A Fluorescent Reporter Mouse for Inflammasome Assembly Demonstrates an Important Role for Cell-Bound and Free ASC Specks during In Vivo Infection

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**Graphical Abstract**

**Highlights**
- The ASC-citrine mouse is a transgenic model that reports inflammasome activation
- ASC-citrine retains the function of endogenous ASC
- Non-hematopoietic cells are capable of forming ASC specks on activation
- The ASC-citrine mouse recapitulates the formation of free specks in vivo

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**In Brief**
In vivo detection of the activated inflammasome complex has been limited by a dearth of tools. Here, Tzeng et al. have developed a reporter mouse model expressing ASC fluorescent protein (ASC-citrine). Mice treated with inflammasome activators have shown increased ASC aggregation, indicating the activation of inflammasome pathways.

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A Fluorescent Reporter Mouse for Inflammasome Assembly Demonstrates an Important Role for Cell-Bound and Free ASC Specks during In Vivo Infection

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SUMMARY

Inflammasome activation is associated with numerous diseases. However, in vivo detection of the activated inflammasome complex has been limited by a dearth of tools. We have developed transgenic mice that ectopically express the fluorescent adaptor protein, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and characterized the formation of assembled inflammasome complexes (“specks”) in primary cells and tissues. In addition to hematopoietic cells, we have found that a stromal population in the lung tissues formed specks during the early phase of inflammation, whereas myeloid cells showed speck formation after 2 days. In a peritonitis and group B streptococcus infection model, a higher percentage of neutrophils formed specks at early phases of infection, while dendritic cells formed specks at later time points. Furthermore, speck-forming cells underwent pyroptosis and extensive release of specks to the extracellular milieu in vivo. These data underscore the importance of free specks during inflammatory processes in vivo.

INTRODUCTION

Inflammasomes are key signaling platforms that detect pathogenic microorganisms and sterile stressors and then control the caspase-1-dependent maturation of the highly pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18. Both IL-1β and IL-18 have numerous functions, including activation of feed-forward pathways that result in the production of more cytokines, such as tumor necrosis factor alpha (TNF-α). Hence, the inflammasome is a central mediator of inflammation. Deregulated inflammasome activity has been linked to several sterile inflammatory diseases, including cryopyrin-associated periodic syndromes (Baroja-Mazo et al., 2014; Hoffman et al., 2001), gout (Martinon et al., 2006), Alzheimer’s disease (Halle et al., 2008; Heneka et al., 2013), and atherosclerosis (Duewell et al., 2010; Samstad et al., 2014; Sheedy et al., 2013). Inflammasome activation has also been shown to play a critical role in the clearance of pathogens, such as influenza A/PR8, group B streptococcus (GBS), and Listeria (Costa et al., 2012; Ichinohe et al., 2009, 2010; Rathinam et al., 2010).

Inflammasomes are multiprotein complexes that assemble in response to microbial triggers and activate caspase-1 and or caspase-11, which, in turn, process the immature proinflammatory cytokines, pro-IL-1β and pro-IL-18, to their mature forms. In general, inflammasomes consist of three proteins: (1) a receptor that recognizes danger signals, (2) the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), and (3) the enzyme caspase-1 (Latz, 2010). After the receptor molecule has sensed a danger signal and undergoes structural rearrangements, the signal transduction domains (PYD domain) induce rapid polymerization of ASC into a protein helix forming large filaments. Filamentous ASC nucleates pro-caspase-1 via a CARD/CARD domain interaction, leading to proximity-induced activation of caspase-1 (Fernandes-Alnemri et al., 2007; Lu et al., 2014). The autocatalytically activated form of caspase-1 then converts pro-IL-1β and pro-IL-18 into the corresponding mature active cytokines.

Many inflammasomes require the assembly of the adaptor protein ASC for activation. ASC aggregation, first described by Masumoto et al. (1999), occurs when ASC molecules form a large aggregate, often called a “speckle” or “speck” during the process of cell death. Because the hallmark of inflammasome activation is speck formation, many tools have been used for examining inflammasome activation using speck formation as a readout. It is relatively common, for example, to stimulate engineered macrophage cell lines that overexpress fluorescent ASC in order to look for specks (Fernandes-Alnemri et al., 2007; Sheedy et al., 2013). Alternatively, one can detect ASC oligomerization by western blot (Ataide et al., 2014) or with anti-ASC antibodies (Franklin et al., 2014).

Although we have several tools to test inflammasome activation in vitro, there is a paucity of in vivo tools to visualize or measure activation of the inflammasome directly. Recently, Sagoo et al. (2016) visualized ASC polymerization and the subsequent
release of ASC specks by transferring hematopoietic cells that had been retrovirally transduced with ASC-GFP. Although the findings were a novel demonstration of the fate of oligomerized ASC complexes in an in vivo setting, the approach required irradiation of recipient mice in order to transplant hematopoietic reporter cells, raising the possibility of radiation-induced inflammation and artifact. Furthermore, assembled inflammasomes in non-hematopoietic lineages were not detectable by this technique. Thus, we generated and exploited a transgenic mouse expressing a mouse ASC-citrine fusion protein in the Rosa26 locus to answer questions concerning inflammasome activation in native tissues during the inflammatory process without these limitations. The vector we used contains a knockin of the ASC-citrine chimera and a proximal floxed stop site, allowing us to conditionally express ASC-citrine in a lineage-specific manner. Bone-marrow-derived macrophages (BMDMs) or bone-marrow-derived dendritic cells (BMDCs) from this reporter mouse showed ASC speck formation after inflammasome stimulation. Speck formation was detectable in the tissues of infected mice, both intracellularly and extracellularly. Both hematopoietic cells and non-hematopoietic cells are capable of expressing specks and stimulating the downstream signal pathway when activated.

RESULTS

Generation of ASC-Citrine Mice

Speck formation is a widely used readout for detecting inflammasome activation (Stutz et al., 2013). However, due to the lack of genetic tools, the detection of inflammasome activation in vivo has thus far been limited. We generated a transgenic mouse expressing mouse ASC-citrine fusion protein in the Rosa26 locus. This system contains a knockin of the ASC-citrine gene and a proximal loxP-flanked stop site, allowing us to conditionally express ASC-citrine in a lineage-specific manner (Figure S1A). The embryonic stem cell (ESC) clones that harbored the expected ASC-citrine fragment were confirmed by Southern blot analysis (Figure S1B), and the targeted ESC clones were injected into blastocysts to generate chimeric mice. Germline transmission of the target gene yielded heterozygous ASC-citrine mice, which were crossed with Cre-expressing mice to generate ASC-citrine/Cre+ expressing mice. We first bred the ASC-citrine mice with ZP3-Cre mice, in which Cre expression is controlled by the regulatory sequences from the mouse zona pellucida 3 (Zp3) gene (de Vries et al., 2000). This promoter normally directs expression exclusively in the growing oocyte prior to the completion of the first meiotic division, and thus we obtained the first generation offspring with deletion of the floxed sequence in the female germine. These females were then crossed with wild-type (WT) males to generate progeny that carry the deleted-floxed allele. Citrine is a variant of yellow fluorescent protein and is very bright when excited with the blue laser (488 nm). The expression of ASC-citrine could be detected by flow cytometry using blood samples from ASC-citrine/Zp3-Cre+ (hereafter referred to as ASC-citrine/Cre+) mice (Figure S1C). ASC-citrine/Cre+ mice showed normal behavior, with no significant difference in survival and no signs of inflammation.

Ectopic-Expressed ASC-Citrine Is Functional

To test whether the ASC-citrine molecule is functional, we bred ASC-citrine/Cre+ mice with ASC knockout (KO) mice to generate mice that only express ectopic ASC-citrine, but lack endogenous ASC. BMDMs were cultured from ASC-citrine/Cre+ mice on a WT and ASC-KO background, as well as the control littermates that were ASC-citrine negative. BMDMs cultured for 8 days were stimulated with lipopolysaccharide (LPS) alone or LPS plus nigericin, a well-known NLRP3 inflammasome stimulator. The expression of ASC-citrine molecules was comparable in both the WT and ASC-KO background (Figure 1A). Cleaved IL-1β and activated caspase-1 p20 were detected in WT BMDMs with or without ectopic expression of ASC-citrine (Figure 1A). In contrast, the activation of IL-1β and caspase-1 was absent in ASC-KO mice. Ectopic expression of ASC-citrine in the ASC-KO background was able to restore the activation of caspase-1 and IL-1β; however, the level of activated molecules was lower compared to that in WT macrophages (Figure 1A). The result was similar when the level of IL-1β was measured by ELISA in the culture supernatant (Figure 1B).

Several studies have demonstrated the formation of specks in macrophage reporter cells stably expressing fluorescent ASC proteins when stimulated with nigericin, ATP, or pathogens (Fernandes-Alnemri et al., 2007; Mariathasan et al., 2006; Masumoto et al., 2001; Silva et al., 2013). The ASC-citrine/Cre+ mice enabled the examination of speck formation in primary cells. Confocal images showed increased speck formation in primary BMDMs from ASC-citrine/Cre+ mice when stimulated with LPS plus nigericin (Figure 1C).

Structural studies of ASC have demonstrated that inflammasome assembly begins with the polymerization of ASC into fibrils by NOD-like receptors (NLRs) or by the HIN 200 family member proteins, such as AIM2 (absent in melanoma 2). These fibrils of flexibly linked ASC serve as a platform for caspase-1, leading to caspase-1 activation (Lu et al., 2014) and the processing of IL-1β and IL-18. We hypothesized that the formation of ASC specks does not require downstream molecules, such as caspase-1. BMDMs from ASC-citrine mice or ASC-citrine mice deficient in caspase-1 expression were stimulated with LPS and nigericin. Confocal images show that even without caspase-1, ASC-citrine was still able to form specks (Figure S2).

Quantitative Method to Detect Inflammasome Activation

We used image-based flow cytometry to characterize the speck-positive cells with a quantitative method. The experiment shown in Figure 2 was performed using BMDCs and demonstrates the technique of quantitation that we developed. Note how in this experiment, speck formation was apparent in BMDCs treated with LPS plus nigericin or LPS plus poly (dA:dT) (Figure 2A). In order to quantify the speck-positive cells, we created a matrix feature to define speck-positive cells using the IDEAS software. Under homeostatic conditions, the distribution of ASC-citrine molecules in the nucleus and cytosol is uniform; indeed, the cell is uniformly fluorescent. Thus, the total area of citrine positivity (represented by a green circle in Figure 2B) in unstimulated cells is similar to the area of the entire cell (i.e., “bright field,” represented by a circle with the letters “BF” in Figure 2B) as measured by bright-field microscopy. Hence, unstimulated cells
have a ratio of BF:area of citrine expression (“citrine”) that is approximately 1.00. On stimulation, ASC-citrine molecules form compact specks, thereby dramatically increasing the ratio of BF:citrine; under these conditions, the ratio of BF:citrine is larger than one. In this context, we used the ratio between BF:citrine as an indicator of speck formation (Figure 2B). The ratio of BF:citrine is between 1 and 3 during homeostasis and increases 10-fold with LPS plus Nigericin treatment. The shift of the fluorescence-activated cell sorting (FACS; flow cytometry) histogram on stimulation allows us to gate on speck-positive cells for purposes of quantification. As seen in Figures 2C and 2D, nearly 50% of BMDCs formed specks when stimulated with LPS plus Nigericin.

Aggregation of ASC molecules promotes the cleavage of pro-caspase-1 to activated caspase-1, which, in turn, processes pro-IL-1β and pro-IL-18 to active cytokines. To test whether speck-forming cells indeed express activated caspase-1, we measured activated caspase-1 with a fluorescent cell-permeable probe that selectively and covalently binds to the catalytic site of caspase-1 (Amstad et al., 2001). This reagent did not lead to any visible staining in caspase-1-deficient cells (data not shown). Overlay histogram analysis of speck-positive cells and speck-negative cells showed a robust increase in catalytically active caspase-1-positive cells in speck-positive cells, whereas speck-negative cells showed no such response (Figure 2E).

Figure 1. ASC-Citrine Can Induce the Maturation of IL-1β on Activation
(A–C) BMDCs were cultured from WT, ASC-KO, ASC-citrine/Cre+, or ASC-KO/ASC-citrine/Cre+ mice. Cells were primed with 100 ng/ml of ultrapure LPS (lipoprotein-free) for 2 hr, followed by stimulation with Nigericin for 1 hr. (A) Concentrated culture supernatants and cell lysates were subjected to western blot with antibodies against mouse IL-1β, mouse caspase-1, and ASC. β-actin was used as a loading control. (B) Concentrations of IL-1β in the culture supernatants were determined by ELISA. Data are presented as mean ± SD of triplicates and are representative of three independent experiments. (C) BMDCs from ASC-citrine mice were primed with 100 ng/ml LPS for 2 hr, followed by stimulation with Nigericin for 1 hr. Living cells were stained with Hoechst nuclear dye (blue) and CellMask Deep Red plasma membrane dye (red), ASC-citrine fusion proteins are shown in green. Speck formation was visualized by confocal microscopy. Scale bars represent 30 µm.

Prolonged inflammasome activation results in a specific type of cell death called “pyroptosis,” characterized by the loss of plasma membrane integrity and the late release of intracellular proteins (Bergsbaken et al., 2009). To test whether the presence of specks invariably is associated with cell death, we stained activated cells with 7-aminoactinomycin D (7AAD), a vital dye with a high affinity for DNA that does not pass through the intact cell membrane. As seen in the histograms plotted in Figures 2A and 2F, 75% of speck-positive cells are 7AAD positive, suggesting that these cells undergo pyroptosis while forming specks, whereas 76% of speck-negative cells are alive, as defined by the absence of staining with 7AAD.

To confirm that speck formation is not due to the consequences of necrotic cell death or represented an artifact, BMDCs from ASC-citrine/Cre+ mice were stimulated with TNFα plus SMAC mimetic in the presence of the caspase inhibitor zVAD, a cocktail known to induce cell necrosis. We detected that 25% of cells were 7AAD+ after 24 hr of stimulation; however, no assembled inflammasome specks were detected (Figure S3A). In contrast, 80% of the 7AAD+ cells with LPS plus Nigericin, which activates the NLRP3 inflammasome, formed specks. These data indicate that speck formation is indeed due to canonical inflammasome activation (Figures S3A and S3B).

Inflammasome Activation In Vivo
To test whether speck formation can be detected in vivo using ASC-citrine/Cre+ mice, we chemically induced peritonitis with a potent activator of the NLRP3 inflammasome, Nigericin. Mice were injected intraperitoneally, and peritoneal exudates were collected 90 min later. Nigericin induced a considerable increase in the recruitment of neutrophils (7-4pos/Ly6Ghigh) to the peritoneal cavity in ASC-citrine/Cre+ mice (Figures 3A and 3C).
In contrast, resident macrophages (F4/80<sup>high</sup>/CD11b<sup>high</sup>) decreased dramatically. Using an image-based flow machine, we gated different populations of cells and analyzed the formation of specks. Unexpectedly, only a small percentage of speck-positive cells were detected in the ASC-citrine/Cre<sup>+</sup> mice (Figure 3B), despite the known potency of nigericin as an activator of Inflammasomes.

Recent publications have demonstrated that inflammasome-activated cells undergo pyroptosis, an event accompanied by the release of ASC specks into the extracellular space. These extracellular ASC specks retain the ability to activate pro-caspase-1 and process pro-IL-1β and pro-IL-18 into mature cytokines (Baroja-Mazo et al., 2014; Franklin et al., 2014). To test the hypothesis that ASC specks are released from the cell, we used ASC-citrine/Cre<sup>+</sup> mice that were bred onto a caspase-1 null-background. As caspase-1 activation is required for nigericin-induced pyroptosis, the release of specks would be prevented. Indeed, there was a 6-fold increase of speck-positive cells.
cells in ASC-citrine/Cre+ mice on the caspase-1 KO background (Figure 3B), suggesting that the lack of specks in nigericin-stimulated cells is due to pyroptosis in WT mice. We further analyzed the ability of different cell types to form specks and have found that the percentage of speck-positive cells was mainly in the neutrophil population, which was 2- to 3-fold higher than in resident macrophages. This is especially evident in the caspase-1 KO background, where 26% of neutrophils contained specks (Figures 3B and S4B).

These data led us to ask whether active inflammasome complexes might be released into the peritoneum of nigericin-treated mice. An analysis of the exudate supernatants that were collected by lavage detected citrine-positive particles only from the ASC-citrine/Cre+ reporter mice treated with nigericin. A citrine signal was conspicuously absent from the exudates of nigericin-treated C57BL/6J (B6) mice or ASC-citrine/Cre+/caspase-1 KOs (Figures 4A and 4B), whose cells would not be expected to undergo pyroptosis. We gated on the citrine-positive population of particles in the exudates from the ASC-citrine/Cre+ mice. This population of particles was between 1–3 μm, which is the expected size for a speck (Figure 4C). Immunoblotting analysis confirmed the presence of oligomerized ASC after chemically crosslinking the exudates with disuccinimidyl suberate (DSS) (Figure 4D, middle lane). Non-crosslinked control macrophage lysates expressed abundant monomeric ASC-citrine (~50 kDa in size) (Figure 4D, right-hand lane).

**Figure 3. Nigericin Induces the Formation of Specks In Vivo in a Caspase-1-Dependent Manner**

(A and B) Mice were injected intraperitoneally with 3 mg/kg of nigericin for 90 min, and the peritoneal exudates were subsequently collected. (A) Numbers of different populations of cells in the exudates. Comparison of the numbers of resident macrophages (F4/80high/CD11bhigh) versus neutrophils (7-4pos/Ly6Ghigh). (B) The percentage of speck-positive cells in different populations is shown by FACS histogram. Bars represent the average of five samples. *p < 0.05, **p < 0.01 (Student’s t test).

**Influenza Virus A/PR8 Induces Inflammasome Activation in Lung Stromal Cells during the Early Phase of Infection**

Different strains of a virus have different capabilities to induce inflammasome activation. We stimulated primary ASC-citrine/Cre+ macrophages with different RNA and DNA viruses, including influenza A/PR8 (NLRP3), vesicular stomatitis virus (VSV; NLRP3 and RIG-I), mouse cytomegalovirus (mCMV; AIM2), and herpes simplex virus (HSV; Iri16 and NLRP3). There was a robust increase in speck-positive cells while stimulating cells for 12 hr, whereas a small percentage of cells formed specks after 4 hr of stimulation. The increased number of speck-positive cells was more obvious when cells were infected with RNA viruses, especially after infection with influenza strain A/PR8 (Figure S5A).

The role of the inflammasome in influenza infection has been studied in detail. Not only are inflammasomes activated during experimental influenza but both ASC and caspase-1 also play essential roles in inducing adaptive immune responses (Ichinohe et al., 2009, 2010). To test for speck formation in vivo, mice were challenged intranasally with influenza strain A/PR8 (Figure 5A). Active IL-1β was detectable in lung tissue as early as day 1 and increased by day 2 (Figure 5A). Confocal microscope imaging of lung tissue of day 1 infected mice showed increased speck-positive cells in every field that was viewed (Figures 5B and 5C). The control mice were treated with PBS and showed a minimal number of specks in each field (Figure 5B). Surprisingly, we did not see speck-positive cells co-localize with
CD45 staining, indicating that after 1 day of infection, the speck-positive cells were the non-hematopoietic cells (Figure 5B). The mAb ERTR7 recognizes antigens that are located in the cytoplasm of reticular fibroblasts and are a component of the extracellular matrix of lymphoid and non-lymphoid organs. Many speck-positive cells were either close to or inside the ERTR7-positive cells (Figure 5D), suggesting that fibroblasts were either able to form specks or to internalize released specks. We confirmed this result using a different fibroblast marker, podoplanin (Figure S6B).

Next, we sought to determine if the speck-positive cells were actually infected with influenza by staining for influenza A nucleoprotein. Many speck-positive cells were positive for influenza A nucleoprotein, indicating that the speck-positive cells were primarily infected with influenza A (Figure 5E). In contrast, we have found that CD11c cells do not form specks until day 3 (Figure 5F). These data suggest a scenario in which the inflammasomes of the non-hematopoietic cells are activated in the early phase of infection, while the inflammasome response is initiated in hematopoietic cells at a later time point.

Figure 4. Nigericin Induces the Release of Specks In Vivo in a Caspase-1-Dependent Manner
(A) Cell-free supernatants of peritoneal exudates were analyzed by flow cytometry for the detection of extracellular specks. Note the large quantity of citrine-positive particles in the supernatants from ASC-citrine-positive animals that were treated with nigericin and the absence of these particles in the caspase-1-deficient mouse (top set of histograms). Citrine was not detected in the exudates from WT B6 mice, which were tested as a control group, while unstimulated ASC-citrine mice had a small quantity of peritoneal specks (bottom set of histograms).
(B) The number of free specks in the exudates was quantified by flow cytometry.
(C) A size comparison of the citrine-positive population shown in (A) with 1-, 3-, 6-, and 10-μm calibrated beads. ASC specks should be approximately 1–3 μm in size.
(D) Immunoblot analysis of released ASC oligomers in the cell-free supernatant of peritoneal exudates from mice that were untreated (−) or treated (+) with nigericin. Filtered exudates were subsequently crosslinked by DSS. Cell lysates from ASC-citrine BMDMs cells were shown as a positive control for the ASC-citrine band (C). Right margin, molecular size in kilodaltons (kDa). The results show an increased number of specks per milliliter of supernatant in WT/ASC-citrine mice after nigericin stimulation.
Figure 5. Influenza Virus A/PR8-Infected Mice Form Specks in the Lung Parenchyma

(A–F) Mice were intranasally challenged with influenza strain A/PR8.
(A) Lung homogenates were prepared in 0.5 ml PBS containing 0.1% BSA. Total IL-1β levels detected from the lung homogenates were measured at different time points by ELISA.
(B) Confocal microscopic images of mouse lung tissue after 1 day of infection. Lung tissue was fixed and stained with CD45 (red) and Hoechst nuclear dye (blue). The white scale bar represents 30 μm.
(C) The tile function was used to photograph multiple images over a defined area using confocal laser microscopy. The total number of specks was counted, and ~20 images were analyzed in each sample. The results represent the mean number of specks per tile in each of two mice.
(D and E) Lung tissue from mice infected for 24 hr was stained with mAb ERTR7 (red in D) or MCA400 (red in E), in combination with Hoechst nuclear dye (blue). The green color represents ASC-citrine. Data are representative of three independent experiments.
To confirm that non-hematopoietic cells from lung tissue can respond to influenza and form specks, we cultured lung primary cells from ASC-citrine/Cre+ mice as described previously (Ichinohe et al., 2009). Under these culture conditions, most of the cells were podoplanin-positive and CD45-negative (Figure 6A). When primary lung fibroblasts were stimulated with LPS plus nigericin or infected with influenza A/PR8, we observed that fibroblasts formed specks 4 hr after stimulation (Figure 6B). Furthermore, there was a significant amount of IL-1β in the culture supernatant, as detected by ELISA (total IL-1β) or western blot (mature IL-1β; Figure 6C).

**Group B Streptococcus Induces Inflammasome Activation as Early as 4 hr after Infection**

Despite advances in intrapartum antibiotic prophylaxis, GBS remains one of the most important causes of neonatal sepsis worldwide (Parks et al., 2015). GBS has been shown to be a potent NLRP3 inflammasome stimulator, and the hemolytic activity is critical for its function for inflammasome activation (Costa et al., 2012; Gupta et al., 2014). Mice were infected with GBS by intravenous injection. ASC-citrine/Cre+ mice produced similar levels of IL-1β in splenic tissue compared to WT B6 mice. The peak for IL-1β level was 4 hr after infection, corresponding to the peak in bacterial colony-forming units (CFUs) (Figures 7A and 7B). Since IL-1β has been shown to play important roles in recruiting neutrophils to inflammatory sites (Kono et al., 2012), we hypothesize that cells with specks should be detectable as early as 4 hr after infection. Speck formation was analyzed by image-based flow cytometry. We observed increased speck formation in neutrophils and F4/80+ macrophages as early as 4 hr after infection. The percentage of speck-positive cells then decreased in the neutrophil population on day 1, whereas the percentage stayed the same in the macrophage population. In contrast, very few speck-positive CD11c+ cells were detected at 4 hr; the number of CD11c+ cells increased by the beginning of day 1 (Figure 7C). Frozen sections of 1-day infected spleens samples were evaluated for speck-forming cells. Confocal images showed that numerous specks were formed in the dense network of stromal cells in the red pulp, where many dendritic cells are located. Some of the specks are close to each other and located right next to the ERTR7+ fibroblast conduit system (Figure 7D). Both intracellular and extracellular specks, presumably arising from pyroptotic cell death, were seen in the splenic samples.

**DISCUSSION**

Speck formation is the hallmark of inflammasome activation and can be studied in vitro using fluorescent ASC-overexpressing macrophage cell lines. However, the lack of an in vivo tool for studying ASC speck formation has limited the understanding of inflammasome activation under conditions of inflammation, especially in the context of an ongoing infection. In the present study, we generated an ASC-citrine reporter mouse that enables us to study speck formation in real time in an in vivo setting.

The primary cells from ASC-citrine mice showed robust speck formation and cleavage of caspase-1 and IL-1β. In addition, expression of ASC-citrine restored caspase-1 activation and IL-1β cleavage in ASC-KO macrophages, although the amount of cleaved caspase-1 was lower compared to WT background. In part, this may have been because there was about one-third fewer total ASC in the knockout background. In addition, the
efficiency of the ASC-citrine chimera to activate caspase-1 appears to be lower than the native molecule. Lu et al. (2014) showed that the CARD domain acts as a platform for caspase-1 molecules. In our mouse model, the citrine was fused to the C terminus of ASC, which is adjacent to the caspase-1 recruitment domain. We hypothesize that the addition of citrine impedes successful assembly of the inflammasome complex, such as the recruitment of caspase-1, and thus decreases its activation. In fact, as studies have shown fluorescent ASC PYD or fluorescent ASC CARD is capable of forming filaments in vitro, we were originally concerned that ectopic expression of fluorescent ASC would lead to autoinflammation in the mouse and that the establishment of the reporter mouse might be difficult or even impossible. To our surprise, no signs of inflammation were observed in ASC-citrine/Cre+ mice due to the lower efficiency of ASC-citrine to activate downstream signals.

The purpose of our in vivo experiments was to learn about inflammasome activation during the course of inflammation in real time. One surprise was that in the peritonitis model (LPS + nigericin), neutrophils were the major speck-forming population detected, as the number of resident macrophages was dramatically decreased. As resident macrophages may adhere to the peritoneal membrane after stimulation or undergo pyroptosis on inflammasome activation (Chen et al., 2014), it is possible that activated resident macrophages were simply not collected as part of the peritoneal exudate. Therefore, we cannot rule out the possibility that resident macrophages are the first wave of speck-forming cells that recruit neutrophils (via the production of IL-1β and IL-18 and subsequent activation of autocrine pathways) and subsequently monocytes. Our results show a high percentage of neutrophils with specks and are consistent with a recent publication showing that neutrophils are resistant to pyroptosis on inflammasome activation (Chen et al., 2014).

Recent reports have demonstrated that, after inflammasome activation, fully formed specks are released from activated cells as a result of pyroptosis (Baroja-Mazo et al., 2014; Franklin et al., 2014). These specks have been reported to become internalized by neighboring cells, of both phagocytic and non-phagocytic origin, potentially changing the character of the stromal milieu into a nest of inflammation. In fact, we observed the release of large quantities of specks into the peritoneal space after inflammasome activation. Similarly, splenic tissue samples from bacterially infected mice were also loaded with free specks in the extracellular space, as well as specks that appeared to have been internalized by non-phagocytic cells. As the specks were observed to be localized close to the ERTR7+ conduit system, one might hypothesize that ASC complexes can travel via...
this conduit system as a means of amplifying systemic inflammation. All of these suggest that releasing specks into extracellular space may be an efficient way of spreading inflammation in a short period of time.

All together, we have shown that ASC-citrine/Cre+ reporter mouse is a useful tool for studying inflammasome activation in vivo. The expression of ASC-citrine in the mouse is beneficial for the study of inflammasome activation in primary cells in real-time, including both hematopoietic and non-hematopoietic cells. In these studies, for example, we have observed a strong in vivo correlate of the recently reported role of extracellular specks as mediators of inflammation in vitro, underscoring the high probability that extracellular specks are far more important than previously thought.

**EXPERIMENTAL PROCEDURES**

**Mice**

Mice between 8 to 10 weeks old were used. C57Bl/6 mice were from The Jackson Laboratory (JAX). The Rosa-26-ASC citrine floxed allele was generated following a strategy previously developed by Sasaki et al. (2006). Namely, the Rosa-26 allele was targeted with a construct containing mouse ASC-citrine cDNA preceded by a loxp-flanked STOP cassette and marked by a signaling deficient truncated version of hCD2 under the control of an internal ribosomal entry site (IRES) downstream of the inserted cDNA. Transgene transcription is controlled by a CAG promoter. ESEs containing targeting genes were injected into blastocysts to generate chimeric mice by the transgenic animal modeling core at UMass medical school.ASC-citrine/Cre+ mice were bred with Caspase-1 KO or ASC KO to generate Caspase-1 KO/ASC-citrine or ASC KO/ASC-citrine. All mice were bred and maintained in specific pathogen-free conditions; mouse protocols were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School.

**Reagents**

Lipofectamine 2000 was obtained from Invitrogen. Poly(dAdT) and nigericin were from R&D Systems. Components of the bacterial culture supernatants were assayed for IL-1α and IL-1β using the ELISA kit for mouse IL-1α from R&D Systems.

**Antibodies**

The following antibodies were used: anti-IL-1β (AF-401-NA) from R&D; mouse monoclonal antibody to NLPR3 (Cry2; AG-20B-0014-C100) and to caspase-1 p20 (caspase-1; AG-20B-0042-C100) from Abcam; anti-ERTR7 from Abcam; anti-podoplanin from Biolegend; polyclonal anti-ASC (sc-2514-R; N-15R) from Santa Cruz; and anti-ASC, anti CD11c, anti-CD3, anti-B220, anti-CD45, anti-CD11b, anti-Ly6G, anti-7-4, and anti-F4/80 from eBioscience. CellMask Plasma membrane stain, DAPI, and Hoechst were obtained from Invitrogen.

**Cell Culture and Stimulation**

BMDMs were prepared by culturing bone marrow cells in the presence of supernatants from L929 cells for 8 days. On day 8, BMDMs were harvested and plated into 12-well plates for immunoblotting. BMDMs were prepared by culturing bone marrow cells in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech 31503) for 7 days. On day 7, BMDMs were harvested and plated into 12-well plates for image-based flow analysis. In order to examine inflammasome activity in primary lung fibroblast, mouse lungs were excised, washed in PBS, cut into small pieces, agitated, and digested enzymatically for 30 min at 37°C. The digestion buffer (10 ml/lung) was composed of 0.25% trypsin solution containing 400 nM EDTA. Ice-cold 10% FBS minimum essential medium (MEM) was added to the resultant cell suspension. After centrifugation (1,500 rpm, 5 min), pellets were resuspended in complete medium and then cultured on dishes. Lung fibroblasts were used for each experiment 10 days after preparation as described previously (Yamamoto et al., 2003).

For stimulation, BMDMs, BMDMs, or lung fibroblasts cells (1 x 10⁶ cells/well in a 12-well plate) were plated with ultrapure LPS 100 ng/ml (from Escherichia coli O111:B4; Invivogen) for 2 hr, followed by stimulation with nigericin (10 μM) for 1 hr or influenza A/PR8 (MOI = 2) for 4 or 16 hr. Cells were transfected with poly(dAdT) DNA through the use of Lipofectamine 2000 at a concentration of 1.5 μg/ml. IL-1β p17 and caspase-1 p20 immunoblots were conducted as described previously (Davaro et al., 2014) with antibodies from Abdirgen (caspase-1 p20) and R&D Systems (IL-1β). The antibodies against I-actin were from Sigma. Speck formation was assessed by confocal microscopy after stimulation, and cell-free supernatant was collected after 24 hr of stimulation and analyzed for IL-1β by ELISA or western blot.

**In Vivo and In Vitro Infections**

Influenza A/PR8 virus for the in vivo infection was purchased from Charles River Laboratories. For intranasal infection, mice were fully anesthetized by isofluorane and then infected by intranasal application of 30 μl of virus suspension (40,000 plaque-forming units [PFUs] in PBS). This procedure leads to the upper and lower respiratory tract infection. For in vitro infection, influenza A/PR8 virus was grown in Madin-Darby canine kidney (MDCK) cells. Supernatants were harvested when at least 75% of the cell monolayer exhibited a cytopathic effect, and standard plaque assays were used to quantify the amount of influenza viruses in the supernatants. The other viruses used in these experiments, including VSV, MCMV, and HSV, were the gifts of by Dr. Katherine Fitzgerald (University of Massachusetts Medical School). For GBS infection, the WT GBS strain (NEM316) was cultured in BBL media (purchased from BD) overnight, and 2 x 10⁷ CFUs of GBS was intravenously injected. Spleens were harvested and embedded in optimal cutting temperature (OCT) medium to prepare frozen sections. Homogenates from spleens were used for bacteria counts and IL-1β measurement.

**Immunoblot Analysis**

BMDMs or primary lung fibroblasts were harvested and lysed in lysis buffer (25 mM Tris-HCL [pH 7.4], 150 mM NaCl, 1 mM EDTA; 1% NP-40, and 5% glycerol) containing protease and phosphatase inhibitor “cocktails” (Roche). Protein concentrations were determined with the BCA Protein Assay Kit (Thermo Scientific). Equal amounts of protein were loaded onto 4%–20% gels (Bio-Rad), separated by electrophoresis, and then transferred to a nitrocellulose membrane. After nonspecific binding was blocked by incubation for 1 hr in 5% nonfat milk, the membranes were incubated overnight with primary antibodies (as described in the antibodies section) in 1% BSA. After three washes with Tris-buffered saline (TBS), the membranes were incubated for 1 hr with secondary antibodies. Finally, the signal was visualized with the SuperSignal West Dura Chemiluminescent Substrate (GE).

**ELISA**

Cell culture supernatants were assayed for IL-1β with ELISA kits from R&D Systems in accordance with the manufacturer’s instructions. Lung or spleen tissues were homogenized with a tissue lyser II (QIAGEN) for 1 min at a speed of 25/s. Homogenates were centrifuged at 16,000 x g for 10 min, and supernatants were collected for detecting IL-1β.

**Immunohistochemistry and Confocal Microscopy**

Isolated tissues were fixed for 4 hr in 4% (v/v) paraformaldehyde (PFA), followed by incubation in 30% (v/v) sucrose for dehydration. Tissues were then embedded in OCT medium, frozen, and cut into 7-μm thick sections. Sections were immunostained (antibodies identified above) and were imaged with a Leica SP8X laser-scanning confocal microscope.

**Flow Cytometry**

Blood samples from ASC-citrine/Cre+ mice were treated with heparin to prevent blood clotting and then red blood cell (RBC) lysis for flow cytometry analysis. BMDMs or BMDMs were treated with inflammasome stimulus and stained for antibodies. Cells were resuspended at a concentration of 10⁶/ml for running the Amnis brand FlowSight Imaging Flow Cytometer (hereafter...
Quantification of Speck-Forming Cells in Tissue

Sections from influenza A/PR8-infected lung tissue were imaged with the Leica SP8X laser-scanning confocal microscope. The tile function of the microscope was used to scan 10–20 fields per section. The average number of specks per field of view was counted.

Quantification of Free ASC Specks

Peritonitis was induced in ASC-citrine/Cre+ mice in the WT or caspase-1+/− background by the intraperitoneal (i.p.) injection of 3 mg/kg of nigericin for 90 min. Animals were euthanized by CO2 exposure, and their peritoneal cavities were lavaged with 6 ml of RPMI with 10% fetal calf serum (FCS) containing 3 mM EDTA and 10 U/ml heparin. The cells collected in these exudates were stained with monocolonal antibodies (mAbs) CD11b APC-Cy7, Ly-6G PE, and 7/4 APC for 30 min on ice in the presence of mAb 2.4G2 (FcγRIIB/III receptor blocker). Labeled cells were analyzed by flow cytometry for the detection of assembled specks. Supernatants from peritoneal exudates were washed through a 0.5 μm filter to eliminate cell and nuclear debris. The specks were concentrated from the supernatants by centrifugation at 21,000 × g for 15 min. Pellets were then resuspended in 200 μl of FACS buffer for FACS analysis. 150 μl of samples were analyzed by FACS, and the number of specks was compared by analyzing dot plots of FSC versus size scatter (SSC). For investigation of the oligomerization of extracellular ASC, the pellets from the supernatants of peritoneal exudate were washed with 0.5 ml CHAPS buffer and were chemically crosslinked for 30 min at room temperature with 2 mM DSS (Pierce), prior to immunoblot analysis.

Statistics

Values are expressed as mean ± SEM. Statistics were calculated with Prism software (GraphPad). For two-group comparisons, a two-tailed unpaired t test was used. Comparisons of multiple groups were analyzed by one-way ANOVA with Bonferroni’s multiple-comparison test or Dunnett’s test for comparison of all groups with the control group. We find that the variance from the compared groups does not present statistically significant differences.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.06.011.

AUTHOR CONTRIBUTIONS

T.-C.J.T. designed and performed the experiments and analyzed the data. S.S. and A.C. performed influenza A/PR8 and GBS injections and helped with some experiments. B.M. performed experiments and provided critical suggestions and discussion. D.W. designed the reporter constructs and provided critical suggestions and discussion throughout the study. K.F. provided critical suggestions and discussion. D.T.G. and E.L. designed the study. T.-C.J.T. designed and performed the experiments and analyzed the data.

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