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DUSP10 Negatively Regulates the Inflammatory Response to Rhinovirus Through IL-1β Signalling.

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Running Head: DUSP10 regulates airway inflammation.

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Abstract

Rhinoviral infection is a common trigger of the excessive inflammation observed during exacerbations of asthma and chronic obstructive pulmonary disease. Rhinovirus (RV) recognition by pattern recognition receptors activates the MAPK pathways, common inducers of inflammatory gene production. A family of dual-specificity phosphatases (DUSPs) can regulate MAPK function, but their roles in rhinoviral infection are not known. We hypothesised that DUSPs would negatively regulate the inflammatory response to RV infection. Our results revealed that p38 and JNK MAPKs play key roles in the inflammatory response of epithelial cells to RV infection. Three DUSPs previously shown to have roles in innate immunity, 1, 4 and 10, were expressed in primary bronchial epithelial cells, one of which, DUSP10, was down regulated by RV infection. Small interfering-RNA knock down of DUSP10 identified a role for the protein in negatively regulating inflammatory cytokine production in response to IL-1β alone and in combination with RV, without any effect on RV replication. This study identifies DUSP10 as an important regulator of airway inflammation in respiratory viral infection.

Importance

Rhinoviruses are one of the causes of the common cold. In patients with asthma or chronic obstructive pulmonary disease, viral infections, including rhinovirus, are the commonest cause of exacerbations. Novel therapeutics to limit viral inflammation are clearly required. The work presented here identifies DUSP10 as an important protein involved in limiting the inflammatory response in the airway without affecting immune control of the virus.

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Introduction

Human rhinoviruses (RV) frequently trigger exacerbations of airway diseases, such as asthma and chronic obstructive pulmonary disease, where excessive inflammation causes worsening airway obstruction and increased symptoms. RV belong to the Picornaviridae family, with positive-sense single-stranded RNA packaged into icosahedral virions. There are over one hundred and fifty serotypes, classified either phylogenetically, into A, B and C, or based on the receptor the virus binds on the cell surface (1, 2). The major group, comprising most of group A and all of group B, bind intracellular adhesion molecule-1 (3), and the minor group, comprising the remainder of group A, bind low-density lipoprotein receptor or related proteins (4, 5). Culture methods for group C have been discovered relatively recently, so investigation into this group has been limited, however it is known to bind cadherin-related family member 3 (6, 7).

RV infect airway epithelial cells, which express several pattern recognition receptors capable of recognising distinct parts of the virus (8). Toll-like receptor 3 (TLR3) and the RIG-like receptors (RLRs), bind double-stranded RNA replication intermediates, and TLR2 on the cell surface binds the rhinoviral capsid (9-12). It has been suggested that TLRs 7 and 8 may also contribute to the response to RV (13, 14), however, we and others have found airway epithelial cells unresponsive to TLR7/8 ligands (10, 15-17). Activation of pattern recognition receptors leads to the production and release of inflammatory cytokines through several pathways, including the NF-κB, interferon regulatory factor (IRF), and mitogen-activated protein kinase (MAPK) pathways. The MAPK pathways, p38, JNK and ERK, consist of a three-tier kinase cascade culminating in phosphorylation of the MAPK on two residues, tyrosine and threonine. The activated MAPKs translocate into the nucleus and activate a range of

transcription factors, including AP-1, ATF, CREB, c/EBP and NF-κB. The p38 pathway can also be activated by binding and internalisation of RV (18-20). Previous work has shown the importance of p38 and ERK MAPK in inducing cytokine release in response to RV infection of airway epithelial cell lines (21-23). This inflammatory response to RV can be further potentiated by IL-1β. IL-1β signals through similar pathways to the TLRs and is known to activate the MAPKs (24, 25). Furthermore, IL-1β is released from RV infected immune cells, such as monocytes and macrophages, and would therefore be present in the infected airway (11). Previous work by our group has shown the importance of IL-1β in the immune response to RV, RV infection induces the release of both IL-1α and IL-1β whilst blocking IL-1 signalling significantly inhibits proinflammatory cytokine release (26), furthermore the addition of IL-1β enhances cytokine production from epithelial cells in response to RV infection (27). Thus, it is imperative that the MAPK pathways are regulated in order to stop over production of cytokines and excessive inflammation.

Dual-specificity phosphatases (DUSPs) are a family of proteins capable of dephosphorylating two residues in one substrate simultaneously. A subgroup of DUSPs, MAPK phosphatases (MKPs), dephosphorylate the MAPK proteins directly. So far ten MKPs have been characterised, three of which have been shown to negatively regulate innate immune signalling: DUSP1 (MKP1), DUSP4 (MKP2), and DUSP10 (MKP5). Knock out mice which lack each of these proteins individually, produce higher levels of inflammatory cytokines in response to TLR4 activation, associated with increased p38 and/or JNK MAPK activation (28-32). It should be noted that another group have shown a conflicting role for DUSP4, with knock out mice producing lower levels of cytokines in response to TLR4 signalling (33). Much of this work has explored the role of DUSPs in bacterial infection, and little is known about

92 the ability of DUSPs to regulate the response to viruses, particularly within epithelial cells.
93 More recently, bone-marrow derived macrophages (BMDMs) and dendritic cells taken from
94 DUSP10 knock out mice have been shown to exhibit increased release of inflammatory
95 cytokines and anti-viral interferons (IFN) in response to influenza infection (34).
96
97 We hypothesised that one or more DUSPs would play a critical role in regulating the
98 inflammatory response to RV infection. We determined that the p38 and JNK pathways were
99 responsible for a large proportion of the CXCL8 produced by primary bronchial epithelial cells
100 (PBECs) in response to RV infection, while ERK did not play as great a role. DUSPs 1, 4 and 10
101 were expressed by PBECs. Expression of DUSPs 1 and 4 was unaltered by RV infection or IL-
102 1β stimulation, however RV decreased expression of DUSP10. Small interfering RNA (siRNA)
103 knock down of DUSP10 identified a role for the protein in regulating the response to IL-1β
104 alone and in combination with RV. These results identify DUSP10 as an important regulator
105 of the inflammatory response in epithelial cells and therefore a potential future therapeutic
106 target for RV induced acute exacerbations.
107

108 ## Results

109 **The p38 and JNK pathways play important roles in cytokine production in response to RV.**
110 It is well documented that the MAPK pathways play roles in inducing cytokine release in
111 response to a variety of stimulants. This has previously been demonstrated for p38 and ERK
112 in response to RV infection, with inhibition of either decreasing the release of CXCL8, a
113 neutrophil chemoattractant (21-23). However, these studies utilised airway epithelial cell
114 lines, BEAS-2B and 16HBE14o-, and the roles of these pathways in the response of PBECs to
115 RV is not well characterised. In addition, the role of JNK in the response to RV is unknown,

116 although it has been shown to be critical in inducing CXCL8 release in a human astroglioma
117 cell line in response to poly(I:C), a synthetic TLR3 ligand (35).
118
119 To explore the contribution of each MAPK pathway to cytokine production in response to viral
120 infection, a panel of MAPK inhibitors were used. PBECs were pretreated with the inhibitors
121 for 1 h prior to stimulation with synthetic double-stranded RNA viral mimic, poly(I:C), and
122 inhibitors remained present throughout the 24 h stimulation. Production of the inflammatory
123 cytokine CXCL8, a downstream target of NF-κB activation, was measured at both the RNA and
124 protein secretion levels using qRT-PCR and ELISA. Poly(I:C) stimulation led to an upregulation
125 of CXCL8 mRNA expression and protein release which was unaffected by inhibition of ERK
126 with PD90859. Inhibition of p38 or JNK reduced CXCL8 levels, however this was only
127 statistically significant at the protein level for SB203580 (Figure 1 A). A similar pattern was
128 observed in response to infection with a major and minor group strain of rhinovirus, RV16
129 and RV1B respectively. PBECs were treated with MAPK inhibitors for 1 h prior to infection
130 with RV, the inhibitors were present for the 1 h RV infection, and remained present for the
131 following 48 h. CXCL8 expression was measured at 48 h as peak cytokine release was observed
132 at this time-point (data not shown). Infection of PBECs with RV induced an increase in CXCL8
133 mRNA and protein secretion. CXCL8 levels were dramatically reduced by inhibition of p38
134 with either SB203580 or SB202190, or JNK with SP600125, although the reduction was less
135 clear at the mRNA level (Figure 1 B and C). Inhibition of ERK by PD90859 did not significantly
136 affect CXCL8 expression or release in response to RV1B infection (Figure 1 B). These data
137 suggest that p38 and JNK have important roles in inducing CXCL8 production in response to
138 infection with major or minor strains of RV, while ERK plays a lesser role.
139

140 **DUSPs are expressed by PBECs.** We therefore went on to investigate the expression and roles
141 of DUSPs, important regulators of the MAPK pathways. To the best of our knowledge, the
142 expression of DUSPs in PBECs has not previously been characterised. We first determined the
143 gene expression of DUSPs 1, 4 and 10 using RT-PCR. Each DUSP was expressed by PBECs, even
144 in unstimulated cells (Figure 2). The regulation of this expression in response to poly(I:C) or
145 IL-1β stimulation was examined over 24 h, however no clear changes were observed in the
146 expression of any of the DUSPs examined using this method (Figure 2).
147
148 In order to examine DUSPs 1, 4 and 10 mRNA expression in more detail, the more sensitive
149 technique of qRT-PCR was utilised. PBECs were infected with RV1B or RV16, or stimulated
150 with IL-1β, over 24 h and qRT-PCR used to measure expression of DUSPs 1, 4 and 10. As no
151 change in DUSP expression was seen in response to 10 ng/ml IL-1β (Figure 2), the
152 concentration was increased to 100 ng/ml. Stimulation with 100 ng/ml IL-1β did not alter
153 expression of any of the DUSPs (Figure 3). Expression of DUSP1 and DUSP4 was unaltered by
154 infection with RV1B (Figure 3 A and B). Infection with RV16 increased DUSP1 mRNA
155 expression at 24 h, however this was variable and non-significant (Figure 3 A). Poly(I:C)
156 stimulation was also found to increase DUSP1 mRNA expression, as found previously (36), but
157 had no effect on expression of DUSPs 4 and 10 (data not shown). Infection with either strain
158 of RV caused a similar regulation of DUSP10 expression, with an initial increase, followed by
159 a consistent and significant downregulation at 8 h post-infection, before returning to baseline
160 by 24 h (Figure 3 C).
161
162 DUSP10 protein expression followed a similar pattern, with a slight increase at 2 h following
163 RV1B infection, before declining to below baseline levels (Figure 4 A). RV16 infection had a

similar effect on DUSP10 protein levels, but was not statistically significant (Figure 4 B). As in the mRNA expression (Figure 3), IL-1β stimulation had no effect on DUSP10 protein expression (Figure 4 C).

DUSP10 does not regulate the response to RV. Out of the proteins examined, DUSP10 was the only one found to be regulated by RV infection, thus it was taken forward for further investigation. siRNA was used to successfully knock down DUSP10 expression in PBECs, reducing DUSP10 mRNA and protein levels to approximately 20% of control levels (Figure 5 A and B). Control and DUSP10 knockdown cells were then infected with RV1B or RV16, or stimulated with poly(I:C), for 24 h and the release of inflammatory protein CXCL8 measured by ELISA. Release of CXCL8 was unaffected by DUSP10 knock down (Figure 5 C).

As DUSP10 has previously been shown to regulate type-I IFN production in response to influenza (34), the level of IFN-β mRNA was measured at 16 h post RV infection. Low levels of IFN-β were detected by qRT-PCR in response to poly(I:C) or either strain of RV, and this was unaffected by DUSP10 knock down (Figure 5 D). DUSP10 knock down also had no effect on levels of release of the interferon stimulated gene, CCL5, in response to RV (Figure 5 E). In response to poly(I:C) stimulation, CCL5 levels were reduced by DUSP10 knock down. However, this may be due to cell death caused by DUSP10 knock down as observed by eye (data not shown). RV replication at 24 h was also unaffected by DUSP10 knock down, with RV RNA levels similar between control and DUSP10 siRNA treatments (Figure 5 F).

DUSP10 regulates the response to IL-1β. Whilst DUSP10 knock down did not affect the response of PBECs to RV, the response to IL-1β was altered. Stimulation of PBECs with a range

188 of IL-1β concentrations induced mRNA production and protein release of CXCL8 (Figure 6 A
189 and B). CXCL8 mRNA and protein levels were significantly increased in cells with reduced
190 DUSP10 levels (Figure 6 A and B). As p38 and JNK were shown to be important inducers of
191 CXCL8 production, the effect of DUSP10 knockdown on IL-1β–induced MAPK activation was
192 investigated. Levels of phosphorylated, activated p38 and JNK in response to IL-1β were
193 measured in cells treated with DUSP10 or control siRNA. IL-1β stimulation upregulated
194 phosphorylation of both proteins, but the level of activation was unaffected by DUSP10 knock
195 down (Figure 6 C).
196
197 In order to gain a wider view of the role of DUSP10 in IL-1β signalling, an array was used to
198 determine the effect of DUSP10 knock down on the release of a variety of cytokines. The
199 chosen array contained antibodies specific for 36 proteins known to be upregulated in
200 response to inflammation (R&D ARY005B). The levels of each protein released by cells from
201 one donor treated with DUSP10 or control siRNA prior to 24 h of stimulation with IL-1β were
202 determined. IL-1β stimulation increased release of several cytokines by PBECs, including
203 CXCL1, CXCL10, G-CSF, GM-CSF, IL-6, CXCL8 and IL-1β itself, and decreased the release of
204 CXCL12 (Figure 7). In keeping with previous data shown above, DUSP10 knock down
205 potentiated the IL-1β induced release of CXCL1, CXCL8 and IL-1β, with IL-1β levels increasing
206 1.71 fold in comparison to cells treated with control siRNA (Figure 7). Intriguingly, DUSP10
207 knock down decreased levels of CXCL10 release in response to IL-1β. These data supported a
208 role for DUSP10 in regulating the inflammatory response of airway epithelial cells.
209
210 **IL-1β is released by PBECs in response to RV infection.** In order to determine whether the
211 role of DUSP10 in the response to IL-1β would be of relevance in a RV infection, the release

of IL-1β in response to RV was quantified. PBECs released around 180 pg/ml IL-1β in response to 24 h infection with both RV1B and RV16 (Figure 8).

DUSP10 regulates the response of PBECs to dual stimulation with RV and IL-1β. IL-1β is an important early signalling molecule in the airway. It has previously been shown to potentiate the response of airway epithelial cells to RV infection, increasing the release of CXCL8 by the bronchial epithelial cell line BEAS-2B (27). We therefore investigated whether IL-1β would potentiate the response of PBECs to RV and determined the role of DUSP10 in this setting. Stimulation with IL-1β or infection with RV16 caused modest increases in CXCL8, whilst the addition of IL-1β to RV16 infected cells significantly augmented CXCL8 release. At the mRNA level only the higher dose of IL-1β, 10 ng/ml, caused observable increases in CXCL8 production compared to RV16 alone (Figure 9 A). However, both concentrations caused incremental increases in CXCL8 release (Figure 9 B). This response was further potentiated by DUSP10 knock down, with significantly higher CXCL8 at the mRNA and protein level in comparison with control siRNA treated cells. A similar pattern was seen in response to infection with the minor group virus RV1B (Figure 9 C). To ensure that the increased CXCL8 in dual-stimulation was not due to an effect of IL-1β on viral replication, the amount of intracellular viral RNA levels were quantified by qRT-PCR. No significant effects were observed between RV16 alone and in combination with IL-1β (data not shown).

In addition to the major and minor classification of rhinoviruses, they are grouped phylogenetically, into A, B and C. Both RV1B and RV16 belong to group A. Therefore, a third serotype, RV14, a major group rhinovirus belonging to group B was examined. In accordance with previous results, infection of PBECs with RV14 led to a small increase in CXCL8 release

236 which was unaffected by DUSP10 knock down. When RV14 infection was combined with IL-
237 1β stimulation, CXCL8 release was increased, and further potentiated by DUSP10 knock down
238 (Figure 9 D). These data demonstrate a role for DUSP10 in negatively regulating the response
239 of PBECs to RV when in combination with IL-1β.
240
241 **DUSP10 has a similar role in PBECs isolated from COPD patients.** In order to ensure that the
242 role of DUSP10 is clinically relevant, its role in PBECs isolated from COPD patients was
243 investigated. Infection of COPD PBECs with RV1B caused a similar pattern of change in
244 expression of DUSP10 mRNA and protein as was seen in normal PBECs, with an initial increase,
245 followed by a downregulation by 8 h (Figure 10). RV16 infection also had a similar effect on
246 DUSP10 mRNA expression as was seen in normal PBECs, but this was not observed at the
247 protein level. In keeping with normal PBECs, IL-1β stimulation did not affect expression of
248 DUSP10 at either the mRNA or protein level. Furthermore, siRNA knock down of DUSP10 in
249 COPD PBECs increased release of CXCL8 in response to a combination of RV16 and IL-1β
250 stimulation, as seen in normal PBECs.
251

252 ## Discussion

253 Rhinoviral infection causes exacerbations of underlying airway disease through excessive
254 inflammatory responses. The MAPKs are known to be activated by rhinoviral infection (18-
255 21, 37), however the roles of each pathway in the inflammatory response of primary cells to
256 RV have not been well characterised. Previous studies have found that inhibition of ERK or
257 p38 reduces production of inflammatory cytokines in response to RV (21-23). In accordance
258 with this, inhibition of the p38 or JNK MAPKs led to a decrease in inflammatory cytokine

production, however, ERK was found to have a lesser role, demonstrating differences between previously studied cell lines (BEAS-2B and 16HBE14o-) and primary cells. Although small molecule inhibitors may have off-target effects (38), the results strongly indicate that p38 and JNK are important inducers of inflammation in RV infection.

Three members of the DUSP family, DUSPs 1, 4 and 10, have been shown to negatively regulate MAPK pathways in innate immune signalling, although their role in RV infection has not yet been studied. All three DUSPs were constitutively expressed by PBECs. DUSPs 1 and 4 have previously been characterised as early response genes, with no constitutive expression of DUSP1 in primary human airway smooth muscle cells, or DUSP4 in mouse BMDMs or embryonic fibroblasts (33, 39, 40). In contrast, DUSP10 is constitutively present in HeLa cells and murine BMDMs, and upregulated by innate immune stimuli (34, 41). The expression of DUSPs 1 and 4 at baseline may be a specific characteristic of bronchial epithelial cells, as opposed to macrophages or fibroblasts. Differentiation of PBECs in air-liquid interface cultures has been shown to alter expression of cellular proteins, however previous gene expression arrays have not shown differences in DUSP1, 4, or 10 expression between submerged and differentiated cultures (42).

Infection with either strain of RV caused a decrease in DUSP10 expression, at the mRNA and protein level, which was not seen in response to IL-1β. In 2008, Proud et al. performed a gene expression array of nasal scrapings after experimental RV16 infection. DUSP10 mRNA expression was unchanged at 8 and 48 h post-infection (43). However, as the downregulation observed in our study was transient, changes in DUSP10 expression may have occurred outside of the two time points investigated in the Proud study. This downregulation of

283 DUSP10 may be a host or viral triggered response. In support of a host-mediated response,
284 previous work has shown DUSPs 1 and 6 are regulated by proteasomal degradation in cells
285 treated with growth factors or carcinogens (44, 45). However, many viruses target host
286 proteins for degradation, either utilising host ubiquitin ligases or expressing their own (46,
287 47). Rhinovirus also encodes its own proteinases 2A and 3C, which have been found to
288 degrade components of the IFN signalling pathway (48), and of relevance the non-structural-
289 1 protein of human immunodeficiency virus has been shown to target DUSP1 for upregulation
290 in order to limit the inflammatory response (49). The extent to which regulation of DUSP10
291 in this context may be a pathogen-driven manipulation of the host immune system remains
292 to be determined.
293
294 In this study, DUSP10 expression was successfully knocked down using siRNA, allowing
295 investigation into the role of this protein in RV infection of PBECs. Reduced DUSP10 levels did
296 not affect RV replication, or IFN-β production in response to RV or poly(I:C). This contrasts
297 with the study by James et al. where influenza replication was decreased in DUSP10 knock
298 out mice due to increased IFN levels (34). This implies specific roles for DUSP10 in individual
299 pathogenic infections, potentially consequent upon differential TLR signalling by each virus
300 (50). Interestingly, DUSP10 knock out BMDMs produced increased mRNA and secreted
301 protein levels of IFN-β in response to poly(I:C) (34). Thus, DUSP10 roles may be species and/or
302 cell-type specific, emphasising the need for studies such as ours examining their role in
303 primary human airway epithelial cells.
304
305 Knock down of DUSP10 did not affect cytokine release in response to RV infection or poly(I:C)
306 stimulation. However, in response to IL-1β, DUSP10 knock down consistently caused an

307 increase in CXCL8 production. In order to gain a wider view of the role of DUSP10 a cytokine
308 array was utilised. Although this technique is semi-quantitative and includes samples from
309 only one donor, it gives an indication of the points at which DUSP10 may be acting.
310 Interestingly, DUSP10 knock down increased release of neutrophil chemoattractants CXCL8
311 and CXCL1, and decreased release of CXCL10, a Th1 cell chemoattractant. The MAPK
312 pathways have previously been shown to downregulate CXCL10 production in response to
313 RV16 through negatively regulating IRF1 activity (51). However, p38 and JNK MAPK activation
314 levels were unchanged by DUSP10 knock down, suggesting a potential novel target of
315 DUSP10. Expression of IL-1β itself was also increased in DUSP10 knock down, which could
316 point towards a role for DUSP10 in inflammasome regulation. Rhinoviral infection of PBECs is
317 known to activate the NLRP3 and NLRC5 inflammasomes leading to IL-1β release (26, 52).
318 More recently, RV infection has been found to increase caspase 1 expression to a greater
319 extent in asthmatic PBECs than normal cells, and in a house dust mite murine model of asthma
320 exacerbations, caspase 1 knock out mice had reduced Th2 responses to poly(I:C) (53). Thus, a
321 potential role of DUSP10 in regulating the inflammasome has significant implications for
322 asthma.
323
324 IL-1β is an important inflammatory molecule shown to have roles in asthma and COPD (54,
325 55) and IL-1β knock out mice have reduced neutrophilic and Th2 responses in a murine
326 asthma model (56). Blocking IL-1β signalling in PBECs decreases the release of inflammatory
327 mediators in response to RV infection (26). Previous work by our group and others has found
328 a key role for IL-1β in cooperative signalling between monocytes/macrophages and epithelial
329 cells. In vitro co-culture models have demonstrated that addition of monocytes to epithelial
330 cells can exacerbate the inflammatory response to lipopolysaccharide, unless IL-1β signalling

is blocked with blocking antibodies or IL-1 receptor antagonist (IL-1Ra) (57-60). Monocytes have been shown to release IL-1β in response to RV infection (11), and cooperative signalling has also been demonstrated in the context of RV infection: addition of primary monocytes to BEAS-2B cells or PBECs increases the production of inflammatory cytokines, CXCL8, CCL2, and CXCL10, in response to RV (27, 61), and IL-1Ra inhibited this increased cytokine generation (27). In accordance with this, PBECs were found to release IL-1β in response to RV infection. Co-stimulating PBECs with RV and IL-1β was found to dramatically potentiate the response to RV alone. The response was further increased by loss of DUSP10. This was true for three serotypes of rhinovirus, including; a major group A, RV16, a minor group A, RV1B, and a major group B, RV14. This suggests that DUSP10 would have a role in the response to rhinoviral infection in the airway; RV inducing IL-1β release by monocytes, which stimulates epithelial cells to release cytokines, regulated by DUSP10. This anti-inflammatory role for DUSP10 was also observed in two independent COPD donors, with increased CXCL8 release in response to RV and IL-1β co-stimulation when DUSP10 was knocked down. COPD and asthma patients have been shown to have increased baseline levels of IL-1β (54, 62, 63), thus DUSP10 may have an increased role in a disease setting. However, this remains to be investigated as it was not possible to directly compare PBECs from healthy and COPD donors in this study, due to differences in isolation techniques. Therefore, any additional role of DUSP10 in inflammatory airway diseases remains to be investigated.

These data demonstrate a novel role for DUSP10 in negatively regulating the inflammatory response of epithelial cells to IL-1β alone and in combination with RV. This suggests DUSP10 has an important role in regulating inflammation of the airway and identifies it as a potential

354 future therapeutic target for exacerbations of asthma and chronic obstructive pulmonary
355 disease.
356

357 ## Materials and Methods

358 **Cells and Viruses.** Primary human bronchial epithelial cells (PBECs) isolated from healthy
359 humans were purchased from Promocell (Heidelberg, Germany) and PBECs isolated from
360 patients with COPD were purchased from Lonza (Basel, Switzerland). Cells were maintained
361 as previously described (27), and all experiments were carried out on at least three
362 independent PBEC donors.
363

364 Human rhinovirus serotypes 1B and 16 were propagated in HeLa Ohio cells (American Type
365 Culture Collection) in DMEM (Gibco), supplemented with 2% FCS (Gibco), 2% hepes (Gibco),
366 1% bicarbonate (Gibco) and penicillin-streptomycin (Invitrogen) as previously described (27).
367 Human rhinovirus serotype 14 was a kind gift from MedImmune Ltd, Cambridge, UK. Viral
368 titres were determined by TCID50 in HeLa Ohio cells.
369

370 **Inhibitor Treatment.** Prior to cell stimulation or infection, cells were treated with the
371 indicated concentration of MAPK inhibitor (Tocris), diluted in DMSO, for one hour. Inhibitors
372 remained present throughout the experiment.
373

374 **siRNA Knock Down**. PBECs were grown in 12 well plates until 80% confluent. Lipofectamine
375 2000 (Invitrogen) and DUSP10 siRNA (Santa Cruz, sc-61048) or control siRNA (Dharmacon, D-
376 001810-02-05) were diluted to the indicated concentrations in Opti-Mem (Gibco) and
377 equilibrated at room temperature for 5 minutes before both solutions were combined and

further equilibrated for 20 minutes. PBECs were washed in PBS and media replaced with fresh supplement-free airway epithelial cell basal media and siRNA mixtures were applied dropwise. Cells were incubated at 37°C, 5% CO_2 for four hours before media was replaced with airway epithelial cell basal media, supplemented with penicillin-streptomycin and the airway epithelial cell supplement pack excepting bovine pituitary extract (recovery media). Cells were incubated at 37°C, 5% CO_2 for 48 h prior to stimulation or infection.

RV Infection of PBECs. PBECs were seeded in 12 well plates and grown to 80-90% confluency. Media was replaced with supplement-free airway epithelial cell basal medium and incubated at 37°C, 5% CO_2 overnight. Cells were incubated with the indicated MOI of RV for one hour at 37°C, 5% CO_2 with agitation. MOIs were selected, based on preliminary concentration-response optimisation experiments, to provide equivalent inflammatory cytokine release, and intracellular RV RNA copies (see also Figure 5C, D, F). Virus was removed and replaced with recovery media and cells were incubated at 37°C, 5% CO_2 for the indicated time point. Cell free supernatants or cell lysates were harvested and stored at -80°C.

IL-1β or Poly(I:C) Stimulation of PBECs. PBECs were seeded in 12 well plates and grown to 80-90% confluency. Media was replaced with supplement-free airway epithelial cell basal medium and incubated at 37°C, 5% CO_2 overnight. Media was replaced with recovery media containing the indicated concentration of IL-1β (Peprotech) or low molecular weight poly(I:C) (Invivogen). Cells were incubated at 37°C, 5% CO_2 for the indicated time point. Cell free supernatants or cell lysates were harvested and stored at -80°C.

401 **Co-treatment of PBECs with RV and IL-1β.** PBECs were infected with RV as described above.
402 After removal of the virus, media was replaced with recovery media containing the indicated
403 concentration of IL-1β (Peprotech). Cells were incubated at 37°C, 5% CO_2 for the indicated
404 time point. Cell free supernatants or cell lysates were harvested and stored at -80°C.
405
406 **PCR.** RNA was extracted using Tri reagent (Sigma-Aldrich) according to the manufacturer's
407 instructions and contaminating DNA removed using a DNase treatment kit (Ambion). cDNA
408 was generated from 1 μg RNA using high-capacity cDNA reverse transcriptase kit (Applied
409 Biosystems).
410
411 PCR was carried out using the GoTaq hot start polymerase kit (Promega) according to the
412 manufacturer's instructions using primers specific to DUSPs 1, 4, and 10, and GAPDH (Sigma-
413 Aldrich). DUSP1 (F: GTCGTGCAGCAAACAGTCGA, R: CGATTAGTCCTCATAAGGTA; (62)), DUSP4
414 (F: TTCAACAGGCATCCATCCCT, R: TGGCTTTGGGAGGGAATGAT), DUSP10 (F:
415 ATGACCAAATGCAGCAAG, R: GGAGCTGGAGGGAGTTGTCAC; (63)), GAPDH (F:
416 GGTGAAGGTCGGTGTGAAC, R: CTCGCTCCTGGAAGATGGTG).
417
418 Quantitative PCR was carried out using primers and probes from Sigma-Aldrich for RV
419 (SY150600935-024, SY150600935-025, HA07878670-002) and IFN-β (SY150506722-061,
420 SY150504450-060, HA07784503-002) and primer-probe sets from Applied Biosystems for
421 DUSP1 (Hs00610256_g1), DUSP4 (Hs01027785_m1), DUSP10 (Hs00200527_m1), CXCL8
422 (Hs00174103_m1), and GAPDH (Hs02758991_g1). Reaction mixtures were made using
423 Promega GoTaq Probe qPCR master mix or Eurogentec qPCR mastermix and run using an
424 ABI7900 fast real-time PCR system (Applied Biosystems) (50°C for 2 minutes, 95°C for 10

minutes, then cycled 40 times through 95°C for 15 seconds, 60°C for 1 minute). CXCL8, IFN-β, and RV were quantified against a standard curve of plasmids containing known copy numbers of target genes. CXCL8, IFN-β, DUSP1, DUSP4, and DUSP10 expression was normalised to GAPDH.

ELISA. Enzyme-linked immunosorbent assay (ELISA) was used to detect CXCL8 and CCL5 in cell-free supernatants using matched antibody pairs from R&D systems. Levels of CXCL8 and CCL5, above the minimum detection level (CXCL8: 78.125 pg/ml, CCL5: 156.25 pg/ml), were quantified against a standard curve from the same plate.

Cytokine Array. The presence of 36 proteins in cell-free supernatants was determined using R&D systems human cytokine array (ARY005B) according to manufacturer's instructions.

Western Blot. PBECs were lysed in buffer containing 1% Triton-X and boiled for 5 minutes in SDS-PAGE buffer. Lysates were subjected to SDS-PAGE and proteins transferred to nitrocellulose membrane. Membranes were blotted with antibodies to DUSP10 (Abcam), phosphorylated p38 (Promega), phosphorylated JNK (Cell Signalling), and actin (Sigma-Aldrich). Antibodies were detected using HRP-conjugated anti-rabbit secondary antibody (Dako). Densitometric analysis was performed using ImageJ software (Version 1.50i; NIH).

Statistical Analysis. All data presented, excluding figure 6, are mean ± SEM (where appropriate) of at least three independent experiments using PBECs from different donors. Data were analysed using the statistical test stated in the figure legend on log data, as data are lognormally distributed. In figures 1, 6 A, and 9 A, normalised data has been presented

due to variability between PBEC donors. For normalised data or ΔΔCt qRT-PCR data, statistical tests were performed on raw data or ΔCt values respectively. Significant differences are indicated by * ≤ p 0.05, ** ≤ p 0.01, *** ≤ p 0.001, and **** p ≤ 0.0001.

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677

678 ## Figure Legends

679 **Figure 1: Inhibition of the p38 or JNK pathways reduces cytokine release in response to RV**
680 **infection.** PBECs were treated with media only, DMSO or MAPK inhibitors (20 μM): PD90859
681 (ERK), SB203580 (p38), SB202190 (p38) and SP600125 (JNK), for one hour prior to stimulation
682 with poly(I:C) (25 μg/ml) for 24 h, n = 3 (A), or infection with RV1B (MOI 3) (B) or RV16 (MOI
683 4) (C) for 48 h, n=4 individual donors. Inhibitors remained present throughout the experiment.
684 Supernatants and cell lysates were collected and levels of CXCL8 mRNA and release were
685 measured by qRT-PCR and ELISA. Data shown are mean ± SEM, normalised to RV + DMSO
686 treated cells. Significance versus RV + DMSO treated cells is indicated by * ≤ p 0.05, ** p ≤
687 0.01, as measured by one-way ANOVA, Dunnett's post-test on log raw data.

688

689 **Figure 2: DUSPs 1, 4 and 10 are expressed in PBECs**. PBECs were stimulated with poly(I:C) (25
690 μg/ml) (P), IL-1β (10 ng/ml) (β) or left untreated (U) over 24 hours. mRNA was collected at the
691 time points indicated and RT-PCR performed using primers for DUSPs 1, 4 and 10 and a GAPDH
692 control. n=2 individual donors with a representative gel shown.

693

694 **Figure 3: DUSP10 expression is decreased by RV infection.** PBECs were infected with RV1B
695 (MOI 3), RV16 (MOI 4), or IL-1β (100 ng/ml) over 24 h and cell lysates collected at the indicated
696 time points. DUSP1 (A), DUSP4 (B) and DUSP10 (C) expression was measured using qRT-PCR.
697 Data shown are mean ± SEM, n=3 individual donors. Significance versus uninfected control (0
698 h) is indicated by * p ≤ 0.05, as measured by one-way ANOVA, Dunnett's post-test on log ΔCt
699 values.

700

Figure 4: DUSP10 protein expression is decreased by RV infection. PBECs were infected with RV1B (MOI 3) (A), RV16 (MOI 4) (B), or IL-1β (100 ng/ml) (C) over 24 h and cell lysates collected at the indicated time points. DUSP10 and actin expression was measured using western blot. Data shown are mean ± SEM of densitometry, n=3 individual donors, with representative blot below. Significance is indicated by * $p \leq 0.05$, as measured by one-way ANOVA, Dunnett's post-test on log values. Please note, blots shown are segments of longer time courses.

Figure 5: DUSP10 knock down does not affect the response of PBECs to RV infection. PBECs were untransfected or treated with DUSP10 (D10) or control (Ctrl) siRNA (100 nM) for 48 h. Cell lysates and supernatants were collected and analysed for DUSP10 expression by qRT-PCR (A) and western blot (B). PBECs were then infected with RV1B (MOI 3), RV16 (MOI 4), or poly(I:C) (25 μg/ml). Cell lysates and supernatants were collected after 24 h and analysed by ELISA for CXCL8 (C) and CCL5 (E) or after 16 h and qRT-PCR performed for IFN-β (D). RV RNA levels after 24 h were measured using qRT-PCR (F). Data shown are mean ± SEM, n=3 individual donors, except F n=4 individual donors. Significance between siRNA treatments is indicated by * $p \leq 0.05$ as measured using one-way ANOVA Dunnett's post-test on log ΔCt values (A) or log protein expression (B) or two-way ANOVA, Sidak's post-test on log data (C-F).

Figure 6: DUSP10 knock down increases cytokine production but not MAPK activation in response to IL-1β. PBECs were treated with DUSP10 (D10) or control (Ctrl) siRNA (100 nM) for 48 h prior to stimulation with IL-1β (1-100 ng/ml) for 24 h. Cell lysates and supernatants were collected and CXCL8 measured by qRT-PCR (A) and ELISA (B), n=4 individual donors. PBECs were treated with siRNA for 48 h prior to stimulation with IL-1β (100 ng/ml) for 30

minutes. Cell lysates were collected and levels of phosphorylated p38 and JNK and total actin were measured using western blot. Data shown are mean ± SEM of band density with representative blots shown, n=3 individual donors (C). Significance between siRNA treatments is indicated by * $p \leq 0.05$ as measured by two-way ANOVA, Sidak's post-test on log data.

Figure 7: The effect of DUSP10 knock down on cytokine expression in response to IL-1β. PBECs were treated with DUSP10 (D10) or control (Ctrl) siRNA (100 nM) for 48 h prior to stimulation with IL-1β (10 ng/ml) for 24 h. Supernatants were collected and cytokine array performed. Data presented are spot density normalised to cell number, n=1.

Figure 8: IL-1β is released in response to RV infection. PBECs were infected with RV1B (MOI 3) or RV16 (MOI 4) for 24 h. Supernatants were collected and levels of IL-1β release were measured by ELISA. Data shown are mean ± SEM, n=3 individual donors. Significance versus uninfected cells is indicated by * $p \leq 0.05$, *** $p \leq 0.001$, as measured by one-way ANOVA, Dunnett's post-test on log data.

Figure 9: IL-1β potentiates the response of PBECs to RV infection. PBECs were treated with control (Ctrl) or DUSP10 (D10) siRNA (100 nM) for 48 h prior to stimulation with IL-1β (1 or 10 ng/ml) and/or infection with RV16 (MOI 4) (A and B), RV1B (MOI 3) (C) or RV14 (D) for 24 h. Cell supernatants and lysates were collected and CXCL8 measured by qRT-PCR (A) and ELISA (B, C, D). Significance is indicated by * $p \leq 0.05$, ** $p \leq 0.01$ *** $p \leq 0.001$, **** $p \leq 0.0001$ between Ctrl and D10 siRNA as measured by two-way ANOVA Sidak's post test, or # between

RV alone and in combination with IL-1β as measured by two-way ANOVA Dunnett's post-test on log raw data, n=3 individual donors.

Figure 10: DUSP10 expression is decreased by RV infection in COPD cells and DUSP10 knock down increases CXCL8 release. COPD PBECs were infected with RV1B (MOI 3), RV16 (MOI 4), or IL-1β (100 ng/ml) over 24 h and cell lysates collected at the indicated time points. DUSP10 expression was measured using qRT-PCR (A) and western blot (B). Data shown are mean ± SEM n=3 individual donors. Densitometry is shown in B with a representative blot in C. Significance was measured by one-way ANOVA, Dunnett's post-test on log ΔCt values (A) or log densitometric values (B). COPD PBECs were treated with control (Ctrl) or DUSP10 (D10) siRNA (100 nM) for 48 h prior to stimulation with IL-1β (1 or 10 ng/ml) and/or infection with RV16 (MOI 4) for 24 h. Cell supernatants were collected and CXCL8 measured by ELISA, n=2 individual donors (D).