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REVIEW ARTICLE

Inflammasomes: a novel therapeutic target in pulmonary hypertension?

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Pulmonary hypertension (PH) is a rare, progressive pulmonary vasculopathy characterized by increased mean pulmonary arterial pressure, pulmonary vascular remodelling and right ventricular failure. Current treatments are not curative, and new therapeutic strategies are urgently required. Clinical and preclinical evidence has established that inflammation plays a key role in PH pathogenesis, and recently, inflammasomes have been suggested to be central to this process. Inflammasomes are important regulators of inflammation, releasing the pro-inflammatory cytokines IL-1β and IL-18 in response to exogenous pathogen- and endogenous damage-associated molecular patterns. These cytokines are elevated in PH patients, but whether this is a consequence of inflammasome activation remains to be determined. This review will briefly summarize current PH therapies and their pitfalls, introduce inflammasomes and the mechanisms by which they promote inflammation and, finally, highlight the preclinical and clinical evidence for the potential involvement of inflammasomes in PH pathobiology and how they may be targeted therapeutically.

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Abbreviations

4-PBA, 4-phenyl butyric acid; 6MWD, 6-min walk distance; AIM, absent in melanoma; ASC, apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD); BALF, bronchoalveolar lavage fluid; CARD, caspase activation and recruitment domain; COPD, chronic obstructive pulmonary disease; DAMP, damage-associated molecular pattern; EC, endothelial cell; ERA, endothelin receptor antagonist; ET-1, endothelin-1; IFI, interferon (IFN)-γ inducible protein; IPF, idiopathic pulmonary fibrosis; LRR, leucine rich repeat; mPAP, mean pulmonary arterial pressure; NACHT/NOD, nucleotide-binding and oligomerization domain; NLR, NOD-like receptor; NLRC, NOD-like receptor family, CARD-containing protein; NLRP, NOD-like receptor family, pyrin domain (PYD)-containing protein; PAMP, pathogen associated molecular pattern; PASMC, pulmonary artery smooth muscle cell; PDTC, pyrrolidine dithiocarbamate; PH, pulmonary hypertension; PPHN, persistent pulmonary hypertension of the new-born; PRR, pattern recognition receptor; PSGL, P-selectin glycoprotein ligand; PVR, pulmonary vascular resistance; PYD, pyrin domain; RV, right ventricular; RVSP, right ventricular systolic pressure; TLR, toll-like receptor; Treg, regulatory T-cell

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Introduction

Pulmonary hypertension (PH), defined as increased mean pulmonary arterial pressure (mPAP) ≥25 mmHg at rest by right heart catheterization (Galie *et al.*, 2015), is a rare but incurable pulmonary vasculopathy, primarily affecting the distal pulmonary arteries. Associated morbidity and mortality are unacceptably high, with 5-year survival standing at almost 50% (Humbert *et al.*, 2010; Benza *et al.*, 2012; Ling *et al.*, 2012; Hoeper *et al.*, 2017). Specifically, PH is characterized by muscularization of the pulmonary vascular wall, resulting in a progressive increase in resistance and right heart pressure-overload, ultimately leading to right heart failure and death (Galie *et al.*, 2015; Ryan *et al.*, 2015). Underpinning the increased mPAP is an interplay between an aberrant vascular endothelial homeostasis and the remodelling that occurs in the vasculature and heart (Lai *et al.*, 2014). Because right ventricular (RV) function is a major determinant of prognosis (Fine *et al.*, 2013), the poor outcomes associated with PH are likely to be due to the fact that currently approved drugs are primarily pulmonary vasodilators with limited ability to address the underlying pathological remodelling, although they almost certainly offer some RV support (Wilkins *et al.*, 2005; Seferian and Simonneau, 2013). Therefore, to advance therapy, new treatments which target remodelling in the pulmonary vasculature and/or RV are urgently required.

An increasing body of evidence suggests that the immune system plays a central role in the pathogenesis of PH, particularly in terms of the pulmonary vascular remodelling (Rabinovitch *et al.*, 2014). Although this phenomenon was recognized more than two decades ago (Tuder *et al.*, 1994; Voelkel *et al.*, 1994; Humbert *et al.*, 1995), advancement in our understanding of the inflammatory pathways involved in PH has been slow. Recently, inflammasomes have emerged as key contributors to disease progression, with supportive evidence from both preclinical and clinical studies (Soon *et al.*, 2010; Ross *et al.*, 2012; Villegas *et al.*, 2013; Groth *et al.*, 2014; Cero *et al.*, 2015; Tang *et al.*, 2015; Morisawa *et al.*, 2016; Parpaleix *et al.*, 2016; Yin *et al.*, 2017). Inflammasomes are key components of macrophage-mediated immunity and important regulators of inflammation (Martinon *et al.*, 2002), releasing the pro-inflammatory cytokines **IL-1β** and **IL-18** in response to pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) (Krishnan *et al.*, 2014). While this line of research is undoubtedly in its infancy, further elucidation of these inflammatory pathways and strategies aimed at alleviating their pathogenicity may represent a novel approach to treat PH and address the current deficit. This review will briefly summarize current PH therapies and their pitfalls, introduce inflammasomes and the mechanisms by which they promote inflammation and highlight both the preclinical and clinical evidence for the potential involvement of inflammasomes in PH pathobiology and how they may be targeted therapeutically.

Classification and current therapies for pulmonary hypertension

Pulmonary hypertension encompasses a large and aetiologically distinct group of pathologies, outlined in the most recent 'updated clinical classification of PH' (Simonneau *et al.*, 2013). In addition to aetiological dissimilarity, four different classes of PH functional severity exist; ranging from mild, class I PH, which does not affect daily activity, to the most severe, class IV PH, where patients are unable to partake in any activity without symptoms and are likely to experience discomfort even at rest (Taichman *et al.*, 2009).

Haemodynamic assessment by right heart catheterization is a prerequisite to confirm PH diagnosis (Rich and Rich, 2014). Other measures such as the 6-min walk distance (6MWD; shown to be significantly reduced in PH patients compared to healthy controls) and echocardiography are used to assess exercise and functional capacity (Bossone *et al.*, 2013; Demir and Kucukoglu, 2015). As treatments are not curative, clinical management of patients is primarily on a symptomatic basis, aiming to improve quality of life. In this sense, the functional class (I-IV) is more important in determining treatment options than the WHO category, despite the fact that most drugs are only officially approved for group 1 PH (Trammell *et al.*, 2015). Group 1 PH, or pulmonary arterial hypertension (PAH) in particular, includes a wide range of idiopathic aetiologies, but despite this, these patients share a collection of clinically presenting pathologies, including pulmonary artery smooth muscle cell (PASMC) and pulmonary endothelial cell (EC) proliferation and dysfunction, as well as vasoconstriction and *in situ* thrombosis (Montani *et al.*, 2014), elements which are present in other PH categories as well. As this diverse group has many similarities in terms of clinical manifestation, the treatment and management of these patients are also similar.

Therapies currently used for the management of PH target one of three pathways and are categorized based on their mechanism of action. The notion that pulmonary vascular remodelling is central to the disease is somewhat of a paradigm-shift compared to initial understanding. Previously, the dysregulation of endothelial mediators was thought more pertinent in PH than remodelling, a concept which led to the subsequent development of all currently used and approved therapies (Montani *et al.*, 2014). Thus, a reduction in the vasodilator mediators, prostacyclin (**PGI_2**; Tuder *et al.*, 1999) and **NO** (Giaid and Saleh, 1995), combined with an up-regulation of vasoconstrictor endothelin-1 (**ET-1**; Giaid *et al.*, 1993), causes an overwhelming pathological shift towards a constricted pulmonary arterial vasculature.

Currently approved PH therapeutics targeting these pathways include PGI_2 analogues (e.g. **epoprostenol**, **treprostinil** and **iloprost**) and prostacyclin **(IP) receptor** agonists (e.g. **selexipag**) which activate the endogenous Gs-coupled **IP receptor** to increase intracellular cAMP and hence induce vasodilatation (Tuder *et al.*, 1999; Mitchell *et al.*, 2014). Secondly, there are two main classes of drugs which exploit the NO pathway; the **PDE5** inhibitors (e.g. **sildenafil**, **tadalafil** and **vardenafil**) and soluble GC (**GC-1**/**GC-2**; Alexander *et al.*, 2017c) stimulators (e.g. **riociguat**). Under normal physiological conditions, NO activates GC-1 and/or GC-2 to increase cGMP in PASMCs. cGMP is subsequently broken down by PDE5, but elevated cGMP levels must be maintained in order for the vessel to remain dilated and patent (Qian and Fulton, 2013). Hence, the main mechanism of action of PDE5 inhibitors is to stop breakdown of

cGMP to induce vasodilatation. As such, PDE5 inhibitors rely mainly on endogenous NO to exert their effects, although they are also able to potentiate natriuretic peptide activity to ameliorate PH (Zhao *et al.*, 2003; Preston *et al.*, 2004; Klinger *et al.*, 2006; Baliga *et al.*, 2008). Conversely, GC-1/GC-2 stimulators increase cGMP production independently of intrinsic NO bioavailability. In addition to these NO modulating drugs, inhaled NO is utilized specifically in a unique category of PH, persistent pulmonary hypertension of the new-born (PPHN). Normally, the high pulmonary vascular resistance (PVR) seen *in utero* falls shortly after birth, but in PPHN infants, there is a sustained increase in PVR with concomitantly normal or low systemic vascular resistance which persists *post-partum* (Nair and Lakshminrusimha, 2014). Inhaled NO is the first line therapy in this setting due to its high specificity as a pulmonary vasodilator with minimal effect on systemic resistance (Nair and Lakshminrusimha, 2014; Abman *et al.*, 2015). Finally, endothelin receptor antagonists (ERAs) block the actions of ET-1 at its two cognate Gq-coupled receptors, **ET_A** and **ET_B**; both of which are present on PASMCs while only ET_B receptors are expressed on pulmonary ECs. Activation of both ET_A and ET_B receptors on the PASMCs initiates a contractile response, *via* inositol triphosphate signalling, leading to sarcoplasmic reticulum-dependent Ca^{2+} release (Giaid *et al.*, 1993). Conversely, binding of ET-1 to ET_B receptors located on the pulmonary ECs aids in vasodilatation by promoting clearance of ET-1 as well as increasing production of NO and PGI_2 (Seo *et al.*, 1994). Despite the fact that ET_B receptor activity can be different depending on the localization of the receptor, in practice, dual antagonists (e.g. **bosentan**) appear to have similar efficacy in PH to those selective for ET_A receptors (e.g. **ambrisentan**), with both non-selective and ET_A receptor selective ERAs used in PH patients (Hoeper *et al.*, 2016; Lajoie *et al.*, 2017).

Therapeutic limitations

The introduction of these drugs has undoubtedly improved treatment over the last 20 years. Indeed, these therapies have proven efficacy to combat the increased mPAP, albeit by only ~10% (Galie *et al.*, 2005), and they do extend survival and diminish morbidity by reducing breathlessness and increasing exercise capacity (Demir and Kucukoglu, 2015). However, despite this, they do not offer a cure, and PH patients must continue to take these drugs for the remainder of their lives. While many agents, including ERAs and PDE5 inhibitors, show benefits on pulmonary vascular remodelling in preclinical studies (Jeffery and Wanstall, 2001), they have negligible impact on remodelling in PH patients (Pogoriler *et al.*, 2012). Therefore, despite years of research, current therapies have reached somewhat of an efficacy plateau. For this reason, it is paramount that new targets and pathways in the setting of PH are identified, to facilitate further improvement in disease outcome.

Inflammation and immunity in pulmonary hypertension: what is known?

Inflammation and immunity have long been considered as major underlying factors in the pathogenesis of PH; indeed, evidence dating back over 20 years hints at this possibility (Table 1). Tuder *et al.* (1994) revealed that plasma levels of the pro-inflammatory cytokines IL-1 and **IL-6** are elevated in PH patients, and Humbert *et al.* (1995) showed that monocrotaline-induced PH in rats was ameliorated by the use of an IL-1 antagonist. The link between PH and altered immunity is also illustrated by the fact that group 1 PH encompasses PAH associated with both autoimmune disorders such as systemic lupus erythematosus and systemic sclerosis (Bazan *et al.*, 2018), as well as human immunodeficiency virus infection (Correale *et al.*, 2015). In addition to this, PAH patients and indeed PH patients more generally (Pugliese *et al.*, 2015; Kuebler *et al.*, 2018) display signs of a chronic inflammatory state even without an associated immune-related condition, most clearly delineated by elevated circulating cytokine levels and perivascular inflammatory infiltrates (Stacher *et al.*, 2012). While it is well established that the pathogenesis, and poor prognosis, of PH is primarily due to the remodelling that occurs in the pulmonary vasculature along with RV dysfunction, it is now becoming evident that this remodelling is not simply a consequence of imbalanced vasoreactivity but may indeed be driven by aberrant immune responses (Rabinovitch *et al.*, 2014). Therefore, in order to advance therapy, researchers have recently sought to identify alternative pathways that may be involved in the remodelling and inflammatory aspects of disease progression.

A growing body of evidence, in the form of both clinical and preclinical studies, links various aspects of inflammation to human and experimental PH (Table 1). In fact, numerous studies have demonstrated that perivascular inflammatory infiltrates comprised of macrophages, T and B lymphocytes, dendritic and mast cells are present in the occlusive plexiform lesions in the pulmonary vasculature of PH patients (Figure 1; Tuder *et al.*, 1994; Humbert *et al.*, 1995; Soon *et al.*, 2010; Stacher *et al.*, 2012; Rabinovitch *et al.*, 2014). In addition to this, Stacher *et al.* (2012) examined the degree of inflammatory cell infiltration in explanted lungs from PH patients and used this information to calculate perivascular inflammation scores. They found that in PH patients, perivascular inflammation scores positively correlated with vascular intima plus media and adventitia thickness as well as a trend towards a correlation with increased mPAP. Taken together, these studies demonstrate the importance of inflammation to pulmonary vascular remodelling. Intriguingly, manipulation of some of the aforementioned immune cell types confers protection or worsens progression in animal models of PH. Specifically, a recent study in rats found that depletion of alveolar macrophages, by **clodronate**-containing liposomes, attenuated hypoxia-induced PH (Zaloudikova *et al.*, 2016), but conversely, regulatory T-cells (Tregs) appear to limit vascular endothelial injury and prevent PH. As such, Tamosiuniene *et al.* (2011) found that T-cell deficient athymic nude rats developed worsened PH in response to VEGF inhibition, which could be rescued by adoptive T-cell transfer. Moreover, Chu *et al.* (2015) revealed that adoptive transfer of Tregs into hypoxic mice protected them from PH as well as vascular and RV remodelling. In addition, these animals displayed reduced expression of pro-inflammatory cytokines, IL-1β and IL-6, and the chemokine **CCL2** in the lungs. Thus, it appears that macrophages are detrimental, whereas certain T-cell populations may be beneficial in the resolution or prevention of PH. In addition, Wang *et al.* (2013) revealed that

Table 1

Clinical and preclinical evidence for inflammation in pulmonary hypertension

Target	Preclinical	Clinical
NF-κB	Inhibition improves PH (Kumar *et al.*, 2012; Hosokawa *et al.*, 2013; Wang *et al.*, 2013; Farkas *et al.*, 2014; Li *et al.*, 2014)	–
IL-1	Inhibition improves PH (Voelkel *et al.*, 1994; Parpaleix *et al.*, 2016)	Increased levels in patients (Humbert *et al.*, 1995; Soon *et al.*, 2010)
IL-18	Inhibition improves PH (Morisawa *et al.*, 2016)	Increased levels in patients (Ross *et al.*, 2012)
IL-6	Inhibition improves PH (Golembeski *et al.*, 2005; Savale *et al.*, 2009; Steiner *et al.*, 2009; Hashimoto-Kataoka *et al.*, 2015; Parpaleix *et al.*, 2016)	Increased levels in patients (Humbert *et al.*, 1995; Selimovic *et al.*, 2009; Soon *et al.*, 2010; Gitto *et al.*, 2012; Matura *et al.*, 2015)
Other cytokines/ chemokines/growth factors	–	Increased levels of MCP-1 (Itoh *et al.*, 2006); VEGF, PDGF, TGF-β1 (Selimovic *et al.*, 2009); IL-2, -4, -8, -10, -12p70, TNF-α (Soon *et al.*, 2010) IL-8, TNF-α in PPHN (Gitto *et al.*, 2012); CXCL10 (Ross *et al.*, 2012); TNF-α (Matura *et al.*, 2015)
Inflammatory cells	Depletion of T-cells worsens PH (Tamosiuniene *et al.*, 2011; Chu *et al.*, 2015); macrophages improves PH (Zaloudikova *et al.*, 2016)	Infiltrates comprised of macrophages, T and B lymphocytes, dendritic and mast cells (Tuder *et al.*, 1994; Savai *et al.*, 2012; Stacher *et al.*, 2012)
P2X7/ATP	Inhibition improves PH (Yin *et al.*, 2017)	–
Inflammasomes	Inhibition improves PH (Villegas *et al.*, 2013; Cero *et al.*, 2015; Tang *et al.*, 2015)	–
Survival	–	Elevated cytokines predict (Soon *et al.*, 2010)
Remodelling	–	Inflammatory infiltrates correlate with vessel wall thickness (Stacher *et al.*, 2012)

inhibition of the pro-inflammatory cytokine **TNF-α** in rats attenuates monocrotaline-induced PH, with the involvement of NF-κB.

Similar links are evident in PH patients, with circulating levels of many cytokines including, IL-1β, **IL-2**, IL-4, IL-6, IL-8, IL-10, **IL-12**, IL-18 and TNF-α, as well as the chemokines CCL2 and **CXCL10** (Humbert *et al.*, 1995; Itoh *et al.*, 2006; Soon *et al.*, 2010; Ross *et al.*, 2012), being elevated compared to healthy controls; a strong indicator that altered immunity is at play. Indeed, Soon *et al.* (2010) found that increased levels of the pro-inflammatory cytokines IL-2, IL-6, IL-8 and IL-12, and perhaps surprisingly, the anti-inflammatory cytokine IL-10, correlate with worse outcome and patient survival. Interestingly, in this study, these cytokines served as a better predictor of prognosis than traditional measures like 6MWD and haemodynamic parameters (Soon *et al.*, 2010), although this remains to be substantiated.

Another emerging concept is that interactions between platelets, leukocytes and endothelial cells contribute to the inflammatory processes underpinning PH. Indeed, these cells are a recognized source of inflammatory mediators (Thomas and Storey, 2015). NO and PGI_2 inhibit platelet activation and aggregation, but both molecules are reduced in PH (Giaid and Saleh, 1995; Tuder *et al.*, 1999), allowing aberrant platelet aggregation and thrombus formation to occur (Thomas and Storey, 2015). In addition to their interactions with NO and PGI_2, platelets may also serve as a source of IL-1β in PH, as they have been shown to process and release IL-1β in dengue fever patients, as well as increase endothelial permeability in this infection (Hottz *et al.*, 2013). Activated platelets also play an integral role in leukocyte adhesion, rolling and subsequent extravasation in atherosclerosis (Lievens and von Hundelshausen, 2011). The adhesion molecule, P-selectin, is expressed on activated platelets, and endothelial cells and its ligand P-selectin glycoprotein ligand 1 (PSGL-1) is expressed on leukocytes (Dole *et al.*, 2005; von Hundelshausen and Weber, 2007). P-selectin–PSGL-1 interactions lead to the formation of platelet-leukocyte multicellular aggregates and contribute to the recruitment of leukocytes to sites of endothelial injury and inflammation (Dole *et al.*, 2005; von Hundelshausen and Weber, 2007; Thomas and Storey, 2015; Coenen *et al.*, 2017). Although many adhesion molecules are involved in this process, P-selectin is of particular interest with respect to PH since P-selectin levels have been shown to be elevated in PH patients (Semenov *et al.*, 2000). The importance of this mechanism in the development of atherosclerosis is demonstrated by the fact that mice genetically deficient in P-selectin are protected from atherosclerotic lesion development (Dong *et al.*, 2000). As atherosclerotic lesions are known to include inflammatory cell infiltrates (Libby, 2012), and a similar inflammatory milieu is present within the plexiform lesions of PH patients (Tuder *et al.*, 1994; Stacher *et al.*, 2012), it is plausible that platelet and endothelial adhesion molecules play a role in the transendothelial migration of immune cells here too (Figure 1), although the precise mechanism in this setting remains to be

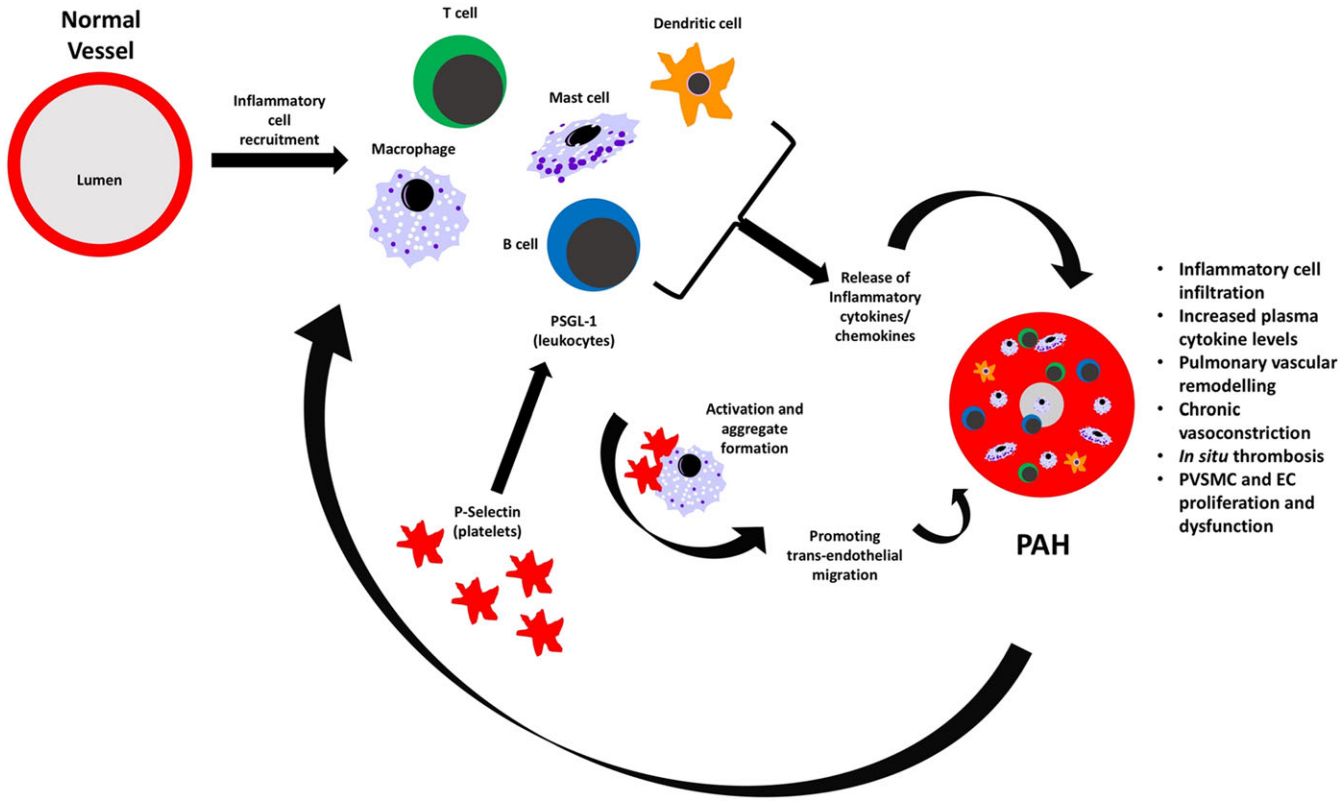

Figure 1

Inflammation in pulmonary hypertension. Proposed mechanisms by which inflammation contributes to PH pathogenesis. Recruitment of inflammatory cells such as macrophages, T and B lymphocytes, dendritic and mast cells leads to the release of pro-inflammatory cytokines IL-1β, IL-2, IL-6, IL-8, IL-12, IL-18 and TNF-α, MCP-1 and CXCL10. Platelet P-selectin binds PSGL-1 expressed on leukocytes to induce multicellular aggregation and trans-endothelial migration. Here, inflammatory cells take up residence to form perivascular inflammatory infiltrates seen in the plexiform lesions of PH patients. This results in further cytokine release, pulmonary vascular remodelling, cell proliferation and thrombosis causing an ongoing exacerbation cycle.

elucidated. Encouragingly, in terms of inflammatory processes and potential translation into new therapies, similar findings are made in animal models of PH, as are observed in PAH and PH patients more generally, and will be described below. This indicates that the use of these models will be instrumental in elucidating inflammatory aspects of disease progression and provides a strong rationale for their continued use.

A new approach to inflammation in pulmonary hypertension

In addition to the clinical data (Soon *et al.*, 2010; Ross *et al.*, 2012; Groth *et al.*, 2014), many preclinical studies, involving depletion or alteration of immune cells, cytokines or their signalling pathways have been shown to modify PH pathobiology (Voelkel *et al.*, 1994; Kumar *et al.*, 2012; Li *et al.*, 2014; Cero *et al.*, 2015; Tang *et al.*, 2015; Morisawa *et al.*, 2016; Parpaleix *et al.*, 2016; Zaloudikova *et al.*, 2016), highlighting the important role of altered immunity in PH (as described above; Table 1). The inflammasome, a key component of the innate immune system, is emerging as a novel target in the treatment of PH, because preclinical studies modulating cells or pathways associated with the inflammasome show particular promise (Gasse *et al.*, 2009; Villegas *et al.*, 2013; Tang *et al.*, 2015; Parpaleix *et al.*, 2016; Yin *et al.*, 2017). Inflammasome components are most highly expressed in macrophages (Heng and Painter, 2008) but have also been described in endothelial cells (Xiang *et al.*, 2011; Xia *et al.*, 2014), neutrophils, dendritic cells (Guarda *et al.*, 2011), platelets (Hottz *et al.*, 2013) and lung epithelial cells (De Nardo *et al.*, 2014). The observation that depletion of macrophages attenuates PH (Zaloudikova *et al.*, 2016) and inflammasome components are most highly expressed in these cells (Heng and Painter, 2008) suggests macrophages may play a critical role in PH development, potentially mediated *via* the inflammasome.

Inflammasome activation and targets in disease

Inflammasomes are a family of multi-protein complexes, first identified by Martinon *et al.* (2002), which revolutionized the understanding of immune function. They are key players in macrophage-related immunity and important regulators of inflammation, responding to danger signals and releasing the pro-inflammatory cytokines IL-1β and IL-18 (Mariathasan *et al.*, 2006; Martinon *et al.*, 2006; Halle *et al.*, 2008).

Inflammasomes comprise of three subunits and, depending upon the particular subunit composition, display selectivity towards a unique array of activation signals. Each inflammasome contains a distinct pattern recognition receptor (PRR) and this defines the inflammasome type and selectivity (Man and Kanneganti, 2015). To date, inflammasome-forming PRRs identified include **NLRP1** and **NLRP3** [nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family, leucine rich repeat (LRR) and pyrin domain (PYD)-containing proteins; Krishnan *et al.*, 2016]. These NLRP-type inflammasomes are comprised of an NLR structure [a central nucleotide-binding and oligomerization domain (NACHT) flanked by leucine-rich repeats at the C-terminus and at the N-terminus a PYD]. In addition to these most widely known NLRP-type inflammasomes, another PRR known as NLRC4 [NOD-like receptor family CARD (caspase activation and recruitment domain)-containing protein 4] also forms an inflammasome. Furthermore, there are non-NLR type inflammasome-forming PRRs such as absent in melanoma (AIM) 2 and IFN-γ inducible protein 16 (IFI16; Figure 2; Coll *et al.*, 2015). Following detection of pathogen- and damage- associated molecular patterns (PAMPs and DAMPs), the PRR is auto-activated which allows it to recruit the heterodimeric adaptor molecule, ASC (apoptosis-associated speck-like protein containing a CARD), which is common to a number of inflammasome varieties (Figure 2; Lu *et al.*, 2014). Cytoplasmic ASC monomers are then reorganized into a single large 'speck', which is considered a hallmark of inflammasome assembly (Man and Kanneganti, 2015). Finally, the ASC speck recruits the cysteine protease pro-caspase-1, which, by means of proximity-induced auto-cleavage yields active **caspase-1** (Malik and Kanneganti, 2017).

Inflammasome assembly and activation

By far the most widely studied and best characterized inflammasome isoform to date, and the focus here, is the NLRP3 inflammasome (also known as NALP3). In order for the NLRP3 inflammasome to initiate its activity, a two-step process is required, beginning with signal I (priming) and then subsequently signal II (activation; Figure 3; Bauernfeind *et al.*, 2009). Priming is induced by engagement of toll-like receptors (TLRs) or inflammatory cytokine receptors on the cell surface by exogenous PAMPs [e.g. microbial components (Ishii *et al.*, 2008), pore-forming toxins (Malik and Kanneganti, 2017), silica crystals or asbestos fibres (Dostert *et al.*, 2008; Franchi *et al.*, 2009)] and/or endogenous DAMPs [e.g. uric acid crystals (Martinon *et al.*, 2006), pro-inflammatory cytokines like TNF-α (Chow *et al.*, 2014)]. Following this initial detection, NF-κB signal transduction up-regulates expression of the inflammasome components NLRP3, ASC and pro-caspase-1, and pro-inflammatory cytokine precursors pro-IL-1β and pro-IL-18, completing the priming (signal I) step (Figure 3; Bauernfeind *et al.*, 2009).

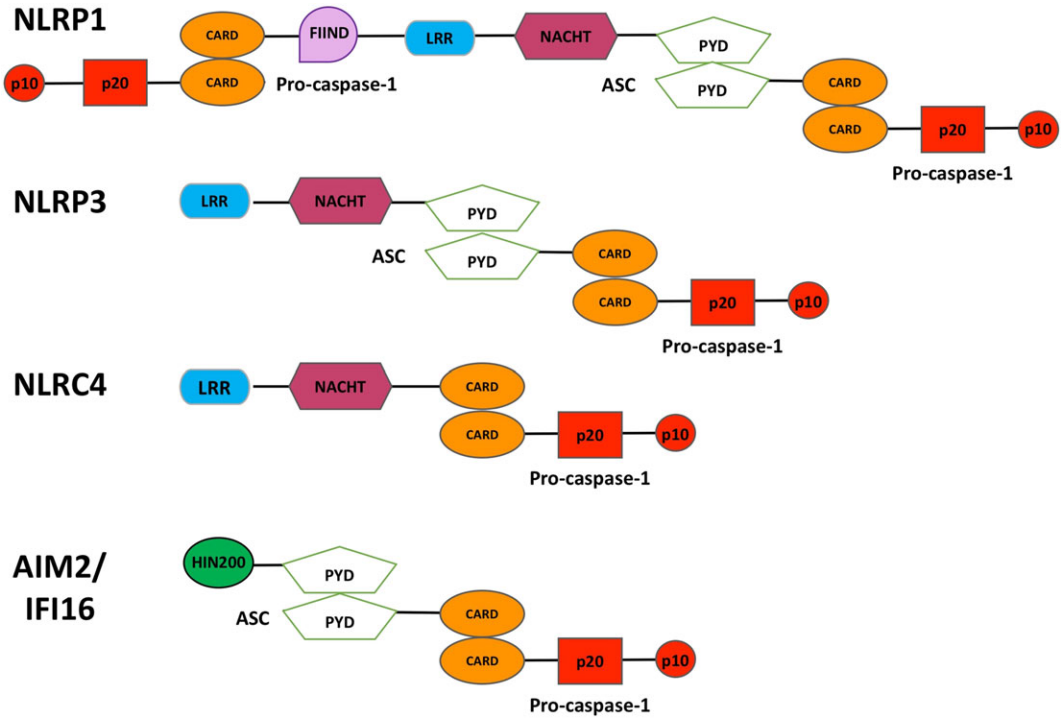

Figure 2

Inflammasome isoforms and subunit compositions. The main inflammasome isoforms described to date include NLRP1, NLRP3, NLRC4, AIM2 and IFI16; the subunit composition differs depending on the variant. NLRP3, AIM2 and IFI16 inflammasomes require the adaptor molecule ASC for homotypic oligomerization. Conversely, NLRC4 contains its own CARD domain and can recruit pro-caspase-1 independently of ASC. NLRP1 contains both a CARD and PYD and can therefore either recruit caspase-1 independently of ASC using its own CARD, or recruit caspase-1 in an ASC-dependent manner, similarly to the NLRP3, AIM2 and IFI16 inflammasomes. FIIND, function to find domain; HIN200, dsDNA binding domain.

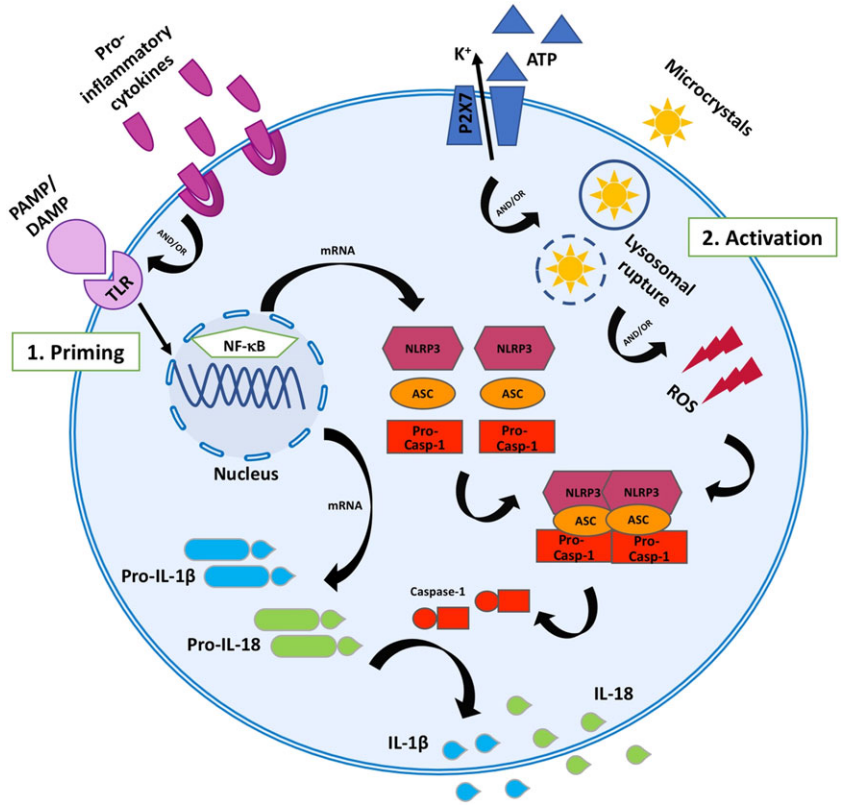

Figure 3

NLRP3 inflammasome activation. Schematic representation of activators and effectors of the NLRP3 inflammasome. The NLRP3 inflammasome comprises the pattern recognition receptor, NLRP3, along with the adaptor molecule, ASC, and pro-caspase-1. NLRP3 inflammasome activation requires two steps. (1) Priming is induced by engagement of TLRs and pro-inflammatory cytokine receptors on the cell surface by exogenous PAMPs (e.g. microbial components, silica crystals, asbestos fibres or pore-forming toxins), endogenous DAMPs (e.g. ATP, microcrystals or ROS) or pro-inflammatory cytokines such as TNF-α; this results in NF-κB-mediated up-regulation of NLRP3, ASC, pro-caspase-1, pro-IL-1β and pro-IL-18 gene expression. (2) Activation occurs when further DAMPs are detected by NLRP3. This leads to oligomerization of NLRP3 subunits and recruitment of ASC and pro-caspase-1. Pro-caspase-1 then undergoes auto-cleavage into p10 and p20 subunits, which heterodimerize to form active caspase-1. Caspase-1 then processes pro-IL-1β and pro-IL-18 into their active, pro-inflammatory cytokine forms IL-1β and IL-18.

The NLRP3 inflammasome is then equipped for activation, an event which involves inflammasome oligomerization. A number of pathways have been postulated to be involved in triggering inflammasome activation including lysosomal rupture and release of enclosed microcrystals (Martinon *et al.*, 2006; Gasse *et al.*, 2009), ROS generation (Zhou *et al.*, 2010) and high extracellular ATP concentrations (resulting from cellular damage) acting at **P2X7** purinoceptors (Mariathasan *et al.*, 2006; Franchi *et al.*, 2009; Riteau *et al.*, 2010; Krishnan *et al.*, 2014; Shao *et al.*, 2015). Once these subsequent danger signals have been recognized, activation of the NLRP3 inflammasome is initiated by oligomerization of NLRP3 monomers *via* homotypic PYD–PYD reactions, which then interact with the PYD of the adaptor molecule ASC (Sborgi *et al.*, 2015). Once activated, the inflammasome recruits the zymogen, pro-caspase-1 using the CARD region in ASC, and several pro-caspase-1 subunits cluster around the inflammasome, resulting in auto-cleavage to active caspase-1 heterodimers which each comprise a p20 (20 kDa) and p10 (10 kDa) subunit (Malik and Kanneganti, 2017). Active caspase-1 then cleaves inactive pro-IL-1β (31 kDa) and pro-IL-18 (24 kDa) to their active forms IL-1β (17.5 kDa) and IL-18 (18 kDa) respectively (Figure 3; Dinarello, 2002). Additionally, inflammasome generated caspase-1 promotes a highly inflammatory type of cell death known as pyroptosis (Maltez *et al.*, 2015). The IL-1 family of cytokines are 'early response' mediators of immunity and promote the release of a number of secondary cytokines which lie further downstream in the inflammasome activation cascade (Labow *et al.*, 1997; Dinarello, 2002; Cahill and Rogers, 2008; Mills *et al.*, 2013). These include IL-6, which is released from macrophages and T-cells in response to IL-1β stimulation (Cahill and Rogers, 2008), as well as IL-2 and IL-12 which are produced downstream of IL-18 (Dinarello, 2002).

NLRP3 inflammasome pathophysiology and pharmacology

The NLRP3 inflammasome has been shown to play a role in a large number of disease states affecting various organ systems, including the kidneys (Anders and Muruve, 2011), liver (Szabo and Csak, 2012) and as a consequence of ageing (Youm *et al.*, 2013). More interestingly, of relevance to PH, increased NLRP3 inflammasome activity has been associated

with cardiovascular and metabolic diseases such as gout (Martinon *et al*., 2006), atherosclerosis (Duewell *et al*., 2010), arteritis (Chen *et al*., 2015) obesity and type 2 diabetes (Wen *et al*., 2012; Liu *et al*., 2015), as well as lung disorders including chronic obstructive pulmonary disease (COPD; Yang *et al*., 2015), asthma (Simpson *et al*., 2014; Kim *et al*., 2017) and cystic fibrosis (Iannitti *et al*., 2016); and fibrotic disorders like idiopathic pulmonary fibrosis (IPF) and substance-induced pulmonary fibrosis (e.g. asbestos and silica crystals; Dostert *et al*., 2008). The level of understanding in each condition varies, but many examples have implications for PH.

As alluded to earlier, endothelial cells play an important role in the inflammatory cell infiltration seen in atherosclerosis, a phenomenon that is likely to parallel what occurs in PH. The actions of the NLRP3 inflammasome may also contribute to the endothelial dysfunction seen in PH patients. In fact, the NLRP3 inflammasome has been associated with endothelial dysfunction in a number of cardiovascular pathologies. Liu *et al*. (2015) found that activation of the NLRP3 inflammasome induces endothelial dysfunction in obese rats, and moreover, Chen *et al*. (2015) revealed that lysosomal destabilization in coronary arteritis induces NLRP3 inflammasome activation which subsequently results in endothelial dysfunction. Finally, cardiovascular insult in the form of homocysteine or inflammatory LPS leads to NLRP3-dependent pyroptosis in endothelial cells, ultimately contributing to endothelial dysfunction (Xi *et al*., 2016). Given that endothelial dysfunction is a major contributor to the pathogenesis of PH and the NLRP3 inflammasome contributes to this in other diseases, it is possible that the NLRP3 inflammasome may contribute to PH pathogenesis in this manner.

Gout is characterized by deposition of **uric acid** crystals in joints and periarticular tissues (Martinon *et al*., 2006) and, as previously mentioned, uric acid crystals are known activator signals for NLRP3 activity. Interestingly, a similar pathology is seen in a murine model of IPF, where bleomycin instillation produces uric acid crystals in the lung which subsequently induce NLRP3 inflammasome activation (Gasse *et al*., 2009). Similarly, inhalation of other particulate substances (e.g. asbestos or silica) induces pulmonary fibrosis and leads to NLRP3 inflammasome activation and IL-1β release in the lung, by means of lysosomal rupture and ROS production (Dostert *et al*., 2008). Furthermore, IPF patients exhibit elevated extracellular ATP (released from injured cells) in their bronchoalveolar lavage fluid (BALF) compared to healthy controls, which acts as a major NLRP3 danger signal (Riteau *et al*., 2010), an observation that can be reproduced by bleomycin administration in mice. Interestingly, dos Santos *et al*. (2015) found that the cytoskeletal intermediate filament, vimentin, is an essential element for bleomycin and asbestos-induced lung injury and NLRP3 inflammasome activation. The link between pulmonary fibrotic disorders and the NLRP3 inflammasome is strong evidence that this inflammasome also contributes to PH. As PH often occurs as a result of pulmonary fibrosis, it is possible the NLRP3 inflammasome plays a similar and synergistic role in the two conditions (Riteau *et al*., 2010). Perhaps the lung disorder most strongly tied to NLRP3 inflammasome activity, and closely linked to the pathology seen in PH, is COPD. Notably, in mice, exposure to cigarette smoke leads to increased caspase-1 activity as well as IL-1β and IL-18 release in the lungs, and genetic disruption or pharmacological inhibition of the P2X7 receptor, a known NLRP3-related activation stimulus, ameliorates this effect (Eltom *et al*., 2011). Moreover, similar findings are noted in patients, with increased caspase-1 detected in the lungs of emphysema patients and smokers compared to non-smokers (Eltom *et al*., 2011). Additionally, the known inflammasome activator, uric acid, is increased in BALF from COPD patients (Wanderer, 2008). Interestingly, mice overexpressing IL-1β exhibit a COPD-like phenotype including lung inflammation and fibrosis, and mice genetically deficient in IL-1β are protected from cigarette smoke induced pathology (Lappalainen *et al*., 2005).

A lack of selective inflammasome inhibitors is one reason these pathways have been so difficult to study, not only in PH, but also other disorders. Compounds which inhibit inflammasome activity, albeit non-selectively include various micro-RNAs, ellagic acid, the SOD mimetic (MnTE-2-PyP), 4-phenyl butyric acid (4-PBA) and type I interferons, which have been used in multiple sclerosis for many years (Villegas *et al*., 2013; Shao *et al*., 2015; Tang *et al*., 2015; Zeng *et al*., 2017). There are a number of other approaches to modulate inflammasome activity at different levels within its signalling cascade. Firstly, targeting inflammasome priming by inhibiting NF-κB-mediated transcription of inflammasome subunits (NLRP3, ASC and caspase-1) and pro-inflammatory cytokine precursors (pro-IL-1β and IL-18), with compounds such as pyrrolidine dithiocarbamate (PDTC), and the selective, synthetic NF-κB inhibitor, IMD-0354 (Hosokawa *et al*., 2013; Farkas *et al*., 2014). Secondly, inflammasome activation signals can be targeted to mitigate oligomerization. Depending on the condition, and particular stimuli, such approaches include P2X7 receptor inhibition with **A-740003** (Yin *et al*., 2017) or limiting production of ROS with a SOD mimetic (Villegas *et al*., 2013). Another potential inflammasome-directed target is caspase-1. Indeed, there are a number of low MW caspase-1 inhibitors that have been trialled in humans for inflammatory conditions such as psoriasis and rheumatoid arthritis. Unfortunately, these trials were terminated early due to the risk of liver toxicity or generally poor efficacy (MacKenzie *et al*., 2010). As such, the newer caspase-1 inhibitor **VX-765** was developed to improve safety and efficacy (MacKenzie *et al*., 2010). Another well-known inflammasome target is IL-1, for which two specific drugs are available to block its activity, **anakinra** and **canakinumab**, have prior approved uses in humans. Indeed, the former is currently used to treat rheumatoid arthritis and the latter is licensed for a number of auto inflammatory syndromes. Canakinumab has additionally undergone recent evaluation in patients with inflammatory vascular (i.e. coronary artery) disease (Van Tassell *et al*., 2017; Ridker *et al*., 2017b). Therefore, both of these drugs may represent a novel opportunity to repurpose for use in PH. Finally, IL-6 is produced downstream of IL-1β (Cahill and Rogers, 2008), and the human monoclonal antibody targeting the IL-6 receptor (**IL-6R**), **tocilizumab**, is currently undergoing early phase testing in human PAH (Hernandez-Sanchez *et al*., 2018). Although all these compounds do interrupt inflammasome activity to some extent or at some level, a truly selective inflammasome inhibitor did not emerge until 2015 when Coll *et al*. (2015) described

a highly selective low MW inhibitor of the NLRP3 inflammasome, **MCC950**. They demonstrated that MCC950 selectively inhibits NLRP3 inflammasome activation both *in vitro* and *in vivo*. Although MCC950 has not been trialled in humans to date, it does show promise as a therapeutic agent in terms of safety and efficacy in several preclinical models of disease, including cardiovascular and metabolic disorders and lung-associated conditions (Krishnan *et al*., 2016; Primiano *et al*., 2016; Kim *et al*., 2017; Pavillard *et al*., 2017; van der Heijden *et al*., 2017; van Hout *et al*., 2017), which will be described in more detail below.

Inflammasomes in pulmonary hypertension

The concept of inflammasome involvement in PH is particularly new, with studies examining this unique aspect of inflammation only emerging over the last 5 years or so. There is currently a lack of evidence for involvement of other inflammasome isoforms, apart from NLRP3, in PH and this review will therefore focus on this isoform. As outlined below, preclinical and clinical evidence for NLRP3 inflammasome activity and downstream signalling in the pathobiology of PH is now strong. Pharmacological manipulation of the priming, assembly, activation and subsequent downstream signalling of the NLRP3 inflammasome in the context of PH will be addressed, beginning at the upstream end and working downward.

Potential sites of inflammasome modulation in pulmonary hypertension

Furthest upstream are the signals that initiate the priming stage of inflammasome activity. These include a variety of exogenous and endogenous signals in the form of PAMPs and DAMPs; the distinct combination of which can initiate activity in different inflammasome isoforms through activation of TLRs (Kahlenberg *et al*., 2005; Becker and O'Neill, 2007). To date, there is no evidence of specifically modulating TLR activity with respect to inflammasomes in PH, but since this pathway is so far upstream, it is probably not the most desirable target. Following the initiation of priming through TLR-mediated detection of PAMPs and DAMPs, an important pathway to complete this step is NF-κB (Figure 3). This transcription factor drives the expression of the inflammasome subunits NLRP3, ASC and pro-caspase-1 as well as the inflammatory cytokine precursors, pro-IL-1β and pro-IL-18 (Bauernfeind *et al*., 2009; Krishnan *et al*., 2016). Interestingly, Kumar *et al*. (2012) found that cardiac-specific genetic deletion of NF-κB in mice is able to prevent RV hypertrophy and increased right ventricular systolic pressure (RVSP; a surrogate for mPAP used in rodents) induced by monocrotaline, and moreover, Li *et al*. (2014) showed that genetic NF-κB deletion in the lung prevented monocrotaline-induced lung fibrosis and attenuated increased RVSP in mice. In addition, pharmacological inhibition of NF-κB with the selective, synthetic inhibitor IMD-0354 or with PDTC decreased RVSP and reversed pulmonary vascular remodelling and RV hypertrophy in monocrotaline (Hosokawa *et al*., 2013) and **sugen**-hypoxic rats (Farkas *et al*., 2014; Figure 4).

Following priming, a number of other pathways promote NLRP3 activation; one such signal is high extracellular ATP levels, acting at the P2X7 purinoceptor (Mariathasan *et al*., 2006; Franchi *et al*., 2009; Riteau *et al*., 2010). Indeed, the P2X7 receptor inhibitor A-740003 has been utilized in a rat model of PH with positive effects, reducing RVSP, reversing RV hypertrophy and pulmonary vascular remodelling as well as attenuating NLRP3 inflammasome up-regulation (Yin *et al*., 2017; Figure 4). Another NLRP3 activating stimulus is ROS, and interestingly, reducing superoxide through the use of the SOD mimetic, MnTE-2-PyP also attenuates PH and NLPR3 expression in hypoxic mice (Villegas *et al*., 2013; Figure 4). It is important to note that, as both the P2X7 receptor and SOD are widespread physiologically and diverse in their actions, these approaches may be simultaneously exerting beneficial effects through mechanisms distinct from NLRP3. An additional NLRP3 inflammasome activation signal is microcrystal lysosomal rupture (Martinon *et al*., 2006). Indeed, Gasse *et al*. (2009) illustrated that minimizing uric acid crystal levels in mice with the inhibitors **allopurinol** or uricase attenuates bleomycin-induced pulmonary fibrosis, inflammation and increased IL-1β levels (Figure 4). Conversely, they revealed that exogenous addition of uric acid microcrystals reproduces the lung injury induced by bleomycin.

Another potential inflammasome target is caspase-1 but, so far, there is no human evidence available for the use of low MW caspase-1 inhibitors in any cardiovascular or lung disorders. However, there is some evidence that the newer caspase-1 inhibitor VX-765 is beneficial in a rat model of myocardial infarction (Yang *et al*., 2017). Excitingly, this orally bioavailable compound has also recently been trialled for the treatment of epilepsy in humans (MacKenzie *et al*., 2010) and it could, therefore, be potentially repurposed for therapeutic utility in PH (Figure 4).

Further support for a role of the NLRP3 inflammasome in PH comes in the form of up-regulated NLRP3 expression and activation in the lungs of mice exposed to a chronic hypoxia model of PH. Such effects that are attenuated by a SOD mimetic, presumably *via* reduced ROS formation, an established activation signal for the NLRP3 inflammasome (Villegas *et al*., 2013). Additionally, inhibition of endoplasmic reticulum stress with 4-PBA prevents monocrotaline-induced PH *via* a reduction in NLRP3 inflammasome activity in rats (Zeng *et al*., 2017). Moreover, NLRP3 inflammasome inhibition by ellagic acid ameliorates monocrotaline-induced PH in rats, as well as vascular remodelling, RV hypertrophy and expression of NLRP3, caspase-1 and IL-1β (Tang *et al*., 2015). It is important to note, however, that while the SOD mimetic, ellagic acid and 4-PBA did inhibit NLRP3 inflammasome activity in these studies, they may have had other non-specific effects, targeting other inflammasomes or inflammatory pathways. Furthermore, NLRP3$^{-/-}$ and Caspase-1$^{-/-}$ mice are protected from acute bleomycin-induced lung fibrosis and inflammation (Gasse *et al*., 2009). Taken together, these findings suggest that selectively inhibiting NLRP3 inflammasome activity may be of therapeutic benefit in PH.

In addition to promising results from modulating the inflammasome itself, other studies have looked at altering the downstream cytokines generated as a result of inflammasome activity (Figures 3 and 4). A pathway of particular recent interest in cardiovascular disease has been the use

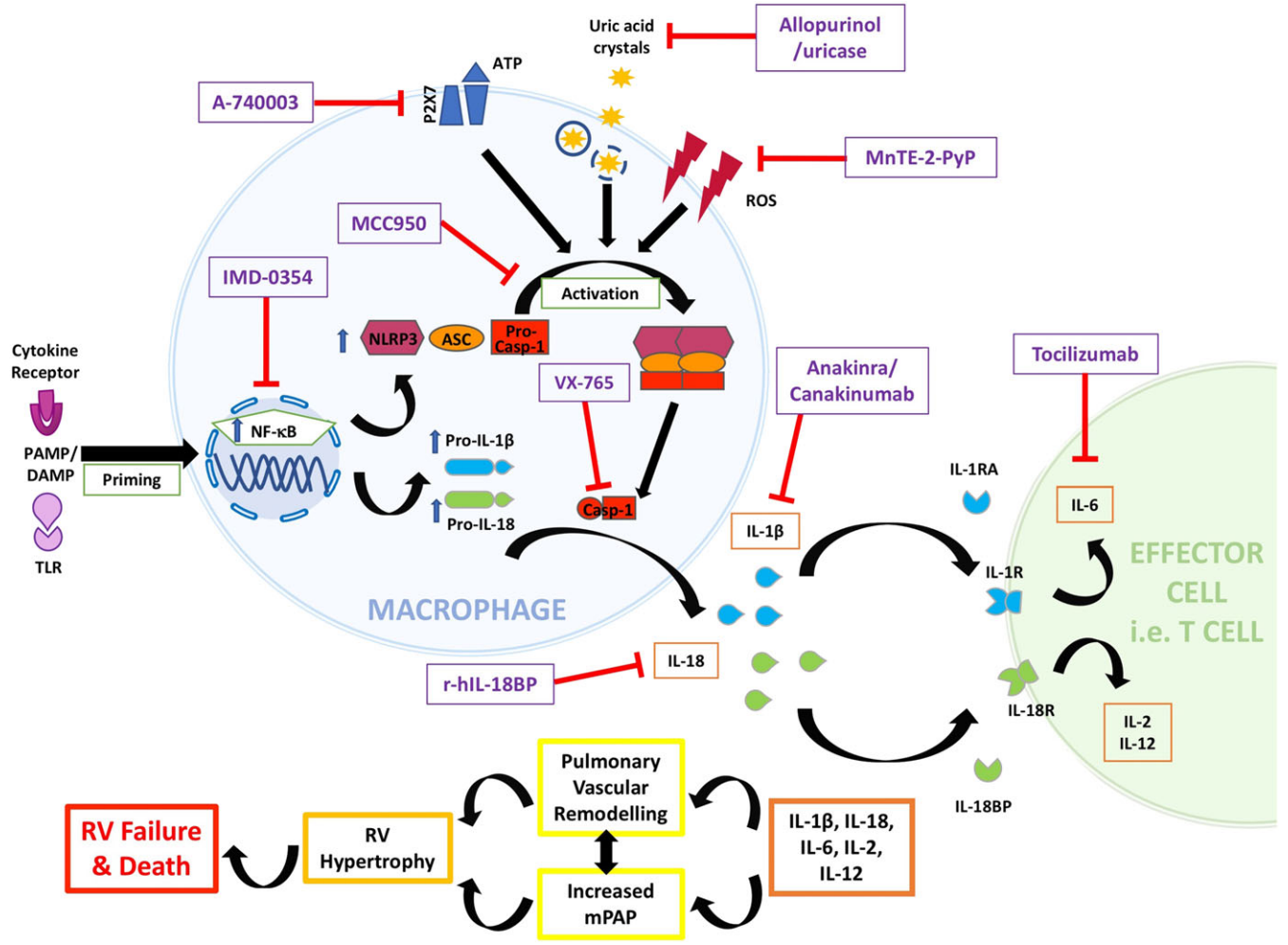

Figure 4

Involvement of the NLRP3 inflammasome and potential sites of modulation in pulmonary hypertension. Numerous drugs are able to modulate NLRP3 inflammasome activity at various levels in PH; from activation to downstream cytokines. Priming is followed by assembly and activation of the NLRP3 inflammasome, resulting in subsequent pro-inflammatory cytokine generation (Figure 3). The actions of these cytokines are thought to contribute to pulmonary vascular remodelling (Figure 1), which occurs alongside increased mPAP. These effects lead to RV compensatory hypertrophy and eventually RV decompensation, failure and death. Inhibitory drugs are in purple squares. IMD-0354, selective NF-κB inhibitor; A-740003, P2X7 receptor inhibitor; Allopurinol/uricase, uric acid inhibitors; MnTE-2-PyP, SOD mimetic; MCC950, selective, low MW NLRP3 inflammasome activation inhibitor; VX-765, caspase-1 inhibitor; Anakinra, recombinant IL-1 decoy receptor, IL1-RA; Canakinumab, human monoclonal antibody targeting IL-1β; r-hIL-18BP, recombinant IL-18 decoy receptor, IL-18BP; Tocilizumab, human monoclonal antibody targeting IL-6 receptor (IL-6R).

of IL-1 receptor activity blockers such as anakinra (a recombinant version of the endogenous IL-1 receptor antagonist, **IL1-RA**; Granowitz *et al.*, 1992) and canakinumab (a human monoclonal antibody targeted at IL-1β; Alten *et al.*, 2008; Figure 4). In contrast to the low MW caspase-1 inhibitors, the structure of these agents necessitates subcutaneous administration (Granowitz *et al.*, 1992; Alten *et al.*, 2008) and implicitly blocks only the IL-1β half of the inflammasome-driven IL-1β/IL-18 cytokine pathway. Nonetheless, the recently published results of the CANTOS (Ridker *et al.*, 2017b) trial support the idea that targeting IL-1β produces an anti-inflammatory effect in patients, at least in the context of secondary prevention following myocardial infarction (despite no reduction in overall mortality). A secondary analysis also revealed that patients receiving canakinumab had a significantly lower incidence of lung cancer compared to placebo, and the associated mortality was also reduced. Interestingly, patients that developed lung cancer exhibited increased IL-6 levels, and these were lowered following treatment with canakinumab (Ridker *et al.*, 2017a). These observations parallel the pathobiology of PH which is characterized by marked increases in circulating IL-6 and is increasingly thought of as a hyper-proliferative, anti-apoptotic, metabolically disturbed disorder (Boucherat *et al.*, 2017). Notwithstanding, there was a higher incidence of fatal infection and sepsis in those patients receiving canakinumab compared to placebo (Ridker *et al.*, 2017b). Given that blocking IL-1β will not only suppress the aberrant inflammation known to exist in cardiovascular disease (Libby, 2012) but also compromise the infection-fighting capacity of the immune system in

general, this is not entirely surprising. Nonetheless, these findings are very promising as they provide a 'proof of concept' for the use of drugs that modulate inflammasome-related cytokines in human vascular disease and endorse further investigation into this pathway. Indeed, a pilot study, run by Virginia Commonwealth University, USA, is currently underway to investigate the efficacy and safety of another IL-1 modulator, anakinra, in PH patients, with results expected by April 2019 (clinical trials US identifier: NCT03057028). Intriguingly, long before inflammasomes were discovered, Voelkel *et al*. (1994) found that treatment with an IL-1 receptor antagonist improved monocrotaline-induced, but not hypoxia-induced PH in rats; more recently, anakinra was found to attenuate both monocrotaline-induced PH in rats and hypoxia-induced PH in mice (Parpaleix *et al*., 2016; Figure 4). Given the differences between experimental models and species seen in animal studies, it will be interesting to see if anakinra produces positive effects in PH patients.

The alternate cytokine pathway triggered by the inflammasome involves IL-18. To date, little information exists on the modulation of IL-18 activity in the setting of PH and that available is somewhat conflicting. On one hand, Morisawa *et al*. (2016) showed that genetic IL-18 disruption suppresses hypoxia-induced PH in mice, but conversely, Bruns *et al*. (2016) concluded that genetic IL-18 deletion alone is not sufficient to prevent RV hypertrophy and increased RVSP in response to hypoxia, suggesting that therapeutic approaches targeting both inflammasome cytokines, IL-18 and IL-1β would be more efficacious. A human recombinant version of the endogenous IL-18 binding protein (IL-18BP), a decoy receptor (r-hIL-18BP), was developed and underwent safety and efficacy evaluation in healthy volunteers and rheumatoid arthritis patients (Tak *et al*., 2006). This compound has shown promise in a rat model of experimental autoimmune myocarditis (Chang *et al*., 2014), but the conflicting results following blockade of IL-18 alone in PH models raises the question as to whether this is a therapeutic avenue worth exploring further. Regardless, such studies do imply the involvement of both of the primary cytokines produced by the NLRP3 inflammasome, IL-1β and IL-18, in the pathogenesis of PH.

The early response IL-1 family of cytokines released by inflammasomes promote the release of secondary cytokines lying further downstream (Labow *et al*., 1997; Dinarello, 2002; Cahill and Rogers, 2008; Mills *et al*., 2013); akin to the primary cytokines, most of these have also been implicated in PH. Firstly, IL-1β stimulation promotes IL-6 release from macrophages and T-cells (Cahill and Rogers, 2008), and secondly, IL-2 and IL-12 are produced downstream of IL-18 (Dinarello, 2002). Interestingly, Parpaleix *et al*. (2016) found that expression of IL-1β, together with IL-6, is up-regulated in hypoxic mice, and IL-1 receptor signalling is necessary for the development and progression of PH, implicating not only IL-1β but also the IL-1β/IL-6 axis in this disease. Moreover, pharmacological IL-6 blockade (Hashimoto-Kataoka *et al*., 2015) or genetic deletion (Savale *et al*., 2009) protects mice from hypoxia-induced PH, and conversely, lung-specific IL-6 overexpression spontaneously produces PH in mice, an effect worsened by chronic hypoxia (Steiner *et al*., 2009). Finally, subcutaneous injection of recombinant IL-6 also induces PH in rats (Miyata *et al*., 2001) and mice (Golembeski *et al*., 2005). Importantly, these experimental studies are supported by clinical findings (as alluded to above), in which plasma concentrations of the inflammasome-associated cytokines, IL-1β, IL-2, IL-6, IL-12 and IL-18 were significantly increased in PH patients compared to healthy controls. Of these cytokines, increased IL-6 and IL-12 levels correlate with worse outcome and survival (Humbert *et al*., 1995; Soon *et al*., 2010; Ross *et al*., 2012; Groth *et al*., 2014). This body of evidence surrounding IL-6 in PH led to the current UK-wide clinical trial (TRANSFORM-UK), examining the efficacy and safety of a 6-month course of tocilizumab, a human monoclonal antibody targeting the IL-6R in a small number of group 1 PH patients (Hernandez-Sanchez *et al*., 2018). If this study proves successful, a large-scale phase II trial will take place to fully elucidate the efficacy of this approach. Although in the very early stages, this is an exciting development in PH research, and it will be interesting to see if the results from this trial support the strong body of preclinical evidence for IL-6 blockade, as a treatment strategy for PH.

As the efficacy of IL-18 blockade alone in PH is unclear, and the CANTOS trial demonstrated that pan IL-1β blockade is associated with an increased incidence of fatal infection (Ridker *et al*., 2017b), it is reasonable to hypothesize that inhibition of a specific inflammasome subtype is likely to be a safer therapeutic strategy. This is one of the reasons that the recently identified selective NLRP3 inflammasome inhibitor, MCC950 (Coll *et al*., 2015), might be ideal as a therapeutic agent. The ability to selectively inhibit activation of NLRP3 whilst sparing the activity of other inflammasomes (and their ability to produce IL-1β and IL-18) may offer therapeutic benefit, but still allow appropriate response to infection if necessary. Moreover, since MCC950 has a relatively short half-life and is orally bioavailable, in the setting of infection, it could be withdrawn to allow immune function to restore quickly. While the precise mechanism of action of MCC950 remains to be elucidated, Coll *et al*. (2015) successfully demonstrated that MCC950 selectively inhibits activation (but not priming) of the NLRP3 inflammasome both *in vitro* and *in vivo*. Since then, it has shown promising results as a therapeutic agent, being utilized in numerous preclinical models of disease. Those of particular interest in the context of PH include cardiovascular and metabolic disorders such as hypertension (Krishnan *et al*., 2016), myocardial infarction (van Hout *et al*., 2017), atherosclerosis (van der Heijden *et al*., 2017) and obesity (Pavillard *et al*., 2017) along with lung-associated conditions like asthma (Kim *et al*., 2017) and pulmonary inflammation (Primiano *et al*., 2016). The fact that the NLRP3 inflammasome is implicated in other lung and cardiovascular diseases and that MCC950 treatment shows promise in these conditions, taken together with the knowledge that inflammasome related cytokines are elevated in PH patients (Soon *et al*., 2010; Ross *et al*., 2012) and that NLRP3 activation occurs in animal models of PH (Villegas *et al*., 2013; Cero *et al*., 2015; Tang *et al*., 2015; Zeng *et al*., 2017), lends heavily to the possibility that specific targeting of NLRP3, may be beneficial in PH.

These studies support a role, in particular, for the NLRP3 inflammasome in PH. By contrast, Cero *et al*. (2015) found that $ASC^{-/-}$, but not $NLRP3^{-/-}$ mice, were protected from hypoxia-induced PH. Additionally, there is some indirect evidence that the NLRP1 inflammasome could contribute to PH

pathogenicity. Kovarova *et al*. (2012) found that administration of a lethal toxin from anthrax in mice led to pyroptosis induced by NLRP1 activation, causing acute lung injury and death, but mice genetically deficient in NLRP1 were protected from these effects. Intriguingly, gain of function polymorphisms in the NLRP1 gene appear to be associated with asthma in a cohort of Brazilian children (Leal *et al*., 2018). Taken together, these data suggest that the NLRP1 inflammasome plays a role in lung disease, but whether this holds true in PH remains to be seen. The observations of Cero *et al*. (2015), along with the indirect evidence for a role of the NLRP1 inflammasome in PH, are interesting as it raises the possibility that even though there is abundantly more information currently available on the NLRP3 inflammasome, it is not the sole contributor to the pathogenesis of PH. Since the ASC component is common to a number of inflammasome complexes aside from NLRP3, namely, NLRP1, AIM2 and IFI16 (Lee *et al*., 2016), these other inflammasomes, or indeed a combination of inflammasomes, may contribute to PH. Unfortunately, due to the lack of selective inhibitors available for other inflammasome varieties, teasing out the contribution of each could be difficult. Mice deficient in NLRP1 have been developed and utilized in other disease models (Kovarova *et al*., 2012), so further studies could use these mice to help substantiate the above findings and uncover the possible contribution of these inflammasomes.

Conclusions

Inflammasomes, particularly NLRP3, have emerged as promising drug targets in the setting of PH. Both clinical and preclinical evidence support the thesis that modulating the inflammasome, at multiple levels, confers beneficial effects in the disease. Such modulation may encompass disrupting upstream signals such as the P2X7 receptors and NF-κB signal transduction, through to depletion of macrophages and interruption of inflammasome-related cytokines (e.g. IL-1β, IL-6 and IL-18), as well as inflammasome components (i.e. NLRP3, ASC and caspase-1). In reality, not all of these targets are therapeutically viable, as targeting too far upstream, such as at the level of priming, is likely to have unpredictable side effects. The effectiveness of anakinra and canakinumab in auto-inflammatory disorders, and proof-of-concept results from the CANTOS trial, coupled with evidence for the pathogenicity of IL-1β in PH, suggests that drugs modifying inflammasome-generated cytokines might be an efficacious strategy to treat the disease. This is an exciting new avenue in the PH field, and as such, future studies should aim to elucidate the precise nature, and function, of inflammasome(s) involved in the pathogenesis of PH to develop novel and more effective therapies.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al*., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al*., 2017a,b,c,d).

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Conflict of interest

A.H. has been a consultant/advisory board member for Bayer AG, Serodus ASA and Palatin Technologies Inc.

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