Inflammasomes in the lung

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Abstract

Innate immune responses act as first line defences upon exposure to noxious stimuli. The innate immune system has evolved numerous intracellular and extracellular receptors that undertake surveillance for noxious stimuli. Inflammasomes are intracellular innate immune multiprotein complexes that form and are activated following interaction with these stimuli. Inflammasome activation leads to the cleavage of pro-IL-1β and release of the pro-inflammatory cytokine, IL-1β, which initiates acute phase pro-inflammatory responses, and other responses are also involved (IL-18, pyroptosis). However, excessive activation of inflammasomes can result in chronic inflammation, which has been implicated in a range of chronic inflammatory diseases. The airways are constantly exposed to a wide variety of stimuli. Inflammasome activation and downstream responses clears these stimuli. However, excessive activation may drive the pathogenesis of chronic respiratory diseases such as severe asthma and chronic obstructive pulmonary disease. Thus, there is currently intense interest in the role of inflammasomes in chronic inflammatory lung diseases and in their potential for therapeutic targeting. Here we review the known associations between inflammasome-mediated responses and the development and exacerbation of chronic lung diseases.

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List of non-standard abbreviations:

AAD	Allergic airways disease
AHR	Airways hyperresponsiveness
AIM	Absent in melanoma
ALI	Acute lung injury
Alum	Aluminium hydroxide
ARDS	Acute respiratory distress syndrome
BALF	Bronchoalveolar lavage fluid
CAP	Community acquired pneumonia
CAPS	Cryopyrin-associated periodic syndrome
Casp1	Caspase-1
CARD	Caspase-recruitment domain
CF	Cystic fibrosis
CFTR	CF transmembrane conductance regulator
COPD	Chronic obstructive pulmonary disease
DAMP	Damage-associated molecular patterns
HDM	House dust mite
HIN	Haemopoietic IFN-inducible nuclear
HMGB1	Chromatin-binding high motility group box 1 protein
IAV	Influenza A virus
LRR	C-terminal leucine rich repeats
NBD	Nucleotide binding domain
NLR	NOD-like receptor
NOD	Nucleotide oligomerisation domain
NLRC	NOD-like receptor containing
NLRP	NOD-like receptor protein

Ova	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
PYD	Pyrin domain
PYHIN	Pyrin and haemopoietic IFN-inducible nuclear
ROS	Reactive oxygen species
SSI	Severe, steroid-insensitive
SLE	Systemic lupus erythematosus
TRAF	TNF receptor-associated factor
TXNIP	Thioredoxin-interacting protein

1. Introduction

The mucosal surface of the lung is continuously exposed to noxious stimuli that can activate host immunity. As a result, the immune system needs to strike a delicate balance between immune-mediated clearance of these stimuli, and avoiding inadvertent self-harm from chronic inflammation. The term 'noxious stimuli' collectively refers to any stimulus that is capable of causing a tissue-damaging event, such as infection and environmental exposures (e.g. allergens, smoke, pollution) (dos Santos et al., 2012; Medzhitov, 2008). The innate immune system is an evolutionarily conserved, non-antigen specific system that is essential for inducing and orchestrating acute inflammatory responses to infection. In the lung, innate immune responses exert these effects through downstream signalling from numerous germline-encoded pattern recognition receptors (PRRs), which are constitutively expressed by airway epithelial cells, alveolar macrophages, antigen presenting cells and neutrophils (dos Santos et al., 2012; Hallstrand et al., 2014; Medzhitov, 2008). Cell surface as well as cytosolic PRRs, such as Toll-Like Receptors (TLR)s and C-type lectins, continuously survey the extracellular milieu and intracellular compartment, respectively, for pathogen-associated molecular patterns (PAMPs).

PAMP-recognising PRRs are split into various families based on their specificity, function and localisation. PRRs can also detect host derived damage-associated molecular patterns (DAMP)s. PRR localisation is important in determining their function, and membrane bound receptors such as Toll-like receptors (TLR)s recognise exogenous extracellular PAMPs or DAMPs. Cytosolic PRRs receptors include the interferon-inducible pyrin and haemopoietic interferon (IFN)-inducible nuclear (PYHIN) family of proteins such as absent in melanoma (AIM)2, which activate type 1 IFN responses, as well as the nucleotide oligomerisation domain (NOD)-like receptors (NLRs) that all recognise PAMPs or DAMPs intracellularly. The recognition of noxious stimuli by these PRRs leads to the induction of innate immune responses.

The NLR family recognise a variety of PAMPs as well as host-derived DAMPs (Becker and O'Neill, 2007; dos Santos et al., 2012; Dowling and O'Neill, 2012; Schroder and Tschopp, 2010). Recent studies have shown that several NLRs, namely NLR protein (NLRP)1, NLRP3 and NLR family caspase recruitment domain (CARD)-containing (NLRC4) can assemble into multi-protein complexes termed inflammasomes that recruit and activate pro-inflammatory caspases, such as Caspase-1 (Casp1). Inflammasome-activated Casp1 in turn cleaves pre-

cursor forms of pro-inflammatory cytokines in the interleukin (IL)-1 family to produce active IL-1β and IL-18, and inactivates IL-33 (Martinon et al., 2002; Martinon et al., 2009; Schroder and Tschopp, 2010; Stutz et al., 2009).

To date, five inflammasomes have been studied in detail; the NLR family members, NLRP1, NLRP3, NLRC4, and retinoic acid-inducible gene 1 (RIG-I), and AIM2. Following assembly and activation, all of these inflammasomes can enzymatically cleave and activate Casp1 in response to DAMP signalling (Dowling and O'Neill, 2012).

Clinical and experimental evidence increasingly and strongly implicates excessive inflammasome activation and production of IL-1β in the pathogenesis of several chronic respiratory diseases, including severe, steroid-insensitive (SSI) asthma and chronic obstructive pulmonary disease (COPD) (Aaron et al., 2001; Baines et al., 2011; Chung, 2001; Hastie et al., 2010; Kim et al., 2014a; Kim et al., 2015; Konno et al., 1996; Simpson et al., 2014a; Wanderer, 2000). Here we review the current literature on the association between inflammasome-mediated host innate immune responses and the development and exacerbation of chronic lung diseases.

1.1 IL-1β activation in the lung

Through respiration the airway lumen is continually exposed to potentially pathogenic micro-organisms (viral, fungal and bacterial) and environmental stimuli (exogenous noxious pollutants such as cigarette, cooking or wood smoke, air pollution, particulate matter, silica, asbestos or elemental metals). The homeostatic regulation of immune responses, and the ability to distinguish self from noxious stimuli, involves complex communication and inter-regulation between the innate and adaptive immune systems. Innate immunity relies on the activation of germline-encoded PRRs to mount immediate responses to infectious and noxious stimuli. Epithelial cells and activated macrophages and neutrophils are the first cells to respond following PRR activation. They play critical roles in mediating the adaptive immune response through the release of cytokines that promote the chemo-attraction of adaptive immune cells. Thus, innate immune responses have significant bearing on the ensuing adaptive immune response and, if unregulated, can result in severe pathological consequences (Hirota and Knight, 2012; Schroder and Tschopp, 2010; Stutz et al., 2009; Yang et al., 2012).

The NLRs are an important family of intracellular PRRs that can be activated by PAMPs such as lipopolysaccharide (LPS) or DAMPs including those of endogenous origin, such as adenosine tri-phosphate (ATP) and uric acid crystals that are released from lysed cells, and/or DAMPs of exogenous origin, including inhaled silica, the adjuvant aluminium hydroxide (alum) and smoke, particulate matter and allergens (Eisenbarth et al., 2008; Hirota et al., 2012; Hornung et al., 2008; Shi et al., 2003). The NLR family is comprised of 22 members that are characterised based on their C-terminal leucine rich repeats (LRR) domains and central NACHT nucleotide-binding domains (NBD). The LRR domains detect NLR-specific ligands and, in the absence of ligand-mediated activation, play an important role in the auto-inhibitory regulation of inflammasomes. NLR family members are also categorised based on their N-terminal domains and the largest group, containing 14 members, possesses distinct N-terminal pyrin domains (PYD) (Dowling and O'Neill, 2012; Martinon et al., 2002; Schroder and Tschopp, 2010; Stutz et al., 2009). To date 14 unique NLRP family members (termed NLRP1-14) have been identified and classified (Zhang et al., 2008). Some NLRs possess a common N-terminal CARD (e.g. NLRC4) and variations of NODs (e.g. NOD1 [NLRC1] and NOD2 [NLRC2]) (Dowling and O'Neill, 2012; Martinon et al., 2002; Schroder and Tschopp, 2010; Stutz et al., 2009). All the members of the NLR family have critical roles in the detection of noxious stimuli in the cytosol and, following ligation, induce the proteolytic cleavage and activation of IL-1β.

IL-1β is a potent, pyrogenic, pro-inflammatory cytokine that plays important roles in initiating acute inflammatory responses to infectious, noxious or cell damage-derived stimuli. In the lung, activation of PRRs such as TLR2 or TLR4 induces the transcription of biologically inactive pro-IL-1β. Pro-IL-1β is primarily produced by macrophages but is also generated in neutrophils, lymphocytes, airway epithelial cells and fibroblasts (Dinarello, 1994). Proteolytic cleavage of pro-IL-1β generates bio-active IL-1β that is then released into the extracellular milieu and exerts its pro-inflammatory effects by binding to the extracellular domain of the ubiquitously expressed IL-1 receptor, type 1 (IL-1R)-1. Ligand-receptor interactions result in the recruitment of the secondary receptor chain IL-1Racp (Sims et al., 1988). This initiates the formation of a receptor complex that recruits the pro-inflammatory adaptor molecules myeloid differentiation primary response gene (MyD)88, IL-1R-associated kinase 1 (IRAK) and tumour necrosis factor (TNF) receptor-associated factor (TRAF)6 (Greenfeder et al., 1995). In turn, this results in the activation of the pro-inflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (Nf-κB). This induces the release of TNF-α and

IL-6, promotes the recruitment and activation of innate immune cells such as neutrophils and macrophages, and further expression of pro-IL-1β (Greenfeder et al., 1995). Since active IL-1β is secreted into the extracellular milieu it can exert pro-inflammatory effects through both autocrine and paracrine mechanisms and, depending on the circulating levels, can lead to severe systemic inflammation (Attur et al., 2000).

Activation and release of IL-1β plays a major role in acute inflammatory responses to respiratory infection and promotes clearance of pathogens. However, prolonged and unmitigated activation and release can lead to chronic systemic inflammation such as that observed in Muckle-Wells syndrome (also known as cryopyrin-associated periodic syndrome [CAPS]) where a gain-of-function mutation in the NLRP3 gene leads to chronic activation of IL-1β causing fever, skin rash, joint pain, and conjunctivitis (Broderick et al., 2015; Coll et al., 2015). Under normal conditions, the activation and release of IL-1β occurs in a tightly controlled two-stage process that requires an initial priming step provided by PAMP-mediated activation of PRRs (e.g. TLR4 ligation following exposure to LPS), and a secondary activating signal provided by DAMPs (e.g. ATP) (dos Santos et al., 2012; Schroder and Tschopp, 2010; Takeda et al., 2003). This causes inflammasome assembly and activation and leads to the proteolytic cleavage and activation of the protease zymogen, pro-Casp1, which was originally described as interleukin-converting enzyme (ICE) (Thornberry et al., 1992). Intriguingly, recent research investigating the therapeutic potential of targeting this inflammasome–Casp1–IL-1β axis has focused on the development of monoclonal antibodies that target IL-1β and/or IL-1R. Anakinra, an IL-1R antagonist, has been trialled in patients with chronic rheumatoid arthritis, however, a study by Listing *et al.*, showed that treatment increased the risk of infection compared to conventional therapy with disease modifying anti-rheumatic drugs (Listing et al., 2005).

Excess activation of the inflammasome can also lead to pyroptosis, which is a form of rapid lytic cell death. Sagulenko *et al.*, identified that dsDNA, another DAMP, increases the activation of the AIM2 inflammasome in a dose-dependent manner, leading to pyroptosis (Sagulenko et al., 2013). It is therefore important that inflammasome responses are tightly regulated in order to prevent the development of chronic inflammatory diseases.

2. The NLRP3 inflammasome in the lung

NLRP3 is the best characterised and most widely implicated inflammasome in inflammatory diseases of the lung. Activation of the NLRP3 inflammasome complex is important for combating respiratory infections, however, excessive activation may contribute to the development of severe disease. It is comprised of an NLRP3 domain that possesses a PYD central NBD and C-terminal LRRs (O'Connor et al., 2003), and an adaptor protein, apoptosis-associated speck-like protein containing a CARD (ASC), which recruits and activates pro-Casp1 (Mariathasan et al., 2004; Masumoto et al., 1999). Active Casp1 subsequently cleaves pro-IL-1β and pro-IL-18 to their active secreted forms (Mariathasan et al., 2004).

In the lung, the NLRP3 inflammasome can be activated by a diverse range of microbial pathogens, including opportunistic bacteria (*Klebsiella pneumoniae*, *Streptococcus pneumoniae* and other spp. and *Haemophilus influenzae* (Mariathasan and Monack, 2007; Rotta Detto Loria et al., 2013; Willingham et al., 2009)), atypical bacteria (*Chlamydia* (Abdul-Sater et al., 2010)) and viruses (including influenza A virus (IAV), vesicular stomatitis virus (VSV) and adenovirus (AdV)) (Table 1) (Muruve et al., 2008; Rajan et al., 2011; Wang et al., 2014). A study by Costa *et al.*, showed that NLRP3-deficient mice are more susceptible to group B *Streptococci* respiratory infection than wild-type mice (Costa et al., 2012). Furthermore, *Streptococcus*-derived pneumolysin has been shown to induce NLRP3 inflammasome-mediated responses and downstream IL-17A and IFN-γ responses to confer protection against infection (McNeela et al., 2010). These studies suggest that the NLRP3 inflammasome plays a critical role in the clearance of respiratory infections. Furthermore, a recent study by Tate *et al.*, examined the temporal effect of NLRP3 inflammasome inhibition using the novel, highly specific NLRP3 inflammasome inhibitor, MCC950, in IAV infected mice. They showed that inhibition of NLRP3 in the early phase of infection led to hyper-susceptibility to lethality, whereas inhibition of NLRP3 at the peak of infection significantly protected mice from severe disease (Tate et al., 2016). These data highlight that NLRP3 activation may be beneficial in acute phase inflammation in IAV infection, but may be detrimental in more chronic inflammatory disease.

Inhaled environmental stimuli (such as smoke, asbestos, silica, metal alloys and particulate matter and air pollution), are also capable of activating the NLRP3 inflammasome (Hirota et al., 2015; Hirota et al., 2012; Kim et al., 2015), and several studies have linked exposure to airway pollutants with the development of chronic airway diseases. A recent study by Hirota *et al.,* showed that particulate matter derived from airway pollutants

collected from developed areas can activate the NLRP3 inflammasome *in vitro* (Hirota et al., 2012). This study showed that particulate matter up to 10μM in diameter (PM_{10}) activated the NLRP3 inflammasome in epithelial cells. The same team also reported that this could occur through the induction of innate immune responses (Hirota et al., 2015). Other studies have shown that endogenous DAMPs, such as extracellular ATP, can also activate the NLRP3 inflammasome (Mariathasan et al., 2006; Zhou et al., 2011). The presence of DAMPs in the airway is a strong indication of prior pathogenic insult resulting in cell lysis and/or chronic inflammation. Aeffner *et al*., and others, recently showed that IAV, which infects the respiratory epithelium and is a known inflammasome activator, causes the release of ATP into the airway lumen, potentially highlighting the mechanism by which this virus activates the NLRP3 inflammasome (Aeffner et al., 2011; Allen et al., 2009; Muruve et al., 2008). Several studies have shown that ATP and K^+ efflux activates the NLRP3 inflammasome through the P2Xpurinoceptor 7 ($P2X_7R$), which is a cation-permeable ligand-gated ion channel. ATP-mediated activation results in $P2X_7R$ pore formation of the pannexin-1 hemi-channel that then facilitates passive migration of extracellular PAMPs and DAMPs into the cytosol where they can induce the assembly and activation of the NLRP3 inflammasome (Chaudhuri et al., 2010; Kanneganti et al., 2007).

Recent studies have also highlighted a potential role for oxidative stress, induced by the production of reactive oxygen species (ROS), in promoting the assembly and activation of the NLRP3 inflammasome. Zhou *et al*., showed that mitochondria-derived ROS (mtROS) induces the activation of the NLRP3 inflammasome in the endoplasmic reticulum (Zhou et al., 2011). They also showed that mtROS contributes to the assembly of the NLRP3 inflammasome *via* the thioredoxin and thioredoxin-interacting (TXNIP) complex. ROS interact with the TXNIP complex and cause it to dissociate from thioredoxin. This allows dissociated TXNIP to bind with NLRP3 that then recruits ASC and Casp1 to form the inflammasome complex (Zhou et al., 2010). It remains possible that one mechanism by which air pollution activates the inflammasome in the lung is via the oxidative capacity of the components of particulate matter (e.g. elemental metals and poly aromatic hydrocarbons). Collectively, these data suggest that exogenously-induced oxidative stress and ROS and can also induce the assembly of the NLRP3 inflammasome.

3. The AIM2 inflammasome and the lung

The AIM2 inflammasome belongs to the PYHIN family of proteins and is comprised of an N-terminal PYD domain and one or two copies of a 200 amino acid repeat haemopoietic IFN-inducible nuclear proteins (HIN) domain at its C-terminus (Dowling and O'Neill, 2012). The HIN motifs detect DNA of microbial origin and induce type 1 IFN responses and the expression of pro-IL-1β (Dowling and O'Neill, 2012).

Several studies have demonstrated that exposure to microbial or mammalian dsDNA leads to Casp1 activation in an AIM2-dependent manner (Fernandes-Alnemri et al., 2010; Hornung et al., 2009; Muruve et al., 2008; Roberts et al., 2009; Schroder et al., 2009) (Table 1). Cytosolic dsDNA is detected by the HIN-200 domain of the AIM2 inflammasome that then, through its PYHIN domain, recruits ASC and pro-Casp1. This results in the proteolytic cleavage and activation of Casp1 and IL-1β (Hornung et al., 2009; Muruve et al., 2008; Roberts et al., 2009; Schroder et al., 2009). Fernades-Almenri *et al.*, recently showed that AIM2-deficient mice exhibit increased mortality following *Francisella tularensis* respiratory infection compared to wild-type controls, which indicated that AIM2 inflammasome-mediated responses are required for effective clearance of infection. Since *F. tularensis* replicates in the cytosol of infected macrophages the authors concluded that pathogen-derived DNA is detected by the AIM2 inflammasome during the process of its escape (Fernandes-Alnemri et al., 2010). Collectively, these data suggest that AIM2 inflammasome-mediated responses are important in immune responses to microbial pathogens that produce dsDNA as part of their replication strategy, and highlight the need for its further exploration. Given that increased IL-1β is a feature of many chronic respiratory conditions that are also associated with respiratory infections, the further examination of the AIM2 inflammasome in these disease contexts is warranted to elucidate whether it plays a pathogenic role in driving disease.

4. Other inflammasomes in the lung

Our understanding of the roles of inflammasomes in the development of chronic lung diseases is in its infancy and research efforts to date have focussed primarily on characterising the roles of the NLRP3 and AIM2 inflammasomes. As a result, the functional roles of other inflammasomes in immune responses to respiratory infections and in the development and/or exacerbation of chronic lung diseases are yet to be resolved. The NLRP1 inflammasome was the first to be described and murine studies showed that it is directly activated by anthrax lethal toxin, which is produced by *Bacillus anthracis*, and is able to cleave

Casp1 in the absence of ASC (Boyden and Dietrich, 2006; Martinon et al., 2002) (Table 1). Activation of NLRP1 by anthrax lethal toxin is required in mice for the maturation and release of IL-1β. This results in rapid cell death and is the causative agent of systemic anthrax toxicity (Banks et al., 2006). The development of therapeutics specifically targeting this inflammasome may be crucial in preventing the lethal toxicity of anthrax.

The NLRC4 inflammasome, also known as CARD12, has been mostly associated with the induction of pyroptosis (Dowling and O'Neill, 2012). However, in the lung it has been shown to be activated by cytosolic flagellin and the basal rod component of the type 3 secretion system found in a range of bacteria such as *Salmonella typhimurium*, *Shigella flexneri* and *Legionella pneumophila* (Sutterwala et al., 2007).

5. Regulation and activation of inflammasomes

The precise mechanisms that lead to NLRP3 inflammasome activation remain poorly defined. However, as mentioned previously this process consists of two distinct events, the first of which involves the expression and assembly of inflammasome components (Schroder and Tschopp, 2010; Stutz et al., 2009). Increasing evidence shows that PAMP-induced TLR ligation and increased levels of TNF-α are important in the initiation of the expression and assembly of NLRP3 inflammasome components as well as inducing pro-IL-1β expression through the activation of NF-κB (He et al., 2013; Hiscott et al., 1993; Martinon et al., 2002; Martinon et al., 2009; Schindler et al., 1990a; Schindler et al., 1990b)) (Table 1). The mechanism of NLRP3 activation involves its translocation to mitochondria and the induction of ROS is these organelles, K^+-efflux and cathepsin release. However, this is an intense area of investigation and recent studies have found new roles for NEK7 an NLRP3 binding proteins and NIMA-related kinase (He et al., 2016). Interestingly, a study by Ikejima *et al.*, demonstrated that rabbits injected with recombinant IL-1β produced endogenous IL-1β, indicating that IL-1β responses can self-perpetuate (Ikejima et al., 1990). The authors also showed that human peripheral blood mononuclear cells produced TNF-α in response to treatment with IL-1β. Thus, in NLRP3 inflammasome-mediated responses, the induction of pro-IL-1β expression constitutes a form of 'priming' that then requires a secondary round of activation of assembled inflammasome components to drive the proteolytic pathway that leads to the activation and release of IL-1β (Hornung and Latz, 2010). This represents an important mechanism of immune regulation given that unmitigated activation and release of

IL-1β and other IL-1 family members can have destructive consequences (Dinarello, 2009). Many studies mimic this priming effect by pre-treating cultured cells *in vitro* with TLR agonists and/or pro-inflammatory cytokines, such as LPS and TNF-α (Martinon et al., 2002; Schroder and Tschopp, 2010; Stutz et al., 2009). Recent research has focused on characterising the role of the NLRP3 inflammasome in disease development.

There is, however, a paucity of information relating to the role of other inflammasomes in disease and it is important to interrogate these further. *Guarda et al.*, showed that type 1 IFNs are capable of suppressing NLRP1 and NLRP3 inflammasome activity, which resulted in diminished Casp1-dependent IL-1β maturation, suggesting that both of these inflammasomes are equally important in activating IL-1β (Guarda et al., 2011). Several studies have also examined the role of autophagy, a normal cellular process that deals with the controlled degradation of defective organelles in the cell, and inflammasome activation. Saitoh *et al.*, showed that cells deficient in the autophagy-specific protein Atg16L1 had elevated endotoxin-induced IL-1β production (Saitoh et al., 2008). Further characterisation of the regulation of the other inflammasomes, such as the AIM2 inflammasome, is essential to increase our understanding of their roles in the pathogenesis of chronic lung diseases. Our understanding of the activation and regulation of inflammasomes is rapidly evolving and therapeutic intervention thorough numerous avenues could be possible once their biology has been elucidated.

6. Inflammasomes in lung diseases

Whilst inflammasomes play crucial roles in the clearance of pathogens, aberrant inflammasome-mediated IL-1β responses are strongly implicated in the pathogenesis of a wide range of human diseases such as metabolic dysregulation, systemic autoimmune diseases such as systemic lupus erethematosus (SLE), inflammatory diseases of the skin and joints such as rheumatoid arthritis and gout, and chronic inflammatory lung diseases such as asthma and COPD (Baechler et al., 2003; Martinon et al., 2006; Simpson et al., 2014a; Strowig et al., 2012; Yang et al., 2015). Nevertheless, the roles of inflammasomes in the development and exacerbation of lung disease are not well understood and further characterisation of the mechanisms of activation of individual inflammasomes will provide greater insight into their roles. Here we review the existing literature that links aberrant inflammasome activation with the development, exacerbation and progression of chronic airway disease.

6.1 Inflammasomes in community acquired pneumonia

Community acquired pneumonia (CAP) refers to any form of pneumonia that is contracted outside a hospital environment. It is a serious life-threatening condition and is the leading cause of infectious disease mortality in many countries (Mizgerd and Skerrett, 2008). Pneumonia is primarily caused by respiratory bacterial infection and results from fluid filling the alveolar spaces. Infection with *S. pnemoniae* is by far the most common cause of CAP (File, 2004). A recent study by Witzenrath *et al.*, examined a *S. pnemoniae* infection murine model and determined that the streptococcal exotoxin pneumolysin was essential for NLRP3 inflammasome activation (Witzenrath et al., 2011). Pneumolysin disrupts the plasma membrane in cells leading to K^+ efflux, which could lead to the activation of the NLRP3 inflammasome (Schroder and Tschopp, 2010). As described previously, mice deficient in NLRP3 had increased mortality following *S. pnemoniae* infection, therefore the activation of the NLRP3 inflammasome by this bacterium is likely to be essential for the clearance of infection and may be an important factor in CAP (McNeela et al., 2010; Witzenrath et al., 2011).

6.2 Inflammasomes in asthma

Asthma is a chronic inflammatory disease of the airways that affects approximately 10% of the population in Westernised countries and 300 million sufferers worldwide (Akinbami et al., 2012; Bateman et al., 2008; Hansbro et al., 2008; Hansbro et al., 2011; Wang et al., 2010). The majority of asthmatic patients exhibit a mild to moderate form of the disease that is characterised by T-helper lymphocyte type 2-mediated, eosinophil-dominated immune responses (Hansbro et al., 2013; Shahidi and FitzGerald, 2010). Most commonly, asthma is characterised as an allergic disorder and >50% of asthmatics have some form of atopy (Arbes et al., 2007). Increasing evidence now shows that asthma is a heterogeneous disease that is associated with a range of phenotypes and endotypes (Hansbro et al., 2011; Hansbro et al., 2013). The majority of asthmatics can control their symptoms through the use of inhaled short-acting β-agonists and inhaled corticosteroids. However, some asthmatics have persistent airflow obstruction, more frequent exacerbations and remain symptomatic despite high doses of these drugs (Bell and Busse, 2013). These asthmatics are more likely to have severe asthma that is insensitive to steroid therapy and are more commonly associated with

non-eosinophilic endotypes of disease (Hansbro et al., 2011; Wang et al., 2010; Wood et al., 2010).

Several studies have highlighted potential roles for the NLRP3 inflammasome in the development of allergic asthma. The inflammasome activator ATP has been shown to be elevated in the airways of asthmatics compared to non-asthmatics, and is further increased following challenge with allergen (Idzko et al., 2007; Muller et al., 2011). Experimental studies using murine models of allergic airways disease (AAD) are being used to elucidate the role of the inflammasome in the development of allergic asthma. These murine models typically involve systemic sensitisation to model protein allergens (e.g. ovalbumin [Ova]) in the presence of the Th2-inducing adjuvant alum, which is a known activator of the NLRP3 inflammasome (Hornung et al., 2008). A study by Eisenbarth *et al.*, used NLRP3-deficient mice to demonstrate that this inflammasome is required for adjuvanticity of alum in allergic antibody responses to antigen (Eisenbarth et al., 2008). Another study demonstrated the importance of NLRP3 in allergic airway inflammation using an adjuvant (alum)-free Ova model. Besnard *et al.*, used mice deficient in NLRP3, IL-1 receptor (IL-1R)1, IL-1β or IL-1α to demonstrate critical roles for NLRP3-mediated IL-1β responses in Ova-induced allergic airway inflammation. They also reported that each of these factor-deficient mice exhibited marked decreases in the production of Ova-induced, Th2-associated cytokines (Besnard et al., 2011). Primiano *et al*., further implicated the NLRP3 inflammasome in driving AAD, by showing that therapy with the NLRP3 specific inhibitor, MCC950 reversed neutrophilic inflammation in AAD (Primiano et al., 2016). In contrast, a different study by Kool *et al.*, demonstrated that uric acid potently induces Th2 cell immunity in an NLRP3-independent, PI3Kδ-dependent manner (Kool et al., 2011). These findings are supported by a study by Allen *et al*., which showed that WT and NLRP3-deficient mice exhibited no differences in the key features of acute or chronic Ova-induced AAD including eosinophilic airway inflammation, mucus hypersecretion and airways hyperresponsiveness (AHR) (Allen et al., 2012). Similar observations have recently been found with a combined particulate matter/Ova-induced model (Hirota et al., 2015). Taken together, the role of the NLRP3 inflammasome in the pathogenesis of allergic asthma remains to be elucidated.

It is likely that additional challenges, such as particulate matter or infections are needed to drive inflammasome activation and more severe disease (Hansbro et al., 2011; Hansbro et al., 2013). Recent clinical studies have identified that moderate to severe asthmatics have

increased Th1 and/or Th17 type, monocyte- or neutrophil-dominated immune responses in their airway secretions (Baines et al., 2011; Simpson et al., 2007; Simpson et al., 2014a). This has led to an increased focus on the development of more targeted therapies, particularly as monocyte/neutrophil dominated asthma is more likely to be resistant to mainstay anti-inflammatory corticosteroid therapy (Hansbro et al., 2011; Hansbro et al., 2013).

Recently, there has been an intense focus on elucidating the mechanisms of SSI asthma and an increasing number of clinical and experimental studies strongly, and specifically, implicate NLRP3 inflammasome activation and/or excess IL-1β production in the pathogenesis of this disease (Baines et al., 2011; Besnard et al., 2012; Essilfie et al., 2011; Hastie et al., 2010; Kim et al., 2014a; Kim et al.; Kim et al., 2016; Kim et al., 2014b; Konno et al., 1996; Simpson et al., 2014a; Starkey et al., 2013a; Starkey et al., 2014). Baines *et al.*, used gene expression profiling of induced sputum to identify distinct gene signatures in different asthmatic inflammatory endotypes. The expression of genes associated with the IL-1β signalling pathway, such as IL-1β, IRAK2, IRAK3, IL-1R2, were significantly increased in the sputum of neutrophilic asthmatics, which are more likely to be associated with severe asthma (Baines et al., 2011). More recently, Simpson *et al.*, showed that neutrophilic asthmatics have increased levels of NLRP3, Casp1 and IL-1β expression in the airways, and macrophages and neutrophils were the dominant cellular sources of NLRP3 and Casp1 in this cohort (Simpson et al., 2014a). These asthmatics also had elevated expression of TLR2, TLR4, and IL-8 and increased levels of LPS suggesting that innate immune activation in asthma may drive aberrant inflammasome activation (Simpson et al., 2014a).

Substantial clinical evidence links bacterial respiratory infections with SSI asthma. *Chlamydia pneumoniae* is an obligate intracellular bacterial pathogen that is associated with SSI asthma. *Chlamydia*-associated asthma is less responsive to steroid treatment, and acute antibody responses to *Chlamydia* strongly predicted the presence of neutrophils in sputum in these patients (Cho et al., 2005; Patel et al., 2010; Wark et al., 2002). Airway neutrophilia also positively predicted the presence of *Chlamydia* infection in SSI asthma (Patel et al., 2010). Studies of *Chlamydia* are difficult due to the complexity of detection and the need to sample the lower respiratory tract, where it prefers to grow. This may explain some negative studies. *H. influenzae* is a Gram-negative bacteria that is the most commonly isolated bacterium from the airways of SSI asthmatics (Simpson et al., 2007; Wood et al., 2010) compared to mild to moderate asthmatics. We have developed and used experimental models of SSIAAD to assess

the effect of *Chlamydia* and *H. influenzae* respiratory infection on the development of SSIAAD, to better understand their potential role in SSI asthma. We have shown that both infections induce neutrophilia, and Th1 and/or Th17 responses and SSIAAD (Essilfie et al., 2015; Essilfie et al., 2012; Essilfie et al., 2011; Horvat et al., 2010a; Horvat et al., 2010b; Kim et al.). Significantly, both *Chlamydia* and *Haemophilus* respiratory infections induce the release of active IL-1β in an NLRP3 inflammasome-dependent, Casp1-mediated manner (Essilfie et al., 2015; Essilfie et al., 2011; He et al., 2010; Horvat et al., 2010b; Kim et al.; Rotta Detto Loria et al., 2013). Collectively, these data suggest that infection-induced, inflammasome-mediated IL-1β responses may play a key role in the development of SSI asthma.

Emerging evidence shows that systemic inflammation (determined through increased serum levels of IL-6 and TNF-α) as a result of high-fat diet and/or obesity is associated with severe asthma. Obese asthmatics are more likely to have severe disease and SSI asthma (Forno et al., 2011; Gibeon et al., 2013; Scott et al., 2016). A recent study by Scott *et al.*, showed that obese asthmatics had increased levels of systemic IL-6 and C-reactive protein, and that these factors were positive predictors of neutrophilia in female obese asthmatics, compared to non-obese female asthmatics (Scott et al., 2016). Importantly, IL-1β levels are significantly increased in the plasma of overweight (BMI of 25-29.9) and obese (BMI ≥30) women, compared to females in the normal weight range (Um et al., 2004), and obese asthmatics, particularly obese females, are more likely to have severe asthma (Forno et al., 2011; Gibeon et al., 2013; Lefaudeux et al., 2016; Scott et al., 2016). Recently, Kim *et al.*, assessed the mechanisms of obesity-induced AHR in an experimental murine model of high fat diet-induced obesity (Kim et al., 2014b). They showed that obese mice had increased AHR in the absence of allergic sensitisation compared to non-obese control mice. They also demonstrated that AHR in this model was driven by aberrant NLRP3 inflammasome-dependent responses in the adipose tissue, which contributed to an induction of innate lymphoid cells and increased IL-17 responses in the lung that led to spontaneous AHR (Kim et al., 2014b). These experimental data highlight a key mechanism by which this inflammasome may contribute to AHR in severe asthma, in the absence of allergic disease. Everaere *et al.*, recently extended these findings by showing that obese mice with house dust mite (HDM)-induced AAD had worsened AHR compared to non-obese, HDM allergen-challenged mice (Everaere et al., 2016). Nevertheless, the roles of inflammasomes in obese AAD are yet to be fully defined.

Further investigations and specific targeting of inflammasomes in the airways of allergen challenged mice, particularly in the context of SSI asthma, is required to improve the understanding of how NLRP3 inflammasomes contribute to the development of severe asthma.

6.3 Inflammasomes in COPD

COPD is a progressive, obstructive disease of the lungs that encompasses several conditions, including chronic bronchitis and emphysema, it is now the third leading cause of death worldwide and its prevalence is increasing (Chapman et al., 2006; Fricker et al., 2014; Keely et al., 2012; Lozano et al.). Significantly, several clinical studies have shown that IL-1β levels are elevated in the lungs of patients with COPD and that these levels increase further during exacerbations of disease (Aaron et al., 2001; Chung, 2001). Clinical studies have also shown that cigarette smoke induces the release of IL-1β in the lung (Kuschner et al., 1996; Pauwels et al., 2011). This is supported by data from mouse models that have shown increased lung IL-1β expression during cigarette smoke-induced experimental COPD (Beckett et al., 2013). Pauwels *et al.,* interrogated a murine model of cigarette smoke-induced pulmonary inflammation and showed that airway inflammation was significantly attenuated through the neutralisation of IL-1β (Pauwels et al., 2011). Cigarette smoke contains over 4,000 toxins, including LPS, which are capable of triggering innate immune responses through PRR activation. These data therefore suggest that aberrant inflammasome activation may play an important role in the pathogenesis of COPD.

Pouwels *et al.* showed that cigarette smoke exposure induces necroptosis, a form of programmed necrosis, of airway epithelial BEAS-2B cells *in vitro* that results in the release of endogenous DAMPs (Pouwels et al., 2016). Indeed, chromatin-binding high motility group box 1 protein (HMGB1) is a DAMP that can occur at high levels in the airways. HMGB1 levels are elevated in the sputum and bronchoalveolar lavage fluid (BALF) of patients with COPD (Ferhani et al., 2010; Hou et al., 2011). Significantly, HMGB1 has been shown to activate the NLRP3 inflammasome in a TLR4-dependent manner in a model of haemorrhagic shock syndrome (Xiang et al., 2011) suggesting that cigarette smoke-induced, HMGB1-mediated activation of the inflammasome may play an important role in the pathogenesis of COPD.

Further mechanistic studies by Franklin *et al*., showed that inflammasome responses may also be mediated by the accumulation of ASC specks in the lungs of patients with COPD

(Franklin et al., 2014). ASC is an essential component of the NLRP3 and AIM2 inflammasomes and is essential for Casp1 recruitment. Chronic activation of the inflammasome that results in pyroptosis leads to the release of ASC specks, which have prion-like activity. They accumulate in extracellular spaces and retain their ability to mature IL-1β in the extracellular environment (Franklin et al., 2014). These ASC specks are then readily phagocytosed by macrophages and induce the production of IL-1β in these cells. Most importantly, ASC specks are upregulated in BALF from COPD patients and murine models of cigarette smoke-induced COPD (Franklin et al., 2014). These studies indicate that ASC specks may drive aberrant inflammasome responses and play an important role in the pathogenesis of COPD. In contrast, Di Stefano *et al.*, found no correlation between NLRP3, Casp1 and IL-1β responses in a cohort of stable COPD patients compared to healthy smokers in a randomised control trial although they proposed that they would be relevant in exacerbations (Di Stefano et al., 2014). Collectively, these data highlight that the inflammasome may not be playing a role in stable COPD, however, these studies do not assess the precise role of the inflammasome during the development, progression or exacerbation of disease, which may be the critical issue. Thus, the role of the NLRP3 inflammasome in COPD is complex and warrants further investigation to delineate its roles. The use of mouse models that accurately replicate the major hallmark features of cigarette smoke-induced COPD and exacerbations in a reasonable time frame and in parallel with complementary human studies will be valuable in elucidating the mechanisms involved, identifying new therapeutic targets and testing new therapies in this and other respiratory diseases (Beckett et al., 2013; Conickx et al., 2016; Franklin et al., 2014; Fricker et al., 2014; Hansbro et al., 2014; Haw et al., 2016; Hsu et al., 2015; Jarnicki et al., 2016; Liu et al.; Simpson et al., 2014b; Starkey et al., 2013b; Tang et al., 2016; Tay et al., 2015) (Franklin et al., 2014). Although not discussed here the role of the lung and gut microbiomes may also play significant roles in inflammasome activity in these diseases, which could be elucidated using similar strategies (Budden et al., 2016; Chambers et al., 2014; Ormerod et al., 2016).

6.4 Inflammasomes in other chronic airway diseases

6.4.1 Pulmonary fibrosis

Pulmonary fibrosis refers to a range of lung disorders characterised by irreversible destruction and remodeling of lung architecture that occurs as a result of excess deposition of collagen and extracellular matrix proteins. This results in scarring of the airways, which

leads to the significant breathing difficulties that are characteristic of the disease (dos Santos et al., 2012). The role of the inflammasome in the pathogenesis of pulmonary fibrosis is unclear, however, it is known that fibrosis-inducing irritants injure the lung epithelium (e.g. silica, asbestos, cigarette smoke and bleomycin), and these are also known to directly activate the NLRP3 inflammasome (Dostert et al., 2008; Gasse et al., 2007; Hornung et al., 2008). IL-1β secretion also promotes the production of TGF-β1, a potent pro-fibrotic cytokine (Liu, 2008), and promotes neutrophil chemoattraction, which may contribute to epithelial damage. However, the precise mechanisms of inflammasome-mediated pathogenesis in pulmonary fibrosis are yet to be elucidated.

6.4.2 Cystic fibrosis

Cystic fibrosis (CF) is a debilitating lung disease that is caused by a genetic mutation in the gene encoding the CF transmembrane conductance regulator (CFTR) (Caplen et al., 1995). CFTR is a chloride ion transport channel that is defective in patients with CF. This results in salt imbalances and excess accumulation of mucus in the lung that significantly increases the risk of infections. The role of the inflammasome is not well understood in this disease, however, the CFTR gene has been implicated as an important regulator of IL-1β release (Reiniger et al., 2007). A recent study by Iannitti *et al.*, used *in vitro* and *in vivo* models to assess the importance of the NLRP3 and NLRC4 inflammasomes in the clearance of infections in CF (Iannitti et al., 2016). However, they show that the deleterious effects of inflammation caused by inflammasome activation are caused by NLRP3, which correlates with defective NLCR4-IL-1R1 responses (Iannitti et al., 2016). These data distinguish important differences in the roles of the NLRP3 and NLRC4 inflammasome in CF. They highlight that the activation of different inflammasomes can contribute to inflammation in different ways, highlighting some uniqueness in inflammasome-specific immune responses.

6.5.2 Acute respiratory distress syndrome

Acute respiratory distress syndrome (ARDS) refers to acute lung injury (ALI) in its most severe form. ARDS can occur as a secondary complication of a range of different disorders such as sepsis, ischemia, and trauma. It is a severe disease and these patients have a survival rate of ~25% (Spragg et al., 2010). IL-18 is significantly increased in the blood of ARDS patients, highlighting a potential role of inflammasome activation in this disease. Murine models of

LPS-induced ALI, which model ARDS, have determined that extracellular ATP is an important neutrophil chemoattractant in the late phase of injury, and specifically targeting this factor could limit damage induced by these cells. It is likely that the events observed in this model are driven by aberrant NLRP3 inflammasome activation (Shah et al., 2014), where extracellular ATP activates the NLRP3 inflammasome via the $P2X_7R$. Significantly, Wang *et al.*, recently showed that pharmacological inhibition of $P2X_7R$ suppresses the production IL-1β and Casp1, and ameliorates the key features of experimental ALI (Wang et al., 2015). These data highlight the importance of the NLRP3 inflammasome in this form of chronic lung disease and further support therapeutically targeting it.

7. Conclusions

Inflammasomes play a critical role in early innate immune responses particularly duirng the resolution of infections in the lung. However, excessive inflammasome activation has been associated with several major chronic inflammatory conditions. Whilst aberrant NLRP3 inflammasome responses are associated with SSI forms of asthma, COPD studies have not clarified the causal nature of the relationship, let alone interrogated the potential for therapeutic targeting of the NLRP3 inflammasome. Much of this is due to the lack of understanding of upstream drivers of inflammasome assembly and activation, and use of representative animal and experimental models that recapitulate the hallmark features of disease. These are crucial in understanding the molecular mechanisms of action of the NLRP3 inflammasome in chronic airway disease and developing and testing new therapies. Furthermore, it will be important to assess the contribution of other inflammasomes, such as the AIM2 inflammasome, in the pathogenesis and exacerbation of these diseases. This is a nascent field of enquiry that requires further investigation in order to elucidate the relative contributions of the different inflammasomes and other IL-1β-activating mechanisms in the pathogenesis of disease. Current therapeutic strategies that globally target IL-1β, such as the Canakinumab, Anakinra and Rilanocept biologics, rather than target excess production it in a pathway-specific way, may predispose to increased infection and are only delivered systemically, rather than tissue-specifically, which will increase off-target effects. For the treatment of excess IL-1β responses, the development of inflammasome-mediated, site-specific therapeutics may be more beneficial in suppressing inflammasome-associated disease, whilst not predisposing to infection. To achieve this we need a greater understanding

of the molecular mechanisms driving inflammasome-associated disease, which may be informed through the development and use of representative *in vivo* models and complementary human studies.

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Table 1. Exogenous activators of the inflammasome in the lung

Inflammasome	Activated by	Lung disease	Reference
NLRP1	Anthrax lethal toxin	Anthrax causing pneumoniae and severe respiratory collapse	(Banks et al., 2006)
NLRP3	*S. pneumoniae* *K. pneumoniae* *Chlamydia* spp. *H. influenzae* Influenza A virus Asbestos Silica Particulate matter (PM_{10}) Cigarette smoke Lipopolysaccharide (LPS)	Resolution of Community acquired pneumonia (CAP) Severe, steroid-insensitive (SSI) asthma Resolution of influenza Asbestosis Silicosis Asthma Chronic obstructive pulmonary disorder (COPD) Acute lung injury (ALI)	(Mariathasan and Monack, 2007) (Essilfie et al., 2015; He et al., 2010; Rotta Detto Loria et al., 2013) (Thomas et al., 2009) (Dostert et al., 2008) (Hornung et al., 2008) (Hirota et al., 2015; Hirota et al., 2012) (Yang et al., 2015) (Wang et al., 2015)
AIM2	*F. tularensis*	Resolution of CAP	(Fernandes-Alnemri et al., 2010)

Fig 1. Proposed model for the triggering of inflammasomes that leads to the development of severe, steroid-insensitive (SSI) asthma and potentially other respiratory diseases. Infection and/or exposure to infections, allergens, cigarette smoke, airway pollutants or other noxious stimuli in the asthmatic airway triggers the assembly and activation of the NLRP3 inflammasome in the lung. This results in the cleavage of pro-IL-1β into active IL-1β leading to increases in Th1- and/or Th17-associated responses and neutrophils in the airways. This contributes to the development of inflammation, mucus hypersecretion and airways hyperresponsiveness (AHR), which are resistant to steroid therapy. In the

obese asthmatic lung, obese adipose tissue contributes to the activation of the NLRP3 inflammasome systemically. This results in an increase in systemic inflammation which activates the NLRP3 inflammasome in the lung, resulting in steroid-insensitive asthma.