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Danger signals, inflammasomes, and the intricate intracellular lives of chlamydiae



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ABSTRACT

Chlamydiae are obligate intracellular bacterial pathogens, and as such are sensitive to alterations in the cellular physiology of their hosts. Chlamydial infections often cause pathologic consequences due to prolonged localized inflammation. Considerable advances have been made in the last few years regarding our understanding of how two key inflammation-associated signaling pathways influence the biology of *Chlamydia* infections: inflammation regulating purinergic signaling pathways significantly impact intracellular chlamydial development, and inflammasome activation modulates both chlamydial growth and infection mediated pro-inflammatory cytokine production. We review here elements of both pathways, presenting the latest developments contributing to our understanding of how chlamydial infections are influenced by inflammasomes and purinergic signaling.

The bacterial family *Chlamydiaceae* includes several species which promiscuously or sporadically infect humans. *Chlamydia trachomatis* is the most common nationally notifiable infection in the USA [1], the most common bacterial cause of sexually transmitted infection worldwide [2], and also a prominent cause of preventable blindness following repeated conjunctival infections in the developing world [3]. *C. trachomatis* can also cause pneumonia in infants following exposure

during birth [4]. *Chlamydomphila pneumoniae* is one of the leading causes of pneumonia in the developed world [5] and may increase the risk of developing atherosclerotic lesions in coronary artery disease [6]. *Chlamydomphila psittaci*, while primarily an avian pathogen, sporadically causes human pneumonia [7,8]. *Chlamydomphila abortus* and *Chlamydomphila caviae* zoonotic infections have been reported in humans but are rare [9,10]. Untreated *C. trachomatis* genital tract infection in women may

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ascend the endometrial endothelium to reach the fallopian tubes, with the associated chronic inflammation leading to pelvic inflammatory disease (PID), which may also cause miscarriage [11], ectopic pregnancy [12], or tubal scarring and infertility [13]. Repeated and chronic infection of the conjunctiva with *C. trachomatis* leads to recruitment of lymphocytes and the formation of follicles and inflammation mediated conjunctival thickening, which subsequently causes the deformation of the eyelids and corneal damage via scraping of the cornea by in-turned eyelashes [14]. Pneumonia caused by *C. pneumoniae* develops slowly and leads to inflammation of the lungs but with limited production of purulent sputum. *C. pneumoniae* also causes infections of the upper respiratory tract including pharyngitis, sinusitis, and bronchitis. Inflammation due to repeated infections with *C. pneumoniae*, or unrecognized and untreated infections, may contribute to chronic obstructive pulmonary disorder (COPD) [15,16]. Human disease following infection with *Chlamydiaceae* species bears a consistent hallmark: chronic, localized inflammation.

Two major pathways relevant to the induction and regulation of localized inflammation have recently received considerable research emphasis and have been demonstrated to be particularly relevant during *Chlamydia* infection: purinergic signaling, and the formation of macro-molecular inflammasomes. Here we review the basic concepts of purinergic signaling in the context of immune function and inflammation regulation, and inflammasome mediated inflammatory cytokine production, followed by an examination of the recent literature evaluating the impact of host purinergic signaling and inflammasome activation on chlamydial infection.

Extracellular purines and purinergic receptors

A wide range of extracellular purine concentrations are physiologically relevant [17], and they are met by a similarly broad spectrum of sensory affinity in the purinergic receptor families [18]. ATP is released from cells under normal physiologic conditions reaching nanomolar to low micromolar concentrations in the immediately adjacent extracellular space [19–21]. Higher concentrations of ATP or ADP result from various forms of cell stress [22], platelet degranulation [23], or are present in tumor microenvironments [24]. The concentration of ATP in cells ranges from 3 to 10 mM, and thus in the context of cell damage or necrosis the neighboring cells are exposed to low millimolar levels of extracellular ATP and purine metabolites. ATP may be released via degranulation in cell types which produce ADP or ATP rich granules, via pannexin channels [25,26], or following cell damage. At colonized mucosal epithelial surfaces, ATP may also be directly released by bacteria [27]. ATP is also released from cervical epithelial cells *in vitro* during *C. trachomatis* infection, particularly during the late stages of inclusion development when there is likely more cellular stress [28].

Receptor mediated purine signaling is an evolutionarily conserved cellular function, and is involved in a wide variety of physiological processes in mammals including neurologic signaling, vascular function, and immune cell regulation.

Receptors which recognize purine nucleotides and nucleosides are termed purinergic receptors. Purinergic receptors are grouped into families based on functional similarity: P1 receptors are engaged by the purine nucleoside adenosine, while P2 receptors are activated by nucleotides and are further subdivided into gated ion channels (P2X) or G-protein coupled seven transmembrane receptors (P2Y).

Adenosine receptors couple via G-proteins to adenylyl cyclase to modulate cAMP generation in cells. A1 and A3 receptors associate with Gi proteins to inhibit adenylyl cyclase and prevent cAMP upregulation, whereas A2a and A2b receptors interact with Gs proteins to activate adenylyl cyclase, leading to elevated intracellular cAMP. Adenosine receptors are expressed in a wide variety of cell types, with particularly well described roles for A1 and A3 receptors in cardiac function, and for A2a receptors in immune cell function and A2b on epithelial and endothelial cells. A2b also mediates intracellular signaling via Gq and phospholipase C [29]. Recent excellent reviews have organized the great depth of studies related to adenosine receptor mediated regulation of immune cells [30,31].

P2X purinergic receptors are ligand gated ion channels which are activated by ATP, and possibly ADP in the case of P2X4, and are increasingly recognized to play a role in inflammation and immune cell function [32]. There are seven described P2X receptors, the best characterized being P2X7. P2X7 plays a critical role in the production of IL-1 β and other inflammatory cytokines both in the context of infection and sterile inflammation [33,34]. Initial stimulation of P2X receptors leads to the opening of small-cation permeable pores (approximately 0.85 nm for P2X7), while prolonged ligation then leads to increased permeability to larger molecules (greater than 1 nm for P2X7) [35], either by P2X7 pore dilation or by P2X7 coupling to pannexin1 and the pannexin pore.

P2Y receptors respond to purine and pyrimidine nucleotides and mediate intracellular signaling via regulation of cAMP or activation of PLC. Eight P2Y receptors have been characterized (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃, and P2Y₁₄), and while their activity relevant to inflammation and immune cell function is less well characterized than for P2X receptors, some P2Y receptors are expressed on immune cells [18,36,37].

While there is nuance to the roles that purine signaling plays in local and systemic immune regulation, in general, ATP mediates pro-inflammatory responses, and adenosine receptor stimulation is anti-inflammatory [38]. Thus soluble or cell surface associated extracellular enzymes play a critical role in determining the response to extracellular purines. Adenine nucleotides released from cells are dephosphorylated by members of several families of purine enzymes to generate adenosine. Enzymes such as CD39 (ENTPD1) and alkaline phosphatase dephosphorylate ATP and ADP to generate AMP, and CD73 (ecto-5'-nucleotidase) or alkaline phosphatase dephosphorylates AMP to generate adenosine. Adenosine can be captured by adjacent cells via nucleoside transporters for purine salvage, or metabolized by adenosine deaminase to produce inosine. Enzymes regulating extracellular purine metabolism have been more completely described in recent reviews [39,40].

Purinergic receptor stimulation during chlamydial infection

In the last 10 years, there have been many investigations into the impact of purinergic receptor stimulation on chlamydial development *in vitro*.

After work by other groups showed that purinergic receptor stimulation altered mycobacterial infection in host cells [41,42], we demonstrated that P2X7 ligation (5 mM ATPe) reduced growth of *C. psittaci* in J774 macrophages, while at the same time the chlamydial infection partially inhibited ATPe mediated apoptosis of host cells [43]. We then demonstrated that ATP stimulation of P2X7 on murine macrophages led to killing of intracellular *Chlamydia muridarum* [44], the murine equivalent of *C. trachomatis*. Ligation of P2X7 receptor with 0.5–5.0 mM ATPe caused fusion of chlamydial inclusions with host-cell lysosomes, leading to chlamydial death, an effect which was dependent on activation of phospholipase D (PLD). Additionally, P2X7 ligation suppresses *C. muridarum* infection in the preferred target cell of genitotropic *Chlamydia* species – cervical epithelial cells [45]. As was the case in murine macrophages, ATPe/P2X7 mediated chlamydial inhibition in murine cervical epithelial cells was at least partially dependent on PLD activation. In animal experiments with P2X7 wild-type and P2X7^{-/-} mice, P2X7 expression reduced the intensity, but not the duration, of vaginal infection, and pathology scores based on histological evaluation of endocervix, oviduct, and mesosalpinx tissue indicated increased inflammation in the P2X7^{-/-} mice following chlamydial infection [45].

While millimolar concentrations of ATPe are required for P2X7 mediated effects on *Chlamydia* infection, micromolar concentrations of ADPe and ATPe reversibly inhibit chlamydial development in human cervical epithelial cells via P2X4 ligation [28]. One hundred μ M ADPe or ATPe applied to *C. trachomatis* infected epithelial cells at 1 h post-infection (hpi) significantly inhibited chlamydial inclusion development through 24 h of infection, although by 48 hpi inclusion development was proceeding normally. If, however, repeated applications of ADPe or ATPe were made, inclusion development did not occur by 48 hpi unless the media was changed at 24 hpi. Thus, duration of exposure to micromolar concentrations of ADP or ATP determined the extent of impairment of chlamydial development. In the context of inflammation, ATPe (pro-inflammatory) and ADOe (anti-inflammatory) often have paradoxical effects; however, both inhibit development of *C. trachomatis* in human cervical epithelial cells at micromolar concentrations. ADOe suppression of chlamydial development is reversible, and is mediated by A2b receptor stimulation [46]. ADOe and ATPe also inhibit the development of *C. trachomatis* serovar E and *Chlamydia pecorum* in human epithelial cells [47]. It should be noted that two independent investigations have also demonstrated that millimolar concentrations of extracellular cyclic adenosine monophosphate (cAMP) inhibit chlamydial development, leading to the production of aberrant chlamydial bodies [47,48]. As no receptor for extracellular cAMP has been described, and cAMP is not cell permeable, this effect may possibly be mediated by purinergic receptor stimulation following degradation of extracellular cAMP. The mechanism by which purinergic

stimulation of host cells alters chlamydial development has not been elucidated, and it is also not clear whether the alterations described *in vitro* would be deleterious or advantageous for chlamydial propagation *in vivo*. As an obligate intracellular bacterium with a substantially reduced genome, chlamydiae are particularly sensitive to changes in the metabolic environment of the host cell. Indeed, in the best described *in vitro* model of chlamydial persistence, it has been demonstrated that IFN- γ stimulation of human epithelial host cells leads to restriction of intracellular tryptophan for which chlamydiae are auxotrophic [49,50]. Future studies are needed to evaluate the molecular basis for the impairment in chlamydial development following host cell exposure to elevated extracellular purines.

Inflammasomes and inflammation

The innate immune system mounts a rapid response to unique molecular signatures from the invading pathogen termed pathogen associated molecular patterns (PAMPs), where it utilizes a set of germ-line encoded pattern recognition receptors (PRRs) to initiate a measured and appropriate inflammatory response in order to slow down the spread of infection and thereby allowing and contributing to the initiation of adaptive immunity. Furthermore, PRRs can detect danger signals termed danger associated molecular patterns (DAMPs) released by damaged host cells (for a review, see Ref. [51]). PRRs can be membrane-bound like the Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), which sample the extracellular milieu from the plasma membrane and within endosomes, or cytosolic like the nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs) and AIM2-like receptors (ALRs), which survey intracellular PAMPs and DAMPs (reviewed in Refs. [52–54]).

Typically, most PRRs (e.g. TLRs), following recognition of their PAMP, initiate distinct intracellular signaling cascades, which lead to induction of various programs of gene transcription including, but not limited to, co-stimulatory molecules which activate components of the adaptive immune response, production of reactive oxygen and nitrogen species, and expression of pro-inflammatory cytokines and chemokines [53,54]. On the other hand, NLRs – a family of 22 genes in humans and 34 genes in mice – can detect not only intracellular PAMPs (e.g. NOD1, NOD2) [55], but also can sense endogenous DAMPs [52,56,57]. These latter NLRs, which include NLRP and NLRC proteins, are components of a macromolecular protein complex – the inflammasome – required for the activation of caspase-1 and the subsequent secretion of potent pro-inflammatory cytokines, including Interleukin (IL)-1 β (IL-1 β) and IL-18 (reviewed in Ref. [58]).

Due to their high pro-inflammatory potency and their significant role in innate defense to invading pathogens, secretion of both IL-1 β and IL-18 is a highly regulated process in order to avoid diseases associated with their excessive and chronic production, like autoimmune disorders and septic shock [59]. Both cytokines are produced as inactive and leaderless forms, pro-IL-1 β and pro-IL-18, and require cleavage by caspase-1 to promote the unconventional secretion of their

mature and biologically active forms [60–62]. Caspase-1, along with caspase-4 and caspase-5 in humans and caspase-11 in mice, constitute a family of inflammatory caspases that exist as inactive zymogens (pro-caspases). Activation of these caspases requires the activation of inflammasomes, which are a multimeric assembly of pro-caspase-1 and an NLR family member, either directly through CARD–CARD interaction or indirectly via the adapter protein ASC [63,64]. These studies led the way for the identification of additional means to nucleate the inflammasome by members of the RLR and ALR families, and the assembly of non-canonical inflammasomes that lead to the activation of caspase-11 in mice and caspase-4/5 in humans.

The NLRP3 inflammasome (also known as NALP3, cryopyrin) is the best-characterized inflammasome to date and can be activated by wide variety of stimuli including PAMPs, DAMPs, pore-forming toxins, whole pathogens and environmental stressors (reviewed in Refs. [65–67]). Due to the large number of triggers and in order to prevent inadvertent activation, the NLRP3 inflammasome requires two signals for its optimal induction. PRR agonists (e.g. LPS) provide the first signal and cause NF- κ B mediated upregulation of pro-IL-1 β and components of the inflammasome, including NLRP3 [68]. The second signal can be provided by a large repertoire of ‘danger signals’ that include host derived DAMPs (e.g. ATP, adenosine, hyaluronan, HMGB1, gout-associated uric acid crystals and amyloid- β fibrils [69–74]), environmental stressors (e.g. asbestos, silica crystals [75–77]), and PAMPs that gain access to the cytosol. These PAMPs can be derived from bacteria (e.g. toxins, RNA:DNA hybrids, type III secretion system effectors, cyclic-dinucleotides [58,67,70,78–85]), viruses (e.g. dsRNA, viral proteins [86–90]), fungi (e.g. hyphae, zymosan, mannan [91–94]), or *Plasmodium*, a malaria causing parasite (e.g. hemozoin [95]). Importantly, some intracellular pathogens can activate the NLRP3 inflammasome by providing both signals (e.g. *C. trachomatis* [58]).

Despite this wide array of NLRP3 activators, researchers in the field generally agree that NLRP3 does not directly sense any of these stimuli, but instead detects specific host-derived molecular event(s), which then cause the assembly of the NLRP3 inflammasome. These upstream molecular events can be summarized into three main categories. (1) Events that perturb the homeostasis of intracellular cations, and include an increase in intracellular Ca²⁺ [79,96,97] or K⁺ efflux, which usually occurs through the activated ATP-gated ion channel P2X₇ or toxin-induced pores in plasma membrane [70,98–100]. (2) Events that cause reactive oxygen species (ROS)-mediated oxidative stress and mitochondrial perturbation. Although the exact mechanism of ROS-mediated NLRP3 inflammasome activation remains controversial, there is a general consensus that mitochondrial ROS (mROS) production is the upstream trigger for NLRP3 activation following stimulation with several inducers (e.g. ATP, uric acid crystals, alum, nigericin, certain pathogens [65,101]). Elevated and prolonged release of mROS by damaged mitochondria [101] leads to oxidation of mitochondrial DNA, which when leaked to the cytosol binds directly to NLRP3, thereby promoting its activation [102,103]. (3) Release of Cathepsins from destabilized lysosomes following ‘frustrated phagocytosis’ of relatively large ‘sterile’ molecules (e.g. silica, alum and uric acid crystals, asbestos, amyloid- β -fibrils,

malarial hemozoin [69,77]) also activate the NLRP3 inflammasome. Again, the exact mechanism of how this leads to NLRP3 inflammasome activation remains poorly understood.

Chlamydiae and inflammasomes

In response to *Chlamydia* infection, epithelial cells produce pro-inflammatory cytokines and chemokines in order to activate and recruit innate immune cells into the site of infection [104]. These immune cells, which include dendritic cells, macrophages, neutrophils and natural killer cells, in turn secrete cytokines including TNF- α and IL-1 β in response to the infection [105–110]. Consistently, IL-1 β deficient mice infected with *C. muridarum* displayed delayed clearance of the infection [111]. However, chronic and excessive production of these pro-inflammatory cytokines has been touted as the dominant culprit causing the pathology associated with chlamydial infections [112–115]. Consistently, caspase-1 deficient mice displayed reduced inflammatory damage in the urogenital tract, suggesting that it may contribute to the pathology of infection by *Chlamydia* [116]. Therefore, revealing the mechanism of IL-1 β production has important implications for understanding pathology associated with *Chlamydia* infection.

Previous studies have shown that caspase-1 could be activated during chlamydial infection [107,109,111,116,117]. Indeed, *C. trachomatis* infection of cervical epithelial cells caused caspase-1 activation in a manner dependent on NLRP3 inflammasome activation [58]. Subsequent studies showed that human monocytic cell lines infected with *C. trachomatis* and *C. muridarum* secreted IL-1 β following caspase-1 activation in an NLRP3-dependent fashion [78], while *C. pneumoniae* was the most potent inducer of IL-1 β secretion in bone marrow derived murine macrophages [118]. These studies showed that *Chlamydia* infection alone was sufficient for inflammasome-dependent caspase-1 activation likely because an active *Chlamydia* infection can provide both signal one via the TLR2/MyD88 axis [119–121] in addition to signal two, activating caspase-1. The mechanism whereby chlamydial infections provide the second signal for NLRP3 inflammasome activation was thoroughly investigated. *C. trachomatis* induced inflammasome activation is dependent on K⁺ efflux [58] although the role of the P2X₇ receptor, which is required for K⁺ efflux mediated inflammasome activation [122] and plays an important role in *Chlamydia* infection [45], remains to be investigated. *Chlamydia*-induced loss of intracellular potassium seems to trigger production of ROS, which appears to be essential for proper activation of the NLRP3 inflammasome [67]. Importantly, the mitochondrial associated protein, NLRX1, plays an important role in mediating ROS production following infection with *C. trachomatis* [78]. Moreover, *C. pneumoniae* mediated caspase-1 activation required lysosomal acidification and cathepsin B release [118]. The ability of chlamydiae to activate the inflammasome is dependent on the chlamydial type III secretion system (T3SS) [58]. Intriguingly, a chlamydial plasmid encoded protein, pORF5 (a.k.a. pgp3), which is secreted into the host cytosol [123], might play a role in inflammasome activation [124]. Finally, the Rho family GTPase, Rac1, was shown to play a role in *C. pneumoniae* induced inflammasome activation [125].

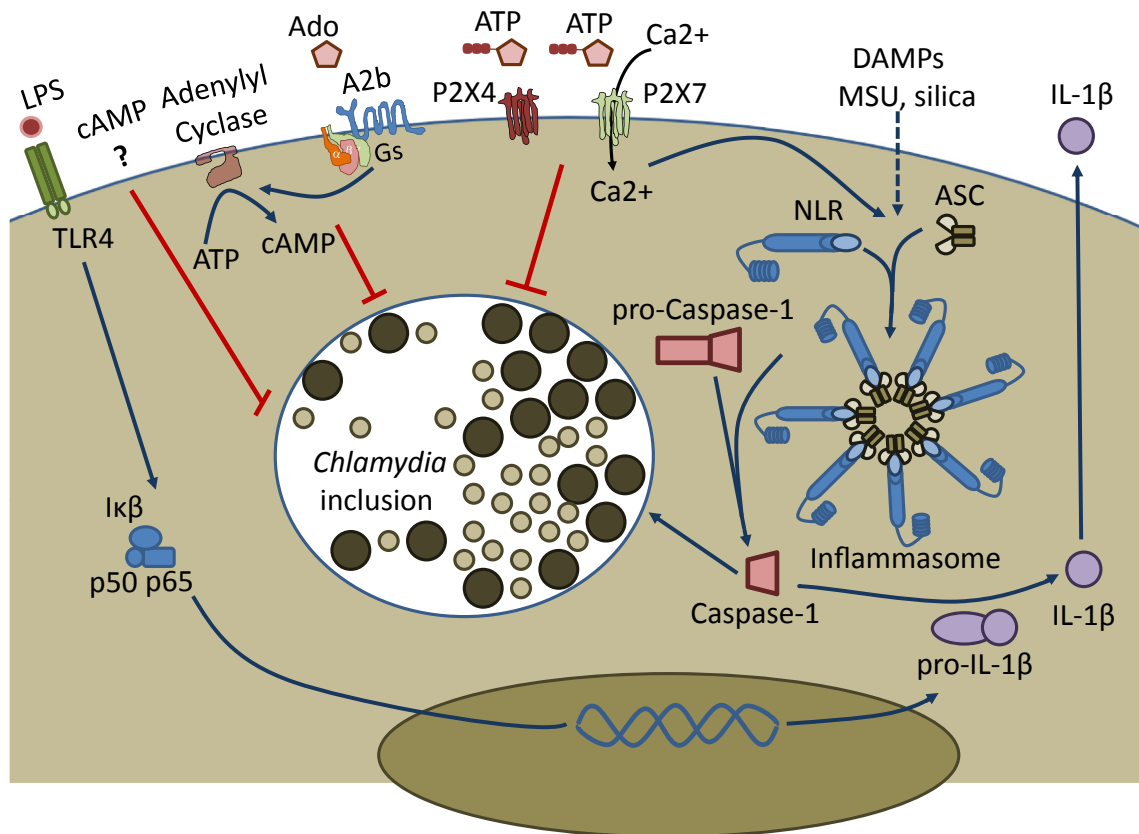


Fig. 1 – Purinergic receptor stimulation and inflammasome activation alter chlamydial development. Prolonged adenosine exposure suppresses chlamydial development via stimulation of host Gs protein- and adenylyl cyclase-coupled A2b receptors, and ADP or ATP mediated activation of P2X4 and P2X7 receptors also leads to reduced chlamydial growth or P2X7 mediated chlamydial killing, depending on the concentration of purine nucleotides and host cell type. Assembly of pro-caspase-1, NLR-family member sensor proteins, and ASC adaptor proteins into multimeric inflammasomes is required to generate active caspase-1, and follows combinatorial signaling events activating multiple upstream pathways, which may include ATP stimulation of P2X7 receptors, or other DAMP-dependent signaling pathways. Inflammatory triggers such as lipopolysaccharide (LPS) activation of TLR4 leads to production of pro-IL-1 β , but inflammasome mediated production of active caspase-1 is required for generation of secreted pro-inflammatory IL-1 β . Inflammasome activation mediated cleavage of pro-caspase-1, yielding active caspase-1, also contributes directly to growth of intracellular chlamydiae.

The role of the inflammasome in the control of chlamydial infection is still controversial. In a mouse model of *C. pneumoniae* lung infection, results showed that caspase-1 deficient mice displayed delayed pulmonary bacterial clearance of infection, which was associated with increased mortality compared to wild type mice, thereby indicating that inflammasome activation has an important role in the host response to the infection [83]. On the other hand, lung fibroblasts from ASC^{-/-} and caspase^{-/-} mice displayed resistance to infection by *C. trachomatis*, a result which was recapitulated by the caspase-1 inhibitor, YVAD [126]. In agreement, a recent report showed that IL-10 mediated increase in tubal damage and infertility in *Chlamydia*-infected mice is mediated by its ability to enhance NLRP3 inflammasome activation in infected DCs [127]. Furthermore, *in vivo* challenge with *C. muridarum* in IL-1R deficient mice showed that, while IL-1 β plays an important role for clearance of genital chlamydial infection,

infection of NLRP3^{-/-} and ASC^{-/-} mice showed that the inflammasome plays a limited role in this model [128].

Chlamydia is described as the ‘perfect pathogen’ due to its ability to evade host-cell defenses and utilize host resources to promote its own growth [129–131]. Consistently, *Chlamydia* subverts host lipids from the Golgi by inducing caspase-mediated fragmentation of the Golgi apparatus [132]. Intriguingly, inflammasome-mediated caspase-1 activation following *C. trachomatis* infection is required for optimal intracellular growth of this pathogen [58]. Similarly, bone-marrow derived macrophages from NLRP3, ASC or caspase-1 deficient mice showed severely impaired intracellular growth of *C. pneumoniae* [133]. Intriguingly, both the caspase-1 inhibitor, YVAD, and caspase-4/5 (murine caspase-11 ortholog) inhibitor, WEHD, inhibited chlamydial growth, suggesting possible roles for both caspase-1 and non-canonical caspases (caspase-4/5 or caspase-11) in this process [58]. In fact, a

recent study suggested that guanylate binding protein (GBP), which promotes caspase-11-dependent pyroptosis, might play a partial role in regulating the kinetics of inflammasome activation and affecting the ratio of IL-1 β vs IL-18 production following *C. muridarum* infection [134]. Furthermore, treatment with probenecid, a pannexin-1 channel blocker that inhibits P2X7 receptor mediated inflammasome activation following K⁺ efflux [135], directly inhibits chlamydial development in a dose-dependent and reversible manner, although a direct role for pannexin-1 is not clear [136].

In conclusion, although results from different laboratories show consistently that an NLRP3 inflammasome is activated by infection with different species of *Chlamydia* and that the inflammasome may be involved in the immune response to infection, much remains to be known of the effects that both canonical and non-canonical inflammasomes may have on metabolism of infected cells [Fig. 1]. In particular, elucidation of the mechanism through which *Chlamydia*-mediated caspase-1 activation may stimulate lipid metabolism for the purpose of aiding chlamydial growth is an unexplored topic and interesting avenue of future research.

Conflicts of interest

None of the authors have a conflict of interest with this manuscript.

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