From atoms to physiology: what it takes to really understand inflammasomes

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Abstract Rapid inflammatory responses to cytosolic threats are mediated by inflammasomes – large macromolecular signalling complexes that control the activation of the pro-inflammatory cytokines interleukin (IL)-1β and IL-18, as well as cell death by pyroptosis. Different inflammasome sensors are activated by diverse direct and indirect signals, and subsequently nucleate the polymerization of the adaptor molecule ASC to form signalling platforms macroscopically observed as ASC specks. Caspase-1 is autocatalytically activated at these sites and subsequently matures pro-inflammatory cytokines and the pore-forming effector molecule gasdermin D. While most molecules and basic assembly principles have been deduced from reductionist experimental systems, we still lack fundamental information on the structure and regulation of these complexes in their physiological environment and in the interplay with other signalling pathways. In this review, novel experimental approaches are proposed, including some that rely on nanobodies and single domain antibodies, to understand inflammasome assembly and regulation in the context of the relevant tissues or cells.

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Inflammasomes

The innate immune system employs a comprehensive selection of receptors and sensors to detect molecular signatures of infection or cell damage, and to mount an appropriate response. A particularly rapid and fierce reaction to cytosolic evidence of threats is orchestrated by inflammasomes – large cytosolic signalling complexes that coordinate maturation and secretion of pre-formed pro-inflammatory cytokines, often accompanied by an inflammatory type of cell death termed pyroptosis (Broz & Dixit, 2016). This response can occur in the absence of gene regulation and is only executed in specialized sentinel cells – often with systemic consequences. Inflammasome signalling is therefore distinct from the pro-inflammatory transcriptional responses elicited by activation of extracellular and intracellular sensors and transcription factors of the nuclear factor-κB (NF-κB), interferon regulatory factor (IRF) or signal transducer and activator of transcription (STAT) families in a broader range of cells.

In response to cytosolic insults, distinct cytosolic inflammasome sensors oligomerize and nucleate the polymerization of the adaptor protein ASC, which in tum recruits pro-caspase-1 (Broz & Dixit, 2016). Some inflammasome sensors bearing a caspase recruitment domain (CARD) can also directly recruit caspase-1, although it is not clear whether this also occurs in physiological situations. Caspase-1 is autoproteolytically activated by local enrichment. It then cleaves and activates pro-interleukin (IL)-1β and pro-IL-18, as well as gasdermin D (GSDMD), the effector molecule of pyroptosis. Cleaved GSDMD assembles into pores in the plasma membrane that facilitate the release of cytokines and ultimately result in cell rupture and release of further cell damage markers. Throughout this article, I will refer to any complex involving a sensor and (pro-)caspases-1 as an inflammasome, although the term itself is not coherently used in the literature. Once ASC is recruited by an inflammasome sensor, hundreds of ASC molecules polymerize into highly crosslinked structures to form 1–2 µm ASC specks (also referred to as ASC foci), which provide the local environment for caspase-1 processing, activation and perhaps regulation (Franklin et al. 2018). While inflammasomes can in principle be nucleated by binding of a single ligand to its sensor, their activation has to be tightly controlled to avoid unnecessary collateral damage (Strowig et al. 2012).

Sensitivity and specificity of the response is achieved by both intrinsic properties of the involved molecules, as well as regulation by post-translational modifications (PTMs) and additional factors (Latz et al. 2013). Aberrant inflammasome activation is involved in a growing list of autoimmune inflammatory conditions, including atherosclerosis, gout, diabetes, Alzheimer’s diseases as well as a number of monogenetic diseases (Strowig et al. 2012).

To fully understand the function of inflammasomes in the context of innate and adaptive immune responses and their contribution to pathological conditions, we need to (1) identify all ligands or triggers, sensors, scaffold proteins and effectors, as well as all their localization, co-factors, and positive or negative regulators; (2) understand the conformational changes and covalent and non-covalent modifications of all involved proteins that alter their properties to permit or fine-tune assembly; (3) reveal which cell types act as sentinel cells and assemble inflammasomes in response to pathogen-mediated or sterile stimuli; (4) elucidate how inflammasome signalling is cross-regulated with other pro- and anti-inflammatory signalling pathways in sentinel cells and the tissue context; and to (5) differentiate in which conditions inflammasome signalling directly affects the invading pathogen, and when the elicited responses orchestrate innate and adaptive immune responses by other cells or effector molecules.

What we know and remaining open questions

In the last two decades, most of the molecules involved in inflammasome signalling have been revealed and many basic principles that govern inflammasome assembly have been elucidated. Much of what we know has been learned from experiments in reductionist systems: the structure of the autoinhibited conformation of NLRC4 (Hu et al. 2013), of individual domains of other sensors (Bae & Park, 2011; Jin et al. 2013; Reubold et al. 2014), of the adaptor ASC (de Alba, 2009) and of caspase-1 (Walker et al. 1994; Wilson et al. 1994) have been solved using ectopically expressed, purified proteins and X-ray crystallography or nuclear magnetic resonance spectroscopy. Electron microscopy-based structures of oligomers or polymers of NAIP 2–NLRC4 (Hu et al. 2015; Zhang et al. 2015), ASC CARD (Lu et al. 2014), ASC CARD (Li et al. 2018) and caspase-1 CARD (Lu et al. 2016) have also been solved using purified protein complexes and have defined quaternary structures and polymerization interfaces. The minimal set...
of necessary proteins for inflammasome assembly has been defined using reconstitution experiments in cell types that do not naturally express any inflammasome components, such as epithelial HEK 293T cells (Martinon et al. 2002; Kofoed & Vance, 2011). Basic signalling cascades and regulatory mechanisms, as well as ligands, have been revealed by experiments in immortalized myeloid cell lines and in in vitro differentiated bone marrow-derived macrophages (Bauernfeind et al. 2009). Where adequate, these efforts have exploited the genome-wide or targeted depletion of factors on the genome or mRNA level (Shi et al. 2015; Schmid-Burgk et al. 2016). The role of inflammasomes in containing infection or causing pathological inflammation has mainly been established in inbred mouse strains challenged with defined pathogens, molecules, or genetic disease models. Such experiments were supported by strong mouse genetics and knockouts of the respective sensors, adaptors, effectors, cytokines or their receptors. In addition to these targeted experimental approaches, further insights were gathered from human patients, human genetics and cytokine signatures associated with known pathological conditions (Hoffman et al. 2001; Canna et al. 2014; Zhong et al. 2016).

The limitations of current approaches become apparent when considering what we still do not understand: while we know the core constituents of inflammasomes, new inflammasome sensors continue to emerge, in part because they are only expressed in specific tissues, cell types or states of a cell that cannot be recapitulated in cell lines. This is exemplified by the recent discovery of NLRP9(b) as a novel inflammasome sensor in intestinal epithelia (Zhu et al. 2017). For similar reasons, the identities of many regulators or co-factors of inflammasome assembly remain unknown or underexplored. Detailed understanding is complicated by the fact that regulatory factors may have tissue-specific functions or may only act in certain situations. The molecular nature of inflammasome activators has only been revealed in the case of AIM2 (double-stranded DNA) (Hornung et al. 2009), NAIP–NLRC4 (type 3 secretion system components and flagellin) (Zhao et al. 2011; Yang et al. 2013), and murine NLRP1b (cleavage by anthrax lethal factor) (Boyden & Dietrich, 2006), partly because many of them are not of proteinaceous nature, or because inflammasome sensors can be activated indirectly, e.g. by loss of homeostasis, rather than by a defined ligand. Direct or indirect ligands for NLRP6 (Hara et al. 2018) and NLRP9 (Zhu et al. 2017) have been proposed based on genetic data, although the reconstitution of inflammasomes from minimal components is pending.

While we know the structure of individual inflammasome components, we lack structural information on their heterologous interactions, native inflammasomes and ASC specks assembled in cells. The latter likely contain additional factors and may exhibit properties that are not faithfully recapitulated when reconstituted from purified components. As a matter of fact, the tendency of inflammasome components to auto-aggregate imposes a substantial challenge for ectopic expression and purification of homogeneous protein preparations, as well as the analysis of well-ordered oligomeric assemblies as they form in the context of the cell (Masumoto et al. 1999).

While lipids exposed on vesicles of the dispersed trans-Golgi network appear to provide the scaffold for NLRP3 inflammasome activation (Chen & Chen, 2018), it is unclear if other inflammasomes are nucleated by soluble monomers in the cytosol, or whether assembly is facilitated by enrichment of sensors on organelle surfaces – a step of activation that may have fundamental implications for its regulation.

It is well established that modification of inflammasome components with ubiquitin-like molecules and other PTMs is critical to control or fine-tune responses (Kattah et al. 2017). Yet, a growing number of seemingly contradicting findings illustrate that the commonly used approaches are not sufficient: most studies rely on the inhibition or depletion of enzymes with multiple functions, as well as the recapitulation of ubiquitination by overexpression of targets and enzymes in HEK 293T cells, or the analysis of cell lysates (Py et al. 2013; Rodgers et al. 2014; Song et al. 2016; Barry et al. 2018; Palazón-Riquelme et al. 2018). Although inflammasome assembly has been described as a step-wise process, we have very little information on the order of events as well as the intermediates of assembly, mostly because we lack the means to stabilize intermediates for more thorough analysis. Some inflammatory signalling pathways, including the activation of interferon and NF-κB responses, seem to positively or negatively regulate inflammasome assembly (Labzin et al. 2016). However, a clearer picture is confounded by the fact that cytokine levels in supernatants from many cells, or mRNA levels from pooled, lysed cells are typically analysed. In addition, strong artificial activators of inflammasomes in tissue culture or in vivo experiments may not recapitulate the more subtle and localized activation in physiological setting, where the activation of multiple pathways will be the rule rather than the exception. The identification of responding cell types and their cross-talk with other immune cells in living organisms is limited by what we can cultivate ex vivo, and by the transient nature of events and the complexity of different cell types and transcriptional states in living organisms.

To gain further mechanistic insights, we must therefore overcome the limitations of the commonly used reductionist systems and develop new experimental systems and tools to asymptotically approach the situation in living organisms. As detailed in the following,
such efforts should include (1) structural information on protein assemblies in the native context of the cell, (2) better methods to detect post-translational modifications and binary interactions while maintaining relevant spatial information, and (3) detailed analysis of cell biological processes in the context of organoids or tissues that better reflect the heterogenous nature of multicellular organisms.

The ideal experiment should be able to track inflammasome assembly in response to natural triggers at endogenous protein levels in the relevant cell types, in which responses occur in the context of all regulatory factors. This demands experiments in untransformed primary cells and should not be limited to myeloid cells. The relevant cell types could be identified by investigating inflammasome assembly in tissues and organoids, which recapitulate the complexities of organs containing multiple cell types to a better extent. To resolve the rapid, multi-step assembly of inflammasomes and the subsequent effector mechanisms, we also need tools that specifically interfere with the individual steps – ideally in a spatio-temporally controlled manner. Such experiments should be extended to more species than merely mice. For practical reasons and to allow immediate transfer to patients, studies have to focus on the human, but should also include other vertebrates to understand common principles of inflammasome assembly. Such studies may thus include pathogen challenges that no longer occur in humans in industrialized societies, but substantially shaped the evolutionary development of our innate immune system.

This article will highlight some novel approaches to study inflammasome assembly and its regulation in experimental systems that better reflect the physiological context of the tissue. Many novel applications described here will benefit from the exceptional specificity and versatility of nanobodies derived from the variable domain of heavy chain-only antibodies found in camels (see Box 1) (Muyldermans, 2013; Helma et al. 2015; Ingram et al. 2018).

Cooperative assembly of inflammasomes – lessons from structural biology

The current model of inflammasome assembly is based on (1) structural information of individual inflammasome components and NAIP–NLRC4 ΔCARD oligomers activated by co-expression of ligands such as flagellin (Hu et al. 2013, 2015; Zhang et al. 2015), (2) in vitro polymerization experiments with individual domains of inflammasomes (Lu et al. 2014), and (3) over-expression experiments of inflammasome components in reconstituted systems (Martinon et al. 2002; Kofoid & Vance, 2011). This model implies that a ligand (or indirect signal) relieves the auto-inhibited conformation of an inflammasome sensor, allowing assembly of a ring-like oligomer with 11–13 monomers in case of NAIP–NLRC4 (see Fig. 1). This step is likely heavily regulated by expression levels, PTMs as well as other regulatory factors that all modulate the likelihood of assembly (Latz et al. 2013). The pyrin domains (PYDs) or CARDs of such an oligomeric sensor assemble into a small helical filament that then nucleates the polymerization of ASCPYD filaments. On their outer surface, individual PYD filaments expose the ASCCARD, which can undergo homotypic interactions that cross-link ASCPYD filaments to coalesce into the observed ASC specks (Dick et al. 2016; Schmidt et al. 2016b). Other free ASCCARD domains recruit and activate pro-caspase-1 in the ASC speck.

Detection of protein interactions in relevant cell types.

We do not know if all inflammasome sensor molecules have to be individually activated by a ligand or indirect signal to assemble in a functional oligomer, or whether a single activated monomer favours recruitment and conformational changes in other sensor molecules, as observed for NAIP–NLRC4 inflammasomes (Hu et al. 2015; Zhang et al. 2015). These unusual inflammasomes only contain a single bona fide receptor molecule, NAIP, which induces cooperative assembly of NLRC4 oligomers once a single molecule is activated by its ligand. We also do not know whether every sensor oligomer nucleates an inflammasome. While CARD-containing inflammasome sensors can directly recruit and activate pro-caspase-1 in the (artificial) absence of ASC (Broz et al. 2010; Van Opdenbosch et al. 2014), it is currently unclear if this interaction also occurs when ASC is present at sufficient levels. To answer these questions, we need to be able to detect sensor oligomerization, recruitment of pro-caspase-1, and perhaps even conformational changes of the involved molecules in situ.

One approach to reveal homotypic and heterogeneous oligomers is proximity ligation assays (see Fig. 2) – a microscopy-based method that only yields an amplified signal when two proteins are present in very close proximity (Soderberg et al. 2006; Leuchowius et al. 2011): Two antibodies modified with different oligonucleotides are used to stain their targets in fixed and permeabilized cells. If these target proteins are in close enough proximity, two additional oligonucleotides that are complementary to the oligos on the antibodies can assemble a nicked circular DNA structure, which is ligated, amplified, and detected with complementary fluorescent probes. This technique can reveal heterologous interactions (Fig. 2A) as well as homo-oligomers (Fig. 2B). While successfully applied to detect complexes of NLRP3 and ASC (Lo et al. 2013; Wu & Lai, 2016), or AIM2 and ASC (Dutta et al. 2015), the full potential of this technique has not yet been fully exploited, in part because it requires highly specific antibodies with
Box I. Nanobodies

In addition to conventional antibodies (top, left), all camelid species including alpacas and llamas produce heavy chain-only antibodies composed of two identical heavy chains (top, middle). The variable domains of heavy chain-only antibodies (VHH), also called nanobodies, can be expressed as stand-alone fragments (top, right) that are 10 times smaller and exhibit specificities and affinities comparable to conventional antibodies. The genetic information for nanobodies can be cloned from immunized animals and stored in plasmid libraries. Nanobodies with desired properties can be identified by phage display, yeast display, or using novel functional screens. Nanobodies can be easily produced in bacteria and modified for fluorescence and electron microscopy, as well as proximity ligation assays (bottom, left). Nanobodies fused to fluorescent proteins, other nanobodies, or enzymes can also be expressed in the cytosol, thus allowing visualization or manipulation of nanobody targets in living cells (bottom, right).

very low background. While oligonucleotide-modified secondary antibodies are commercially available, the stringency of detection (i.e. the required proximity) can be increased by using directly labelled antibodies, or even the 10 times smaller nanobodies (see Box I) (Ingram et al. 2018). Importantly, proximity ligation assays (PLAs) are suitable for primary cells or tissues, and do not require any genetic modifications. They will thus extend information that can be derived from bioluminescence resonance energy transfer-based reporters that, for example, evaluate NLRP3 conformations (Martín-Sánchez et al. 2016). Once a comprehensive collection of such reagents is established, all the molecular steps of inflammasome sensor oligomerization, recruitment of ASC, and further downstream effectors could be individually quantified. More importantly, PLAs would be suitable to test the contribution of different inflammasome sensors to ASC specks in tissues or primary cells. In cases where inflammasome assembly is observed by immunostaining, PLAs with nanobodies against ASC combined with reagents against each possible inflammasome sensors are expected to only yield a signal for the sensor that nucleates the inflammasome – a method that may well have value for the diagnosis of human inflammatory diseases.

Analyses of protein–protein interactions in situ will overcome limitations imposed by current immunoprecipitation studies, which rely on interactions that remain stable after substantial experimental manipulation. What is more, most immunoprecipitation experiments invoke the solubilization of all membranes, and are prone to artificial interactions caused by the loss of compartmentalization, exposure of hydrophobic surfaces, and artificial intermolecular disulfide bonds by reactive cysteines.
Detection of novel protein–protein interactions in relevant cell types. While PLAs will allow the sensitive detection of suspected protein–protein interactions, novel approaches will be required to define the interactome of inflammasome complexes and identify crucial components or regulatory factors that have escaped identification by classical methods such as genetic screens and immunoprecipitation experiments. Two proximity labelling methods allow the specific biotinylation of proteins in very close proximity to a protein of interest in living cells (Trinkle-Mulcahy, 2019). These approaches are particularly powerful as they preserve spatial information beyond cell lysis. Both rely on the expression of fusion proteins of a cellular protein to an enzyme that, temporally controlled, can biotinylate all proteins in very close proximity (Fig. 2D). Upon cell lysis, biotinylated proteins are purified under very stringent conditions, followed by identification by mass spectrometry. Promiscuous biotin ligases based on the bacterial biotin ligase BirA, including BioID and its improved derivatives TurboID and MiniTurbo, biotinylate proteins in close proximity after exogenous addition of excess biotin under mild conditions.

Figure 1. Mechanism of inflammasome assembly
Insights from structural biology (blue boxes) and fluorescence microscopy (green boxes) are highlighted; dashed boxes indicate steps that could be investigated in more detail as proposed in the text. A, auto-inhibited conformation of NLRC4 (PDB: 4KXF) determined by X-ray crystallography (Hu et al. 2013). B and C, quaternary structure of activated NAIP2–NLRC4 oligomer (B; PDB: 3JBL) (Zhang et al. 2015) and ASCPYD filaments (C; PDB: D3J63) (Lu et al. 2014) revealed by electron microscopy. D, endogenous ASC filaments indirectly visualized by ectopically expressed VHHASC–EGFP in human THP-1 cells. The nanobody masks ASCCARD and thus allows ASCPYD polymerization, but prevents ASCCARD-mediated cross-linking of filaments to form specks. E, ASC specks visualized with fluorescently labelled VHHASC. A–C were generated with NGL Viewer (Rose et al. 2018), D–E are from Schmidt et al. (2016b); scale bars represent 10 µm.
conditions that do not compromise viability (Roux et al. 2012; Branon et al. 2018). The engineered ascorbate peroxidase APEX2 can be temporally controlled by the addition of \( \text{H}_2\text{O}_2 \), and catalyses the oxidation of exogenously supplied biotin–phenol (Lam et al. 2014). This generates a short-lived radical that will only biotinylate molecules in close proximity to the enzyme, allowing similar purification strategies. Of note, APEX2 can also oxidize diaminobenzidine and thus deposit electron dense material suitable for detection by electron microscopy. Unlike biotin ligase derivatives, which biotinylate lysine side chains of proteins, phenoxyl radicals of biotin–phenol can in principle form covalent adducts with other side chains or molecules other than proteins, including nucleic acids. Both methods have been successfully applied to define the proteome of macroscopic structures such as nuclear pore complexes (Kim et al. 2014), or the time-resolved proteomic profile of \( \text{G} \) protein-coupled receptors at different critical time points after activation and subsequent relocation (Lobingier et al. 2017; Paek et al. 2017). These tools thus seem suitable to define the cellular proteins that interact with assembling, signalling or degrading inflammasomes. To avoid the risk of artifacts when endogenous inflammasome components are overexpressed, APEX2 and promiscuous biotin ligases could also be delivered as fusions to camelid nanobodies (Fig. 2D). Interestingly, nanobody mutants that are unstable in the absence of their target have been generated, and will thus specifically only deliver their enzymatic payload to sites where their endogenous targets are localized (Tang et al. 2016; Ariotti et al. 2018).

**Structural analysis of native inflammasome complexes.** While larger inflammasome-related structures solved in atomic detail typically contain few components or domains (Lu et al. 2014; Hu et al. 2015; Zhang et al.

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**Figure 2. Novel assays to probe spatial information in intact cells**

A–C, proximity ligation assay-based methods in fixed and permeabilized cells visualize close spatial proximity of two antibody or nanobody targets, thus revealing non-covalent binary interactions (A), sensor oligomerization (B), and covalent modification with ubiquitin-like molecules (C). D, nanobodies fused to derivates of APEX2 or promiscuous mutants of BirA covalently label molecules in close proximity to the nanobody target in living cells. Biotinylated proteins are subsequently purified under stringent conditions using streptavidin affinity matrices, and identified by mass spectrometry.
2015), they do not contain any combination of sensors with adaptors or other (regulatory) factors. We can therefore only speculate on the exact quaternary structure of naturally assembled inflammasomes. Complete assemblies of sensors, ASC, and pro-caspase-1 have not been subjected to structural analysis, as the inherent propensity of all components to aggregate or polymerize increases the likelihood of non-natural assemblies. It will therefore be crucial to visualize the endogenous structure of ASC foci or intermediates of inflammasome assembly. The revolutionary developments in electron microscopy in the last decade may render this scenario much more realistic in the near future (Asano et al. 2016; Beck & Baumeister, 2016): endogenous complexes in their native environment can be preserved in vitrified ice generated by plunge-freezing of cells into liquid ethane. Focused ion beam milling is able to generate sufficiently thin samples to record tilt series of inflammasomes by cryo-electron microscopy. From such datasets, the electron density in 3D, the tomogram, can be reconstructed. The quality of images will benefit from dramatically improved direct detection of electrons, as well as enhanced contrast by use of electron phase plates. Electron tomography is particular powerful for recording pleomorphic structures, as recently shown for the translocon (Pfeffer et al. 2015) or enveloped viruses (Grünewald et al. 2003). Identifying the object of interest in the cell will require fluorescent markers that guide sample preparation by correlative light and electron microscopy (CLEM). Detection of ASC specks could, for example, be achieved by incorporation of minimal amounts of fluorescent ASC monomers, or by recruitment of fluorescent co-factors or suicide substrates of caspase-1. Similarly, intermediates of inflammasome assembly with simpler structure could be observed. We have recently shown that expression of a nanobody against the ASC-CARD fused to enhanced green fluorescent protein (EGFP) stabilizes a filamentous intermediate of inflammasome assembly (Schmidt et al. 2016b). This construct likely masks the free CARD emanating from ASC-PYD filaments that are nucleated from NLRP3, NLRC4, or AIM2 oligomers. This prevents the further coalescence of individual ASC filaments into specks. The simpler and likely symmetric intermediate can be easily visualized by fluorescence microscopy and should allow the analysis of endogenous assemblies of inflammasome sensors and polymerized ASC by CLEM. Tomograms could be subjected to template matching algorithms to identify known repetitive structures, including oligomerized NAIP–NLRC4 or ASC-PYD filaments, for which higher resolutions structures are available. Structural analysis of endogenous inflammasome assemblies could reveal whether inflammasome sensors form ring or short filament structures, determine the stoichiometry of sensors and ASC, validate the postulated mode of nucleation, and reveal if the recruitment of ASC is different in the case of CARD- or PYD-containing sensors. Higher resolution structures of the ASC specks would reveal to what degree ASC-PYD filaments are cross-linked by ASC-CARD and whether higher order ASC-CARD assemblies are present. Importantly, such analyses may reveal the exact arrangement of ASC-CARD necessary to recruit and activate pro-caspase-1. Such studies could thus answer the open question whether dimers of pro-caspase-1 are simply recruited to two ASC-CARD in close proximity, potentially all over the surface of an ASC-PYD filaments, or whether small filaments of ASC-CARD nucleate pro-caspase-1 filaments, as postulated based on in vitro studies (Lu et al. 2014; Zhang et al. 2015).

Analysis of endogenous inflammasome structures will overcome the inherent difficulties of obtaining homogenous preparations of inflammasome components of sufficient quality. This will not only allow us to validate and refine current models, but will in itself inspire novel models or hypotheses. These approaches will also inform further structural investigations of sub-complexes with other methods, which can be fit into the low-resolution structures that are expected to be obtained from cells. Such endeavours may in the future benefit from cross-linking mass spectrometry methods, which can define maximal distances between inter- and intramolecular amino acid side chains and thus preserve information on protein interactions and protein conformations (Sin, 2018).

**Regulation of inflammasomes – lessons from cell biology**

Inflammasome assembly is tightly regulated by non-covalent interactions with regulatory proteins, and by covalent modifications with ubiquitin, small ubiquitin-like modifier (SUMO), or related ubiquitin-like proteins, as well as by inorganic moieties such as phosphate and acetyl groups (Latz et al. 2013; Kattah et al. 2017; Yang et al. 2017). Dephosphorylation, deubiquitination and desumoylation of inflammasome sensors, as well as phosphorylation and linear ubiquitination of ASC have been implicated in inflammasome assembly among other things, with often contradicting or conflicting data. While thousands of molecules of an inflammasome component may be present in a cell, the localized assembly of inflammasomes will only modify and activate some of them. Despite clear implication for activation, the location of inflammasome assembly itself also remains unclear for all sensors but NLRP3 (Chen & Chen, 2018). In addition, only a fraction of all cells in a stimulated culture typically respond to the ligand or trigger, thus increasing the percentage of molecules in an inactive state that are not relevant for activation. To evaluate the role of protein modifications in inflammasome regulation, the localization of the molecule in the cell should thus ideally
be preserved, and best be combined with visual markers for successful inflammasome assembly.

**Detection of post-translational modifications and subcellular localization by microscopy.** Microscopic methods retain spatial information and allow the cell-based quantification of inflammasome responses from small cultures of primary cells. Antibodies or other affinity reagents that specifically recognize the modified or unmodified form of a given target will in some cases be sufficient to visualize modified proteins and their location. Single domain antibodies may be a particularly worthwhile approach for such visualization efforts, as smart selection and counterselection strategies can be applied (Schmidt et al. 2016a; Ingram et al. 2018). Established screening methods such as phage and yeast display, as well as novel functional screening techniques, may identify affinity reagents specific for distinct protein modifications. The successful identification of single domain antibodies and conventional antibodies that report on the conformational and oligomeric states, as well as PTMs, attest to the versatility of such detection reagents (Zameer et al. 2008; Manglik et al. 2016; Hattori & Koide, 2018). To generate affinity reagents against distinct conformations or oligomeric states, point mutants of the respective proteins alone, or the proteins in complex with ligands can be used for immunization and selection. While modified peptides can easily be synthesized to elicit conventional antibodies, immunization to generate nanobodies will require intact proteins exhibiting desired post-translational modifications, relying on the in vitro modification with purified enzymes. Where the respective enzymes are not available or known, novel methods to generate artificially modified proteins using non-natural amino acids and semi-synthetic approaches to achieve site-specific ubiquitination or phosphorylation will prove useful (Stanley & Virdee, 2016; Mukai et al. 2017).

The number of modified target proteins will often not be sufficient for reliable detection with antibodies, demanding methods that include a more substantial amplification step. These may include, for example, tyramide amplification, in which horse-radish peroxidase-coupled secondary reagents catalyse the deposition of fluorophore-coupled tyramides in the microscopy samples (Faget & Hnasko, 2015). PLAs (see above) using a combination of affinity reagents specific for the target protein, as well as the modification, will prove particularly useful to visualize the modification of a protein of interest (Fig. 2C) (Ristic et al. 2016): Signal amplification only occurs if both the target protein and its modification are recognized by affinity reagents in a very low distance. This approach has been successfully used to detect ubiquitination and SUMOylation of proteins (Sahin et al. 2016; Peeler et al. 2017), and has already been applied to study PTMs of inflammasome components (Barry et al. 2018).

Microscopy analysis of PTMs may on the long run overcome limitations of current assays. These often rely on cell lysates or even the reconstitution of modification with ubiquitin(-like) molecules by overexpression in unrelated cell types. The observed spatial distribution of modified target proteins of interest will help resolve where crucial regulatory steps take place, and ultimately determine which modifications are relevant for activation, inhibition, or other steps. Combination with activity-based probes, such as fluorescent suicide protease substrates, may prove particularly useful to unravel how PTMs control activity of caspase-1.

Most current genetic screens aim at the discovery of genes whose depletion causes a selectable phenotype. Yet, screens that identify distinct amino acids with crucial functions will be an elegant way to more rationally address the role of PTMs. Recent advances allow enzymatically catalysed base changes (Eid et al. 2018). Further, near-haploid cell lines as well as murine and human haploid stem cells permit the genome-wide generation of point mutations by chemical mutagenesis (Sagi et al. 2016; Horn et al. 2018). If modified cells can be differentiated into cell types expressing inflammasome components, it will be possible to identify point mutants that prevent conformational changes or post-translational modifications.

The location of inflammasome sensors may have implications for the activation mechanism, or could even initiate sensor activation itself. NLRP3 inflammasome assembly is scaffolded by phosphatidylinositol 4-phosphate (PtdIns4P) exposed on dispersed trans-Golgi network vesicles, which occur in response to various NLRP3 triggers (Chen & Chen, 2018). It is conceivable that other inflammasome sensors are also enriched on organelle surfaces to facilitate their activation, or to ensure better access to ligands. However, this has not been addressed in sufficient detail. Co-localization with abundant or dispersed organelles is not trivial to assess, in particular if the entire pool of a specific sensor is visualized rather than those sensor molecules that are activated by ligand binding or indirect signals. Challenges in the analysis of inflammasome locations are illustrated by alternative models for NLRP3 activation based on overexpression studies, which had previously proposed that NLRP3 is recruited and activated on mitochondria via mitochondrial antiviral-signalling protein (MAVS; Subramanian et al. 2013). Improved microscopy-based methods to visualize active conformations or modifications of endogenous inflammasome sensors, or interactions of sensors with ligands, scaffold proteins, or organelle markers, will thus improve our understanding of spatiotemporal regulation of inflammasomes.
Novel methods that assess or manipulate protein conformations and modifications in situ will be crucial to understand the complex and transient regulation of inflammasome components. The opportunity to define those molecules of a given protein species that are critical for the observed biological activity will greatly increase the signal to noise ratio of their analysis: rather than averaging over the entire pool of a given protein in a (heterogenous) cell mixture, the few molecules that are actually involved in the process can be specifically analysed.

Inflammasome assembly in tissues and primary cells

Inflammasome assembly has been extensively studied in a few myeloid cell lines as well as some murine and human myeloid primary cells. Yet, we continue to identify additional cell types capable of assembling inflammasomes, including keratinocytes and intestinal epithelial cells (Sellin et al. 2015; Sand et al. 2018). One reason for this may be that only a small fraction of cell types in the body can be cultivated outside their tissue context, let alone be recapitulated in immortalized cell lines. A few selected cell types of interest can be artificially derived from induced pluripotent stem cells with the right combination of cytokines. Yet, absent the regulatory environment of the tissue, it is not clear to what extent behaviour in the tissue is reflected.

Microscopic detection of inflammasomes in tissues. A full understanding of the diversity of sentinel cell types capable of assembling inflammasomes will require the analysis of infection or sterile inflammation models in vivo and in cultivated tissue explants, or the microscopic analysis of biopsy samples from patients. Intravital imaging of selected accessible organs in genetically altered reporter mice expressing fluorescent inflammasome components have revealed and will continue to reveal the cell types, dynamics, and context of inflammasome assembly in living organisms (Sagoo et al. 2015; Tzeng et al. 2016).

Cultivation of organoids derived from pluripotent or tissue-specific stem cells may help to more realistically assess the behaviour of cells capable of assembling inflammasomes in the context of their complex tissue environment and architecture (Rios & Clevers, 2018). This was, for example, shown for intestinal organoids recapitulating the four major cell types of the intestinal epithelium, enterocytes, goblet cells, Paneth cells and enteroendocrine cells, in which inflammasome-dependent IL-18 secretion after stimulation of NLRC4 (Rauch et al. 2017), NLRP9 (Zhu et al. 2017) and pyrin (Alimov et al. 2019) inflammasomes was investigated.

Importantly, quantifying the mere expression levels of inflammasome components under steady state conditions will not be sufficient to identify the relevant cell types that assemble inflammasomes, as expression may only be upregulated in response to other cytokines or in the context of infection. As the most prominent example, NLRP3 is only substantially upregulated after Toll-like receptor stimulation in macrophages (Bauernfeind et al. 2009). All attempts to identify cell types assembling inflammasomes will rely on the ability to detect responses in the cells themselves, rather than the detection of secreted cytokines. Thus, staining and detection of ASC specks with antibodies, or active caspase-1 with activity-based probes in combination with microscopy experiments, flow cytometry, or the hybrid ImageStream analysis will be particularly powerful approaches. Detection of ASC specks in mouse primary cells will be simplified by reporter mice that express fluorescent fusions of ASC (Tzeng et al. 2016). Once inflammasome assembly has been observed, the involved inflammasome sensor could be detected by PLAs using antibodies or nanobodies against ASC as well as individual candidate sensors. Importantly, PLAs have previously been shown to be amenable to staining of tissue samples (Soderberg et al. 2006).

Once reagents are available that allow detection of inflammasome assembly in species that are not susceptible to straightforward genetic manipulation, analysis of inflammasome assembly in primary cells and tissues should be expanded to vertebrate species other than mouse and human, as only more systematic comparative studies will reveal the evolutionary contribution of inflammasomes in the defence against infections or intrinsic dangers. Such analyses will reveal which aspects of inflammasome responses are universal, and which are specific for certain species and thus the result of more recent evolutionary pressure. The evolution of multiple paralogues of the inflammasome sensors NLRP1 and NAIP in rodents, likely responding to different ligands or signals, is one example of such species-specific differences (Endrizzi et al. 2000; Sastalla et al. 2013). Studying more than only one or two species will therefore contribute to the evolutionary understanding of inflammasomes as they evolved in vertebrates. This is particularly important as inbred mice in specific pathogen-free conditions as well human beings with average hygiene habits are not necessarily exposed to all the infectious challenges that vertebrates have been facing in the course of evolution, including infections with multicellular parasites of the skin and the intestine.

Exciting new developments in the cultivation of tissues, organoids and primary cells – combined with in vivo experiments and microscopic analysis of preserved tissue samples from biopsies, autopsies or necropsies (from non-human species) – will provide important insights in the inflammasome response in complex tissues. This
information should inform the choice of cell types for experiments that investigate inflammasome assembly with a more molecular focus.

Concluding remarks

Less than 20 years since their initial discovery, we have come a long way to understand many details of the assembly of inflammasomes, including most of the involved molecules, basic rules of assembly and the invoked cellular responses. Yet, several crucial aspects of their assembly, regulation and downstream effects remain enigmatic, since they require experiments in the native environment of the cell or the tissue, and therefore cannot be analysed with conventional methods. This article has highlighted a few novel experimental approaches to overcome some of these challenges and to study inflammasome assembly in more physiological settings of primary cells or tissues. To understand the full spectrum of consequences of inflammasome assembly for immune responses on a systems level, we will have to continue to develop and apply new approaches. These should ideally aim at both observation of endogenous processes and precise manipulation of involved molecules in their endogenous context.

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Additional information

Competing interests

Florian I. Schmidt works as a consultant to IFM Therapeutics. There are no competing interests.

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Sole author.

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Keywords

cell biology, inflammation, inflammasomes, nanobodies, single domain antibodies

Abbreviations

ASC apoptosis-associated speck-like protein containing a caspase recruitment domain
AIM2 absent in melanoma 2
NLR nucleotide-binding domain (NBD)- and leucine-rich repeat (LRR)-containing
NAIP nucleotide-binding oligomerization domain-like receptor family apoptosis inhibitory protein
NLRC4 NLR family CARD-containing protein 4
NLRP3 NLR family Pyrin domain-containing protein 3
NLRP9 NLR family Pyrin domain-containing protein 9

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