A Novel Flow Cytometric Method To Assess Inflammasome Formation


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A Novel Flow Cytometric Method To Assess Inflammasome Formation

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Inflammasomes are large protein complexes induced by a wide range of microbial, stress, and environmental stimuli that function to induce cell death and inflammatory cytokine processing. Formation of an inflammasome involves dramatic relocalization of the inflammasome adapter protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) into a single speck. We have developed a flow cytometric assay for inflammasome formation, time of flight inflammasome evaluation, which detects the change in ASC distribution within the cell. The transit of ASC into the speck is detected by a decreased width or increased height of the pulse of emitted fluorescence. This assay can be used to quantify native inflammasome formation in subsets of mixed cell populations ex vivo. It can also provide a rapid and sensitive technique for investigating molecular interactions in inflammasome formation, by comparison of wild-type and mutant proteins in inflammasome reconstitution experiments. *The Journal of Immunology, 2015, 194: 455–462.

Inflammasomes recruit and activate procaspase-1, which subsequently processes the proinflammatory cytokines IL-1β and IL-18 prior to their release from cells, and also initiates a rapid lytic form of cell death termed pyroptosis (1–3). Recent work has shown that inflammasome formation can also lead to the recruitment and activation of procaspase-8, leading to initiation of apoptotic cell death (4, 5). There is great interest in inflammasome processes, due to their relevance to a wide range of diseases, including gout, atherosclerosis, Alzheimer’s disease, and type II diabetes (6–9).

Formation of inflammasomes is initiated by the induced oligomerization of one of a number of Nod-like receptor proteins such as NLRP3 or NLRC4, or by absent in melanoma 2 (AIM2). NLRC4 is activated by cytosolic flagellin, AIM2 by cytosolic DNA, and NLRP3 by diverse stimuli, including extracellular ATP, the K+ ionophore nigericin, and a range of particulate substances, such as monosodium urate crystals, cholesterol crystals, β-amylloid, and alum (1). A central factor in inflammasome structures is the adapter molecule, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). ASC consists of two domains of the death-fold superfamily, a pyrin domain (PYD) and a caspase recruitment domain (CARD), which are both involved in self-association of ASC within the inflammasome (10). The oligomerization of ASC provides a platform for recruitment of caspases; procaspase-1 is recruited via homotypic CARD-CARD interactions, and procaspase-8 via the PYD of ASC (5).

ASC was first observed as a protein forming a prominent speck in tumor cells undergoing chemotherapy-induced apoptosis (11). This ASC speck is a distinguishing feature of inflammasome activation. ASC is diffuse throughout the cytoplasm and nucleus of resting macrophages. Upon treatment with inflammasome stimuli such as the NLRP3 agonist nigericin, a potassium ionophore, ASC rapidly relocates to form the inflammasome speck. This appears to be an “all or nothing” response, with most of the ASC in the cell accumulating in the speck within minutes of initiation (12). We have taken advantage of this rapid and striking relocalization of ASC to develop a flow cytometric assay for inflammasome activity. This technique provides a direct and quantitative measure of early events in inflammasome formation.

Materials and Methods

Materials

ATP, nigericin, and LPS (L9764, Salmonella minnesota) were purchased from Sigma-Aldrich. ATP was reconstituted in water at 150 mM and used fresh or after no more than one freeze-thaw cycle. Nigericin was reconstituted in ethanol at 5 mM and stored at 4°C. LPS was prepared as a 10 mg/ml stock in PBS/0.1% triethylamine and sonicated before freezing of stocks. Alum was purchased from Pierce Biotechnology. Abs used were as follows: rabbit polyclonal anti-ASC (N-15)-R (Santa Cruz Biotechnology), Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Life Technologies), PerCP rat PE-conjugated anti-mouse CD19 (1D3) (BD Pharmingen), rat PE-conjugated anti-mouse CD19 (1D3) (BD Pharmingen), and allophycocyanin-conjugated F4/80 (BMS) (eBioscience). RPMI 1640, DMEM, FCS, penicillin/streptomycin, GlutaMAX, HEPES, and propidium iodide (PI) were purchased from Life Technologies.

Culture, inflammasome activation, and fixation of mouse macrophages

Mice were used under approval from a University of Queensland Animal Ethics Committee. Bone marrow–derived macrophages (BMMs) were ob-
tained from wild-type C57BL/6, Casp1<sup>−/−</sup> (13), Asc<sup>−/−</sup> (14), and Nlrp3<sup>−/−</sup> (6) mice. BMMs were differentiated in CSF-1 for 7 d, as described previously (15). BMMs, in RPMI 1640 with 10% heat-inactivated FCS (HI-FCS), 50 U/ml penicillin, and 50 µg/ml streptomycin, 1× GlutaMAX, and 25 mM HEPES (referred to as complete RPMI 1640), were treated with 10 ng/ml LPS for 4 h at 37˚C (unless otherwise stated in figure legends), and then harvested from 100-mm-square bacteriological plates (Sterilin) using PBS, and resuspended in complete RPMI 1640 lacking HI-FCS (2×10<sup>6</sup> cells/ml). A total of 1×10<sup>6</sup> cells in polypropylene tubes was treated with either nothing, 5 mM ATP, or 10 mM nigericin and incubated at 37˚C for 15 min prior to fixation. Although activation was done under serum-free conditions, inclusion of 10% HI-FCS did not affect the efficiency of staining. However, serum caused a delay in the response to nigericin, but not ATP, both for ASC speck formation and cell death, and longer incubations were necessary in the presence of serum. Cells were fixed by addition of 4 vol of 100% ethanol, followed by incubation at room temperature for 15 min. Fixed cells were pelleted at 600×g for 10 min, and resuspended as outlined below. Procedures were designed to avoid cell harvesting and repeated centrifugation after inflammasome triggering, due to the fragility of pyroptotic cells. However, extended incubation in polystyrene tubes can lead to loss of cells by adherence.

**Production of Casp1<sup>−/−</sup> immortalized BMM–mouse ASC–enhanced GFP**

Casp1<sup>−/−</sup> immortalized BMMs (5) were stably transfected with pEF6–mouse ASC–enhanced GFP (EGFP) (mouse ASC with a C-terminal EGFP fusion), and clones were established by single-cell deposition using a FACS Aria II.

**Activation of BMMs with alum and DNA**

For activation with alum, BMMs were primed with 10 ng/ml LPS for 3 h in complete RPMI 1640 and then treated for an additional 3.5 h with either nothing, 5 mM ATP or 10 µM nigericin and incubated at 37˚C for 15 min prior to fixation. 

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**Table I. Comparison of percentage of Casp1<sup>−/−</sup> BMM with ASC specks as determined by flow cytometry and microscopy**

<table>
<thead>
<tr>
<th>Method of Analysis</th>
<th>0</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>50:50% Mix of 0 and 10 µM</th>
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<td>11.3</td>
<td>39.4</td>
<td>70.7</td>
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<tr>
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<td>40.6</td>
<td>68.7</td>
<td>44.6</td>
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<tr>
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<td>10.1</td>
<td>34.0</td>
<td>63.4</td>
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</tr>
<tr>
<td>Accuri C6</td>
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<td>9.9</td>
<td>41.2</td>
<td>70.2</td>
<td>45.7</td>
</tr>
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</table>

LPS-primed cells were treated with the indicated dose of nigericin for 15 min. Samples were analyzed by the cytometers listed and by fluorescence microscopy.

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**FIGURE 1.** Principle and use of TOFIE for detection of native ASC speck formation in primary mouse BMMs. (**A**) Fluorescence microscopy of ASC specks in LPS-primed Casp1<sup>−/−</sup> BMMs, either resting or activated with nigericin. Cells were stained for ASC (green), and nuclei with DAPI (blue). Scale bar, 20 µm. (**B**) The shape of the fluorescence pulse detected by a flow cytometer as a cell moves through the laser beam depends on fluorophore distribution within the cell. Diagram of theoretical fluorescent emission profiles for uniform ASC staining and speck formation. (**C**) Analysis of LPS-primed Casp1<sup>−/−</sup> BMMs either untreated or treated with 10 µM nigericin for 15 min and immunostained for ASC, on a BD FACS CantoII cytometer showing a population with a low W:A profile for staining of ASC in activated cells, and (**D**) analysis on an Accuri C6 displaying a population with a high H:A ratio for fluorescent staining of ASC in activated cells. (**E**) Stable expression of mouse ASC-EGFP in a Casp1<sup>−/−</sup> immortalized BMM clone, showing ASC-EGFP expression in live PI-negative cells, and (**F**) TOFIE analysis of LPS-primed (100 ng/ml LPS 3 h) mouse ASC-EGFP<sup>+</sup> cells that had been treated with either nothing or 10 µM nigericin for an additional 90 min.
complete RPMI 1640, as outlined previously (5), with or without 10 μg calf thymus DNA, washed immediately following electroporation, and incubated for an additional 45 min. Cells were then fixed and pelleted as above.

**Infection of BMMs with Salmonella**

BMMs were primed with 10 ng/ml LPS for 4 h in complete RPMI 1640 lacking penicillin and streptomycin and harvested, and 4 × 10^6 cells were exposed to *Salmonella enterica* (SL1344) at a multiplicity of infection of 10 in a volume of 50 μl, centrifuged at 700 × g for 10 min, and then incubated at 37˚C for an additional 5 min. Following this, cells and bacteria were resuspended and incubated for an additional 20 min at 37˚C, after which cells were resuspended in 2 ml complete RPMI 1640 lacking HI-FCS, penicillin, or streptomycin, but supplemented with 50 μg/ml gentamicin and incubated at 37˚C for an additional 90 min. BMMs were fixed and pelleted, as above.

**Activation of resident peritoneal cells ex vivo**

Resident peritoneal cells were obtained by lavage with ice-cold Ca^2+/-Mg^2+/-free PBS and primed with 10 ng/ml LPS for 4 h in complete RPMI 1640 at 37˚C. Cells were then harvested and treated in complete RPMI 1640 lacking HI-FCS with either nothing or 10 μM nigericin for 30 min, after which cells were fixed and pelleted, as above.

**Preparation of PBMCs**

Blood was obtained with informed consent from healthy volunteers under approval of the University of Queensland Human Research Ethics Committee. A total of 90 ml fresh blood was diluted with 210 ml sterile PBS. A quantity amounting to 12.5 ml Ficoll-Paque PLUS (GE Healthcare) was added to 8 × 50-ml tubes, and then 37.5 ml diluted blood was carefully layered on top. Tubes were centrifuged for 45 min at 400 × g, room temperature, no brake, and minimum acceleration. The interface containing the PBMCs was removed and transferred into eight new tubes. PBS was added to the tubes to 50 ml and centrifuged for 10 min, 400 × g, 10˚C with the brake on. The pellet was resuspended in PBS, resup in four tubes of 50 ml, and repeated after pooling cells into one tube. Cells were pelleted and resuspended in complete RPMI 1640.

**Treatment of PBMCs**

A total of 750,000 unprimed and primed PBMCs (100 ng/ml LPS for 4 h at 37˚C) was treated with either nothing for an additional 20 min or 5 mM ATP at 37˚C. Cells were fixed with paraformaldehyde (PFA; Sigma-Aldrich) at 1% final concentration rather than ethanol to facilitate sur-
face staining of the myeloid marker CD14. Whereas both ethanol and PFA fixation permit analysis of ASC specks in human PBMCs, ethanol generally gave superior results. Cells with PFA were incubated for 5 min on ice, and then 1 ml PBS/0.1% sodium azide/0.1% BSA/1% HI-FCS was added and cells were pelleted at 500 × g for 5 min and resuspended as outlined below.

**Immunostaining cells for ASC for flow cytometric analysis**

Supernatant was gently removed from pelleted fixed cells, and cell pellets were resuspended in 250 μl PBS/0.1% sodium azide/0.1% BSA/3% HI-FCS (supplemented with 0.1% saponin for samples fixed with PFA). For mouse cells, 10% (v/v) of 2.4G2 hybridoma supernatant (anti-mouse FcRγII/III) was included and samples were incubated for 20 min. Rabbit anti-ASC (N15)-R was added to samples in an additional 250 μl to achieve a final dilution of 1:1500 and incubated for 90 min, followed by addition of 1 ml PBS/0.1% sodium azide/0.1% BSA/1% HI-FCS, and cells were pelleted at 600 × g for 10 min. Supernatant was removed, and cells were resuspended in 100 μl 1:1500 Alexa Fluor 488 goat anti-rabbit IgG in PBS/0.1% sodium azide/0.1% BSA/3% HI-FCS (supplemented with 0.1% saponin for samples fixed with PFA) and incubated for 45 min. Where lineage-specific Abs were used, they were included at this stage with the secondary Ab. CD14 was used at manufacturer’s recommendations, whereas F4/80 and Abs were used, they were included at this stage with the secondary Ab.

**Plasmids used for transfections**

Plasmids used included pEF6 mammalian expression vector (Life Technologies), pEF6-mouse ASC–EGFP (mouse ASC with a C-terminal EGFP fusion), pEF6–human ASC–EGFP (human ASC with a C-terminal EGFP fusion), pEF6-mouse AIM2, pCDNA3.1 expression vector, pCDNA3.1-human AIM2 (provided by R. Johnstone, Peter MacCallum Cancer Centre, Melbourne, VIC, Australia), and pCDNA3.1–human AIM2–PYD (F27A,F28A) (human AIM2 with F27A and F28A point mutations).

**HEK293 cell transfection**

HEK293 cells (350,000) were seeded into 24-well plates (Corning) in 1 ml DMEM with 4.5 g/L glucose, 110 mg/L sodium pyruvate, Glutamax-1, and 10% HI-FCS, and cultured overnight until ~90% confluency. Transfections for each well were conducted using 2 μl Lipofectamine 2000 (Life Technologies) in 50 μl additive-free DMEM, and a total of 1600 ng Qiagen Endo-free Maxi-Prep DNA in 50 μl additive-free DMEM prepared as per manufacturer’s instruction. When pEF6 and pCMV expression vectors (pCDNA3.1) did not fully account for 1600 ng DNA, empty pEF6 expression vector was added to make the final amount of DNA 1600 ng. All assays used 100 ng ASC-EGFP expression vector, unless otherwise indicated. Prior to transfection, culture volume was reduced to 100–150 μl, and 100 μl transfection mix was added immediately, followed by plate centrifugation at 1000 × g for 10 min to enhance transfection. Transfections were then routinely allowed to proceed for an additional 16–24 h before cells were harvested with PBS, counterstained with 1 μg/ml PI to discriminate dead cells, and analyzed by flow cytometry.

**Protein extracts and immunoblot analysis**

Protein extracts and immunoblot analysis were conducted, as previously described (16). AIM2 was detected using the anti-AIM2 MAb 3B10 (17), and loading was assessed with immunoblotting for tubulin using anti-α-tubulin, clone B-5-1-2 (Sigma-Aldrich).

**Flow cytometry**

Flow cytometers used were a BD Accuri C6 equipped with a 488 nm laser and 530/30 nm, 585/40 nm, and 670 nm LongPass filters; a BD FACSCantoII equipped with a 488 nm laser and 530/30 nm, 585/42 nm, and 670 nm LongPass filters and a 633 nm laser and 660/20 nm filter; a BD LSRII using the 488 nm laser and 530/30 nm laser; and a Beckman Coulter Gallios equipped with a 488 nm laser and 530/30 nm filter. Analysis was conducted with either Kaluza version 1.2 (Beckman Coulter), FlowJo 10.X.7 (Tree Star), or FACSDiva 6.1.3 (BD Biosciences). Samples were gated to exclude debris (forward light scatter [FSC]-area versus side scatter-area), and then any cell doublets were excluded using FSC-area versus FSC-width analysis. For HEK293 cells, only ASC-EGFP+ PI− (or free EGFP+ PI−) cells were gated and used for further analysis of inflammasome activation state by either pulse width to pulse area profile (W:A) or high pulse height to area (H:A) analysis. Sorting was conducted on a BD Aria using the 488 nm laser and 530/30 nm filter running at 20 μl using a 100 μm nozzle with a detailed overview presented in Supplemental Fig. 2.
Immunofluorescence microscopy for detection of ASC

For Fig. 1A, Casp1−/− BMMs grown on glass coverslips were treated for 4 h with 10 ng/ml LPS prior to inflammasome induction using 5 μM nigericin for 45 min. The cells were fixed with 1% PFA, permeabilized, and stained for ASC using rabbit polyclonal anti-ASC (N-15) (Santa Cruz Biotechnology) and nuclei stained with DAPI, all as described previously (5). To assess the number of cells with ASC specks in samples used in flow cytometric analysis in Table I, a sample was taken and counterstained with PI, to identify all cells, and fluorescence microscopy images were captured for EGFP and PI. Images of each field of cells were taken in several focal planes to ensure that specks were found in focus. Specks were manually counted as a percentage of cell nuclei from the images. A total of 250 cells was included in each analysis and counted in a blinded manner.

Results

Principle and validation of time of flight inflammasome evaluation for inflammasome detection

The flow cytometric assay described in this work is dependent upon the rapid relocalization of ASC within the cell upon inflammasome activation (Fig. 1A). We expected a reduction in the fluorescent pulse width, with a concomitant increase in the pulse height (Fig. 1B). To investigate this, LPS-primed BMMs were treated with or without nigericin for 15 min and then fixed with ethanol and stained for ASC under conditions demonstrated to be specific for ASC (Supplemental Fig. 1A). On cytometers capable of evaluating pulse width in fluorescence channels (BD FACScan-II, BD LSRII, and BC Gallios), a distinct population exhibited a substantially decreased pulse width to pulse area profile (W:A) (Fig. 1C, Supplemental Fig. 1B, 1C), and pulse width to pulse height profile (data not shown), in nigericin-stimulated samples. Analysis on a machine lacking detection of pulse width in fluorescence channels (BD Accuri C6) effectively discriminated activated cells with a H:A profile (Fig. 1D). The total amount of ASC stained per cell (pulse area) was higher in cells with specks (Fig. 1C, 1D), which appears to be due to more efficient ethanol fixation and retention of ASC when in a compact speck, than in the diffuse state in the untreated cells. PFA fixation gave greater retention of diffuse ASC in the cell, but ethanol fixation provided the best resolution of the two populations (data not shown). To highlight the time-of-flight principle underlying this assay, we engineered immortalized BMMs to express a fusion protein of mouse ASC-EGFP (Fig. 1E) that allows direct analysis and avoids the issue of differential retention of ASC following fixation and intracellular immunostaining. Exposure of these cells to LPS followed by nigericin resulted in a defined population with similar levels of ASC-EGFP but with clearly increased H:A ratio (Fig. 1F).

Validating the assay for staining native inflammasomes, the quantitative results obtained using various cytometers were very similar to results from microscopy with manual counting of inflammasomes (Table I). We also flow sorted the speck-containing (low-width) and speck-negative (high-width) populations and subsequently reanalyzed them on different flow cytometers to confirm that the low width population and high H:A populations are equivalent (Supplemental Fig. 2). As a final val-

*FIGURE 5.* Detection of ASC specks by TOFIE as a tool for studying inflammasome activation in minor cell populations. PFA-fixed and stained human PBMC samples were analyzed on a BD CantoII. (A) Cells were first gated using FSC-area versus side scatter-area to exclude debris (black events). (B) Doublets were then excluded (red events) using FSC-area versus FSC-width profile characteristics. (C) PBMC samples gated as per (A) and (B), stained with secondary Ab alone acting as a negative control to define ASC expression indicated by the marker boundary. (D) PBMC samples gated as per (A) and (B), stained for ASC, which demonstrated intermediate (green) and high (blue) ASC-expressing cells. The percentage shown is the percentage of high ASC-expressing cells (blue), within the total cells (blue and green). (E) Costaining with anti-CD14 revealed the majority of high ASC-expressing cells (blue) coexpressed high levels of the monocyte marker CD14. (F) Detection of ASC specks in human PBMCs from two donors either left untreated (control), or treated ex vivo with LPS for 4 h, followed by ATP for 15 min. Colors define intermediate ASC-expressing (green), high ASC-expressing (blue), and high ASC-expressing cells with an ASC speck (low W:A profile, purple). The percentage of ASC-high cells forming specks is indicated.
We examined responses in LPS-primed BMMs from mice deficient in inflammasome pathway components. The high H:A population indicative of cells with an inflammasome was noted in nigericin-stimulated wild-type and caspase-1–deficient BMMs, but not in ASC- and NLRP3-deficient cells (Fig. 2A). Efficient induction of ASC specks required LPS priming, as expected (18) (Fig. 2B). The formation of functional inflammasomes under these conditions was confirmed in wild-type cells by rapid pyroptotic death, whereas BMMs deficient in either ASC, caspase 1, or NLRP3 remained viable (Supplemental Fig. 3). The reproducibility of the assay within an experiment was confirmed by independent treatment, staining, and analysis of quadruplicate samples (Fig. 2C). Thus, although the level of specks sometimes differs between experiments (comparing Fig. 2A and 2C), this just reflects the biological response, affected by factors such as cell condition.

Detection of inflammasome formation in response to particulate NLRP3 agonists and AIM2 and NLRC4 activators

To confirm the utility of this assay to detect activation with particulate NLRP3 agonists, we exposed mouse BMMs to alum, which resulted in a population of cells with a high H:A profile, indicative of ASC specks (Fig. 3A). The assay was also applied to treatments that activate either AIM2 or NLRC4. Time of flight inflammasome evaluation (TOFIE) of BMMs that had either been electroporated with DNA or infected with Salmonella enterica Typhimurium revealed cells containing ASC specks (Fig. 3B, 3C).

Detection of inflammasome formation in lineage-stained cell subpopulations

One application of TOFIE is the definition of inflammasome-competent cells in populations of mixed cell types. Using ex vivo inflammasome activation, resident peritoneal cells were collected, LPS primed, and either left untreated or exposed to nigericin. Cells were immunostained for ASC in combination with the macrophage marker F4/80 and B cell marker CD19 (Fig. 4A). CD19+ cells stained moderately for ASC, but did not form ASC specks after treatment with nigericin (Fig. 4B). However, the vast majority of F4/80high cells displayed a striking transition to the high H:A profile. F4/80int cells displayed some propensity to form ASC specks, and a small, yet to be defined, population of cells within the F4/80neg/CD19neg population was capable of inflammasome formation. These data demonstrate the power of TOFIE in identifying inflammasome-competent cells from within mixed populations, and may allow the rapid identification of cell types not formerly recognized to be capable of forming inflammasomes.

Analysis of ASC speck formation in subsets of human PBMCs

Immunostaining of native ASC in human PBMCs revealed intermediate and high ASC-expressing cells, with the vast majority of the latter expressing the monocyte marker CD14 (Fig. 5A–E). We examined speck formation in these populations of ASC-expressing cells, stimulated ex vivo with and without LPS and ATP (Fig. 5F). CD14 could not be used as a marker to define speck-containing monocytes, as it was lost from the stimulated cells (data not shown). Analysis revealed that only the predominantly monocytic ASC-high cells formed an ASC speck. The vast majority of CD14-negative PBMCs expressed moderate levels of ASC (Fig. 5E) but did not form inflammasome specks with a NLRP3 stimulus (Fig. 5F). The moderate staining of ASC in CD14-negative cells, which within PBMCs are predominantly T lymphocytes, is likely to be bona fide given that a comparison of staining of mouse wild-type and ASC knockout cells confirms that lymphocytes express intermediate levels of ASC (data not shown) and a functional role for ASC has recently been revealed in human T lymphocytes (19).

Analysis of inflammasome reconstitution in HEK293 cells

In addition to staining native ASC, this system can be exploited for convenient and quantitative analysis of reconstituted inflammasomes in HEK293 cells. In cells transfected with an ASC-EGFP

**FIGURE 6.** Analysis of spontaneous and AIM2-induced ASC speck formation in HEK293 cells using TOFIE. (A) Spontaneous ASC speck formation occurs at a defined threshold of expression in the HEK293 reconstitution model. Analysis of mouse ASC-EGFP+ PI− cells after transfection with 25–400 ng expression vector revealed a threshold of expression for speck formation (high H:A profile) shown by the gray vertical line. (B) HEK293 cells transfected with 200–400 ng plasmid encoding EGFP (pEF-d2GFP or pEGFPN-1) or mouse and human ASC-EGFP-fusion proteins (pEF6-mouse ASC–EGFP and pEF6-human ASC–EGFP) to achieve similar levels of expression. Cells were subsequently gated for EGFP+ PI− HEK293 cells and analyzed for low W:A profiles (boxed) indicative of ASC specks. (C) Coexpression of mouse AIM2 with mouse ASC-EGFP (100 ng both plasmids) decreases the ASC expression level at which specks form (gray dashed line).
expression plasmid, spontaneous ASC speck formation was highly probable once cells reached a critical threshold of expression (gray line in Fig. 6A). A similar observation was made when expression of either mouse or human ASC-EGFP was analyzed by W:A, whereas EGFP alone did not show this transition to a low W:A profile with high levels of expression (Fig. 6B). The spontaneous ASC speck formation is truly dependent on the level of ASC, as these cells are not fixed and permeabilized. This effect should not be confused with the apparent higher level of ASC in native speck-containing cells (Fig. 1C, 1D), which, as noted earlier, is largely an effect of ethanol fixation. Transient coexpression of the inflammasome initiator AIM2 and its presumed recognition of the encoding plasmid DNA have been shown by microscopy to initiate ASC speck formation in HEK293 cells (20). Analysis by TOFIE revealed that AIM2 coexpression increases the fraction of cells containing specks by decreasing the threshold of ASC concentration at which speck formation occurs (gray to dashed gray line, Fig. 6C).

When using transient expression to reconstitute the inflammasome response, the level of ASC expression and hence the percentage of cells forming specks spontaneously may vary between samples. Using microscopy, there is no means to eliminate variable spontaneous speck formation from the analysis. Using flow cytometry, we can analyze data in a window below the threshold of ASC expression at which specks tend to form spontaneously, and also exclude poorly transfected cells in which ASC levels are not sufficient to permit speck formation. This minimizes effects of variable transfection efficiency and the effects of any subsequent cell treatments that alter the expression of the ASC plasmid, as well as reducing background speck numbers. Using this approach of selecting a window of moderate ASC expression, we analyzed interactions between AIM2 and ASC involved in ASC speck formation. Increasing amounts of human AIM2 cotransfected with human ASC-EGFP revealed dose-dependent induction of ASC specks (Fig. 7A, 7B, Supplemental Fig. 4). Previous work showed by in vitro binding studies and yeast two-hybrid analysis that two phenylalanines of the human AIM2 PYD are important for the interaction with the PYD of ASC (21). In this study, we showed that AIM2 with F27A and F28A mutations could not initiate ASC clustering (Fig. 7A, 7B), although it was expressed at similar level to wild type (Fig. 7C). The advantage of selecting a window of moderate ASC expression to increase sensitivity of the assay is shown in Supplemental Fig. 4. This demonstrates the utility of this assay for molecular analysis of protein–protein interactions in inflammasome formation and is reproducible between independent experiments (Fig. 7D).

Discussion

In this work, we have described, validated, and demonstrated utility of a convenient flow cytometric assay for directly assessing formation of both native and reconstituted inflammasomes. The analysis of inflammasome formation is generally conducted by monitoring downstream processes and outcomes, including the assessment of caspase activity, cell death, or release of IL-1β. These methods are not direct assessments of the formation of an inflammasome structure, and in some cases can be outcomes of noninflammasome processes. Quantification of released IL-1β by ELISA is commonly used as an assay of inflammasome activity. This is confounded by the detection of unprocessed pro–IL-1β and can be misleading if cells have lysed without inflammasome activation. Immunoblot analysis is required to confirm cleavage to its bioactive form. In addition, IL-1β cleavage can occur by inflammasome-independent processes (22). Caspase-1 cleavage measured by Western blot provides good evidence for inflammasome activation, but is generally only semiquantitative. Analysis of caspase activity by means of fluorogenic inhibitor-based substrates is handicapped by the limited specificity of the substrates for caspase-1 (23) and the possibility of measuring other noninflammasome-related caspase or protease activities. Inflammasome activation leads to pyroptotic and apoptotic cell death, but only pyroptosis is unique to the inflammasome. Pyroptotic death can be used to measure inflammasome responses, but its differentiation from other cell-death modalities can be problematic. Determination of inflammasome activity is best done by a combination of techniques, and cells with gene knockouts are useful for confirming that responses require inflammasome function. The assessment of ASC specks in cells has the obvious advantage of being a direct measure of inflammasome formation, and detects an early step in inflammasome formation, at a single cell level.

Direct assessment of ASC speck formation in the past has been conducted by microscopy and counting of speck-containing cells. The flow cytometric assay TOFIE has a number of advantages over microscopic examination of cells for inflammasome specks, including the following: 1) provision of more accurate data by objective analysis of large populations; 2) ready assessment of...
inflammusome formation by a minority population of cells defined by lineage markers; 3) lack of laborious manual counting of samples; 4) no potential loss of populations of cells that may be less adherent to slides; and 5) ability to control for the effect of variable transfected ASC expression level on spontaneous speck formation in inflammasome reconstitution assays. The quantitative nature of this assay may be useful in other situations in which proteins undergo dramatic relocalization. Indeed, analysis of fluorescence pulse shape has also been used to determine aggregation of Huntington protein in cells (24).

The assay described allows rapid and direct quantification of native inflammasomes in cells stimulated in vitro or ex vivo, and convenient quantitative assessment of inducers and regulators of inflammasome formation. In combination with lineage markers, it facilitates detection of inflammasome formation in minor populations of mixed cell types. In addition, TOFIE provides a rapid quantitative technique for assessing molecular interactions governing inflammasome initiation, when using reconstitution of cells with wild-type and mutated proteins. TOFIE will be a sensitive and powerful assay for assessment of inflammasome function in health and disease.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

References
**SUPPLEMENTAL FIGURE 1.** Specificity of ASC immunostaining in mouse BMM, and ASC pulse width:pulse area analysis on additional flow cytometers. A) Wild-type (WT) or Asc<sup>−/−</sup> BMM were immunostained for ASC with anti-ASC and secondary antibody, or with secondary antibody alone, demonstrating specificity of ASC detection. B-C) Analysis of untreated (left panels) and 10 μM nigericin activated (right panels) LPS-primed Casp<sup>1<sup>−/−</sup></sup> BMM analysed on (B) BD LSRII or (C) Beckman-Coulter Gallios flow cytometers.
SUPPLEMENTAL FIGURE 2. Sorting regime for low pulse width:height profile cells on a BD FACS Aria II, and confirmation of identity of low pulse width and high pulse height populations. 

A) Gating within SSC-Height vs SSC-Width and FSC-Height vs FSC-Width plots was used to exclude doublets and debris. B) LPS-primed Casp1−/− BMM were left untreated or treated with 10 µM nigericin for 15 min, before fixation. A portion of the untreated and nigericin treated samples were mixed in a 1:1 ratio prior to immunostaining for ASC and sorting. Presumably due to higher operating pressure of the BD FACS Aria II, discrimination of low and high W:H (and W:A) profiles was not as effective as that observed on other cytometers. Nonetheless, gates were employed to sort high W:H cells (P4 Purple events) and low W:H (P3 Blue events). C) After two rounds of purification, samples were used to confirm that the low W:H profile cells were equivalent of the high H:A cells observed on the Accuri C6 cytometer.
SUPPLEMENTAL FIGURE 3. Formation of functional inflammasomes as measured by downstream pyroptosis. Unprimed and LPS-primed wild-type (WT), Casp1^−/−, Asc^−/− and Nlrp3^−/− BMMs were treated as in Figure 2 with either nothing (control), 5 mM ATP or 10 µM nigericin for 30 minutes and then analysed for cell death after addition of 1 µg/ml propidium iodide (PI), on a BD Accuri C6 flow cytometer. Wildtype, but not other cells formed a functional inflammasome leading to cell death measured by staining with PI.
SUPPLEMENTAL FIGURE 4. Increased sensitivity and decreased background, by elimination of spontaneous speck formation, is an advantage of TOFIE-based analysis of reconstituted inflammasomes in HEK293 cells. A) HEK293 cells were transfected as per Figure 5. Examination of cells transfected with hASC-EGFP alone (“Empty Vector” sample) allows a region (hASC-EGFP WINDOW) to be defined which excludes high hASC-EGFP expressing cells with spontaneous speck formation, and also cells with lower levels of hASC-EGFP never capable of forming an ASC speck. B) Analysis of the proportion of cells in high H:A form (ASC-EGFP speck) using only cells with intermediate expression of hASC-EGFP (defined by the hASC-EGFP WINDOW region in panel A). This demonstrates the processing of data for Figure 5A-B. C) Quantification of the proportion of cells with a high H:A profile (ASC-EGFP Speck) in either all hASC-EGFP+ cells, or cells with intermediate hASC-EGFP expression levels (hASC-EGFP WINDOW) as done in Figure 5B, demonstrates that analysis of the latter reduces the background level of specks, and increases sensitivity for detection of inflammasome responses. Data represent the mean and range of duplicate transfections within the same biological experiment; where error bars are not shown data falls within the symbol. In other experiments we have found that some conditions can change the level of ASC-EGFP expression, giving variation in the number of cells with spontaneous speck formation. Imposition of a window as described above eliminates this as a confounding variable.