1 **Research article**

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3 MicroRNA-125a/b Inhibits A20 and MAVS to Promote Inflammation and 4 Impair Antiviral Response in Chronic Obstructive Pulmonary Disease

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ABSTRACT

Influenza A virus (IAV) infections lead to severe inflammation in the airways. Patients with chronic obstructive pulmonary disease (COPD) characteristically have exaggerated airway inflammation and are more susceptible to infections with severe symptoms and increased mortality. The mechanisms that control inflammation duirng IAV infection and the mechanisms of immune dys-regulation in COPD are unclear. We found that IAV infections lead to increased inflammatory and antiviral reponses in primary bronchial epithelial cells (pBECs) from healthy non-smoking and smoking subjects. In pBECs from COPD patients, infections resulted in an exaggerated inflammatory but deficient antiviral responses. A20 is an important negative regulator of nuclear factor-kappaB (NF-κB)-mediated inflammatory but not antiviral response, and A20 expression was reduced in COPD. IAV infection increased the expression of micro(miR)-125a/b, which directly reduced the expression of A20 and mitochondrial antiviral signaling (MAVS), and caused exaggerated inflammation and impaired antiviral responses. These events were replicated *in vivo* in a mouse model of experimental COPD. Thus, miR-125a/b and A20 and may be targeted therapeutically to inhibit excessive inflammatory responses and enhance antiviral immunity in IAV infections and in COPD.

41 **INTRODUCTION**

42 Influenza A viruses (IAVs) are amongst the most important infectious human pathogens that
43 cause enormous morbidity and mortality worldwide. This largely results from seasonal
44 influenza but an important feature of the biology of IAVs is the frequent emergence of novel
45 pandemic strains/subtypes. Infections cause symptoms ranging from mild to severe viral
46 pneumonia, with uncontrolled inflammation in the airways.

47 Bronchial epithelial cells (BECs) are the primary site of IAV infection, and innate
48 immune responses produced by these cells are important in the early protection against the
49 viruses (1, 2). During infection viral RNAs are recognized by toll-like receptor 3 (TLR3) and
50 retinoic acid-inducible gene-I (RIG-I). Upon binding of TLR3 to viral RNAs signalling
51 pathways are initiated that activate receptor interacting protein 1 (RIP1) by ubiquitination.
52 Activated RIP1 indirectly phosphorylates IκBα, leading to the release of active p65 and p50
53 subunits of nuclear factor-kappaB (NF-κB) into the nucleus where they induce the transcription
54 of inflammatory genes including of cytokines such as interleukin-6 (IL-6), tumor necrosis
55 factor-α (TNF-α), and IL-1β, and chemokines such as CXC chemokine ligand-8 (CXCL-8/IL-
56 8) (3-5). These inflammatory cytokines recruit immune cells, in particular macrophages and
57 neutrophils, to the site of infection that phagocytose pathogens and apoptotic cells (6, 7). RIG-
58 I interacts with mitochondrial antiviral-signaling protein (MAVS), which activates interferon
59 regulatory factor 3 (IRF3) by phosphorylation. Activated IRF3 then translocates into the
60 nucleus where it initiates the production of type I and III interferons (IFNs) (8, 9). These innate
61 cytokines induce the transcription of over 300 IFN-stimulated genes (ISGs) including the Mx1
62 protein that disrupts virus replication (10).

63 The control of inflammation is critical to achieving optimal inflammatory responses
64 that clear viruses without excessive damage to host tissues and airways. We have previously
65 shown that A20, also known as TNF-α-inducing protein 3 (TNFAIP3), is a negative regulator

66 of NF-κB-mediated inflammation that functions by targeting RIP1 for degradation, and
67 therefore suppresses NF-κB activation (11-14). Micro-RNAs (miRNAs; miRs) are another
68 important class of immune signaling regulators that silence gene expression by degradation
69 (15). miR-125a and b have recently been shown to directly inhibit A20, leading to increased
70 NF-κB activation (16). It is currently unknown if A20 or miR-125a/b regulates type I and III
71 IFNs during IAV infections.

72 Chronic obstructive pulmonary disease (COPD) is the 3rd leading cause of illness and
73 death globally and is characterized by progressive airway inflammation, emphysema, and
74 reduced lung function (17). The most important risk factor for COPD in Western societies is
75 cigarette smoking (18). COPD patients have increased susceptibility to IAV infections that
76 cause acute exacerbations and result in more severe symptoms, disease progression, and
77 increased mortality (19-21). Current therapeutics remain limited to vaccination and antiviral
78 drugs. These have major issues with the constant need for developing new vaccines, COPD
79 patients respond poorly to vaccination, IAVs have become drug resistant and all therapeutics
80 have questions surrounding availability and efficacy in future pandmics (22, 23). There is
81 therefore an urgent need to develop novel therapeutics for influenza, especially for those most
82 susceptible to infection.

83 Despite inflammatory signalling pathways being well-characterized, the mechanisms
84 underlying the exaggerated inflammatory responses to IAV, including in COPD are unclear. It
85 is known that increased NF-κB activation is elevated in biopsies from COPD patients (24). We
86 have previously shown that human influenza H3N2 infection induced heightened inflammatory
87 responses (25), and high pathogenic avian H5N1is known to induce severe cytokine storms in
88 the lung (9, 26). We also showed that primary BECs (pBECs) from COPD subjects and our
89 established *in vivo* model of experimental COPD have increased inflammatory and impaired
90 antiviral responses to IAV infections, leading to more severe infection (27-29). Furthermore,

91 miRNAs are known to be altered in COPD (30, 31). However, the molecular mechanisms
92 underpinning the heightened inflammatory response in IAV infections and defective immune
93 responses in COPD remain unclear. In this study, we investigated the mechanisms involved
94 using our established experimental systems (27, 32-34). We found that COPD pBECs and mice
95 with experimental COPD infected with IAV have higher levels of inflammatory cytokines but
96 reduced antiviral responses (30, 35). We uncovered that NF-κB-mediated inflammation in IAV
97 infection and in COPD was also exaggerated, which resulted from decreased levels of A20
98 protein, which in turn was caused by elevated levels of miR-125a/b. Treatment with specific
99 antagomiRs against miR-125a or b reduced NF-κB activation but also increased type I and III
100 IFNs production and suppressed infection. We then found that miR-125a and b directly targets
101 MAVS 3′ untranslated region (UTR), thereby suppressing the induction of type I/III IFNs. This
102 study therefore discovers a novel miR-125-mediated pathway that reduces A20 and MAVS
103 and promotes excessive inflammation and increases susceptibility to IAV infection in COPD.
104 It also identifies novel potential therapeutic options that reduce IAV-mediated inflammation
105 and reverse immune signaling abnormalities in COPD.
106 Some of the data has been previously reported in abstract form (36).
107
108 **RESULTS**
109
110 **IAV infection induces increased inflammatory but reduced antiviral responses *ex vivo* in**
111 **human COPD pBECs**
112 pBECs from healthy non-smoking control subjects, COPD patients (ex-smoker) or smoking
113 (smoker) controls and were infected with IAV H3N2 or H1N1 (MOI 5). Virus replication was
114 measured 24hr after infection. Virus titers increased at 24hr (Fig. 1A), and was two-fold greater
115 in COPD pBECs compared to controls. Infection resulted in the production of the pro-

116 inflammatory cytokines/chemokines IL-6, CXCL-8, TNF-α, and IL-1β, and antiviral cytokines
117 type I (IFN-β) and type III interferons (IFN-λ1) (Fig. 1B). In COPD, the induction levels of
118 cytokines were substantially higher (2.5-10 fold) compared with healthy control and smoker
119 pBECs (Fig. 1B). In contrast, the induction of IFN-β and IFN-λ1 proteins were reduced in
120 COPD.
121 We then measured the levels of activity of NF-κB by assessing the levels of phosphorylated
122 p65 at Ser536 (phospho-p65) (35, 37, 38). Infection significantly increased the activation of
123 p65 (phospho-p65) in both healthy and smoker pBECs at 6hr, which was further increased at
124 24hr (Fig. 1C; Supplementary Fig. S1A). In COPD pBECs the protein levels of phospho-p65
125 was elevated at baseline (media controls) at 6hr and significantly increased with infection at
126 24hr compared to healthy and smoker controls.
127
128 **IAV infection also induces increased inflammatory but reduced antiviral responses *in***
129 ***vivo* in experimental COPD**
130 We then demonstrated these events also occur *in vivo*. BALB/c mice were exposed to either
131 normal air (Air) or cigarette smoke (Smk) for eight weeks. The Smk group develops hallmark
132 features of COPD as previously described extensively (27, 28, 32-35, 39). Mice were then
133 infected with IAV A/PR/8/34, and viral titers, airway inflammation (histopathological score),
134 and inflammatory and antiviral cytokines were determined at 7 days post infection (dpi) (Fig.
135 2A). Infection in Air-exposed controls leads to virus replication (Fig. 2B) that was
136 accompanied by significant airway inflammation (histopathological score, Supplementary Fig.
137 S1B). Infection in Smk-exposed mice resulted in a significantly higher virus titers (four-fold)
138 and airway histopathological score (three fold) compared to Air-exposed mice. In support of
139 these data, the levels of the pro-inflammatory cytokines/chemokines IL-6, KC (mouse
140 equivalent of CXCL-8), TNF-α, and IL-1β were also increased by infection in Air- and to a

141 greater extent in Smk-exposed groups (Fig. 2C). Antiviral cytokines were increased in infected
142 Air-exposed controls but were either not induced (IFN-β) or were induced to a much reduced
143 level (IFN-λ3) in infected Smk-exposed groups (Fig. 2D). The exaggerated release of pro-
144 inflammatory cytokines was associated with significantly increased levels of phospho-p65
145 protein in infected Smk-exposed compared to Air-exposed controls (Fig. 2E; Supplementary
146 Fig. S1C). In all experiments, ultraviolet-inactivated virus did not have any effects compared
147 to media controls (data not shown).
148 Taken together these human *ex vivo* and experimental *in vivo* data demonstrate that IAV
149 infections result in increased airway inflammation, pro-inflammatory and antiviral responses.
150 However, COPD is associated with exaggerated inflammation and reduced antiviral responses,
151 leading to increased virus replication.
152
153 **A20 is an important negative regulatory of NF-κB-mediated inflammatory but not**
154 **antiviral responses, and its expression is reduced in human COPD and experimental**
155 **COPD**
156 We have previously shown that A20 is an important negative regulator of NF-κB activation
157 (11-14), but its roles during IAV infection and whether it also regulates the induction of type I
158 and III IFNs is unclear. We hypothesized that A20 protein expression would be down-regulated
159 and would contribute to the increased activation of NF-κB in response to IAV infection in
160 COPD. IAV infection led to a significant induction of A20 protein at 6hr and 24hr in healthy
161 and smoker controls, but this increase was impaired in COPD pBECs (Fig. 3A; Supplementary
162 Fig. S2A). Similarly in Smk-exposed mice, A20 protein expression was reduced in airway
163 epithelial cells compared to Air-exposed controls (Supplementary Fig. S2B).
164 We then investigated if A20 was important in NF-κB-mediated inflammatory responses, and if
165 exaggerated p65 activation was the direct result of reduced A20 protein levels during infection

166 in COPD pBECs. We inhibited A20 expression using A20-specific siRNA 24hr before
167 infection, and measured the activation of p65 and the production of pro-inflammatory
168 cytokines/chemokines 24hr after infection. Inhibition of A20 expression (Fig. 3B;
169 Supplementary Fig. S2C) resulted in significant increases in the protein levels of phospho-p65
170 (Fig. 3C; Supplementary Fig. S2C), and pro-inflammatory cytokines/chemokines IL-6, CXCL-
171 8, TNF-α, and IL-1β (Fig. 3D) compared to un-treated controls, whether pBECs were infected
172 or not. Conversely, ectopic (ecto-) expression using a pcDNA-A20 expression vector reduced
173 the phosphorylation of p65 (Supplementary Fig. S2D). Nevertheless, inhibition or ecto-
174 expression of A20 did not affect IFN-β and IFN-λ1 induction (Fig. 3E; Supplementary Fig.
175 S2D). siRNA negative control or control vector did not affect the induction of A20 or phospho-
176 p65 protein (Supplementary Fig. S2E-F).
177 Collectively these data indicate that A20 is an important negative regulator of NF-κB but is
178 dispensible in the induction of type I and III IFNs. A20 protein expression is dysregulated in
179 COPD.
180
181 **Elevated miR-125a and b levels decrease A20 levels, increase inflammation and impair**
182 **antiviral responses in COPD pBEC and experimental COPD**
183 miR-125a and b have recently been shown to directly target and inhibit A20 expression (16),
184 but its roles during IAV infection and in COPD are unknown. Thus, we measured the levels of
185 miR-125a and b induced by IAV infection. H3N2 and H1N1 infections resulted in significant
186 increases in the levels of these miRs at 24hr in pBECs from all groups (Fig. 4A). However,
187 their levels were substantially greater at baseline and during infection (2-4 fold) in COPD
188 pBECs compared to healthy controls. We then confirmed the direct link between increased
189 miR-125a and b levels and reduced A20 protein induction using specific antagomiRs and
190 mimetics. pBECs were pre-treated with either miR-125a or b specific antagomiRs or mimetics

191 for 24hr before infection, and A20, phospho-p65, inflammatory and antiviral cytokines and
192 were assessed 24hr after infection. AntagomiR treatment inhibited miR-125a or b expression
193 (Supplementary Fig. S3A), and this resulted in significant increases in A20 protein production,
194 reduced phosphorylation of p65, subsequent induction of pro-inflammatory
195 cytokines/chemokines, and enhanced antiviral IFN-β and λ1 responses (Fig. 4B and
196 Supplementary Fig. S3B-E) compared to un-treated controls. Conversely miR-125a or b
197 mimetics decreased A20 protein induction, increased phospho-p65protein levels and reduced
198 IFN-β responses (Supplementary Fig. S3F). Treatment with scrambled miR or mimetic
199 controls did not affect A20, phospho-p65, or IFN-β production (Supplementary Fig. S3G-H).
200 We then assessed whether similar events occurred *in vivo*. IAV infection significantly increased
201 the levels of miR-125a and b in both groups, with the levels in Smk group significantly higher
202 compared to Air-exposed controls (Fig. 4C). We then inhibited miR-125a or b before and
203 during infection (Fig. 4D). We also extended the *ex vivo* data by inhibiting both miR-125a and
204 b together. Treatment with miR-125a or b antagomiR, alone or in combination, reduced
205 histopathological scores (Fig. 4E and Supplementary Fig. S4A) and improved lung function
206 (reduced lung volume determined during a pressure-volume loop manoeuvre) in Air and Smk-
207 exposed groups compared to infected scrambled antagomiR-treated controls (Supplementary
208 Fig. S4B). Inhibition of miR-125a, b, or a and b, also increased A20 protein expression in the
209 airway epithelium and decreased the levels of phospho-p65 compared to the controls (Fig. 4F;
210 Supplementary Fig. S4C-D). Importantly while we could only detect reductions in TNF-α and
211 KC with combined treatment (Supplementary Fig. S4E), antagomiR treatment, either alone or
212 in combination, also significantly increased IFN-β and IFN-λ3 protein induction (Fig. 4F and
213 Supplementary Fig. S4D).

214 Collectively, these data show that miR-125a and b are directly involved in the regulation of
215 both inflammatory cytokines, through the control of A20, and antiviral cytokine production
216 through an unknown target.
217
218 **miR-125a and b target MAVS**
219 To determine the mechanism of miR-125a and b-mediated regulation of antiviral IFN-β/λ, we
220 performed miRNA prediction analysis using TargetScan (www.targetscan.org). miR-125a and
221 b have a putative binding site in the 3′-UTR of human and mouse *MAVS* (Fig. 5A-B). To
222 examine these putative interactions we first assessed the protein levels of MAVS in pBECs.
223 MAVS protein levels were significantly increased 24hr after IAV infection in healthy control
224 and smoker, but notably not in COPD pBECs (Fig. 5C; Supplementary Fig. S5A). Similarly,
225 infection in Smk-exposed mice was also associated with signficantly impaired production of
226 MAVS compared to infected Air-exposed controls at 7dpi (Fig. 5D; Supplementary Fig. S5B).
227 To confirm the potential interaction of miR-125a/b and MAVS, we cloned the putative binding
228 region of miR-125a and b in wild-type (MAVS-WT) or mutant (MAVS-MT) *MAVS* 3′-UTR
229 into a luciferase reporter construct. The construct was co-transfected into HEK293 cells along
230 with miR-125a or b mimetics, or scrambled controls, and then luciferase activity was assessed.
231 Co-transfection of miR-125a or miR-125b mimetics with MAVS-WT resulted in a significant
232 decrease in luciferase activity compared to scrambled controls (Fig. 5E). There was no
233 reduction in activity with co-transfection with MAVS-MT. We then determined if *MAVS* gene
234 is present with the miR-125a or b mimetics in the silencing complex. To do this we
235 immunoprecipitated Argonaute 2 (Ago2), a core component of RNA-induced silencing
236 complex (RISC) that binds to the miRNAs and their target mRNA, with a specific antibody
237 and detected the presence of both *A20* and *MAVS* by qPCR, which could not be detected with

238 immunoprecipitation with IgG control (Fig. 5F). This confirmed that miR-125a and b directly
239 bind to the endogenous 3′-UTR of *MAVS*.
240
241 **miR-125a and b targeting of MAVS regulates antiviral responses in COPD pBEC and**
242 **experimental COPD**
243 We then investigated whether inhibition of miR-125 has a functional outcome. We showed that
244 miR-125a and b antagomiR treatment lead to significant increases in MAVS (Fig. 6A and
245 Supplementary Fig. S6A-B), IFN-β and IFN-λ1 protein induction (Fig. 4B and Supplementary
246 Fig. S3C), and reduced viral replication in both healthy and control pBECs (Fig. 6B). In
247 contrast, mimetics suppressed the induction of antiviral cytokines and increased virus titers
248 (Supplementary Fig. S3F). Similarly in Smk-exposed mice, inhibition of miR-125a, b, or a+b
249 resulted in increased induction of MAVS (Fig. 6C and Supplementary Fig. S6C), IFN-β and
250 IFN-λ3 (Fig. 4F and Supplementary Fig. S4D-E), and inhibited virus replication (Fig. 6D).
251 Collectively these data demonstrate that miR-125a and b negatively regulate MAVS expression
252 and suppress the induction of IFN-β/λ, and may potentially be targeted therapeutically in the
253 prevention and/or treatment of IAVs and COPD.
254
255 **DISCUSSION**
256
257 Here we discover that IAV infections induce airway inflammation and antiviral responses,
258 however in COPD pBECs and experimental COPD inflammatory responses and activation of
259 NF-κB are exaggerated but antiviral responses are impaired. We show that A20 is a negative
260 regulator of NF-κB-mediated induction of inflammatory but not antiviral cytokines, and that
261 A20 protein levels were impaired in COPD. The impaired induction of A20 and antiviral
262 responses in COPD was attributed to increased expression of miR-125a and b. Elevated levels

263 of these miRNAs suppressed A20 expression, leading to heightened NF-κB activity and
264 inflammation and reduced antiviral responses. Inhibition with miR-125a and b antagomiRs
265 increased A20 levels and reduced NF-κB activity, and also promoted IFN production. We then
266 demonstreated that miR-125a and b modulated IFN induction by targeting MAVS translation.
267 MAVS protein levels were reduced in COPD, but could be increased with specific miR-125a
268 and b antagomiR treatment that also induced IFN production. Thus, IAV infection induces the
269 expression of miR-125a/b that suppress A20 and MAVS, in turn promoting NF-κB-induced
270 inflammation and attenuating anitviral IFN production, respectively, increasing viral
271 replication. All these events are exaggerated in COPD (Fig. 7).

272 IAV is a major infectious pathogen that poses serious health concerns worldwide.
273 Infections, particularly with highly pathogenic influenza viruses, cause severe airway
274 inflammation and a cytokine storm with high morbidity and mortality. COPD is a major global
275 health problem that is underpinned by exaggerated inflammatory responses in the airways (40).
276 IAV infections frequently result in acute exacerbations of COPD, leading to accelerated
277 declines in lung function (41, 42) and increased mortality (20). The mechanisms of exaggerated
278 inflammation and severe outcomes in COPD are poorly understood, and there are no effective
279 therapies for these events.

280 Here we show that IAV-mediated inflammatory response are dampened with ectopic
281 expression of A20 that reduces NF-κB activity and inflammatory responses, without affecting
282 type I and III IFN responses. A20 is a de-ubiquitinating enzyme that degrades RIP1 and inhibits
283 NF-κB activation (13), and has been shown to suppress the induction of IFN-β (43). We found
284 that A20 modulated NF-κB activity and inflammation, but did not affect type I and III IFNs
285 production.

286 Consistent with our previous findings (27), IAV infections in COPD pBECs and
287 experimental COPD led to heightened inflammation and production of inflammatory cytokines

288 but impaired antiviral responses (IFN-β and IFN-λ), which were associated with greater viral
289 replication. Increased inflammation, inflamamtory cytokines and activation of NF-κB are well-
290 known in COPD (24, 44). Here we show that these are the result of reduced induction of A20,
291 leading to uncontrolled activation of NF-κB and subsequent induction of inflammatory
292 cytokines. A20 is a pleiotropic protein involved in various ubiquitin-dependent pathways
293 including NF-κB (16) and mitogen-activated protein (MAP) kinase pathway (45), and has also
294 been shown to negatively regulate type I IFN inductions (43, 46, 47). Surprisingly inhibition
295 or ectopic expression of A20 did not affect IFN-β production. The precise roles of A20 during
296 viral infections therefore require further investigation. We could not rule out that other factors
297 may also contribute to the regulation of A20 expression and of NF-κB activity, including other
298 un-identified miRNAs, which may also be dys-regulated in COPD.

299 Forced expression of A20 may be a novel therapeutic option that reduces IAV-
300 mediated inflammation and cytokine storm, particularly from high pathogenic IAVs such as
301 H5N1, or in COPD where airway inflammation is already persistently heightened.

302 The lack of the induction of A20 protein during IAV infection in COPD was attributed
303 to increased levels of miR-125a and b. These miRNAs down-regulate A20 expression by
304 directly binding to its 3′-UTR, leading to constitutive activation of NF-κB (16). We found that
305 heightened levels of miR-125a/b resulted in increased activation of NF-κB in COPD. Inhibition
306 of miR-125a or b in both healthy and COPD pBECs and in experimental COPD increased A20
307 protein levels and reduced NF-κB activation during IAV infection.

308 We also found that miR-125a and b modulated the induction of type I and III antiviral
309 IFNs. This occurred by the direct targeting of MAVS 3′-UTR, therefore down-regulating the
310 subsequent induction of IFN-β and IFN-λ. MAVS is an important adaptor protein on
311 mitochondria that facilitates the production of IFNs (8), however there was an impaired
312 induction of MAVS by IAV infections in COPD pBECs and in experimental COPD. Inhibition

313 of miR-125a and/or b increased the levels of MAVS and antiviral IFNs, which lead to reduced
314 virus replication both *in vivo* and *in vivo*. Interestingly antagomiRs against miR-125a and/or b
315 (either alone or in combination) in experimental COPD, partially reduced the release of
316 inflammatory cytokines, and substantially suppressed virus replication. This may indicate that
317 miR-125a and b may preferentially target MAVS over A20 during IAV infection in COPD,
318 although such binding preferences of miRNAs have not been widely investigated. Furthermore,
319 as MAVS is transcriptionally driven by IFN-sensitive response element (ISRE) as part of the
320 IFN-stimulated genes (48), and miR-125a/b have been reported to be induced by NF-κB (49),
321 it is possible that reduced MAVS partly attributed to impaired IFNs in COPD, and with
322 enhanced expression of miR-125a/b (NF-κB-inducible) this then leads to continuous cycle of
323 exaggerated inflammation and impaired antiviral immunity in COPD.

324 Although miR-125a/b appears to be NF-κB-inducible, the exact molecular
325 mechanisms of enhanced miR-125a/b expression in COPD require further investigation. In
326 colorectal cancer tissues the levels of miR-125a have been shown to be reduced, which is
327 asoociated with hyper-methylation at the CpG island within the promoter region of miR-125a
328 (50). Similarly in breast cancer cell line reduced miR-125a has also been shown to be associated
329 with tri-methylation at H3K9 and H3K27 at the promoter region of miR-125a (51). It is
330 therefore possible that the methylation status of miR-125a/b promoter site is altered in COPD,
331 leading to increased expression of miR-125a/b. Nevertheless, our data also demonstrate that
332 specific inhibition of miR-125a/b may be novel therapeutic options against IAV infections and
333 for those whom are most vulnerable.

334 Cigarette smoke is the major risk factor for COPD. Acute exposure results in
335 oxidiative stress and NF-κB activation (52-54). However the effects we have observed in
336 COPD appear to be independent of acute eposure to cigarette smoking, as the pBECs obtained
337 from subjects with COPD were all abstinent from smoking for at least 10 years. It is likely that

338 chronic exposure progressively leads to persistent induction of miR-125a and b and NF-κB
339 activation (55, 56), that then reduces the induction of A20 and MAVS in COPD.
340 Collectively, our results demonstrate that A20 regulates NF-κB activation and
341 subsequently the production of inflammatory cytokines but not antiviral IFNs. COPD pBECs
342 and mice with experimental COPD responded to IAV infection with an exaggerated
343 inflammatory but impaired antiviral responses. Increased levels of miR-125a and b by IAV and
344 in COPD suppressed protein inductions of A20 and MAVS, leading to heightened airway
345 inflammation and reduced IFN production. Inhibition of miR-125a and b reduced the induction
346 of inflammatory cytokines and enhanced antiviral responses to IAV infection in both healthy
347 and COPD states. This study therefore identifies a novel potential therapeutic target for IAV
348 infection in general and in COPD.
349
350 **Materials and Methods**
351 **Ex vivo:**
352 COPD patients (10) and healthy non-smoking (10) and smoking (5) controls were recruited
353 and their characteristics are shown in Table 1. Subject recruitment, viruses, cell culture and
354 viral infection, A20 plasmid, siRNA, and miR-125a and b antagomiR/mimetic treatment,
355 cloning and mutagenesis of miR-125a and b binding sites in the MAVS 3′-UTR, reporter
356 assays, immunoblotting, cytometric bead array, immunoprecipitation, miR extraction and
357 analysis, and statistical tests were performed as previously described and/or as in the online
358 supplement (27, 31, 57, 58).
359
360 **In vivo:**
361 Experimental COPD and influenza infection were induced, miR-125a and b was inhibited using
362 specific antagomiRs, histopathology, immunohistochemistry, immunoblotting and cytometric

363 bead array and data analyses were performed as previously described and/or as in online
364 supplement (33, 35, 39, 59-67).
365
366 **Study Approvals**
367 All procedures were approved by The University of Newcastle Human and Animal Ethics
368 Committees.
369
370 **Author contributions**
371 A. C-Y. H. conceived and designed the study. A. C-Y. H. and K. P. performed all *in vitro*
372 experiments. K. D., T-J. H., and P. M. N. performed all in vivo experiments. All authors
373 participated in the completion of the manuscript.
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379
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590

591 **Table 1. Subject characteristics**

	Healthy	COPD	Smoker	P – value
Number	15	15	5	NA
Sex (Male:Female ratio)	1.14	1.2	1.5	p = 0.6
Mean Age (SD)	62 (9.9)	68 (4.1)	64.33 (12.82)	p = 0.06
Mean FEV_1 (SD) *	105% (13.5)	40% (7.75)	97.66% (12.66)	p < 0.001
FEV1/FVC ratio (SD) *	886x (14.50)	40.20 (13.50)	77.80 (12.28)	p < 0.001
Cigarette (Packs/year; SD)	0	53.70 (15.90)	30 (17.32)	p < 0.001
Years abstinent (SD)	0	13.0 (4.64)	0	NA
ICS (percent treated)	0	Seretide (10%) Spiriva (10%) Tiotropium (10%) Spiriva/Salbutamol (10%) Seretide/Tiotropium (20%) Seretide/Tiotropium/Ventolin (20%) Seretide/Spiriva/Ventolin (20%)	0	NA

592 *FEV_1 and FEV_1/FVC ratios are % predicted values. FEV_1 is the forced expiratory volume in

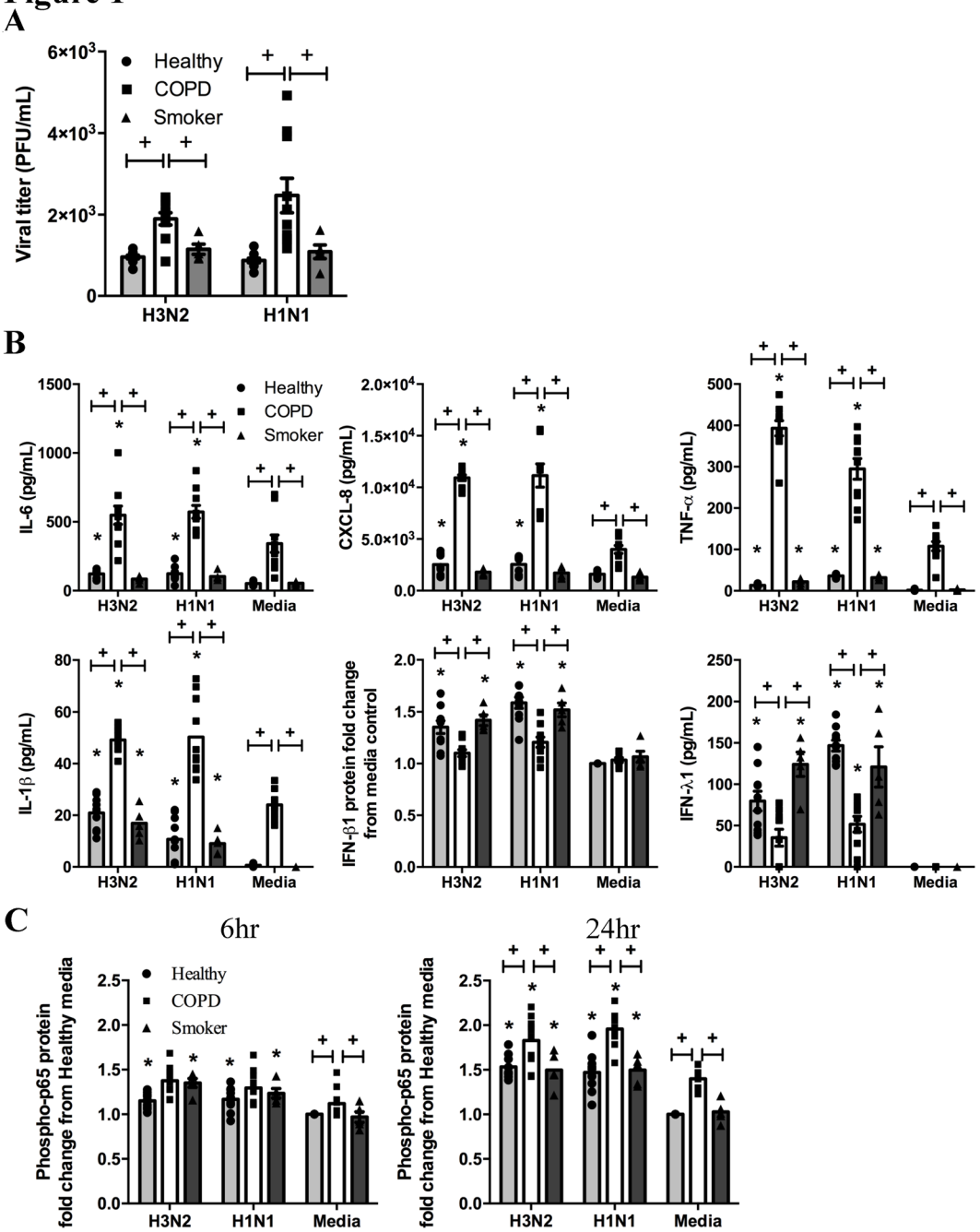
593 1s expressed as a percentage of the predicated value. FVC is forced vital capacity. The

594 statistical analysis used was ANOVA for multiple groups. NA = Not applicable.

595

596 **FIGURE AND FIGURE LEGENDS**

Figure 1



597

598 **Fig. 1.** IAV infection is more severe and results in exaggerated inflammatory but impaired
599 antiviral responses in pBECs from patients with COPD. pBECs from healthy controls, COPD
600 patients and healthy smokers were infected with human IAV H3N2 or H1N1, and (A) virus
601 replication was measured at 24hr. (B) Pro-inflammatory cytokines/chemokines IL-6, CXCL-
602 8, TNF-α, and IL-1β, and antiviral cytokines IFN-β and IFN-λ1 were measured in culture

603 supernatants at 24hr. (C) Phospho-p65 was assessed at 6hr and 24hr, densitometry results (from
604 Supplementary Fig. S1A, representative immunoblot) were calculated as phospho-
605 p65:GAPDH ratios, and expressed as fold change from healthy media control. Data are mean
606 ± SEM, n = 15 (healthy controls and COPD patients) or 5 (healthy smokers). *$P \leq 0.05$ versus
607 respective un-infected media control, + $P \leq 0.05$ versus infected or un-infected healthy controls.
608 Statistical differences were determined with one-way ANOVA followed by Bonferroni post-
609 test.
610

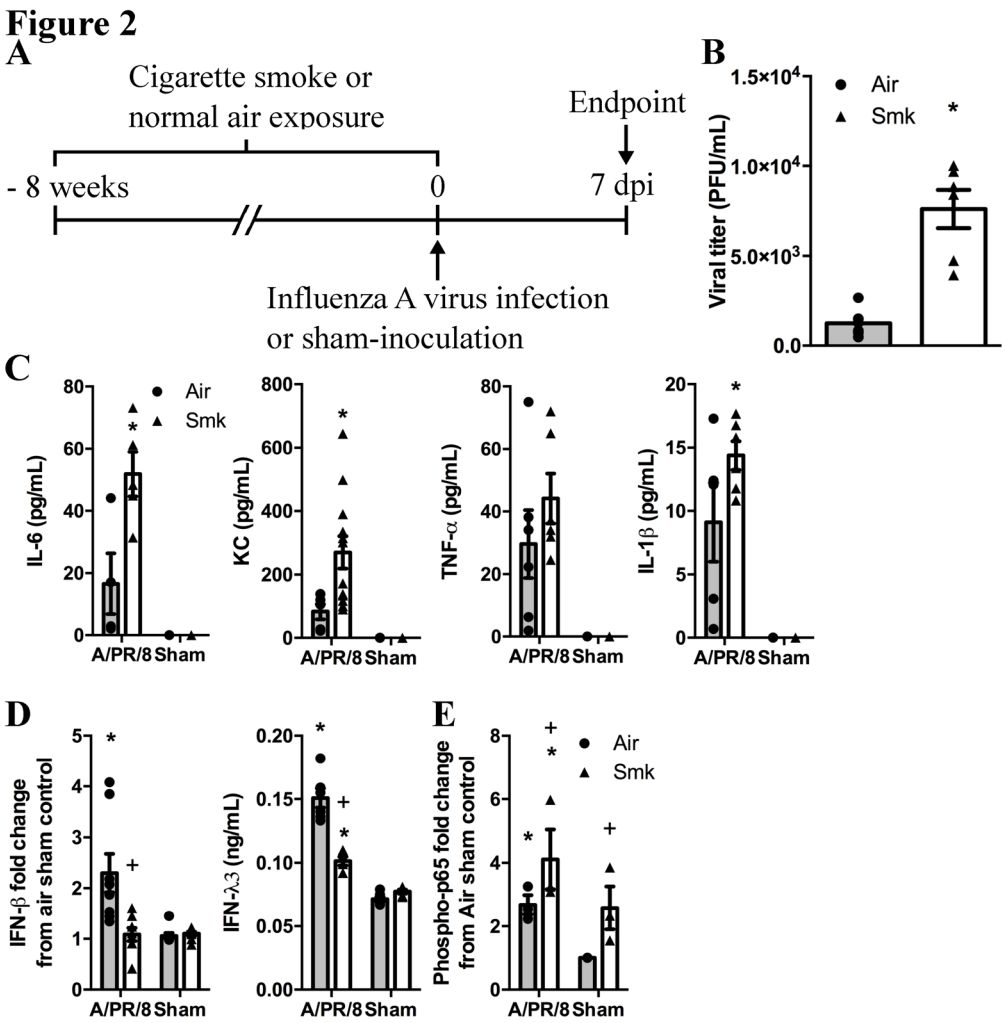


Fig. 2. IAV infection is more severe and results in exaggerated inflammatory and impaired antiviral responses in experimental COPD. (A) BALB/c mice were exposed to cigarette smoke (Smk) or normal air (Air) for eight weeks, infected with IAV H1N1 (A/PR/8/34, 8pfu) or media (Sham) on the last day of smoke exposure and sacrificed 7 days post infection (dpi). (B) Virus titers were measured in bronchoalveolar lavage fluid. (C) IL-6, KC, TNF-α, and IL-1β, and (D) IFN-β and IFN-λ3 were assessed in lung homogenates. (E) Phospho-p65 protein was determined in lung homogenates, densitometry results (from Supplementary Fig. S1D, representative immunoblot) were calculated as phospho-p65 or IFN-β:β-actin ratios, and expressed as fold change from Air sham control. Data are mean ± SEM, n = 6-8 per group,

621 *$P \leq 0.05$ versus Sham control, +$P \leq 0.05$ versus Air control. Statistical differences were

622 determined with one-way ANOVA followed by Bonferroni post-test.

Figure 3

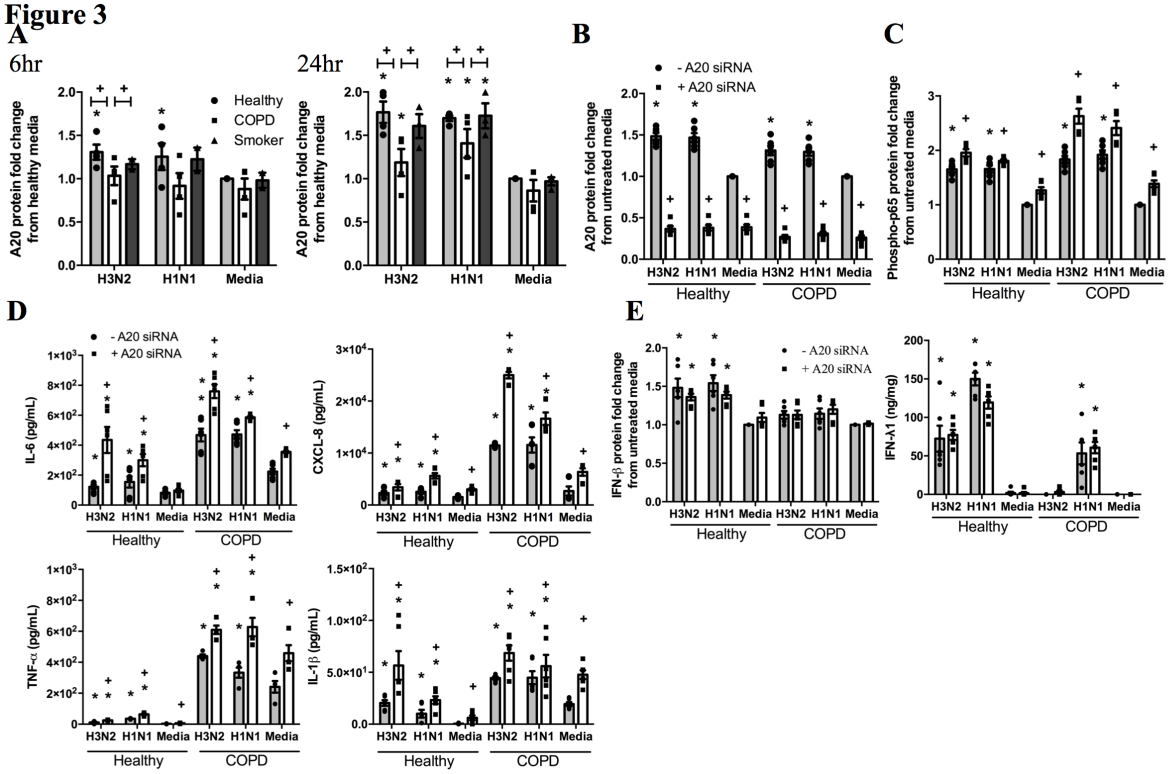


Fig. 3. A20 expression is reduced and it negatively regulates inflammatory but not antiviral responses in pBECs from patients with COPD. (A) pBECs were infected with human IAV H3N2 or H1N1 and the protein levels of A20 were determined at 6hr and 24hr. Densitometry results (from Supplementary Fig. S2A, representative immunoblot) were calculated as A20 or phospho-p65:GAPDH ratios, and expressed as fold change from healthy media control. Data are mean ± SEM, n = 15 per group. *$P \leq 0.05$ versus respective un-infected media control, + $P \leq 0.05$ versus healthy control. A20 expression was inhibited with a specific siRNA, pBECs were infected with IAVs and (B) protein levels of A20 and (C) phospho-p65 and of (D) cytokines/chemokines IL-6, CXCL-8, TNF-α, and IL-1β, and antiviral (E) IFN-β and IFN-λ1 were measured 24hr later. Densitometric ratios (from Supplementary Fig. S2C, representative immunoblot) were expressed as fold change from un-treated media control. Data are mean ± SEM, n = 3 per group. *$P \leq 0.05$ versus un-treated, un-infected media control, +$P \leq 0.05$ versus un-treated infected or un-infected control. Statistical differences were determined with one-way ANOVA followed by Bonferroni post-test.

Figure 4

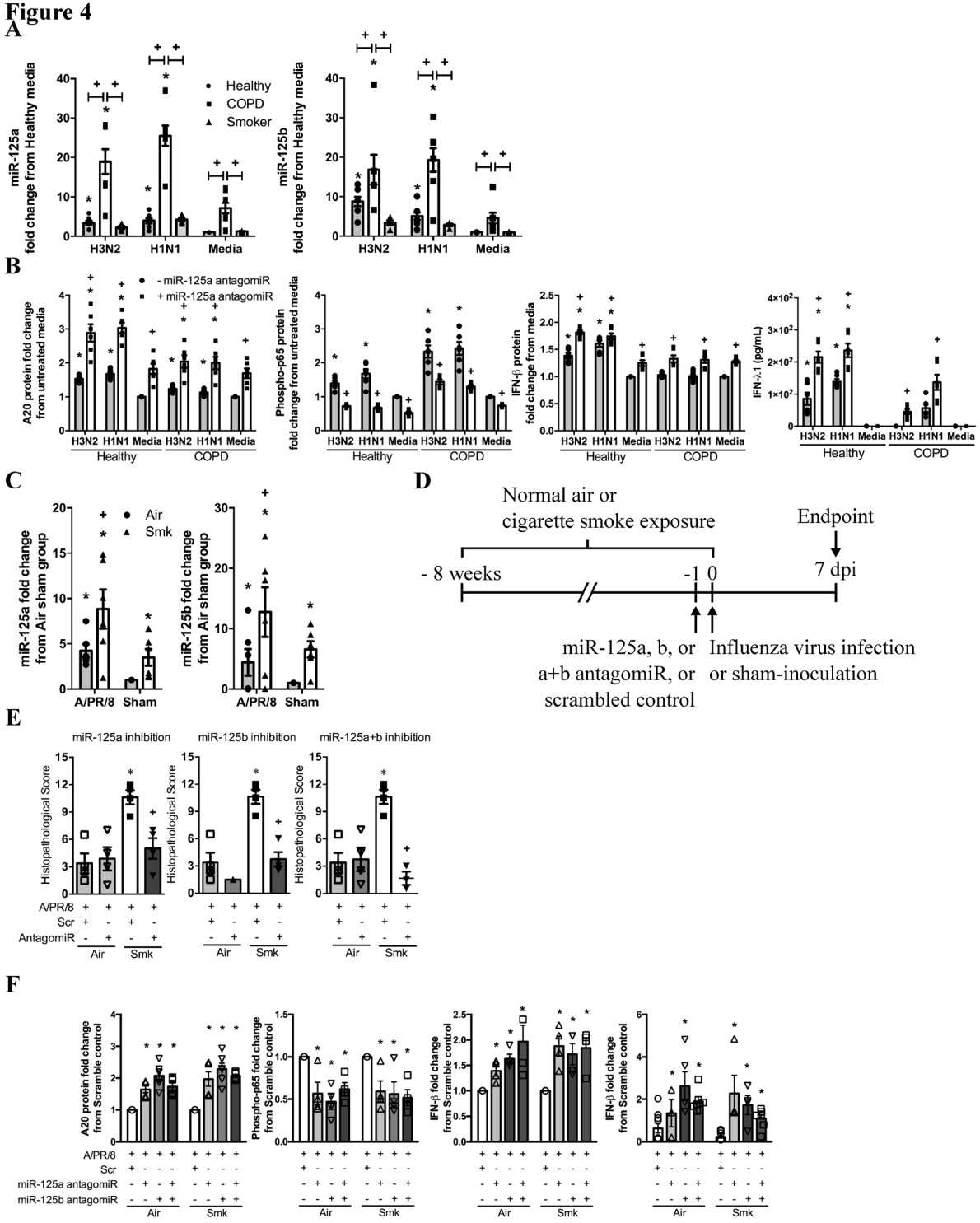


Fig. 4. IAV infection increases the levels of miR-125 and b that suppress the production of A20, increase inflammatory and reduce antiviral responses in human COPD pBECs and experimental COPD. pBECs were infected with human IAV H3N2 or H1N1 and (A) miR-125a

642 and b levels were assessed 24hr. Data are mean ± SEM, n = 15 per group, *P≤0.05 versus un-
643 infected media control. +P≤0.05 versus healthy or smoker control. (B) pBECs were treated
644 with miR-125a or b antagomiR, infected, and the levels of A20, phospho-p65, IFN-β and IFN-
645 λ1 were assessed. Densitometry results (Supplementary Fig. S3B, representative immunoblot)
646 were calculated as A20 or phospho-p65:GAPDH ratios and expressed as fold change from un-
647 treated, un-infected control. Data are mean ± SEM, n = 3 per group, *P≤0.05 versus un-treated,
648 un-infected media control, +P≤0.05 versus un-treated infected or un-infected group. (C)
649 BALB/c mice were exposed to cigarette smoke (Smk) or normal air (Air) for eight weeks,
650 inoculated with IAV H1N1 (A/PR/8/34, 8pfu) or media (Sham) on the last day of smoke
651 exposure, sacrificed 7 days post infection (dpi) and the levels of miR-125a and b were
652 measured. Data are mean ± SEM, n = 6-8 per group, *P≤0.05 versus Sham group, +P≤0.05
653 versus Air infected or un-infected group. (D) In other groups on the last day of smoke exposure
654 mice were treated with miR-125a or b antagomiR alone or in combination, infected with IAV,
655 and (E) airway histological scores were assessed. Data are mean ± SEM, n = 6-8 per group,
656 *P≤0.05 versus infected and scrambled treated Air controls, +P≤0.05 versus infected and
657 scramble-treated Smk group. (F) The protein levels of A20, phospho-p65, and IFN-β in lung
658 homogenates were also measured. Densitometry results (Supplementary Fig. S4D) were
659 calculated as A20 or phospho-p65:β-actin ratios and expressed as fold change from un-treated,
660 un-infected control. Data are mean ± SEM, n = 6-8 per group, *P≤0.05 versus infected
661 scrambled-treated Air group, +P≤0.05 versus infected scrambled Smk group. Statistical
662 differences were determined with one-way ANOVA followed by Bonferroni post-test.

663

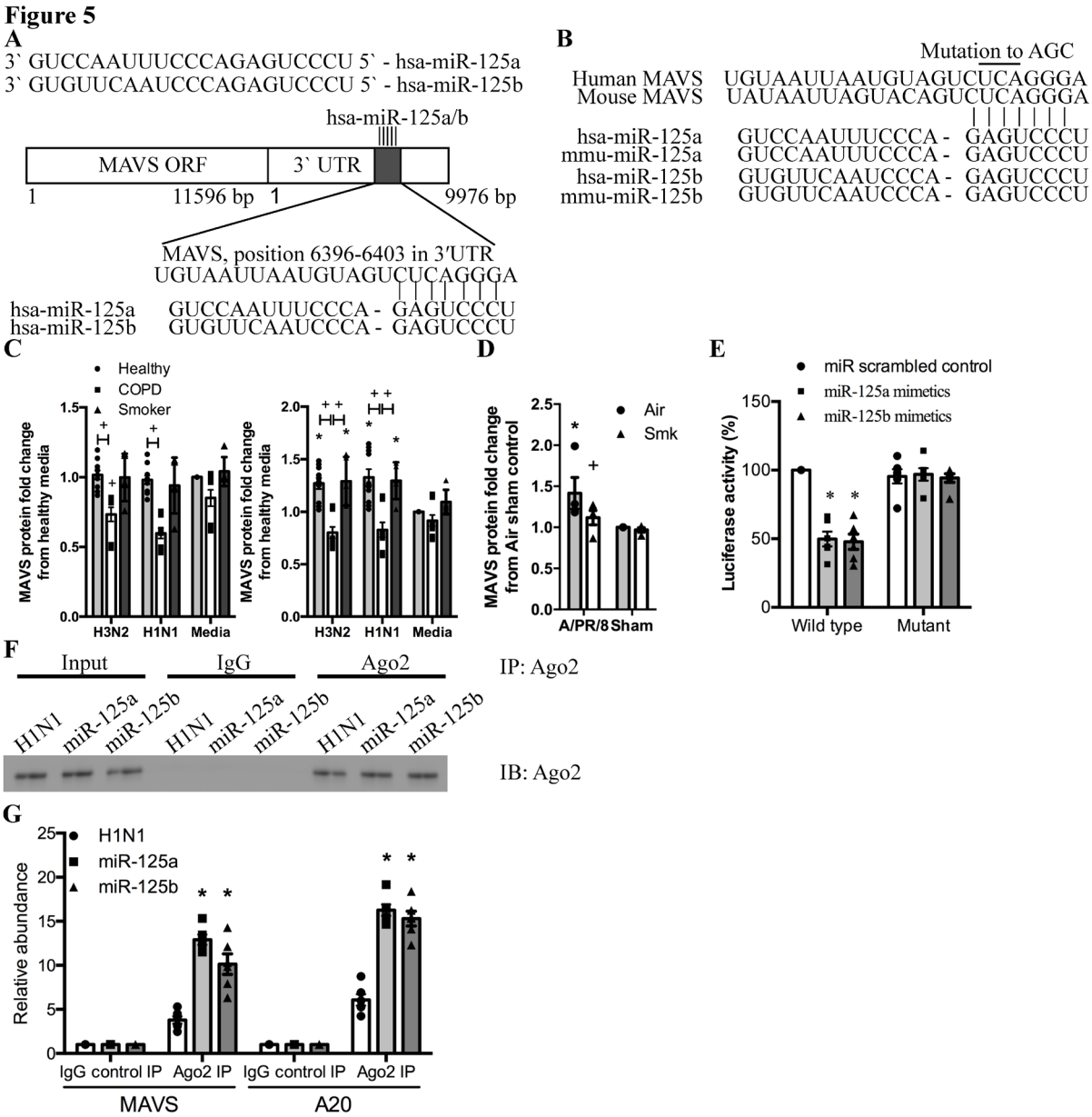


Fig. 5. miR-125a and b target a functional binding site of the 3′-UTR of the mRNA of MAVS to suppress its expression. (A) Representation of *MAVS* gene structure and location of miR-125a and b binding site. (B) The binding site on 3′-UTR of *MAVS* is 100% conserved between human and mouse *MAVS*. (C) pBECs were infected with H3N2 or H1N1 and MAVS protein was detected at 6hr and 24hr. Densitometry results (Supplementary Fig. S5A, representative immunoblot) were calculated as MAVS:GAPDH ratios and expressed as fold change from un-treated, un-infected controls. Data are mean ± SEM, n = 15 per group, *$P \leq 0.05$ versus un-infected healthy or smoker controls, +$P \leq 0.05$ versus infected or un-infected healthy controls.

673 (D) BALB/c mice were exposed to cigarette smoke (Smk) or normal air (Air) for eight weeks,
674 inoculated with IAV H1N1 (A/PR/8/34, 8pfu) or media (Sham) on the last day of smoke
675 exposure, sacrificed 7 days post inoculation (dpi) and the levels of MAVS protein were
676 measured in lung homogenates. Densitometry results (Supplementary Fig. S5B, representative
677 immunoblot) were calculated as MAVS:β-actin ratios in mouse, and expressed as fold change
678 from un-treated, un-infected controls. Data are mean ± SEM, $n = 6$ per group, *$P \leq 0.05$ versus
679 Sham treated controls. + $P \leq 0.05$ versus infected Air controls. (E) The miR-125a and b binding
680 site on 3′-UTR was cloned into a pMIR luciferase reporter construct and transfected into
681 HEK293 cells with miR-125a or b mimetics. The luciferase reporter assay was performed to
682 determine binding Data are mean ± SEM, $n = 3$ per group, *$P \leq 0.05$ versus miR scrambled
683 controls. (F) Ago2 was immunoprecipitated from miR-125a or b mimetic-transfected HEK293,
684 and (G) A20 and MAVS mRNA was detected by qPCR in Ago2-immunoprecipitate. Data are
685 mean ± SEM, $n = 3$ per group, *$P \leq 0.05$ versus IgG control IP. Statistical differences were
686 determined with one-way ANOVA followed by Bonferroni post-test.

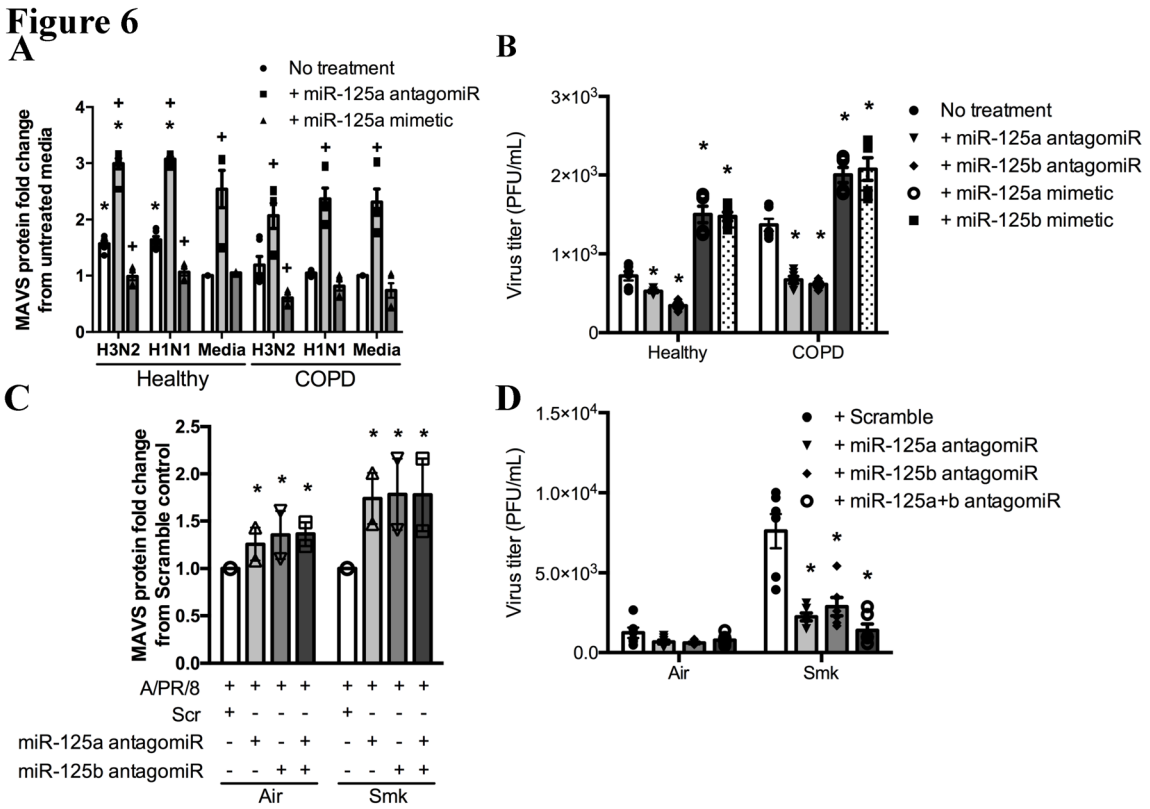


Fig. 6. miR-125a and b suppresses the induction of MAVS and promote virus replication in human COPD pBECs and experimental COPD. (A) miR-125a and b antagomiR or mimetics were added to pBECs before infection with human IAV H3N2 or H1N1 and mitochondrial antiviral signaling (MAVS) protein were assessed 24hr after infection. Densitometry results (Supplementary Fig. S6A, representative immunoblot) were calculated as MAVS:GAPDH ratios and expressed as fold change from un-treated, un-infected controls. Data are mean ± SEM, $n = 3$, *$P \leq 0.05$ versus un-treated, un-infected media controls, +$P \leq 0.05$ versus un-treated, infected or un-infected controls. (B) Virus replication was also measured. Data are mean ± SEM, $n = 3$. *$P \leq 0.05$ versus un-treated, infected controls. (C) BALB/c mice were exposed to cigarette smoke (Smk) or normal air (Air) for eight weeks, treated with mir125a and/or b antagomiR, infected with IAV H1N1 (A/PR/8/34, 8pfu) or media (Sham) on the last day of smoke exposure, sacrificed 7 days post inoculation (dpi) and MAVS protein was measured. Densitometry results (Supplementary Fig. S6C, representative immunoblot) were calculated as

701 MAVS:β-actin ratios and expressed as fold change from un-treated, un-infected controls. Data
702 are mean ± SEM, n = 6, *$P \leq 0.05$ versus infected, scramble treated Air or Smk controls. (D)
703 Virus replication was assessed. Data are mean ± SEM, n = 6, *$P \leq 0.05$ versus infected,
704 scramble-treated controls. Statistical differences were determined with one-way ANOVA
705 followed by Bonferroni post-test.
706

Figure 7

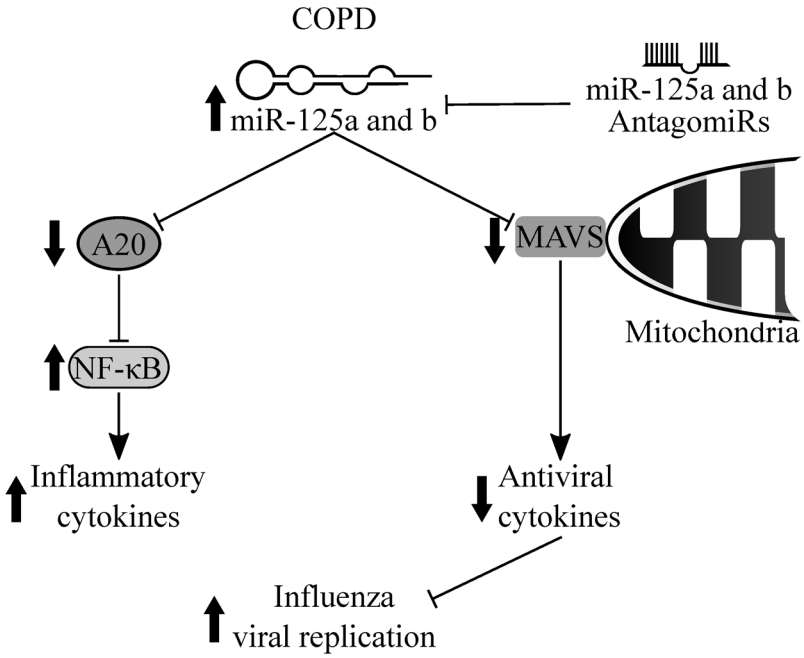


Fig. 7. Roles of miR-125a and b in the regulation of inflammatory and antiviral responses in IAV infection. Increased levels of miR-125a and b, for example in COPD, reduces the protein expression of A20 that results in uncontrolled NF-κB activation, leading to exaggerated induction of pro-inflammatory cytokines. miR-125a and b also targets and reduces MAVS and antiviral type I and III IFN production. Inhibition of miR-125a and b enhances MAVS and antiviral responses and suppresses viral infection.