UNIVERSITY OF CALIFORNIA,
MERCED

COMPARATIVE ANALYSIS OF INFLAMMATION INDUCED BY *ASPERGILLUS.FUMIGATUS* AND *CHLAMYDIA.TRACHOMATIS.*

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Quantitative and Systems Biology

By

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DEDICATION

This thesis is dedicated to the memory of my father **Mounir Said** who believed in me and encouraged me to pursue my dream of reaching the highest degree in education.

It is also dedicated to my mother, **Ferial Said** who taught me that even the hardest tasks can be accomplished if it is done with a lot of determination, bravery and perseverance.

To my brother and sisters especially **Nisrine Said** who through their endless love and support I manage to reach my goals and realize my dream.

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Najwane Saïd-Sadier, Eduardo Padilla, Gordon Langsley, David M. Ojcius. *Aspergillus fumigatus* stimulates the NLRP3 inflammasome through a pathway requiring ROS production and the Syk tyrosine kinase. *PLoS One. 2010 Apr 2;5(4):e10008.*

Abdul-Sater AA, Saïd-Sadier N, Ojcius DM, Yilmaz O, Kelly KA. Inflammasomes bridge signaling between pathogen identification and the immune response. *Drugs Today (Barc). 2009 Nov;45 Suppl B:105-12.*

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ABSTRACT OF THE DISSERTATION

COMPARATIVE ANALYSIS OF INFLAMMATION INDUCED BY *ASPERGILLUS.FUMIGATUS* AND *CHLAMYDIA.TRACHOMATIS.*

By

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Doctor of Philosophy in Quantitative and Systems Biology

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The elaboration of an effective immune response is a key factor in fighting against invading pathogenic microbes and eliminating them. Upon microbial sensing, the innate immune system initiate the inflammatory response by secreting cytokines and chemokines responsible for recruiting immune cells to the site of infection and inducing the establishment of adaptive immunity. During my thesis, I focused my studies on a cytoplamic protein complex called "inflammasome" which had been found to regulate the inflammation process by activating the cystein protease, caspase-1 which has as a substrate the potent pro-inflammatory cytokine IL-1β. During infection, monocytes and macrophages challenged with different microbial molecules get activated and induce the expression and the synthesis of pro- IL-1β waiting for a second signal called Danger signal essential for pro-IL-1β cleavage by caspase-1 and its secretion. Growing number of studies reveal the importance of inflammasomes assembly during several bacterial, viruses and fungal infections as they sense different signals characteristic of a pathogenic infection. The activation of an inflammasome during the fungus *Aspergillus.fumigatus* and the bacteria *Chlamydia.trachomatis* infection have never been investigated so far. Therefore I

investigated whether these two pathogens are able to induce inflammasome assembly in a human monocyte cell line and identified the molecules and the signals triggering this activation. The results demonstrated that both *Aspergillus.fumigatus* and *Chlamydia.trachomatis* induce the activation of an inflammasome in which the Nod Like Receptor 3 (NALP3) is involved requiring the presence of the adaptor ASC that bridges NALP3 to caspase-1. Additionally, I proved using Knocked down cells and different inhibitors that Reactive oxygen species, potassium efflux as well as the activation of the spleen tyrosine kinase (Syk) are required for this activation. As a conclusion, we showed for the first time the activation of NALP3 inflammasome during infection with both pathogens, highlighted how different microbes presenting distinct antigenic molecules, exhibiting different infectious strategies are capable of inducing common intracellular modifications that converge to the activation of the same inflammasome.

CHAPTER 1: Introduction

1. Innate Immunity

1.1 Pattern Recognition Receptors:

The eradication of pathogenic microbes which are themselves constantly evolving relies on the development of adequate immune response to ensure survival of all mutlicellular organisms. Central to a successful elimination is the ability to initiate a robust innate immune response that calibrates microbial, toxic, or allergenic structures removal while avoiding excessive damage of self-tissues. Only vertebrates have developed an adaptive immune system responsible for specific tailoring against a particular pathogen and the ability of long term protective immunity. Over the past decade the field of immunology has witnessed tremendous advances in our understanding of microbial clearance via their recognition by germ-line-encoded pattern-recognition receptors (PRR) along with the activation of their downstream signaling pathways. PRR are evolutionarily conserved receptors that detect relatively invariant molecular patterns found in most microorganisms of a given class called pathogen-associated-molecular patterns (PAMPs) such as lipopolysaccharide (LPS), flagellin, bacterial, viral nucleic-acid motifs and peptidoglycan etc…[1] . The best characterized PRRs are the Toll-Like receptors (TLRs) family members which constitute at least 12 proteins either expressed on the plasma membrane or in endosomal/lysosomal organelles [2] which are composed of a leucin-rich repeats (LRRs) domain

involved in ligand binding and microbial sensing and a cytoplasmic Toll/interleukin-1 (IL-1) receptor (TIR) domain that interacts with TIR domain containing adaptor molecules such as myeloid differentiation –primary response gene 88(Myd88) [3]. The expression of TLRs is cell specific assuring an adequate response depending on each cell type and the microbial challenges that they encounter [4]. The stimulation of these TLRs (except TLR3) converges to nuclear factor-κB (NF-κB) activation through a pathway involving Myd88. Inactive NF-κB is held into the cytoplasm complexed with the inhibitory protein IκBα. Signaling through TLRs induces the activation of IκB kinase(IKK) which in turn, phosphorylates the IκBα protein leading to its ubiquitination, dissociation from NF-κB, and its degradation by the proteosome[5]. Subsequently NF-κB translocates from the cytoplasm into the nucleus and drives the transcription of specific genes involved in inflammatory, cell survival or cell proliferation responses [6]. On the other hand TLR3 or TLR4 induce through their adaptor protein TRIF, the activation of the transcription factor IRF3 required for the production of type I interferon response during viral infection [7]. The outcome of TLRs signaling depends on their distribution on the innate immune cells, the ligands exhibited by the invading microorganisms and the sum of the coordinated cross-talk between these different receptors.

Immune response relies also on the contribution of non-TLR PRR families such as C-lectin type receptors that recognize complex carbohydrates expressed on the cell surface of multiple pathogens especially fungi whose activation triggers endocytosis and phagocytosis of the microbe as well as cell

signaling regulating the innate and adaptive immune system [8]. Of these, Dectin-1, receptor activated by β-glucans molecules, has been characterized with the most molecular detail; it is expressed primarily by cells of myeloid origin, including macrophages, DCs, and neutrophils and its signaling contribute to a variety of cellular responses such phagocytosis, oxidative burst, neutrophil degranulation for fungal killing, and the production of inflammatory cytokines, and chemokines that recruit other immune cells to the site of infection [9-11]. Signal transduction downstream of dectin-1 relies mainly on the activation of Syk and Src family kinases that get recruited to the phosphorylated intracellular tail of dectin-1[12-13].The role of Syk-dependant dectin-1 activation had been extensively studied and results had shown its crucial involvement in mediating phagocytosis, oxidative burst, NF-κB activation and cytokines production [14-18]. Dectin-1 had been shown to cooperate with TLRs particularly TLR2 to increase the production of pro-inflammatory cytokines such as TNF and IL-12 indicating that under high level of infectious stress, different PRRs synergize together for better microbial control and effective host resistance [19]. Recently, identification of a novel class of intracellular PRRs enlightened our understanding of the host innate immunity along with inflammatory pathogenesis. These are the nucleotide binding and oligomerisation domain (NOD)-like receptors (NLRs) and retinoid acid inducible gene-I (RIG)-Like receptors (RLRs) which are soluble intracellular proteins that survey the cytoplasm for signs revealing the presence of not only pathogen-encoded molecules (PAMPs), but also pathogen encoded activities termed “patterns of pathogenesis” [20-21]. Studies focusing on RIG-I

reveal its function in detecting viral PAMPs and subsequent production of antiviral cytokines such as type-I interferons (IFNs) [22]. Up until then, TLR3 was the first receptor involved in viral double stranded RNA (ds) RNA recognition and anti-viral signaling via NFκB and IRF activation [23]. RIG-I structure consists of two N-terminal caspase recruitment domains (CARDs) followed by an RNA helicase domain. Once activated by dsRNA, RIG-1 interacts through its CARDs domain with CARD containing adaptor called CARD adaptor inducing IFN-β (Cardif). Subsequently, this whole platform recruits appropriate signaling intermediates, such as IKKs to activate NF-kB and IRF transcription factors in order to modulate anti-viral responses [24-25].

On the other hand, the NLR family of proteins is rapidly emerging as dominant regulators of immunity in response to huge variety of pathogens. Members of this family share a similar pattern of domain organization which consists of an N-terminal effector region such as CARD, pyrin (PYD) or baculovirus inhibitor repeat (BIR) involved in protein-protein interaction; a central nucleotide binding domain (NBD) required for self oligomerization and finally an array of C-terminal Leucine-rich repeat (LRR) motifs which are believed to be involved in sensing microbial patterns and modulating NLR activity by folding back onto the NOD domain inhibiting thereby spontaneous oligomerization [26-27]. Yet the precise mechanism of how NLR LRRs sense their ligands is still unknown. Bioinformatic studies pointed out the presence of 23 NLR proteins in the human genome; however their functions as well as their respective activators are still under investigation [28]. Based on their N-terminal

domains, NLRs can be classified into three subfamilies which mediate signal transduction to downstream targets, leading to activation of inflammatory caspases by inflammasomes or NF-κB activation by NOD signalosomes. The PYD-containing NALP which represents 14 members in Human (NALP1-NALP14) and are the largest family; the CARD containing NODs such as NOD (1-5), NLRX1 and CIITA; the BIR-containing NAIPs such as NAIP (a-g) [29-31]. The current widely accepted model proposes that when NLRs sense directly or indirectly the presence of microbes via their C-terminal LRRs, this latter unfold exhibiting the NOD domains which become free to oligomerize exposing the N-terminal effector domains responsible for the recruitment and activation of their compatible adaptors leading to the initiation of an adapted signaling pathway [32].Under most circumstances, when the cell host is confronted with a microbe which usually expresses a battery of different PAMPs, activation of different families of PRRs governs the combined effort in order to successfully defeat the microbial challenge with minimum side effects on the host itself. Tissue distribution and sub-cellular localization of these PRRs are a great example of the potential strategy that the host has developed in order to discriminate not only pathogens from commensals but also distinguishing a virulent organism from one that has lower disease-causing potential. Therefore in the next part, I will present an overview of all the studies that had been conducted on NLR families summarizing their roles in microbial sensing, cell injury and their importance as key contributors of effective innate immune response.

1.2 NOD-Like Receptors in Host Defense and Disease:

Keeping the body free from invading pathogens is an intricate task that requires cooperation between different PRRs. While responses to extracellular PAMPs are mediated by membrane bound receptors such as TLRs and C-Lectin type receptors, NLRs are specialized in detecting PAMPs that had reached the sub-cellular compartments. Several members of the NLR gene family are involved in the assembly of a macromolecular protein complex termed the 'inflammasome" that leads to the activation of the inflammatory cysteine protease, caspase-1 (also known as interleukin-1 converting enzyme or ICE), which in turn cleaves pro-IL-1β or pro-IL-18, resulting in secretion of the mature cytokines ([33-34]. NALP3, NALP1 and IPAF are among the NLR members demonstrated to activate the inflammasome in cells, while other NLR members have been shown to activate the inflammasome in a cell free system. Caspase-1 is recruited to those inflammasomes through homotypic interaction with CARD domain expressed either on the NLR itself or on the adaptor protein called ASC (apoptosis-associated speck-like protein containing a CARD domain) which bridges the association of caspase-1 to the NLR proteins [35-36]. To investigate the role of ASC, several studies have revealed that TLR-stimulated macrophages from ASC-deficient mice fail to process caspase-1 and release IL-1β and IL-18 [37]. Therefore ASC plays a central role in the formation of the inflammasome as it links caspase-1 with the NLR proteins

1.3 The requirement of two separate signals for IL-1β secretion

Stimulation of PRRs by pathogens induces the activation of signaling pathways that culminates in translocation of NF-κB into the nucleus and upregulation of the expression of pro-inflammatory cytokines, including IL-1β. However, increased transcription of the IL-1β gene and synthesis of IL-1β proteins does not automatically correlate with an increase in IL-1β activity [38]. A second danger signal (DS) is usually needed to control the processing of IL-1β by a human cysteine protease termed caspase-1, which converts the 31-kDa inactive IL-1β precursor (pro-IL-1β) to the 17.5-kDa mature active IL-1β by cleaving between Asp-116 and Ala-117 [39]. The requirement for two signals thus insures that IL-1β is secreted by macrophages only if they are stimulated by PAMPs and the PAMPs are produced under circumstances that could be viewed as potentially dangerous to the host organism [40].

Recently, it became clear that in order to assure maximal protection, our immune cells had developed series of carefully orchestrated, highly evolved molecular mechanisms that sense not only PAMPs but also danger or warning signals that are delivered by damaged host tissues. Examples of DS include host-cell components released from dying, infected or stressed cells such as ATP, adenosine, uric acid, misfolded/glycated proteins or exposed hydrophobic portions of molecules or chromosomal proteins; it could also be microbial PAMPs that are located in “threatening” locations, such as flagellin in the cytosol of an infected cell [21].

In general it is believed that a first signal is required to promote expression and synthesis of pro-IL1β, and then a second signal is needed to induce activation of caspase-1 leading to the cleavage and secretion of active IL-1β.

1.4 Caspase-1 activation:

Caspase-1 is required for processing and subsequent secretion of IL-1β, but caspase-1 is synthesized as an inactive form that undergoes self-cleavage and activation usually when it is present within a complex of several interacting proteins, named an "inflammasome" [36]. The few studies performed on the inflammasome until now have shown that, depending on the DS sensed by the infected cell, caspase-1 can be activated through separate pathways that involve three different Nod-like receptor (NLR) proteins (NALP1, NALP3 and Ipaf,) [41-43]. The possibility that other DS could be detected through other NLR family members cannot be excluded yet.

1.5 Inflammasomes and signalosomes assembly:

Listed below are the different inflammasomes and signalosomes that had been described so far, their activation by pathogens and other triggers and their fascinating role in regulating the inflammatory response which is essential for microbes 'elimination and for establishment of an effective adaptive immune response for long-lasting immunity.

The NALP3 Inflammasome. The NALP3 Inflammasome increasing number of studies has demonstrated the activation of the NALP3 inflammasome in response to microbial infections. Bacteria including *S. aureus* and *L.monocytogenes* can activate the NALP3 inflammasome by triggering K^+ efflux due to their toxins

listeriolysin O, and α-toxin, β -toxin or γ-toxin [44]. *M. tuberculosis* and *M. marinum* use their ESX-1 secretion system to regulate secretion of IL-1β and IL-18 in a process that requires the NALP3 inflammasome [45]. Interestingly a recent study showed that *K.pneumonia* requires the NALP3 inflammasome not only for caspase-1 dependent IL-1β secretion, but also for inducing macrophage necrosis. Similarly, *N.gonorrhoeae* infection of monocytes causes IL-1β production, pyronecrosis, and HMGB1 release in an NALP3 dependent manner that requires the activity of the cysteine protease cathepsin B [46]. *C. trachomatis*, utilizing its type III secretion system, can stimulate NALP3 inflammasome dependent caspase-1 activation by causing K^+ efflux-dependent and ROS production in epithelial cells – moreover, caspase-1 was deemed crucial for chlamydial growth and survival [47]. In contrast, *P.gingivalis* infection on its own cannot activate an inflammasome: instead it induces synthesis of pro-IL-1β, which can be secreted following NALP3-dependent activation in infected cells that are treated with the danger signal ATP [48]. Conversely, *Mycobacterium tuberculosis* subverts the innate immune response by inhibiting NALR3 inflammasome activation [49].

The NALP3 inflammasome can also be activated by viruses such as the Sendai virus and influenza A virus, whose infections can cause caspase-1 dependent IL-1β secretion [41, 50].Recently, Modified Vaccinia Virus Ankara (MVA), an attenuated poxvirus used as a vector for AIDS vaccines, was shown to activate the NALP3 inflammasome, leading to secretion of IL-1β [51].

Furthermore, the fungus *C. albicans* signaling through the tyrosine kinase Syk, causes NALP3Inflammasome mediated caspase-1 activation and IL-1β secretion in a process triggered by K^+ efflux and ROS production [52-53]. The transition of *Candida* from the yeast to the filamentous form (hyphae formation) is important for activation of the NALP3 inflammasome [54]. Heat killed *S. cerevisiae* can also activate NALP3 inflammasome [55]. Moreover, both abiotic and biological crystals can also activate the NALP3 inflammasome such as asbestos, silica and amyloid-β fibrils that induce NALP3-dependent caspase-1 activation through "frustrated phagocytosis" of the large crystals or lysosomal leakage [56-58] . In this context, aberrant NALP3 activation could play deleterious roles, by inducing inflammation in response to environmental particles; this is the case of auto-inflammatory diseases such as gout, pseudogout, silicosis, and asbestosis. More recently, hemozoin, a heme crystal produced by malaria causing parasite *Plasmodium* was shown to similarly activate NLRP3 inflammasome by acting as a danger signal through the Lyn/Syk kinase pathway [59-60] .

The NLRP3 inflammasome initially gave the impression of being rather promiscuous, as it could be activated by a wide variety of stimuli, ranging from toxins, ATP and uric acid crystals. However, these disaparate stimuli activate NLRP3 through a small number of shared mechanisms (Fig. 2). Thus, the K+ efflux is induced by microbial toxins [44, 61], imidazoquinoline derivatives [42], infection by *C. albicans* or *C. trachomatis* [47, 52-53], and extracellular ATP [62-63]. ATP can stimulate K+ efflux due to the association of the purinergic receptor, P2X7, with the recently-discovered hemichannel, pannexin-1 [64-65],

which allows formation of large non-selective pores in the membrane. Pannexin-1 in fact has been shown to be necessary for ATP-induced IL-1β secretion [65-66].

ATP stimulation of macrophages can also induce formation of reactive oxygen species, which lead to caspase-1 activation [67]. The antioxydant N-acetylcysteine can inhibit both ATP mediated caspase-1 activation and, interestingly, NLRP3 inflammation activation upon treatment with ionophores such as nigericin [61, 67].

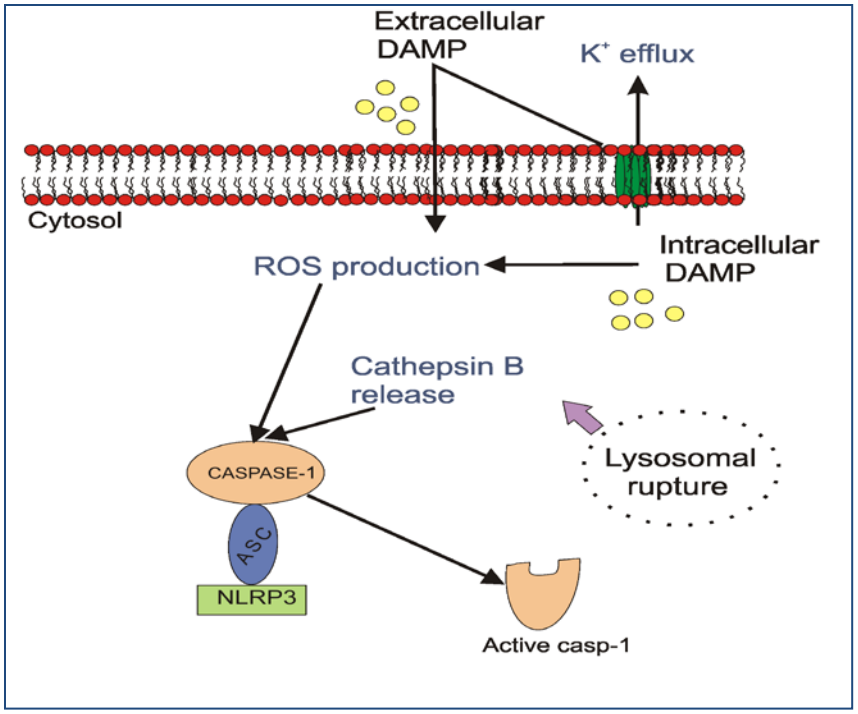

Figure 2 Diverse danger signals lead to NLRP3 inflammasome activation through common mechanisms. Extracellular damage-associated molecular patterns (DAMPs) can lead to K+ efflux and then ROS production, or directly to ROS production. Large particles can also cause lysosomal destabilization and release of enzymes such as cathepsin B. Either high ROS levels or cytosolic cathepsin B can stimulate the NLRP3 inflammasome, leading to caspse-1 activation.

Finally, destabilization of lysosomes by silica crystals, amyloid-β fibrils and alum salts has been shown to activate the NLPR3 inflammasome [57-58].This rupture of lysosomes causes release of the lysosomal protease, cathepsin B, which is somehow sensed by the NLRP3 inflammasome inducing its assembly and activation.

The NALP1 Inflammasome. NALP1 forms an inflammasome containing caspase-1 and ASC. It is stimulated by the presence of cytosolic muramyl

dipeptide (MDP), which results in activation of caspase-1 [36]. NALP1 inflammasome in a cell-free system demonstrated that caspase-1 activation occurs in a two-step mechanism whereby MDP triggers a conformational change in NALP1, allowing it to oligomerize into the inflammasome after binding nucleotides [26]. Separate studies on the NALP1 inflammasome were carried out in mice, which possess three NALP1 paralogues (NALP1a, NALP1b and NALP1c), compared to the sole NALP1 gene in humans. Susceptibility of macrophages to lethal toxin (LT), a metalloproteinase crucial for *B. anthracis* pathogenesis, is mediated by an NALP1b inflammasome through an unknown mechanism [68] (53). Transfection of fibroblasts with NALP1b and caspase-1 conferred susceptibility of these cells to anthrax LT [69].

The IPAF Inflammasome. The IPAF inflammasome is activated by some Gram-negative bacteria possessing type III or type IV secretion systems. These bacteria include *S. typhimurium*, *S. flexneri*, *P. aeruginosa* and *L. pneumophila* [37, 70-74]. An initial study showed that caspase-1 activation and subsequent IL-1β and IL-18 maturation in response to *S. typhimurium* infection requires the assembly of the IPAF inflammasome [37], and later work demonstrated that IPAF senses the presence of flagellin in the cytosol aided by the type III secretion apparatus [75-76]. Similarly, *L. pneumophila* utilizes its Dot- Icm type IV secretion system to activate the IPAF inflammasome and caspase-1, which then restricts growth of *Legionella* in macrophages [74]. Flagellin from *Legionella* was shown to be the trigger for assembly of the IPAF inflammasome [77] . A recent study showed that *L.pneumophila* can also induce caspase-1 activation

independently of IPAF, through a pathway involving ASC and triggered by loss of intracellular K+ [78]. Unlike *Salmonella* and *Legionella, S. flexneri* does not possess flagellin, yet is still capable of activating the IPAF inflammasome through a process that requires an intact type III secretion system [73]. Although initial studies showed that IPAF inflammasome activation following *P. aeruginosa* infection requires flagellin and an intact type III secretion system [70-71], it was also found that either the non-flagellated strain PA103ΔU or the flagellin-deficient mutant strain PAKΔfliC can also activate the IPAF inflammasome, leading to IL-1β secretion [72].These results suggest the existence of a flagellin-independent mechanism for IPAF inflammasome assembly.

NOD Signalosomes. Studies had shown that overexpression of both NOD1 and NOD2 leads to their auto-oligomerization leading to the exposure of their CARD domains that recruit a CARD containing serine/threonine kinase called RIP2 or RICK which interacts with the regulatory subunits of IKK complex triggering NF-κB activation [79-80]. Recently, the CARD-containing protein CARD9 has also been found to interact with NOD2 and RIP2 and to be involved consequently in the activation of JNK and p38 [81]. Both NOD1 and NOD2 sense muropeptides released from intracellular or phagocytosed bacterila peptidoglycan (PGN) which is a major component of the Gram-positive and Gram-negative bacteria cell wall. NOD1 and NOD2 were shown to detect different motifs within this structure [82-83].

Impotantly, mutations in NOD1 and NOD2 encoding genes are associated with auto inflammatory diseases; mutation in the LRR domains of NOD2 is translated into loss of bacterial sensing leading to decreased tolerance toward commencal bacteria seen Crohn's disease [84] ; on the other hand a gain of function mutations in the NACHT domain of NOD2 have been shown to be responsible for Blau syndrome, a rare autoinflammatory disorder affecting the eye and joints [85].

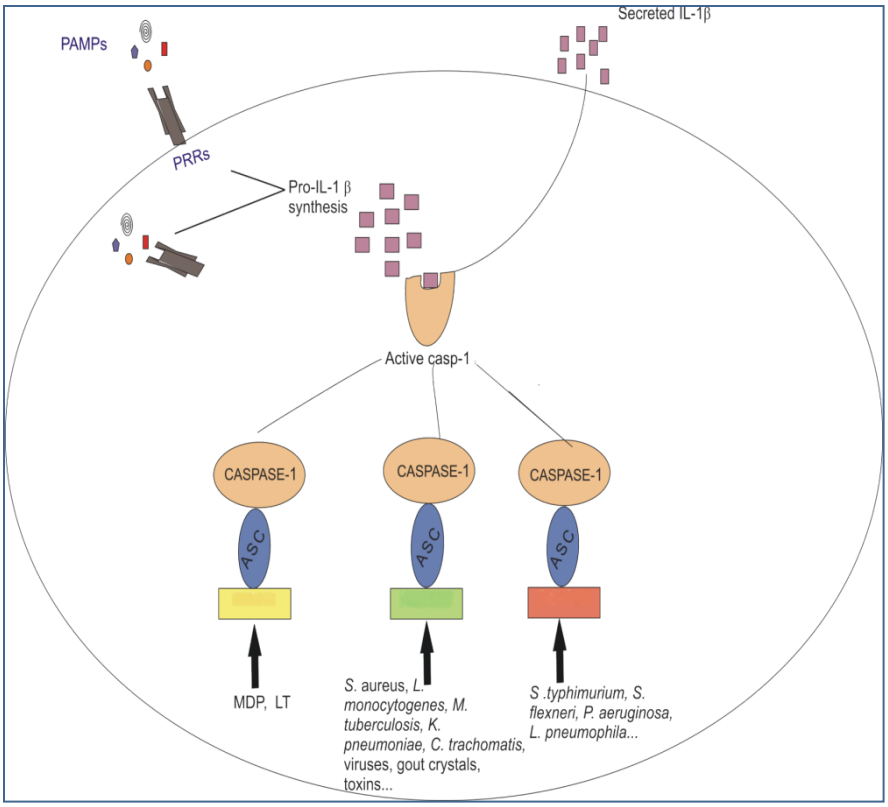

Figure 2 : Both a pathogen recognition receptor (PRR) and Nod-like receptor (NLR) family member are usually required for secretion of IL-1β. Binding of a pathogen-associated molecular patterns (PAMP) to its PRR results in pro-IL-1β synthesis, but not always secretion of IL-1β. A second signal, derived from an extracellular "danger signal" such as ATP or gout crystals, or an intracellular PAMP such as muramyl-dipeptide (MDP) from peptidoglycan activate an inflammasome consisting of caspase-1, the adaptor protein ASC, and a NLR family member or AIM2. LT, lethal toxin from *Bacillus anthracis*; dsDNA, double-stranded DNA. Inflammasome-dependent caspase-1 activation results in processing and secretion of the mature IL-1β.

The function of these different inflammasomes and signalosomes in trigerring the inflammation process in response to a huge variety of microbes is now well established (Figure 2).

Indeed, the identification of the inflammasome role in several disease to which caspase-1 activation has been linked had brought tremendous advances understanding the critical importance of NLR and their mutations which has rapidly led to specific and effective therapy for these disorders.

Therefore, during my PhD, I focused on studying the involvement of inflammasome activation and the mechanisms that governs inflammasome-associated cellular responses to two different pathogens known to cause diseases with severe consequences on Human health. I conducted a comparative analysis of inflammation induced by the *Aspergillus.fumigatus* and the bacterium *Chlamydia.trachomatis* and studied their similarities and their discrepancies in terms of inflammasome activation.

2. *Aspergillus.fumigatus* and Invasive Aspergillosis:

Aspergillus is a filamentous, ubiquitous fungus found in air, plants and soil worldwide.The genus *Aspergillus* includes about 200 species, of which 20 have been reported as human pathogens causing opportunistic infections, allergic states and invasive aspergillosis (IA).

Recently, *Aspergillus fumigatus* has been identified as the second most common causative agent of fungal infection after *Candida albicans*.

2.1 *Aspergillus* life cycle:

The life cycle of the filamentous ascomycete *Aspergillus fumigates* is marked by three important developmental events. Under favorable extrinsic environmental growth conditions i.e. when temperature and humidity are right and when enough nutriments and oxygen are provided, the haploid, uninucleate conidia (spores) starts the germination process giving rise to an elongated, septate and hyaline filament called hyphae that undergo a growth phase that is accomplished through spatial organization of differentiated cell types which end up forming

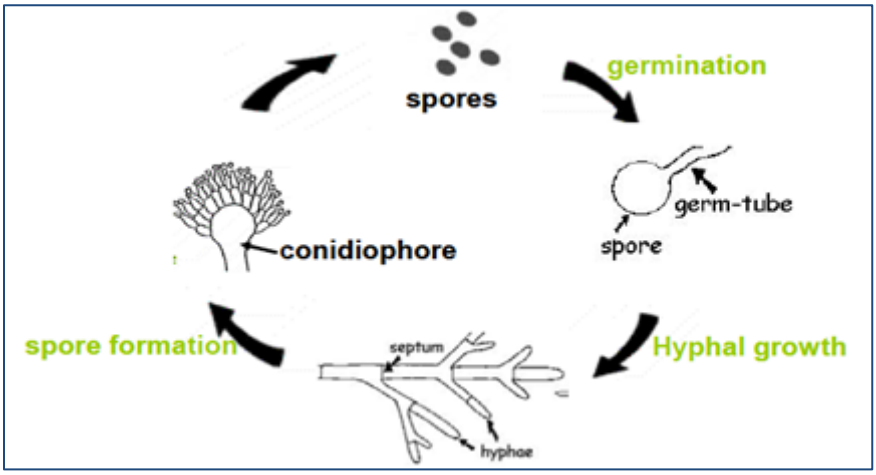

Figure 3: Cell cycle of *A. fumigatus* which includes 3 phases: **germination,** where the dormant spores become competent for growth and start swelling; **hyphal growth,** which consists in a long branching filament; and **spore formation** in the conidiophores that originate from the basal cell located on the supporting hyphae and terminate in a vesicle full of spores at the apex.

multicellular differentiated structures, called conidiophores in which newly synthesized pigmented haploid conidia are stocked and released subsequently into the air [86]. For many years *A. fumigatus* was thought to only reproduce asexually, however in 2009, O'Gorman CM *et al* had shown for the first time that *A. fumigatus* possesses a fully functional sexual reproductive cycle that occurs under very specific conditions [86-87].

2.2 Aspergillus.fumigatus in immunodepressed patients:

Due to their small size (2-3 μm in diameter), the conidia can penetrate deeply into the respiratory airways by simple inhalation and adhere to epithelial cells before infection starts [88-89].

These fungi are efficiently eliminated by the immune system in healthy individuals; however it can trigger a severe IA responsible for high rates of morbidity and mortality in immunocompromised patients [90-91]. In these patients, *Aspergillus* spores start to germinate in the lungs, forming branching hyphal filaments which break off and enter the bloodstream, leading to vascular invasion of hyphal fragments throughout the body [92]. Almost all organs can be infected after fungal dissemination: the pathogen has been found in the skin, oesophagus, blood, spleen kidneys and brain of patients with IA [93-94]. In bone marrow recipients, cerebral aspergillosis is the most common cause of brain abscess, which can result in epileptic fits and focal neurological signs [95].

Very few survivors from IA have been reported in immunodepressed patients. These may be due to two main reasons. First, there are no clinical symptoms that are specific for IA. The common symptoms of IA, such as fever, chest pain, and

breathlessness, are shared by other pulmonary diseases and make the diagnosis of IA a real challenge for clinicians. Secondly, there is still no antifungal therapy that is effective in controlling IA.

2.3 Immune response to *Aspergillus fumigatus* infection:

During the last 20 years, as the number of immunodepressed patients has increased, IA has become a more common life-threatening disease, with a death rate higher than 60% [96]. Hence, there has been a steady increase in the number of publications on this fungal infection, but we are still far from a thorough understanding of the disease, especially with regards to initiation of inflammation during *Aspergillus* infection. As mentioned above, inflammation results in large part to stimulation of PRRs that serve as sensors of conserved motifs expressed on microbial pathogens called PAMPs. Studies on the interactions between *A. fumigatus* and TLRs have begun a few years ago. The findings show that conidia and hyphae from *A. fumigatus* can activate murine peritoneal macrophages via TLR-2 and -4 [97]. Moreover, it appears that conidia can stimulate both TLR-2 and -4, whereas hyphae are only recognized by TLR-2 [97].This is believed to be a strategy adopted by the fungus to evade the innate immune response during germination. Nevertheless, the immunostimulatory molecule(s) of *A. fumigatus* that are recognized by TLRs are still under investigation. Characterization of the hyphal cell wall showed that it contains four major carbohydrate polymers, of which one of them, the β-glucans, can activate dectin-1 in alveolar macrophages [98]. Stimulation of these PRRs (TLR-2, TLR-4, and dectin-1) during infection with *A. fulmigatus* subsequently leads to activation of transcription factors such as

NF-κB whose translocation into the nucleus stimulates upregulation of the pro-inflammatory cytokines.

Secretion of pro-inflammatory cytokines (TNF α , IL-12 and especially IL-1 β) and chemokines (Mip-2 in mice, IL-8 in humans) helps to recruit neutrophils and lymphocytes to the pulmonary infection site and insure clearance of the fungus. Neutrophils and macrophages are the two main cell types responsible for the innate host defence against aspergillosis. The risk of infection is higher in subjects presenting an inadequate number or anomalies of these cell types [99]. The use of immunosuppressive treatment such as high-doses of steroids had been reported to cause a failure in the production of the oxidative burst and in the initiation of cytokine and chemokine production required to eliminate the fungal spores and hyphae present in the lungs airways [100]. This might explain why immunocompromised patients who encounter conidias in their respiratory tract are predisposed to subsequent invasive pulmonary aspergillosis.

The functional assembly of an inflammasome has not been described yet during aspergillosis. Caspase-1 activation during *A. fumigatus* infection has yet to be investigated, but studies showing secretion of IL-1 β by the human monocyte/macrophage cell line, THP1, following stimulation by *A. fumigatus* [101] suggest that caspase-1 must be activated in the cells, either directly by the fungal pathogen or in combination with a host-cell derived DS. Therefore, **we hypothesize that *A. fumigatus* infection induces caspase-1 activation in an inflammasome-dependent manner.**

The Specific Aims of the *Aspergillus* project were to:

1. Evaluate if *A. fumigatus* infection induces caspase-1 activation in human monocytes.

2. Characterize the "danger signal" produced and identify the "inflammasome" activated during *A. fumigatus* infection that leads to IL-1β secretion.

SIGNIFICANCE:

When I started my PhD, inflammasome-dependant caspase-1 activation has never been investigated in fungal diseases. Thus, the characterization of the molecular basis for *A. fumigatus*-induced inflammation may help us to better understand the biological basis for the immune response to *Aspergillus* infection. Additionally it will provide us with new insights for understanding the predisposition of immunocompromised patients to develop IA. Finally, since synthetic ligands for various NLR family members are being developed [102-103], they could be used to stimulate an inflammasome in IA patients, with the goal of ameliorating the debilitating symptoms of this fungal disease.

3. *Chlamydia trachomatis* and Chlamydial diseases

Chlamydia is a Gram negative obligate intracellular bacterium. Three different species of *Chlamydia* are pathogenic to human beings: *Chlamydia trachomatis, Chlamydia psittaci*, and *Chlamydia pnemoniae*.

Chlamydia trachomatis has many different sub-groups called serovars which are divided into sub-group depending on the diseases caused upon infection [104]. Certain serovars A-C infect ocular tissues and give rise to trachoma, the leading cause of preventable blindness worldwide while other serovars D-K, L1-L3 infect urogenital tissues leading to sexually transmitted disease including pelvic inflammatory disease (PID) in women, where 11% develop infertility and 9% develop ectopic pregnancy due to scarring respectively of the ovaries and the fallopian tubes [105-108]. Over 90 million people each year get infected with *C.trachomatis* with higher prevalence among young sexually active adults aged 18 to 26 years [109]. Even though chlamydial infection is the most common bacterial sexually transmitted infection worldwide, the biggest challenge to control this infection is that as many as 80% of women and 50% of men are asymptomatic; however within their infected cells Chlamydia multiplies silently and cause irreversible scarring and tissues damages [110].

C. pneumoniae is a common cause of pneumonia around the world exhibiting symptoms that are indistinguishable from pneumonia caused by other infectious agents. These include cough, fever, and difficulties breathing. Studies have shown that around 80% of the population is infected sometime during their lives with *C.*

pneumoniae, which is responsible for 10% of all community-acquired pneumonia [111]

C. psittaci infects birds that represent an excellent mobile vector capable of transmitting chlamydial infection to humans by contact, ingestion or inhalation of aerosols of their droppings. The symptoms may vary from mild “flu-like” symptoms that might turn into a life-threatening pneumonia [112].

C. muridarum (MoPn) is the trachomatis biovar that naturally infects members of the Muridae family which includes both mice and hamsters. Conveniently, MoPn infection was shown to be capable of producing a pathology similar to what is seen in humans, and therefore is used in vivo and in vitro models of chlamydial pathogenesis [113].

3.1 Cycle of Chlamydiae*:*

Although the different *C.trachomatis* serovars exhibit different tissue tropisms, they all infect preferentially epithelial cells in which they undergo a distinct developmental cycle converting between two morphologically and functionally separate forms, the elementary body (EB) and the reticulate body (RB) [114]. The elementary body is the dispersal form and is analogous to a spore of 300nm in diameter; it is the infectious form that induces its own endocytosis when exposed to target cells. They are able to survive in the extracellular environment and display little or almost no metabolic activity. Once inside the endosome it prevents phagolysosomal fusion and hence allows its intracellular survival. Within 2 hours, EBs differentiate into metabolically active but non infectious RBs

[114]. Once established in this niche, the inclusion selectively recruits factors from the host cell essential for chlamydial development such as sphingolipids [115], cholesterol [116] and glycerophospholipids [117]. Rapidly, reticulate bodies start replicating by binary fission inside inclusion boundaries and after division, transform back to the elementary form which lyses the cell after Developmental cycle completion to initiate subsequent rounds of infection [114] (figure 4).

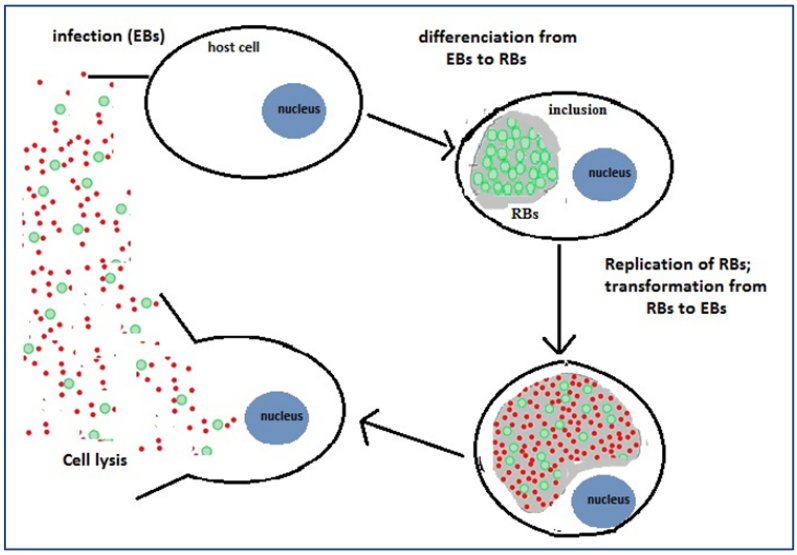

Figure 4: ***Chlamydia*** **cycle consists of : infection** where the elementary body infects the epithelial host cells and **differenciation** within few hours from EBs to RBs followed by **Replication** of RBs within the vesicles. RBs differentiate back into EB which are released into the extracellular milieu after **cell lysis**.

3.2 Host response to Clamydial infection

Most information on the immune response to *Chlamydia* has been obtained from work with mice. In general, mouse models have proven to be excellent systems to study the immune mechanisms that are thought to control *Chlamydia* in humans. During *Chlamydia* infections, the immune system of the infected host encounters antigens expressed at various stages of the chlamydial developmental cycle. Even though epithelial cells are not considered to be immune cells they are capable of initiating and propagating innate immune responses [118] as they express anti-microbial factors such as epithelial defensins and produce a wide range of

cytokines and chemokines such as interleukin (IL)-6, IL-1α, IL-8, tumor necrosis factor-α(TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF) which attract and direct immune cells to the challenged site [119]. The recruited cells produce in turn more inflammatory cytokines through the recognition of chlamydial LPS by TRL4 and other still unknown chlamydial PAMPs by TLR2 which contributes to the progression of a chronic inflammatory stage responsible for irreversible tissue damage associated with *Chlamydia* – induced pathology.

The adaptive immune response to *Chlamydia* relies primordially on B cells activation which assures the recognition of soluble antigen via B cell receptor during secondary infection [120]. In addition to anti-chlamydial antibody production by B cells that help in neutralizing chlamydial infectivity and enhancing phagocytosis, $CD4^+$ Th1 and $CD8^+$ T_c cells play a greater role in clearing the infection first by producing IFNγ which inhibits chlamydial replication and second by direct killing of infected cells [121].

Previous studies demonstrated that dendritic cells, macrophages, and monocytes, secrete the pro-inflammatory cytokine IL-1β in response to chlamydial infection [122-125].Although, the secretion of this potent inflammatory cytokine is necessary for chlamydial clearance, when excessive it becomes detrimental for the host as it contributes to the development to genital tract pathology accompanied by the infection [126]. Inflammasome activation during chlamydial infection had never been investigated so far. Ali Abdul Sater el al from our lab had published in 2009 that *Chlamydia.trachomatis* induces caspase-1 activation

through NALP3 inflammasome assembly in cervical epithelial cells [47]. This study is very interesting since it was the first to show that epithelial cells do express NLRs and are capable of assembling a functional inflammasome responsible for caspase-1 activation. Therefore, since caspase-1 activation in immune cells during chlamydial infection is required for IL-1β secretion**, I hypothesized that *C.trachomatis* is able to induce NALP3-mediated caspase-1 activation in human monocytes**.

The Specific Aims of the *Chlamydia* project were to:

1. Evaluate if *C.trachomatis* infection induces caspase-1 activation in human monocytes in a NALP3 dependant manner.

2. Characterize the "danger signals" required and identify the mechanisms by which it induces the inflammasome activation.

Significance:

In his study Ali showed that although epithelial cells are not known to secrete large amounts of IL-1β, inflammasome dependent caspase-1activation in epithelial cells is crucial for chlamydial growth. This reveals the importance of caspase-1 activation in a non-inflammation context where it is involved in lipid metabolism and membrane regeneration in epithelial cells [127]. However further insights are needed in order to understand whether the bulk of IL-1β secreted by the macrophages and neutrophils is inflammasome-dependant and which of the NLRs is being involved. Studying the mechanisms for caspase-1 activation in immune cells challenged with *Chlamydia* will bring more knowledge in understanding how chronic inflammation is established leading to the immune

mediated pathology and may eventually lead to the development of an anti-*Chlamydia* vaccine that can control chlamydial challenge while avoiding host irreversible tissue damage.

Chapter 2. Results

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Aspergillus fumigatus Stimulates the NLRP3 Inflammasome through a Pathway Requiring ROS Production and the Syk Tyrosine Kinase

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Abstract

Invasive aspergillosis (IA) is a life-threatening disease that occurs in immunodepressed patients when infected with *Aspergillus fumigatus*. This fungus is the second most-common causative agent of fungal disease after *Candida albicans*. Nevertheless, much remains to be learned about the mechanisms by which *A. fulmigatus* activates the innate immune system. We investigated the inflammatory response to conidia and hyphae of *A. fumigatus* and specifically, their capacity to trigger activation of an inflammasome. Our results show that in contrast to conidia, hyphal fragments induce NLRP3 inflammasome assembly, caspase-1 activation and IL-1β release from a human monocyte cell line. The ability of *Aspergillus* hyphae to activate the NLRP3 inflammasome in the monocytes requires K^+ efflux and ROS production. In addition, our data show that NLRP3 inflammasome activation as well as pro-IL-1β expression relies on the Syk tyrosine kinase, which is downstream from the pathogen recognition receptor Dectin-1, reinforcing the importance of Dectin-1 in the innate immune response against fungal infection. Furthermore, we show that treatment of monocytes with corticosteroids inhibits transcription of the gene encoding IL-1β. Thus, our data demonstrate that the innate immune response against *A. fumigatus* infection involves a two step activation process, with a first signal promoting expression and synthesis of pro-IL-1β; and a second signal, involving Syk-induced activation of the NLRP3 inflammasome and caspase-1, allowing processing and secretion of the mature cytokine.

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Introduction

Invasive aspergillosis (IA) is a life-threatening disease that occurs in patients with hematological malignancies [1,2], solid organ transplants [3], or immunodeficiency syndromes or patients receiving immunosuppressive treatment [4,5]. The genus *Aspergillus* includes about 200 species, of which 20 have been reported as human pathogens causing opportunistic infections, allergic states and invasive aspergillosis.

Aspergillus fumigatus is considered as the second most-common causative agent of fungal infection after *Candida albicans*. *A. fumigatus* grows at physiological temperature (37°C), has a stable haploid genome, and undergoes asexual reproduction, forming conidiospores that are released into the environment. Due to their small size (2–3 μm in diameter), the conidia can penetrate deeply into the respiratory airway by simple inhalation and adhere to epithelial cells before infection starts [6–8].

Normally, this fungus is efficiently eliminated by the immune system in healthy individuals; however it can trigger a severe IA responsible for high rates of morbidity and mortality in immunocompromised people [9,10]. In these patients, *Aspergillus* spores begin to germinate in the lungs, forming branching hyphal filaments that break off and enter the bloodstream, leading to vascular invasion throughout the body [11]. Almost all organs can be infected after fungal dissemination. Co-infection with other pathogens such as cytomegalovirus (CMV) or *Candida* is very common and complicates IA, making it harder to cure.

The innate immune response against *A. fumigatus* plays a crucial role in controlling infection [12]. Several pattern recognition receptors (PRRs) such as Toll-like receptor (TLR)-2, TLR-4 and dectin-1 [13] have been observed to play a role in recognition and clearance of the fungus [14–17]. These studies have shown that host resistance to *A. fumigatus* involves the induction of pro-inflammatory cytokines including INFγ, interleukin (IL)-12, TNFα, and significantly, IL-1β [18,19]. Nevertheless, the immunostimulatory molecule(s) of *A. fumigatus* that are recognized by PRRs and the molecular basis for inflammation initiation are still under investigation.

PRRs sensors of conserved motifs expressed on microbial pathogens called "pathogen-associated molecular patterns" (PAMPs) [20]. PAMPs stimulate PRRs such as surface-bound and endosomal TLRs, but also dectin-1 and cytosolic NOD-like receptor (NLR) family members. Stimulation of these PRRs (TLR-2, TLR-4, and dectin-1) during infection with A. *fumigatus* subsequently leads to activation of transcription factors such as NF-κB, whose translocation into the nucleus stimulates the

upregulation of pro-inflammatory cytokines. Secretion of pro-inflammatory cytokines (TNFα, IL-12 and IL-1β) and chemokines (Mip-2 in mice, IL-8 in humans) helps to recruit neutrophils and lymphocytes to the pulmonary infection site and insure clearance of the fungus. Neutrophils and macrophages are the two main cell types responsible for the innate host response against aspergillosis, therefore the risk of infection is higher in subjects presenting an inadequate number or anomalies of these cell types [21].

The pro-inflammatory cytokine, IL-1β, is synthesized as an inactive cytoplasmic precursor, pro-IL-1β, which is processed into a biologically active, secreted form by caspase- 1, a cysteine protease [22,23]. The latter is synthesized as an inactive form that is self-activated by cleavage, generating an enzymatically active heterodimer composed of 10 and 20 kDa chains [22]. Recent studies have implicated members of the NLR family of proteins in the regulation of caspase-1 activation [22,24]. The NLR family is composed of 23 cytosolic proteins, some of which recognize PAMPs. The family includes nucleotide binding oligomerization domain 1 (NOD1), NOD2 [12,16], the NLRP3/cryopyrin/Nalp3 "inflammasome" component [25,26], and the NLRC4/Ipaf inflammasome component [27,28].

Upon infection, stimulation of TLRs or the cytosolic NOD1 or NOD2 receptors activates transcription, synthesis, and secretion of pro-inflammatory cytokines such as INFγ, IL-12, and TNFα [29,30]. Given the key role played by IL-1β in fever and inflammatory disease [31], its production and secretion is tightly controlled and requires typically two separate signals [32–34]. The first signal comes from PAMPs and promotes transcription, production and intracellular accumulation of the immature cytokine. The second signal, usually derived from a "danger signal" (DS), leads to the activation of an inflammasome, activation of caspase-1, and secretion of the mature cytokine. The requirement for two signals thus insures that IL-1β is secreted by macrophages only if they are stimulated by PAMPs and the PAMPs are produced under circumstances that could be viewed as potentially dangerous to the host organism [25]. Examples of DSs include host-cell components released from dying, infected or stressed cells such as ATP, adenosine, uric acid, or chromosomal proteins; but they could also be microbial PAMPs that are located in "threatening" locations, such as flagellin in the cytosol of an infected cell [33].

Several studies have recently described stimulation of the NLRP3 inflammasome in the innate immune response to *C. albicans* infection [35–37]. These were the first reports to show the involvement of an inflammasome during a fungal infection. However, stimulation of an inflammasome has not been described yet during *A. fulmigatus* infection. Although caspase-1 activation during *A. fumigatus* infection has not been investigated, studies showing secretion of IL-1β by the human monocyte/macrophage cell line, THP-1, following stimulation by *A. fumigatus* [19] suggested that caspase-1 must be activated in these cells, either directly by the fungal pathogen or in combination with a host-cell derived DS.

The goal of this study was therefore to determine whether *A. fumigatus* induces IL-1β secretion in a caspase-1 dependent manner by THP-1 cells, and evaluate whether this fungus activates an inflammasome. Our results show that *A. fumigatus* spores fail to induce caspase-1 activation, unlike hyphal fragments, which upregulate pro-IL-1β synthesis and stimulate caspase-1 activation. Importantly we revealed the requirement of an NLRP3 inflammasome and its adaptor protein, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), in activating caspase-1, thus revealing NLRP3 and ASC as key regulators of inflammation during *A. fumigatus* infection.

Results

A. fumigatus hyphae upregulate pro-IL-1β expression and induce IL-1β secretion in human monocytes

To assess whether *A. fumigatus* could induce directly the synthesis or secretion of IL-1β, we examined the effect of different morphological forms of this fungus. The human monocyte cell line, THP-1, was infected with either conidia at a multiplicity of infection (MOI) of 10, or hyphal fragments (HFs) for 6 hours. As a positive control, the cells were primed with 10 ng/ml of lipopolysaccharide (LPS) for 6 hours in order to stimulate pro-IL-1β protein synthesis, with or without subsequent treatment with an NLRP3 inflammasome stimulator, the bacterial toxin nigericin for 1 hour. Real time PCR analysis showed that a 6 hour incubation with HFs induced a drastic increase in transcription of this pro-inflammatory cytokine gene, while spores provoked only a 2-fold increase (Figure 1A). Analysis of the supernatants by ELISA revealed that mature IL-1β was secreted from cells treated with LPS and nigericin, or only infected with HFs (Figure 1B). However, no significant secretion, beyond basal cytokine secretion levels, was seen when the cells were incubated with conidia (Figure 1B), consistent with the inability of this fungal form to induce transcription of the cytokine. To further investigate the potential of conidia to induce a pro-inflammatory cellular response, we incubated THP-1 cells in the presence of *Aspergillus* spores for a longer period of time, long enough to initiate the germination process. IL-1β and TNFα secretion was measured in the supernatants 12 hours after infection with spores, compared to 6 hour stimulation with HFs. Interestingly, swollen conidia were unable to induce any significant IL-1 β or TNFα secretion whereas the HFs caused noticeable monocyte activation (Figure 1C,D). Thus, we conclude that in contrast to HFs, *A. fumigatus* spores are unlikely to be critically involved in initiating IL-1β-dependent inflammatory responses. We decided therefore to perform the subsequent experiments only with the HFs of *A. fumigatus*.

A. fumigatus-induced caspase-1 activation correlates with ROS production and K^+ efflux

Caspase-1 activation is essential for pro-Il-1 β cleavage and subsequently IL-1β secretion. In fact, cells stimulated only with HFs activate caspase-1, as detected by Western Blot analysis of the cell lysates and supernatant by the appearance of the active p20 subunit of caspase-1 (Figure 2A, inset). This result was confirmed by measuring the presence of caspase-1 p20 subunits whose activated form is secreted into the supernatant of infected THP1 cells, as detected by ELISA (Figure 2A).

Caspase-1 activation is remarkably reduced in the presence of the irreversible caspase-1 inhibitor (Z-WEHD-FMK) (Figure 2A). Consistent with this result, Il-1β secretion induced by *Aspergillus* HFs was significantly decreased when monocytes were pretreated with Z-WEHD-FMK, again confirming the requirement for caspase-1 activation for Il-1 β secretion (Figure 2B).

A common feature of NLRP3 inflammasome activation by diverse stimuli is the cell-signaling pathway relying on K^+ efflux and, concomitantly, production of reactive oxygen species (ROS) [34,38]. To test the role of each of these variables, we first blocked K^+ efflux by increasing the concentration of extracellular potassium, before stimulating the cells with HFs for 6 hours. IL-1 β secretion (Figure 2C) and caspase-1 activation (Figure 2A) were both significantly impaired by preventing K^+ efflux. Comparable results were obtained when we used the antioxidant, N-acetyl-cysteine (NAC), as both IL-1β secretion and caspase-1 activation were strongly inhibited by incubating cells with NAC during exposure to *Aspergillus* HFs (Figure 2A,C). Thus, we

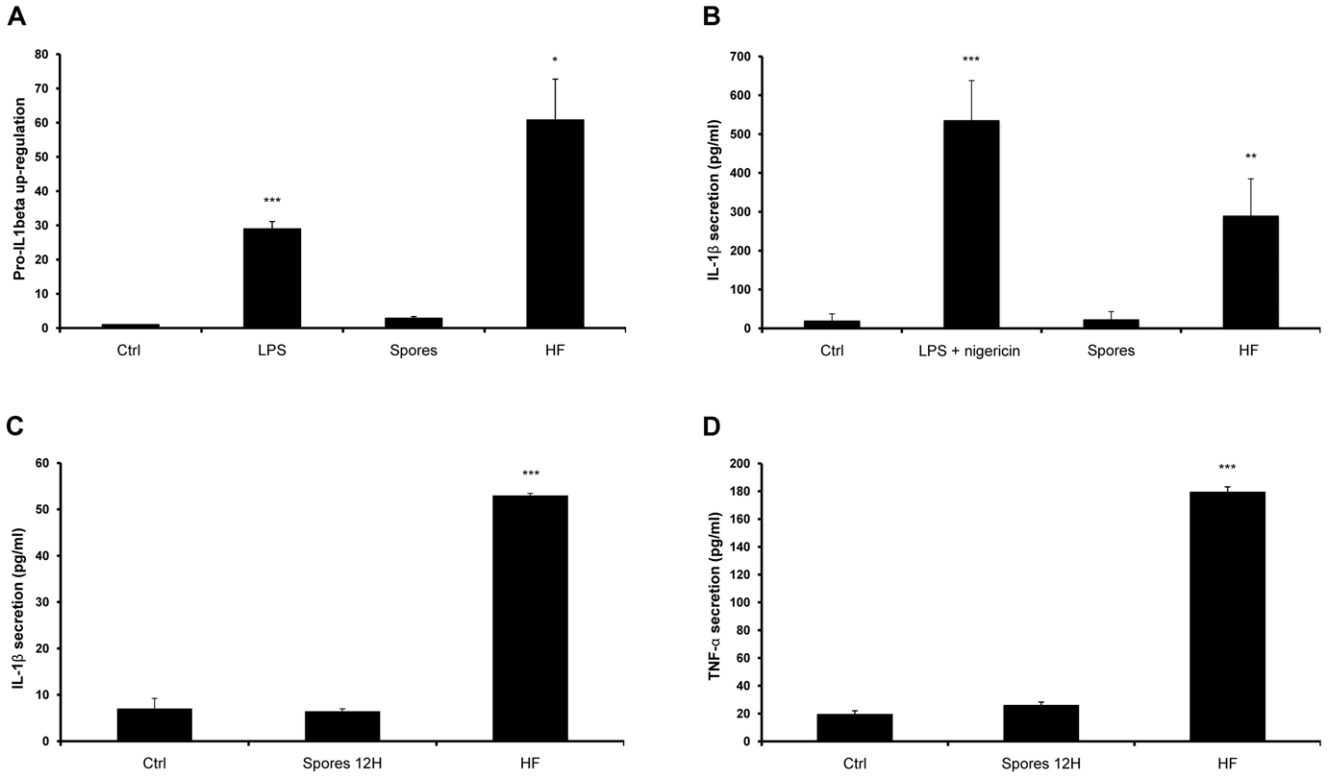


Figure 1. *A. fumigatus* hyphae upregulate pro-IL-1β transcription and induce IL-1β secretion in monocytes. One million THP-1 cells/ml were treated with 10 ng/ml of LPS with and without nigericin, spores or HFs at an MOI = 10 for 6 hours (A, B), or spores for 12 hours and HFs for 6 hours (C, D). (**A**) Intracellular IL-1β gene transcription was quantified by real-time PCR and compared to control. (**B, C**) The amount of secreted IL-1β was quantified by ELISA. (**D**) TNFα secretion was measured in supernatants by ELISA. Error bars represent standard deviation of at least three separate experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$, compared to infected untreated cells.
doi:10.1371/journal.pone.0010008.g001

conclude that ROS production and K^+ efflux are essential for HF-induced caspase-1 activation and IL-1β secretion.

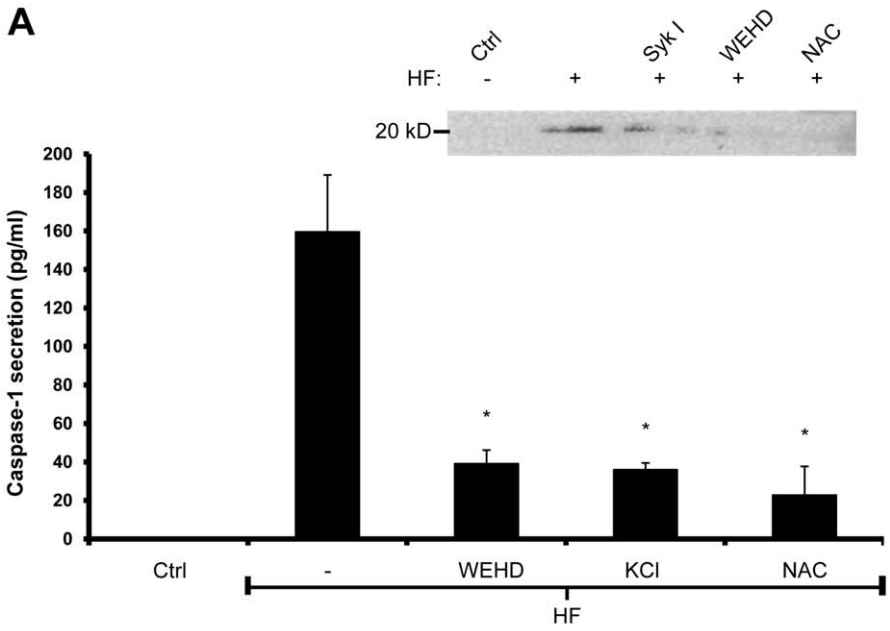
The NLRP3 inflammasome in monocytes is stimulated by *A. fumigatus*

At least four inflammasomes have been described, based on: NLRP1 (Nalp1), NLRC4 (Ipaf), NLRP3 (Nalp3/cryopyrin), and AIM2 [34,38,39]. Murine Nalp1b detects anthrax LT, while NLRC4 recognizes mainly flagellin, and AIM2 is activated in response to cytosolic double-stranded RNA. Human Nalp1 is sensitive to peptidoglycan fragments [40]. Until now, the only inflammasome reported to be sensitive to cytosolic K^+ concentrations and ROS contains NLRP3. We reasoned therefore that the NLRP3 inflammasome may be responsible for caspase-1 activation in response to *A. fumigatus* infection.

The role of NLRP3 and its adaptor protein, ASC, was determined by gene silencing in THP1 cells. The mRNA expression levels of either inflammasome component was significantly reduced in knocked down (KD) cells, in comparison to non-target shRNA, as measured by real-time PCR (Figure 3A). Protein depletion was also confirmed using Western blot analysis (Figure 3A, inset). Because secretion of mature IL-1β after stimulation of primed THP-1 cells with nigericin relies primarily on NLRP3/ASC inflammasome activation, we examined IL-1β secretion in ASC KD and NLRP3 KD cells as a functional control. THP-1 KD cells secreted significantly less IL-β when stimulated with LPS and nigericin, demonstrating the efficiency of NLRP3 and ASC gene silencing (Figure 3B). In addition, these cells showed dramatic reductions in IL-1β secretion and caspase-1 activation in response to HFs, when compared to cells transfected with non-target shRNA (Figure 3C,D,E), which also correlated with the extent of mRNA depletion in the KD cells. The decrease in caspase-1 activation and IL-1β secretion in the KD cells implies that *A. fumigatus* infection induces caspase-1 activation through a process that requires, at least partially, the assembly of the NLRP3 inflammasome.

Syk kinase provides both the first signal for IL-1β synthesis and the second signal for caspase-1 activation during *A. fumigatus* infection

Previous studies have shown that *Aspergillus* hyphae and conidia have morphologically distinct and complex features that undergo several modifications during swelling. The composition of the conidial cell wall is complex and has not been completely defined, whereas hyphae contain mainly four major carbohydrate polymers of which one, the β-glucans, can activate dectin-1 in alveolar macrophages [41]. This receptor uses an intracellular ITAM motif to initiate signaling through a tyrosine kinase, Syk, in a MyD88-independent manner [42,43]. Recent studies have revealed the importance of Syk in inducing NF-κB activation and controlling NLRP3-dependent caspase-1 activation during *C. albicans* infection. To examine whether Syk is involved in signaling during *A. fumigatus* infection, we blocked its signaling with a specific Syk inhibitor (Syk I) prior to HF exposure and measured IL-1β and caspase-1 secretion into the supernatant. The results suggest that Syk activation is indispensable for IL-1β secretion and caspase-1 activation (Figure 4A,B).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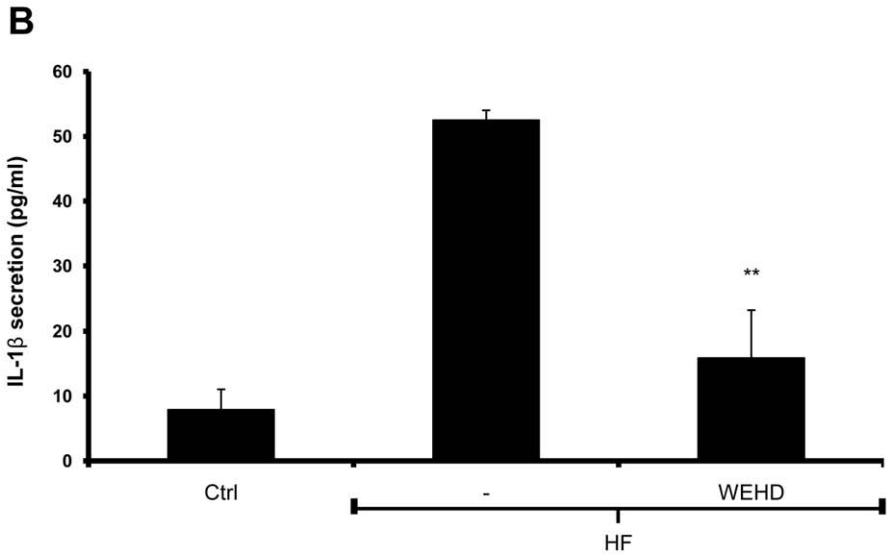
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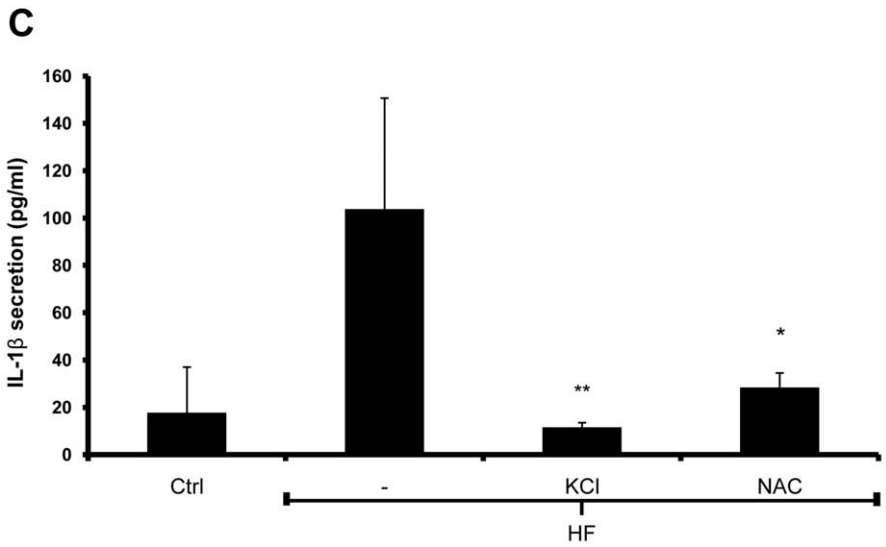
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Figure 2. *A. fumigatus* induced-caspase-1 activation depends on ROS production and K^+ efflux. THP-1 cells were incubated with HFs for 6 hours in the presence or absence of 130 mM KCl, 25 mM NAC, 100 μM caspase-1/caspase-5 inhibitor (Z-WEHD-FMK), or pretreated for 30 min with 1 μM of Syk kinase inhibitor (Syk I). (**A inset**) Caspase-1 activation was analyzed by Western blot, using an antibody against the Caspase-1 p20 cleavage product. Each band intensity was measured by NIH ImageJ software (Ctrl = 1, HF = 4.848, HF + Z-WEHD-FMK = 2.92, HF + Syk I = 1.67, and HF + NAC = 1.54). (**A**) Secreted Caspase-1 p20 and (**B, C**) mature IL-1β p17 in the supernatant of infected cells, compared to the control, was assessed by ELISA. Error bars represent the standard deviation of at least three separate experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$, compared to infected untreated cells.
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In order to further investigate the role of Syk, we used shRNAs to knock down separately Syk and MyD88. The adaptor protein MyD88 acts downstream of TLRs and is responsible for NF-κB activation. MyD88-specific gene silencing was confirmed in THP1 KD cells by real-time PCR (not shown). Since *A. fumigatus* stimulates TLR2/4 and Dectin-1 [12], knocking down Syk and MyD88 resulted in a large decrease in transcription of the gene encoding IL-1β, as shown by real time PCR (Figure 4C), and simultaneously an abrogation of IL-1β secretion by THP-1 cells (Figure 4D), compared to the SH control cells. However, only Syk KD cells presented a significant reduction in caspase-1 activation when stimulated with HFs for 6 hours (Figure 4E). These results imply that signaling through Syk and MyD88 both converge on NF-κB activation during innate responses against *A. fumigatus* infection, but only Syk signaling results in NLRP3 inflammasome activation.

A. fumigatus PAMP recognition is impaired in the presence of corticosteroids

In a large cohort study, *A. fumigatus*-infected patients who were under corticosteroid treatment were found to be at increased risk of subsequent invasive aspergillosis, suggesting a deleterious effect of these compounds on host anti-fungal resistance [44]. Therefore, it was of interest to determine whether corticosteroid treatment affects the ability of *A. fumigatus* to trigger secretion of IL-1β. Pre-incubation of THP-1 cells with β-methasone, a potent anti-inflammatory corticosteroid, followed by infection with HFs resulted in a significant drop in IL-1β secretion (Figure 5A). To distinguish between the ability of β-methasone to interfere with pro-IL-1β gene expression or caspase-1 activation, we observed that pro-IL-1β transcription induced by LPS is severely defective when THP-1 cells were pretreated with β-methasone (Figure 5B). These results show that corticosteroids inhibit primarily the ability of monocytes to transcribe the gene for IL-1β, and may partially explain why patients treated with corticosteroids fail to produce pro-inflammatory cytokines, which are crucial for recruitment of other immune cells to clear infections.

Discussion

Stimulation of PRRs (TLR-2, TLR-4, and dectin-1) during infection with *A. fumigatus* leads to activation of transcription factors such as NF-κB, whose translocation into the nucleus stimulates upregulation of pro-inflammatory cytokines. In contrast, inflammasome assembly during aspergillosis has never been described, although previous studies showing secretion of IL-1β by THP-1 during infection by *A. fumigatus* [19] suggest that an inflammasome and caspase-1 must be activated. Our studies reveal that *A. fumigatus* does in fact stimulate both pro-IL-1β production and caspase-1 activation, leading to mature IL-1β secretion into the supernatant. However, our results suggest that only *Aspergillus* hyphae, and not conidia, are capable of inducing inflammasome assembly and caspase-1 activation in monocytes. Furthermore, we showed that a NLRP3 inflammasome is involved in caspase-1 activation, since there was a profound suppression of IL-1β release from NLRP3 and ASC knocked-down cells. We also showed that the adaptor protein, ASC, is required for inflammasome activity. The list of NLRP3 inflammasome activators is growing, but the mechanisms by which this NLR family member senses its activators seem to converge on a small number of intracellular perturbations such as K^+ efflux and ROS production [38]. Our data confirmed that *A. fumigatus*-induced NLRP3 inflammasome activation in monocytes is associated with K^+ efflux and ROS production, since their inhibition resulted in a significant decrease of caspase-1 activation and IL-1β secretion.

Since *A. fumigatus* expresses ligands for several PRRs, it is likely that these ligands cooperate in transducing diverse signals. Our studies with HFs are consistent with synergy between TLR-2, TLR-4 and dectin-1 signaling, since depletion of MyD88 and Syk significantly reduced pro-IL-1β production. Moreover, our results highlighted the role of the Syk kinase as an inflammasome activator in *Aspergillus* infection, and in contrast, ruled out any involvement of MyD88 signaling in caspase-1 activation.

Disease caused by *A. fumigatus*, which is mostly nonpathogenic for humans, is closely associated with the status of the host immune system, particularly the innate immune system, rather than the pathogenicity of the fungal pathogen. In fact, immunodeficiency is a primary factor predisposing patients and animals to severe IA. Here, we show that treatment with the corticosteroid, beta-methasone, which induces immunosuppression, translated into failure of human monocytes to produce IL-1β in response to LPS or *Aspergillus* hyphae. Pro-inflammatory cytokines are crucial for stimulating an effective immune response to *A. fumigatus* infection, which includes recruitment of neutrophils to the alveolar spaces, where they constitute more than 90% of the phagocytic cells [12].

Taken together, our data thus demonstrate that the innate immune response against *A. fumigatus* infection involves a two step activation process, with a first signal, due to TLR and dectin-1 ligation, promoting expression and synthesis of pro-IL1β; and a second signal, involving Syk-induced activation of the NLRP3 inflammasome and caspase-1. Both signals, together, allow secretion of mature IL-1β. In many immunosuppressed patients, susceptibility to *A. fumigatus* infection could be caused by failure to provide an effective response to the first signal.

Materials and Methods

Aspergillus growth and culture

A. fumigatus strain AFCOH1, isolated from patients at the City of Hope National Medical Center (Duarte, CA) and kindly provided by Drs. Joseph Lyons and Markus Kalkum (City of Hope), was grown 5 to 7 days at 37°C in potato dextrose agar (BD/Difco). Conidia were extracted from agar slants by gentle tapping and resuspended into PBS containing 0.1% Tween 80 (PBS/Tw). Clumps of conidia were dispersed with 3 mm glass beads, washed with PBS/Tw and suspended in 30% glycerol. Aliquots were frozen at −80°C and thawed to 37°C prior to use as described previously [45].

To induce hyphal growth, 10^7 spores/ml were inoculated in 50 ml of potato broth (BD/Difco) and incubated for 24 hours under 200 rpm agitation at 37°C. The mycelium was then dried down onto Whatmann 54 paper using a Buckner funnel and a

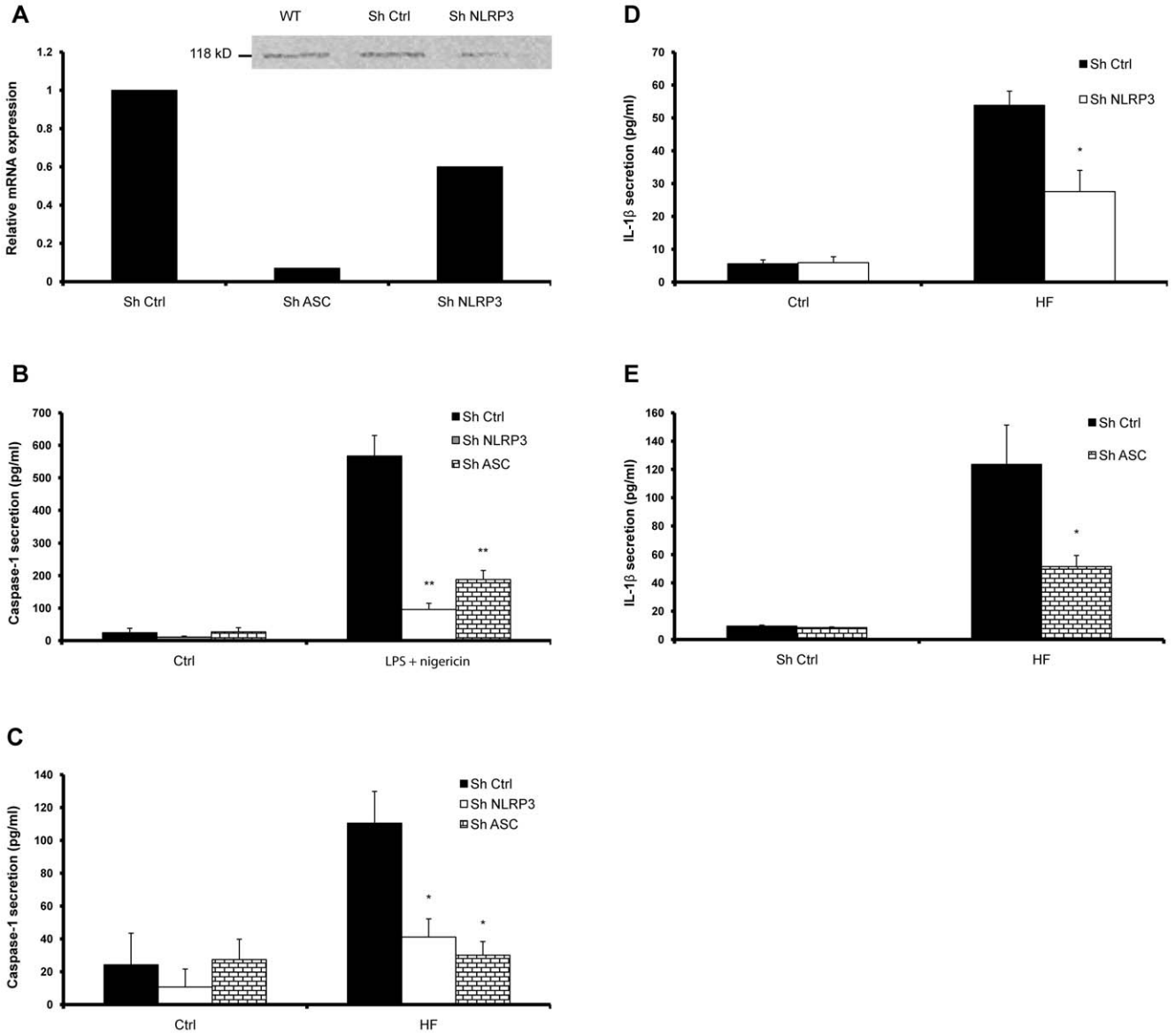

Figure 3. The NLRP3 inflammasome controls the anti-*A. fumigatus* innate immune response. THP-1 cells were stably transfected with shRNA targeting NLRP3 or ASC in order to induce gene silencing. (**A inset**) Western blot analysis of wildtype (WT) cells, cells treated with non-target control (SH control), and cells treated with shNLRP3, confirming decreased expression of the NLRP3 protein after mRNA depletion. Western blot was performed with an anti-NLRP3 antibody, which detects the 118 kDa protein. (**A**) mRNA levels of NLRP3 and ASC were quantified by real-time PCR and compared to wild type (WT) and non-target control (SH Ctrl). Supernatants of each of the knocked down (KD) cells treated with nigericin after LPS priming, or HFs for 6 hours was analyzed by ELISA for the presence (**B, C**) caspase-1 p20 and (**D, E**) mature IL-1β. All values are representative of at least three independent experiments. The error bars represent the standard deviation of at least three separate experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$, compared to infected untreated cells.
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side-arm flask attached to a vacuum pump. Hyphae were washed 3 times with 0.6 M $MgSO_4$, and resuspended in PBS/Tw. To yield hyphal fragments (HFs), this mycelium suspension was broken down under vigorous vortexing in the presence of 3 mm glass beads and stored at 4°C for up to one week.

Reagents and cell line

The human acute monocytic leukemia cell line (THP-1) was obtained from American Type Culture Collection (ATCC). N-acetyl cysteine (NAC), glibenclamide, beta-methasone and *Escherichia coli* LPS were from Sigma (St. Louis, MO). KCl was from Fisher Scientific, Syk inhibitor was from Calbiochem (Cat. No. 574711) and Z-WEHD-FMK was purchased from R&D Systems (Minneapolis, MN).

Cell culture infection and treatments

THP-1 cells were cultured in tissue culture flasks (Costar, Corning, NY) using RPMI medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Gibco) and incubated in a humidified incubator at 37°C with 5% CO_2. One million cells/ml were plated in medium and conidia or HFs were added at a multiplicity of infection (MOI) of 10 and incubated for 6 hours at 37°C with 5% CO_2. Cells were spun down at 1200 rpm, 4°C for 5 min, and supernatants were stored at −80°C for cytokine assay

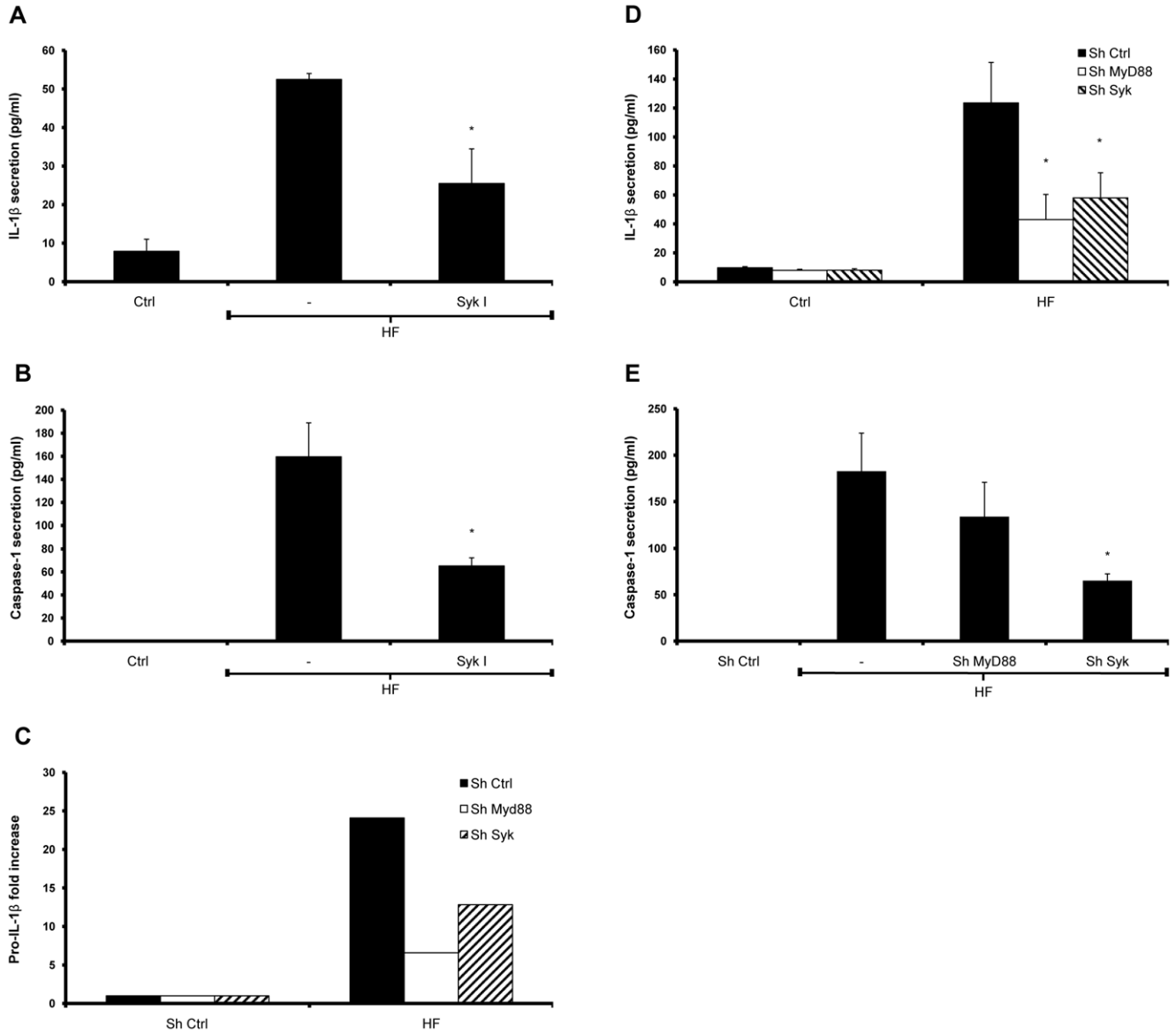

Figure 4. Syk kinase signaling provides the stimulus for both IL-1β synthesis and caspase-1 activation during *A. fumigatus* infection. THP-1 cells were pretreated with 1 μM of the Syk kinase inhibitor (Syk I) for 30 min prior to challenge with HFs, and (**A**) mature IL-1β and (**B**) active caspase-1 p20 subunit were measured by ELISA. MyD88 and Syk were stably silenced by RNA interference using shRNA. (**C**) Transcript levels of pro-IL-1β in MyD88 KD and Syk KD cells treated with HFs was measured using real-time PCR. Representative real-time PCR values representative of three independent experiments are shown. The secretion (**D**) of IL-1β and (**E**) caspase-1 p20 into the supernatants of MyD88 KD and Syk KD cells treated with HFs was assessed by ELISA. All values are representatives of at least three independent experiments. Error bars represent standard deviation of at least three separate experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$, compared to infected untreated cells.
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use and pellets are resuspended in the appropriate lysis buffer for RNA extraction or Western blot analysis. Treatment with inhibitors or other reagents was performed at the indicated times and concentrations.

Generation of THP-1 cells expressing shRNA

THP-1 cells stably expressing shRNA against NLRP3 and ASC were obtained by transducing THP-1 cells with lentiviral particles. The sequences 5′- CCGGGCGTTAGAAACACTTCAAGAACTCGAGTTCTTGAAGTGTTTCTAACGCTTTTTG-3′ for human NLRP3 (Sigma; Cat. No. NM_004895), 5′-CCGGCGGAAGCTCTTCAGTTTCACACTCGAGTGTGAAACTGAAGAGCTTCCG TTTTTG-3′ for human ASC (Sigma; Cat. No. NM_013258), 5′-CCGGCCTGTCTCTGTTCTTGAACGTCTCGAGACGTTCAAGAACAGAGACAGGTTTTT-3′for human MyD88 (Sigma; Cat No. NM_002468), and 5′-CCGGGCAGGCCATCATCAGTCAGAACTCGAGTTCTGACTGATGATGGCCTGCTTTTT-3′ for human spleen tyrosine kinase (Syk) (Sigma; Cat #: NM_003177) were used separately to silence gene expression following the manufacturer's instructions. Non-target shRNA control cells were also generated using an irrelevant sequence (Sigma; Cat. No. SHC002V).

Western blotting

Samples were lysed using RIPA Lysis Buffer (Millipore) and loaded onto a 15% SDS-PAGE gel, and then transferred to a

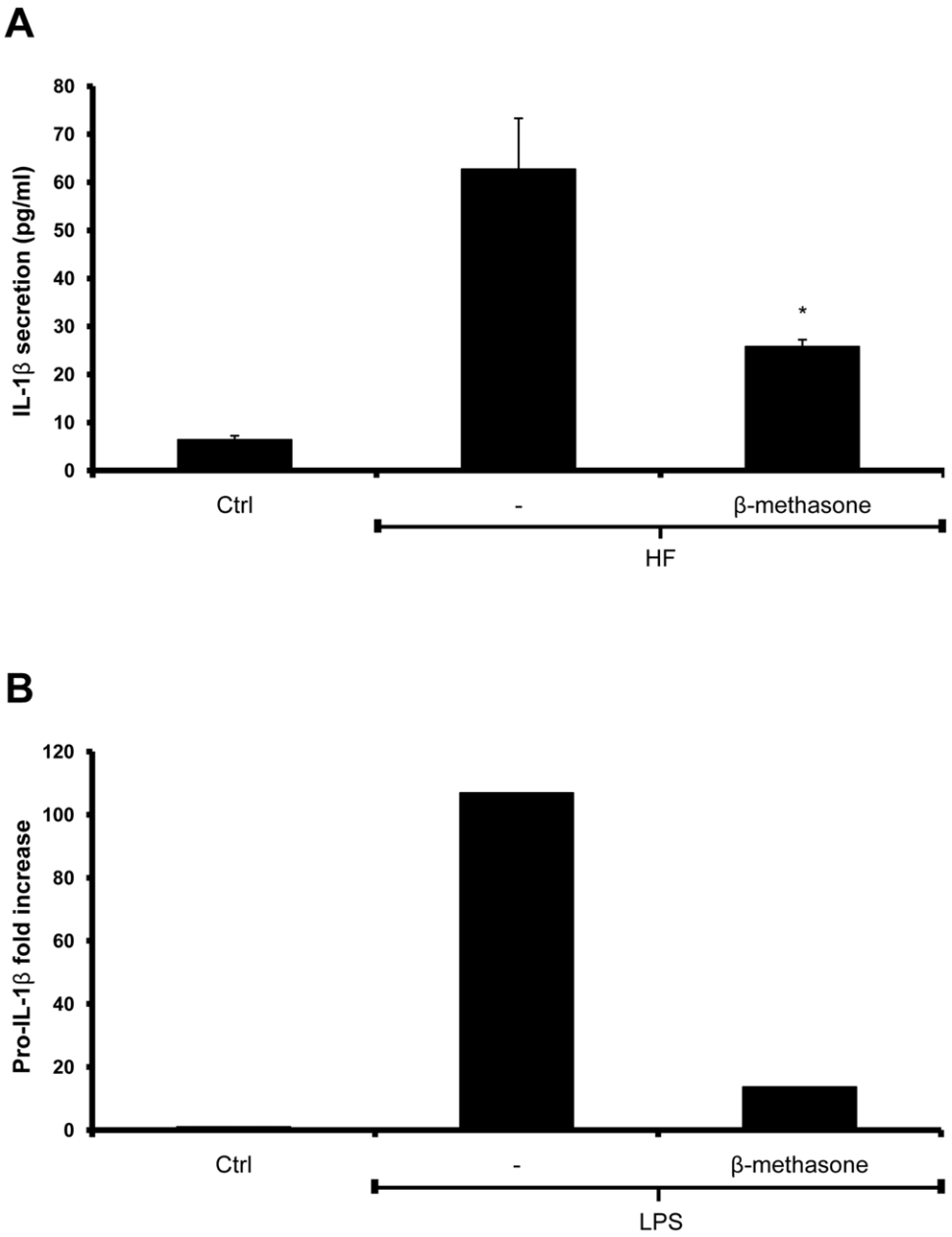

Figure 5. The inflammatory response against *A. fumigatus* is impaired in immunosuppressed monocytes. (**A**) THP-1 cells were stimulated for 10 min with 30 μM β-methasone prior to stimulation with HFs for 6 hours. IL-1β secretion was measured by ELISA. (**B**) THP-1 cells were stimulated for 10 min with 30 μM β-methasone prior to stimulation with 10 ng/ml LPS for 6 hours. IL-1β mRNA was quantified by real-time PCR.
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polyvinylidene difluoride membrane (Millipore) as we previously described [46]. Blots were blocked for 1 hr with 5% (w/v) nonfat dried milk in TBST. The membrane was incubated overnight at 4°C with rabbit antihuman caspase-1 antibody (Millipore) followed by an incubation with a conjugated anti-rabbit IgG horseradish peroxidase (Millipore). For confirmation of NLRP3 depletion by RNA interference, a 9% gel was used and the blot was incubated with rabbit anti-human NLRP3 antibody (Sigma; Cat. No. HPA012878). Immunoreactive proteins were detected with ECL Plus Western Blotting Detection Reagents (Amersham, Scituate, MA) using a gel doc system (Biorad, Hercules, CA). Intensity of bands was determined using NIH ImageJ software [47].

RNA isolation and real-time PCR

mRNA was isolated from THP-1 cells using the Qiagen RNeasy kit (Qiagen, Valencia, CA) following manufacturer's instructions, and total RNA was converted into cDNA by standard reverse transcription with Taqman® reverse transcriptase kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed with 1/50 of the cDNA preparation in an Mx3000P (Stratagene, La Jolla, CA) in a 25 μl final volume with Brilliant QPCR Master Mix (Stratagene). The primers for human GAPDH were: 5′-CTTCTCTGATGAGGCCCAAG-3′ forward, 5′GCAGCAAA-CTGGAAAGGAAG-3′ reverse. Primers for human NLRP3: 5′- CTTCCTTTCCAGTTTGCTGC-3′ forward, 5′-TCTCG-CAGTCCACTTCCTTT-3′ reverse. Primers for human ASC:

5′-AGTTTCACACCAGCCTGGAA-3′ forward, 5′- TTTTC-AAGCTGGCTTTTCGT-3′ reverse. Primers for Syk: 5′-AGA-GCGAGGAGGAGCGGGTG-3′ forward, 5′-CCGCTGAC-CAAGTCGCAGGA-3′ reverse. Primers for MyD88: 5′AGCG-CTGGCAGACAATGCGA-3′ forward, 5′-TCCGGCGGCA-CCTCTTTTCG-3′ reverse. Primers for Il-1β: 5′-CAGC-CAATCTTCATTGCTCA-3′ forward, 5′-TCGGAGATTCG-TAGCTGGAT-3′ reverse. The real-time PCR included an initial denaturation at 95°C for 10 min, followed by 40 cycle of 95°C for 30 s, 55°C for 1 min, 72°C for 1 min, and one cycle of 95°C for 1 min, 55°C for 30 s, 95°C for 30 s.

ELISA measurement of cytokine and caspase

Commercially available ELISA kits for human IL-1β (Ebioscience) and human caspase-1 (R&D systems) were used according to the manufacturers' instructions.

Statistical analysis

The difference between groups was performed using GraphPad Instat software (GraphPad Software Inc, La Jolla, CA) by Student's test. The level of significance between groups was set at $P<0.05$. All experiments were performed at least 3 times (unless stated otherwise) and the data was presented as the cumulative result of all the experiments done.

Author Contributions

Conceived and designed the experiments: DO. Performed the experiments: NSS EP. Analyzed the data: NSS. Contributed reagents/materials/analysis tools: GL DO. Wrote the paper: NSS GL DO.

References

1. Wiederhold NP, Lewis RE, Kontoyiannis DP (2003) Invasive aspergillosis in patients with hematologic malignancies. Pharmacotherapy 23: 1592–1610.
2. Herbrecht R, Moghaddam A, Mahmal L, Natarajan-Ame S, Fornecker LM, et al. (2005) Invasive aspergillosis in the hematologic and immunologic patient: new findings and key questions in leukemia. Med Mycol 43 Suppl 1: S239–242.
3. Almyroudis NG, Holland SM, Segal BH (2005) Invasive aspergillosis in primary immunodeficiencies. Med Mycol 43 Suppl 1: S247–259.
4. Latge JP (1999) Aspergillus fumigatus and aspergillosis. Clin Microbiol Rev 12: 310–350.
5. Silveira F, Paterson DL (2005) Pulmonary fungal infections. Curr Opin Pulm Med 11: 242–246.
6. Wasylnka JA, Moore MM (2002) Uptake of *Aspergillus fumigatus* Conidia by phagocytic and nonphagocytic cells in vitro: quantitation using strains expressing green fluorescent protein. Infect Immun 70: 3156–3163.
7. Zhang Z, Liu R, Noordhoek JA, Kauffman HF (2005) Interaction of airway epithelial cells (A549) with spores and mycelium of Aspergillus fumigatus. J Infect 51: 375–382.
8. Paris S, Boisvieux-Ulrich E, Crestani B, Houcine O, Taramelli D, et al. (1997) Internalization of Aspergillus fumigatus conidia by epithelial and endothelial cells. Infect Immun 65: 1510–1514.
9. Medoff G, Kobayashi GS (1991) Systemic fungal infections: an overview. Hosp Pract (Off Ed) 26: 41–52.
10. Denning DW (1998) Invasive aspergillosis. Clin Infect Dis 26: 781–803; quiz 804–785.
11. Filler SG, Sheppard DC (2006) Fungal invasion of normally non-phagocytic host cells. PLoS Pathog 2: e129.
12. Balloy V, Chignard M (2009) The innate immune response to Aspergillus fumigatus. Microbes Infect 11: 919–927.
13. Gersuk GM, Underhill DM, Zhu L, Marr KA (2006) Dectin-1 and TLRs permit macrophages to distinguish between different Aspergillus fumigatus cellular states. J Immunol 176: 3717–3724.
14. Gersuk GM, Underhill DM, Zhu L, Marr KA (2006) Dectin-1 and TLRs permit macrophages to distinguish between different *Aspergillus fumigatus* cellular states. J Immunol 176: 3717–3724.
15. Hohl TM, Van Epps HL, Rivera A, Morgan LA, Chen PL, et al. (2005) Aspergillus fumigatus triggers inflammatory responses by stage-specific beta-glucan display. PLoS Pathog 1: e30.
16. Bretz C, Gersuk G, Knoblaugh S, Chaudhary N, Randolph-Habecker J, et al. (2008) MyD88 signaling contributes to early pulmonary responses to *Aspergillus fumigatus*. Infect Immun 76: 952–958.
17. Werner JL, Metz AE, Horn D, Schoeb TR, Hewitt MM, et al. (2009) Requisite role for the dectin-1 beta-glucan receptor in pulmonary defense against Aspergillus fumigatus. J Immunol 182: 4938–4946.
18. Netea MG, Warris A, Van der Meer JW, Fenton MJ, Verver-Janssen TJ, et al. (2003) Aspergillus fumigatus evades immune recognition during germination through loss of toll-like receptor-4-mediated signal transduction. J Infect Dis 188: 320–326.
19. Simitsopoulou M, Roilides E, Likartsis C, Ioannidis J, Orfanou A, et al. (2007) Expression of immunomodulatory genes in human monocytes induced by voriconazole in the presence of Aspergillus fumigatus. Antimicrob Agents Chemother 51: 1048–1054.
20. Carneiro LA, Travassos LH, Philpott DJ (2004) Innate immune recognition of microbes through Nod1 and Nod2: implications for disease. Microbes Infect 6: 609–616.
21. Gerson SL, Talbot GH, Hurwitz S, Strom BL, Lusk EJ, et al. (1984) Prolonged granulocytopenia: the major risk factor for invasive pulmonary aspergillosis in patients with acute leukemia. Ann Intern Med 100: 345–351.
22. Martinon F, Burns K, Tschopp J (2002) The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell 10: 417–426.
23. Pirhonen J, Sareneva T, Kurimoto M, Julkunen I, Matikainen S (1999) Virus infection activates IL-1 beta and IL-18 production in human macrophages by a caspase-1-dependent pathway. J Immunol 162: 7322–7329.
24. Inohara, Chamaillard, McDonald C, Nunez G (2005) NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. Annu Rev Biochem 74: 355–383.
25. Franchi L, McDonald C, Kanneganti TD, Amer A, Nunez G (2006) Nucleotide-binding oligomerization domain-like receptors: intracellular pattern recognition molecules for pathogen detection and host defense. J Immunol 177: 3507–3513.
26. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, et al. (2006) Cryopyrin activates the inflammasome in response to toxins and ATP. Nature 440: 228–232.
27. Geddes BJ, Wang L, Huang WJ, Lavellee M, Manji GA, et al. (2001) Human CARD12 is a novel CED4/Apaf-1 family member that induces apoptosis. Biochem Biophys Res Commun 284: 77–82.
28. Poyet JL, Srinivasula SM, Tnani M, Razmara M, Fernandes-Alnemri T, et al. (2001) Identification of Ipaf, a human caspase-1-activating protein related to Apaf-1. J Biol Chem 276: 28309–28313.
29. Janeway CA, Jr., Medzhitov R (2002) Innate immune recognition. Annu Rev Immunol 20: 197–216.
30. Takeda K, Kaisho T, Akira S (2003) Toll-like receptors. Annu Rev Immunol 21: 335–376.
31. Dinarello CA (2006) Interleukin 1 and interleukin 18 as mediators of inflammation and the aging process. Am J Clin Nutr 83: 447S–455S.
32. Kahlenberg JM, Lundberg KC, Kertesy SB, Qu Y, Dubyak GR (2005) Potentiation of caspase-1 activation by the P2X7 receptor is dependent on TLR signals and requires NF-kappaB-driven protein synthesis. J Immunol 175: 7611–7622.
33. Meylan E, Tschopp J, Karin M (2006) Intracellular pattern recognition receptors in the host response. Nature 442: 39–44.
34. Abdul-Sater AA, Said-Sadier N, Ojcius DM, Yilmaz O, Kelly KA (2009) Inflammasomes bridge signaling between pathogen identification and the immune response. Drugs Today (Barc) 45 Suppl B: 105–112.
35. Hise AG, Tomalka J, Ganesan S, Patel K, Hall BA, et al. (2009) An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen Candida albicans. Cell Host Microbe 5: 487–497.
36. Joly S, Ma N, Sadler JJ, Soll DR, Cassel SL, et al. (2009) Cutting edge: Candida albicans hyphae formation triggers activation of the Nlrp3 inflammasome. J Immunol 183: 3578–3581.
37. Gross O, Poeck H, Bscheider M, Dostert C, Hannesschlager N, et al. (2009) Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. Nature 459: 433–436.
38. Schroder K, Zhou R, Tschopp J (2010) The NLRP3 inflammasome: a sensor for metabolic danger? Science 327: 296–300.
39. Lamkanfi M, Dixit VM (2009) Inflammasomes: guardians of cytosolic sanctity. Immunol Rev 227: 95–105.
40. Faustin B, Lartigue L, Bruey JM, Luciano F, Sergienko E, et al. (2007) Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation. Mol Cell 25: 713–724.
41. Hohl TM, Van Epps HL, Rivera A, Morgan LA, Chen PL, et al. (2005) Aspergillus fumigatus triggers inflammatory responses by stage-specific beta-glucan display. PLoS Pathog 1: e30.
42. Rogers NC, Slack EC, Edwards AD, Nolte MA, Schulz O, et al. (2005) Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. Immunity 22: 507–517.

43. Brown GD, Gordon S (2005) Immune recognition of fungal beta-glucans. Cell Microbiol 7: 471–479.
44. Wald A, Leisenring W, van Burik JA, Bowden RA (1997) Epidemiology of Aspergillus infections in a large cohort of patients undergoing bone marrow transplantation. J Infect Dis 175: 1459–1466.
45. Ito JI, Lyons JM, Hong TB, Tamae D, Liu YK, et al. (2006) Vaccinations with recombinant variants of Aspergillus fumigatus allergen Asp f 3 protect mice against invasive aspergillosis. Infect Immun 74: 5075–5084.
46. Abdul-Sater AA, Koo E, Hacker G, Ojcius DM (2009) Inflammasome-dependent caspase-1 activation in cervical epithelial cells stimulates growth of the intracellular pathogen Chlamydia trachomatis. J Biol Chem 284: 26789–26796.
47. Yilmaz O, Yao L, Maeda K, Rose TM, Lewis EL, et al. (2008) ATP scavenging by the intracellular pathogen Porphyromonas gingivalis inhibits P2X7-mediated host-cell apoptosis. Cell Microbiol 10: 863–875.

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Original Article

Chlamydial infection of monocytes stimulates IL-1β secretion through activation of the NLRP3 inflammasome

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Abstract

Chlamydia trachomatis infections represent the leading cause of bacterial sexually-transmitted disease in the United States and can cause serious tissue damage leading to infertility and ectopic pregnancies in women. Inflammation and hence the innate immune response to chlamydial infection contributes significantly to tissue damage, particularly by secreting proinflammatory cytokines such as interleukin (IL)-1β from monocytes, macrophages and dendritic cells. Here we demonstrate that *C. trachomatis* or *Chlamydia muridarum* infection of a monocytic cell line leads to caspase-1 activation and IL-1β secretion through a process requiring the NLRP3 inflammasome. Thus, secretion of IL-1β decreased significantly when cells were depleted of NLRP3 or treated with the anti-inflammatory inhibitors parthenolide or Bay 11-7082, which inhibit inflammasomes and the transcription factor NF-κB. As for other infections causing NRLP3 inflammasome assembly, caspase-1 activation in monocytes is triggered by potassium efflux and reactive oxygen species production. However, anti-oxidants inhibited IL-1β secretion only partially. Atypically for a bacterial infection, caspase-1 activation during chlamydial infection also involves partially the spleen tyrosine kinase (Syk), which is usually associated with a pathogen recognition receptor for fungal pathogens. Secretion of IL-1β during infection by many bacteria requires both microbial products from the pathogen and an exogenous danger signal, but chlamydial infection provides both the pathogen-associated molecular patterns and danger signals necessary for IL-1β synthesis and its secretion from human monocytes. Use of inhibitors that target the inflammasome in animals should therefore dampen inflammation during chlamydial infection.

Keywords: Inflammasome; Nod-like receptor; Innate immunity; Monocytes; *Chlamydia*

1. Introduction

Chlamydia trachomatis is responsible for the most common bacterial sexually-transmitted diseases and is the main cause of preventable blindness worldwide [1–4]. The Centers for Disease Control reported an increase in diagnosed cases of sexually-transmitted chlamydial infections in the U.S. from 102.5 to 401.3 cases per 100,000 populations from 1998 to 2008 respectively, with the highest prevalence being among young adults (18–26 years of age). A *C. trachomatis* strain, lymphogranuloma venereum (LGV2), causes inflammation and swelling of lymph nodes, and is becoming more common in North America and Europe [5–7]. This strain is commonly used in cellular studies of *Chlamydia* infection. *Chlamydia muridarum*, the chlamydial biovar that infects mouse species, gives a pathology similar to that seen in humans, and is used in in vivo and in vitro models of chlamydial pathogenesis [8].

Both innate and adaptive immune systems coordinate their responses in an attempt to eliminate chlamydial infection. At the same time, much of the pathogenesis due to *C. trachomatis* infection is due to chronic inflammation [9,10]. Epithelial cells, in response to chlamydial infection, secrete interleukin (IL)-6, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF) [11]. These chemokines recruit and activate immune cells including neutrophils, macrophages, dendritic

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cells (DCs) and natural killer (NK) cells, which in turn secrete more proinflammatory cytokines such as TNF-α. Moreover, previous studies demonstrated that dendritic cells, macrophages, and monocytes, secrete the proinflammatory cytokine IL-1β in response to chlamydial infection [12–15]. Excessive IL-1β secretion plays a role in tubal pathology associated with chlamydial infection [16]. More recently, the bulk of IL-1β secreted during chlamydial infection in genitally-infected mice was shown to be produced by macrophages and neutrophils, with very little produced by epithelial cells [17].

Infection by chlamydiae and other bacteria stimulate an innate immune response when pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS), upregulate the expression of proinflammatory mediators following ligation to pathogen recognition receptors (PRRs), including Toll-like receptors (TLRs) and Nod-like receptors (NLRs) [18–21]. Most cytokines are secreted following ligation of PRRs; however, secretion of the key inflammatory cytokine interleukin IL-1β is under a stringent regulatory process. A first signal, following recognition of PAMP by its PRR, causes the production of the immature pro-IL-1β, and a second "danger signal", derived from host-cell molecules that are released from stressed or infected cells, or detected as a PAMP such as flagellin that is present in the cytosol, can stimulate the assembly of an inflammasome that activates the protease caspase-1. Caspase-1, in turn, is responsible for processing and secretion of the mature IL-1β [21,22].

Previous studies have shown that caspase-1 could be activated during chlamydial infections [13,14,17,23,24]. Moreover, we recently demonstrated that *C. trachomatis* dependent caspase-1 activation in cervical epithelial cells is dependent on the NLRP3 inflammasome and requires K^+ efflux and reactive oxygen species (ROS) production. Epithelial cells secrete little IL-1β [17], and we showed that caspase-1 activation in epithelial cells during *C. trachomatis* infection is required instead for chlamydial growth [25]. On the other hand, caspase-1 activation in immune cells during chlamydial infection is required for IL-1β secretion, which plays an important role in clearance and pathology associated with infection. However, the mechanism by which caspase-1 is activated, and whether an inflammasome is required, for IL-1β secretion in immune cells had yet to be investigated. In this study, we therefore demonstrated that unprimed human monocytes (THP-1 cells) secrete IL-1β in response to *C. trachomatis* and *C. muridarum* infections in a process that requires NLRP3-mediated caspase-1 activation. We also find that ROS production and K^+ efflux are required for inflammasome activation in human monocytes.

Spleen tyrosine kinase (Syk) is a cytosolic tyrosine kinase that is expressed in immune and non-immune cells. Syk plays a key role in transmitting signals from a variety of cell surface receptors, such as FcγR, CR3, Dectin-1 and apoptotic cell-recognizing receptor [26]. Several recent studies have shown that Syk can be coupled to NLRP3 inflammasome to activate caspase-1 and induce NF-κB activation in response to fungal infection [27–30], which involves Syk recruitment to lipid raft domains [31]. As chlamydiae have been reported to enter host cells by lipid raft domains [32,33] and a protein secreted by *Chlamydia* is regulated by Syk phosphorylation [34], we also investigated whether Syk signaling is required for NLRP3-dependent caspase-1 activation and IL-1β secretion following chlamydial infection. Indeed we find that the ability of chlamydiae to activate caspase-1 relies at least partially on Syk kinase signaling.

2. Materials and methods

2.1. Cells, bacteria, and chemical reagents

The human acute monocytic leukemia cell line, THP-1, was from American Type Culture Collection (ATCC). The LGV/L2 strain of *C. trachomatis* and *C. muridarum* Nigg strain (also known as the mouse pneumonitis biovar, MoPn) were obtained from Roger Rank, University of Arkansas. THP-1 cells were cultured in a humidified incubator at 37 °C with 5% CO_2 in RPMI medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen). *C. trachomatis* and *C. muridarum* were grown in infected HeLa cell monolayer cultures to determine the number of bacterial inclusion forming units as described previously [35,36]. After 2 days of infection, chlamydiae were harvested from infected cells by combining cells and supernatant and performing a freeze thaw cycle at −80 °C, and then centrifuging at 2000 rpm for 5 min at 4 °C to remove cell debris, followed by a high speed centrifugation at 15,000 rpm for 45 min at 4 °C. The resulting pellet was resuspended in an appropriate volume of DMEM with 10% FBS medium, and aliquoted and stored at −80 °C until ready for use. Diphenyliodonium chloride (DPI), parthenolide and bay 11-7082 were purchased from Enzo Life Sciences (Plymouth Meeting, PA), N-acetyl cysteine (NAC) was from Sigma (St. Louis, MO), KCl was from Fisher.

2.2. Cell culture, infection, and treatments

THP-1 cells were plated at 10^6 cells per well and infected with LGV/L2 *C. trachomatis* at a multiplicity of infection (m.o.i.) of 5.0 or infected with *C. muridarum* Nigg strain at an m.o.i. of 1.0, and incubated for 24 h in an incubator at 37 °C with 5% CO_2. Treatment with inhibitors or other reagents was performed at the indicated times and concentrations.

2.3. Enzyme-linked immunosorbent assay (ELISA) for IL-1β secretion

Following 24 h of infection with *Chlamydia*, supernatant from cultured cells was collected and stored at −80 °C until ready for use in the assay. Measurement of IL-1β from THP-1 cells was carried out using Human IL1-β ELISA kit (eBioscience, San Diego, CA), following manufacturer's instructions.

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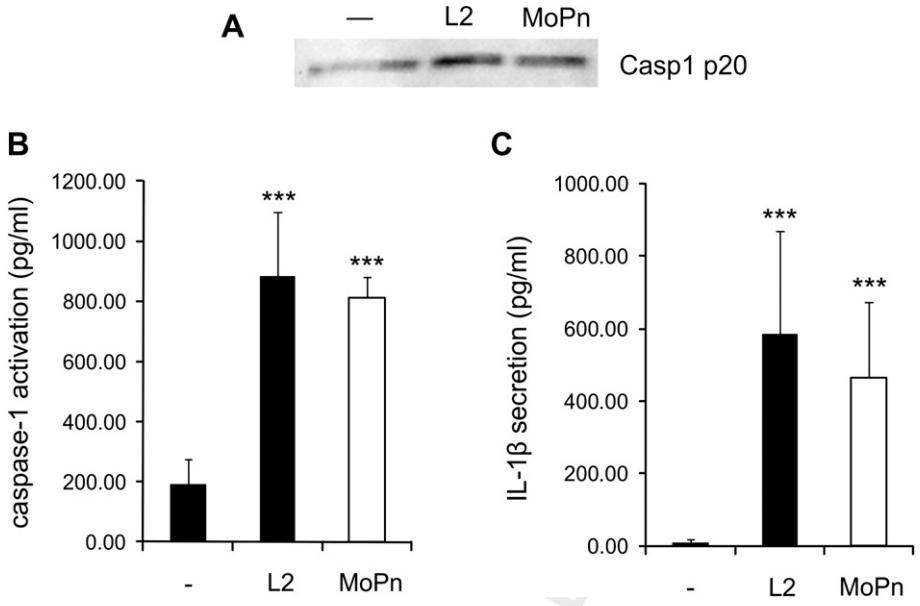

Fig. 1. *Chlamydia* induces caspase-1 activation in monocytes. THP-1 cells treated with non-target control (sh Control) were infected with *C. trachomatis* (L2) at an m.o.i. of 5 or *C. muridarum* (MoPn) at an m.o.i. of 1 for 24 h. (A) Western blot analysis of the lysates was performed to monitor caspase-1 (Casp1) activation using an antibody that detects active caspase-1 (p20). (B) Caspase-1 ELISA was used to quantitatively measure caspase-1 activity in the supernatant of cultured cells. (C) IL-1β ELISA was used to measure IL-1β secretion in supernatants of cultured cells. Error bars represent standard deviation of at least three separate experiments. *** indicates $p < 0.001$.

2.4. Generation of cells stably expressing shRNA

THP-1 stably expressing shRNA against NLRP3, ASC, Syk, MyD88 and caspase-1 were obtained by transducing THP-1 cells with lentiviral particles. The sequences 5′- CCGGGCGTTAGAAACACTTCAAGAACTCGAGTTCTTGAAGTGTTTCTAACGCTTTTTG-3′ for human NLRP3 (Sigma; catalog number NM_004895), 5′-CCGGCGGAAGCTCTTCAGTTTCACACTCGAGTGTGAAACTGAAGATTCCG TTTTTG-3′ for human ASC (Sigma; catalog number NM_013258), 5′-CCGGCCTGTCTCTGTTCTTGAACGTCTCGAGACGTTCAAGAACAGAGACAGGTTTTT-3′ for human MyD88 (Sigma; catalog number NM_002468), 5′-CCGGGCAGGCCATCATCAGTCAGAACTCGAGTTCTGACTGATGATGGCCTGCTTTTT-3′ for human spleen tyrosine kinase (Syk) (Sigma; catalog number NM_003177), and five sequences for caspase-1 (Sigma; catalog number NM_001223): 5′- CCGGGAAGAGTTTGAGGATGATGCTCTCGAGAGCATCATCCTCAAACTCTTCTTTTT-3′, 5′- CCGGTGTATGAATGTCTGCTGGGCACTCGAGTGCCCAGCAGACATTCATACATTTTT-3′, 5′- CCGGCACACGTCTTGCTCTCATTATCTCGAGATAATGAGAGCAAGACGTGTGTTTTT-3′, 5′- CCGGCTACAACTCAATGCAATCTTTCTCGAGAAAGATTGCATTGAGTTGTAGTTTTT-3′, 5′- CCGGCCAGATATACTACAACTCAATCTCGAGATTGAGTTGTAGTATATCTGGTTTTT-3′ were used separately to silence gene expression following the manufacturer's instructions. Nontarget shRNA control cells were also generated using an irrelevant sequence (Sigma; catalog number SHC002 V). Briefly, cells were plated at 35% confluency 24 h prior to transduction and then the corresponding lentiviral transduction particles were added at m.o.i. of 3 overnight. Fresh media were added the next day, and transduced cells were selected by addition of media containing 2 μg/ml puromycin (Sigma).

2.5. RNA isolation, PCR and real-time PCR

mRNA was isolated from cells after the indicated treatments or infections using the Qiagen RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Quantitative PCR was performed with 1:50 of the cDNA preparation in the Mx3000P (Stratagene, La Jolla, CA) in a 25 μl final volume with Brilliant QPCR master mix (Stratagene). The primers for human GAPDH were 5′-CTTCTCTGATGAGGCCCAAG-3′ forward and 5′-GCAGCAAACTGGAAAGGAAG-3′ reverse. Primers for human NLRP3 were 5′-CTTCCTTTCCAGTTTGCTGC-3′ forward and 5′-TCTCGCAGTCCACTTCCTTT-3′ reverse. Primers for human ASC were 5′-AGTTTCACACCAGCCTGGAA-3′ forward and 5′-TTTTCAAGCTGGCTTTTCGT-3′ reverse. Primers for Syk were 5′-AGAGCGAGGAGGAGCGGGTG-3′ forward, 5′-CCGCTGACCAAGTCGCAGGA-3′ reverse. Primers for MyD88 were 5′AGCGCTGGCAGACAATGCGA-3′ forward, 5′-TCCGGCGGCACCTCTTTTCG-3′ reverse. Primers for caspase-1 were 5′- GCCCAAGTTTGAAGGACAAA-3′ forward, 5′- GGTGTGGAAGAGCAGAAAGC-3′ reverse. Real-time PCR included initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min, and one cycle of 95 °C for 1 min, 55 °C for 30 s, 95 °C for 30 s.

2.6. Western blotting

Samples were lysed using 1x RIPA Lysis Buffer (Millipore) with 1x protease inhibitor cocktail (Biovision) and loaded onto

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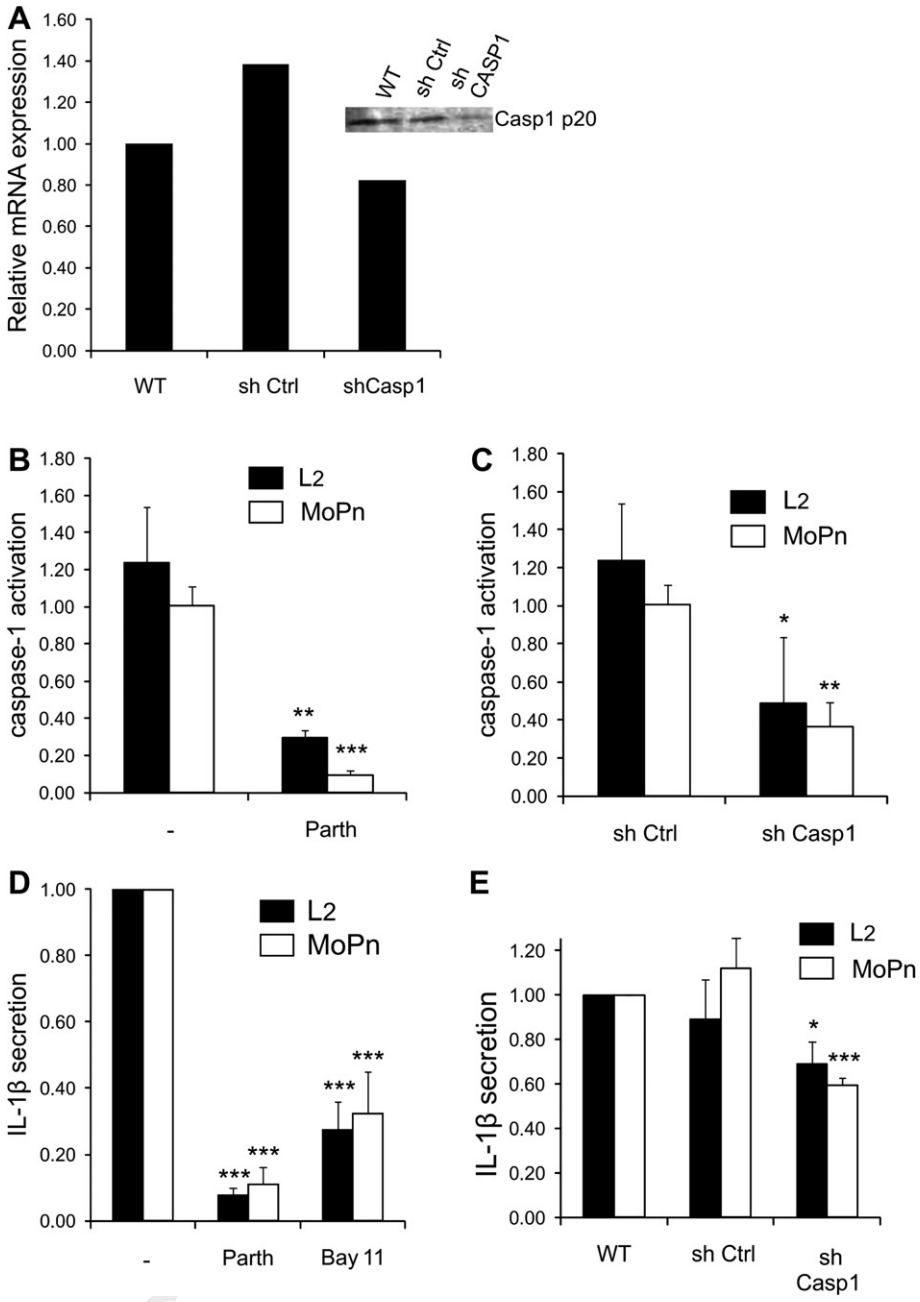

Fig. 2. Caspase-1 activation is required for *Chlamydia*-induced IL-1β secretion. (A) THP-1 cells were stably transfected with shRNAs that target caspase-1, and mRNA expression of caspase-1 was quantified by real-time PCR and compared with wild-type (WT) and non-target control (sh Ctrl) (one representative experiment is shown). Inset: Western blot analysis of wild-type THP-1 cells, cells treated with non-target control, and cells treated with shCasp1, confirming the decreased expression of the caspase-1 protein. The Western blot was performed with an anti-caspase-1 antibody that detects the p20 fragment of the enzyme. (B and D) THP-1 cells were infected with *C. trachomatis* (L2) at an m.o.i. of 5 or *C. muridarum* (MoPn) at an m.o.i. of 1 for 24 h and treated with control buffer or 10 μM parthenolide (Parth) or 12 μM Bay 11-7082 (Bay 11) for 3 h p.i. (B) Caspase-1 activation or (D) IL-1β secretion in the supernatants were measured by ELISA and plotted as a bar graph. (C and E) Caspase-1 (Casp1) or non-target control THP-1 cells were infected with L2 (m.o.i. 5) or MoPn (m.o.i. 1) for 24 h, and *Chlamydia*-induced (C) caspase-1 activation or (E) IL-1β secretion were measured by ELISA. The fold increase in IL-1β secretion in infected non-target controls (shCasp1-treated cells) with respect to uninfected cells was compared with the increase in 24 h-infected (C) non-target control treated cells or (E) wild-type cells. Error bars represent standard deviations from at least three separate experiments. * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$ compared with untreated infected cells.

a 15% SDS-polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (Millipore). For detection of the active caspase-1 subunit (p20), the blot was probed with 1 μg/ml rabbit anti-human caspase-1 antibody (Millipore), and then incubated again with conjugated 1:2000 dilution of anti-rabbit IgG horseradish peroxidase (Millipore). For confirmation of NLRP3 depletion by RNA interference, a 9% gel was used, and the blot was incubated with rabbit anti-human NLRP3 antibody (Sigma; cat. # HPA012878) at a dilution of 1:200 for 1 h. Immunoreactive proteins were detected by adding ECL Plus

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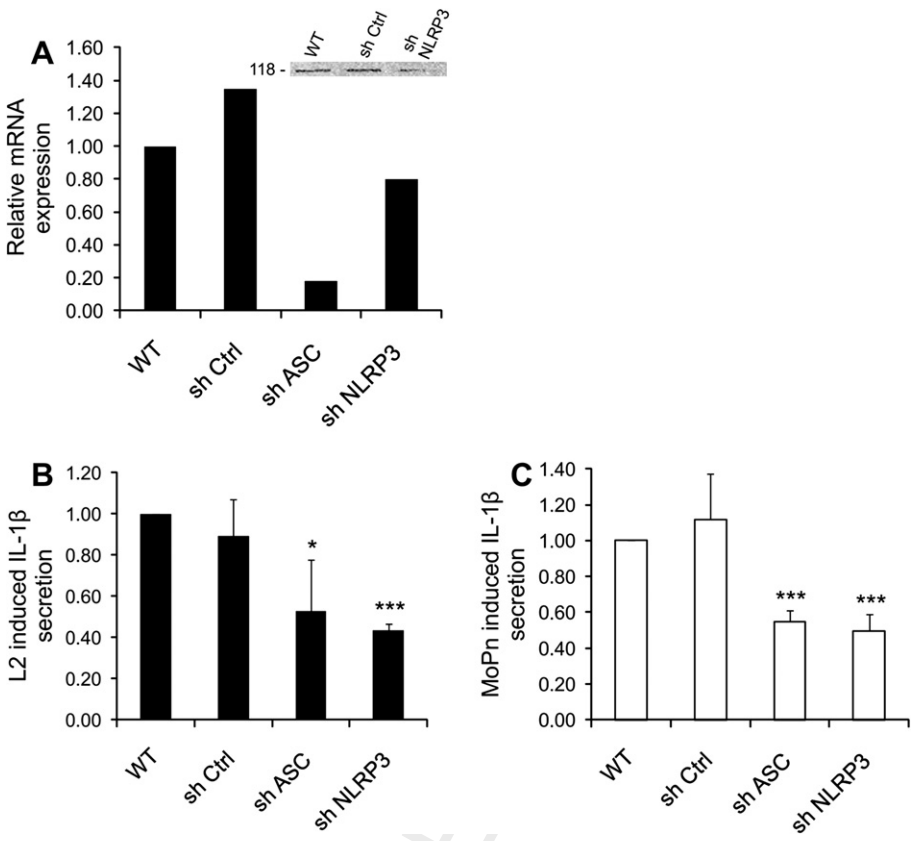

Fig. 3. *Chlamydia*-induced IL-1β secretion requires the NLRP3 inflammasome. (A) THP-1 cells were stably transfected with shRNAs that target NLRP3 or ASC, and mRNA expression of NLRP3 and ASC was quantified by real-time PCR and compared with wild-type (WT) and non-target control (sh Ctrl). Inset: Western blot analysis of wild-type THP-1 cells, cells treated with non-target control, and cells treated with shNLRP3, confirming decreased expression of the NLRP3 protein after mRNA knockdown (one representative experiment is shown). The Western blot was performed with an anti-NLRP3 antibody, which detects the 118-kDa protein. (B) NLRP3, ASC, or non-target control knockdown cells were infected with L2 at an m.o.i. of 5 for 24 h, and *C. trachomatis*-induced IL-1β secretion in the supernatants was measured by ELISA. The fold increase in IL-1β secretion in infected non-target controls, shNLRP3-treated cells, and shASC-treated cells with respect to uninfected cells was compared with the increase in 24 h-infected wild-type cells. (C) Same as in panel B, but cells were infected with *C. muridarum* at an m.o.i. of 1 for 24 h. Error bars represent standard deviations of an experiment performed on three separate occasions. * indicates $p < 0.05$; *** indicates $p < 0.001$.

Western blotting detection reagents (Amersham Biosciences) following manufacturer's instructions and chemiluminescence was detected using a gel doc system (Bio-Rad).

2.7. Statistical and flow cytometric analyses

The statistical analysis was performed using GraphPad Instat software (GraphPad Software Inc, La Jolla, CA) by Student's *t* test and was considered significant at $p < 0.05$. Flow cytometry data were analyzed using FlowJo software (Tree Star Inc, Ashland, OR).

3. Results

3.1. Chlamydia induces caspase-1 activation in monocytes

Previous studies have shown that monocytes, macrophages and dendritic cells secrete IL-1β during chlamydial infection, although the mechanisms were not elucidated. Therefore we used a human monocytic cell line to determine whether IL-1β secretion in *Chlamydia*-infected cells requires inflammasome-dependent caspase-1 activation. In fact, cells that were infected with either *C. trachomatis* (L2) at m.o.i of 5 or *C. muridarum* (MoPn) at m.o.i of 1 for 24 h exhibited an increase in the amount of active caspase-1 subunit (p20), compared to non-infected cells as shown by Western Blot analysis (Fig. 1 A). This result was quantitatively assessed by measuring the amount of active caspase-1 in the supernatant of infected THP-1 cells using a caspase-1 ELISA kit (Fig. 1 B). Furthermore, high levels of mature IL-1β were detected in the supernatant of these cells 24 h post infection (Fig. 1 C).

To confirm that the IL-1β secretion was dependent on caspase-1 activation, we silenced caspase-1 gene expression by RNA interference. When compared with wild-type (WT) THP-1 cells or to cells treated with non-target shRNA (Sh Ctrl), cells treated with shRNA for caspase-1 showed a 40% reduction in mRNA expression as well as significantly lower protein expression (Fig. 2 A). When these cells were infected with *C. trachomatis* or *C. muridarum* for 24 h, they secreted significantly less IL-1β and activated caspase-1 into the supernatant, confirming that *Chlamydia*-induced IL-1β secretion relies on caspase-1 activation (Fig. 2 C,E).

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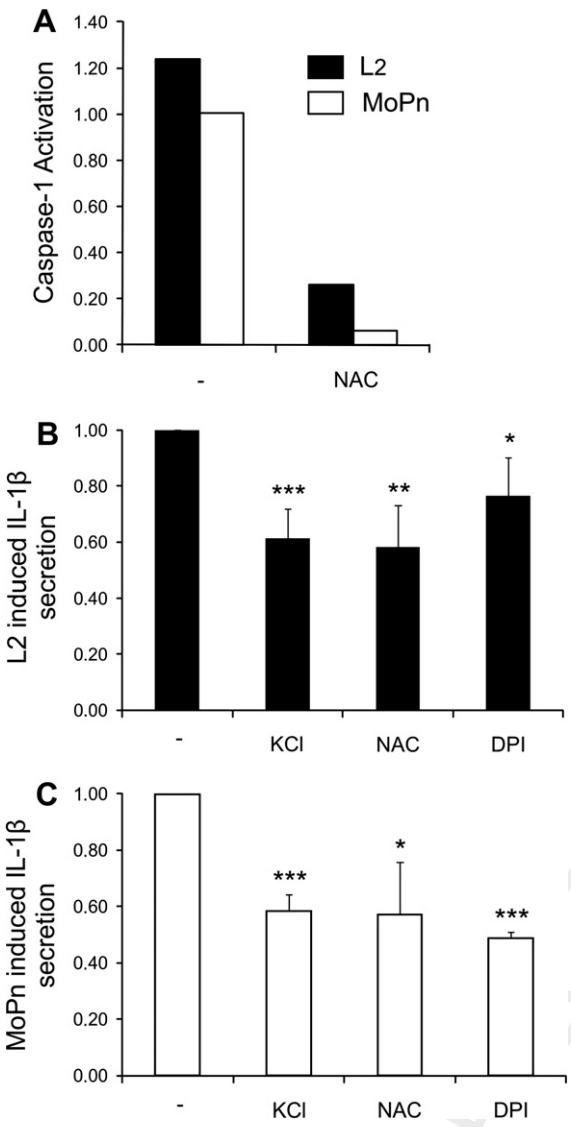

Fig. 4. IL-1β secretion during *Chlamydia* infection is caused by K^+ efflux and, partially, by ROS production. (A) THP-1 cells were infected with *C. trachomatis* (L2) at an m.o.i. of 5 or *C. Muridarum* (MoPn) at an m.o.i. of 1 for 24 h, and treated with control buffer or 10 mM NAC. Caspase-1 activation was measured by ELISA (one representative experiment). (B and C) THP-1 cells were infected with *C. trachomatis* (L2) at an m.o.i. of 5 (A) or *C. muridarum* (MoPn) at an m.o.i. of 1 (B) for 24 h, and treated with control buffer or 10 mM NAC, 250 nM diphenyliodonium chloride (DPI), or 70 mM KCl during the last 15 h of infection. IL-1β levels in the supernatant were measured by ELISA and plotted as a bar graph. Error bars represent standard deviations from at least three separate experiments. * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$, compared with uninfected cells.

A recent study shed light on the mechanisms of action of two known NF-κB pathway inhibitors: parthenolide and Bay 11-7082 [37]. Independently of its inhibitory effect on NF-κB activity, parthenolide also acts as a direct inhibitor of caspase-1 and a potent inhibitor of multiple inflammasomes, whereas Bay 11-7082 selectively inhibits NLRP3 inflammasome activity. Therefore, to further confirm a caspase-1 requirement for IL-1β secretion, we incubated THP-1 cells with parthenolide for 3 h during chlamydial infection and measured the concentration of IL-1β and activated caspase-1 in the supernatant by ELISA. In fact, parthenolide induced a drastic reduction in both caspase-1 activation and IL-1β secretion in the supernatant of infected cells (Fig. 2 B, D). Since parthenolide inhibits both NF-κB and caspase-1 activation, we determined whether this result is due predominantly to inflammasome inhibition by treating THP-1 cells with Bay 11-7082, which selectively inhibits the NLRP3 inflammasome independently of its effect on NF-κB activity. Although the effect of Bay 11-7082 was less potent than parthenolide, IL-1β secretion was still remarkably diminished in the presence of Bay 11-7082 (Fig. 2 D). We therefore conclude that both *C. trachomatis* and *C. muridarum* induce caspase-1 dependent IL-1β secretion, and more importantly these results imply that the NLRP3 inflammasome is required for caspase-1 activation.

3.2. NLRP3 inflammasome is activated in response to chlamydial infection

To further investigate the role of NLRP3 in activating caspase-1, we used shRNA to knockdown separately NLRP3 and its adaptor protein, ASC, in THP-1 cells. mRNA expression of either inflammasome component was significantly reduced in comparison with non-target shRNA, as measured by real-time PCR (Fig. 3 A), and NLRP3 protein depletion was confirmed by Western blotting (Fig. 3 A inset). We previously showed that *C. trachomatis* induces NLRP3/ASC inflammasome-dependent caspase-1 activation in epithelial cells [25], and we find here that *Chlamydia* uses the same effectors in monocytes, since both NLRP3 and ASC knockdown cells exhibited almost 45% reduction in the amount of secreted IL-1β when compared to wild-type and non-target (Sh Ctrl) cells during infection with *C. trachomatis* or *C. muridarum* (Fig. 3 B,C). The reduction of IL-1β secretion was partial, probably due to the partial mRNA depletion that was achieved by shRNA.

3.3. K^+ efflux and ROS production are key elements in Chlamydia*-induced NLRP3 activation*

Despite the large variety of ligands that can induce NLRP3 inflammasome activation, the cell-signaling pathways often converge on K^+ efflux and production of reactive oxygen species (ROS) [21,38–40]. To test the role of these elements, we neutralized ROS by adding the anti-oxidant reagent, N-acetyl cysteine (NAC), or by treating with an NADPH oxidase inhibitor, diphenyliodonium chloride (DPI). Treatment of *C. trachomatis* or *C. muridarum* infected cells with NAC diminished caspase-1 activation (Fig. 4 A). Consistent with this result, addition of DPI 9 h post infection (p.i.) caused a reduction in *Chlamydia*-induced IL-1β secretion (Fig. 4 B,C). However, the effects of anti-oxidants on secretion of IL-1β from monocytes were not as dramatic as we previously observed for caspase-1 activation during *C. trachomatis* infection of epithelial cells [25].

Next, we blocked K^+ efflux by increasing the extracellular KCl concentration, and checked the secretion of IL-1β into the supernatant of infected cells 24 h post infection. Limiting K^+ efflux was in fact able to significantly reduce IL-1β secretion from cells infected with *C. trachomatis* or *C. muridarum*

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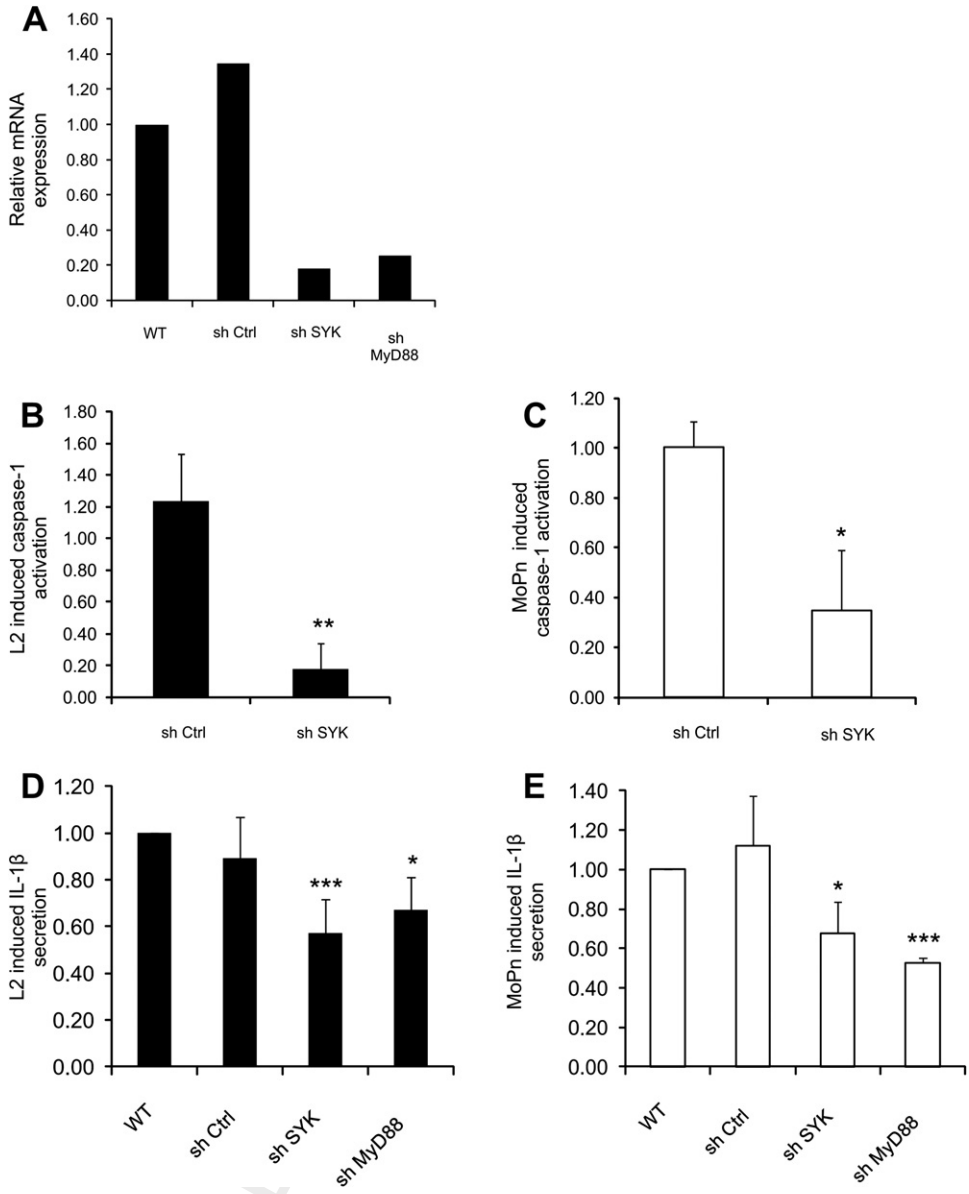

Fig. 5. Caspase-1 dependent IL-1β secretion during *Chlamydia* infection involves MyD88 and Syk signaling. (A) THP-1 cells were stably transfected with shRNAs that target Syk or MyD88, and mRNA expression of Syk and MyD88 was quantified by real-time PCR and compared with wild-type (WT) and non-target control (sh Ctrl) (one representative experiment is shown). (B and D) Syk, MyD88, or non-target control knockdown THP-1 cells were infected with L2 at an m.o.i. of 5 for 24 h, and *C. trachomatis*-induced (B) caspase-1 activation or (D) IL-1β secretion in the supernatants was measured by ELISA. The fold increase in IL-1β secretion in infected non-target controls, shSyk-treated cells, and shMyD88-treated cells with respect to uninfected cells was compared with the increase in 24 h-infected (B) sh Ctrl treated cells or (D) wild-type cells. (C and E) Same as in panels B and D, except that THP-1 cells were infected with MoPn at an m.o.i. of 1 for 24 h. (E) Error bars represent standard deviations of an experiment performed on three separate occasions. * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$.

(Fig. 4 B,C). Taken together, these results demonstrate that NLRP3 activation during chlamydial infection relies on a cell-signaling pathway involving, at least partially, K^+ efflux and ROS production.

3.4. Syk kinase signaling couples with the NLRP3 inflammasome for inducing IL-1β release during chlamydial infection

Syk, through its interaction with immunoreceptor tyrosine-based activation motif (ITAMs)-based receptors, plays a major role in MyD88-independent signaling pathways of several receptors such as FcγR, CR3, Dectin-1 and apoptotic cell-recognizing receptor [26]. Recently, Syk signaling had been shown to be involved in NLRP3 inflammasome activation by fungi such as *Candida albicans* and *Aspergillus fumigatus* [27,30]. Interestingly, chlamydial translocated actin recruiting phosphoprotein (Tarp), a type three secretion (T3S) effector protein, is regulated by Syk phosphorylation [34]. At the same time, the TLR-adaptor protein MyD88 has been shown to participate in cytokine production during chlamydial infection [41–45]. To examine whether Syk and MyD88 are involved in NLRP3 inflammasome activation during *Chlamydia* infection, we depleted Syk and MyD88 in THP-1 cells using RNA

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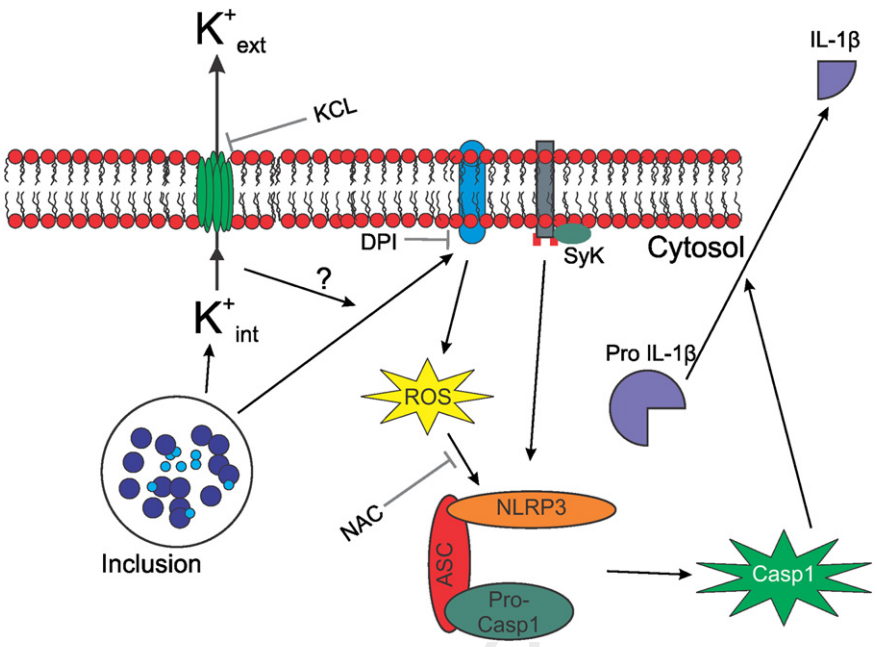

Fig. 6. In monocytes, *C. trachomatis* and *C. muridarum* trigger inflammasome-mediated caspase-1 activation and IL-1β secretion through K^+ efflux and ROS production, through a mechanism involving the Syk kinase. Chlamydial infection induces K^+ efflux and ROS production, which promote assembly of the NLRP3 inflammasome through a pathway involving the Syk kinase. The inflammasome in turn activates caspase-1, which processes pro-IL-1β into the mature IL-1β form.

interference. mRNA expression of Syk and MyD88 was significantly reduced in comparison with non-target shRNA, as measured by real-time PCR (Fig. 5 A). Individual knockdowns of Syk or MyD88 resulted in a reduction of ~40% in the amount of IL-1β secretion after 24 h of *C. trachomatis* (L2) infection (Fig. 5 D) or *C. muridarum* (MoPn) infection (Fig. 5 E), when compared to wild-type or non-target (Sh ctrl) THP-1 cells. Secretion of activated caspase-1 into the supernatants of infected Syk knockdown cells exhibited a ~60% reduction, when compared to control cells (Fig. 5 B,C). Our results thus demonstrate for the first time that NLRP3 activation can be coupled, at least partially, to tyrosine kinase Syk signaling in response to a bacterial infection in human monocytes.

4. Discussion

Previous studies have demonstrated that chlamydial infection of monocytes and macrophages leads to IL-1β secretion, and a requirement for caspase-1 was shown [13,14,17,23,24]. Despite the important role that IL-1β plays in pathogenesis of chlamydial infection, the mechanism of caspase-1 activation and the subsequent secretion of IL-1β had not been revealed yet. In this study, we demonstrate that the NLRP3 inflammasome is required for *C. trachomatis* or *C. muridarum* induced caspase-1 dependent IL-1β secretion by monocytes. The adaptor protein ASC was also needed for NLRP3-dependent caspase-1 activation.

Although the NLRP3 inflammasome could be activated by a wide range of triggers, most of these activators converge on a small number of shared mechanisms, including K^+ efflux and ROS production [21,38,39]. We previously reported that K^+ efflux precedes ROS production in *C. trachomatis*-induced NLRP3-dependent caspase-1 activation in epithelial cells [25]. Here we show that K^+ efflux and ROS production are also required for NLRP3-dependent caspase-1 activation and IL-1β secretion in monocytes infected with *C. trachomatis* or *C. muridarum*. Blocking K^+ efflux by increasing extracellular KCl caused a reduction in the amount of IL-1β secretion and caspase-1 activation in response to infection with both chlamydial strains, and scavenging ROS with the anti-oxidant NAC also led to lower levels of IL-1β production and caspase-1 activation, although the ability of anti-oxidants to block IL-1β secretion from monocytes was not as striking as for the effect of anti-oxidants on caspase-1 activation in epithelial cells, suggesting the existence of ROS-independent mechanisms for IL-1β secretion in human monocytes. We further demonstrated that ROS production induced by chlamydial infection of monocytes is produced, at least partially, by NADPH oxidase, because inhibiting NADPH oxidase with DPI caused a reduction in both IL-1β levels and caspase-1 activation following infection with either *C. trachomatis* or *C. muridarum*. In monocytes, our data further demonstrate that Syk signaling contributes to caspase-1 activation and IL-1β secretion during chlamydial infection.

The mechanism of chlamydial entry into the host cell is not well understood, although previous studies have suggested that entry might taken place through lipid raft domains [32,33]. Independently of the mechanism of chlamydial entry, the Syk kinase is recruited to lipid raft domains [31], where the NADPH oxidase is also assembled [46–50]. Given the role that the NADPH oxidase plays in inflammasome activation in monocytes infected with chlamydiae, and recent reports that Syk signaling is coupled with NLRP3 inflammasome to activate caspase-1 and induce NF-κB activation [27,30], we investigated whether the Syk kinase might be involved in *Chlamydia*-induced NLRP3 inflammasome activation in monocytes. Indeed, we showed that both caspase-1 activation

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and IL-1β secretion following chlamydial infection of monocytes requires Syk signaling. Although the exact mechanism for Syk kinase involvement in *Chlamydia*-induced inflammasome activation will require further studies, we propose that K^+ efflux during chlamydial infection leads to NADPH oxidase activation and ROS production, consistent with previous reports that plasma membrane depolarization can lead to ROS production [51–53]. ROS production results in NLRP3 inflammasome activation [40], which activates caspase-1. In parallel, chlamydial infection, through a process at least partially dependent on MyD88 [41,44,45], induces synthesis of pro-IL-1β. Activated caspase-1 cleaves pro-IL-1β, allowing its secretion (Fig. 6).

References

[1] A.C. Gerbase, J.T. Rowley, T.E. Mertens, Global epidemiology of sexually transmitted diseases. Lancet 351 (1998) 2–4.

[2] W.C. Miller, C.A. Ford, M. Morris, M.S. Handcock, J.L. Schmitz, M.M. Hobbs, M.S. Cohen, K.M. Harris, J.R. Udry, Prevalence of chlamydial and gonococcal infections among young adults in the United States. JAMA 291 (2004) 2229–2236.

[3] J. Schachter, Infection and disease epidemiology. in: R.S. Stephens (Ed.), Chlamydia: Intracellular Biology, Pathogenesis, and Immunity. ASM Press, Washington, D.C, 1999, pp. 139–169.

[4] B. Thylefors, A.D. Negrel, R. Pararajasegaram, K.Y. Dadzie, Global data on blindness. Bull. World Health Organ. 73 (1995) 115–121.

[5] M. Klint, M. Lofdahl, C. Ek, A. Airell, T. Berglund, B. Herrmann, Lymphogranuloma venereum prevalence in Sweden among men who have sex with men and characterization of *Chlamydia trachomatis* ompA genotypes. J. Clin. Microbiol. 44 (2006) 4066–4071.

[6] C. Bebear, B. de Barbeyrac, Genital *Chlamydia trachomatis* infections. Clin. Microbiol. Infect. 15 (2009) 4–10.

[7] G. Stary, T. Meyer, C. Bangert, N. Kohrgruber, B. Gmeinhart, R. Kirnbauer, C. Jantschitsch, A. Rieger, A. Stary, A. Geusau, New *Chlamydia trachomatis* L2 strains identified in a recent outbreak of lymphogranuloma venereum in Vienna, Austria. Sex. Transm. Dis. 35 (2008) 377–382.

[8] T.W. Cotter, G.I. Byrne, Immunity to *Chlamydia*: comparison of human infections and murine models. Res. Immunol. 147 (1996) 587–595.

[9] T. Darville, C.W. Andrews, J.D. Sikes, P.L. Fraley, R.G. Rank, Early local cytokine profiles in strains of mice with different outcomes from chlamydial genital tract infection. Infect. Immun. 69 (2001) 3556–3561.

[10] M.E. Ward, Mechanisms of *Chlamydia*-induced disease. in: R.S. Stephens (Ed.), Chlamydia: Intracellular Biology, Pathogenesis, and Immunity. ASM Press, Washington, D.C, 1999, pp. 171–210.

[11] S.J. Rasmussen, L. Eckmann, A.J. Quayle, L. Shen, Y.X. Zhang, D.J. Anderson, J. Fierer, R.S. Stephens, M.F. Kagnoff, Secretion of proinflammatory cytokines by epithelial cells in response to *Chlamydia* infection suggests a central role for epithelial cells in chlamydial pathogenesis. J. Clin. Invest. 99 (1997) 77–87.

[12] G. Entrican, R. Wilkie, P. McWaters, J. Scheerlinck, P.R. Wood, J. Brown, Cytokine release by ovine macrophages following infection with *Chlamydia psittaci*. Clin. Exp. Immunol. 117 (1999) 309–315.

[13] A. Gervassi, M.R. Alderson, R. Suchland, J.F. Maisonneuve, K.H. Grabstein, P. Probst, Differential regulation of inflammation cytokine secretion by human dendritic cells upon *Chlamydia trachomatis* infection. Infect. Immun. 72 (2004) 7231–7239.

[14] D.M. Ojcius, P. Souque, J.L. Perfettini, A. Dautry-Varsat, Apoptosis of epithelial cells and macrophages due to infection with the obligate intracellular pathogen *Chlamydia psittaci*. J. Immunol. 161 (1998) 4220–4226.

[15] C.D. Rothermel, J. Schachter, P. Lavrich, E.C. Lipsitz, T. Francus, *Chlamydia trachomatis*-induced production of interleukin-1 by human monocytes. Infect. Immun. 57 (1989) 2705–2711.

[16] M. Hvid, A. Baczynska, B. Deleuran, J. Fedder, H.J. Knudsen, G. Christiansen, S. Birkelund, Interleukin-1 is the initiator of Fallopian tube destruction during *Chlamydia trachomatis* infection. Cell. Microbiol. 9 (2007) 2795–2803.

[17] D. Prantner, T. Darville, J.D. Sikes, C.W. Andrews Jr., H. Brade, R.G. Rank, U.M. Nagarajan, Critical role for interleukin-1beta (IL-1beta) during *Chlamydia muridarum* genital infection and bacterial replication-independent secretion of IL-1beta in mouse macrophages. Infect. Immun. 77 (2009) 5334–5346.

[18] N. Inohara, M. Chamaillard, C. McDonald, G. Nunez, NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. Ann. Rev. Biochem. 74 (2005) 355–383.

[19] F. Martinon, J. Tschopp, NLRs join TLRs as innate sensors of pathogens. Trends. Immunol. 26 (2005) 447–454.

[20] L.L. Bourhis, C. Werts, Role of nods in bacterial infection. Microbes Infect. 9 (2007) 629–636.

[21] J. Tschopp, K. Schroder, NLRP3 inflammasome activation: the convergence of multiple signalling pathways on ROS production? Nat. Rev. Immunol. 10 (2010) 210–215.

[22] A.A. Abdul-Sater, N. Said-Sadier, D.M. Ojcius, O. Yilmaz, K.A. Kelly, Inflammasomes bridge signaling between pathogen identification and the immune response. Drugs Today (Barc)(45 Suppl. B) (2009) 105–112.

[23] H. Lu, C. Shen, R.C. Brunham, *Chlamydia trachomatis* infection of epithelial cells induces the activation of caspase-1 and release of mature IL-18. J. Immunol. 165 (2000) 1463–1469.

[24] W. Cheng, P. Shivshankar, Z. Li, L. Chen, I.T. Yeh, G. Zhong, Caspase-1 contributes to *Chlamydia trachomatis*-induced upper urogenital tract inflammatory pathologies without affecting the course of infection. Infect. Immun. 76 (2008) 515–522.

[25] A.A. Abdul-Sater, E. Koo, G. Hacker, D.M. Ojcius, Inflammasome-dependent caspase-1 activation in cervical epithelial cells stimulates growth of the intracellular pathogen *Chlamydia trachomatis*. J. Biol. Chem. 284 (2009) 26789–26796.

[26] Y. Tohyama, H. Yamamura, Protein tyrosine kinase, syk: a key player in phagocytic cells. J. Biochem. 145 (2009) 267–273.

[27] O. Gross, H. Poeck, M. Bscheider, C. Dostert, N. Hannesschlager, S. Endres, G. Hartmann, A. Tardivel, E. Schweighoffer, V. Tybulewicz, A. Mocsai, J. Tschopp, J. Ruland, Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. Nature 459 (2009) 433–436.

[28] A.G. Hise, J. Tomalka, S. Ganesan, K. Patel, B.A. Hall, G.D. Brown, K.A. Fitzgerald, An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen *Candida albicans*. Cell Host Microbe 5 (2009) 487–497.

[29] S. Joly, N. Ma, J.J. Sadler, D.R. Soll, S.L. Cassel, F.S. Sutterwala, Cutting edge: *Candida albicans* hyphae formation triggers activation of the Nlrp3 inflammasome. J. Immunol. 183 (2009) 3578–3581.

[30] N. Said-Sadier, E. Padilla, G. Langsley, D.M. Ojcius, Aspergillus fumigatus stimulates the NLRP3 inflammasome through a pathway requiring ROS production and the Syk tyrosine kinase. PLoS One 5 (2010) e10008.

[31] S. Xu, J. Huo, M. Gunawan, I.H. Su, K.P. Lam, Activated dectin-1 localizes to lipid raft microdomains for signaling and activation of phagocytosis and cytokine production in dendritic cells. J. Biol. Chem. 284 (2009) 22005–22011.

[32] I. Jutras, L. Abrami, A. Dautry-Varsat, Entry of the lymphogranuloma venereum strain of Chlamydia trachomatis into host cells involves cholesterol-rich membrane domains. Infect. Immun. 71 (2003) 260–266.

[33] L.C. Norkin, S.A. Wolfrom, E.S. Stuart, Association of caveolin with *Chlamydia trachomatis* inclusions at early and late stages of infection. Exp. Cell. Res. 266 (2001) 229–238.

[34] A. Mehlitz, S. Banhart, S. Hess, M. Selbach, T.F. Meyer, Complex kinase requirements for *Chlamydia trachomatis* Tarp phosphorylation. FEMS Microbiol. Lett. 289 (2008) 233–240.

[35] J.L. Perfettini, D.M. Ojcius, C.W. Andrews, S.J. Korsmeyer, R.G. Rank, T. Darville, Role of proapoptotic BAX in propagation of *Chlamydia muridarum* (the mouse pneumonitis strain of *Chlamydia trachomatis*) and the host inflammatory response. J. Biol. Chem. 278 (2003) 9496–9502.

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[36] D.M. Ojcius, H. Degani, J. Mispelter, A. Dautry-Varsat, Enhancement of ATP levels and glucose metabolism during an infection by *Chlamydia*. J. Biol. Chem. 273 (1998) 7052−7058.

[37] C. Juliana, T. Fernandes-Alnemri, J. Wu, P. Datta, L. Solorzano, J.W. Yu, R. Meng, A.A. Quong, E. Latz, C.P. Scott, E.S. Alnemri, Anti-inflammatory compounds parthenolide and Bay 11-7082 are direct inhibitors of the inflammasome. J. Biol. Chem. 285 (2010) 9792−9802.

[38] C. Dostert, V. Pétrilli, R. Van Bruggen, C. Steele, B.T. Mossman, J. Tschopp, Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. Science 320 (2008) 674−677.

[39] L. Franchi, T.D. Kanneganti, G.R. Dubyak, G. Núñez, Differential requirement of P2X7 receptor and intracellular K+ for caspase-1 activation induced by intracellular and extracellular bacteria. J. Biol. Chem. 282 (2007) 18810−18818.

[40] Zhou R, Tardivel A, Thorens B, Choi I, Tschopp J. Thioredoxin-interacting protein links oxidative stress to inflammasome activation. Nat. Immunol;11:136−140.

[41] W.A. Derbigny, M.S. Kerr, R.M. Johnson, Pattern recognition molecules activated by *Chlamydia muridarum* infection of cloned murine oviduct epithelial cell lines. J. Immunol. 175 (2005) 6065−6075.

[42] U.M. Nagarajan, D.M. Ojcius, L. Stahl, R.G. Rank, T. Darville, *Chlamydia trachomatis* induces expression of IFN-gamma-inducible protein 10 and IFN-beta independent of TLR2 and TLR4, but largely dependent on MyD88. J. Immunol. 175 (2005) 450−460.

[43] K.R. Buchholz, R.S. Stephens, The cytosolic pattern recognition receptor NOD1 induces inflammatory interleukin-8 during *Chlamydia trachomatis* infection. Infect. Immun. 76 (2008) 3150−3155.

[44] L. Chen, L. Lei, X. Chang, Z. Li, C. Lu, X. Zhang, Y. Wu, I.T. Yeh, G. Zhong, Mice deficient in MyD88 develop a Th2-dominant response and severe pathology in the upper genital tract following *Chlamydia muridarum* infection. J. Immunol. 184 (2010) 2602−2610.

[45] X. Zhang, L. Gao, L. Lei, Y. Zhong, P. Dube, M.T. Berton, B. Arulanandam, J. Zhang, G. Zhong, A MyD88-dependent early IL-17 production protects mice against airway infection with the obligate intracellular pathogen *Chlamydia muridarum*. J. Immunol. 183 (2009) 1291−1300.

[46] W. Han, H. Li, V.A. Villar, A.M. Pascua, M.I. Dajani, X. Wang, A. Natarajan, M.T. Quinn, R.A. Felder, P.A. Jose, P. Yu, Lipid rafts keep NADPH oxidase in the inactive state in human renal proximal tubule cells. Hypertension 51 (2008) 481−487.

[47] S. Jin, F. Zhou, Lipid raft redox signaling platforms in vascular dysfunction: features and mechanisms. Curr. Atheroscler. Rep. 11 (2009) 220−226.

[48] H. Li, W. Han, V.A. Villar, L.B. Keever, Q. Lu, U. Hopfer, M.T. Quinn, R.A. Felder, P.A. Jose, P. Yu, D1-like receptors regulate NADPH oxidase activity and subunit expression in lipid raft microdomains of renal proximal tubule cells. Hypertension 53 (2009) 1054−1061.

[49] S.J. Park, H.Y. Kim, H. Kim, S.M. Park, E.H. Joe, I. Jou, Y.H. Choi, Oxidative stress induces lipid-raft-mediated activation of Src homology 2 domain-containing protein-tyrosine phosphatase 2 in astrocytes. Free. Radic. Biol. Med. 46 (2009) 1694−1702.

[50] M. Ushio-Fukai, Compartmentalization of redox signaling through NADPH oxidase-derived ROS. Antioxid. Redox. Signal. 11 (2009) 1289−1299.

[51] R. Liu, J.L. Garvin, Y. Ren, P.J. Pagano, O.A. Carretero, Depolarization of the macula densa induces superoxide production via NAD(P)H oxidase. Am. J. Physiol. Renal. Physiol. 292 (2007) F1867−F1872.

[52] I. Matsuzaki, S. Chatterjee, K. Debolt, Y. Manevich, Q. Zhang, A.B. Fisher, Membrane depolarization and NADPH oxidase activation in aortic endothelium during ischemia reflect altered mechanotransduction. Am. J. Physiol. Heart. Circ. Physiol. 288 (2005) H336−H343.

[53] T.E. DeCoursey, D. Morgan, V.V. Cherny, The voltage dependence of NADPH oxidase reveals why phagocytes need proton channels. Nature 422 (2003) 531−534.

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Chapter3.Discussion

1. Innate immune response to *Aspergillus fumigatus.*

Pathogenic fungi interact with a variety of host cells during the induction of disease. In order to cross the tissue barrier, they normally invade non-phagocytic host cells such as epithelial and endothelial cells by triggering their own uptake. During invasive aspergillosis, the inhaled conidia are deposited in the alveoli and induce their own endocytosis by type II pneumocytes and tracheal epithelial cells [89]. Once internalized, they traffic to late endosomes/lysosomes, germinate and the resultant hyphae escape the endosome and penetrate into the plasma membrane of the host cell [128]. Infected epithelial cells play a key position participating in local airway inflammation via their production of cytokines and chemokines such as IL-6, IL-8, IL-1β and others that link innate to adaptive immune systems [129]. The interaction between *A.fumigatus* and host cells have been studied thoroughly and recent efforts have focused on describing the cellular mechanisms by which hosts avoid invasive infection caused by frequently inhaled conidia while modulating associated inflammation which represents a key element in clearing this fungus. These findings show evidence that immune recognition relies on TLRs (2, 4, 9) and C-type lectins such as dectin-1, to sense the presence and respond to *Aspergillus* ligands [98, 130-131]. Netea et.al showed that conidia and hyphae from *A. fumigatus* can activate murine peritoneal macrophages via TLR-2 and -4 [97]. However, only TLR2 and dectin-1

recognize the hyphal form of this fungus and trigger intracellular pathways mediating hypha-exposed macrophages responses [98, 130-131]. In vitro results suggest that these responses are at least partially mediated by MyD88-dependent signaling pathways although the in vivo studies in MyD88 knock out (KO) mice are sometimes conflicting and hard to define [132-133]. Activation of TLR2, TLR4 and dectin-1 have an important impact on the generation of host antifungal immunity but since *A.fumigatus* induce their stimulation in a morphotype-specific fashion, it does not come as surprise given the complexity of the different morphological states that the immunostimulatory ligands of those receptors except for dectin-1 are still unknown. In fact, during maturation, conidia cell surface undergoes several modifications that involve loss of a thin proteinaceous hydrophobic cell wall before switching to the hyphal form. The latter is composed primarily of β (1,3)-glucans, chitin and galactomannan [134-135]. The innate immune system senses differently these distinct forms of *A.fumigatus* and presents a remarkable functional plasticity in response to each form using different effector mechanisms in terms of maturation, cytokine production and Th cell response both in vitro and in vivo. In healthy individuals, *A.fumigatus* can infect the lungs but the establishment of disease is prevented by the host immune system; however any changes in the host defense components may lead to abnormal response and the development of pulmonary infections. Therefore, in immunocompromised patients, invasive aspergillosis is a major cause of morbidity and mortality which despite the development of new treatments, is beyond 50% and reaches up to 95% in certain cases [136-137]. It is well

established that immunosuppression whether due to corticosteroid treatment or secondary to another disease impairs host defences and predisposes immunosuppressed patients to high risk of developing invasive aspergillosis [138-139]. The exact mechanisms by which corticosteroids suppress the ability of the host to clear infection are less known but could be related to the inhibition and the delay of a local inflammatory cytokine expression which will be translated into a significant decrease in mediated inflammatory cell recruitment to the site of infection allowing therefore the fungus to germinate and expand [140]. The regulation of cytokine production is a crucial mechanism by which infected cells trigger and modulate inflammation. Therefore, my study was focused on the production and the secretion of the potent pro-inflammatory cytokine IL-1β during *A.fumigatus* infection. Taking into account the diversity of cytokines secreted during inflammation, I focused my interest on the elaboration and the production of IL-1β in the context of host response to the conidial and the hyphal form of *A.fumigatus. S*ince no study up to now had revealed the pattern of IL-1β secretion upon stimulation of these two different forms and whether any functional inflammasome is being activated during aspergillosis, we believed that our investigation will be greatly appreciated as it will clarify how the innate immune response modulates cytokine expression according to the severity of the infectious form of this fungus.

In our study, we wanted to determine which of *A.fumigatus* morphotype is capable of inducing the expression of pro-IL-1β gene and the secretion of its mature form by the human monocyte cell line (THP-1). Our results showed that

only the mycelium form is provoking a significant release of IL-1β whereas stimulation with *Aspergillus* conidia failed to be seen as potential danger and therefore exhibited comparable amount of IL-1 β secretion as the unstimulated monocytes. In order to rule out any monocytic activation by conidia we incubated THP-1 with conidia for 12hours and assessed IL-1 β secretion using as a control TNF-α secretion which is another pro-inflammatory cytokine whose secretion do not require caspase-1 and inflammasome activations and we proved that even when stimulated for longer period with spores, THP-1 cells do not secrete significant amount of IL-1β and TNF-α. This finding shows that although monocytes bind and ingest *Aspergillus* resting conidia efficiently, there is very little inflammation response; however maturing hyphae stimulate NF-κβ, secretion of pro-inflammatory cytokines such as IL-1β and TNF-α demonstrating that the inflammatory response is tailored toward metabolically active and invading fungal form, thereby avoiding unnecessary tissue damage with frequent inhalation of ubiquitous spores.

When we started the project our hypothesis was to show that ***A. fumigatus* infection induces caspase-1 activation in an inflammasome-dependent manner,** therefore in order to achieve our first specific aim which was to **evaluate if *A. fumigatus* infection induces caspase-1 activation in human monocytes,** we tested and proved by Western blot and Elisa analysis that caspase-1 is being activated and cleaved into the p20 subunit which is secreted to the supernatant of the infected cells with hyphal fragments. Our next step was to **characterize the "danger signal" produced and identify the "inflammasome"**

activated during *A. fumigatus* infection that leads to IL-1β secretion.

Therefore, using RNA interference we knocked down the NLR NALP3 and its adaptor ASC in THP-1 and showed that hyphal fragments mediating caspase-1 activation is depending on NALP3 inflammasome assembly. In order to characterize the danger signals responsible for this activation we looked at intracellular modifications that were already reported to occur during *A. fumigatus* infection and checked their involvement in inflammasome activation. Reactive oxygen species production was shown upon *A. fumigatus* infection and is believed to play a major role in anti-fungal defense. B-glucans recognition by dectin-1 was shown to be involved in nicotinamide adenine dinucleotide phosphate (NADPH) oxidase stimulation and ROS induction [141]. On the other hand, several studies had already pointed the role of ROS in NALP3 inflammasome activation; indeed several microbial infections were proved to induce NALP3 inflammasome assembly in a ROS dependant manner [52-53]. Thus, using NAC we proved that ROS production is needed to NALP3 inflammasome activation by HF. Furthermore, we showed that K^+ efflux was also a part of the required intracellular changes that activate NALP3 during HF stimulation. We also have found using RNA interference that the intracellular adaptor kinase Syk that is activated downstream of dectin-1 is involved in NALP3 inflammasome activation as well as in NF-κβ signalling whereas Myd88 is partially required for NF-κβ signalling but do not interfere with inflammasome activation. Finally, to bring more physiological relevance to our study we checked the effect of corticosteroids on pro-IL-1β expression and our data supported previous studies that

immunosuppressive treatment is translated into failure of inducing adequate pro-inflammatory cytokine production; however we did not manage to show any interference or inhibition of the inflammasome activation post corticosteroids treatments.

Conclusion and future perspective

Collectively, our results provide a mechanism by which monocytes distinguish between inhaled resting forms of this fungus and maturing mycelium. Since the latter presents a threat for invasive disease, selective secretion of IL-1β and TNF-α are produced only when innate immune cells fail to eradicate conidia which will lead to fungal growth. Limiting inflammasome activation to the hyphal form is a smart strategy minimizing lung damage that would take place with constant inhalation of ubiquitous fungal spores. Our data provide a compelling argument that collaborative responses triggered by TLRs and dectin-1 are required for controlling fungal infection through synergistic induction of cytokines as shown in Myd88 and Syk KD cells whose stimulation with HF induced statistically significant reduction in pro-IL-1β expression. Furthermore, we confirmed that corticosteroids treatment abrogates host defence against *A. fumigatus* by reducing the capacity of monocytes to establish a rapid and effective inflammatory response.

Better understanding of the innate immune response during invasive aspergillosis becomes necessary as the complex interplay between cytokines, chemokines and the cells of the immune system needs to be deeply studied and clearly elucidated. On the other hand, the identification of the immunostimulatory

fungal components characteristic of each morphotype is an important research priority. In turn, revelation of activated PRRs by these components will definitely yield experimental strategies such as the use of specific agonist or antagonists or recombinant cytokines that may offer promising approach to understand invasive aspergillosis pathogenesis and develop new antifungal, anti-inflammatory and immunomodulatory drugs.

2. Innate immune response to *Chlamydia Trachomatis*.

The pathogenic chlamydiae are obligate intracellular bacterial pathogens that cause serious human diseases. We focused our study on *Chlamydia. trachomatis* which represents the leading cause of preventable blindness in developing countries and the most commonly reported sexually transmitted infection worldwide [109, 142]. The biphasic developmental cycle and the obligate intracellular growth of the organism have complicated the study of the host-pathogen interplay. Elucidation of microbial pathogenesis derives from a better understanding of the complex relationships between pathogenic microbes and their hosts. Hence, a rapid expansion of studies involving the characterization of the stimulated PRRs, their ligands and signalling pathways that govern the innate immune response to *C.trachomatis* had shed the light on host defense against chlamydial infection. As I stated before, the first line of protection from mucosal pathogens is the selective epithelial barrier that prevents *Chlamydia*'s entry through the formation of tight intercellular junctions. Epithelial cells are preferentially targeted by *Chlamydia*; they play an early and important role in host defence as they secrete a battery of pro-inflammatory cytokines and anti-microbial agents such as β-defensins which permeabilize the bacterial cell membrane [143] and act as chemoattractant agents linking between the innate and the acquired immune system [144]. Several studies described TLR2-and-4 dependent cytokine production from inflammatory cells exposed to chlamydial EBs [145-146]; however only live, actively replicating *Chlamydia* were shown to

be capable of producing an inflammatory response whereas UV-inactivated EBS fail to induce cytokine release. This finding emphasizes one more time how the immune system “samples” pathogens, discriminates and reacts against the dangerous form of these microbes [147] while avoiding exaggerated and unnecessary responses against dormant, non replicating microbes which might harm the host tissues. Cytokines secreted by cervical epithelial cells infected with *C.trachomatis* include IL-8, growth regulated oncogene-alpha (GROα), Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), IL-6, IL-1 (α and β) [148] which are logical inducers of rapid activation and migration of leukocytes to the infected sites. Macrophages and their circulating precursors, monocytes reside in subepithelial connective tissues and are therefore strategically placed at the sites where chlamydiae gain entry into the host. Studies had shown that chlamydial-infected macrophages are important players in chlamydial eradication as they ingest and phagocytose the bacterium on one hand and contribute in establishing pro-inflammatory environment by secreting huge quantities of IL-1β and TNF-α [149-150]. Since the massive secretion of these pro-inflammatory cytokines especially IL-1 β are major contributors of the development of chlamydial pathology, it was of big interest to study whether this secretion is inflammasome dependent and which of the NLRs protein is sensing chlamydial infection. Our Lab demonstrated NALP3 inflammasome activation in epithelial cells upon *C.trachomatis* infection [47] therefore **I hypothesized that *C.trachomatis* is able to induce NALP3-mediated caspase-1 activation in human monocytes**. In order to **evaluate if *C.trachomatis* infection induces**

caspase-1 activation in human monocytes in a NALP3 dependant manner, we infected THP-1 cells with two chlamydial strains L2 and MoPn and confirmed by Western blot and ELISA analysis respectively the activation and the secretion of the caspase-1 p20 subunits. We also proved using caspase-1 inhibitors and caspase-1 KD THP-1 cell line the requirement of caspase-1 activation for IL-1β secretion. We furthermore investigated the involvement of NALP3 inflammasome and its adaptor ASC and showed that THP-1 KD for those two genes exhibit significant reduction in IL-1β and caspase-1 secretion. These findings showed that the same inflammasome is being activated in both epithelial and monocytic cell lines. Our second specific aim was to **characterize the "danger signals" required and identify the mechanisms by which they induce the inflammasome activation**. Using a NADPH oxidase inhibitor, NAC treatment and blocking potassium efflux, our result revealed the importance of ROS production and K^+ efflux in NALP3 inflammasome assembly upon chlamydial infection. Surprisingly, Syk KD cells showed a significant drop in IL-1β secretion and caspase-1 activation hinting a role of this kinase in activating NALP3 inflammasome during bacterial infection. Syk was already demonstrated to have an effect on NALP3 inflammasome during fungal infection [52, 151] ; our paper on *C.trachomatis* is the first to link Syk to NALP3 inflammasome in a bacterial context. An explanation of this involvement could be that stimulation of mannose receptor which recruits and phosphorylates Syk was reported to be necessary for chlamydial ingestion. Chlamydial major outer membrane protein (MOMP) is glycosylated and the structure of its carbohydrates is N-linked high mannose type

and this makes *C.trachomatis* infect mannose-receptor positive cells more efficiently than cells lacking its expression [152].

Conclusion and future perspective

Our chlamydial project was the first to show that *C.trachomatis* infection is inducing NALP3/ASC inflammasome assembly in human monocytes, and thus activates caspase-1 which in turn leads to massive IL-1β secretion. This process requires Syk activation, ROS production and K^+ efflux. Direct comparison between *C.trachomatis* and *A.fumigatus* confirms the availability of multiple PRRs and adaptor molecules that allow the innate immune system to tailor responses to a specific pathogen with exquisite refinement. In addition, we showed for the first time the activation of NALP3 inflammasome during infection with both pathogens, highlighted how different microbes presenting distinct antigenic molecules, exhibiting different infectious strategies are capable of inducing common intracellular modifications that converge to the activation of the same inflammasome.

Despite the enormous cost of treatment and growing number of people affected by this disease, questions on *Clamydia* pathogenesis remain unanswered because of the limitation of genetic manipulations of this bacterium. However, *C.trachomatis* 'genome had been sequenced [153]; transcriptomics and proteomics can be used in the analysis of the biology and the pathogenesis of this bacteria. Identifying and analyzing the battery of genes expressed within the infected host and *Chlamydia* could be representative of which host pathways are being activated on one hand and on other hand, which chlamydial genes with

pathogenic properties are involved in the establishment and progression of the disease. Alternatively, the study of genetic polymorphisms may also contribute to elucidate and interpret the variations in the immune responses toward clamydial infection. Thus, a better understanding of the cellular mechanisms will provide valuable knowledge that will be critical for investigating host-pathogen interaction and developing of effective vaccines.

3. Inflammasomes at the intersection of inflammation and immunity.

Our papers and previous studies bring evidence that innate immunity is able to discriminate pathogenic microbes from nonpathogenic microbes; but this raises the question of how the immune system interprets the microbial environment allowing the discrimination between a virulent organism from one that has lower disease-causing potential [154]. Beside the recognition of microbial PAMPs, the immune system senses other signals associated to infection such as host components released from infected, necrotic cells that activate and intensify the immune response [155]. Even though, the initiation of an inflammatory response is due to the presence of pathogens, the tissue damage that occurs during infection is mostly caused by the host response rather than the pathogen itself. Up until the discovery of inflammasomes, scientists believed that the major role of the immune system is to differentiate self from non-self; this led to multiple studies identifying molecules and signals that are released by host damaged or stressed tissues that trigger and engage inflammasomes assembly [155-156] leading to an innate immune response induction. Interestingly, some PAMPs are sensed by the immune system only when they are detected at specific locations such as flagellin, dsRNA, DNA that activate the inflammasome only upon delivery to the cytosol [76-77, 157-158]. This indicates that the immune system initiates responses based not only on whether PAMPs are present, but on where and under what cellular context these PAMPs are presented!

Moreover, many bacterial pathogens produce toxins that contribute to virulence by modifying intracellular host environment such as altering the intracellular ionic balances leading mostly to a drop of intracellular potassium or an increase of intracellular calcium level which was shown to activate the NALP3 inflammasome [61, 63]; Additional studies revealed that the most striking features associated with NALP3 inflammasome activation are the induction of frustrated phagocytosis, ROS production and potassium efflux [159]. It is now believed that most NALP3 activators induce a common triggering mechanism that involves and requires simultaneously ROS production and K^+ efflux; studies had shown that these two elements are tightly correlated since inhibition of K^+ efflux induces a reduction of ROS production and consequently leads to less NALP3 inflammasome activation [47, 159]. However the interplay between ROS production and K^+ efflux is currently unclear, further investigations are needed to clarify their effects one on the other. This unifying pathway suggests that inflammasome activation by microbes is indirect and thus explain how different pathogens with no obvious homology activate the same NLR protein.

Reactive oxygen species (ROS) are highly reactive molecules originating from molecular oxygen which has been implicated to be involved in a broad variety of pathologies and ageing; the major source of ROS is originated during oxidative phosphorylation within the mitochondria where electrons are passed through a series of proteins until it reaches its final destination on an oxygen molecule [160]. In phagocytic cells, upon microbial ingestion NADPH oxidase is activated and generates ROS for the direct purpose of killing invading microbes;

beside the damaging properties of these oxidant molecules, several studies had highlighted their importance at lower concentration as cell signalling molecules mediating tyrosine kinase receptor signalling in specific cellular subdomains [161]. All NLRP3 activators that have been examined, including ATP, bacterial pore-forming toxins, asbestos and silica trigger the generation of short-lived ROS, and treatment with various ROS scavengers blocks NLRP3 activation [61, 159]. The molecular mechanism whereby ROS induce the activation of NALP3 inflammasome is not fully understood but it may involve a thioredoxin-interacting protein (TXNIP; also known as VDuP1) which associates to NALP3 in a ROS dependent manner [162].

The participation and the critical importance of NALP3 inflammasome in initiating inflammatory response is now clearly accepted; however the molecular players that mediate this process and the mechanisms by which NALP3 sense its activators have not yet been completely identified. Certain studied confirmed the tight relation linking NALP3inflammasome to inflammation since gain-of-function mutations in NALP3 gene give rise to an hyperactive NALP3 inflammasome and are responsible for causing auto-inflammatory diseases associated with an massive secretion of IL-1β [163-164]. Patients develop symptoms ranging from a regular fever, skin rashes, arthralgia to very serious neurological impairment caused by chronic polymorphonuclear meningitis; these observations highlight the potent effects of IL-1β in inducing fever and acting on the central nervous system [164-165]. The studies mentioned above had brought, just by revealing the involvement of NALP3 and its crucial role in secreting IL-

1β, a lot of news hopes for patients suffering from various auto-inflammatory diseases. Lately, considerable research studies are focusing on the development of new drugs, new adjuvants and effective inflammasomes inhibitors. A new IL-1β antibody is currently in Phase II trials for rheumatoid and arthritis; additionally, various inflammatory diseases, such as gout, are now successfully treated with an IL-1 inhibitor [34]. Furthermore, the discovery of two new natural inflammasome inhibitors, Bay 11-7082 inhibiting specifically NALP3 and Parthenolide inhibiting several inflammasomes, provide a new strategy for the future development of specific anti-inflammasome drugs [166].

Beside their roles in inducing inflammatory responses, inflammasomes were reported to induce under certain circumstances cell death that does not rely on the classical pro-apoptotic caspases (caspase-3, 8 and 9) but rather on caspase-1, called pyroptosis. This form of cell death shares with apoptosis nuclear condensation and membrane blebbing but is unlike apoptosis caspases-3 independent [74, 157]. Other studies demonstrated that NLR are involved in necrotic cell death induction in a capsase-1 independent manner and this process was name pyronecrosis [27, 167]. Although the mechanism is still unclear, the data shows the requirement of NLR in inducing apoptosis-like or necrosis-like cell death induced by microbial component which adds an additional level of complexity to NLR modulating cellular processes. Future studies should aim to clarify the roles of NLR proteins beyond caspases-1 activation in inducing cell death and how this latter contribute to immunity.

Concluding Remarks:

Despite substantial progress in understanding of the activation, regulation and the function of the different inflammasomes, many gaps are to be filled for full understanding of this sophisticated system. Given the abundance of NLRs, their adaptors, their regulators and the various pathogens and danger signals that trigger the innate immune system, there are many obstacles to overcome. For example, generating KO mice for different NLRs will definitely aid in elucidating the roles and the physiological functions of these receptors upon infection and during auto-inflammatory diseases. Based on sequence studies, inflammasomes 'regulators are divided in two families: those containing a CARD domain and those with a PYD domain. The PYD containing domain will interfere with the PYD-PYD interactions between the NLRs and their adaptors, and the CARD containing domain will affect the recruitment of caspase-1 to the inflammasome by modulating the CARD-CARD interactions. Overexpression or gene silencing of those regulators will provide a new approach clarifying the synergisms, feedback loops, and checkpoints that ultimately control inflammasome activation.

All of these studies combined together shows that the host innate immune system, through the activation of NLR proteins, is able to respond to patterns of pathogenesis, signals that derive from the strategies that live pathogens use to invade, manipulate, replicate within, or spread among their hosts. Regardless of what might also be learned in the future, the discovery of the NLRs will be always viewed as a critical element in renovating our knowledge of how an effective innate immune response is established.

Bibliography

1. Janeway, C.A., Jr., *Approaching the asymptote? Evolution and revolution in immunology.* Cold Spring Harb Symp Quant Biol, 1989. 54 Pt 1: p. 1-13.
2. Takeda, K. and S. Akira, *Toll-like receptors in innate immunity.* Int Immunol, 2005. 17(1): p. 1-14.
3. Takeda, K., T. Kaisho, and S. Akira, *Toll-like receptors.* Annu Rev Immunol, 2003. 21: p. 335-76.
4. Iwasaki, A. and R. Medzhitov, *Toll-like receptor control of the adaptive immune responses.* Nat Immunol, 2004. 5(10): p. 987-95.
5. Gilmore, T.D., *Introduction to NF-kappaB: players, pathways, perspectives.* Oncogene, 2006. 25(51): p. 6680-4.
6. Brasier, A.R., *The NF-kappaB regulatory network.* Cardiovasc Toxicol, 2006. 6(2): p. 111-30.
7. Sato, S., et al., *Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling.* J Immunol, 2003. 171(8): p. 4304-10.
8. Apostolopoulos, V. and I.F. McKenzie, *Role of the mannose receptor in the immune response.* Curr Mol Med, 2001. 1(4): p. 469-74.
9. Saijo, S., et al., *Dectin-1 is required for host defense against Pneumocystis carinii but not against Candida albicans.* Nat Immunol, 2007. 8(1): p. 39-46.
10. Werner, J.L., et al., *Requisite role for the dectin-1 beta-glucan receptor in pulmonary defense against Aspergillus fumigatus.* J Immunol, 2009. 182(8): p. 4938-46.
11. Willment, J.A., et al., *The human beta-glucan receptor is widely expressed and functionally equivalent to murine Dectin-1 on primary cells.* Eur J Immunol, 2005. 35(5): p. 1539-47.
12. Fuller, G.L., et al., *The C-type lectin receptors CLEC-2 and Dectin-1, but not DC-SIGN, signal via a novel YXXL-dependent signaling cascade.* J Biol Chem, 2007. 282(17): p. 12397-409.
13. Underhill, D.M. and H.S. Goodridge, *The many faces of ITAMs.* Trends Immunol, 2007. 28(2): p. 66-73.
14. Herre, J., et al., *Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages.* Blood, 2004. 104(13): p. 4038-45.
15. Slack, E.C., et al., *Syk-dependent ERK activation regulates IL-2 and IL-10 production by DC stimulated with zymosan.* Eur J Immunol, 2007. 37(6): p. 1600-12.
16. LeibundGut-Landmann, S., et al., *Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17.* Nat Immunol, 2007. 8(6): p. 630-8.

17. **Dennehy, K.M., et al., *Syk kinase is required for collaborative cytokine production induced through Dectin-1 and Toll-like receptors.* Eur J Immunol, 2008. 38(2): p. 500-6.**
18. **Underhill, D.M., et al., *Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production.* Blood, 2005. 106(7): p. 2543-50.**
19. **Rogers, N.C., et al., *Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins.* Immunity, 2005. 22(4): p. 507-17.**
20. **Vance, R.E., R.R. Isberg, and D.A. Portnoy, *Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system.* Cell Host Microbe, 2009. 6(1): p. 10-21.**
21. **Meylan, E., J. Tschopp, and M. Karin, *Intracellular pattern recognition receptors in the host response.* Nature, 2006. 442(7098): p. 39-44.**
22. **Yoneyama, M., et al., *The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses.* Nat Immunol, 2004. 5(7): p. 730-7.**
23. **Alexopoulou, L., et al., *Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3.* Nature, 2001. 413(6857): p. 732-8.**
24. **Kawai, T., et al., *IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction.* Nat Immunol, 2005. 6(10): p. 981-8.**
25. **Meylan, E., et al., *Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus.* Nature, 2005. 437(7062): p. 1167-72.**
26. **Faustin, B., et al., *Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation.* Mol Cell, 2007. 25(5): p. 713-24.**
27. **Duncan, J.A., et al., *Cryopyrin/NALP3 binds ATP/dATP, is an ATPase, and requires ATP binding to mediate inflammatory signaling.* Proc Natl Acad Sci U S A, 2007. 104(19): p. 8041-6.**
28. **Harton, J.A., et al., *Cutting edge: CATERPILLER: a large family of mammalian genes containing CARD, pyrin, nucleotide-binding, and leucine-rich repeat domains.* J Immunol, 2002. 169(8): p. 4088-93.**
29. **Martinon, F. and J. Tschopp, *NLRs join TLRs as innate sensors of pathogens.* Trends Immunol, 2005. 26(8): p. 447-54.**
30. **Proell, M., et al., *The Nod-like receptor (NLR) family: a tale of similarities and differences.* PLoS One, 2008. 3(4): p. e2119.**
31. **Tschopp, J., F. Martinon, and K. Burns, *NALPs: a novel protein family involved in inflammation.* Nat Rev Mol Cell Biol, 2003. 4(2): p. 95-104.**
32. **Inohara, N., et al., *Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB.* J Biol Chem, 1999. 274(21): p. 14560-7.**
33. **Mariathasan, S., *ASC, Ipaf and Cryopyrin/Nalp3: bona fide intracellular adapters of the caspase-1 inflammasome.* Microbes Infect, 2007. 9(5): p. 664-71.**
34. **Martinon, F., A. Mayor, and J. Tschopp, *The inflammasomes: guardians of the body.* Annu Rev Immunol, 2009. 27: p. 229-65.**
35. **Wang, L., et al., *PYPAF7, a novel PYRIN-containing Apaf1-like protein that regulates activation of NF-kappa B and caspase-1-dependent cytokine processing.* J Biol Chem, 2002. 277(33): p. 29874-80.**

36. Martinon, F., K. Burns, and J. Tschopp, *The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta.* Mol Cell, 2002. 10(2): p. 417-26.
37. Mariathasan, S., et al., *Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf.* Nature, 2004. 430(6996): p. 213-8.
38. Dinarello, C.A., *Biologic basis for interleukin-1 in disease.* Blood, 1996. 87(6): p. 2095-147.
39. Kostura, M.J., et al., *Identification of a monocyte specific pre-interleukin 1 beta convertase activity.* Proc Natl Acad Sci U S A, 1989. 86(14): p. 5227-31.
40. Franchi, L., et al., *Nucleotide-binding oligomerization domain-like receptors: intracellular pattern recognition molecules for pathogen detection and host defense.* J Immunol, 2006. 177(6): p. 3507-13.
41. Kanneganti, T.D., et al., *Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA.* J Biol Chem, 2006. 281(48): p. 36560-8.
42. Kanneganti, T.D., et al., *Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3.* Nature, 2006. 440(7081): p. 233-6.
43. Poyet, J.L., et al., *Identification of Ipaf, a human caspase-1-activating protein related to Apaf-1.* J Biol Chem, 2001. 276(30): p. 28309-13.
44. Mariathasan, S., et al., *Cryopyrin activates the inflammasome in response to toxins and ATP.* Nature, 2006. 440(7081): p. 228-32.
45. Koo, I.C., et al., *ESX-1-dependent cytolysis in lysosome secretion and inflammasome activation during mycobacterial infection.* Cell Microbiol, 2008. 10(9): p. 1866-78.
46. Duncan, J.A., et al., *Neisseria gonorrhoeae activates the proteinase cathepsin B to mediate the signaling activities of the NLRP3 and ASC-containing inflammasome.* J Immunol, 2009. 182(10): p. 6460-9.
47. Abdul-Sater, A.A., et al., *Inflammasome-dependent caspase-1 activation in cervical epithelial cells stimulates growth of the intracellular pathogen Chlamydia trachomatis.* J Biol Chem, 2009. 284(39): p. 26789-96.
48. Yilmaz, O., et al., *ATP-dependent activation of an inflammasome in primary gingival epithelial cells infected by Porphyromonas gingivalis.* Cell Microbiol, 2010. 12(2): p. 188-98.
49. Master, S.S., et al., *Mycobacterium tuberculosis prevents inflammasome activation.* Cell Host Microbe, 2008. 3(4): p. 224-32.
50. Ichinohe, T., et al., *Inflammasome recognition of influenza virus is essential for adaptive immune responses.* J Exp Med, 2009. 206(1): p. 79-87.
51. Delaloye, J., et al., *Innate immune sensing of modified vaccinia virus Ankara (MVA) is mediated by TLR2-TLR6, MDA-5 and the NALP3 inflammasome.* PLoS Pathog, 2009. 5(6): p. e1000480.
52. Gross, O., et al., *Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence.* Nature, 2009. 459(7245): p. 433-6.
53. Hise, A.G., et al., *An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen Candida albicans.* Cell Host Microbe, 2009. 5(5): p. 487-97.
54. Joly, S., et al., *Cutting edge: Candida albicans hyphae formation triggers activation of the Nlrp3 inflammasome.* J Immunol, 2009. 183(6): p. 3578-81.

55. Lamkanfi, M., R.K. Malireddi, and T.D. Kanneganti, *Fungal zymosan and mannan activate the cryopyrin inflammasome.* J Biol Chem, 2009. 284(31): p. 20574-81.
56. Franchi, L., T. Eigenbrod, and G. Nunez, *Cutting edge: TNF-alpha mediates sensitization to ATP and silica via the NLRP3 inflammasome in the absence of microbial stimulation.* J Immunol, 2009. 183(2): p. 792-6.
57. Halle, A., et al., *The NALP3 inflammasome is involved in the innate immune response to amyloid-beta.* Nat Immunol, 2008. 9(8): p. 857-65.
58. Hornung, V., et al., *Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization.* Nat Immunol, 2008. 9(8): p. 847-56.
59. Dostert, C., et al., *Malarial hemozoin is a Nalp3 inflammasome activating danger signal.* PLoS One, 2009. 4(8): p. e6510.
60. Shio, M.T., et al., *Malarial hemozoin activates the NLRP3 inflammasome through Lyn and Syk kinases.* PLoS Pathog, 2009. 5(8): p. e1000559.
61. Petrilli, V., et al., *Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration.* Cell Death Differ, 2007. 14(9): p. 1583-9.
62. Ferrari, D., et al., *The P2X7 receptor: a key player in IL-1 processing and release.* J Immunol, 2006. 176(7): p. 3877-83.
63. Franchi, L., et al., *Differential requirement of P2X7 receptor and intracellular K+ for caspase-1 activation induced by intracellular and extracellular bacteria.* J Biol Chem, 2007. 282(26): p. 18810-8.
64. Pelegrin, P. and A. Surprenant, *Pannexin-1 couples to maitotoxin- and nigericin-induced interleukin-1beta release through a dye uptake-independent pathway.* J Biol Chem, 2007. 282(4): p. 2386-94.
65. Pelegrin, P. and A. Surprenant, *Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor.* EMBO J, 2006. 25(21): p. 5071-82.
66. Kanneganti, T.D., et al., *Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling.* Immunity, 2007. 26(4): p. 433-43.
67. Cruz, C.M., et al., *ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages.* J Biol Chem, 2007. 282(5): p. 2871-9.
68. Boyden, E.D. and W.F. Dietrich, *Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin.* Nat Genet, 2006. 38(2): p. 240-4.
69. Liao, K.C. and J. Mogridge, *Expression of Nlrp1b inflammasome components in human fibroblasts confers susceptibility to anthrax lethal toxin.* Infect Immun, 2009. 77(10): p. 4455-62.
70. Miao, E.A., et al., *Pseudomonas aeruginosa activates caspase 1 through Ipaf.* Proc Natl Acad Sci U S A, 2008. 105(7): p. 2562-7.
71. Franchi, L., et al., *Critical role for Ipaf in Pseudomonas aeruginosa-induced caspase-1 activation.* Eur J Immunol, 2007. 37(11): p. 3030-9.
72. Sutterwala, F.S., et al., *Immune recognition of Pseudomonas aeruginosa mediated by the IPAF/NLRC4 inflammasome.* J Exp Med, 2007. 204(13): p. 3235-45.

73. Suzuki, T., et al., *Differential regulation of caspase-1 activation, pyroptosis, and autophagy via Ipaf and ASC in Shigella-infected macrophages.* PLoS Pathog, 2007. 3(8): p. e111.
74. Zamboni, D.S., et al., *The Birc1e cytosolic pattern-recognition receptor contributes to the detection and control of Legionella pneumophila infection.* Nat Immunol, 2006. 7(3): p. 318-25.
75. Miao, E.A., et al., *Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf.* Nat Immunol, 2006. 7(6): p. 569-75.
76. Franchi, L., et al., *Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages.* Nat Immunol, 2006. 7(6): p. 576-82.
77. Lightfield, K.L., et al., *Critical function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of flagellin.* Nat Immunol, 2008. 9(10): p. 1171-8.
78. Case, C.L., S. Shin, and C.R. Roy, *Asc and Ipaf Inflammasomes direct distinct pathways for caspase-1 activation in response to Legionella pneumophila.* Infect Immun, 2009. 77(5): p. 1981-91.
79. Bertin, J., et al., *Human CARD4 protein is a novel CED-4/Apaf-1 cell death family member that activates NF-kappaB.* J Biol Chem, 1999. 274(19): p. 12955-8.
80. Inohara, N., et al., *RICK, a novel protein kinase containing a caspase recruitment domain, interacts with CLARP and regulates CD95-mediated apoptosis.* J Biol Chem, 1998. 273(20): p. 12296-300.
81. Hsu, Y.M., et al., *The adaptor protein CARD9 is required for innate immune responses to intracellular pathogens.* Nat Immunol, 2007. 8(2): p. 198-205.
82. McDonald, C., N. Inohara, and G. Nunez, *Peptidoglycan signaling in innate immunity and inflammatory disease.* J Biol Chem, 2005. 280(21): p. 20177-80.
83. Girardin, S.E. and D.J. Philpott, *Mini-review: the role of peptidoglycan recognition in innate immunity.* Eur J Immunol, 2004. 34(7): p. 1777-82.
84. Vignal, C., et al., *How NOD2 mutations predispose to Crohn's disease?* Microbes Infect, 2007. 9(5): p. 658-63.
85. Tanabe, T., et al., *Regulatory regions and critical residues of NOD2 involved in muramyl dipeptide recognition.* EMBO J, 2004. 23(7): p. 1587-97.
86. Latge, J.P., *Aspergillus fumigatus and aspergillosis.* Clin Microbiol Rev, 1999. 12(2): p. 310-50.
87. O'Gorman, C.M., H.T. Fuller, and P.S. Dyer, *Discovery of a sexual cycle in the opportunistic fungal pathogen Aspergillus fumigatus.* Nature, 2009. 457(7228): p. 471-4.
88. Zhang, Z., et al., *Interaction of airway epithelial cells (A549) with spores and mycelium of Aspergillus fumigatus.* J Infect, 2005. 51(5): p. 375-82.
89. Paris, S., et al., *Internalization of Aspergillus fumigatus conidia by epithelial and endothelial cells.* Infect Immun, 1997. 65(4): p. 1510-4.
90. Denning, D.W., *Invasive aspergillosis.* Clin Infect Dis, 1998. 26(4): p. 781-803; quiz 804-5.
91. Medoff, G. and G.S. Kobayashi, *Systemic fungal infections: an overview.* Hosp Pract (Off Ed), 1991. 26(2): p. 41-52.
92. Filler, S.G. and D.C. Sheppard, *Fungal invasion of normally non-phagocytic host cells.* PLoS Pathog, 2006. 2(12): p. e129.

93. Morrison, V.A., R.J. Haake, and D.J. Weisdorf, *Non-Candida fungal infections after bone marrow transplantation: risk factors and outcome.* Am J Med, 1994. 96(6): p. 497-503.
94. Denning, D.W., et al., *An EORTC multicentre prospective survey of invasive aspergillosis in haematological patients: diagnosis and therapeutic outcome. EORTC Invasive Fungal Infections Cooperative Group.* J Infect, 1998. 37(2): p. 173-80.
95. Hagensee, M.E., et al., *Brain abscess following marrow transplantation: experience at the Fred Hutchinson Cancer Research Center, 1984-1992.* Clin Infect Dis, 1994. 19(3): p. 402-8.
96. Ho, P.L. and K.Y. Yuen, *Aspergillosis in bone marrow transplant recipients.* Crit Rev Oncol Hematol, 2000. 34(1): p. 55-69.
97. Netea, M.G., et al., *Aspergillus fumigatus evades immune recognition during germination through loss of toll-like receptor-4-mediated signal transduction.* J Infect Dis, 2003. 188(2): p. 320-6.
98. Hohl, T.M., et al., *Aspergillus fumigatus triggers inflammatory responses by stage-specific beta-glucan display.* PLoS Pathog, 2005. 1(3): p. e30.
99. Gerson, S.L., et al., *Prolonged granulocytopenia: the major risk factor for invasive pulmonary aspergillosis in patients with acute leukemia.* Ann Intern Med, 1984. 100(3): p. 345-51.
100. Roilides, E., et al., *Prevention of corticosteroid-induced suppression of human polymorphonuclear leukocyte-induced damage of Aspergillus fumigatus hyphae by granulocyte colony-stimulating factor and gamma interferon.* Infect Immun, 1993. 61(11): p. 4870-7.
101. Simitsopoulou, M., et al., *Expression of immunomodulatory genes in human monocytes induced by voriconazole in the presence of Aspergillus fumigatus.* Antimicrob Agents Chemother, 2007. 51(3): p. 1048-54.
102. Pan, Q., et al., *MDP-induced interleukin-1beta processing requires Nod2 and CIAS1/NALP3.* J Leukoc Biol, 2007. 82(1): p. 177-83.
103. Jiang, Z.H. and R.R. Koganty, *Synthetic vaccines: the role of adjuvants in immune targeting.* Curr Med Chem, 2003. 10(15): p. 1423-39.
104. Black, C.M., *Current methods of laboratory diagnosis of Chlamydia trachomatis infections.* Clin Microbiol Rev, 1997. 10(1): p. 160-84.
105. Cohen, C.R. and R.C. Brunham, *Pathogenesis of Chlamydia induced pelvic inflammatory disease.* Sex. Trans. Infect., 1999. 75: p. 21-24.
106. Schachter, J., *Infection and disease epidemiology.*, in *Chlamydia: Intracellular Biology, Pathogenesis, and Immunity.*, R.S. Stephens, Editor. 1999, ASM Press.: Washington, D.C. p. 139-169.
107. Miller, W.C., et al., *Prevalence of chlamydial and gonococcal infections among young adults in the United States.* JAMA, 2004. 291: p. 2229-2236.
108. Belland, R., D.M. Ojcius, and G.I. Byrne, *Chlamydia.* Nature Rev. Microbiol., 2004. 2: p. 530-531.
109. Gerbase, A.C., J.T. Rowley, and T.E. Mertens, *Global epidemiology of sexually transmitted diseases.* Lancet, 1998. 351: p. 2-4.
110. Cates, W., Jr. and J.N. Wasserheit, *Genital chlamydial infections: epidemiology and reproductive sequelae.* Am J Obstet Gynecol, 1991. 164(6 Pt 2): p. 1771-81.
111. Campbell, L.A. and C.C. Kuo, *Chlamydia pneumoniae--an infectious risk factor for atherosclerosis?* Nature Rev. Microbiol., 2004. 2: p. 23-32.

112. Vanrompay, D., R. Ducatelle, and F. Haesebrouck, *Chlamydia psittaci infections: a review with emphasis on avian chlamydiosis.* Vet Microbiol, 1995. 45(2-3): p. 93-119.
113. Cheng, W., et al., *Caspase-1 contributes to Chlamydia trachomatis-induced upper urogenital tract inflammatory pathologies without affecting the course of infection.* Infect Immun, 2008. 76(2): p. 515-22.
114. Moulder, J.W., *Interaction of chlamydiae and host cells in vitro.* Microbiol. Rev., 1991. 55: p. 143-190.
115. Moore, E.R., et al., *The chlamydial inclusion preferentially intercepts basolaterally directed sphingomyelin-containing exocytic vacuoles.* Traffic, 2008. 9(12): p. 2130-40.
116. Carabeo, R.A., D.J. Mead, and T. Hackstadt, *Golgi-dependent transport of cholesterol to the Chlamydia trachomatis inclusion.* Proc Natl Acad Sci U S A, 2003. 100(11): p. 6771-6.
117. Wylie, J.L., G.M. Hatch, and G. McClarty, *Host cell phospholipids are trafficked to and then modified by Chlamydia trachomatis.* J Bacteriol, 1997. 179(23): p. 7233-42.
118. Quayle, A.J., *The innate and early immune response to pathogen challenge in the female genital tract and the pivotal role of epithelial cells.* J Reprod Immunol, 2002. 57(1-2): p. 61-79.
119. Rasmussen, S.J., et al., *Secretion of proinflammatory cytokines by epithelial cells in response to Chlamydia infection suggests a central role for epithelial cells in chlamydial pathogenesis.* J. Clin. Invest., 1997. 99: p. 77-87.
120. Su, H., et al., *Chlamydia trachomatis genital tract infection of antibody-deficient gene knockout mice.* Infect Immun, 1997. 65(6): p. 1993-9.
121. Roan, N.R. and M.N. Starnbach, *Immune-mediated control of Chlamydia infection.* Cell Microbiol, 2008. 10(1): p. 9-19.
122. Entrican, G., et al., *Cytokine release by ovine macrophages following infection with Chlamydia psittaci.* Clin. Exp. Immunol., 1999. 117: p. 309-315.
123. Gervassi, A., et al., *Differential regulation of inflammation cytokine secretion by human dendritic cells upon Chlamydia trachomatis infection.* Infect. Immun., 2004. 72: p. 7231-7239.
124. Ojcius, D.M., et al., *Apoptosis of epithelial cells and macrophages due to infection with the obligate intracellular pathogen Chlamydia psittaci.* J. Immunol., 1998. 161: p. 4220-4226.
125. Rothermel, C.D., et al., *Chlamydia trachomatis-induced production of interleukin-1 by human monocytes.* Infect. Immun., 1989. 57: p. 2705-2711.
126. Prantner, D., et al., *Critical role for interleukin-1beta (IL-1beta) during Chlamydia muridarum genital infection and bacterial replication-independent secretion of IL-1beta in mouse macrophages.* Infect Immun, 2009. 77(12): p. 5334-46.
127. Gurcel, L., et al., *Caspase-1 activation of lipid metabolic pathways in response to bacterial pore-forming toxins promotes cell survival.* Cell, 2006. 126(6): p. 1135-45.
128. Wasylnka, J.A. and M.M. Moore, *Aspergillus fumigatus conidia survive and germinate in acidic organelles of A549 epithelial cells.* J Cell Sci, 2003. 116(Pt 8): p. 1579-87.

129. Kauffman, H.F., *Interaction of environmental allergens with airway epithelium as a key component of asthma.* Curr Allergy Asthma Rep, 2003. 3(2): p. 101-8.
130. Gersuk, G.M., et al., *Dectin-1 and TLRs permit macrophages to distinguish between different Aspergillus fumigatus cellular states.* J Immunol, 2006. 176(6): p. 3717-24.
131. Steele, C., et al., *The beta-glucan receptor dectin-1 recognizes specific morphologies of Aspergillus fumigatus.* PLoS Pathog, 2005. 1(4): p. e42.
132. Wang, J.E., et al., *Involvement of CD14 and toll-like receptors in activation of human monocytes by Aspergillus fumigatus hyphae.* Infect Immun, 2001. 69(4): p. 2402-6.
133. Mambula, S.S., et al., *Toll-like receptor (TLR) signaling in response to Aspergillus fumigatus.* J Biol Chem, 2002. 277(42): p. 39320-6.
134. Fontaine, T., et al., *Molecular organization of the alkali-insoluble fraction of aspergillus fumigatus cell wall.* J Biol Chem, 2000. 275(52): p. 41528.
135. Paris, S., et al., *Conidial hydrophobins of Aspergillus fumigatus.* Appl Environ Microbiol, 2003. 69(3): p. 1581-8.
136. Pannuti, C.S., et al., *Nosocomial pneumonia in adult patients undergoing bone marrow transplantation: a 9-year study.* J Clin Oncol, 1991. 9(1): p. 77-84.
137. Wingard, J.R., et al., *Aspergillus infections in bone marrow transplant recipients.* Bone Marrow Transplant, 1987. 2(2): p. 175-81.
138. Guiot, H.F., W.E. Fibbe, and J.W. van 't Wout, *Risk factors for fungal infection in patients with malignant hematologic disorders: implications for empirical therapy and prophylaxis.* Clin Infect Dis, 1994. 18(4): p. 525-32.
139. Yuen, K.Y., et al., *Stage-specific manifestation of mold infections in bone marrow transplant recipients: risk factors and clinical significance of positive concentrated smears.* Clin Infect Dis, 1997. 25(1): p. 37-42.
140. Duong, M., et al., *Kinetic study of host defense and inflammatory response to Aspergillus fumigatus in steroid-induced immunosuppressed mice.* J Infect Dis, 1998. 178(5): p. 1472-82.
141. Gantner, B.N., et al., *Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2.* J Exp Med, 2003. 197(9): p. 1107-17.
142. Thylefors, B., et al., *Global data on blindness.* Bull World Health Organ, 1995. 73(1): p. 115-21.
143. Lehrer, R.I., A.K. Lichtenstein, and T. Ganz, *Defensins: antimicrobial and cytotoxic peptides of mammalian cells.* Annu Rev Immunol, 1993. 11: p. 105-28.
144. Yang, D., et al., *Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6.* Science, 1999. 286(5439): p. 525-8.
145. Prebeck, S., et al., *The Gram-negative bacterium Chlamydia trachomatis L2 stimulates tumor necrosis factor secretion by innate immune cells independently of its endotoxin.* Microbes Infect, 2003. 5(6): p. 463-70.
146. Prebeck, S., et al., *Predominant role of toll-like receptor 2 versus 4 in Chlamydia pneumoniae-induced activation of dendritic cells.* J Immunol, 2001. 167(6): p. 3316-23.
147. Underhill, D.M., et al., *The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens.* Nature, 1999. 401(6755): p. 811-5.

148. Rasmussen, S.J., et al., *Secretion of proinflammatory cytokines by epithelial cells in response to Chlamydia infection suggests a central role for epithelial cells in chlamydial pathogenesis.* J Clin Invest, 1997. 99(1): p. 77-87.
149. Rothermel, C.D., et al., *Chlamydia trachomatis-induced production of interleukin-1 by human monocytes.* Infect Immun, 1989. 57(9): p. 2705-11.
150. La Verda, D. and G.I. Byrne, *Interactions between macrophages and chlamydiae.* Immunol Ser, 1994. 60: p. 381-99.
151. Said-Sadier, N., et al., *Aspergillus fumigatus stimulates the NLRP3 inflammasome through a pathway requiring ROS production and the Syk tyrosine kinase.* PLoS One, 2010. 5(4): p. e10008.
152. Kuo, C., et al., *An N-linked high-mannose type oligosaccharide, expressed at the major outer membrane protein of Chlamydia trachomatis, mediates attachment and infectivity of the microorganism to HeLa cells.* J Clin Invest, 1996. 98(12): p. 2813-8.
153. Stephens, R.S., et al., *Genome sequence of an obligate intracellular pathogen of humans: Chlamydia trachomatis.* Science, 1998. 282(5389): p. 754-9.
154. Seong, S.Y. and P. Matzinger, *Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses.* Nat Rev Immunol, 2004. 4(6): p. 469-78.
155. Kono, H. and K.L. Rock, *How dying cells alert the immune system to danger.* Nat Rev Immunol, 2008. 8(4): p. 279-89.
156. Rock, K.L. and H. Kono, *The inflammatory response to cell death.* Annu Rev Pathol, 2008. 3: p. 99-126.
157. Molofsky, A.B., et al., *Cytosolic recognition of flagellin by mouse macrophages restricts Legionella pneumophila infection.* J Exp Med, 2006. 203(4): p. 1093-104.
158. Ren, T., et al., *Flagellin-deficient Legionella mutants evade caspase-1- and Naip5-mediated macrophage immunity.* PLoS Pathog, 2006. 2(3): p. e18.
159. Dostert, C., et al., *Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica.* Science, 2008. 320(5876): p. 674-7.
160. Han, D., E. Williams, and E. Cadenas, *Mitochondrial respiratory chain-dependent generation of superoxide anion and its release into the intermembrane space.* Biochem J, 2001. 353(Pt 2): p. 411-6.
161. Woo, H.A., et al., *Inactivation of peroxiredoxin I by phosphorylation allows localized H(2)O(2) accumulation for cell signaling.* Cell, 2010. 140(4): p. 517-28.
162. Zhou, R., et al., *Thioredoxin-interacting protein links oxidative stress to inflammasome activation.* Nat Immunol, 2010. 11(2): p. 136-40.
163. Agostini, L., et al., *NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder.* Immunity, 2004. 20(3): p. 319-25.
164. Masters, S.L., et al., *Recent advances in the molecular pathogenesis of hereditary recurrent fevers.* Curr Opin Allergy Clin Immunol, 2006. 6(6): p. 428-33.
165. Dinarello, C.A., *Infection, fever, and exogenous and endogenous pyrogens: some concepts have changed.* J Endotoxin Res, 2004. 10(4): p. 201-22.
166. Juliana, C., et al., *Anti-inflammatory compounds parthenolide and Bay 11-7082 are direct inhibitors of the inflammasome.* J Biol Chem, 2010. 285(13): p. 9792-802.

167. **Willingham, S.B., et al.,** ***Microbial pathogen-induced necrotic cell death mediated by the inflammasome components CIAS1/cryopyrin/NLRP3 and ASC.*** **Cell Host Microbe, 2007. 2(3): p. 147-59.**