Biochemical and Biophysical Research Communications 426 (2012) 486–491

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Viral Toll Like Receptor activation of pulmonary vascular smooth muscle cells results in endothelin-1 generation; relevance to pathogenesis of pulmonary arterial hypertension

Peter M. George [a,1], Rekha Badiger [a,1], Dongmin Shao [b], Michael R. Edwards [c,d,e], Stephen J. Wort [b], Mark J. Paul-Clark [a,*], Jane A. Mitchell [a]

[a] Cardiothoracic Pharmacology, National Heart and Lung Institute, Imperial College London, Dovehouse Street, London, United Kingdom
[b] Unit of Critical Care, Royal Brompton Hospital, National Heart and Lung Institute, Faculty of Medicine, Imperial College London, London, United Kingdom
[c] Department of Respiratory Medicine, National Heart Lung Institute, Imperial College London, United Kingdom
[d] MRC & Asthma UK Centre in Allergic Mechanisms of Asthma, United Kingdom
[e] Centre for Respiratory Infections, United Kingdom

ARTICLE INFO

Article history:
Received 17 August 2012
Available online 28 August 2012

Keywords:
Pulmonary hypertension
Endothelin-1
Toll Like Receptor
TLR3
Virus
Interferon

ABSTRACT

Pulmonary arterial hypertension (PAH) is a rare but fatal condition in which raised pulmonary vascular resistance leads to right heart failure and death. Endothelin-1 is a potent endogenous vasoconstrictor, which is considered to be central to many of the events that lead to PAH, and is an important therapeutic target in the treatment of the condition. In many cases of PAH, the aetiology is unknown but inflammation is increasingly thought to play an important role and viruses have been implicated in the development of disease. The Toll Like Receptors (TLRs) play a key role in innate immune responses by initiating specific anti-bacterial and anti-viral defences in recognition of signature molecular motifs on the surface of invading pathogens. In this study, we set out to examine the expression of bacterial and viral TLRs in human pulmonary artery smooth muscle cells and to establish whether their activation could be relevant to PAH. We found that the viral TLR3 and bacterial TLRs 4 and 6 were most abundantly expressed in human pulmonary artery smooth muscle cells. Using specific TLR ligands, we found that activation of TLRs 3 and 4 resulted in IL-8 release by human pulmonary artery smooth muscle cells but that only TLR3 stimulation resulted in IP10 and endothelin-1 release. These data suggest that human pulmonary artery smooth muscle cells express significant levels of viral TLR3 and respond to its activation by releasing endothelin-1. This may have importance in understanding the association between viruses and the development of PAH.

1. Introduction

Pulmonary arterial hypertension (PAH) describes a collection of rare but devastating conditions that are characterised by increased pulmonary vascular resistance, raised pulmonary arterial pressure and consequent right heart failure. In PAH, a cascade of pathological vascular events occur that are thought to be initiated by endothelial injury and dysfunction and culminate in remodelling of the vascular smooth muscle cells of the pulmonary vessels. However, work from our group suggests that the vascular smooth muscle may be a direct target for inflammatory insults, which in turn leads to vasoconstriction and vascular remodelling [1,2]. Indeed, proliferation of the smooth muscle layer contributes significantly to increasing the pulmonary vascular resistance - thus, understanding how muscularisation of the smooth muscle layer is initiated is critical to our ability to identify new therapeutic targets to treat PAH.

The mechanisms underlying idiopathic PAH (IPAH) are still poorly understood and while there is evidence of a genetic predisposition to the disease, in the form of abnormalities in the bone morphogenetic protein receptor (BMPR) 2 in patients with IPAH [3], penetrance is low suggesting additional factors must be relevant. There is a growing body of evidence associating inflammation and more specifically viral infection with the development of PAH and that these events may represent a second "hit". Inflammatory cytokines such as IL-1β and IL-6 are known to be raised in patients with IPAH and those with other conditions associated with PAH such as scleroderma, mixed connective tissue disease, Eisenmenger syndrome and HIV infection [4]. In fact, it is estimated that the prevalence of PAH in HIV infection is as high as 0.5% [5]. Furthermore, other viruses such as human herpes virus (HHV)-8 have also

* Corresponding author. Address: Cardiothoracic Pharmacology, National Heart and Lung Institute, Imperial College, Dovehouse Street, London SW36LY, United Kingdom.
E-mail address: m.paul-clark@imperial.ac.uk (M.J. Paul-Clark).
[1] Joint first authors.

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.08.106

been implicated in the development of PAH [6]. Like other pathogens, viruses are sensed by pattern recognition receptors including Toll Like Receptors (TLRs). Each of 9 TLRs recognises a specific molecular motif (pathogen associated molecular pattern (PAMP)) on the surface of pathogens and has a defined role in anti-bacterial, anti-fungal and anti-viral defences [7]. Ligation of these receptors initiates signalling cascades that results in the expression of inflammatory mediators, which are an integral part of host defences.

Endothelin-1 (ET-1) is a potent endogenous vasoconstrictor that is a critical therapeutic target in PAH and is physiologically released by the vascular endothelium [8]. However, work from our group over the past 10 years has shown that ET-1 is released from the vascular smooth muscle of both systemic [9,10] and pulmonary vessels [1] under inflammatory conditions. The mechanism of increased expression of ET-1 involves enhanced NF-κB binding and histone acetylation at specific κB sites on the ET-1 promoter [11]. We have also shown that ET-1 acts in an autocrine fashion to stimulate smooth muscle cell proliferation [1] and this may well have relevance to the pathogenesis of PAH. The pathological role of ET-1 in PAH has long been established – levels positively correlate with disease severity [12] and ET-1 receptor blockade is a mainstay of therapy. On a cell by cell basis, the endothelium is the main cell type to produce ET-1. However, the endothelial layer is one cell thick and the cumulative release of ET-1 by the vascular smooth muscle component of large pulmonary vessels which is many layers thick, is likely to reach pathological levels conferring a central role in the pathogenesis of PAH.

Thus far no studies have been published defining the relative expression and function of bacterial versus viral TLRs in pulmonary vascular smooth muscle cells or the relationship this may have with ET-1 release. Given the postulated association between infection/inflammation and the development of PAH and the important role of the vascular smooth muscle in its pathogenesis, in this study we investigated the gene expression of TLRs in human pulmonary artery smooth muscle cells (HPASMCs). We then went on to investigate the functional effect of stimulating TLRs in HPASMCs on ET-1 release. We measured IL-8 and IP10 as surrogates for the MyD88 and TRIF pathways respectively.

2. Materials and Methods

2.1. Cell Culture

Human pulmonary arteries from healthy segments of lung were obtained from patients undergoing pulmonary resection at the Royal Brompton Hospital, London, UK. The adventitia and endothelial cell layer were dissected away under sterile conditions and 3–4 mm^2 sections of vessel were cultured in Dulbecco's modified Eagle media (DMEM) supplemented with 15% foetal calf serum (FCS), penicillin, streptomycin, L-Glutamine and non essential amino acids. HPASMCs were seeded onto 96 well plates for treatment at a concentration of 8,000 cells per well between passages 2 and 9. Cells were then serum starved for 24 h with DMEM containing penicillin, streptomycin, L-Glutamine, non essential amino acids and 0.1% bovine serum albumin and were then treated in DMEM with all of the above supplements and 10% FCS. Supernatant was collected after 24 h and ET-1 and selected cytokines were measured by ELISA (R&D systems, Abingdon, UK) according to manufacturer's instructions.

2.2. PCR for gene expression

For gene expression experiments, HPASMCs were seeded at 150,000 cells per well in 6 well plates. Cells were serum deprived for 24 h and lysates were collected 6 h after cell treatments. RNA was extracted from cell lysates using a commercial RNA extraction kit (Quiagen). A real time polymerase chain reaction (RT-PCR) array obtained from Quiagen was used to establish gene expression of TLRs and other inflammatory mediators. 5 house keeping genes were included in the array and these were 2 β-microglobulin, hypoxanthine phosphoribosyltransferase 1, Ribosomal protein L13a, Glyceraldehyde-3-phosphate dehydrogenase and β-actin. An average of the C_T values for the 5 house keeping genes was used to subtract from the C_T value of the gene of interest to generate a ΔC_T value.

2.3. Materials

DMEM, penicillin, streptomycin, L-Glutamine, non essential amino acids and FCS were obtained from Sigma. TLR agonists were obtained from InvivoGen USA.

3. Results

3.1. Gene expression in HPASMCs

The mRNA expression of TLRs, inflammatory cytokines and chemokines, and relevant signalling proteins in HPASMCs were assessed using a focused RT-PCR array (Fig. 1). The most abundantly expressed TLRs were TLRs 3, 4 and 6 (Fig 1a). IL-6 and CCL2 were by far the most abundantly expressed cytokines (Fig 1b) but interferon-α1 (IFNA1), IL-1 (a and b), TNF receptor superfamily member 1a (TNFRSF1A), IL-8 and IL-12A were also highly expressed in HPASMCs (Fig. 1b). Furthermore, a number of signalling proteins were expressed in HPASMCs – the most highly expressed were interferon regulatory factor 3 (IRF 3), interleukin-1 receptor-associated kinase (IRAK 1), transforming protein p21 (HRAS) and Ubiquitin-conjugating enzyme E2N (UBE2N) (Fig. 1c).

3.2. Effects of TLR activation in HPASMCs

HPASMCs were stimulated for 24 h with a range of bacterial TLR agonists – Pam3CSK4, which activates the TLR2/TLR1 heterodimer; heat-killed Gram positive *Staphylococcus aureus*, which activates TLR2; heat-killed Gram negative *Esherichia coli*, which activates TLR4; Lipopolysaccahride (LPS), which activates TLR4; flagellin, which activates TLR5 and FSL-1, which activates the TLR2/TLR6 heterodimer (Fig. 2a–c) and viral TLR agonists – Poly(I:C), which activates TLR3; Imiquimod, which activates TLR7 and clo 75, which activates TLR8 (Fig. 2d–f) after which conditioned media was analysed for IL-8, IP10 and ET-1 release. Under control culture conditions HPASMCs release relatively low levels of IL-8 (Fig. 2a and d), IP10 (Fig. 2b and e) or ET-1 (Fig. 2c and f). Of the TLR agonists tested, only TLR3 and TLR4 agonists induced significantly raised levels of IL-8 (Fig. 2a and b). Only the TLR3 agonist Poly(I:C) induced significantly raised levels of IP-10 and ET-1 (Fig. 2 d and f). It should be noted that the putative TLR8 agonist *E. coli* RNA/Lyovec (10 μg/ml) induced raised levels of ET-1from a basal level of 1.92 pg/ml (±0.42)–7.10 pg/ml (±0.17), IL-8 from 655.46 pg/ml (±190.32) to 956.56 pg/ml (±416.55) and IP10 from 94.18 pg/ml (±27.33) to 1585.21 pg/ml (±118.12). As this molecule is no longer commercially available, we were unable experiment on it further and could not reproduce these finding using any of the 3 other commercially available putative TLR8 agonists (clo 75; Fig. 2d–f), clo 97 or ssPolyU/Lyovec (data not shown).

3.3. Effect of TNF-α on IL-8, IP10 and ET-1 release induced by Poly(I:C) from HPASMCs

TNF-α synergises with Type I and Type II IFN to release ET-1 from HPASMCs [13] or IP10 from human airway smooth muscle

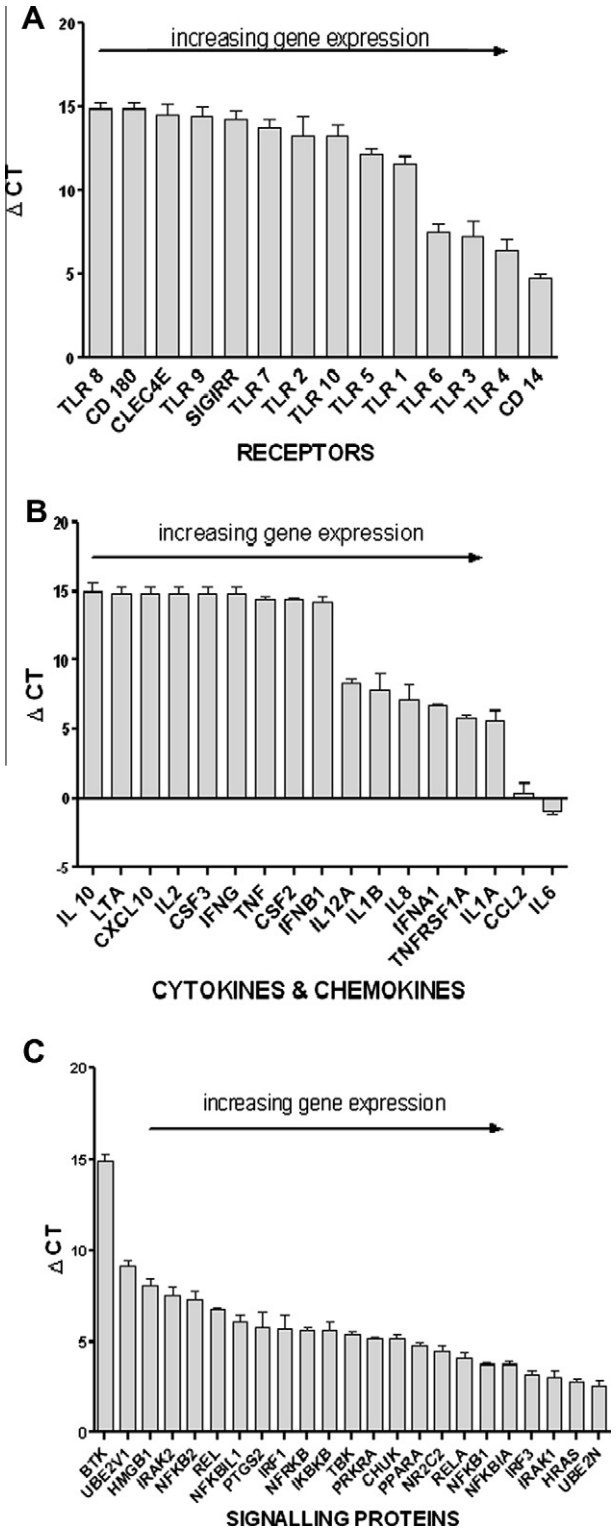

Fig. 1. Gene expression of Toll Like Receptors (A), cytokines and chemokines (B) and signalling proteins (C) in human pulmonary artery smooth muscle cells. Data is mean ± SEM for $n = 3$. ΔC_T values of 15 indicates no or minimal gene expression. The lower the ΔC_T, the greater the level of gene expression.

cells [14]. Since TLR3 activates IFN signalling pathways in cells, we performed separate experiments to investigate the effect of co-stimulating HPASMCs with TNF-α and Poly(I:C) on release of IL-8, IP10 and ET-1 (Fig.3 A–C). In these experiments, as was seen above, Poly(I:C) induced IL-8 (Fig. 3A), IP10 (Fig. 3B) and ET-1 (Fig. 3C) release from HPASMCs after 24 h stimulation. TNF-α had a direct effect on IL-8 release which did not appear to be additive with that induced by Poly(I:C) (Fig. 3A). By contrast, TNF-α had no direct effect on IP10 (Fig. 3B) or ET-1 (Fig. 3C) but provided a clear synergistic effect when co-administered with Poly(I:C).

4. Discussion

In this study we have demonstrated that HPASMCs express relatively high levels of TLR3 mRNA and respond avidly to TLR3 agonists by releasing ET-1, the endogenous therapeutic target for PAH. TLR3 is a viral sensing pattern recognition receptor and, as such, our findings support the hypothesis that viral infection may be an important pro-inflammatory stimulus for smooth muscle activation, pulmonary artery vascular remodelling and eventually the development of PAH. TLR3 is an endosomal TLR that plays a vital role in anti-viral host defences by recognising viral double stranded RNA molecules and initiating interferon signalling pathways [15]. Others have demonstrated TLR3 gene expression in pulmonary arterial smooth muscle cells [16] but our work is the first to profile the relative expression of TLRs (amongst other related genes) or a role in ET-1 generation in this important cell type.

TLRs mediate their effect via two well-defined adapter protein/signalling pathways; these are MyD88 and TRIF [7]. ET-1 is not a typical 'pathogen associated gene' and is generally regulated in endothelial cells by angiogenic factors such as VEGF, angiotensin II or serum [17]. In order to understand how TLR induced ET-1 might profile with better studied genes we measured IL-8 and IP10 release as they are considered surrogate markers of the MyD88 and TRIF pathway respectively [7,18]. It was interesting to note that whilst Poly(I:C) induced IL-8, IP10 and ET-1, TLR4 activation with heat killed *E. coli Coli* or LPS, stimulated HPASMCs to release IL-8 only. This validates our observations that, along with TLR3, TLR4 is highly expressed in these cells and indicates that ET-1 is a product of TLR3/TRIF rather than TLR4/MyD88 pathways. This is in keeping with what we know of TRIF genes and their strong association with IFN signalling. While TLR3 senses double stranded RNA viruses, TLR8 senses single stranded RNA viruses. In early work, we found that the putative TLR8 agonist *E. coli* RNA/Lyovec could induce IL-8, IP10 and ET-1 in HPASMCs. However, as this molecule is no longer commercially available, we could not perform confirmatory experiments nor look for evidence of synergy with TNF-α. We performed similar experiments with three further putative TLR8 agonists - clo 75, clo 97 and ssPolyU/Lyovec (data only shown for clo 75 as representative of all three). On balance, and taking into account the low levels of TLR8 expression in these cells, we conclude that the effects of *E. coli* RNA/Lyovec could be a result of TLR8 independent effects, possibly via TLR3 activation.

We have previously shown that type I and II IFN can stimulate HPASMCs to release ET-1, an effect that is synergistically enhanced by TNF-α [1,11,13]. Interestingly, for ET-1 or IP10 release, we found a similar relationship between TNF-α and Poly(I:C) as we had found with IFN. TLR3 stimulation leads to activation of TRIF dependent signalling cascades that result in the generation of Types I,II and III IFN. Work from our group has previously demonstrated that TNF-α and IFN-γ act synergistically to stimulate the induction of preproET-1 mRNA and release of mature ET1- protein through cooperative p65 binding and histone acetylation at distinct κB sites in the preproET-1 [11]. As TLR3 stimulation strongly induces IFN production, we hypothesise that endogenously produced IFN acts in an autocrine manner to synergise with TNF-α and induce the production of ET-1. TNF-α is a key cytokine that is largely produced by activated macrophages but, importantly, is also released by vascular smooth muscle cells [19] and endothelial cells [20]. The ob-

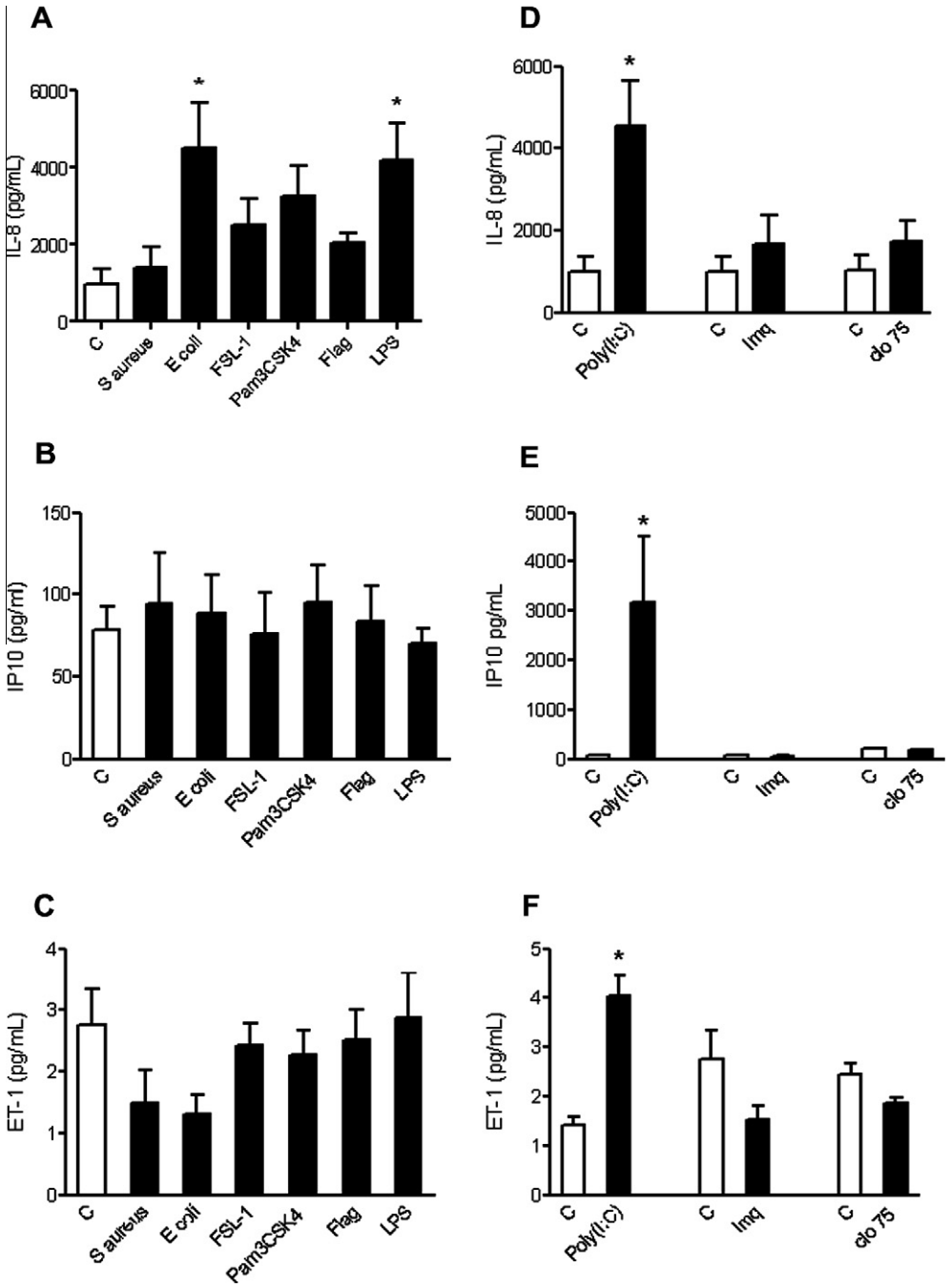

Fig. 2. Effect of bacterial (A–C) and viral (D–F) Toll Like Receptor (TLR) agonists on IL-8 (A,D), IP10 (B,E) and ET-1 (C,F) release by human pulmonary artery smooth muscle cells. Bacterial TLR agonists: TLR1/2 agonist – Pam3CSK4 (300 ng/ml), TLR2 agonist – *Staphylococcus aureus* (10^8 cfu/ml), TLR4 agonists – *Escherichia coli* (10^8 cfu/ml) and Lipopolysaccharide (LPS) (1 μg/ml), TLR5 agonist – Flagellin (Flag) (1 μg/ml), TLR2/6 agonist – FSL-1 (1 μg/ml). Viral TLR agonists – TLR3 agonist Poly(I:C), (10 μg/ml), TLR7 agonist imiquimod (10 μg/ml) and TLR8 agonist clo 75 (5 μg/ml). Data is the mean ± SEM for $n = 8–9$. Data in (A–C) was analysed by one-way ANOVA followed by Dunnett's post-test. Data in (D–F) was analysed using two-way ANOVA followed by the Bonferroni post test; $^*p < 0.05$.

served synergy between Poly(I:C) and TNF-α may be particularly important in our understanding of how cytokines network in viral infection particularly in relation to vascular inflammation and PAH. Indeed, in clinical studies of patients with PAH, serum levels of TNF-α and other pro-inflammatory cytokines IL-1β, IL-2, IL-4 IL-6, IL-8, IL-10 and IL-12p70 are raised as compared to healthy controls [21].

It is interesting to note that genes for these cytokines and chemokines were amongst the most highly expressed in HPASMCs. IL-6 gene expression was abundant in HPASMCs and this is of particular relevance as it has a well established and central role in vascular inflammation [22] and is elevated in the serum of patients with PAH [4]. In addition, IFN-α and TNFRSF1A (tumour necrosis factor receptor superfamily 1 A, one of the major receptors for TNF-α) were also highly expressed in HPASMCs. Again, at this stage we can only speculate on the relevance of gene expression in these cells to PAH, but it is interesting to note that IFN-α stimulates HPASMCs to release ET-1 in vitro, an effect that is increased by TNF-α [13].

This study is the first to demonstrate that TLR3 stimulation of HPASMCs results in the release of ET-1 and this may provide important clues in explaining the pathogenesis of IPAH. While

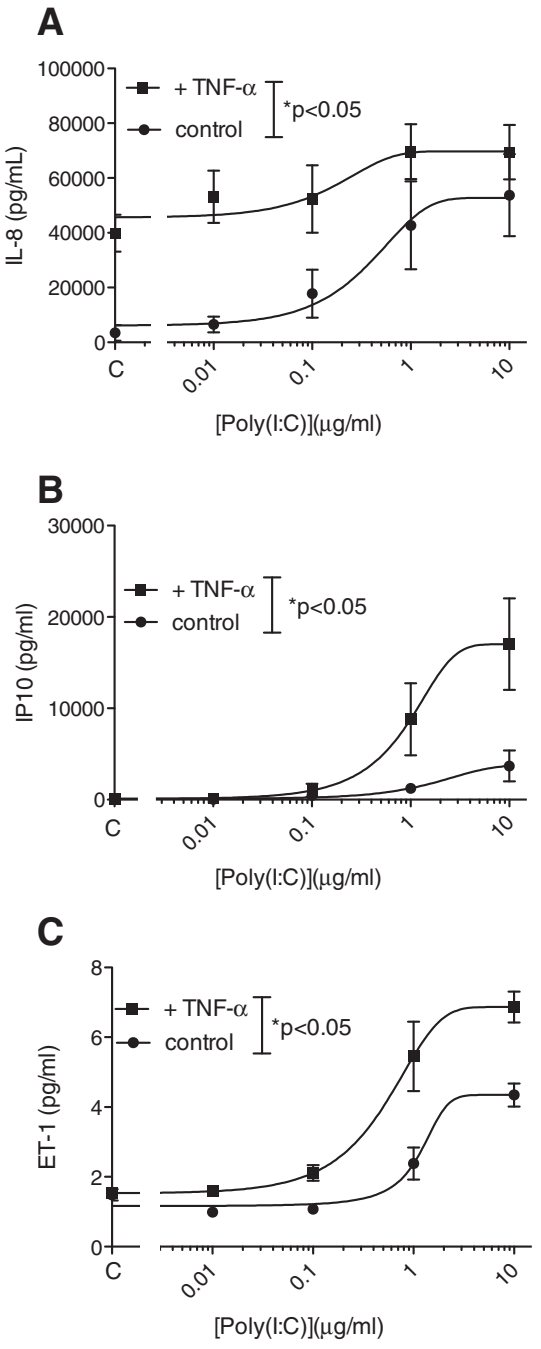

Fig. 3. Effect of the TLR3 agonist Poly(I:C) on IL-8 (A), IP10 (B) and ET-1 (C) release from human pulmonary artery smooth muscle cells. Cells were also stimulated with or without TNFα (10 ng/ml). Data is the mean ± SEM for $n = 3$–4. Data in the presence versus absence of TNFα was analysed using two-way ANOVA; $^{*}p < 0.05$.

we do not yet know the clinical relevance of our in-vitro work, other investigators have found that of all the TLRs, only TLR3 stimulation of endothelial cells and dermal fibroblasts resulted in upregulation of ET-1 protein and mRNA [23]. In confirmatory in-vivo mouse experiments, subcutaneous Poly(I:C) administration resulted in significant increase of ET-1 and Endothelin Converting Enzyme (ECE) mRNA. This growing body of in-vivo and in-vitro evidence appears to implicate TLR3 in PAH but further studies in lung tissue from humans with PAH are now required to allow a full appreciation of the impact of this data in man. As previously discussed, the vascular smooth muscle layer of pulmonary arteries is likely to play a key role in the development of PAH. As the vascular smooth muscle is the target cell for ET-1, generation within the vascular wall, at the site of action is likely to be at least as important as (potentially) higher levels generated by the endothelium. In addition, previous work from our group has demonstrated that endogenous ET-1 release drives proliferation of HPASMCs [1].

In summary, this study is the first to reveal a link between TLR3 and ET-1 release by human lung vascular cells. HPASMCs express TLR3 abundantly and respond avidly to the viral mimetic Poly(I:C) by releasing inflammatory cytokines and ET-1. These findings may provide some mechanistic insight to the theory that viruses may be latent 'second hit' candidates for the development of PAH.

Role of funding sources

No funding source was involved in any of the following; study design, data collection, analysis, interpretation of data; writing of the report or in the decision to submit this paper for publication.

Acknowledgments

PMG is an MRC funded Clinical Research Training Fellow.

References

[1] S.J. Wort, M. Woods, T.D. Warner, T.W. Evans, J.A. Mitchell, Endogenously released endothelin-1 from human pulmonary artery smooth muscle promotes cellular proliferation: relevance to pathogenesis of pulmonary hypertension and vascular remodeling, Am. J. Respir. Cell Mol. Biol. 25 (2001) 104–110.

[2] D. Shao, J.E. Park, S.J. Wort, The role of endothelin-1 in the pathogenesis of pulmonary arterial hypertension, Pharmacol. Res. 63 (2011) 504–511.

[3] R.D. Machado, M.A. Aldred, V. James, R.E. Harrison, B. Patel, E.C. Schwalbe, E. Gruenig, B. Janssen, R. Koehler, W. Seeger, O. Eickelberg, H. Olschewski, C.G. Elliott, E. Glissmeyer, J. Carlquist, M. Kim, A. Torbicki, A. Fijalkowska, G. Szewczyk, J. Parma, M.J. Abramowicz, N. Galie, H. Morisaki, S. Kyotani, N. Nakanishi, T. Morisaki, M. Humbert, G. Simonneau, O. Sitbon, F. Soubrier, F. Coulet, N.W. Morrell, R.C. Trembath, Mutations of the TGF-beta type II receptor BMPR2 in pulmonary arterial hypertension, Hum. Mutat. 27 (2006) 121–132.

[4] M. Humbert, G. Monti, F. Brenot, O. Sitbon, A. Portier, L. Grangeot-Keros, P. Duroux, P. Galanaud, G. Simonneau, D. Emilie, Increased interleukin-1 and interleukin-6 serum concentrations in severe primary pulmonary hypertension, Am. J. Respir. Crit. Care Med. 151 (1995) 1628–1631.

[5] R. Speich, R. Jenni, M. Opravil, M. Pfab, E.W. Russi, Primary pulmonary hypertension in HIV infection, Chest 100 (1991) 1268–1271.

[6] C.D. Cool, P.R. Rai, M.E. Yeager, D. Hernandez-Saavedra, A.E. Serls, T.M. Bull, M.W. Geraci, K.K. Brown, J.M. Routes, R.M. Tuder, N.F. Voelkel, Expression of human herpesvirus 8 in primary pulmonary hypertension, N. Engl. J. Med. 349 (2003) 1113–1122.

[7] M.J. Paul-Clark, P.M. George, T. Gatheral, K. Parzych, W.R. Wright, D. Crawford, L.K. Bailey, D.M. Reed, J.A. Mitchell, Pharmacology and therapeutic potential of pattern recognition receptors, Pharmacol. Ther. 135 (2012) 200–215.

[8] M. Yanagisawa, H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto, T. Masaki, A novel potent vasoconstrictor peptide produced by vascular endothelial cells, Nature 332 (1988) 411–415.

[9] M. Woods, J.A. Mitchell, E.G. Wood, S. Barker, N.R. Walcot, G.M. Rees, T.D. Warner, Endothelin-1 is induced by cytokines in human vascular smooth muscle cells: evidence for intracellular endothelin-converting enzyme, Mol. Pharmacol. 55 (1999) 902–909.

[10] M. Woods, D. Bishop-Bailey, J.R. Pepper, T.W. Evans, J.A. Mitchell, T.D. Warner, Cytokine and lipopolysaccharide stimulation of endothelin-1 release from human internal mammary artery and saphenous vein smooth-muscle cells, J. Cardiovasc. Pharmacol. 31 (Suppl. 1) (1998) S348–S350.

[11] S.J. Wort, M. Ito, P.C. Chou, S.K. Mc Master, R. Badiger, E. Jazrawi, P. de Souza, T.W. Evans, J.A. Mitchell, L. Pinhu, K. Ito, I.M. Adcock, Synergistic induction of endothelin-1 by tumor necrosis factor alpha and interferon gamma is due to enhanced NF-kappaB binding and histone acetylation at specific kappaB sites, J. Biol. Chem. 284 (2009) 24297–24305.

[12] C. Rubens, R. Ewert, M. Halank, R. Wensel, H.D. Orzechowski, H.P. Schultheiss, G. Hoeffken, Big endothelin-1 and endothelin-1 plasma levels are correlated with the severity of primary pulmonary hypertension, Chest 120 (2001) 1562–1569.

[13] P.M. George, R. Badiger, W. Alazawi, G.R. Foster, J.A. Mitchell, Pharmacology and therapeutic potential of interferons, Pharmacol. Ther. 135 (2012) 44–53.

[14] D.L. Clarke, R.L. Clifford, S. Jindarat, D. Proud, L. Pang, M. Belvisi, A.J. Knox, TNFα and IFNγ synergistically enhance transcriptional activation of CXCL10 in human airway smooth muscle cells via STAT-1, NF-κB, and the transcriptional coactivator CREB-binding protein, J. Biol. Chem. 285 (2010) 29101–29110.

[15] L. Slater, N.W. Bartlett, J.J. Haas, J. Zhu, S.D. Message, R.P. Walton, A. Sykes, S. Dahdaleh, D.L. Clarke, M.G. Belvisi, O.M. Kon, T. Fujita, P.K. Jeffery, S.L. Johnston, M.R. Edwards, Co-ordinated role of TLR3, RIG-I and MDA5 in the innate response to rhinovirus in bronchial epithelium, PLoS Pathog. 6 (2010) e1001178.
[16] X. Yang, V. Murthy, K. Schultz, J.B. Tatro, K.A. Fitzgerald, D. Beasley, Toll-like receptor 3 signaling evokes a proinflammatory and proliferative phenotype in human vascular smooth muscle cells, Am. J. Physiol. Heart Circ. Physiol. 291 (2006) H2334–H2343.
[17] F. Rodriguez-Pascual, O. Busnadiego, D. Lagares, S. Lamas, Role of endothelin in the cardiovascular system, Pharmacol. Res.: Official J. Italian Pharmacol. Soc. 63 (2011) 463–472.
[18] T. Hirotani, M. Yamamoto, Y. Kumagai, S. Uematsu, I. Kawase, O. Takeuchi, S. Akira, Regulation of lipopolysaccharide-inducible genes by MyD88 and Toll/IL-1 domain containing adaptor inducing IFN-beta, Biochem. Biophys. Res. Commun. 328 (2005) 383–392.
[19] W.H. Newman, L.M. Zhang, S.K. Leeper-Woodford, M.R. Castresana, Human blood vessels release tumor necrosis factor-alpha from a smooth muscle cell source, Crit. Care Med. 24 (1996) 294–297.
[20] V. Ranta, A. Orpana, O. Carpén, U. Turpeinen, O. Ylikorkala, L. Viinikka, Human vascular endothelial cells produce tumor necrosis factor-alpha in response to proinflammatory cytokine stimulation, Crit. Care Med. 27 (1999) 2184–2187.
[21] E. Soon, A.M. Holmes, C.M. Treacy, N.J. Doughty, L. Southgate, R.D. Machado, R.C. Trembath, S. Jennings, L. Barker, P. Nicklin, C. Walker, D.C. Budd, J. Pepke-Zaba, N.W. Morrell, Elevated levels of inflammatory cytokines predict survival in idiopathic and familial pulmonary arterial hypertension, Circulation 122 (2010) 920–927.
[22] M. Klouche, S. Bhakdi, M. Hemmes, S. Rose-John, Novel path to activation of vascular smooth muscle cells: up-regulation of gp130 creates an autocrine activation loop by IL-6 and its soluble receptor, J. Immunol. 163 (1999) 4583–4589.
[23] G. Farina, M. York, C. Collins, R. Lafyatis, DsRNA activation of endothelin-1 and markers of vascular activation in endothelial cells and fibroblasts, Ann. Rheum. Dis. 70 (2011) 544–550.