

**Type I interferon signaling deficiency results in  
dysregulated innate immune responses to SARS-CoV-2 in a  
mouse model**

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4 1 **Type I interferon signaling deficiency results in dysregulated innate immune**  
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6 2 **responses to SARS-CoV-2 in mice**  
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**Abstract:**

SARS-CoV-2 is a newly emerged coronavirus, causing the global pandemic of respiratory coronavirus disease (COVID-19). The type I interferon (IFN) pathway is of particular importance for anti-viral defence and recent studies identified that type I IFNs drive early inflammatory responses to SARS-CoV-2. Here, we use a mouse model of SARS-CoV-2 infection, facilitating viral entry by intranasal recombinant Adeno-Associated Virus (rAAV) transduction of *hACE2* in wildtype (WT) and type I IFN-signalling-deficient (*Ifnar1*<sup>-/-</sup>) mice, to study type I IFN signalling deficiency and innate immune responses during SARS-CoV-2 infection. Our data show that type I IFN signaling is essential for inducing anti-viral effector responses to SARS-CoV-2, control of virus replication and to prevent enhanced disease. Furthermore, *hACE2-Ifnar1*<sup>-/-</sup> mice had increased gene expression of the chemokine *Cxcl1* and airway infiltration of neutrophils as well as a reduced and delayed production of monocyte-recruiting chemokine CCL2. *hACE2-Ifnar1*<sup>-/-</sup> mice showed altered recruitment of inflammatory myeloid cells to the lung upon SARS-CoV-2 infection, with a shift from Ly6C<sup>+</sup> to Ly6C<sup>-</sup> expressing cells. Together, our findings suggest that type I IFN deficiency results in a dysregulated innate immune response to SARS-CoV-2 infection.

**Keywords:**

Innate Immune Response/ type I IFN / In vivo / SARS-CoV-2/ myeloid cells

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## 70 Introduction

71 The ongoing coronavirus disease (COVID-19) pandemic caused by severe acute respiratory syndrome-  
72 coronavirus 2 (SARS-CoV-2) has resulted in over 400 million cases in the first two years of the  
73 pandemic. The estimated fatality rate lies between 1-2%, however this is considerably higher for  
74 elderly patients over 80 years of age (~10%) and nursing home residents (>20%) (1). Type I interferons  
75 (IFNs) are one of the first responses elicited against viral infection and they induce anti-viral defense  
76 mechanisms by binding to the IFN- $\alpha/\beta$  receptor (IFNAR) and signaling through the JAK-STAT pathway.  
77 This induces expression of IFN stimulated genes (ISG), resulting in expression of anti-viral effector  
78 proteins that restrict viral replication and activation of immune cells via induction of chemokine and  
79 cytokine production (2), including CXCL10 and CCL2 (3). Early *in vitro* studies using human bronchial  
80 epithelial cell lines infected with SARS-CoV-2 showed decreased production of type I and III IFNs  
81 coupled with low anti-viral defense signals and a pro-inflammatory environment compared to  
82 infection with influenza A virus (IAV) (4). Furthermore, in severe and critically ill COVID-19 patients, an  
83 impaired type I IFN response has been observed, resulting in decreased viral clearance (5). A lack of  
84 an efficient type I IFN response in these patients is in part due to inborn errors of type I IFN immunity  
85 (6) or circulating auto-antibodies neutralizing type I IFNs (7). Also, a recent animal study has identified  
86 that type I IFN signaling is required for the recruitment of pro-inflammatory cells into the lungs  
87 following SARS-CoV-2 infection (8). These findings highlight the importance of functional type I IFN  
88 responses for anti-viral defenses against SARS-CoV-2.

89 Using a mouse model of SARS-CoV-2 infection, facilitated by intranasal recombinant Adeno-  
90 Associated Virus (rAAV) induced expression of human angiotensin converting enzyme 2 (hACE2), this  
91 study investigated the dynamics of innate immune responses to infection with SARS-CoV-2 in the  
92 context of type I IFN signaling impairment. Overall, the data show that type I IFN signaling is essential  
93 to induce anti-viral responses and control viral replication and disease severity during SARS-CoV-2  
94 infection. Furthermore, type I IFN signaling-deficient mice show dysregulated innate immune  
95 responses to SARS-CoV-2 infection, marked by increased neutrophil recruitment into the airways and  
96 delayed recruitment of myeloid inflammatory cells.

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## 98 Results and discussion

99 To investigate the dynamics of innate, anti-viral immune responses to SARS-CoV-2 infection in the  
100 context of type I IFN-signaling deficiency, 8–12-week-old C57BL/6 wildtype (WT) or *interferon alpha*  
101 *receptor-1*<sup>-/-</sup> (*Ifnar1*<sup>-/-</sup>) mice were intranasally transduced with rAAV9 containing either *hACE2* or *eGFP*  
102 (control) as published recently (9), followed by intranasal infection with 2x10<sup>6</sup> PFU SARS-CoV-2

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3 103 (D614G, first wave isolate) 20 days later and study endpoints were at 2, 4 and 8 days post infection  
4 104 (d.p.i.) (Figure 1A). Gene expression analysis of *hACE2* in lung tissue 20 days post administration of  
5 105 rAAV9 (before infection) showed similar expression in WT and *Ifnar1*<sup>-/-</sup> mice relative to *Gapdh* (Figure  
6 106 1B). Cryo-sectioning of lung tissue 20 days post administration of rAAV9-*eGFP* (before infection)  
7 107 furthermore showed similar distribution of *eGFP* in WT and *Ifnar1*<sup>-/-</sup> mice (Supp. Figure 1A), suggesting  
8 108 similar rAAV transduction efficacy in both groups of mice. Upon infection with SARS-CoV-2, *hACE2*-  
9 109 *Ifnar1*<sup>-/-</sup> mice showed increased weight loss, peaking at 6 d.p.i., compared with *hACE2*-WT mice (Figure  
10 110 1C). Furthermore, plaque assays on Vero cells overexpressing SARS-CoV-2 binding receptors ACE2 and  
11 111 transmembrane protease serine 2 precursor TMPRSS2 (VAT cells) showed that IFNAR1-deficiency  
12 112 results in significantly higher viral loads at day 2 and 4 post infection with SARS-CoV-2 compared to  
13 113 *hACE2*-WT mice (Figure 1D). Gene expression of SARS-CoV-2 nucleocapsid (N) and envelope (E) genes  
14 114 was significantly higher in *hACE2-Ifnar1*<sup>-/-</sup> mice compared with *hACE2*-WT mice from 2 d.p.i. (Figure 1E  
15 115 and Supp. Figure 2B). Since AAV-*eGFP* transduced *Ifnar1*<sup>-/-</sup> mice did not become infected with SARS-  
16 116 CoV-2 and did overall not differ significantly from AAV-*eGFP* transduced WT mice (Supp. Figure 2), this  
17 117 group was not included in all experiments to reduce animal numbers. Together, these data show  
18 118 increased viral load in *hACE2* expressing IFNAR1-deficient mice upon SARS-CoV-2 infection, measured  
19 119 both by plaque assay and N and E gene expression. Previous studies have shown similar trends (8),  
20 120 although differences between WT and *Ifnar1*<sup>-/-</sup> are more pronounced in the model used here. This  
21 121 could be due to several factors: here, Vero cells overexpressing *hACE2* and TMPRSS2 were used for  
22 122 plaque assays, allowing for better viral replication, while the isolate used for infection was hCoV-  
23 123 19/England/IC19/2020, which harbors the D614G spike mutation as opposed to USA-WA1/2020,  
24 124 enhancing viral replication (10). Together, these factors might explain why we detected higher viral  
25 125 loads and more prominent differences emerged between *hACE2*-WT and *hACE2-Ifnar1*<sup>-/-</sup> mice.

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43 126 To investigate the impact of impaired type I IFN signaling during SARS-CoV-2 infection, we first  
44 127 assessed type I IFN expression upon infection. At 2 d.p.i. both IFN- $\alpha$  and IFN- $\beta$  were significantly  
45 128 increased in BAL fluid of *hACE2*-WT compared to *eGFP*-WT mice (Supp. Figure 1B). In the *hACE2-Ifnar1*<sup>-/-</sup>  
46 129 <sup>-/-</sup> group, expression of IFN- $\alpha$  was significantly lower, while IFN- $\beta$  levels were higher compared to the  
47 130 *hACE2*-WT group. We next investigated ISG expression after SARS-CoV-2 infection, since in *Ifnar1*<sup>-/-</sup>  
48 131 mice limited amounts of type I IFN cytokines can be produced but cannot signal for downstream ISGs  
49 132 induction. Chemokine *Cxcl10* and anti-viral effectors *Mx1*, *Oas1* and *Viperin* were quantified. The  
50 133 expression of these ISGs was increased in *hACE2*-WT mice upon infection with SARS-CoV-2 at 2 d.p.i.,  
51 134 however in *hACE2-Ifnar1*<sup>-/-</sup> mice, ISG expression was significantly reduced, but not completely absent  
52 135 (Figure 1F and Supp. Figure 3 C-F). These results suggest an initial increase of IFN- $\beta$  in *hACE2-Ifnar1*<sup>-/-</sup>  
53 136 mice, which may be due to higher viral titers, but that is not translated into ISG expression due to the

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3 137 lack of signaling through the IFNAR1. We therefore investigated the expression of type III IFNs, IFN-  
4 138  $\lambda$ 2-3, which can contribute to ISG expression. IFN- $\lambda$  expression was induced upon infection in *hACE2*-  
5 139 WT mice at 2 d.p.i. while remained at baseline levels in the *hACE2-Ifnar1*<sup>-/-</sup> mice (Supp. Figure 1C).  
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7 140 Furthermore, *Ifng* gene expression was significantly increased in *hACE2-Ifnar1*<sup>-/-</sup> mice later during the  
8 141 infection, by 4 d.p.i. (Supp. Figure 4A). This correlated with CD3<sup>+</sup> T cell recruitment to the airways  
9 142 (Supp. Figure 4B-D). Our data suggest that limited levels of type I or III IFNs are produced early during  
10 143 infection in the *Ifnar1*<sup>-/-</sup> mice resulting in some ISG expression but overall, these data suggest that type  
11 144 I IFN signaling is the main driver for inducing cell intrinsic anti-viral responses.  
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18 146 We next assessed the gene expression of inflammatory mediators and found that expression of the  
19 147 chemokine *Cxcl1*, which is not dependent on type I IFN signaling (3), was increased in *hACE2-Ifnar1*<sup>-/-</sup>  
20 148 mice at 2 and 4 d.p.i. compared to *hACE2*-WT (Figure 2A and Supp. Figure 3G). As CXCL1 plays an  
21 149 essential role in early host immune responses by recruiting neutrophils (11), we next analysed  
22 150 infiltration of neutrophils (gated as live, CD45<sup>+</sup>, Ly6G<sup>+</sup>, Supp. Figure 5A) into the airways at 2 d.p.i. In  
23 151 line with highly increased gene expression of *Cxcl1*, neutrophil recruitment to the airways (BAL) was  
24 152 significantly increased in *hACE2-Ifnar1*<sup>-/-</sup> mice at 2 d.p.i., both proportional of leukocytes (CD45<sup>+</sup> cells)  
25 153 and in total numbers (Figure 2B and C). This was recapitulated in lung tissue with increased  
26 154 proportions of neutrophils in type I IFN signaling-impaired mice at 2 d.p.i. (Figure 2D and Supp. Figure  
27 155 3H), decreasing over time. Taken together, these findings suggest that during SARS-CoV-2 infection,  
28 156 type I IFN signaling deficiency results in increased neutrophil recruitment via CXCL1, thereby  
29 157 contributing to a pro-inflammatory environment. Indeed, *Cxcl1* is also increased in *Ifnar1*<sup>-/-</sup> mice during  
30 158 influenza A with secondary pneumococcal infection (12), but decreased during RSV infection in mice  
31 159 (13), highlighting a pathogen specific CXCL1 response. Furthermore, since we show similar trends for  
32 160 viral load and neutrophil recruitment upon SARS-CoV-2 infection (both significantly increased in  
33 161 *hACE2-Ifnar1*<sup>-/-</sup> mice), which is a mechanism present in other respiratory viral infections such as  
34 162 respiratory syncytial virus (RSV) (13, 14), it will be important to further investigate the link between  
35 163 neutrophil recruitment and viral load in this model.  
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50 165 Since monocyte recruitment to the airways and lungs is key to early host responses to viral infection,  
51 166 we next investigated the expression of monocyte recruiting chemokine CCL2 and the recruitment of  
52 167 inflammatory myeloid cells. CCL2 protein expression was increased in BAL fluid of *hACE2*-WT mice at  
53 168 2 d.p.i. with SARS-CoV-2 (Supp. Figure 3I). However, in *hACE2-Ifnar1*<sup>-/-</sup> mice CCL2 expression was  
54 169 significantly lower at 2 d.p.i., peaking at 4 d.p.i. at lower levels than in IFNAR1-sufficient mice (Figure  
55 170 3A). These findings are in line with a report identifying early CCR2 signaling essential to restrict viral  
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3 171 burden in the lung in a mouse model of SARS-CoV-2 infection (15). The recruitment of CD64<sup>+</sup>CD11b<sup>+</sup>  
4 172 inflammatory myeloid cells to the lung followed similar kinetics, as in *hACE2*-WT mice proportions  
5 173 were highest at 2 d.p.i. and subsequently decreased, while in IFNAR1-deficient mice proportions and  
6 174 total numbers of CD64<sup>+</sup>CD11b<sup>+</sup> inflammatory myeloid cells strongly increased between 2 and 4 d.p.i.  
7 175 and were highest at 8 d.p.i. (Figure 3B and Supp. Figure 3J). We next assessed expression of the  
8 176 monocyte/macrophage differentiation antigen Ly6C within this population, since previous studies  
9 177 reported the infiltration of CD64<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>+</sup> inflammatory myeloid cells into the lung during SARS-  
10 178 CoV-2 infection (8, 15). This showed highly increased proportions of CD64<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>+</sup> in *hACE2*-WT  
11 179 but not IFNAR1-deficient mice at 2 d.p.i. in the BAL (Supp. Figure 5D) and lung (Figure 3C-D and Supp.  
12 180 Figure 3K), suggesting type I IFN dependency for recruitment. However, as we have previously shown  
13 181 that Ly6C is gradually downregulated on monocytes during response to respiratory viral infection (16),  
14 182 we also analyzed CD64<sup>+</sup>CD11b<sup>+</sup> Ly6C<sup>-</sup> cells. The presence of CD64<sup>+</sup>CD11b<sup>+</sup> Ly6C<sup>-</sup> inflammatory myeloid  
15 183 cells in the airways was not type I IFN signaling dependent, since both proportions and total numbers  
16 184 were significantly increased in *hACE2-Ifnar1*<sup>-/-</sup> mice at 4 and 8 d.p.i. (Figure 3C and E), while at 2 d.p.i.  
17 185 in the airways no significant differences emerged (Supp. Figure 5F). This accounts for the delayed  
18 186 emergence of inflammatory myeloid cells in the lung during type I IFN signaling impairment shown in  
19 187 Figure 3B and overall indicates altered recruitment dynamics of inflammatory myeloid cells. Taking  
20 188 these data together, our model recapitulates the deficiency of type I interferon responses seen in  
21 189 severe SARS-CoV-2 infection, which in patients is marked by decreased IFN- $\alpha$ , type I IFN activity and  
22 190 ISG score, as well as neutrophilia and increased CCL2 (5). Our data suggest that the lack of type I IFN  
23 191 signaling results in dysregulated innate immune responses in the lung during SAR-CoV-2 infection.  
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## 40 193 **Concluding Remarks**

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42 194 In summary, using a mouse model of SARS-CoV-2 infection we show that type I IFN signaling is  
43 195 essential for inducing anti-viral effector responses, control of virus replication and disease severity.  
44 196 Our data indicate that type I IFN signaling-deficient mice express increased levels of *Cxcl1* in the lung  
45 197 and increased infiltration of neutrophils to the airways compared to WT controls. Furthermore, we  
46 198 found reduced and delayed production of CCL2 and altered recruitment of inflammatory myeloid cells  
47 199 during IFNAR1-deficiency. This, together with an increased viral burden is associated with more severe  
48 200 disease in type I IFN signaling-deficient mice. The data shown here will be valuable for better  
49 201 understanding how impaired type I IFN signaling drives SARS-CoV-2 pathology and disease severity,  
50 202 which is highly relevant considering the large contribution of impaired type I IFN responses on life-  
51 203 threatening SARS-CoV-2 infections (6, 7) and deaths (17) and for the development of type I IFN-based  
52 204 treatment options for COVID-19 in vulnerable populations. To conclude, our findings show that type I  
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205 IFN deficiency results in dysregulated innate immune responses to SARS-CoV-2 infection in the rAAV-  
206 *hACE2* mouse model.

## 207 **Materials and Methods**

### 208 **Mice**

209 C57BL/6 mice were purchased from Charles River UK Inc. *Ifnar1*<sup>-/-</sup> mice on a C57BL/6 background were  
210 bred in-house. All mice were bred and maintained in pathogen-free conditions and 8–12-week-old  
211 mice were used for experiments. All animal experiments were reviewed and approved by the Animal  
212 Welfare and Ethical Review Board (AWERB) at Imperial College London and approved by the UK Home  
213 Office in accordance with the Animals Act 1986 (Scientific Procedures) and ARRIVE guidelines. Both  
214 male and female mice were used for experiments after excluding sex bias in preliminary experiments.  
215 All experiments were performed twice, independently, per time point.

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### 217 **rAAV vector production**

218 The production, purification, and titration of rAAV2/9-*eGFP* or *hACE2* vectors were performed as  
219 previously described (9). Briefly, the respective rAAV vector was produced by polyethylenimine (PEI,  
220 PolySciences)-based triple transfection of human embryonic kidney (HEK) 293T/17 cells (ATCC, CRL-  
221 11268). The AAV plasmids transfected included the Adenovirus helper plasmid (pAdDeltaF6), AAV  
222 Rep-Cap pAAV2/9 plasmid and the transgene plasmid. The transgene plasmid containing *eGFP* or  
223 *hACE2* was engineered to include a lung-optimized hCEFI (human Cytomegalovirus  
224 enhancer/elongation factor 1 alpha) promoter (18), Woodchuck Hepatitis Virus Post-transcriptional  
225 Regulatory Element (WPRE) (19) and mir142-3pT (20). rAAV particles were concentrated and  
226 formulated into PBS using 100 kDa Ultra centrifugal filters (Amicon, Merck) after iodixanol gradient  
227 centrifugation. Physical titre (DNase-resistant genome copies, DRGC/mL) was determined by  
228 quantitative polymerase chain reaction (qPCR) analysis with primers and a probe against WPRE (21).  
229 Purity of vectors was confirmed by analyzing 20 µl of diluted vector on 4-12% SDS polyacrylamide gels,  
230 where total protein was visualized using Coomassie stain according to the manufacturer's protocols  
231 (Life Technologies).

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### 233 **hACE2 transduction**

234 For transduction, WT or *Ifnar1*<sup>-/-</sup> mice were lightly anesthetized and instilled i.n. with 1x10<sup>11</sup> DNase  
235 Resistant Gene Copies (DRGC) rAAV9-*eGFP* or rAAV9-*hACE2* in 100 µl PBS. *hACE2* gene expression in  
236 whole lung homogenate was assessed at day 20 post instillation by relative quantification to *Gapdh*  
237 using primers and probes for *hACE2* listed in the key resource table.

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### 239 **Cryosectioning and native eGFP detection**

240 Mice were sacrificed 20 days post instillation of AAV-eGFP or PBS and lungs were removed after  
241 inflation with 4% PFA. After 24-hour fixation in 4% PFA, lungs were inflated with 30% sucrose and  
242 submerged in 30% sucrose for 24 hours. Lungs were subsequently inflated with 1:1 cryo embedding  
243 matrix (OCT)/30% sucrose and individual lobes were submerged in OCT/30% sucrose in plastic molds  
244 and frozen at -80 °C. Left lungs were cryosectioned to produce 7 µm thick sections, mounted using  
245 DAPI-supplemented mounting media with coverslip, and eGFP expression was detected by fluorescent  
246 microscopy using the EVOS FL Auto 2 system (Thermo Scientific).

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### 248 **Virus and infections**

249 First wave SARS-CoV-2 (D614G, isolate of hCoV-19/England/IC19/2020) was grown in African green  
250 monkey kidney cells overexpressing human ACE2 and TMPRSS2 (Vero-ACE2-TMPRSS2; VAT cells) (22).  
251 For infection 20 days post transduction with rAAVs, mice were lightly anesthetized and instilled i.n.  
252 with  $2 \times 10^6$  plaque forming units (PFU) of SARS-CoV-2 in 100 µl volume. SARS-CoV-2 titre was assessed  
253 in lungs at 2, 4 and 8 d.p.i. using a plaque assay. In brief, serial dilutions of lung homogenate in serum-  
254 free Dulbecco's Modified Eagle Medium (DMEM, containing 1% non-essential amino acids (NEAA),  
255 100U/ml Penicillin and 100 µg/ml Streptomycin) were performed and inoculated onto VAT cells for 1  
256 h at 37°C. The inoculum was then removed and replaced with overlay medium (1x MEM, 0.2% w/v  
257 BSA, 0.16% w/v NaHCO<sub>3</sub>, 10 mM HEPES, 2 mM L-Glutamine, 100 U/ml penicillin, 100 µg/ml  
258 streptomycin and 0.84% agarose). Plates were incubated for 3 days at 37°C before overlay was  
259 removed and cells were stained for 1 h at room temperature in 2x crystal violet solution. Virus plaques  
260 were counted and multiplied by the dilution factor to calculate titer as PFU/ml.

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### 262 **Isolation of lung cells**

263 Mice were sacrificed at 0.75, 2, 4 and 8 d.p.i. and lungs were perfused with PBS. To obtain lung  
264 leukocytes, lung lobes were cut into smaller pieces and incubated in complete DMEM (cDMEM,  
265 supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml  
266 streptomycin), 1mg/ml Collagenase D (Roche) and 30 µg/ml DNase I (Invitrogen) for 1h at 37°C and  
267 then mashed through a 100-µm filter (BD). Red blood cells were lysed using Ammonium-Chloride-  
268 Potassium buffer.

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### 270 **BAL cell processing**

271 BAL was collected by flushing the lungs three times with 1 ml PBS supplemented with 5 mM EDTA (Life  
272 Technologies). BAL cells and supernatant were separated by centrifugation and BAL supernatants

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3 273 were exposed to UV light for 2 min to inactivate SARS-CoV-2. Red blood cells were lysed using  
4 274 Ammonium-Chloride-Potassium buffer.

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### 7 8 276 **Flow cytometry**

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10 277 After red blood cell lysis, lung and BAL cells were incubated for 30 min with fixable live-dead Aqua dye  
11 278 (Invitrogen), followed by fixation for 30 minutes with 4% paraformaldehyde (PFA) to inactivate virus.  
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13 279 Cells were then incubated for 20 min with a purified rat IgG<sub>2b</sub> anti-mouse CD16/CD32 receptor  
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15 280 antibody (BD) to block Fc binding, followed by staining with fluorochrome-conjugated antibodies  
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17 281 against CD45 (30-F11, BV605), CD26 (H194-112, BV711), Siglec-F (E50-2440, BV786), Ly6G (1A8,  
18 282 AF488), Ly6C (12HK1.4, PE), CD11c (HL3, PE-CF594), CD64 (X54-5/7.1, APC) and CD11b (M1/70, AF700)  
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20 283 in PBS containing 1% BSA and 5 mM EDTA for 25 min at 4°C. For the adaptive immune cells, they were  
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22 284 stained with CD11c (HL3, V450), Siglec-F (E50-2440, BV786), CD19 (6D5, AF488), CD45 (30-F11, PerCP-  
23 285 Cy5.5), Ly6G (1A8, PE-Cy7) and CD3 (17A2, AF700). Samples were analysed on a BD-Fortessa Flow  
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25 286 Cytometer equipped with 50-mW 504-nm, 50-mW 488-nm, 50-mW 561-nm and 20-mW 633-nm lasers  
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27 287 and an ND1.0 filter in front of the FSC photodiode. All antibodies were purchased from BD, Biolegend  
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29 288 or eBioscience. Data were analyzed with FlowJo software (Tree Star).

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### 31 32 290 **RNA isolation and quantitative RT-PCR**

33 291 Lung tissue was homogenized in TRIzol and RNA extraction performed according to manufacturer's  
34 292 instructions. After the chloroform step, the aqueous phase containing RNA was further processed  
35 293 using the RNeasy Mini Kit (QIAGEN) according to manufacturer's instructions. 2 µg RNA was reverse  
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37 294 transcribed using a High-Capacity RNA-to-cDNA kit (Applied Biosystems) according to manufacturer's  
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39 295 instructions. To quantify mRNA levels in lung tissue, quantitative RT-PCR reactions for *Oas1*, *Viperin*  
40 296 and *Ifnl* were performed using primers and probes as previously described (23). Analysis was  
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42 297 performed using the QuantiTect Probe PCR Master Mix (QIAGEN) and the 7500 Fast real-Time PCR  
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44 298 System (Applied Biosystems). For absolute quantification, the exact number of copies of the gene of  
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46 299 interest was calculated using a plasmid DNA standard curve, and the results were normalized to levels  
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48 300 of *Gapdh* (Applied Biosystems). For relative quantification, the expression of *Cxcl1*, *Cxcl10*, *hACE2*,  
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50 301 *Mx1* and SARS-CoV-2 *N* and *E* gene was expressed relatively to the expression of *Gapdh*. First, the  $\Delta$ CT  
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52 302 (CT = cycle threshold) between the target gene and *Gapdh* was calculated for each sample, followed  
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54 303 by calculation of  $2^{-\Delta$ CT. Analysis was performed using 7500 Fast System SDS Software (Applied  
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56 304 Biosystems).

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### 58 306 **Chemokine and IFN detection**

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3 307 CCL2 and IFN- $\lambda$ 2/3 quantifications were performed on BAL fluid using mouse DuoSet ELISA (R&D  
4 308 Systems) according to the manufacturer's instructions. Data were acquired on a SpectraMax Plus plate  
5 309 reader (Molecular Devices) and analysed using SoftMax software (version 5.2). The concentration of  
6 310 IFN- $\alpha$  and IFN- $\beta$  was measured in BAL fluid using the Mouse ProCartaPlex Immunoassay (Invitrogen)  
7 311 according to the manufacturer's instructions. Data were acquired and analysed with a Bio-Plex 200  
8 312 system (Bio-Rad Laboratories).  
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### 11 314 **Statistical analysis**

12 315 Statistical analysis was performed using Prism 9.2 (Graph-Pad Software). One-way ANOVA with  
13 316 Tukey's post-hoc test was used to compare multiple groups. Data are expressed as mean  $\pm$  SEM, and  
14 317 for all tests a value of  $P < 0.05$  was considered significant. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.005$ , \*\*\*\*  
15 318  $P < 0.001$   
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17 319

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28

29 330

### 30 331 **Author contribution**

31 332 P.P.O. designed, performed, and analyzed the experiments and wrote the paper. M.G.M. and C.M.  
32 333 performed specific experiments and reviewed the paper. Y.D., D.R.G., S.C.H., O.H., and K.M.M.  
33 334 designed, manufactured and provided rAAV9-*hACE2* and rAAV9-*eGFP* and reviewed the paper,  
34 335 additionally Y.D. and K.M.M. performed specific experiments. J.Z., J.C.B. and W.S.B. provided advice  
35 336 and the SARS-CoV-2 WT isolate (D614G) and reviewed the paper. C.J. supervised the project, designed  
36 337 the experiments, and wrote the paper.  
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### 39 339 **Conflict of interest**

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1  
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3 340 The authors declare no commercial or financial conflict of interest.  
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## 6 342 **Data availability statement**

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9 343 The data that support the findings of this study are available from the corresponding author upon  
10 344 reasonable request.  
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## 14 345 **Ethics approval**

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17 346 All animal experiments were reviewed and approved by the Animal Welfare and Ethical Review Board  
18 347 (AWERB) at Imperial College London and approved by the UK Home Office in accordance with the  
19 348 Animals (Scientific Procedures) Act 1986 Amendment Regulations (PPL P3AFFFODD Johansson).  
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432 activation are differentially dependent on MyD88/TRIF and MAVS signaling during RSV  
433 infection. *Mucosal Immunology* 2019;12:1244–1255.

## 436 Figure legends

### 437 Figure 1: Increased viral load, weight loss and lower expression of ISGs in *Ifnar1*<sup>-/-</sup> mice during 438 infection with SARS-CoV-2

439 A) Recombinant Adeno-associated virus (rAAV) containing human angiotensin converting enzyme 2  
440 (*hACE2*) or *eGFP* genes was administered intranasally to *Ifnar1*<sup>-/-</sup> or wildtype (WT) mice (1x10<sup>11</sup> Dnase  
441 resistant gene copies/mouse). 20 days later mice were intranasally infected with SARS-CoV-2 (D614G,  
442 2x10<sup>6</sup> PFU/mouse). Lungs and bronchoalveolar lavage (BAL) were harvested at 2, 4 and 8 days post  
443 infection (d.p.i). B) Expression of *hACE2* in lung tissue relative to *Gapdh*, measured by RT-PCR before  
444 infection (d20 post transduction with rAAV). WT/rAAV-*eGFP* n = 3, *Ifnar1*<sup>-/-</sup> /rAAV-*eGFP* n = 4,  
445 WT/rAAV-*hACE2* n = 4, *Ifnar1*<sup>-/-</sup> /rAAV-*hACE2* n = 5). C) Weight loss post infection with SARS-CoV-2. D)  
446 Viral load measured by plaque assay on Vero cells overexpressing *hACE2* and *TMPRESS2*. E) Expression  
447 of SARS-CoV-2 *N gene* (nucleocapsid phosphoprotein) and *E gene* (envelope protein) in lung tissue  
448 relative to *Gapdh*, measured by RT-PCR. F) Gene expression analysis of IFN stimulated genes (ISG)  
449 *Cxcl10*, *Mx1*, *Oas1* and *Viperin* measured by RT-PCR, relative to expression of *Gapdh* or total copy  
450 numbers normalized to expression of *Gapdh* (*Oas1* and *Viperin*). Data are shown as mean ± SEM. B)  
451 WT/rAAV-*eGFP* n = 3, WT/rAAV-*hACE2* n = 4, *Ifnar1*<sup>-/-</sup> /rAAV-*hACE2* n = 5 C – F) Two independent  
452 experiments per time point, data pooled, n = 6-8 per group. One Way ANOVA + Tukey's multiple

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3 453 comparison test per time point; \* indicates significant difference between WT rAAV-*hACE2* and *Ifnar1*<sup>-/-</sup>  
4 454 rAAV-*hACE2*, \* P < 0.05, \*\* P < 0.01, \*\*\* p < 0.005, \*\*\*\* p < 0.001.

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8 456 **Figure 2: Increased neutrophil recruitment to airways in IFNAR1-deficient mice during SARS-COV-2**  
9 **infection**

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11 458 A) Gene expression of *Cxcl1* in lung tissue relative to *Gapdh* at 2, 4 and 8 d.p.i. with SARS-CoV-2  
12 459 (D614G), measured by RT-PCR. B) Proportions of live, CD45<sup>+</sup> and total numbers of neutrophils in BAL  
13 460 at 2 d.p.i. C) Representative flow cytometry plots of lung cells gated on live, CD45<sup>+</sup> Ly6G<sup>+</sup> D) Proportions  
14 461 of live, CD45<sup>+</sup> and total numbers of neutrophils in lung tissue at 2, 4 and 8 d.p.i. Data are shown as  
15 462 mean ± SEM. Two independent experiments per time point, data pooled, n = 6-8 per group. One Way  
16 463 ANOVA + Tukey's multiple comparison test per time point; \* indicates significant difference between  
17 464 *hACE2*-WT and *hACE2-Ifnar1*<sup>-/-</sup>, \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.005, \*\*\*\* P < 0.001.

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20 466 **Figure 3: Type I interferon signaling deficiency results in dysregulated inflammatory myeloid cell**  
21 **recruitment during SARS-CoV-2 infection**

22 467  
23 468 A) Protein expression of CCL2 in BAL fluid at 2, 4 and 8 d.p.i. measured by ELISA. B) Proportions of live,  
24 469 CD45<sup>+</sup> and total numbers of CD64<sup>+</sup> CD11b<sup>+</sup> inflammatory myeloid cells in lung tissue at 2, 4 and 8 d.p.i.  
25 470 C) Representative flow cytometry plots of lung cells gated on live, CD45<sup>+</sup> Ly6G<sup>-</sup> SigF<sup>-</sup> CD11b<sup>+</sup>. D)  
26 471 Proportions of live, CD45<sup>+</sup> and total numbers of CD64<sup>+</sup> CD11b<sup>+</sup> Ly6C<sup>+</sup> inflammatory myeloid cells in  
27 472 lung tissue at 2, 4 and 8 d.p.i. E) Proportions of live, CD45<sup>+</sup> and total numbers of CD64<sup>+</sup> CD11b<sup>+</sup> Ly6C<sup>-</sup>  
28 473 inflammatory myeloid cells in lung tissue at 2, 4, and 8 d.p.i. Data are shown as mean ± SEM. Two  
29 474 independent experiments per time point, data pooled, n = 6-8 per group. One Way ANOVA + Tukey's  
30 475 multiple comparison test per time point; \* indicates significant difference between *hACE2*-WT and  
31 476 *hACE2-Ifnar1*<sup>-/-</sup>, \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.005, \*\*\*\* P < 0.001. Dotted line = limit of detection.

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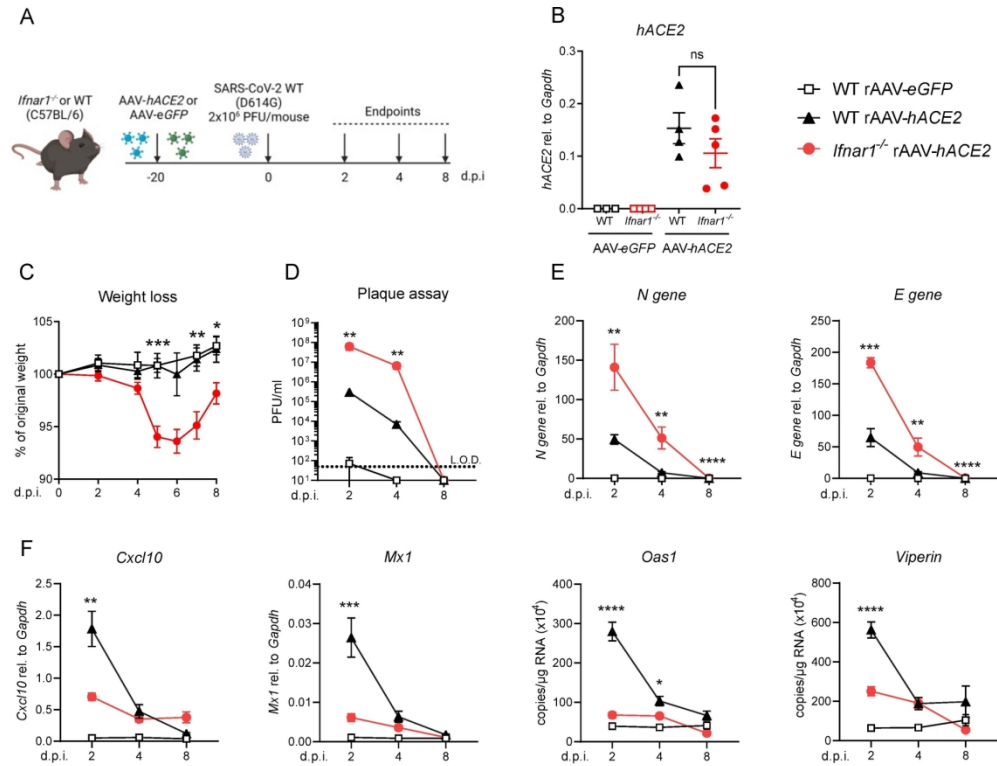


Fig1 eps format

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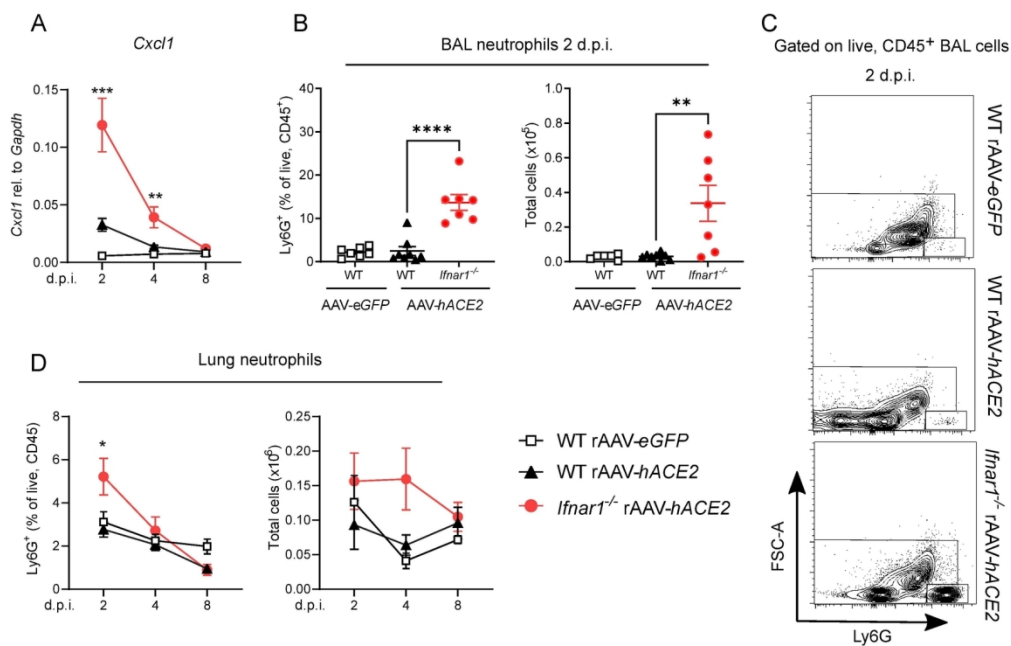


Fig2 eps format

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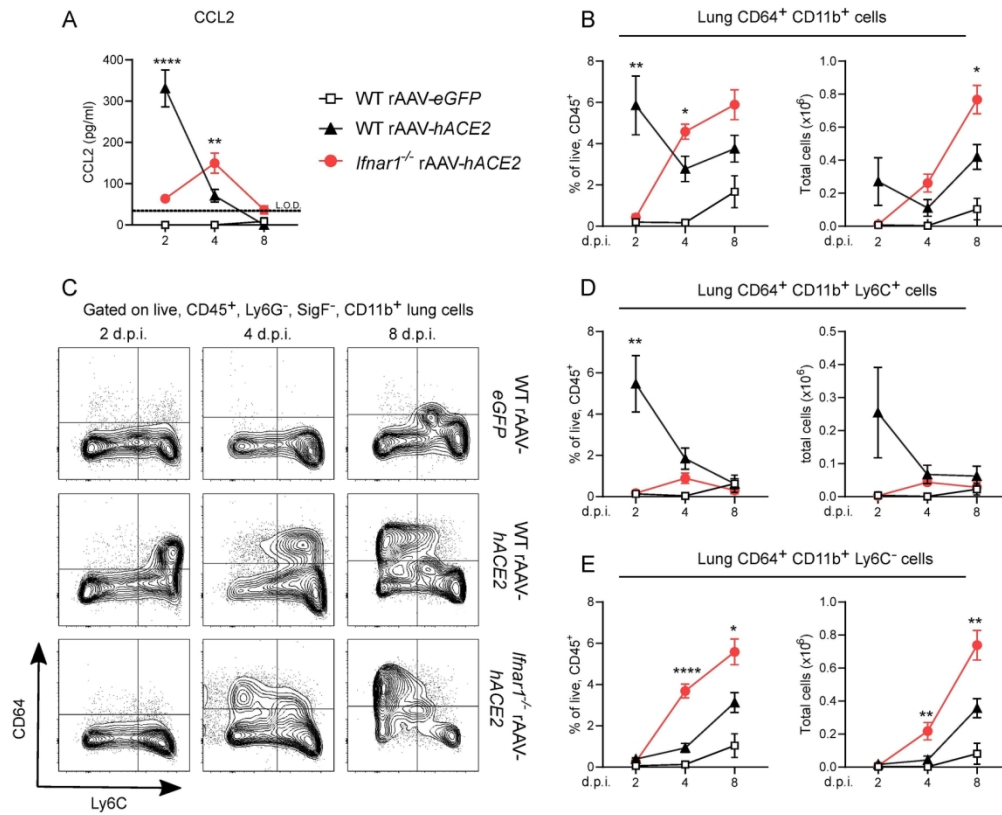
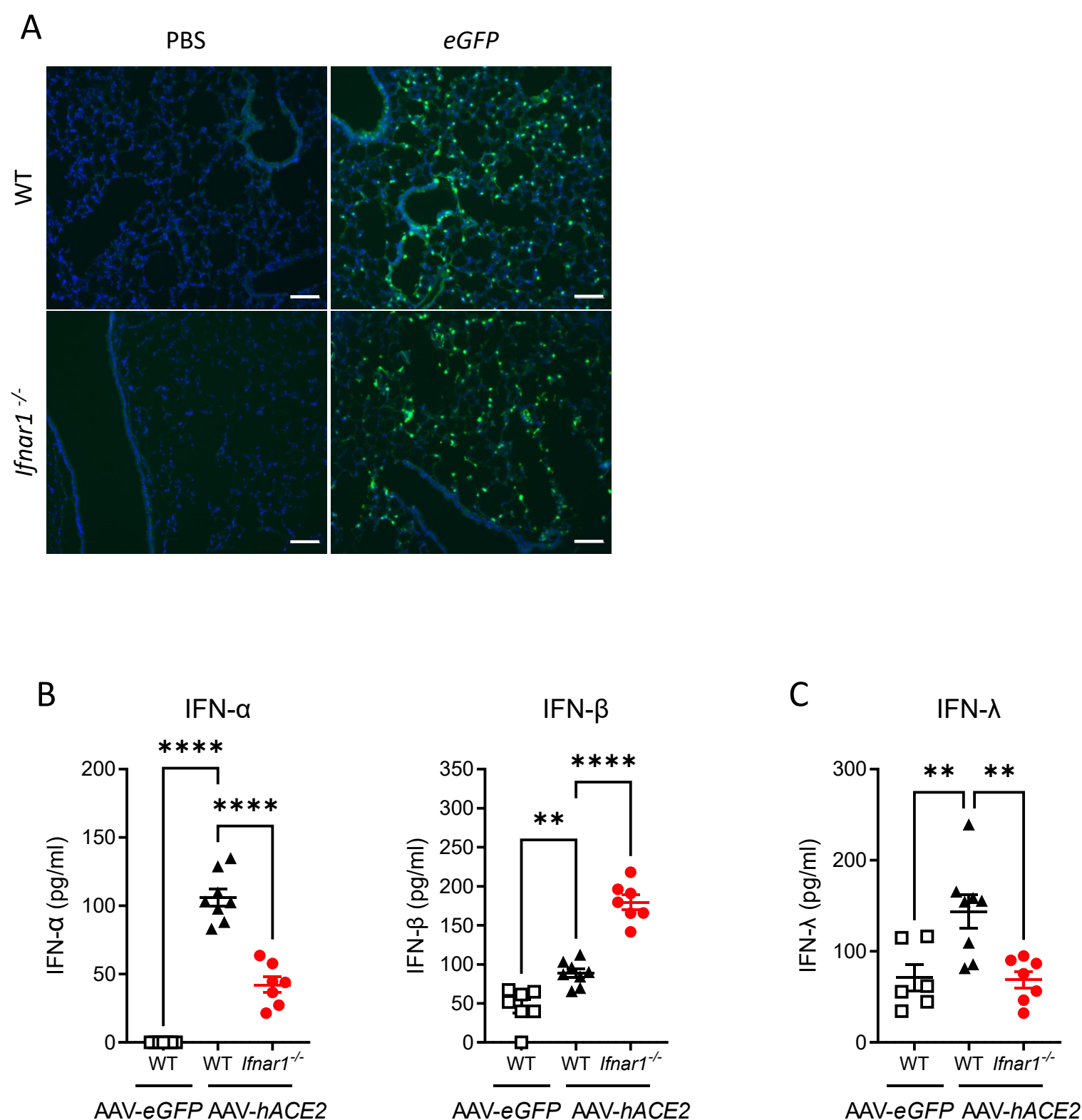


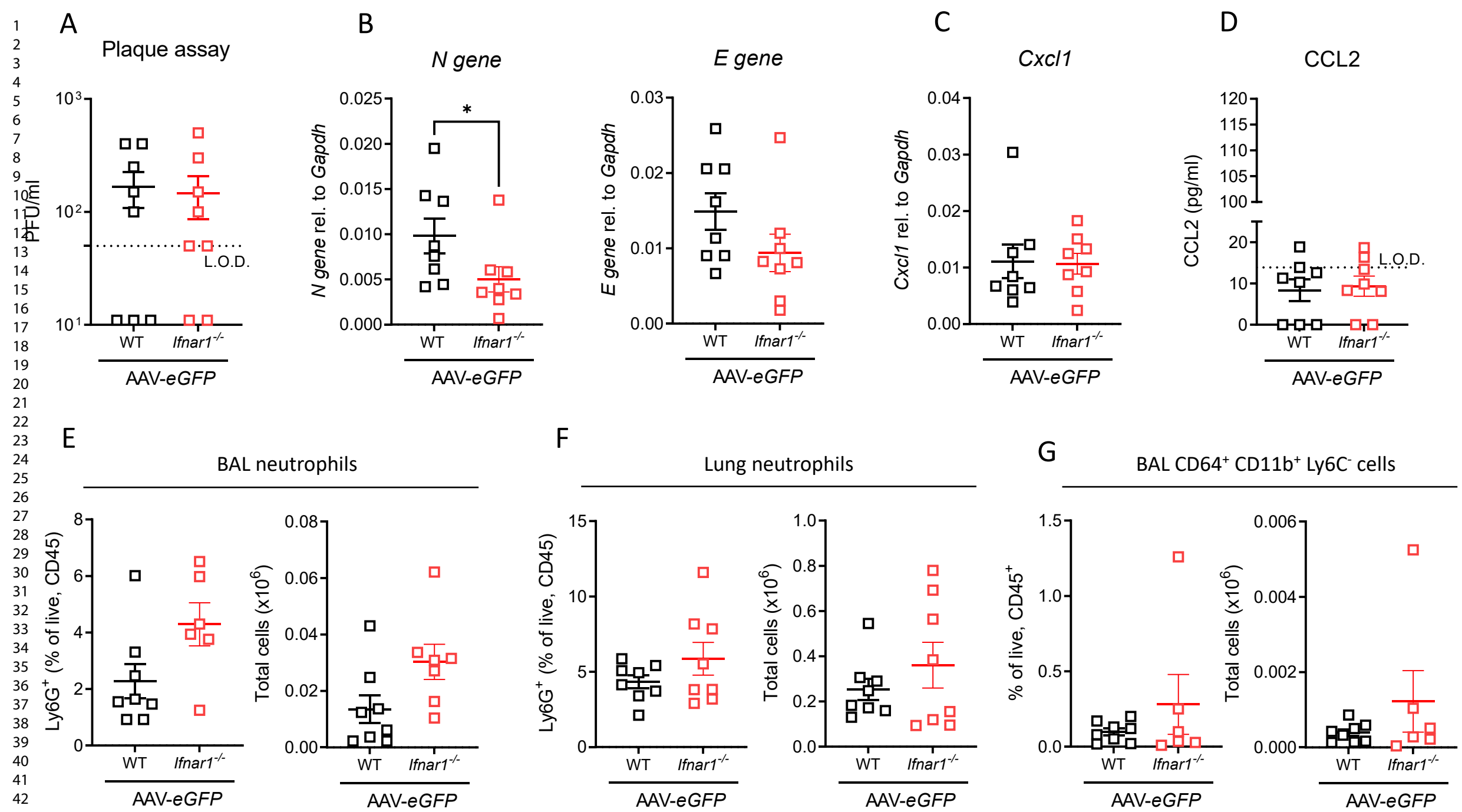
Fig3 eps format

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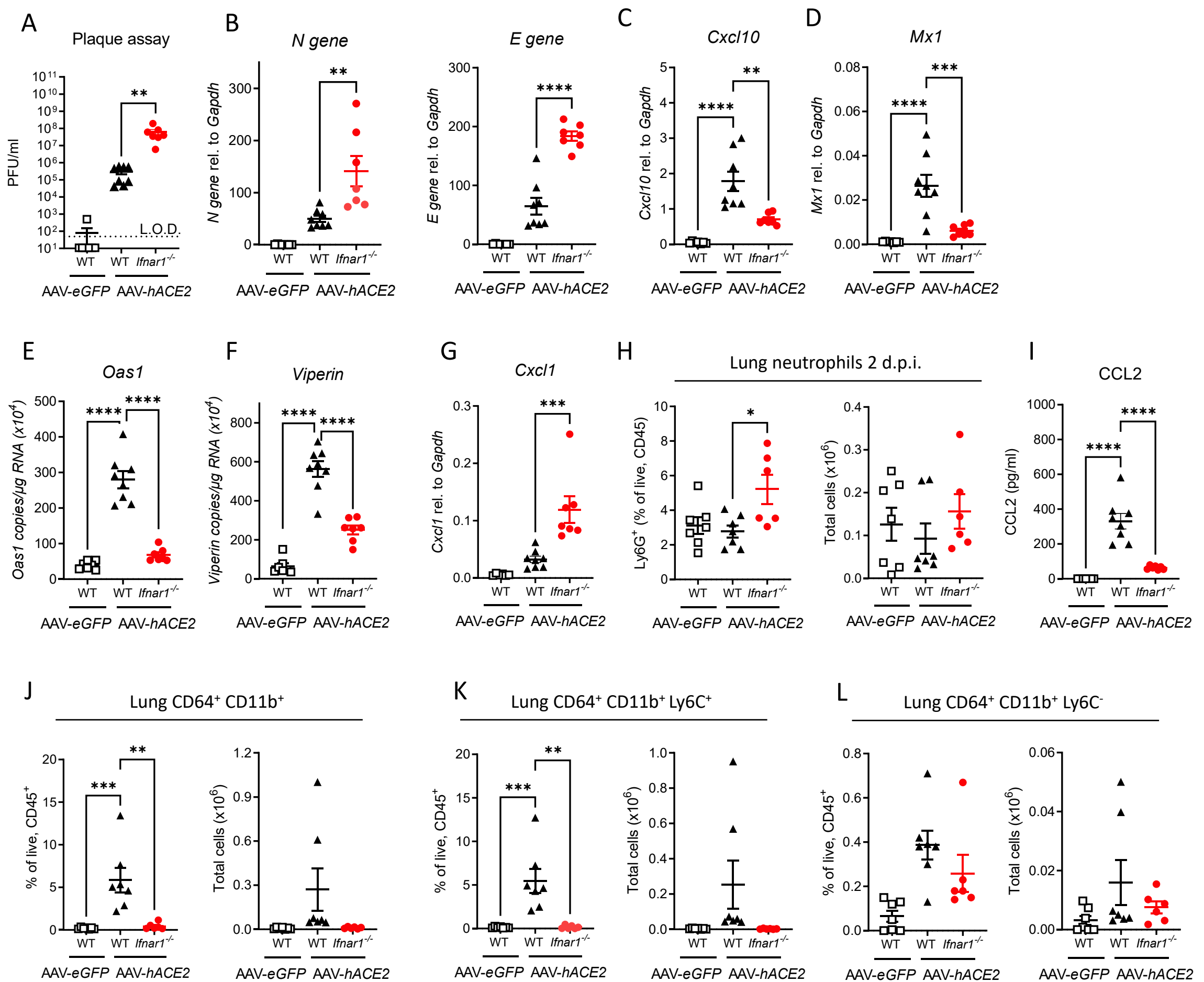
### Supplemental Figure 1: Validation of *hACE2* transduction and interferon expression upon SARS-CoV-2 infection

A) *eGFP* signal in lung cryosections of WT or *Ifnar1*<sup>-/-</sup> transduced with rAAV-*eGFP* (20 days post transduction), or vehicle (PBS). Representative images shown for each group (2-3 mice per group). Scale bar = 100  $\mu$ m. B) Expression of IFN- $\alpha$  and IFN- $\beta$  in BAL fluid at 2 d.p.i. measured by Luminex Procarta assay. C) Expression of IFN- $\lambda$  in BAL fluid at 2 d.p.i. measured by ELISA. Data are shown as mean  $\pm$  SEM. Two independent experiments per time point, data pooled, n = 6-8 per group. One Way ANOVA + Tukey's multiple comparison test; \* indicates significant difference between WT rAAV-*hACE2* and *Ifnar1*<sup>-/-</sup> rAAV-*hACE2*, \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.005, \*\*\*\* P < 0.001.



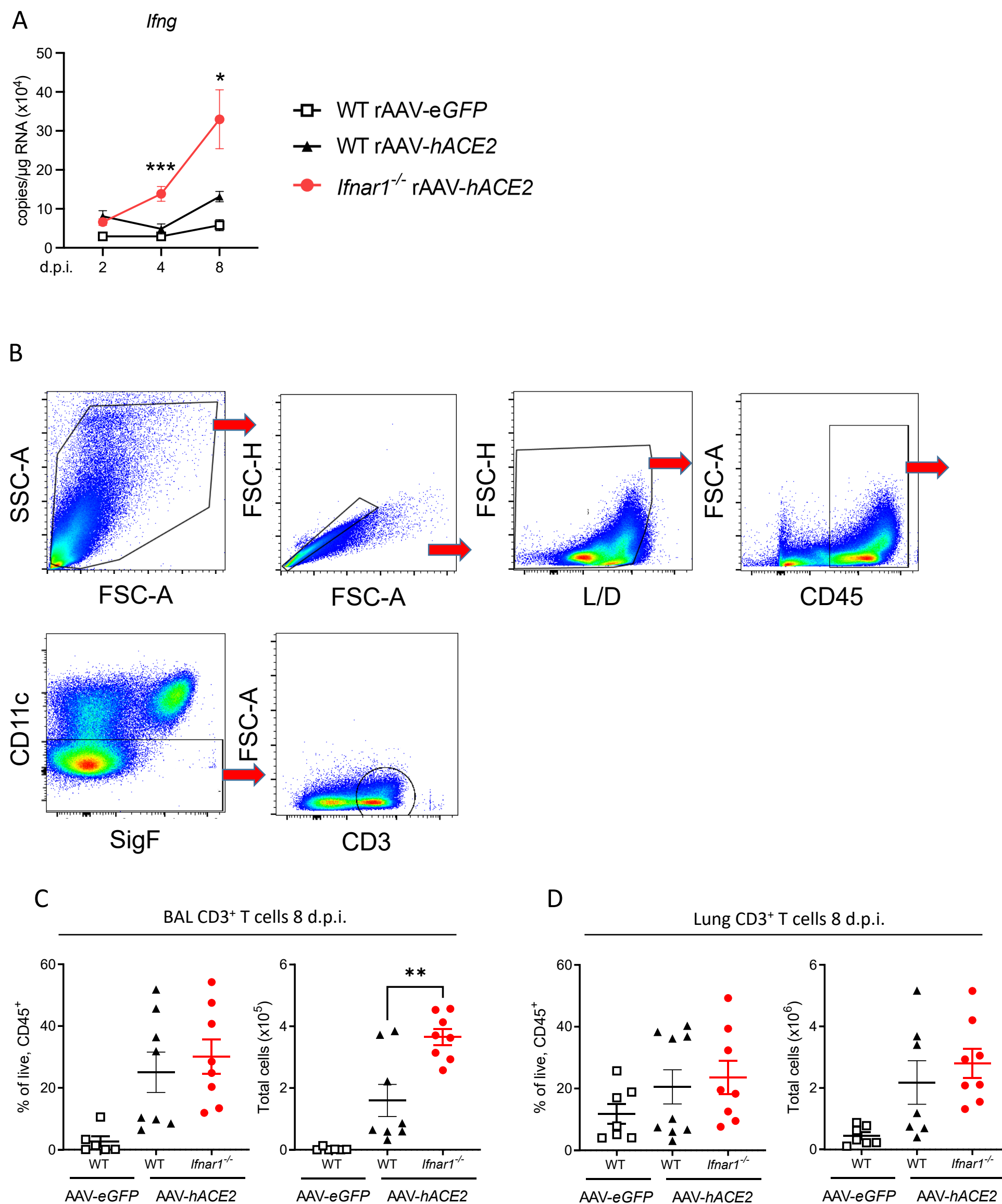
**Supplemental Figure 2: Baseline viral load and immune response at 0.75 d.p.i. with SARS-CoV-2 in rAAV-eGFP transduced WT and *Ifnar1*<sup>-/-</sup> mice**

WT and *Ifnar1*<sup>-/-</sup> mice were transduced with rAAV-eGFP and after 20 days infected with with SARS-CoV-2 (D614G, 2x10<sup>6</sup> PFU/mouse). BAL and lungs were analysed at 0.75 d.p.i. A) Viral load measured by plaque assay on Vero cells overexpressing *hACE2* and *TMPRESS2* B) Expression of SARS-CoV-2 *N gene* (nucleocapsid phosphoprotein) and *E gene* (envelope protein) in lung tissue relative to *Gapdh*, measured by RT-PCR C) Gene expression of *Cxcl1* in lung tissue relative to *Gapdh*. D) Protein expression of CCL2 in BAL fluid measured by ELISA. E-F) Proportions of live, CD45<sup>+</sup> and total numbers of neutrophils in BAL (E) and lung (F) at 0.75 d.p.i. G) Proportions of live, CD45<sup>+</sup> and total numbers of CD64<sup>+</sup> CD11b<sup>+</sup> Ly6C<sup>+</sup> monocytes in BAL at 0.75 d.p.i. Data are shown as mean ± SEM. Two independent experiments, data pooled, n = 6-8 per group. One Way ANOVA + Tukey's multiple comparison test; \* indicates significant difference between WT AAV-*hACE2* and *Ifnar1*<sup>-/-</sup> AAV-*hACE2*, \* P < 0.05.



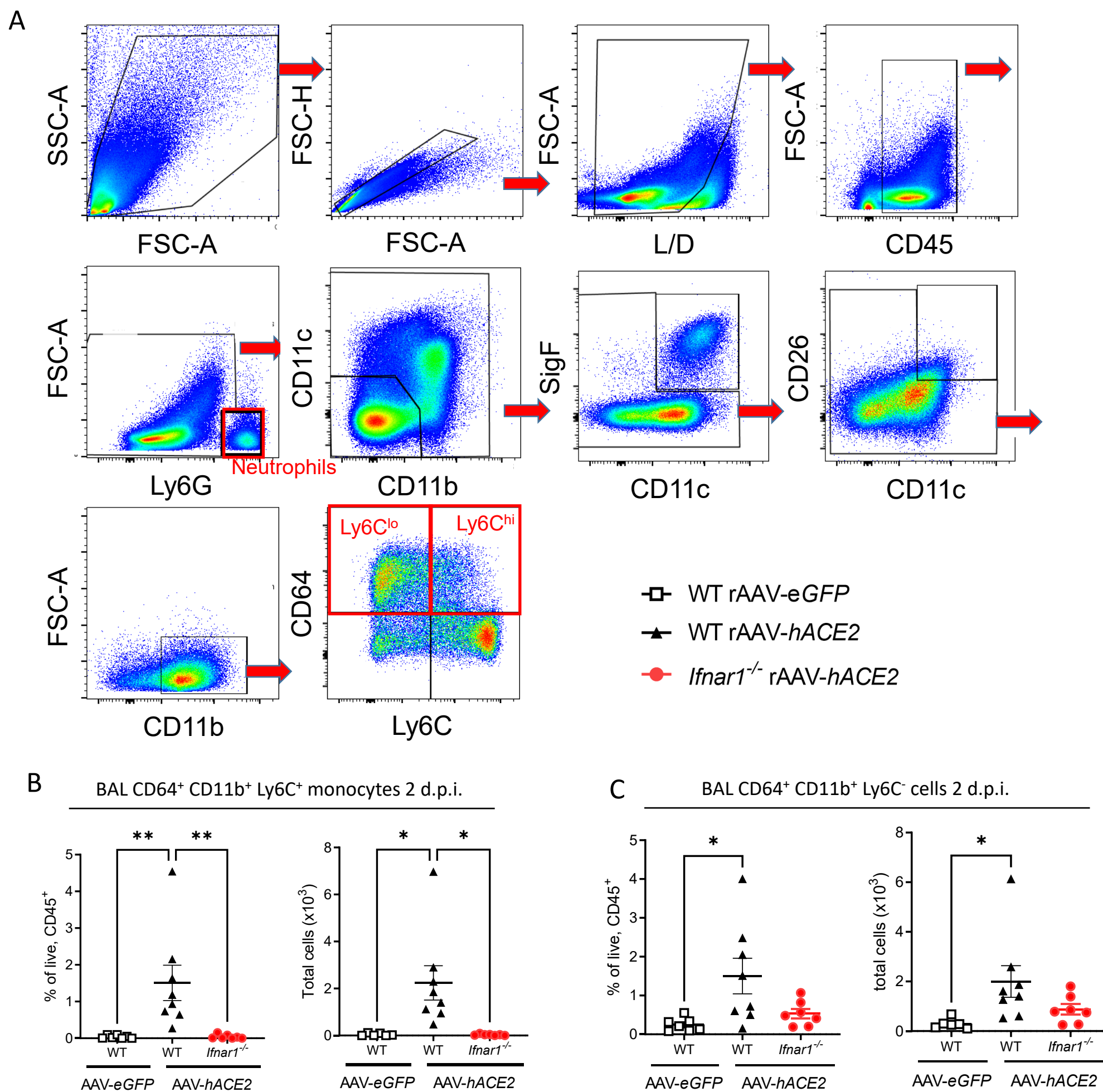
### Supplemental Figure 3: Viral load and immune responses at 2 d.p.i. with SARS-CoV-2 in rAAV-hACE2 mouse model

WT and *Ifnar1*<sup>-/-</sup> mice were transduced with rAAV-eGFP or rAAV-hACE2 and after 20 days infected with SARS-CoV-2 (D614G, 2x10<sup>6</sup> PFU/mouse). BAL and lungs were analysed at 2 d.p.i. A) Viral load measured by plaque assay on Vero cells overexpressing hACE2 and TMPRESS2 B) Expression of SARS-CoV-2 *N gene* (nucleocapsid phosphoprotein) and *E gene* (envelope protein) in lung tissue relative to *Gapdh*, measured by RT-PCR C-F) Gene expression analysis of IFN stimulated genes (ISG) *Cxcl10* (C), *Mx1* (D), *Oas1* (E) and *Viperin* (F) measured by RT-PCR, relative to expression of *Gapdh* or total copy numbers normalized to expression of *Gapdh* (*Oas1* and *Viperin*). G) Gene expression of *Cxcl1* in lung tissue relative to *Gapdh*. H) Protein expression of CCL2 in BAL fluid measured by ELISA. I) Proportions of live, CD45<sup>+</sup> and total numbers of neutrophils in lung tissue J - L) Proportions of live, CD45<sup>+</sup> and total numbers of CD64<sup>+</sup> CD11b<sup>+</sup> inflammatory myeloid cells (J), CD64<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>+</sup> cells (K) and CD64<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>-</sup> cells (L) in lung tissue. Data are shown as mean ± SEM. Tissues were collected at 2 d.p.i. Two independent experiments per time point, data pooled, n = 6-8 per group. One Way ANOVA + Tukey's multiple comparison test; \* indicates significant difference between WT AAV-hACE2 and *Ifnar1*<sup>-/-</sup> AAV-hACE2, \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.005, \*\*\*\* P < 0.001.



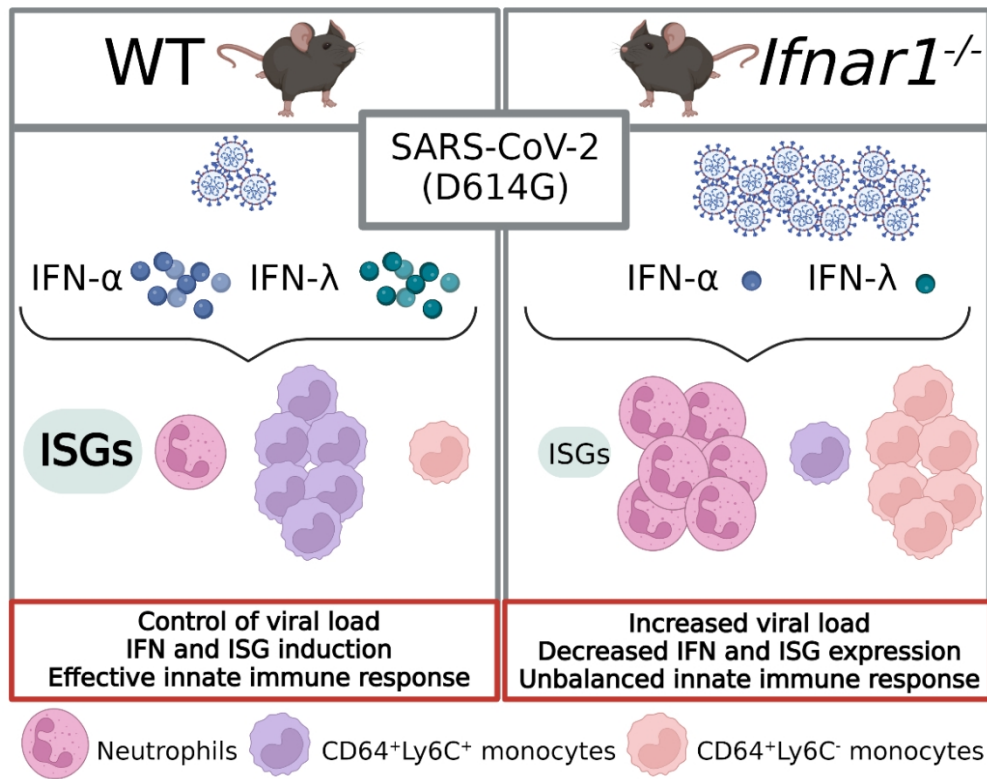
#### Supplemental Figure 4: Interferon- $\gamma$ and T cell recruitment to the lungs upon infection SARS-CoV-2 in rAAV-*hACE2* mouse model

A) Gene expression analysis of *Ifng* measured in lung homogenates by RT-PCR, total copy numbers normalized to expression of *Gapdh* at 2, 4 and 8 days. B) Gating strategy to detect CD3<sup>+</sup> T cells in BAL and lung 8 days after infection with SARS-CoV-2. C – D) Proportions of live, CD45<sup>+</sup> and total numbers of T cells in BAL (C) and lung (D) at 8 d.p.i. Data are shown as mean  $\pm$  SEM. Two independent experiments per time point, data pooled, n = 6-8 per group. One Way ANOVA + Tukey's multiple comparison test per time point; \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.005, \*\*\*\* P < 0.001.



### Supplemental Figure 5: Myeloid cell recruitment into the airways upon infection SARS-CoV-2 in rAAV-hACE2 mouse model

A) Gating strategy to detect innate immune cell populations in BAL and lung after infection with SARS-CoV-2. Populations of interest (highlighted in red) are Ly6G<sup>+</sup> neutrophils, CD11b<sup>+</sup>CD64<sup>+</sup>Ly6C<sup>-</sup> and CD11b<sup>+</sup>CD64<sup>+</sup>Ly6C<sup>+</sup> inflammatory myeloid cells. B-C) Proportions of live, CD45<sup>+</sup> and total numbers of CD64<sup>+</sup> CD11b<sup>+</sup> Ly6C<sup>+</sup> monocytes (C) and CD64<sup>+</sup> CD11b<sup>+</sup> Ly6C<sup>-</sup> monocytes (D) in BAL at 2 d.p.i. Data are shown as mean  $\pm$  SEM. Two independent experiments per time point, data pooled, n = 6-8 per group. One Way ANOVA + Tukey's multiple comparison test; \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.005, \*\*\*\* P < 0.001.



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3 During SARS-CoV-2 infection in mice, IFNAR1-deficiency results in dysregulated innate immune  
4 responses, such as increased neutrophil recruitment to the lung and an altered monocyte population  
5 profile. Together with decreased expression of anti-viral effector interferon-stimulated genes *Mx1*,  
6 *Oas1* and *Viperin* this results in higher viral loads and overall worsened disease phenotype.  
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For Peer Review

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3 **Type I interferon signaling deficiency results in dysregulated innate immune**  
4 **responses to SARS-CoV-2 in mice**  
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**Abstract:**

SARS-CoV-2 is a newly emerged coronavirus, causing the global pandemic of respiratory coronavirus disease (COVID-19). The type I interferon (IFN) pathway is of particular importance for anti-viral defence and recent studies identified that type I IFNs drive early inflammatory responses to SARS-CoV-2. Here, we use a mouse model of SARS-CoV-2 infection, facilitating viral entry by intranasal recombinant Adeno-Associated Virus (rAAV) transduction of *hACE2* in wildtype (WT) and type I IFN-signalling-deficient (*Ifnar1*<sup>-/-</sup>) mice, to study type I IFN signalling deficiency and innate immune responses during SARS-CoV-2 infection. Our data show that type I IFN signaling is essential for inducing anti-viral effector responses to SARS-CoV-2, control of virus replication and to prevent enhanced disease. Furthermore, *hACE2-Ifnar1*<sup>-/-</sup> mice had increased gene expression of the chemokine *Cxcl1* and airway infiltration of neutrophils as well as a reduced and delayed production of monocyte-recruiting chemokine CCL2. *hACE2-Ifnar1*<sup>-/-</sup> mice showed altered recruitment of inflammatory myeloid cells to the lung upon SARS-CoV-2 infection, with a shift from Ly6C<sup>+</sup> to Ly6C<sup>-</sup> expressing cells. Together, our findings suggest that type I IFN deficiency results in a dysregulated innate immune response to SARS-CoV-2 infection.

**Keywords:**

Innate Immune Response/ type I IFN / In vivo / SARS-CoV-2/ myeloid cells

## Introduction

The ongoing coronavirus disease (COVID-19) pandemic caused by severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) has resulted in over 400 million cases in the first two years of the pandemic. The estimated fatality rate lies between 1-2%, however this is considerably higher for elderly patients over 80 years of age (~10%) and nursing home residents (>20%) (1). Type I interferons (IFNs) are one of the first responses elicited against viral infection and they induce anti-viral defense mechanisms by binding to the IFN- $\alpha/\beta$  receptor (IFNAR) and signaling through the JAK-STAT pathway. This induces expression of IFN stimulated genes (ISG), resulting in expression of anti-viral effector proteins that restrict viral replication and activation of immune cells via induction of chemokine and cytokine production (2), including CXCL10 and CCL2 (3). Early *in vitro* studies using human bronchial epithelial cell lines infected with SARS-CoV-2 showed decreased production of type I and III IFNs coupled with low anti-viral defense signals and a pro-inflammatory environment compared to infection with influenza A virus (IAV) (4). Furthermore, in severe and critically ill COVID-19 patients, an impaired type I IFN response has been observed, resulting in decreased viral clearance (5). A lack of an efficient type I IFN response in these patients is in part due to inborn errors of type I IFN immunity (6) or circulating auto-antibodies neutralizing type I IFNs (7). Also, a recent animal study has identified that type I IFN signaling is required for the recruitment of pro-inflammatory cells into the lungs following SARS-CoV-2 infection (8). These findings highlight the importance of functional type I IFN responses for anti-viral defenses against SARS-CoV-2.

Using a mouse model of SARS-CoV-2 infection, facilitated by intranasal recombinant Adeno-Associated Virus (rAAV) induced expression of human angiotensin converting enzyme 2 (hACE2), this study investigated the dynamics of innate immune responses to infection with SARS-CoV-2 in the context of type I IFN signaling impairment. Overall, the data show that type I IFN signaling is essential to induce anti-viral responses and control viral replication and disease severity during SARS-CoV-2 infection. Furthermore, type I IFN signaling-deficient mice show dysregulated innate immune responses to SARS-CoV-2 infection, marked by increased neutrophil recruitment into the airways and delayed recruitment of myeloid inflammatory cells.

## Results and discussion

To investigate the dynamics of innate, anti-viral immune responses to SARS-CoV-2 infection in the context of type I IFN-signaling deficiency, 8–12-week-old C57BL/6 wildtype (WT) or *interferon alpha receptor-1*<sup>-/-</sup> (*Ifnar1*<sup>-/-</sup>) mice were intranasally transduced with rAAV9 containing either *hACE2* or *eGFP* (control) as published recently (9), followed by intranasal infection with 2x10<sup>6</sup> PFU SARS-CoV-2

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3 (D614G, first wave isolate) 20 days later and study endpoints were at 2, 4 and 8 days post infection  
4 (d.p.i.) (Figure 1A). Gene expression analysis of *hACE2* in lung tissue 20 days post administration of  
5 rAAV9 (before infection) showed similar expression in WT and *Ifnar1*<sup>-/-</sup> mice relative to *Gapdh* (Figure  
6 1B). Cryo-sectioning of lung tissue 20 days post administration of rAAV9-*eGFP* (before infection)  
7 furthermore showed similar distribution of *eGFP* in WT and *Ifnar1*<sup>-/-</sup> mice (Supp. Figure 1A), suggesting  
8 similar rAAV transduction efficacy in both groups of mice. Upon infection with SARS-CoV-2, *hACE2*-  
9 *Ifnar1*<sup>-/-</sup> mice showed increased weight loss, peaking at 6 d.p.i., compared with *hACE2*-WT mice (Figure  
10 1C). Furthermore, plaque assays on Vero cells overexpressing SARS-CoV-2 binding receptors ACE2 and  
11 transmembrane protease serine 2 precursor TMPRSS2 (VAT cells) showed that IFNAR1-deficiency  
12 results in significantly higher viral loads at day 2 and 4 post infection with SARS-CoV-2 compared to  
13 *hACE2*-WT mice (Figure 1D). Gene expression of SARS-CoV-2 nucleocapsid (N) and envelope (E) genes  
14 was significantly higher in *hACE2-Ifnar1*<sup>-/-</sup> mice compared with *hACE2*-WT mice from 2 d.p.i. (Figure 1E  
15 and Supp. Figure 2B). Since AAV-*eGFP* transduced *Ifnar1*<sup>-/-</sup> mice did not become infected with SARS-  
16 CoV-2 and did overall not differ significantly from AAV-*eGFP* transduced WT mice (Supp. Figure 2), this  
17 group was not included in all experiments to reduce animal numbers. Together, these data show  
18 increased viral load in *hACE2* expressing IFNAR1-deficient mice upon SARS-CoV-2 infection, measured  
19 both by plaque assay and N and E gene expression. Previous studies have shown similar trends (8),  
20 although differences between WT and *Ifnar1*<sup>-/-</sup> are more pronounced in the model used here. This  
21 could be due to several factors: here, Vero cells overexpressing *hACE2* and TMPRSS2 were used for  
22 plaque assays, allowing for better viral replication, while the isolate used for infection was hCoV-  
23 19/England/IC19/2020, which harbors the D614G spike mutation as opposed to USA-WA1/2020,  
24 enhancing viral replication (10). Together, these factors might explain why we detected higher viral  
25 loads and more prominent differences emerged between *hACE2*-WT and *hACE2-Ifnar1*<sup>-/-</sup> mice.  
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43 To investigate the impact of impaired type I IFN signaling during SARS-CoV-2 infection, we first  
44 assessed type I IFN expression upon infection. At 2 d.p.i. both IFN- $\alpha$  and IFN- $\beta$  were significantly  
45 increased in BAL fluid of *hACE2*-WT compared to *eGFP*-WT mice (Supp. Figure 1B). In the *hACE2-Ifnar1*  
46 <sup>-/-</sup> group, expression of IFN- $\alpha$  was significantly lower, while IFN- $\beta$  levels were higher compared to the  
47 *hACE2*-WT group. We next investigated ISG expression after SARS-CoV-2 infection, since in *Ifnar1*  
48 <sup>-/-</sup> mice limited amounts of type I IFN cytokines can be produced but cannot signal for downstream ISGs  
49 induction. Chemokine *Cxcl10* and anti-viral effectors *Mx1*, *Oas1* and *Viperin* were quantified. The  
50 expression of these ISGs was increased in *hACE2*-WT mice upon infection with SARS-CoV-2 at 2 d.p.i.,  
51 however in *hACE2-Ifnar1*<sup>-/-</sup> mice, ISG expression was significantly reduced, but not completely absent  
52 (Figure 1F and Supp. Figure 3 C-F). These results suggest an initial increase of IFN- $\beta$  in *hACE2-Ifnar1*  
53 <sup>-/-</sup> mice, which may be due to higher viral titers, but that is not translated into ISG expression due to the  
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3 lack of signaling through the IFNAR1. We therefore investigated the expression of type III IFNs, IFN-  
4  $\lambda$ 2-3, which can contribute to ISG expression. IFN- $\lambda$  expression was induced upon infection in *hACE2*-  
5 WT mice at 2 d.p.i. while remained at baseline levels in the *hACE2-Ifnar1<sup>-/-</sup>* mice (Supp. Figure 1C).  
6 Furthermore, *Ifng* gene expression was significantly increased in *hACE2-Ifnar1<sup>-/-</sup>* mice later during the  
7 infection, by 4 d.p.i. (Supp. Figure 4A). This correlated with CD3<sup>+</sup> T cell recruitment to the airways  
8 (Supp. Figure 4B-D). Our data suggest that limited levels of type I or III IFNs are produced early during  
9 infection in the *Ifnar1<sup>-/-</sup>* mice resulting in some ISG expression but overall, these data suggest that type  
10 I IFN signaling is the main driver for inducing cell intrinsic anti-viral responses.  
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18 We next assessed the gene expression of inflammatory mediators and found that expression of the  
19 chemokine *Cxcl1*, which is not dependent on type I IFN signaling (3), was increased in *hACE2-Ifnar1<sup>-/-</sup>*  
20 mice at 2 and 4 d.p.i. compared to *hACE2*-WT (Figure 2A and Supp. Figure 3G). As CXCL1 plays an  
21 essential role in early host immune responses by recruiting neutrophils (11), we next analysed  
22 infiltration of neutrophils (gated as live, CD45<sup>+</sup>, Ly6G<sup>+</sup>, Supp. Figure 5A) into the airways at 2 d.p.i. In  
23 line with highly increased gene expression of *Cxcl1*, neutrophil recruitment to the airways (BAL) was  
24 significantly increased in *hACE2-Ifnar1<sup>-/-</sup>* mice at 2 d.p.i., both proportional of leukocytes (CD45<sup>+</sup> cells)  
25 and in total numbers (Figure 2B and C). This was recapitulated in lung tissue with increased  
26 proportions of neutrophils in type I IFN signaling-impaired mice at 2 d.p.i. (Figure 2D and Supp. Figure  
27 3H), decreasing over time. Taken together, these findings suggest that during SARS-CoV-2 infection,  
28 type I IFN signaling deficiency results in increased neutrophil recruitment via CXCL1, thereby  
29 contributing to a pro-inflammatory environment. Indeed, *Cxcl1* is also increased in *Ifnar1<sup>-/-</sup>* mice during  
30 influenza A with secondary pneumococcal infection (12), but decreased during RSV infection in mice  
31 (13), highlighting a pathogen specific CXCL1 response. Furthermore, since we show similar trends for  
32 viral load and neutrophil recruitment upon SARS-CoV-2 infection (both significantly increased in  
33 *hACE2-Ifnar1<sup>-/-</sup>* mice), which is a mechanism present in other respiratory viral infections such as  
34 respiratory syncytial virus (RSV) (13, 14), it will be important to further investigate the link between  
35 neutrophil recruitment and viral load in this model.  
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50 Since monocyte recruitment to the airways and lungs is key to early host responses to viral infection,  
51 we next investigated the expression of monocyte recruiting chemokine CCL2 and the recruitment of  
52 inflammatory myeloid cells. CCL2 protein expression was increased in BAL fluid of *hACE2*-WT mice at  
53 2 d.p.i. with SARS-CoV-2 (Supp. Figure 3I). However, in *hACE2-Ifnar1<sup>-/-</sup>* mice CCL2 expression was  
54 significantly lower at 2 d.p.i., peaking at 4 d.p.i. at lower levels than in IFNAR1-sufficient mice (Figure  
55 3A). These findings are in line with a report identifying early CCR2 signaling essential to restrict viral  
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3 burden in the lung in a mouse model of SARS-CoV-2 infection (15). The recruitment of CD64<sup>+</sup>CD11b<sup>+</sup>  
4 inflammatory myeloid cells to the lung followed similar kinetics, as in *hACE2*-WT mice proportions  
5 were highest at 2 d.p.i. and subsequently decreased, while in IFNAR1-deficient mice proportions and  
6 total numbers of CD64<sup>+</sup>CD11b<sup>+</sup> inflammatory myeloid cells strongly increased between 2 and 4 d.p.i.  
7 and were highest at 8 d.p.i. (Figure 3B and Supp. Figure 3J). We next assessed expression of the  
8 monocyte/macrophage differentiation antigen Ly6C within this population, since previous studies  
9 reported the infiltration of CD64<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>+</sup> inflammatory myeloid cells into the lung during SARS-  
10 CoV-2 infection (8, 15). This showed highly increased proportions of CD64<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>+</sup> in *hACE2*-WT  
11 but not IFNAR1-deficient mice at 2 d.p.i. in the BAL (Supp. Figure 5D) and lung (Figure 3C-D and Supp.  
12 Figure 3K), suggesting type I IFN dependency for recruitment. However, as we have previously shown  
13 that Ly6C is gradually downregulated on monocytes during response to respiratory viral infection (16),  
14 we also analyzed CD64<sup>+</sup>CD11b<sup>+</sup> Ly6C<sup>-</sup> cells. The presence of CD64<sup>+</sup>CD11b<sup>+</sup> Ly6C<sup>-</sup> inflammatory myeloid  
15 cells in the airways was not type I IFN signaling dependent, since both proportions and total numbers  
16 were significantly increased in *hACE2-Ifnar1*<sup>-/-</sup> mice at 4 and 8 d.p.i. (Figure 3C and E), while at 2 d.p.i.  
17 in the airways no significant differences emerged (Supp. Figure 5F). This accounts for the delayed  
18 emergence of inflammatory myeloid cells in the lung during type I IFN signaling impairment shown in  
19 Figure 3B and overall indicates altered recruitment dynamics of inflammatory myeloid cells. Taking  
20 these data together, our model recapitulates the deficiency of type I interferon responses seen in  
21 severe SARS-CoV-2 infection, which in patients is marked by decreased IFN- $\alpha$ , type I IFN activity and  
22 ISG score, as well as neutrophilia and increased CCL2 (5). Our data suggest that the lack of type I IFN  
23 signaling results in dysregulated innate immune responses in the lung during SAR-CoV-2 infection.  
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## 41 Concluding Remarks

42 In summary, using a mouse model of SARS-CoV-2 infection we show that type I IFN signaling is  
43 essential for inducing anti-viral effector responses, control of virus replication and disease severity.  
44 Our data indicate that type I IFN signaling-deficient mice express increased levels of *Cxcl1* in the lung  
45 and increased infiltration of neutrophils to the airways compared to WT controls. Furthermore, we  
46 found reduced and delayed production of CCL2 and altered recruitment of inflammatory myeloid cells  
47 during IFNAR1-deficiency. This, together with an increased viral burden is associated with more severe  
48 disease in type I IFN signaling-deficient mice. The data shown here will be valuable for better  
49 understanding how impaired type I IFN signaling drives SARS-CoV-2 pathology and disease severity,  
50 which is highly relevant considering the large contribution of impaired type I IFN responses on life-  
51 threatening SARS-CoV-2 infections (6, 7) and deaths (17) and for the development of type I IFN-based  
52 treatment options for COVID-19 in vulnerable populations. To conclude, our findings show that type I  
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3 IFN deficiency results in dysregulated innate immune responses to SARS-CoV-2 infection in the rAAV-  
4 *hACE2* mouse model.

## 6 **Materials and Methods**

### 8 **Mice**

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10 C57BL/6 mice were purchased from Charles River UK Inc. *Ifnar1*<sup>-/-</sup> mice on a C57BL/6 background were  
11 bred in-house. All mice were bred and maintained in pathogen-free conditions and 8–12-week-old  
12 mice were used for experiments. All animal experiments were reviewed and approved by the Animal  
13 Welfare and Ethical Review Board (AWERB) at Imperial College London and approved by the UK Home  
14 Office in accordance with the Animals Act 1986 (Scientific Procedures) and ARRIVE guidelines. Both  
15 male and female mice were used for experiments after excluding sex bias in preliminary experiments.  
16 All experiments were performed twice, independently, per time point.

### 23 **rAAV vector production**

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25 The production, purification, and titration of rAAV2/9-*eGFP* or *hACE2* vectors were performed as  
26 previously described (9). Briefly, the respective rAAV vector was produced by polyethylenimine (PEI,  
27 PolySciences)-based triple transfection of human embryonic kidney (HEK) 293T/17 cells (ATCC, CRL-  
28 11268). The AAV plasmids transfected included the Adenovirus helper plasmid (pAdDeltaF6), AAV  
29 Rep-Cap pAAV2/9 plasmid and the transgene plasmid. The transgene plasmid containing *eGFP* or  
30 *hACE2* was engineered to include a lung-optimized hCEFI (human Cytomegalovirus  
31 enhancer/elongation factor 1 alpha) promoter (18), Woodchuck Hepatitis Virus Post-transcriptional  
32 Regulatory Element (WPRE) (19) and mir142-3pT (20). rAAV particles were concentrated and  
33 formulated into PBS using 100 kDa Ultra centrifugal filters (Amicon, Merck) after iodixanol gradient  
34 centrifugation. Physical titre (DNase-resistant genome copies, DRGC/mL) was determined by  
35 quantitative polymerase chain reaction (qPCR) analysis with primers and a probe against WPRE (21).  
36 Purity of vectors was confirmed by analyzing 20 µl of diluted vector on 4-12% SDS polyacrylamide gels,  
37 where total protein was visualized using Coomassie stain according to the manufacturer's protocols  
38 (Life Technologies).

### 49 **hACE2 transduction**

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51 For transduction, WT or *Ifnar1*<sup>-/-</sup> mice were lightly anesthetized and instilled i.n. with 1x10<sup>11</sup> DNase  
52 Resistant Gene Copies (DRGC) rAAV9-*eGFP* or rAAV9-*hACE2* in 100 µl PBS. *hACE2* gene expression in  
53 whole lung homogenate was assessed at day 20 post instillation by relative quantification to *Gapdh*  
54 using primers and probes for *hACE2* listed in the key resource table.



### Cryosectioning and native eGFP detection

Mice were sacrificed 20 days post instillation of AAV-eGFP or PBS and lungs were removed after inflation with 4% PFA. After 24-hour fixation in 4% PFA, lungs were inflated with 30% sucrose and submerged in 30% sucrose for 24 hours. Lungs were subsequently inflated with 1:1 cryo embedding matrix (OCT)/30% sucrose and individual lobes were submerged in OCT/30% sucrose in plastic molds and frozen at -80 °C. Left lungs were cryosectioned to produce 7 µm thick sections, mounted using DAPI-supplemented mounting media with coverslip, and eGFP expression was detected by fluorescent microscopy using the EVOS FL Auto 2 system (Thermo Scientific).

### Virus and infections

First wave SARS-CoV-2 (D614G, isolate of hCoV-19/England/IC19/2020) was grown in African green monkey kidney cells overexpressing human ACE2 and TMPRSS2 (Vero-ACE2-TMPRSS2; VAT cells) (22). For infection 20 days post transduction with rAAVs, mice were lightly anesthetized and instilled i.n. with  $2 \times 10^6$  plaque forming units (PFU) of SARS-CoV-2 in 100 µl volume. SARS-CoV-2 titre was assessed in lungs at 2, 4 and 8 d.p.i. using a plaque assay. In brief, serial dilutions of lung homogenate in serum-free Dulbecco's Modified Eagle Medium (DMEM, containing 1% non-essential amino acids (NEAA), 100U/ml Penicillin and 100 µg/ml Streptomycin) were performed and inoculated onto VAT cells for 1 h at 37°C. The inoculum was then removed and replaced with overlay medium (1x MEM, 0.2% w/v BSA, 0.16% w/v NaHCO<sub>3</sub>, 10 mM HEPES, 2 mM L-Glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.84% agarose). Plates were incubated for 3 days at 37°C before overlay was removed and cells were stained for 1 h at room temperature in 2x crystal violet solution. Virus plaques were counted and multiplied by the dilution factor to calculate titer as PFU/ml.

### Isolation of lung cells

Mice were sacrificed at 0.75, 2, 4 and 8 d.p.i. and lungs were perfused with PBS. To obtain lung leukocytes, lung lobes were cut into smaller pieces and incubated in complete DMEM (cDMEM, supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin), 1mg/ml Collagenase D (Roche) and 30 µg/ml DNase I (Invitrogen) for 1h at 37°C and then mashed through a 100-µm filter (BD). Red blood cells were lysed using Ammonium-Chloride-Potassium buffer.

### BAL cell processing

BAL was collected by flushing the lungs three times with 1 ml PBS supplemented with 5 mM EDTA (Life Technologies). BAL cells and supernatant were separated by centrifugation and BAL supernatants

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3 were exposed to UV light for 2 min to inactivate SARS-CoV-2. Red blood cells were lysed using  
4 Ammonium-Chloride-Potassium buffer.  
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### 8 **Flow cytometry**

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10 After red blood cell lysis, lung and BAL cells were incubated for 30 min with fixable live-dead Aqua dye  
11 (Invitrogen), followed by fixation for 30 minutes with 4% paraformaldehyde (PFA) to inactivate virus.  
12 Cells were then incubated for 20 min with a purified rat IgG<sub>2b</sub> anti-mouse CD16/CD32 receptor  
13 antibody (BD) to block Fc binding, followed by staining with fluorochrome-conjugated antibodies  
14 against CD45 (30-F11, BV605), CD26 (H194-112, BV711), Siglec-F (E50-2440, BV786), Ly6G (1A8,  
15 AF488), Ly6C (12HK1.4, PE), CD11c (HL3, PE-CF594), CD64 (X54-5/7.1, APC) and CD11b (M1/70, AF700)  
16 in PBS containing 1% BSA and 5 mM EDTA for 25 min at 4°C. For the adaptive immune cells, they were  
17 stained with CD11c (HL3, V450), Siglec-F (E50-2440, BV786), CD19 (6D5, AF488), CD45 (30-F11, PerCP-  
18 Cy5.5), Ly6G (1A8, PE-Cy7) and CD3 (17A2, AF700). Samples were analysed on a BD-Fortessa Flow  
19 Cytometer equipped with 50-mW 504-nm, 50-mW 488-nm, 50-mW 561-nm and 20-mW 633-nm lasers  
20 and an ND1.0 filter in front of the FSC photodiode. All antibodies were purchased from BD, Biolegend  
21 or eBioscience. Data were analyzed with FlowJo software (Tree Star).  
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### 32 **RNA isolation and quantitative RT-PCR**

33 Lung tissue was homogenized in TRIzol and RNA extraction performed according to manufacturer's  
34 instructions. After the chloroform step, the aqueous phase containing RNA was further processed  
35 using the RNeasy Mini Kit (QIAGEN) according to manufacturer's instructions. 2 µg RNA was reverse  
36 transcribed using a High-Capacity RNA-to-cDNA kit (Applied Biosystems) according to manufacturer's  
37 instructions. To quantify mRNA levels in lung tissue, quantitative RT-PCR reactions for *Oas1*, *Viperin*  
38 and *Ifnl* were performed using primers and probes as previously described (23). Analysis was  
39 performed using the QuantiTect Probe PCR Master Mix (QIAGEN) and the 7500 Fast real-Time PCR  
40 System (Applied Biosystems). For absolute quantification, the exact number of copies of the gene of  
41 interest was calculated using a plasmid DNA standard curve, and the results were normalized to levels  
42 of *Gapdh* (Applied Biosystems). For relative quantification, the expression of *Cxcl1*, *Cxcl10*, *hACE2*,  
43 *Mx1* and SARS-CoV-2 *N* and *E* gene was expressed relatively to the expression of *Gapdh*. First, the  $\Delta$ CT  
44 (CT = cycle threshold) between the target gene and *Gapdh* was calculated for each sample, followed  
45 by calculation of  $2^{-\Delta$ CT. Analysis was performed using 7500 Fast System SDS Software (Applied  
46 Biosystems).  
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### 58 **Chemokine and IFN detection**

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3 CCL2 and IFN- $\lambda$ 2/3 quantifications were performed on BAL fluid using mouse DuoSet ELISA (R&D  
4 Systems) according to the manufacturer's instructions. Data were acquired on a SpectraMax Plus plate  
5 reader (Molecular Devices) and analysed using SoftMax software (version 5.2). The concentration of  
6 IFN- $\alpha$  and IFN- $\beta$  was measured in BAL fluid using the Mouse ProCartaPlex Immunoassay (Invitrogen)  
7 according to the manufacturer's instructions. Data were acquired and analysed with a Bio-Plex 200  
8 system (Bio-Rad Laboratories).  
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### 15 **Statistical analysis**

16 Statistical analysis was performed using Prism 9.2 (Graph-Pad Software). One-way ANOVA with  
17 Tukey's post-hoc test was used to compare multiple groups. Data are expressed as mean  $\pm$  SEM, and  
18 for all tests a value of  $P < 0.05$  was considered significant. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.005$ , \*\*\*\*  
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### 45 **Author contribution**

46 P.P.O. designed, performed, and analyzed the experiments and wrote the paper. M.G.M. and C.M.  
47 performed specific experiments and reviewed the paper. Y.D., D.R.G., S.C.H., O.H., and K.M.M.  
48 designed, manufactured and provided rAAV9-*hACE2* and rAAV9-*eGFP* and reviewed the paper,  
49 additionally Y.D. and K.M.M. performed specific experiments. J.Z., J.C.B. and W.S.B. provided advice  
50 and the SARS-CoV-2 WT isolate (D614G) and reviewed the paper. C.J. supervised the project, designed  
51 the experiments, and wrote the paper.  
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### 58 **Conflict of interest**

The authors declare no commercial or financial conflict of interest.

## Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Ethics approval

All animal experiments were reviewed and approved by the Animal Welfare and Ethical Review Board (AWERB) at Imperial College London and approved by the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986 Amendment Regulations (PPL P3AFFFODD Johansson).

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## Figure legends

### Figure 1: Increased viral load, weight loss and lower expression of ISGs in *Ifnar1*<sup>-/-</sup> mice during infection with SARS-CoV-2

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36 A) Recombinant Adeno-associated virus (rAAV) containing human angiotensin converting enzyme 2  
37 (hACE2) or *eGFP* genes was administered intranasally to *Ifnar1*<sup>-/-</sup> or wildtype (WT) mice (1x10<sup>11</sup> Dnase  
38 resistant gene copies/mouse). 20 days later mice were intranasally infected with SARS-CoV-2 (D614G,  
39 2x10<sup>6</sup> PFU/mouse). Lungs and bronchoalveolar lavage (BAL) were harvested at 2, 4 and 8 days post  
40 infection (d.p.i). B) Expression of *hACE2* in lung tissue relative to *Gapdh*, measured by RT-PCR before  
41 infection (d20 post transduction with rAAV). WT/rAAV-*eGFP* n = 3, *Ifnar1*<sup>-/-</sup> /rAAV-*eGFP* n = 4,  
42 WT/rAAV-*hACE2* n = 4, *Ifnar1*<sup>-/-</sup> /rAAV-*hACE2* n = 5). C) Weight loss post infection with SARS-CoV-2. D)  
43 Viral load measured by plaque assay on Vero cells overexpressing hACE2 and TMPRESS2. E) Expression  
44 of SARS-CoV-2 *N gene* (nucleocapsid phosphoprotein) and *E gene* (envelope protein) in lung tissue  
45 relative to *Gapdh*, measured by RT-PCR. F) Gene expression analysis of IFN stimulated genes (ISG)  
46 *Cxcl10*, *Mx1*, *Oas1* and *Viperin* measured by RT-PCR, relative to expression of *Gapdh* or total copy  
47 numbers normalized to expression of *Gapdh* (*Oas1* and *Viperin*). Data are shown as mean ± SEM. B)  
48 WT/rAAV-*eGFP* n = 3, WT/rAAV-*hACE2* n = 4, *Ifnar1*<sup>-/-</sup> /rAAV-*hACE2* n = 5 C – F) Two independent  
49 experiments per time point, data pooled, n = 6-8 per group. One Way ANOVA + Tukey's multiple  
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comparison test per time point; \* indicates significant difference between WT rAAV-*hACE2* and *Ifnar1*<sup>-/-</sup> rAAV-*hACE2*, \* P < 0.05, \*\* P < 0.01, \*\*\* p < 0.005, \*\*\*\* p < 0.001.

**Figure 2: Increased neutrophil recruitment to airways in IFNAR1-deficient mice during SARS-CoV-2 infection**

A) Gene expression of *Cxcl1* in lung tissue relative to *Gapdh* at 2, 4 and 8 d.p.i. with SARS-CoV-2 (D614G), measured by RT-PCR. B) Proportions of live, CD45<sup>+</sup> and total numbers of neutrophils in BAL at 2 d.p.i. C) Representative flow cytometry plots of lung cells gated on live, CD45<sup>+</sup> Ly6G<sup>+</sup> D) Proportions of live, CD45<sup>+</sup> and total numbers of neutrophils in lung tissue at 2, 4 and 8 d.p.i. Data are shown as mean ± SEM. Two independent experiments per time point, data pooled, n = 6-8 per group. One Way ANOVA + Tukey's multiple comparison test per time point; \* indicates significant difference between *hACE2*-WT and *hACE2-Ifnar1*<sup>-/-</sup>, \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.005, \*\*\*\* P < 0.001.

**Figure 3: Type I interferon signaling deficiency results in dysregulated inflammatory myeloid cell recruitment during SARS-CoV-2 infection**

A) Protein expression of CCL2 in BAL fluid at 2, 4 and 8 d.p.i. measured by ELISA. B) Proportions of live, CD45<sup>+</sup> and total numbers of CD64<sup>+</sup> CD11b<sup>+</sup> inflammatory myeloid cells in lung tissue at 2, 4 and 8 d.p.i. C) Representative flow cytometry plots of lung cells gated on live, CD45<sup>+</sup> Ly6G<sup>-</sup> SigF<sup>-</sup> CD11b<sup>+</sup>. D) Proportions of live, CD45<sup>+</sup> and total numbers of CD64<sup>+</sup> CD11b<sup>+</sup> Ly6C<sup>+</sup> inflammatory myeloid cells in lung tissue at 2, 4 and 8 d.p.i. E) Proportions of live, CD45<sup>+</sup> and total numbers of CD64<sup>+</sup> CD11b<sup>+</sup> Ly6C<sup>-</sup> inflammatory myeloid cells in lung tissue at 2, 4, and 8 d.p.i. Data are shown as mean ± SEM. Two independent experiments per time point, data pooled, n = 6-8 per group. One Way ANOVA + Tukey's multiple comparison test per time point; \* indicates significant difference between *hACE2*-WT and *hACE2-Ifnar1*<sup>-/-</sup>, \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.005, \*\*\*\* P < 0.001. Dotted line = limit of detection.