

Tristetraprolin and its role in regulation of airway inflammation

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Non-standard abbreviations: ARE, adenosine/uridine-rich element; AP-2, activating protein 2; COPD, chronic obstructive pulmonary disease; EGR-1, early growth response protein 1; ERK, extracellular signal related kinase; FEV1, forced expiratory volume in one second; FVC, forced vital capacity; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; miR, microRNA; MKP-1, mitogen-activated protein kinase phosphatase 1; NF- κ B, nuclear factor κ B; PKC, protein kinase C; PP2A, protein phosphatase 2A; SP-1, specificity protein 1; TPE1, TTP promoter element 1; TTP, tristetraprolin; UTR, untranslated region.

Abstract

Chronic inflammatory diseases, such as asthma and chronic obstructive pulmonary disease (COPD), are clinically and socio-economically important diseases globally. Currently the mainstay of anti-inflammatory therapy in respiratory diseases is corticosteroids. Although corticosteroids have proven clinical efficacy in asthma, many asthmatic inflammatory conditions (e.g. infection, exacerbation, severe asthma) are not responsive to corticosteroids. Moreover, despite an understanding that COPD progression is driven by inflammation we currently do not have effective anti-inflammatory strategies to combat this disease. Hence alternative anti-inflammatory strategies are required. p38 mitogen-activated protein kinase (p38 MAPK) has emerged as an important signalling molecule driving airway inflammation, and pharmacological inhibitors against p38 MAPK may provide potential therapy in chronic respiratory disease. In this review we discuss some of the recent *in vitro* and *in vivo* studies targeting p38 MAPK to date, but suggest that p38 MAPK inhibitors may prove less effective than originally considered due to the fact that they may block anti-inflammatory molecules along with pro-inflammatory responses. We propose that an alternative strategy may be to target an anti-inflammatory molecule further downstream of p38 MAPK, i.e. tristetraprolin (TTP). TTP is an mRNA-destabilising RNA-binding protein that enhances the decay of mRNAs, including those encoding proteins implicated in chronic respiratory diseases. We suggest that understanding the molecular mechanism of TTP expression and its temporal regulation will guide future development of novel anti-inflammatory pharmacotherapeutic approaches to combat respiratory disease.

Introduction

Many chronic inflammatory diseases are a consequence of overactive inflammatory signalling pathways. Hence, repressing these pathways as potential drug targets represents a way in which to re-establish control and attenuate the severity of various chronic inflammatory diseases. However, these pathways are not exclusively responsible for pro-inflammatory signalling and targeting these pathways may cause the ablation of other vital, anti-inflammatory signals necessary for restoring normal physiology. We propose that this may be the case with targeting one of the members of the mitogen-activated protein kinase (MAPK) superfamily - p38 MAPK - in respiratory disease. That is, while p38 MAPK inhibitors may repress pro-inflammatory cytokines, they may also inhibit the expression of crucial anti-inflammatory proteins, such as tristetraprolin (TTP). TTP is a destabilising RNA binding protein that promotes mRNA decay of many proteins, including those implicated as playing a role in chronic respiratory diseases. In this review we focus on the role of the p38 MAPK signalling pathway in respiratory disease and highlight some *in vitro* and *in vivo* evidence with p38 MAPK inhibitors. We outline the role of TTP in the regulation of inflammation in other disease settings and highlight the potential of understanding the molecular mechanism of TTP expression and its temporal regulation towards future development of novel anti-inflammatory pharmacotherapeutic approaches in respiratory disease.

MAPK

Members of the mitogen-activated protein kinase (MAPK) superfamily play important roles in the regulation of airway inflammation in respiratory disease pathogenesis. Three well-defined phosphoproteins; ERK (extracellular signal related kinase), JNK (c-Jun N-terminal kinase) and p38 MAPK, are the MAPKs central to this signalling cascade. The signalling cascade that results in the activation of MAPKs is derived from a relatively linear framework

(see Figure 1). Stimuli such as cellular stressors or mitogens interact in a predominately receptor-mediator manner to trigger the phosphorylation of a specific MAPKKK (MAPK kinase kinase) which then causes the phosphorylation of its specific downstream MAPKK (MAPK kinase). Once MAPKK is phosphorylated it is subsequently able to phosphorylate its own substrate MAPK. Activated MAPKs then represent a point of divergence leading to the phosphorylation of numerous downstream transcription factors, protein kinases and proto-oncogenes (Ashwell, 2006; Balakathiresan et al., 2009; Hu et al., 2007). In this review we will focus on p38 MAPK and its downstream signalling effectors. The p38 MAPK-mediated pathway regulates a plethora of cellular processes that drive chronic airway inflammatory disease (Chung, 2011). Extensive investigation of the p38 MAPK-driven molecular mechanisms responsible for respiratory disease pathogenesis has led to drug discovery programmes that target this pathway as a potential anti-inflammatory therapy. Moreover, p38 MAPK has also been significantly implicated in corticosteroid insensitivity (Ammit, 2013).

p38 MAPK: role in airway inflammation and corticosteroid insensitivity

There are four isoforms of p38 MAPK - α , β , δ , γ - transcribed by four separate genes and characterised by different expression patterns. p38 α is expressed ubiquitously and along with p38 β is considered to represent the major isoform responsible for inflammatory responses. Inflammation is a central component of many respiratory diseases, especially asthma and COPD, and the use of selective pharmacological inhibitors has positioned p38 MAPK as playing a major role in disease severity and progression. The majority of the *in vitro* studies have utilized the inhibitor SB203580, a pyridinyl imidazole that binds competitively at the ATP-binding site of p38 MAPK. It is widely considered to predominately target p38 α and p38 β , but not the δ and γ isoforms of p38 MAPK, although questions regarding selectively have been raised, especially at higher doses (Bain et al., 2007). Nevertheless, extensive

investigations utilizing this first-generation inhibitor have yielded much valuable information regarding the role of p38 MAPK in chronic respiratory disease. p38 MAPK regulates the immunomodulatory function of various airway cells through the control of chemokine and cytokine release (Amrani et al., 2001; Hallsworth et al., 2001; Henness et al., 2006; Quante et al., 2008). p38 MAPK is also responsible for neutrophil and eosinophil migration (Rousseau et al., 1997), altering the airway smooth muscle contractility, causing obstruction of air flow (Bhavsar et al., 2008; Lakser et al., 2002), releasing degradative enzymes, like matrix metalloproteinases (Woo et al., 2004), and causing the remodelling of airway architecture by increasing smooth muscle proliferation (Nath et al., 2006).

p38 MAPK has also been significantly implicated in corticosteroid insensitivity (Bhavsar et al., 2008; Chang et al., 2012). Inhaled corticosteroids are the mainstay of chronic asthma treatment and are used effectively at low doses for the majority of asthmatics. However, there is a subset of the asthmatic population with severe asthma who need maximal doses of inhaled corticosteroids and another small subset of the asthmatic population who are resistant to corticosteroids. A detailed account of the molecular mechanisms thought responsible for corticosteroid insensitivity and resistance have been outlined in recent reviews (Ammit, 2013; Barnes, 2013). Interestingly, p38 MAPK isoforms, p38 γ (Bhattacharyya et al., 2011) and p38 α/β (Mercado et al., 2012) in particular, may serve as legitimate targets towards restoring corticosteroid responsiveness. Thus, targeting p38 MAPK, and perhaps in an isoform-specific manner, might prove to have clinical efficacy as an anti-inflammatory treatment in chronic respiratory disease, especially in patients who respond very poorly to inhaled corticosteroids.

Use of p38 MAPK inhibitors to repress inflammatory disease: what have we learned?

First-generation inhibitors of p38 MAPK include pharmacological inhibitors like SB203580, BIRB 796, SB202190 and SB239063. These pyridinyl imidazole compounds competitively

antagonize the ATP binding site thereby blocking the activity of p38 MAPK (Lee et al., 1994). Recent studies of p38 MAPK inhibitors have used these compounds as a template to create a new set of slightly remodelled and more selective p38 MAPK inhibitors. These inhibitors offer a broad range of anti-inflammatory effects since p38 MAPK is responsible for a large portion of inflammatory responses. Inhibiting the p38 MAPK pathway in animal models showed a dramatic reduction in levels of pro-inflammatory cytokines and thereby reasonably attenuated chronic inflammatory responses (Ronkina et al., 2010; Young et al., 1993). These compounds have not yet been used in the treatment of asthma but they have been used marginally in clinical trials for rheumatoid arthritis (Haddad, 2001) and Crohn's disease (Schreiber et al., 2006).

More recently, three molecules have been recently trialled as potential anti-inflammatory molecules in COPD. Losmapimod, an oral inhibitor of p38 MAPK was shown to be effective in improving plasma fibrinogen levels and levels of hyperinflation in patients with COPD (Lomas et al., 2012). However it was also revealed to be ineffective in reducing IL-6, IL-8 and C-reactive protein. Similarly another p38 MAPK inhibitor, dilmapimod, was administered as a single oral dose in a 4 week clinical trial and demonstrated reduction in phosphorylated heat shock protein 27 and TNF α production (Singh et al., 2010). Similar to losmapimod, dilmapimod was also able to reduce serum fibrinogen and FVC (forced vital capacity) but unable to alter levels of IL-6 or IL-8. Another 6 week clinical study has suggested that a small molecule p38 MAPK inhibitor, PH-797804 (Xing et al., 2012), was effective in increasing some measurable airway criteria like FEV1 (forced expiratory volume in one second). This study also reported that PH-797804 reduced systemic fibrinogen and circulating cytokines in patients with moderate to severe COPD in comparison to the placebo patients. A randomised clinical trial assessing safety and efficacy of PH-797804 in COPD has been conducted (MacNee et al., 2013) demonstrating improvements over placebo, albeit with limited sample

size. This has led to “cautious optimism” (see Editorial (Singh, 2013)) while noting the possibility of unwanted effects and potential for transient efficacy of p38 MAPK inhibition, as observed with rheumatoid arthritis.

Efficacy of these trials can be used as a yard stick to determine if any generation of p38 MAPK inhibitors can be used as monotherapy or in combination with corticosteroids. But there are important lessons to be learned from trials of first generation p38 MAPK inhibitors in rheumatoid arthritis. The trials have been largely unsuccessful in rheumatoid arthritis due to the unexpected inefficacy and side effects. The side effects associated with the use of p38 MAPK inhibitors, such as hepato-toxicity, dizziness and skin rash, may occur as a result of potential non-specific inhibition (Heinrichsdorff et al., 2008). The unexpected inefficacy could also be attributed to the activation of alternate pathways to induce pro-inflammatory molecules including nuclear factor κ B (NF- κ B) (Hammaker and Firestein, 2010). Notably, Clark and Dean (Clark and Dean, 2012) have raised the important issue of p38 MAPK-driven anti-inflammatory proteins to explain the apparent failure of p38 MAPK inhibitors in the rheumatology clinic. They state ...“It is likely that the failure of p38 inhibitors is connected to the important role of the p38 pathway in the negative feedback control of inflammatory responses” (Clark and Dean, 2012). That is, inhibiting p38 MAPK also blocks the endogenous anti-inflammatory proteins present that naturally curtail the inflammatory response. Important anti-inflammatory molecules, including IL-10, mitogen-activated protein kinase phosphatase 1 (MKP-1) and TTP, are upregulated in response to increased p38 MAPK activation. Hence p38 MAPK inhibitors represent a double-edged sword. Going further upstream in the MAPK signalling pathway (see Figure 1) could be an alternative option as the therapeutic efficacy could be higher, but there is a greater risk of incurring further non-specific side effects. Another possible option could be to target the anti-inflammatory molecules that regulate the MAPK pathway. Preventing the suppression or prolonging the activation of the transiently

activated anti-inflammatory molecules could be of therapeutic benefit. This is the promise of TTP.

Alternative anti-inflammatory molecule: TTP

TTP is a critical anti-inflammatory protein that functions to promote mRNA decay. Many clinically important proteins, including cytokines, are controlled by post-transcriptional mRNA stabilizing mechanisms (see recent review (Brooks and Blackshear, 2013)). Notably, many of these proteins have been implicated in asthma (Table 1, adapted from (Brooks and Blackshear, 2013)). We assert that an important consideration that will likely affect success of p38 MAPK inhibitors in respiratory disease is that the expression and function of this critical anti-inflammatory protein is controlled by p38 MAPK. Because of this, inhibiting p38 MAPK to repress pro-inflammatory proteins will also repress important anti-inflammatory molecules such as TTP.

TTP target implicated in asthma	Function in asthma pathogenesis	Shown to be regulated by TTP in cell lines/cell types
IL-17A	Inducing and mediating pro-inflammatory responses	Human T cell lines (Lee et al., 2012)
IL-6	Pro-inflammatory (neutrophil production)	Various (Brahma et al., 2012; Chafin et al., 2013; Jalonen et al., 2006; Mercado et al., 2012; Banerjee, 2014 #1604; Neininger et al., 2002; Patil et al., 2008; Tudor et al., 2009; Van Tubergen et al., 2011; Zhao et al., 2011)
IL-1 β	Many pro-inflammatory functions	Mouse RAW264.7 cells (Chen et al., 2006)
TNF α	Many pro-inflammatory functions	Mouse macrophages (Carballo et al., 1998; Taylor et al., 1996a)
CCL2	Recruitment of inflammatory mediators	Mouse macrophages, fibroblasts (Sauer et al., 2006)
CXCL8	Recruitment of neutrophils	Various cell lines (Al Ghouleh and Magder, 2012; Balakathiresan et al., 2009;

		Bhattacharyya et al., 2011; Colin et al., 2014; Galbiati et al., 2012; Mercado et al., 2012; Suswam et al., 2008; Suswam et al., 2013)
GM-CSF	Increases levels of neutrophils and eosinophils	Mouse bone-marrow derived stromal cells (Carballo et al., 2000)
VEGF	Promotes angiogenesis	Various (Banerjee et al., 2014; Essafi-Benkhadir et al., 2007; Essafi-Benkhadir et al., 2010; Lee et al., 2010; Bhattacharyya, 2011 #1594; Suswam et al., 2008)
IL-13	Involved in airway remodelling	Mast cells (Barnstein et al., 2006)
IL-23	Neutrophil infiltration	Mouse dendritic cells, macrophages (Molle et al., 2013; Qian et al., 2011)
CCL5	Chemotaxis of chemokines and cytokines	Mouse dendritic cells, peripheral blood mononuclear cells (Jin et al., 2012; Rosenberger et al., 2012)
ICAM-1	Recruitment of circulating pro-inflammatory mediators	Human pulmonary microvascular endothelial cells (Colin et al., 2014; Mercado et al., 2012)
COX-2	Regulates PGD ₂	Various (Bhattacharyya et al., 2011; Hammaker and Firestein, 2010; Phillips et al., 2004; Sully et al., 2004; Tudor et al., 2009)
iNOS	Modulation airway and vascular smooth muscle tone	DLD-1 cells, rat cortical astrocytes (Linker et al., 2005; Lisi et al., 2011)
IL-2	Inducing and mediating pro-inflammatory responses	Mouse splenocytes (Ogilvie et al., 2005)
CXCL1	Neutrophil chemoattractant	Mouse macrophages (Datta et al., 2008)
MMP-9	Remodelling of the extra cellular matrix	3D cancer model, chick chorioallantoic membrane (Van Tubergen et al., 2013)
CCL3	Chemotaxis of chemokines and cytokines, inflammatory mediator release	Mouse macrophages, fibroblasts (Kang et al., 2011; Sauer et al., 2006)

TTP is a destabilising RNA binding protein that enhances mRNA decay

A role for TTP as a *trans*-acting anti-inflammatory protein was first elucidated when the TTP knockout mouse developed a pro-inflammatory phenotype due to the overexpression of the

cytokine TNF α in macrophages resulting in cachexia, myeloid hyperplasia and a host of other inflammatory conditions (Taylor et al., 1996a). Based on evidence from earlier work (Caput et al., 1986) it was concluded that TTP confers mRNA instability and degradation by binding the conserved adenosine/uridine-rich element (ARE) present within the 3'-untranslated region (UTR) of mRNA transcripts containing the nonameric sequence UUAUUUAUU (Carballo et al., 1998; Lai et al., 1999). This promotes the poly(A) tail shortening shown in transcripts of GM-CSF and TNF α (Carballo et al., 2000; Marchese et al., 2010). The importance of this *cis*-element is highlighted by its presence in approximately 8% of the human transcriptome (Bakheet et al., 2006).

Regulation of inflammatory mediators can occur at the transcriptional level, controlling gene expression, or it can occur at the post transcriptional level, whereby mRNA stability determines whether a particular transcript is translated to protein or is rapidly degraded. Regulation can also occur at the post-translational level by modulating the stability and longevity of translated proteins. This typically involves either prevention of the degradation of the protein (via the proteasome) or modifying it to increase its stability. Although all three levels of regulatory control can be targeted to modulate an inflammatory response, post-transcriptional modification presents a feasible opportunity to exert fine control over the pro-inflammatory process.

The mRNA transcripts of many inflammatory mediators are unstable and are regulated post-transcriptionally; this ensures that low protein levels are maintained under homeostatic conditions. However, they are rapidly induced and then degraded depending on the nature of the extracellular cues. In this way immunomodulatory cells are able to react with the appropriate physiological responses to match the stimuli. Inflammatory conditions arise when inflammatory proteins are induced at a time they are not needed, or in an amount that is excessive. Hence, mRNA stability plays a crucial role in the pathogenesis of inflammatory

diseases. This is evident in the pathogenesis of asthma. Many of the characteristics associated with asthma, such as basement membrane thickening, increased interstitial matrix deposition, airway smooth muscle mass increasing, increased airway fibrosis, and neovascularization in the subepithelial mucosa can be attributed to elevated levels of the inflammatory mediators that give rise to these characteristics (Ammit, 2005). As outlined in Table 1, many of the TTP target mRNAs encode proteins responsible for these disease characteristics (Banerjee et al., 2014; Barnes, 2008; Batra et al., 2007; Boonpiyathad et al., 2013; Hirst, 2003; Ishmael, 2011; Kang et al., 2011; Kato and Schleimer, 2007; Masaki et al., 2014; Mattos et al., 2002; Rael and Lockey, 2011; Tang and Fiscus, 2001).

TTP in respiratory disease: what is known?

The important anti-inflammatory role of TTP has been extensively studied in mouse models and cell lines such as peripheral blood monocytes, bone marrow-derived macrophages (Carballo et al., 2001), HeLa cells and embryonic fibroblasts (Chen et al., 2012) and a plethora of other cell lines to elucidate the molecular mechanisms that drive these anti-inflammatory functions (Brooks and Blackshear, 2013) (Table 1). To date, the potential of TTP as an anti-inflammatory therapy has primarily focussed on its role in rheumatoid arthritis, leaving investigations into the anti-inflammatory function of TTP in airway disease relatively unexplored. Lessons may also be learned from studies examining tumours, as many inflammatory tumours have features that parallel chronic inflammatory diseases like asthma. Among the similarities are structural remodelling, inflammatory cell migration, cell proliferation, the induction of proteinases and dysregulation of endogenous anti-inflammatory proteins. The latter characteristic is perhaps the most important similarity, as there is a prolific induction of inflammatory mediators in both inflammatory tumours and chronic inflammatory diseases. This suggests that the endogenous feedback machinery present within

the cell is being dysregulated in cancer. TTP acted as a tumour suppressor in a model of a specific mast cell tumour by reducing the pivotal cytokine IL-3 that propagated that tumour (Stoecklin et al., 2003). Similar to this, IL-6, a pro-inflammatory mediator, shown to maintain a pro-cancerous inflammatory state, was reduced by TTP when cells were stimulated with lipopolysaccharide (Sauer et al., 2006). This along with studies that showed TTP was attenuated in many cancerous cell lines relative to non-cancerous lines (Brennan et al., 2009; Carrick and Blackshear, 2007) presents compelling evidence to suggest TTP could be a viable therapeutic target. This is however just a guide, and caution should must be exercised when using tumour models as they have many limitations. Among the main limitations, we see that tumours under resistance mechanisms and induce a host of neoplastic characteristics not mentioned here. It is not just in cancer models however that TTP has shown to be effective, TTP was shown to down-regulate cytokines in acute respiratory disease (Colin et al., 2014), arthritis (Carrick et al., 2004) and psoriasis (Colin et al., 2014) to highlight a few. It was also shown to be effective in reducing immune cell driven cytokine IL-17A in the presence of TTP overexpression and increased when TTP was silenced (Lee et al., 2012). TTP was also a key anti-inflammatory protein upregulated in response to anti-asthma drugs in cellular models utilizing human bronchial epithelial BEAS-2B cells and airway smooth muscle cells (Kaur et al., 2008).

TTP function: multiple levels of regulatory control

To assess the potential of TTP as an anti-inflammatory molecule we have to first gain an understanding of the mechanisms and molecules that control its regulation. Tristetraprolin, also known as Nup475 (DuBois et al., 1990), Tis 11 (Varnum et al., 1991), G0S24 (Heximer and Forsdyke, 1993) or Zfp36 (Taylor et al., 1991), is the prototypical member of the family of Cys-Cys-Cys-His class tandem zinc finger proteins. It is an immediate early response gene

with low levels expressed within quiescent cells. It can be rapidly expressed and translated into protein to exert regulatory control upon mRNA decay. Various studies have shown that TTP is regulated at different stages of its life cycle. As with many critical molecules, TTP regulation is possible at the transcriptional level, the post-transcriptional level and the post-translational level.

Transcriptional regulation: At the transcriptional level it was shown that the deletion of a single intron from the promoter region in the Zfp36 5'-UTR caused an 85% drop in serum-induced TTP mRNA (Lai et al., 1995). Structural analysis of the 5'-UTR region revealed promoter element binding sites identified as transcription factor binding sites (Lai et al., 1998; Lai et al., 1995), including: EGR-1 (early growth response protein 1), TPE1 (TTP promoter element 1), AP-2-like (activating protein 2), and SP-1 (specificity protein 1). These binding sites in the Zfp36 promoter region were shown to be partially responsible for TTP transcriptional activation through mutational analysis of splice variants in the presence of serum (DuBois et al., 1990; Kaneda et al., 2000; Lai et al., 1998; Lai et al., 1995). The same studies also showed that NF- κ B had a binding site on the intron suggesting that an interaction could exist between transcription factors to induce TTP mRNA. A strong interaction was not proven, only that the intron had to remain in close proximity to the promoter elements. This data, taken in concert with studies showing the ability of TTP mRNA to be upregulated by molecules such as TNF α , LPS, IL-1 β , IL-4, TGF- β , INF- γ , mitogenic serum stimuli, cAMP and phorbol esters has allowed us to gain insight into the molecular pathways that drive TTP mRNA upregulation. Inhibitor studies involving inhibition of NF- κ B, in the presence of IL-1 β and LPS, have shown that TTP mRNA is a direct substrate of the NF- κ B signalling pathway (Chen et al., 2013; King et al., 2009). Other signalling pathways include the protein kinase C (PKC) pathway, via transcription factor AP-2, which was shown to regulate TTP mRNA in the presence of a phorbol ester (Chen et al., 1998; Jalonen et al., 2007; Leppanen et

al., 2008; Leppanen et al., 2010; Tan and Parker, 2003), and the EGF pathway via the transcription factor, ETS domain-containing protein ELK-1 ((Florkowska et al., 2012).

The signalling molecule most extensively studied in relation to TTP mRNA regulation is p38 MAPK. The p38 MAPK-mediated pathway exerts most of its control over TTP via the downstream protein kinase MK2 (Chrestensen et al., 2004; Hitti et al., 2006; Mahtani et al., 2001; Marchese et al., 2010; Stoecklin et al., 2004; Tudor et al., 2009); this will be discussed in greater detail in later sections. TTP mRNA is expressed with an immediate early peak followed by a decline back to near baseline levels after approximately 4 hours (Hitti et al., 2006; Tchen et al., 2004). In the presence of a p38 MAPK inhibitor, this peak at the early time point was reduced suggesting p38 MAPK-dependent regulation of TTP mRNA expression. It has also been suggested that since TTP mRNA is increased via the p38 MAPK pathway along with other pathways, it may be increased in a temporally specific manner with NF- κ B responsible for the early mRNA production and p38 MAPK responsible for later time point (Chen et al., 2013).

Taken together, these studies demonstrate that targeting TTP at the transcriptional level represents an opportunity to inhibit multiple inhibitory pathways at a downstream level since there are a number of signalling molecules and pathways that regulate TTP mRNA expression.

Post-transcriptional regulation: It has been widely reported that the p38 MAPK pathway is responsible for post-transcriptional regulation of TTP by influencing mRNA stability. Actinomycin D chase experiments performed in the presence of a p38 MAPK inhibitor demonstrated direct reliance on the p38 MAPK in TTP mRNA stability (Carballo et al., 2001; Tchen et al., 2004). TTP mRNA transcripts were more stable in the presence of the p38 MAPK inhibitor. The ERK pathway has also been synergistically linked with p38 MAPK to induce TTP mRNA stability (Brook et al., 2006; Deleault et al., 2008; Essafi-Benkhadir et al.,

2010). Indirectly p38 MAPK is able to destabilise TTP mRNA through TTP self-regulated mRNA decay. Examination of TTP 3'-UTR constructs in the presence of TTP protein revealed that TTP has a high affinity for its own mRNA, and as such it is able to negatively regulate itself (Brooks et al., 2004; Tchen et al., 2004). This differential regulation is representative of the complex interaction between these molecules. Moreover, actinomycin D analysis of TTP mRNA stability in the presence of a PKC pathway inhibitor showed that PKC had a similar effect on TTP mRNA stability to p38 MAPK thereby suggesting that part of its impact on TTP may be due to an increase in TTP mRNA stability (Leppanen et al., 2010).

Another interesting area of focus on the regulation and function of TTP has been with microRNA (miR) and their ability to impact TTP and bind 3'-UTR of specific mRNA transcripts. MicroRNAs are small, approximately 20 nucleotides in length, they are regulators of mRNA decay. Accumulating evidence demonstrates that miRs can regulate TTP; miR-346 for example was shown to be important in the regulation of TNF α in rheumatoid arthritis, by controlling release of TNF α directly via TTP (Semaan et al., 2011), while miR29 was observed to bind TTP mRNA in a cancer model (Al-Ahmadi et al., 2013; Gebeshuber et al., 2009). Another study proposed that TTP does not directly bind to miR-16 but interacts through association with Ago/eIF2C family members (proteins required for miR activity) in a complex with miR16 to assist with the targeting of AREs (Jing et al., 2005). Moreover, it was reported that miR-466 bound competitively to IL-10 and conferred anti-inflammatory properties by antagonizing TTP in TLR triggered macrophages (Ma et al., 2010). These reports reveal that miRs have an impact on TTP both directly or indirectly. So far, whether miR present within the airways and implicated in asthma are linked to TTP remains an open question, however data from rheumatoid arthritis and cancer models provides us with an inflammatory model in which miRs have been shown to alter TTP binding. Further studies

exploring the regulation of TTP by miR in the context of asthmatic inflammation are warranted.

Post-translational control: Once the stable mRNA transcripts are translated into protein there is a level of control that is exercised over the translated protein. Being an immediate early response gene, TTP protein is rapidly expressed after gene transcription; hence TTP protein expression bears a similar temporal profile to mRNA expression (Brook et al., 2006; Mahtani et al., 2001). Post-translational regulation of TTP protein occurs predominately in the form of phosphorylation. TTP protein appears in two distinct forms, a phosphorylated form and an unphosphorylated form. Phosphorylation of TTP is regulated post-translationally via the ERK and p38 MAPK pathways (Brook et al., 2006; Deleault et al., 2008; Essafi-Benkhadir et al., 2010). Other kinase pathways such as the PKC pathway were shown not to have an impact on TTP protein expression. Post-translational regulation of TTP is also able to alter its localisation to the cytoplasm with the aid of 14-3-3 proteins (Benjamin et al., 2006; Brook et al., 2006; Johnson et al., 2002; Sun et al., 2007). Post-translational control of TTP function represents a promising means to harness the anti-inflammatory capacity of TTP, since its phosphorylation status dictates its activity. Hence this will be explored in greater depth in the following sections.

TTP is a substrate for MK2

TTP's function as an mRNA destabilising factor is mainly controlled by the p38 MAPK - MK2 axis. Critically, p38 MAPK also activates the downstream kinase MK2, which phosphorylates murine TTP protein on two key serine residues (Ser52 and Ser178) (Chrestensen et al., 2004). MK2 is a substrate of the p38 MAPK pathway and has been implicated in post-translational control of cytokines. This was demonstrated by using MK2 knockout mice, which had attenuated production of TNF α protein when compared to wild

type equivalents. However, the mRNA levels in wild type mice were very similar to knockout mice, suggesting that the control exerted was at the translational level. This also suggested that control may be imparted via a downstream substrate of MK2. TTP was shown to be a substrate for MK2 in primary cells by the use of MK2/TTP double knockout mice, p38 MAPK inhibitors and by reconstituted p38 MAPK transduction pathway using recombinant kinases to detect TTP phosphorylation (Hitti et al., 2006; Mahtani et al., 2001; Tudor et al., 2009). Production of cytokines was also impaired compared to wild type, with IL-6 mRNA half-life in particular, markedly attenuated (Kotlyarov et al., 1999; Neininger et al., 2002). *In vitro* studies revealed that TTP is phosphorylated by MK2 (Chrestensen et al., 2004; Mahtani et al., 2001) and phosphorylation of MK2 is able to modulate TTP function (Stoecklin et al., 2004).

Phosphorylation of TTP protein

As detailed above, the main function of TTP protein is decay target mRNAs however its activity is also profoundly affected by post-translational modification (Cao et al., 2007). Post-translational phosphorylation appears to be the main factor responsible for determining its stability, activity and its ability to form protein complexes. Phosphorylation of TTP has been extensively studied and mapped revealing that TTP is a hyper-phosphorylated protein *in vivo* with its mRNA destabilizing activity depending on its phosphorylation status. When TTP protein is phosphorylated it is unable to effect the stability of target mRNA, however when unphosphorylated it is active and therefore able to confer its mRNA destabilizing ability. TTP protein is more stable when phosphorylated and unstable when unphosphorylated. This allows TTP protein to be rapidly degraded by the proteasome when unphosphorylated (Brook et al., 2006; Hitti et al., 2006). It has also been shown that Ser220 on mouse TTP is phosphorylated via the ERK MAPK pathway (Taylor et al., 1995) suggesting that dual

MAPK involvement may be required to confer post-translational modifications to TTP (Brooks et al., 2004). The functions of all the various TTP phosphorylation sites are not completely understood, however the sites for MK2 protein binding have been characterized at Ser52 and Ser178 on murine TTP (Chrestensen et al., 2004; Stoecklin et al., 2004). Phosphorylation at these sites enables TTP to form complexes with multifunction adaptor 14-3-3 proteins that ablate its function as an mRNA destabilizing protein (Chrestensen et al., 2004; Marchese et al., 2010; Sun et al., 2007). This complex also serves to protect TTP from dephosphorylation in a protein phosphatase 2A (PP2A)-dependent manner (Sun et al., 2007) and prevent degradation of TTP via the proteasome (Deleault et al., 2008; Pfeiffer and Brooks, 2012). There is however, some data to suggest that the role of 14-3-3 proteins might be limited to chaperoning TTP and only preventing its dephosphorylation (Marchese et al., 2010; Ngoc et al., 2014). The additional role as a block on proteosomal degradation is challenged, as recombinant 14-3-3 in the presence of TTP did not affect TTP stability and degradation by the proteasome (Ngoc et al., 2014). This is in line with data that showed TTP levels increased when cells were in the presence of a proteasome inhibitor. This complex that forms alters the functionality of TTP without altering its ability to bind mRNA (Cao et al., 2003; Clement et al., 2011; Marchese et al., 2010; Rigby et al., 2005) suggesting that phosphorylated TTP remains inactive whilst being bound to target mRNA until it is dephosphorylated. It is then able to recruit the Ccr4-CAF-1 deadenylase components via Not 1, which are then able to rapidly deadenylate the target mRNA resulting in their destabilization (Colin et al., 2014; Sandler et al., 2011). Phosphorylation of TTP also has an impact on its subcellular localization, with studies showing that upon stimulation with a mitogen, TTP is rapidly translocated from the nucleus to the cytoplasm (Brook et al., 2006; Johnson et al., 2002; Taylor et al., 1996b). This is in line with data suggesting that degradation of ARE-containing occurs at different locations within the cytoplasm (Franks

and Lykke-Andersen, 2007). Taken together, these studies demonstrate the importance of the phosphorylation status of TTP in controlling TTP activity and thus represent a novel area to target in future pharmacotherapeutic strategies.

Regulation of the temporal kinetics of TTP by p38 MAPK

Knowing the temporal kinetics of TTP may allow us to tailor pharmacological intervention to increase the anti-inflammatory efficiency of the process. This would be beneficial as it might allow us to target specific events within the TTP temporal regulation rather than the molecule as a whole. The kinetics of TTP originates with the p38 MAPK pathway or other such inflammatory pathways. When p38 MAPK is phosphorylated it becomes active, at which point it then phosphorylates downstream targets like MK2. This p38 MAPK upregulation occurs very soon after the cell is stimulated by some extracellular trigger. Our own studies in human airway smooth muscle cells indicate that activation of p38 MAPK occurs as early as 10-15 minutes after stimulation (Moutzouris et al., 2010; Quante et al., 2008) and could be even earlier. TTP is a known substrate for p38 MAPK and MK2, both of which are required for the full induction of TTP expression. As an immediate early gene TTP is rapidly expressed, with our preliminary studies indicating an increase in mRNA as early as 30 minutes and with one study showing upregulation as early as 10 minutes (Taylor et al., 1995). This causes the initial accumulation of TTP mRNA within the nucleus followed by efficient translation (Brook et al., 2006; Johnson et al., 2002; Taylor et al., 1996b). During this period when TTP is inactive, the cytokine mRNA is stabilized and results in a wave of cytokine upregulation, peaking at 1 hour (Balakathiresan et al., 2009). This is derived from the fact that active p38 MAPK has a peak at 15-30 minutes with activation returning to basal levels after about 1 hour (Moutzouris et al., 2010; Quante et al., 2008). Once p38 MAPK activation has subsided, MK2 is no longer phosphorylated and hence it is unable to phosphorylate TTP.

To determine the phosphorylation status of TTP using Western blotting can be challenging however, earlier studies have used phosphatases to identify and confirm the phosphorylated immunoreactive band from the unphosphorylated band (Balakathiresan et al., 2009; Carballo et al., 2001; Mahtani et al., 2001). Hence, at the 1 hour time point, the unphosphorylated immunoreactive band of about 40-45 kDa appears, suggesting that TTP is active (King et al., 2009). The literature suggests that this may be in part due to the action of PP2A dephosphorylating phosphorylated TTP, which leads to the rapid deadenylation and degradation of cytokine mRNAs thereby potentially attenuating the degree of inflammation. MKP-1 regulation of p38 MAPK phosphorylation also plays an important role. Since there appears to be a sequential order to the control of TTP activity, it can be refined and modified to aid in the fine-tuning of a potential anti-inflammatory agent.

Regulation of TTP by PP2A

To fully explore TTP and its potential as an anti-inflammatory agent it is important to highlight the key molecules that may positively affect TTP function. We know that TTP is a hyper-phosphorylated protein, and any protein that could reverse its inactivation would be of therapeutic value. This is where PP2A may play a role. PP2A is a heterotrimeric serine threonine protein phosphatase consisting of a scaffold subunit (A), a catalytic subunit (C) for its phosphatase activity, and regulatory subunit (B) to confer substrate specificity (Janssens et al., 2008; Shi, 2009). PP2A is a master controller of cell signalling and regulates many cellular functions, including cell growth, inflammation and apoptosis (Santoro et al., 1998; Yang et al., 2001). Many of these functions have been attributed to PP2A-mediated regulation of MAPKs (Junttila et al., 2008). PP2A is able to act on p38 MAPK and its upstream kinase MKK3 (Prickett and Brautigan, 2007). Inhibitors of PP2A, like okadaic acid, calyculin A and simian viral protein SV40, were shown to induce phosphorylation and

activate upstream kinases of p38 MAPK and ERK *in vitro* (Junttila et al., 2008). It was also shown that inhibition of PP2A increases mRNA stability of cytokine mRNA by increasing the activity of p38 MAPK and its downstream kinase MK2 (Clement et al., 2011; Sun et al., 2007). These studies are evidence of indirect regulation of TTP by PP2A. More directly, Brook *et al.* (Brook et al., 2006) showed that the PP2A inhibitor (calyculin A) caused accumulation of hyper-phosphorylated and stable TTP protein and highlighted a MK2-Ser52/Ser178-PP2A-dependent mechanism for controlling TTP stability. Since PP2A is able to regulate p38 MAPK as well as dephosphorylate TTP, TTP is both an indirect and direct target of PP2A. Thus PP2A activation offers promise as a means to control the activity of TTP, although the fine temporal control needs to be delineated. Most of the work on PP2A regulation has been performed in cancer cells (Janssens et al., 2008; Perrotti and Neviani, 2008; Tay et al., 2012) with some work on mast cells (Kranias et al., 2010) and respiratory epithelial cells (Cornell et al., 2009). Notably, PP2A has emerged as playing an important role in *in vivo* models of asthma (Collison et al., 2013; Hatchwell et al., 2014) and PP2A inhibition (with okadaic acid) resulted in corticosteroid insensitivity *in vitro* (Kobayashi et al., 2011). Strategies targeting PP2A could become therapeutically important as an anti-inflammatory agent in asthma.

Summary and future directions

The substantial *in vitro* evidence implicating the p38 MAPK signalling pathway in the pathogenesis of respiratory diseases driven by inflammation has propelled studies targeting p38 MAPK as a novel anti-inflammatory strategy *in vivo*. However, as discussed in this review, p38 MAPK activation also results in repression of critical anti-inflammatory proteins, in addition to inhibition of pro-inflammatory molecules. The RNA destabilising molecule TTP is one such key anti-inflammatory protein whose function is significantly repressed by

p38 MAPK inhibition. We propose that a greater understanding of TTP upregulation and its activation by phosphatases such as PP2A in airway inflammation represent an alternative anti-inflammatory strategy in chronic respiratory diseases.

Authorship contributions

Wrote or contributed to the writing of the manuscript: PP and AJA

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MOL #95984

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Figure Legend

Figure 1. Current and potential inhibitory targets to control p38 MAPK-dependent signalling in respiratory disease. The three members of the MAPK superfamily - ERK, JNK and p38 MAPK - are stimulated via a series of steps involving MAPKKK phosphorylating downstream MAPKK, then ultimately MAPKs. This figure focuses on downstream effectors of the p38 MAPK pathway and the numbers indicate inhibitory strategies along this pathway. While 1) and 2) will repress p38 MAPK, we propose that blocking p38 MAPK may have a negative impact on anti-inflammatory proteins, as TTP (tristetraprolin) is regulated downstream of p38 MAPK. Thus we propose 3) to control the phosphorylation status and activity of TTP. PP2A (protein phosphatase 2A) is an endogenous protein able to activate TTP. Once active TTP is able to elicit anti-inflammatory actions by destabilising cytokine mRNA after recruiting CAF 1 and CNOT 1 (see text for abbreviations).

