shRNA cells relative to their controls (Figure 5f, 4th vs. 6th bars in 1205Lu-ASC-shRNA cells and 1st vs. 3rd bars in 1205Lu-control-shRNA cells), suggesting that IL-1-mediated IL-1R stimulation is already

Figure 3. Effects of Apoptosis-associated Speck-like protein containing a CARD (caspase recruitment domain) (ASC) short hairpin RNA (shRNA) on primary melanoma. (a) Quantitative RT-PCR (qRT-PCR) (left panel) and western blot (right panel) of ASC in cells transduced with control or ASC shRNA. mRNA expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). β-Actin served as a loading control. (b) Cell viability in cells transduced with shRNA. (c) Cell cycle analysis in cells transduced with control and ASC shRNA. (d) Annexin V/propidium iodide (PI) staining in cells transduced with control and ASC shRNA. (e) Effects of ASC knockdown in vivo. Nude mice injected with WM35-control-shRNA or WM35-ASC-shRNA cells (upper panel). Tumor growth curve (lower panel). (f) Representative tumor sections stained with hematoxylin and eosin (H&E) (upper panel), Ki-67 (middle panel), and CD31 (lower panel). Bar = 100 μm. Data represent mean ± SE (n = 3 except in e, and n = 8 in e). **P < 0.01; ***P < 0.001. OD, optical density.
inhibited by ASC knockdown in metastatic melanoma. Taken together, these results indicate that ASC suppresses IL-1R signaling by inhibiting NF-κB activation in primary melanoma cells, whereas it contributes to an inflammasome-mediated positive feedback loop of IL-1R signaling in metastatic melanoma cells.

**DISCUSSION**

Previous studies have indicated ASC as a tumor suppressor gene that induces apoptosis in certain tumor cell lines. This study demonstrates, however, that although ASC suppresses tumor growth in primary melanoma, it promotes the growth of metastatic melanoma.

ASC contains PYD and CARD, and is thus a central adaptor protein to recruit PYD-containing NLRP3 to CARD-containing pro-caspase-1 for inflammasome activation and subsequent processing of IL-1β. We have previously shown the coexpression of ASC and NLRP3 in human melanoma cells and demonstrated that constitutively activated inflammasomes are present in both primary and metastatic melanoma (Okamoto et al., 2010). Congruent with our previous findings, ASC knockdown was shown here to decrease caspase-1 activity and impair IL-1β processing/secretion in human melanoma cells.

Only metastatic melanoma cells, however, spontaneously produce and secrete high levels of IL-1β, resulting in angiogenesis and tumor promotion (Okamoto et al., 2010). A previous report has shown that ASC can either inhibit or activate NF-κB, depending on the cellular context (Stehlik et al., 2002). Consistent with this, we found dual roles of ASC in human melanoma cells. Although ASC is necessary for inflammasome function and maturation of IL-1β in human melanoma, ASC differentially regulates NF-κB activity in primary and metastatic melanoma. NF-κB is pivotal for transactivation of cell cycle regulation, cytokine production, and expression of adhesion molecules, and is dysregulated in many cancers (Van Waes, 2007). We show here that ASC downregulates NF-κB activity in primary melanoma cells, despite the coexpression of ASC and NLRP3. In metastatic melanoma cells, however, NF-κB activity is enhanced by ASC. These seemingly contradictory functions of ASC may depend on the cellular context (primary vs. metastatic) (Figure 6). For example, ASC expression in primary melanoma cells inhibits NF-κB activity by inhibiting IKK-mediated degradation of IκB. This may explain why primary melanoma cells do not secrete IL-1β despite constitutively assembled and activated inflammasomes. On the other hand, the inhibitory effect of ASC on NF-κB is diminished in metastatic melanoma. As ASC associates with multiple PYD- and CARD-containing proteins, it is tempting to speculate that decreased ASC expression in metastatic melanoma cells may result in competition among various pathways for a limited supply of ASC protein. However, overexpressing ASC in metastatic melanoma cells did not alter NF-κB activity (Supplementary Figure S2a online), suggesting that constitutively active IL-1R signaling and NF-κB activity in metastatic melanoma are present in both primary and metastatic cells and demonstrated that constitutively activated inflammasome 

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**Figure 4. Inflammasome function in melanoma cells after Apoptosis-associated Speck-like protein containing a CARD (caspase recruitment domain) (ASC) knockdown.** (a) Intracellular and secreted IL-1β in 24 hours in WM35, WM115, HS294T, and 1205Lu cells transduced with control (ctrl) or ASC short hairpin RNA (shRNA). Levels of intracellular IL-1β from WM35 and WM115 cells transduced with control or ASC shRNA are shown above the line. ND, not detected. (b) Western blot of caspase-1 in WM35, WM115, HS294T, and 1205Lu cells transduced with control or ASC shRNA. β-Actin served as a loading control. (c) Caspase-1 activity in WM35, WM115, HS294T, and 1205Lu cells transduced with control or ASC shRNA. Data represent mean ± SE (n=3). **P<0.01 compared with cells transduced with control shRNA.
melanoma (autoactive Signal 1) make cells less vulnerable to the inhibitory effect of ASC on NF-κB. It is noteworthy that overexpressing ASC in metastatic melanoma cells did not affect autocrine IL-1β secretion (Supplementary Figure S2b) or caspase-1 activity (Supplementary Figure S2c), suggesting that there is enough biologically functional ASC to mediate caspase-1-dependent maturation and secretion of IL-1β, and the inflammasome is constitutively active in metastatic melanoma (autoactive Signal 2), resulting in a positive feedback mechanism of IL-1 signaling to further upregulate NF-κB activity in the absence of the inhibitory effect of ASC on IκB. ASC may thus contribute to a positive feedback loop of autoinflammation in metastatic melanoma, in which upregulated NF-κB induces transcription of pro-IL-1β, which is subsequently processed and secreted by constitutively active inflammasome, leading to further NF-κB activation through autocrine IL-1 signaling. Alterations in ASC and associated protein interactions may thus result in varying tumor-dependent responses, implying that ASC could be a promising therapeutic target in metastatic cancers.

Figure 5. The NF-κB pathway in melanoma cells after Apoptosis-associated Speck-like protein containing a CARD (caspase recruitment domain) (ASC) knockdown. (a) NF-κB activity in melanoma cells transduced with control or ASC short hairpin RNA (shRNA). (b) Immunoprecipitation interaction of IκB kinase (IKKα/β) with ASC in cells transduced with control or ASC shRNA. Lysates were immunoprecipitated (IP) and immunoblotted (IB) with antibodies. (c) Western blot of ASC, IKKα/β, phosphor-IKKα/β, and IκBα in cells transduced with control or ASC shRNA. β-Actin served as a loading control. (d) Representative xenografted tumor sections stained with IκBα. Bar = 100 μm. (e, f) NF-κB activity in cells transduced with control or ASC shRNA treated with IL-1α (10 ng ml⁻¹) or IL-1 receptor antagonist (IL-1Ra) (10 μg ml⁻¹) for 24 hours. Data represent mean ± SE (n = 3). **P < 0.01; ***P < 0.001 compared with cells transduced with control shRNA.
In conclusion, ASC appears to have cell-dependent roles in tumor pathogenesis by differentially regulating NF-kB activity and IL-1β processing. In metastatic melanoma, ASC induces tumorigenic pathways, most likely by activating caspase-1-dependent IL-1β secretion and enhancing auto-inflammatory NF-kB activity. On the other hand, higher levels of ASC expression in primary melanoma reduce IKKα/β phosphorylation and inhibit NF-kB activity. In fact, it appears that ASC transforms from a protein with antitumor properties in primary melanoma to a protumorigenic factor in metastatic melanoma. To our knowledge, no previous studies have examined stage-dependent roles of ASC in cancer cells. Further studies of ASC and its upstream and downstream intermediaries may enhance our understanding of the molecular mechanisms governing tumorigenesis and reveal new molecular targets for designing anti-cancer drugs.

MATERIALS AND METHODS

Cell culture

Human melanoma cell lines were obtained from the American Type Culture Collection (Manassas, VA): WM1552c and WM35 are from radial growth phase primary melanoma; WM115, WM75, and WM278 are from vertical growth phase primary melanoma; and A375, WM852c, HS294T, and WM1617 are from metastatic melanoma. 1205Lu is from a lung metastasis after subcutaneous injection of mice with a primary melanoma line WM793. Cells were maintained in RPMI 1640 (Mediatech, Manassas, VA) (except HS294T) or Dulbecco’s modified Eagle’s medium (Mediatech) (HS294T), supplemented with 10% fetal bovine serum (Mediatech), 100 μg ml⁻¹ streptomycin, and 100 U ml⁻¹ penicillin (Mediatech).

Western blotting analysis

Western blotting was carried out as described previously (Okamoto et al., 2010). Anti-caspase-1, IκBα/IκBβ, phospho-IκBα/ IκBβ, and IκBα antibodies were from Cell Signaling Technology (Boston, MA). Anti-ASC antibody was from Alexis Biochemicals (San Diego, CA). Horseradish peroxidase-conjugated anti-mouse IgG was from Jackson Immuno-Research Laboratories (West Grove, PA), and horseradish peroxidase-conjugated anti-rabbit IgG and anti-goat IgG were from Sigma-Aldrich (St Louis, MO). Signals were detected by SuperSignal West Femto maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL) and analyzed using GelDoc 200 (Bio-Rad, Hercules, CA).

DNA transfection

Plasmid pCMV-GFP-ASC or pCMV-GFP empty Vector (OriGene Technologies, Rockville, MD) was transfected at a concentration of 0.5 μg ml⁻¹ by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 48 hours, cells were collected and analyzed.

RNA extraction and quantitative RT-PCR analysis

RNA was extracted using the RNAqueous-Micro kit (Ambion, Austin, TX) and reverse transcribed. Quantitative PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on the MX3000P PCR system (Stratagene, La Jolla, CA). Primers were designed to generate a PCR product of 50 to 150 bp. The following primer sequences were used: human ASC, forward: 5’-CATGAAGTCGACACAGGGATG-3’, reverse: 5’-GGAAC TCTTCCAATGTTC-3’; human GAPDH, forward: 5’-CAGGGCT GTTTTTACTCTGG-3’, reverse: 5’-TGGGTGGAATCATATTGGAA CA-3’.

Figure 6. Hypothetical roles of Apoptosis-associated Speck-like protein containing a CARD (caspase recruitment domain) (ASC) in melanoma tumor progression. Solid lines indicate constitutive/autonomous pathways. Dashed lines indicate signal-dependent pathways. Arrow thickness indicates the relative strength of signaling pathways. ASC inhibits phosphorylation of IκB kinase IKKα/β and decreases NF-kB in primary melanoma cells. Synthesis of pro-IL-1β is thus low and IL-1β secretion is minimal despite autoactive inflammasome in primary melanoma cells. In metastatic melanoma, however, autoactive IL-1R signaling and other signaling pathways result in reduced inhibition of NF-kB from ASC in the presence of sustained autoactive inflammasome, leading to the spontaneous synthesis and secretion of IL-1β from melanoma cells. IL-1β secreted from melanoma cells further augments the autoinflammatory loop of metastatic melanoma cells to activate NF-kB. NLRP, NOD-like receptor family containing pyrin domain.
Cell viability assay
Cell viability was measured using the CellTiter 96 AQUeous One Solution Cell Proliferation Assay kit (Promega, Madison, WI). A total of 1–5 × 10^3 cells in 100 μl of media were plated per well in 96-well plates and cultured. Viability was determined using the ELX808 Ultra Microplate Reader (Bio-Tek Instruments, Winooski, VT).

shRNA transduction
Cells were transduced with shRNA lentiviral particles against control or ASC (Santa Cruz Biotechnology, Santa Cruz, CA) in cell culture medium containing 5 μg ml⁻¹ of Polybrene (Santa Cruz Biotechnology). After overnight transduction, cells were incubated in medium with 1 μg ml⁻¹ of puromycin (Santa Cruz Biotechnology) to select for stable clones expressing transduced shRNA, and maintained in medium with 1 μg ml⁻¹ of puromycin at 37 °C.

Cell cycle analysis
Cells were stained at 4 °C with Krishan’s staining buffer containing 70 μl propidium iodide (Sigma-Aldrich), 3.8 μl trisodium citrate (Sigma-Aldrich), 0.01% Nonidet P-40 (Sigma-Aldrich), and 0.01 mg of RNase A (Roche Diagnostics, Indianapolis, IN), and analyzed using flow cytometer FC500 (Beckman-Coulter, Brea, CA). The Modfit LT program (Verity Software House, Topsham, ME) was used for data analysis.

Dead cell apoptosis assay
Cells were stained with Annexin-V and propidium iodide (Invitrogen) following the manufacturer’s instructions for flow cytometric analysis. Cell death was measured from apoptotic (Annexin-V-positive) and/or necrotic (PI-positive) cells.

Immunohistochemistry
Human melanoma tissues were obtained from surgical specimens with written consent from the patients under institutional review board-approved protocols, adhering to Health Insurance Portability and Accountability Act regulations and to the Declaration of Helsinki Principles. Paraffin sections were treated with a low pH solution such as citrate buffer (pH 6) (Leica Microsystems, Buffalo Grove, IL). ASC and IκBα were stained with rabbit anti-human ASC (Proteintech Group, Chicago, IL) and rabbit anti-human IκBα (Abcam, Cambridge, MA), respectively, followed by rat anti-rabbit IgG link (DAKO, Carpinteria, CA) and Bond Polymer Refine Red Detection kit (Leica Microsystems). CD31 was stained with rat anti-mouse CD31 (Dianova, Rodeo, CA) and rabbit anti-rat IgG link (DAKO), followed by detection with the Bond Polymer Refine Red Detection kit. Ki-67 was stained with rabbit anti-human Ki-67 (Thermo Scientific) and rat anti-rabbit IgG link, followed by detection with the Bond Polymer Refine Detection kit (Leica Microsystems). Sections were counterstained with hematoxylin (Leica Microsystems), and were reviewed by two observers.

Tumor formation in nude mice
The 6-week-old female athymic nu/nu mice (Jackson Laboratory, Bar Harbor, ME) were used. Animals were kept under specific pathogen-free conditions, according to National Institutes of Health Animal Care Guidelines. Experimental protocols were approved by the Institutional Animal Care and Use Committee of University of Colorado Denver. A total of 1 × 10^6 1205Lu-control-shRNA or 1205Lu-ASC-shRNA cells or 2.5 × 10^6 WM35-control-shRNA or WM35-ASC-shRNA were resuspended in 0.1 ml of Matrigel Basement Membrane Matrix (BD Biosciences, San Jose, CA) diluted 1:1 with phosphate-buffered saline and injected intradermally into the flank of mice. Tumor growth was monitored bi-weekly with an electronic digital caliper (Control, Friendswood, TX). Tumor volume was calculated by the following formula: tumor volume = (longest diameter) × (shortest diameter)^2/2.

ELISA
Human IL-1β ELISA kit was obtained from R&D Systems (Minneapolis, MN) (Okamoto et al., 2010). A total of 1 × 10^5 per ml of cells were seeded in 6-well plates, and the medium was changed to OPTIMEM (Invitrogen) when cells reached 80% confluence. After 24 hours, supernatants were collected to assess secreted IL-1β. Intracellular cytokines were assessed by lysing cells with 0.5% Triton X-100 in phosphate-buffered saline followed by the freeze–thaw cycle.

Caspase-1 activity assay
Caspase-1 Flica kit (ImmunoChemistry Technologies, LLC, Bloomington, MN) was used. Cells (1.5 × 10^7/100 μl per well in 96-well plates) were trypsinized, washed in ice-cold phosphate-buffered saline, and incubated with caspase-1 inhibitor probe (FAM-YVAD-FMK) for 1 hour at 37 °C, 5% CO₂. The fluorescence was measured using the fluorescence plate reader (Promega). A recombinant caspase-1 was used as a positive control.

NF-κB activity assay
The Ready-to-Glow Secreted Luciferase pNfX-B-MetLuc Vector Kit (Clontech Laboratories, San Francisco, CA) was used. A total of 5 × 10^4 per ml of melanoma cells were seeded in a 24-well plate, transfected with a control vector (pMetLuc2-Reporter) or an NF-κB vector (pNfX-B-MetLuc2 Reporter) in OptiMEM, and treated with IL-1α (10 ng ml⁻¹) and/or IL-1 receptor antagonist (10 μg ml⁻¹). The cell culture supernatant was collected 24 hours later, and luciferase activity was measured using a luminometer (Promega).

Immunoprecipitation and immunoblot analysis
Immunoprecipitation was carried out as described previously (Okamoto et al., 2010). Briefly, cell lysates were incubated with primary antibodies or preimmune serum and immunoprecipitated with protein A/G plus agarose (Santa Cruz Biotechnology). The precipitates were washed, separated by SDS-PAGE, and analyzed with western blotting.

Statistical analysis
The values in the figures are expressed as the means ± SE. The figures in this study are representatives of more than two different experiments. Statistical analysis of the data between two groups was performed by a Student’s t-test. Values of P<0.05 were considered as statistically significant.

CONFLICT OF INTEREST
The authors state no conflict of interest.
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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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