

1 **The *In Vivo* Source of Type I and Type III IFNs is Pathogen Dependent**

2 Marvin J. Sandoval^{1,4}, Hsiang-Chi Tseng², Heidi P. Risman², Sergey Smirnov³ Qing Li³,
3 Jian-Da Lin^{3,5}, Amariliz Rivera⁶, Russell K. Durbin², Sergei V. Kotenko³ and Joan E.
4 Durbin^{2*},

5

6 ¹. Immunology and Inflammation Graduate Program, New York University School of
7 Medicine, 550 1st Ave., New York, NY, USA.

8 ². Department of Pathology, Immunology and Laboratory Medicine, Rutgers – New
9 Jersey Medical School, 185 South Orange Ave., Newark, NJ, USA

10 ³. Department of Microbiology, Biochemistry and Molecular Genetics, Rutgers – New
11 Jersey Medical School, 205 S. Orange Ave., Newark, NJ, USA

12 ⁴. Current Address: Autoimmunity and Inflammation Program, Hospital for Special
13 Surgery, New York, NY 10021

14 ⁵Current Address: Department of Biochemical Science and Technology, College of Life
15 Science, National Taiwan University, Taipei City, Taiwan.

16 ⁶. Department of Pediatrics, Rutgers – New Jersey Medical School, 205 S. Orange Ave.,
17 Newark, NJ, USA

18 * Corresponding author

19

20 durbinje@njms.rutgers.edu (JED)

21

22 **Abstract**

23 Type I ($-\alpha$, β) and type III ($-\lambda$) interferons (IFNs) are produced in response to virus
24 infection and upregulate a largely overlapping set of IFN stimulated genes which
25 mediate the protective effects of these antiviral cytokines. *In vitro* studies have
26 demonstrated the redundancy of these two cytokine families which activate the same
27 transcription factor, IFN stimulated gene factor 3 (ISGF3), via distinct ligands and
28 receptors. However, *in vivo*, these IFN types do have distinct functions based on
29 receptor distribution, but also ligand availability. Using a newly generated IFN- λ reporter
30 mouse strain we have observed that both type I and type III IFNs are produced in
31 response to respiratory tract infection by Newcastle disease virus (NDV) and influenza
32 A virus (IAV). In the case of NDV these IFNs are synthesized by different cell types.
33 Type I IFNs are produced primarily by alveolar macrophages, type III IFNs are made
34 only by epithelial cells, and production of either is dependent on MAVS. While epithelial
35 cells of the respiratory tract represent the primary target of IAV infection, we found that
36 they did not significantly contribute to IFN- λ production, and IFN- λ protein levels were
37 largely unaffected in the absence of MAVS. Instead we found that pDCs, a cell type
38 known for robust IFN- α production via TLR/MyD88 signaling, were the major producers
39 of IFN- λ during IAV infection, with pDC depletion during influenza infection resulting in
40 significantly reduced levels of both IFN- α and IFN- λ . In addition, we were able to
41 demonstrate that pDCs rely on type I IFN for optimal IFN- λ production. These studies
42 therefore demonstrate that the *in vivo* producers of Type III IFNs in response to
43 respiratory virus infection are pathogen dependent, a finding which may explain the
44 varying levels of cytokine production induced by different viral pathogens.

45 Introduction

46 The antiviral activity of the type I interferons (IFN- α/β) has been appreciated since their
47 discovery in 1957 [1, 2]. The more recent discovery of type III IFNs (IFN- λ) [3] [4], a new
48 IFN family with its own distinct receptor but mediating the same antiviral response, has
49 led to a reevaluation of our understanding of innate immune responses. The IFN- λ
50 receptor, comprised of the IFN-lambda receptor (IFNLR) chain and the IL-10R2 chain, is
51 expressed on most epithelial cells, but not ubiquitously found on all cell types like the
52 IFN-alpha receptors (IFNAR) 1 and 2. The type III IFNs act to protect mucosal surfaces,
53 the point of entry for many viral pathogens, but their source(s), and the stimuli leading to
54 their production are not yet well characterized [7, 8]. To better understand the relative
55 contributions of type I and type III IFNs to antiviral protection, we generated an IFN- λ
56 reporter mouse, *Ifnl2^{gfp/gfp}*, and used this strain to examine the relative contribution of
57 various cell types to the production of these cytokines. This approach was inspired by
58 the work of Kumagai *et al.* [5] who demonstrated that alveolar macrophages produce
59 the bulk of IFN- α following pulmonary infection with Newcastle disease virus (NDV)
60 using mice expressing GFP under the control of the *Ifna6* promoter. Similar results
61 were obtained following infection of these *Ifna6^{gfp/+}* mice with respiratory syncytial virus
62 (RSV) [6].

63

64 Multiple studies have demonstrated that type I and type III IFNs are induced *in vitro* by
65 the same stimuli, e.g. virus infection and TLR ligand exposure [7] [8], but it is not yet
66 known if induction consistently follows the same pathways following virus infection *in*
67 *vivo*. Making use of *Ifnl2^{gfp/gfp}* mice we have confirmed Kumagai's observation that

68 alveolar macrophages are the major source of IFN- α during NDV infection, but also
69 observed that this cell type produces no IFN- λ . IFN- λ in this infection is entirely derived
70 from respiratory epithelial cells in a MAVS-dependent manner. The finding that IFN- λ is
71 preferentially produced by polarized epithelial cells *in vitro* [9] has also been observed in
72 mouse models [10]. These published results support the notion that, despite their
73 activation of the same antiviral pathway, IFN- α/β and IFN- λ are produced by different
74 cell types in response to the same stimulus.

75

76 In this study we used the *Ifnl2^{gfp/gfp}* reporter mice to test the generality of this hypothesis
77 with a number of viruses. In both the NDV and rhesus rotavirus (RRV) infections we
78 observed IFN- λ production by the IFN- λ responsive cells lining the target tissue,
79 emphasizing the important role for this cytokine in protection of the epithelial barrier.
80 This pattern of IFN expression was not recapitulated in the murine model of influenza A
81 virus (IAV) infection, where a significant proportion of IFN- λ induced by infection was
82 produced by pDCs in a MAVS-independent manner. Taken together our studies show
83 that the source of type I and type III IFNs *in vivo* is pathogen dependent, and not
84 defined by the route of infection or the tropism of the infecting virus.

85

86 **Results**

87 **Generation and Characterization of the IFN- λ reporter mouse**

88 To identify IFN- λ -producing cell populations *in vivo*, we generated an IFN- λ reporter
89 mouse by replacing the coding region of *ifn12* with an *eGFP-Neo* cassette through
90 homologous recombination, while maintaining the *ifn12* promoter and UTR regions intact
91 (Fig 1A). The FRT sites flanking the neo gene allowed its removal by crossing mice
92 carrying the targeted allele with mice expressing the FLP-recombinase to generate
93 animals lacking the Neo gene while simultaneously carrying the GFP-targeted allele. Mice
94 heterozygous for the eGFP-targeted allele were crossed to generate mice homozygous
95 for the targeted allele. Homozygous *ifn12*^{eGFP/eGFP} knockin mice can no longer produce
96 IFN- λ 2, but still retain functional *ifn13* gene loci. Genotypes were determined by
97 conventional PCR using primers targeting the IFN- λ locus and the eGFP transgene (Fig
98 1A, B).

99 Human pDCs secrete high levels of both type I and type III IFNs when stimulated by
100 TLR ligands or virus infection [11], so characterization of the reporter strain was initiated
101 by Newcastle disease virus (NDV) infection of Flt3-ligand (FLT3L) cultured bone marrow
102 derived dendritic cells. NDV is an avian paramyxovirus known to induce high levels of
103 type I IFNs in many cell types including murine pDCs [5, 12], so we used this system to
104 ask whether GFP expression correlated with IFN- λ production upon induction by virus
105 infection.

106 GFP expression following virus inoculation was detected by fluorescence microscopy
107 (Fig 1C), as well as flow cytometry (Fig 1D). A Fluorescence Activated Cell Sorter (FACS)
108 was used to separate NDV-treated, FLT3L-cultured DCs into GFP- and GFP+ populations

109 for RNA extraction and subsequent qRT-PCR analysis of IFN- λ 3 and eGFP transcripts
110 (Fig 1D). This analysis confirmed that IFN- λ 3 and eGFP transcripts were enriched only
111 in GFP+ cells, demonstrating that GFP fluorescence from reporter cells reflected IFN- λ
112 production.

113 We also explored GFP induction following virus infection of epithelial cells. Primary
114 kidney epithelial cells derived from *ifnl2^{gfp/gfp}* reporter mice were cultured *in vitro* and
115 infected with NDV, respiratory syncytial virus (RSV), rhesus rotavirus (RRV) or influenza
116 A virus (Fig 1E). At 24 hours post-infection, all infected cultures contained GFP+ cells,
117 but for all viruses the percentage of GFP+ cells was relatively small, even at higher MOIs.
118 A similar effect was observed in FLT3L cultured DCs (data not shown), suggesting that
119 during a virus infection, not all cells capable of producing IFN- λ go on to do so as has
120 been found for IFN- α [13].

121

122 **A small population of CD8 α DCs produce IFN- λ in response to poly I:C treatment *in*** 123 ***vivo***

124 Production of IFN- λ by different cell types has been detected in response to a wide
125 variety of stimuli or pathogens *in vitro* [14-16]. Taken together, these publications
126 demonstrate that many cell types have the capacity to produce type III IFNs under the
127 appropriate conditions. However, reports investigating the cellular sources of IFN- λ in a
128 whole animal in response to TLR ligands or virus infections are few. One such study
129 identified splenic CD8 α + dendritic cells as the main producers of IFN- λ in mice that were
130 treated intravenously (i.v.) with the TLR3 agonist, poly I:C [17]. We carried out a similar
131 experiment using our IFN- λ reporter mouse model to determine whether we could confirm

132 that result by following GFP expression. Cohorts of *Ifn12^{gfp/gfp}* reporter mice were injected
133 i.v. with 100 µg of poly I:C or PBS, and their splenocytes harvested six hours later. Using
134 antibodies to the cell surface markers CD11b, CD11c, CD8α, B220, and Siglec H, we
135 identified pDC, CD8α DC, and cDC subsets, and found, as expected, that GFP
136 expression was mainly confined to the CD8α DC populations (Fig 2). Interestingly, IFN-
137 λ-producing (GFP+) CD8α DCs made up only 6% of the entire CD8α DC population; GFP
138 expression by pDCs and cDCs was minimal. This result is consistent with the observation
139 of Lauterbach *et al.* [17] that CD8α DCs are by the far the most significant contributor to
140 IFN-λ production in response to poly I:C, supporting the use of the *Ifn12^{gfp/gfp}* mouse strain
141 to determine the source of IFN-λ *in vivo*. No upregulation of GFP was found in animals
142 receiving PBS.

143

144 **Intestinal epithelial cells produce IFN-λ in response to rotavirus infection *in vivo***

145 As both type I and type III IFNs are produced in response to many different virus
146 infections *in vivo* [14] [7], we wished to investigate the cellular sources of type III IFNs
147 during virus infection of mucosal surfaces such as the gastrointestinal tract and the
148 respiratory tract. Rotavirus is a double-stranded RNA virus that primarily infects intestinal
149 epithelial cells and is one of the leading causes of severe diarrhea in young children in
150 the developing world [18]. Several studies have shown the importance of IFN-λ responses,
151 along with type I IFNs, in controlling rotavirus replication [19, 20]. Here, we explored the
152 *in vivo* source of IFN-λ during rhesus rotavirus (RRV) infection of suckling mice. The
153 kinetics of IFN induction by RRV was first determined using Mx2-Luciferase mice, a
154 reporter mouse strain that allows visualization and quantification of IFN responses by

155 measuring luciferase activity driven by the promoter of the interferon-stimulated gene Mx2
156 [21]. By this approach, a robust interferon response was detected as early as 12 hours
157 post-infection, peaking at 24 hours, and subsiding after 48 hours (Fig 3A&B). Consistent
158 with this observation, IFN- λ protein was detected in the small intestine of rotavirus-
159 infected WT 129SvEv mice 24 hours post-infection by ELISA (Fig 3C). To determine the
160 source of IFN- λ induced by RRV, intestines were harvested from infected IFN- λ reporter
161 mice 24 hours post-inoculation and analyzed by immunohistochemistry. Formalin-fixed,
162 paraffin-embedded tissue sections from mock-infected or RRV-infected IFN- λ reporter
163 mice were stained with antibodies against GFP and RRV antigens (Fig 3E). GFP+ cells
164 were present only in tissues harvested from RRV-infected IFN- λ reporter mice, and
165 absent from the intestines of mock-infected IFN- λ reporter mice, or RRV-infected WT
166 129SvEv animals. GFP expression in RRV-infected IFN- λ reporter mice was found
167 exclusively in the intestinal epithelial cells. RRV-infected epithelial cells were found in
168 both RRV-infected IFN- λ reporter mice and RRV-infected WT controls, confirming
169 successful infection of these animals. These results support the hypothesis that epithelial
170 cells are the primary source of IFN- λ during RRV infection. As IFN- λ reporter mice lack
171 the *ifn12* locus but have an intact *ifn13* locus, we compared RRV titers from the small
172 intestine of IFN- λ reporter and WT 129SvEv animals, and found no difference in the
173 amount of virus detected (Fig 3D). Thus the reporter mice are phenotypically normal,
174 without enhanced susceptibility to virus infection or elevated virus loads.

175

176

177

178 **Airway epithelial cells produce IFN- λ in response to NDV infection *in vivo***

179 Next, we sought to investigate IFN- λ production during virus infection of the respiratory
180 tract, another mucosal compartment that serves as a major portal for virus entry. Our lab
181 and others have reported that both type I and type III IFNs are produced in response to
182 respiratory virus infections [22, 23]. In the lung, as in the intestine, both IFN types appear
183 to be important for antiviral protection of the respiratory tract, as mice deficient in both
184 type I and type III IFN receptors demonstrate significantly elevated viral burdens upon
185 virus challenge [24, 25]. To determine whether the airway lining cells are major IFN- λ
186 producers during respiratory virus infection *in vivo*, we inoculated IFN- λ reporter mice with
187 Newcastle disease virus (NDV), a virus known to induce high levels of type I IFNs in
188 mammals [26, 27]. At 24 hours post-infection, lungs from mock-infected or NDV-infected
189 mice were harvested and processed to generate single cell suspensions. IFN- λ -producing,
190 epithelial cell adhesion molecule (EpCAM)+ epithelial cells were assayed for GFP
191 expression by flow cytometry. Only epithelial cells from NDV-infected reporter mice were
192 GFP+ (Fig 4A), with approximately 15% of lung epithelial cells in the IFN- λ reporter mice
193 expressing IFN- λ in response to infection *in vivo* (Fig. 4B). ELISA measurements of IFN-
194 λ protein present in bronchoalveolar lavage samples (BALs) from these same animals
195 also demonstrated robust IFN- λ secretion in response to NDV infection, with > 10 ng/ml
196 of IFN- λ protein detected (Fig 4C).

197 As with our rotavirus studies, we looked for GFP+ cells in tissue sections prepared from
198 IFN- λ reporter mice that were mock or NDV-infected. As before, lung tissue sections were
199 stained using antibodies against GFP and viral antigens (Fig 4D). This histological
200 analysis confirmed our flow cytometry results, showing that GFP expression was limited

201 to the columnar epithelial cells that line the bronchi and bronchioles of the lung. The same
202 cell type expressed both NDV and GFP protein in infected lungs. Interestingly, while rare
203 cells stained with antibodies to both GFP and NDV proteins, most cells in the infected
204 airway showed either GFP or NDV staining. To be certain that elimination of IFN- λ 2 in our
205 reporter mouse was not skewing our results, we compared total IFN- α and IFN- λ
206 production in BAL samples from NDV-infected WT and reporter mice and saw no
207 significant difference between the strains (Fig 5).

208

209 **Immune cells are not a significant source of IFN- λ during NDV infection *in vivo***

210 IFN- λ synthesis by dendritic cells and monocytes exposed to viruses and pattern
211 recognition receptor (PRR) agonists *in vitro* has been reported [7, 8, 11, 28]. So while
212 results from our flow cytometry and histology studies using the IFN- λ reporter mice clearly
213 demonstrated GFP expression from bronchial epithelial cells during NDV infection *in vivo*,
214 we wished to determine whether there was also a contribution from innate immune cells
215 in this model. To do this we used a panel of antibodies targeting cell surface markers to
216 assess IFN- λ production, or GFP expression, by neutrophils (CD45⁺ F4/80⁻ Ly6G⁺),
217 eosinophils (CD45⁺ F4/80⁺ SiglecF⁺ CD11c⁻), monocyte-derived macrophages (CD45⁺
218 F4/80⁺ SiglecF⁻ CD11c⁺) and alveolar macrophages (CD45⁺ F4/80⁺ Siglec F⁺ CD11c⁺)
219 purified from the lungs of NDV-infected IFN- λ reporter mice 24 hours post-infection. GFP
220 expression was minimal in all of these cell types (Fig 6), and therefore it is unlikely that
221 they are contributing significantly to IFN- λ production in this setting.

222 We were particularly intrigued by the lack of GFP expression in alveolar macrophages
223 from NDV-infected IFN- λ reporter mice. Previously published reports using an IFN- α

224 reporter mouse have identified alveolar macrophages as the major IFN- α -producing cell
225 population during NDV and RSV infections *in vivo* [5, 6]. The results of our experiment
226 demonstrate that alveolar macrophages do not contribute to IFN- λ production stimulated
227 by NDV infection *in vivo*, suggesting that alveolar macrophages preferentially upregulate
228 type I IFNs. To confirm this observation we carried out NDV infections in WT 129 SvEv
229 mice and looked for induced IFN- λ expression in both epithelial cells and alveolar
230 macrophages. Lungs from mock- or NDV-infected mice were harvested 24 hrs post-
231 infection, and epithelial cells (CD45⁻ EpCAM⁺) and alveolar macrophages (CD45⁺
232 F4/80⁺ SiglecF⁺ CD11c⁺) were sort purified by FACS. RNA from the sorted cell
233 populations was used to synthesize cDNA for qPCR analysis which showed that IFN- λ
234 transcripts were present only in the epithelial cell fraction isolated from NDV-infected mice
235 (Fig 7). IFN- λ transcripts were not detected in the alveolar macrophage fraction from
236 either mock-infected or NDV-infected mice, further confirming the readout from the IFN- λ
237 reporter mouse strain. As an alternative approach to assessing IFN- λ production induced
238 by virus, we used *in situ* hybridization to detect IFN- λ mRNA in lung sections from infected
239 animals (Fig 8). IFN- λ RNA was found in bronchial epithelial cells of NDV-infected mice
240 and not mock-infected animals. Taken together, the results from our qRT-PCR and *in situ*
241 hybridization studies lead to the conclusion that only epithelial cells, and not alveolar
242 macrophages, are responsible for IFN- λ production during NDV infection.

243 To ensure that our study produced results consistent with the work of Kumagai *et al.*
244 [5], we also assayed RNA purified from the sorted epithelial cell and alveolar macrophage
245 populations (Fig 8A) for the presence of IFN- α transcripts. As expected, induction of IFN-
246 α mRNAs were observed only in alveolar macrophages (Fig 8C&D).

247

248 **IFN production by alveolar macrophages *ex vivo***

249 Noting the inability of the alveolar macrophage population to synthesize type III IFNs
250 in the course of NDV infection, we wished to determine whether these cells were
251 intrinsically unable to produce IFN- λ in response to NDV, or whether extrinsic factors in
252 the lung microenvironment were restricting their production of IFN- λ . To ask this question,
253 alveolar macrophages were purified from the lungs of naïve 129 SvEv mice by
254 collagenase digestion and FACS sorting (Fig 9A) then mock-infected, or infected with
255 NDV at an MOI=10, for 24 hours. The cells were then collected for RNA extraction and
256 assayed by qPCR for the presence of IFN- λ transcripts. RNA isolated from a rodent cell
257 line constitutively expressing FLAG-tagged mIFN- λ 2, was used as a positive control. This
258 analysis failed to detect IFN- λ expression from either mock-infected or NDV-infected
259 alveolar macrophages (Fig 9B), a result confirmed by ELISA assay of media from the
260 infected alveolar macrophage cultures (Fig 9C). Assay of the same samples showed high
261 levels of IFN- α in media harvested from NDV-infected alveolar macrophages (Fig 9C).
262 These results demonstrate that the same stimulus results in the production of either type
263 I or type III IFN in a cell-type specific manner.

264

265 **Plasmacytoid dendritic cells, and not epithelial cells, are the major producers of** 266 **IFN- λ during influenza A virus (IAV) infections *in vivo***

267 Intranasal administration of the non-replicating NDV allowed us to examine type I and
268 type III IFN production following initial exposure, without regard to virus spread. However
269 we also wished to use the *Ifn12^{gfp/gfp}* reporter mouse strain to characterize IFN- λ induction

270 by influenza A virus (IAV), a virus capable of robust replication and spread in this species.
271 Published studies have reported co-production of type I and type III IFNs from both pDCs
272 [11] and epithelial cells [23, 29] exposed to IAV *ex vivo*, and we wished to characterize
273 the *in vivo* source(s) of these cytokines. Lung homogenates from *Ifnl2^{gfp/gfp}* mice infected
274 with the WSN strain of IAV (10⁶ pfu) were prepared 48 hours post infection, and assayed
275 by flow cytometry for GFP expression. By this method, measurable GFP expression was
276 detected only in the pDC subset (CD45+/CD11c+/CD11b-/B220+/PDCA1+) and not in
277 epithelial cells (Fig 10A-C).

278 Given the data obtained with NDV, and a recent study which concluded that epithelial
279 cells are the primary source of IFN- λ in IAV infection [30], we considered the possibility
280 that the more numerous epithelial cells might produce lower levels of IFN- λ on a per cell
281 basis, below the level of detection by GFP expression, but still contribute to overall
282 production of this cytokine. We approached this question in two ways. Using the sorting
283 strategy shown in Figure 10 B & C, epithelial cells and pDCs from lungs of IAV-infected
284 WT mice and controls were obtained, and RNA harvested from these populations was
285 assayed by qRT-PCR for the presence of *Ifnl2/3* transcripts. As shown in Figure 10D,
286 while IFN- λ 2/3 mRNA could be detected in EpCAM+ cells from infected animals, pDCs
287 appeared to be the predominant source of both IFN- α and IFN- λ . This result was
288 consistent with ISH hybridization studies using formalin-fixed lung tissues from IAV-
289 infected WT and reporter mice. Immunofluorescence studies showed diffuse positive
290 staining for influenza antigen in airway lining epithelium, and oligonucleotide probes did
291 detect rare IFN- λ 2/3 expressing epithelial cells (Fig 10E) in animals infected with the WSN
292 strain of IAV. A similar GFP expression pattern in pDCs and epithelial cells was observed

293 by flow cytometry in animals infected with the PR8 strain, in the presence or absence of
294 the NS1 gene (Fig 11) 48 hrs post-infection, but we were not able to detect *ifnl* transcripts
295 by ISH in PR8-infected epithelial cells (data not shown).

296 To determine the relative contribution of these sources, we generated marrow chimeras
297 using bone marrow harvested from newly generated IFN- λ ligand deficient mice, *ifnl*^{-/-},
298 lacking both the *ifnl2* and *ifnl3* alleles (See Supplemental Fig. 2). Lethally irradiated WT
299 mice were reconstituted with bone marrow harvested from either WT or *ifnl*^{-/-} animals.
300 Chimeric mice were then challenged with IAV, 10⁵ pfu of WSN, and BALs collected 48
301 hours post infection were assayed for the presence of IFN- λ by ELISA. IAV-infected mice
302 reconstituted with *ifnl*^{-/-} bone marrow showed significantly reduced levels of IFN- λ protein
303 compared with IAV-infected animals which received WT bone marrow (Fig 12)
304 demonstrating that ~ 30%, of IFN- λ produced in response to IAV is derived from the
305 epithelial compartment. To further pinpoint the source of this cytokine, WT mice were
306 depleted of pDCs prior to IAV infection with anti-PDCA1 antibody, or a Rat IgG Isotype
307 control (Fig 13A). Post infection measurements of BAL IFN- λ show a significant decrease
308 in IFN- λ production in the absence of pDCs at 24 hrs post-infection, ~ 60% (Fig 13B),
309 consistent with the data obtained by generation of bone marrow chimeras.

310

311

312 **Pathway activation by virus**

313 As IFN- λ induction by NDV occurred only in CD45⁻ EpCAM⁺ cells, and IFN- α induction
314 only in alveolar macrophages in NDV infection, we hypothesized that production of either
315 cytokine would depend on cytoplasmic PRRs. Many single stranded RNA viruses,

316 including NDV and influenza [31], have been found to activate retinoic acid inducible
317 gene-I (RIG-I). RIG-I bound to viral RNA interacts with the adaptor protein called
318 mitochondrial antiviral-signaling protein (MAVS), triggering the events leading to IFN
319 synthesis. To determine the role of MAVS activation in the production of each IFN type,
320 we made use of MAVS^{-/-} mice, available on the C57BL/6 background. WT and MAVS
321 deficient animals were inoculated with NDV, and both IFN- α and IFN- λ levels were
322 determined by ELISA of BALs collected 24 hours post-infection. As expected, there was
323 no induction of either cytokine in the absence of MAVS, demonstrating that the events
324 triggered by NDV recognition are dependent on cytoplasmic pattern recognition pathways
325 in both cell types (Fig 14).

326

327 Since our studies of IFN- λ induction by IAV suggested that pDCs are the predominant
328 type III IFN producing cell population *in vivo*, we wished to determine which signaling
329 pathways were triggered by this infection. Several reports have previously shown that
330 pDC recognition of the influenza genomic ssRNA by the TLR7 endosomal pathway that
331 signals through the protein myeloid differentiation primary response 88 protein (MyD88)
332 [32-34] results in robust type I IFN expression. Based on our finding that pDCs are also
333 the major source of type III IFNs in response to IAV infection, we suspected that this
334 pathway was also required for optimal IFN- λ production. To test this assumption, MAVS-
335 ^{-/-} mice on the C57BL6 background were infected with the WSN and PR8 strains of IAV,
336 and IFN- λ protein levels were measured in BALs. We saw no reduction in IFN- λ
337 expression following PR8 infection of MAVS^{-/-} mice at 24, 48 and 72 hours post infection
338 (Fig 15A), but a ~ 40% reduction following WSN infection at 48 hours post infection,

339 consistent with data from our pDC depletion study. Conversely, in the absence of MyD88
340 (Fig 15B), a more significant reduction in IFN- λ was seen in the BALs of WSN-infected
341 mice. Taken together, our data show that while both pathways can contribute to IFN- λ
342 production following IAV infection, the major contribution is pDC derived, particularly for
343 the PR8 strain of IAV.

344

345 We further explored this IAV strain dependence *ex vivo* using WT murine tracheal
346 epithelial cells (mTEC) cultured at an air-liquid interface on transwell filters. IFN- λ protein
347 levels in culture supernatants from virus infected mTECs showed robust production by
348 NDV or WSN infected cultures, but no detectable IFN- λ following PR8 infection (Fig 16).
349 This result is consistent with our inability to detect epithelial IFN- λ production following
350 PR8 infection *in vivo*, and the absence of a requirement for functional MAVS protein.

351

352 **Plasmacytoid DC production of IFN- λ requires type I IFN signaling**

353 We and others have observed no significant difference in the resistance of wild type
354 and IFNAR^{-/-} mice to IAV infection [22, 35]. In the absence of the type I IFN pathway,
355 IFN- λ production is sufficient to inhibit virus replication and spread. Assuming that pDCs
356 were a major source of this cytokine, GFP expression was assayed in pDCs isolated from
357 the lungs of PR8-infected *Ifnl2^{gfp/gfp}* mice on WT or IFNAR^{-/-} 129SvEv strain backgrounds.
358 In this experiment, pDCs from IAV-infected *Ifnl2^{gfp/gfp}* reporter mice with intact type I IFN
359 signaling expressed GFP, but strongly reduced expression was detected in pDCs from
360 IFNAR deficient *Ifnl2^{gfp/gfp}* animals (Fig 17A). *In vitro* WSN infection of FLT3L cultured
361 pDCs derived from bone marrow of wild type or IFNAR^{-/-} mice produced the same result,

362 with ELISA-detectable amounts of IFN- λ protein found only in medium from IAV-infected
363 IFNAR^{+/+} pDC cultures.

364 Plasmacytoid DCs are unique for their constitutive expression of interferon regulatory
365 factor 7 (IRF7), a transcription factor essential for the induction of IFN- α genes [36]. IRF7
366 has also been implicated as a driver for IFN- λ transcriptional activation [37] [38]. Given
367 the importance of IRF7 in the induction of IFN genes, we looked to see whether IRF7
368 levels were altered in the absence of IFNAR. As shown in Figure 17C, qPCR analysis
369 demonstrated a substantial reduction in basal levels of IRF7 mRNA in IFNAR^{-/-} pDCs.
370 Since IRF7 is itself an ISG, we conclude that pDCs require some level of tonic activation
371 of the type I IFN signaling pathway to maintain sufficient IRF7 for type III IFN induction in
372 response to IAV infection. Interestingly, there was no decrease in GFP⁺ epithelial cells in
373 NDV-infected IFNAR^{-/-} IFN- λ reporter mice (Fig 18), supporting our conclusion that type
374 I IFN-dependent maintenance of IRF7 levels in pDCs is essential for optimal type III IFN
375 induction by IAV.

376

377 **Discussion**

378 Since the discovery of type III IFNs in 2003 [3] [4] and the demonstration that identical
379 sets of ISGs are induced by type I and type III IFNs in sensitive cells [39, 40], many groups
380 have sought to determine whether these cytokines play a unique role in antiviral immunity.
381 It has been found that type I and type III IFN receptors are differentially expressed by
382 different cell types. While most immune cells in mouse and man are IFN- α responsive,
383 only human pDCs, neutrophils and B cells have been found to consistently respond to
384 IFN- λ [11, 30, 41, 42]. Until recently it was thought that all epithelial cells respond to both

385 IFN- α/β and IFN- λ , but recent *in vivo* studies have demonstrated that intestinal epithelial
386 cells become IFN- α insensitive soon after birth, and respond only to IFN- λ *in situ* [19].
387 These observations point to distinct roles for type I and type III IFNs in innate immune
388 responses, determined primarily by the responsiveness and sensitivity of specific cell
389 types to IFN- α/β and IFN- λ , but also by the availability of their ligands.

390 Our goal in generating an IFN- λ reporter mouse was to investigate the source of this
391 cytokine as a viral infection of an epithelial surface progressed. This was done by
392 replacing the *IFNL2* coding sequence with *eGFP*, a reporter gene whose expression is
393 then regulated by the *IFNL2* promoter, UTR regions and other elements surrounding the
394 *IFNL2* gene. Our first aim was to determine the extent to which GFP expression correlated
395 with IFN- λ production by various cell types. *In vitro* virus infection of both epithelial and
396 dendritic cells derived from the reporter mice induced GFP expression, and only GFP+
397 cells were found to express IFN- λ transcripts. Equally important were studies to confirm
398 GFP and IFN- λ co-expression *in vivo*. Lauterbach *et al.* [17] had previously demonstrated
399 that splenic CD8 α DCs were the major producers of IFN- λ following systemic treatment
400 with poly(I:C), a TLR3 ligand, and we established that GFP expression in the IFN- λ
401 reporter mouse was limited to that cell type (Fig 3) following i.v. administration of poly(I:C).
402 We next asked whether GFP expression by the *Ifnl2^{gfp/gfp}* reporter mouse would
403 recapitulate the pattern of IFN- λ expression previously described for heterologous
404 rotavirus infection. Hernandez *et al.* [43] reported that IFN- λ transcripts were present only
405 in CD45- EPCAM+ intestinal epithelial cells, but not expressed by cells isolated from
406 lamina propria during RV infection. We repeated this study in WT and IFN- λ reporter
407 suckling mice, harvesting tissue at the time point corresponding to maximal IFN

408 responsiveness post-infection. Elevated, and equivalent, levels of IFN- λ were detected in
409 intestinal homogenates from both strains following infection, with GFP expression
410 detectable only in the epithelial compartment (Fig 6). We concluded from these results
411 that GFP expression is an accurate indicator of IFN- λ expression in this reporter mouse
412 strain and, importantly, that IFN- λ expression levels are equivalent in WT and *Ifnl2^{gfp/gfp}*
413 mice where production of IFN- λ 3 protein compensates for the lack of the functional *IFNL2*
414 gene.

415 The mucosal surfaces of the gastrointestinal and respiratory tracts are major portals of
416 virus entry, and infection of these organs induces the secretion of both type I and type
417 III IFNs [19, 22, 25, 43]. While both IFN types contribute to an effective antiviral
418 response, receptor distribution suggests that type III IFNs have a primary role in
419 protecting epithelial cells and that IFN- λ production is therefore crucial to an effective
420 antiviral response at these barrier surfaces. While it is established that epithelial cells
421 are the major IFN- λ producers during RNA virus infection of intestinal epithelial cells [10,
422 19], we wished to determine whether this was also a feature of respiratory virus
423 infection. Using an IFN- α reporter mouse, Kamagai *et al.* [5] had demonstrated that IFN-
424 α production following NDV infection was limited to alveolar macrophages. This was a
425 surprising and important finding as it had been widely understood that essentially all cell
426 types produced type I IFNs in response to NDV infection *in vitro*. As many respiratory
427 virus infections result in the production of both type I IFNs and type III IFNs, we wished
428 to determine whether alveolar macrophages were the source of both cytokines, or
429 whether these cytokines were produced by different cell types in response to the same
430 viral pathogen.

431 NDV infection of the IFN- λ reporter mouse resulted in GFP expression only by
432 EPCAM+ epithelial cells as measured by flow cytometry and immunohistochemistry,
433 and *in situ* hybridization studies detected IFN- λ transcripts only in bronchial epithelial
434 cells of NDV-infected animals. This result is consistent with other published studies that
435 have described IFN- λ production by mouse and human airway epithelial cells in
436 response to respiratory viruses *in vivo* [23, 44-47]. Like Kumagai *et al.*, we observed
437 IFN- α mRNA induction in alveolar macrophages isolated from the lungs of NDV-infected
438 animals, but no IFN- λ transcripts were detected in these cells. Therefore, NDV
439 simultaneously induces both type I and type III IFNs upon respiratory infection, but each
440 IFN type is produced by a different cell type.

441 Cellular recognition of RNA viruses generally occurs through recognition of viral
442 genomes or viral transcripts by TLRs and RIG-I-like receptors, which signal through
443 MyD88 and MAVS, respectively, to induce the expression of IFNs. TLRs are generally
444 expressed by hematopoietic cells and RIG-I-like receptors by epithelial cells, although
445 TLR3 expression at the apical surface of polarized murine and human tracheal epithelial
446 cell cultures has been demonstrated [29]. Our results in MAVS^{-/-} mice indicate that
447 both IFN- α and IFN- λ production following NDV infection requires MAVS activation, but
448 we do not yet understand how activation of this pathway by the same virus leads to
449 production of one or the other IFN type in a cell type dependent manner. The most likely
450 explanation is suggested by work showing that MAVS is located on the surface of
451 mitochondria and peroxisomes [48], and further, that signaling via MAVS on
452 peroxisomes induces type III, but not type I IFN production [9]. Preferential IFN- λ
453 synthesis following MAVS activation was found to depend upon the relative abundance

454 of peroxisomes in a given cell type, and polarization of intestinal epithelial cells resulted
455 in increased numbers of these organelles. While these data predict a shift in the relative
456 levels of type I and type III IFN production by different cell types, the complete absence
457 of IFN- λ synthesis by alveolar macrophages in infected animals was unexpected.
458 Further investigation is required to understand this compartmentalization of IFN
459 production that we observe *in vivo*.

460 As NDV infection is abortive in mammalian cells [49], it was of interest to repeat these
461 studies using IAV, which replicates and causes disease in the murine host. Based on
462 our NDV data, and published reports which suggested that infected epithelial cells were
463 the primary source of type III IFNs in IAV infection [30, 45], we expected our influenza
464 studies to confirm this conclusion, but this was not the case. By all approaches taken it
465 appeared that, for IAV infection, the bulk of IFN- λ was produced by pDCs rather than
466 the respiratory epithelium. When pDC-depleted mice were infected with IAV, there was
467 a 60% reduction in BAL levels of both type I and type III IFNs. Infection of bone marrow
468 chimeras generated by the transfer of *Ifnl*^{-/-} bone marrow into WT mice showed a
469 similar decrease in type III IFN induction by IAV. In our study, ~ 40% of the IFN- λ
470 induced by IAV was from non-pDC sources, but this was dependent on virus strain.
471 While GFP⁺ pDCs were detected using either the PR8 or WSN strains of IAV, only in
472 WSN infections was epithelial IFN- λ synthesis detected *in vivo* or *ex vivo*. The
473 conclusion that type III IFN synthesis induced by PR8 came primarily from pDCs was
474 further supported by studies in MAVS^{-/-} animals which showed no impact on IFN- λ
475 levels in the absence of this pathway in PR8 infection.

476 Also of interest was our finding that IFN- λ production by IAV-exposed pDCs is type I
477 IFN dependent. Our data suggest that the tonic signaling through the type I IFN receptor
478 may be required to maintain the elevated basal levels of IRF7 which is a hallmark of this
479 cell type [50]. While this result was unexpected, it is consistent with the observation that
480 human subjects with a deficiency of IRF7 expression are more likely to experience life-
481 threatening infections of influenza [51] as well as SARS-CoV-2 [52]. As both type I and
482 type III IFNs are known to play a role in protection from respiratory viruses, this
483 observation is further support of the hypothesis that pDCs are the major source of both
484 of these cytokines during IAV infection. The demonstration that both IFN- α and IFN- λ
485 are produced by IAV-exposed human pDCs [11] also supports this possibility.

486 In summary, we have used an IFN- λ reporter mouse model to determine the source of
487 type III IFNs in two mouse models of respiratory virus infection. For NDV, type I and
488 type III IFNs are simultaneously induced through the engagement of the same virus
489 sensing pathway, but from two distinct cell types with respiratory epithelium as the sole
490 source of IFN- λ . In IAV infection, pDCs appear to produce the bulk of type III IFN, and
491 its production by this cell type requires type I IFN signaling. This study suggests that
492 IFN induction by viruses *in vivo* is pathogen specific, as is the source of these cytokines.

493

494 **Methods**

495 **Generation of the IFN- λ Reporter Mouse**

496 Generation of the IFN- λ reporter mouse was carried out by ingenious Targeting
497 Laboratory (www.genetargeting.com). The targeting vector was constructed using an
498 11 kb region of a C57BL/6 BAC clone (RP23: 24B20), containing the *Irf1* locus, sub-
499 cloned into the pSP72 backbone vector (Promega). A GFP/FRT-flanked neomycin

500 cassette was inserted into the BAC subclone using Red/ET recombineering technology,
501 resulting in the complete replacement of the *IFNλ2* coding region of exons 1-5 (including
502 intron sequences), with a long homology arm extending approximately 6.91 kb 5' to the
503 site of the cassette insertion and a short homology arm that extends approximately 2.68
504 kb 3' to the site of the cassette insertion. The GFP-Neomycin targeting vector was
505 linearized by Not I enzymatic digestion and electroporated into BA1 (C57B/6 x
506 129SvEv) hybrid embryonic stem cells. G418 resistant ES cell clones expanded for
507 PCR analysis to identify homologous recombinants. Clones found to carry the GFP
508 transgene were sequenced to confirm insertion, sequence fidelity, the genome/5'
509 cassette junction and the genome/3' cassette junction. Southern blot analysis of
510 positive clones was carried out using Apal digestion of PCR products hybridized with a
511 probe targeted against the 5'external region. Targeted BA1 hybrid embryonic stem cells
512 were microinjected into C57BL/6 blastocysts and resulting chimeras with a high
513 percentage agouti coat color were mated to C57BL/6 FLP mice to remove the neomycin
514 cassette. Tail DNA from pups with agouti or black coat color was analyzed by PCR to
515 confirm presence of transgene. Animals heterozygous for the *Ifnl2^{+gfp}* allele
516 were backcrossed onto the 129SvEv background for 10 generations, then bred to
517 produce homozygous animals. Genotyping was carried out by two PCR reactions: One
518 reaction using Primer A (5'-CAGAGCTGGAAACTCAGAGCC-3') and Primer B (5'-
519 GACCGAGTCTGAGACCCACAAG-3') and another reaction using Primer C (5'-
520 CAGAGCTGGAAACTCAGAGCC-3') and Primer B. Thermocycler conditions for those
521 reactions were as follows: 95° C for 15 minutes, followed by 35 cycles of (94° C for 45
522 minutes, 65° C for 1 minute, and 72° C for 1.5 minutes), with an end step of 72° C for 5

523 minutes. The amplicon generated by Primers A and B, which encompasses the *Ifn12* or
524 *gfp* region, was then digested with the NcoI restriction endonuclease. NcoI treatment of
525 the wildtype allele yields a 1100 bp band and a 700 bp band, while digestion of the
526 amplicon containing the *Ifn12^{+gfp}* allele, which lacks NcoI restriction sites, yields a single
527 1110 bp band.

528

529 **Additional Mice**

530 Mice on the 129SvEv background lacking the type I IFN receptor (IFNAR^{-/-}) were
531 originally derived by Michel Aguet [53] and strain matched controls were purchased
532 from Taconic Farms. MAVS^{-/-} and MYD88^{-/-} mice on the C57BL/6 background were
533 obtained from Jackson Labs. Mx2-Luc Mx2 luciferase reporter mice were obtained from
534 Hansjörg Hauser and Mario Köster [21]. Generation of the IFN- λ ^{-/-} mice, lacking both
535 the IFN- λ 2 and - λ 3, sequences is described in supplemental figure 2. All mice were
536 maintained under specific pathogen-free conditions in the vivarium of Rutgers-New
537 Jersey Medical School.

538

539 **Cell culture**

540 Bone-marrow derived FLT3-ligand dendritic cell cultures were generated from the
541 bone marrow of IFN- λ reporter mice, *ifn1*^{-/-} mice and WT controls. Bone marrow from
542 femurs and tibia of 6-10 week old mice was washed, depleted of red cells with ACK
543 lysis buffer, and cultured in RPMI+10% FBS containing 100 ng/mL human FLT3-ligand
544 (PeproTech), 50 μ M β -mercaptoethanol (Sigma), penicillin (100 IU/ml.) and streptomycin
545 (100 μ g/ml) at a cell density of 3×10^6 cells/ml for 7 – 8 days.

546

547 For preparation of kidney epithelial cells, kidney capsules were removed, renal
548 parenchyma was minced into 1 mm³ pieces and digested with collagenase IV
549 (Worthington) at 37°C for 30 min. Following red blood cell lysis, digested tissue was
550 pressed through a 100 µM, then a 40 µM cell strainer, and the recovered cells were
551 washed and then plated in DMEM/HamsF12 with 10% FBS. After 1-2 hours of
552 incubation, non-adherent cells were collected and plated on collagen-coated dishes.
553 Cells were allowed to reach 70% confluence prior to virus infection.

554

555 Murine tracheal epithelial cells were derived from WT or IFN-λ reporter mice at 6 to 10
556 weeks of age following the procedures outlined by You et al. [54].

557

558 **Poly I:C Treatment and Splenic DC Analysis**

559 6-10 week old IFN-λ reporter mice were injected intravenously with sterile PBS or 100
560 µg polyI:C (InVivoGen). Splenocytes were harvested 6 hours after treatment, incubated
561 with anti-mouse CD32/Fc-Block (BD Pharmingen) for 10 minutes at 4°C, then stained
562 with the following antibodies: anti-mouse CD45-BUV395 (BD Horizon), anti-mouse
563 CD3-APC Cy7 (BioLegend), anti-mouse CD11c-APC (eBioscience), anti-mouse CD11b-
564 PerCP Cy5.5 (ebioscience), anti-mouse CD8α-V450 (BD Horizon), and anti-mouse
565 Siglec-H-PE (eBioscience) antibodies. Analysis was carried out using the LSRII (BD
566 Biosciences) flow cytometer.

567

568

569

570 **Virus infection and assay**

571 Six-day-old suckling mice were infected with 4×10^6 PFU of the simian RRV strain by
572 oral gavage. Intestines were harvested 24 hours post-infection. These were either
573 formalin fixed or homogenized in medium for virus quantitation. RRV quantitation was
574 done using a focus forming assay as previously described [19].

575 Six-10 week old mice, under isoflurane anesthesia, were infected intranasally with 10^7
576 PFU of NDV (Hitchner B1 strain) in 50 μ l of PBS. Mice were euthanized 24 hours post-
577 infection for collection of bronchoalveolar lavage fluid and lung tissue.

578 *In vitro* infection of primary kidney epithelial cells was carried out at a multiplicity of
579 infection (MOI) of 2 and 20. Infections were done with RRV, the Hitchner B1 strain of
580 NDV, the A2 strain of respiratory syncytial virus (RSV), and the WSN strain of influenza
581 A virus (IAV).

582 A/PR8/34 and A/WSN/33 influenza viruses were grown in embryonated chicken eggs.
583 Allantoic fluid was assayed for infectious viral particles by focus forming assay on MDCK
584 cells. Serial dilutions of allantoic fluid were prepared and incubated on MDCK cells at
585 37°C, in 5% CO₂ for 24 hours. Cells were then fixed in phosphate-buffered formalin, and
586 viral plaques were visualized by incubating fixed cells with polyclonal rabbit anti-IAV
587 serum (US Biologicals), followed by HRP-conjugated secondary antibodies for
588 visualization of plaques via a colorimetric assay.

589

590

591

592 **Ethics statement**

593 All mouse studies were approved by the Institutional Animal Care and Use
594 Committees of Rutgers-New Jersey Medical School (protocols 999900760 and
595 13009C0316), performed in compliance with relevant institutional policies, local, state,
596 and federal laws, and conducted following National Research Council Guide for the
597 Care and Use of Laboratory Animals, Eighth Edition.

598

599 ***In vivo* luciferase detection**

600 Six-day old Mx2-Luc mice were injected intraperitoneally with D-Luciferin (15 µg per
601 pup), (Perkin Elmer), anesthetized with isoflurane, and imaged using the IVIS-200 Vivo
602 Vision system (Caliper). Gray-scale images followed by bioluminescent images were
603 then acquired and superimposed using the Living Image software (Caliper).
604 Bioluminescent images represent luciferase signal intensity, with red as highest and
605 blue as lowest, respectively. Regions of interest with detectable luciferase activity were
606 measured quantitatively as relative light units (RLUs) at 3, 6, 12, 24, 48, 72, and 96
607 hours post-RRV infection.

608

609 **Immunostaining**

610 Harvested tissues were fixed in 10% neutral buffered formalin, and submitted for
611 processing and paraffin embedding. Deparaffinized sections underwent antigen retrieval
612 and were subsequently blocked with Superblock (ScyTek) for 5 minutes at room
613 temperature, washed in wash buffer (PBS+0.05% Tween) and then stained using anti-
614 NDV antibodies (US Biological), anti-RRV antibodies (Meridian), anti-eGFP (Life

615 Technologies) antibodies or anti-IAV antibodies (US Biological) overnight at 4°C. The
616 following day, slides were washed in wash buffer and stained using anti-DyLight488
617 (Abcam) or anti-DyLight564 (Abcam) secondary antibodies for 1 hour at room
618 temperature in the dark, washed in wash buffer, and incubated with DAPI for 6 minutes.
619 After washing in distilled water, mounting medium (Vectashield) was applied, and slides
620 were coverslipped. Images were obtained using an Axiovert 200M inverted
621 fluorescence microscope (Zeiss) using the AxioVision LE64 software (Zeiss).

622

623 **Lung digestion and flow cytometry analysis**

624 Perfused lungs were finely minced and digested in 5 mL of a 1.78mg/mL Collagenase
625 IV (Worthington) solution in PBS (with Ca²⁺, Mg²⁺) for 45 minutes at 37°C. Lung digests
626 were further dissociated by passage through a 20G needle, then filtration through a 100
627 µm filter. ACK lysis buffer was used to remove red blood cells, and remaining cell
628 suspension was washed twice with PBS. Lung cell suspensions were first blocked with
629 anti-CD32/Fc-Block (BD Pharmingen) for 10 minutes at 4°C, then stained with cocktail
630 of primary antibodies including: anti-mouse CD45-BUV395 (BD Horizon), anti-mouse
631 EPCAM-PECy7 (BioLegend), anti-mouse Ly6G-Alexa Fluor 700 (BioLegend), anti-
632 mouse F4/80-APC (BioLegend), anti-mouse CD11c-PerCPCy5.5 (BioLegend), anti-
633 mouse CD11b-APC-Cy7 (BioLegend), and anti-mouse Siglec F-PE (BD Pharmingen).
634 Cells were then washed in FACS buffer (PBS+2% FBS and sodium azide),
635 resuspended in FACS Buffer containing DAPI, and analyzed using an LSRII flow
636 cytometer (BD Biosciences). Sorting of stained cell populations was carried out on an
637 Aria II Cell sorter (BD Biosciences).

638

639 **Plasmacytoid DC depletion**

640 Mice were injected i.p. with 500 µg of anti-PDCA1 or Rat IgG 24 and 48 hours before
641 IAV infection. Mice were sacrificed 24 hours post-infection to collect BALS, and blood
642 and spleens were collected to stain for pDC surface marker (CD11c+, CD11b-, B220+,
643 PDCA1+).

644

645 ***In situ* hybridization**

646 *In situ* detection of IFN-λ transcripts was carried out using the RNA Scope® HD 2.5
647 Detection Reagent-Red kit (Advanced Cell Technologies) according to manufacturer's
648 instructions. Briefly, formalin-fixed, paraffin-embedded tissue sections were heated at
649 60°C for 1 hour, deparaffinized in xylene and 100% alcohol, and incubated with
650 hydrogen peroxide for 10 minutes at room temperature. Antigen retrieval was then
651 carried out for 1 hour in RNA Scope antigen retrieval buffer, and slides were washed in
652 distilled water and 100% ethanol, and then left to dry overnight. The following day,
653 slides were washed in RNA Scope proprietary wash buffer and incubated in protease-
654 plus buffer, washed again, and then incubated with mouse IFNλ2/3 DNA probes
655 (Advanced Cell Technologies), followed by a series of RNA Scope adaptor probes,
656 streptavidin-HRP, and chromogen solution to visualize IFN-λ transcripts. Slides were
657 counterstained with 50% Lillie Mayer's hematoxylin (American Master Tech Scientific
658 Inc).

659

660

661 **RNA isolation and qRT-PCR**

662 RNA from cells was obtained using the RNA Easy Kit (Qiagen) incorporating the
663 DNase I step to remove contaminating genomic DNA. cDNA was synthesized using
664 iScript cDNA Synthesis kit (Biorad) using 10 ng RNA, with 10% of the cDNA product
665 used for qPCR analysis. The following primer pairs were used to carry out qPCR
666 reactions in a CFX96 Real Time System (BioRad) using SYBR Green (BioRad):

667 IFN λ 2/3 Forward 5'-TCAAGC ACCTCTTCTCGATGG-3'

668 IFN λ 2/3 Reverse 5'-AGCTGCAGGCCTTCAAAAAG-3'

669 IFN α Forward 5'-TCTGATGCAGCAGGTGGG-3'

670 IFN α Reverse 5'-AGGGCTCTCCAGACTTCTGCTCTG-3'

671 18s rRNA Forward 5'-GTAACCCGTTGAACCCCAT-3'

672 18s rRNA Reverse 5'-CCATCCAATCGGTAGTAGCG-3'

673 eGFP Forward 5'-GACGTAAACGGCCACAAGTT-3'

674 eGFP Reverse 5'-ATG CCGTTCTTCTGCTTGT-3'

675 Thermocycler conditions were set with the following parameters for amplification of
676 IFN λ 2/3 transcripts: 95°C for 3 minutes, followed by 40 cycles of 95°C for 10 seconds
677 and a gene-specific annealing temperature for 30 seconds. Gene-specific annealing
678 temperatures for IFN λ 2/3, IFN α , eGFP were 58.4°C, 61.3°C, and 58.4°C, respectively.
679 18s rRNA qPCR reactions using the same annealing temperature as the target gene of
680 interest were carried out simultaneously. Relative normalized expression was
681 determined by normalizing amplification of IFN- λ , IFN- α , or eGFP expression to 18s
682 rRNA levels by the $\Delta\Delta$ Ct method [PMID 11846609].

683

684 **Data analysis**

685 Data were analyzed using GraphPad Prism version 7 (graphPad Software). Mean and
686 SEM were calculated by the same.

687
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- 925

926 **Figure Legends**

927 **Figure 1. Generating the IFN- λ Reporter Mouse.**

928 (A) The targeting vector carrying *eGFP* and *Neo* genes was inserted into the *Ifnl2*
929 locus by homologous recombination, resulting in complete replacement of the *Ifnl2*
930 coding region with the *eGfp* and *Neo* genes. Excision of the *Neo* gene was carried out
931 by crossing mice carrying the targeted allele with Flipase mice, resulting in deletion of
932 the *Neo* gene and generation of the *Ifnl2^{+/gfp}* allele. (B) PCR products generated with
933 primers shown in (A), and digested with *Nco1*, yielded unique banding patterns for tail
934 DNA obtained from WT 129 SvEv mice (+/+), heterozygous *Ifnl2^{+/gfp}* (+/-), and
935 homozygous *Ifnl2^{gfp/gfp}* IFN- λ reporter animals. (C) Pictured are phase contrast and
936 GFP immunofluorescence images (200x) of uninfected or NDV-infected, FLT3L
937 cultured, bone marrow derived DCs from *Ifnl2^{gfp/gfp}* mice. Images are representative of
938 three independent experiments. (D) NDV-infected FLT3L cultured, bone marrow derived
939 DCs were cell sorted into GFP+ and GFP- fractions that were then assayed by qRT-
940 PCR for the presence of GFP and IFN- λ 3 transcripts. Data are representative of two
941 independent experiments. (E) Flow cytometry dot plots show GFP expression in
942 cultures of primary kidney epithelial cells (gated as EPCAM+ cells) derived from
943 *Ifnl2^{gfp/gfp}* mice that were mock-infected or infected with Newcastle disease virus (NDV),
944 rhesus rotavirus (RRV), respiratory syncytial virus (RSV), or influenza A virus (IAV) at
945 the indicated MOIs for 24 hrs. Data are representative of two independent experiments.
946
947

948 **Figure 2. CD8 α DCs are the predominant IFN- λ -producing cells in spleens of mice**
949 **treated intravenously with poly I:C.**

950 (A) Flow cytometry analysis of splenic populations from IFN- λ reporter mice mock-
951 treated (left) or i.v. treated with poly I:C (right) for 6 hrs. Cells were gated on CD11c
952 while simultaneously excluding CD3⁺ cells to exclude T cells. Dendritic cell populations
953 were then identified as follows: pDCs (CD11c⁺/Siglec-H⁺/CD11b⁻); CD8 α DCs (Siglec-
954 H⁻/CD11b⁻/CD8 α ⁺); cDCs (Siglec-H⁻/CD11b⁺/CD11c⁺). (B) Mean percentages, with
955 SEM, of GFP⁺ cells in populations shown in (A). Data are representative of two
956 independent experiments.

957

958 **Figure 3. Epithelial cells are the predominant IFN- λ -producing cell population in**
959 **the small intestine in response to RRV.**

960 (A) Interferon responses of 6-day old, RRV-infected Mx2-Luciferase transgenic mice
961 were quantitated by IVIS at the indicated timepoints. (B) Graphical representation of
962 mean luciferase activity values and SEM from two independent experiments. (C) ELISA
963 measurements of IFN- λ protein in homogenates of intestinal tissue harvested from 6-
964 day old WT C57BL/6 pups, 24 hours after RRV infection. (D) Comparison of RRV viral
965 titers, measured by fluorescent-focus assay, in the small intestine of 6-day old WT 129
966 SvEV mice and IFN- λ reporter mice 3 days post-RRV infection. Data are pooled from
967 two independent experiments. (E) Images of formalin-fixed paraffin-embedded (FFPE)
968 small intestinal tissue sections from mock- or RRV-infected IFN- λ reporter pups, and
969 RRV-infected WT pups, were captured 24 hours post-infection. Sections were probed

970 with anti-GFP antibodies (green), anti-RRV sera (red) and DAPI (blue) and visualized by
971 fluorescence microscopy. Images are representative of three independent experiments.

972

973 **Figure 4. Epithelial cells are the predominant IFN- λ producers in response to NDV**
974 **infection *in vivo*.**

975 (A) GFP expression was assayed by flow cytometry of cells obtained from collagenase-
976 digested lungs of IFN- λ reporter mice which were mock-infected or infected intranasally
977 with NDV for 24 hrs. Epithelial cells were defined as CD45⁻, EPCAM⁺. WT 129SvEV
978 mice were included as controls for GFP expression, gating in the same manner. (B)
979 Graphical representation of mean percentage of GFP⁺ epithelial cells from each
980 treatment cohort. Error bars represent SEM. (C) IFN- λ protein levels in BALs harvested
981 at 24 hours from mock-infected or NDV-infected reporter mice were determined by
982 ELISA. (D) Lung sections from uninfected IFN- λ reporter mice, and NDV-infected WT
983 and NDV-infected IFN- λ reporter mice were stained with anti-GFP antibodies (green),
984 anti-NDV antibodies (red), and DAPI (blue). Data are representative of three
985 independent experiments.

986

987 **Figure 5. WT and IFN- λ reporter mice produce similar levels of type I and type III**
988 **IFNs following NDV infection *in vivo*.**

989 (A) Levels of IFN- λ and (B) IFN- α proteins in BAL samples from WT or IFN- λ reporter
990 mice 24 hours after mock- or NDV (10^7 pfu) infection were quantified by ELISA. Data
991 are pooled from two independent experiments.

992

993 **Figure 6. Alveolar macrophages and other myeloid cells do not produce IFN- λ**
994 **during NDV infections *in vivo*.** GFP expression was assayed by flow cytometry
995 analysis of cells obtained from collagenase-digested lungs of mock-infected or NDV-
996 infected IFN- λ reporter mice or infected WT animals. Cell populations are defined as
997 follows: alveolar macrophages (F4/80+, Ly6G-, SiglecF+, CD11c+), neutrophils (F4/80-,
998 LY6G+), monocyte-derived macrophages (F4/80+, Ly6G-, SiglecF-, CD11c-) , and
999 eosinophils (F4/80+, Ly6G-, SiglecF+, CD11c-). Data is representative of three
1000 independent experiments.

1001
1002 **Figure 7. Kinetics of IFN- λ induction by NDV *in vivo***
1003 GFP expression by (A) alveolar macrophages and (B) respiratory epithelial cells
1004 obtained from the lungs of mock- or NDV-infected IFN- λ reporter mice was assayed by
1005 flow cytometry. Samples were obtained at post-infection intervals of 10, 24, and 48
1006 hours. (C) Graphical representation of mean % of GFP+ cells at each time point. Data
1007 are representative of two independent experiments.

1008
1009 **Figure 8. Distinct cell sources of IFN- α and IFN- λ *in vivo*.**
1010 (A) Cell sorting strategy for isolation of alveolar macrophage and epithelial cell
1011 populations from collagenase-digested lungs obtained from mock- or NDV-infected WT
1012 mice 24 hours post-infection mice. Isolated populations of epithelial cells (CD45-
1013 /EPCAM+) and alveolar macrophages (as CD45+/F4/80+/Siglec F+/CD11c+) are
1014 indicated with an asterisk. (B) RNA harvested from FACS-sorted epithelial cells and
1015 alveolar macrophages and used to synthesize cDNA for detection of endogenous

1016 IFN λ 2/3 transcripts by qPCR. qPCR detection of endogenous pan-IFN α transcripts
1017 (non-IFN α 4) was also performed on FACS-sorted (C) alveolar macrophages and (D)
1018 epithelial cells. Expression is shown normalized to 18s rRNA. Data are pooled from two
1019 independent experiments. (E) *In situ* hybridization assays to detect endogenous IFN- λ
1020 transcripts were carried out on FFPE lung sections from NDV-infected WT and IFN- λ
1021 reporter mice at the 24 hour time point using probes specific for IFN- λ 2/3 transcripts
1022 (seen in red). Images are representative of two independent experiments.

1023

1024 **Figure 9. NDV infection of alveolar macrophages *in vitro*.**

1025 (A) Sorting strategy for the isolation of alveolar macrophages from collagenase-digested
1026 lungs of uninfected WT mice. Isolated alveolar macrophages were then infected with
1027 NDV *in vitro* at the indicated MOIs. Supernatants and RNA samples were collected at
1028 24 hours post-infection. (B) cDNA was synthesized from the RNA of mock- or NDV-
1029 infected cells and used to detect *Ifnl2/3* expression by qPCR. RNA isolated from cells
1030 stably transfected with a construct expressing FLAG-tagged murine IFN- λ 2 (FL-mIFN-
1031 λ 2 cells) was included as a positive control. (C) Levels of IFN- λ and IFN- α proteins
1032 secreted into the medium by cultured alveolar macrophages were determined by ELISA.
1033 Culture medium from FL-mIFN- λ 2 cells was used as a positive control. Data are
1034 representative of two independent experiments

1035

1036 **Figure 10. Plasmacytoid dendritic cells are the major producers of IFN- λ during**

1037 **IAV infections *in vivo***

1038 (A) BAL samples collected from mock-infected or IAV-infected IFN- λ reporter mice were
1039 assayed by ELISA for the measurement of IFN- λ protein in airways. (B) Representative
1040 flow cytometry plots evaluating GFP expression specifically in epithelial cells,
1041 characterized as EPCAM⁺ cells (initially gated from DAPI-/CD45- and single cell
1042 populations), from single-cell suspensions of collagenase-digested lungs from mock-
1043 infected and IAV-infected (WSN; 10⁶ PFUs, 48 hrs) IFN- λ reporter mice or WT 129
1044 SvEv controls. (C) Flow cytometry plots demonstrating gating scheme of pDCs from the
1045 lungs of the same mice as in (A). From initial gating of DAPI-/CD45⁺ and single cells,
1046 pDCs were gated as CD11c⁺/CD11b⁻, B220⁺/PDCA1⁺, from which GFP expression
1047 was assessed. Graphs on the right represent SEM of %GFP⁺ pDCs, and number of
1048 pDCs from each cohort. Data are pooled from three independent experiments. (D)
1049 qPCR analysis of IFN- λ and IFN- α transcripts in epithelial cells and pDCs that were
1050 FACS-sorted from the single cell suspension of collagenase-digested lungs from of
1051 mock-infected and IAV (WSN)-infected WT 129 SvEv mice (10⁵ PFUs, 48 hrs). Data are
1052 representative of two independent experiments. (E) Formalin fixed lung tissue sections
1053 from mock-infected or IAV-infected (10⁶ PFUs WSN) IFN- λ reporter mice, harvested at
1054 the indicated time points, were stained with IFN- λ oligonucleotide probes to detect *ifnl*
1055 RNA transcripts. Data are representative of two independent experiments.

1056

1057 **Figure 11. Minimal IFN- λ expression by epithelial cells during IAV infections is not**
1058 **due to a NS1-mediated inhibition**

1059 Representative flow cytometry plots showing GFP expression from (A) epithelial cells
1060 (gated from DAPI-/CD45-/EPCAM⁺) and (B) pDCs (gated from DAPI-

1061 /CD45+/CD11c+/CD11b-/B220+/PDCA1+) from wildtype 129 SvEv mice and IFN- λ
1062 reporter mice mock-infected or infected with 10^4 PFUs of either IAV (PR8) or IAV Δ NS1
1063 (PR8 Δ NS1) for 48 hrs. (C) SEM of %GFP epithelial cells and pDCs from each cohort.

1064

1065 **Figure 12. The hematopoietic compartment is the source of the bulk of IFN- λ**
1066 **protein produced during IAV infection.**

1067 Bone marrow chimera mice, generated by reconstituting lethally irradiated wildtype
1068 C57BL6/J mice with bone marrow from either wildtype C57BL6/J mice or *ifnl*^{-/-} mice,
1069 were infected with 10^5 PFUs IAV (WSN) for 48 hrs, at which point BAL samples were
1070 harvested and assayed for IFN- λ protein by ELISA. The graph represents the mean and
1071 SEM of IFN- λ protein levels measured from each cohort. Data were pooled from two
1072 independent experiments

1073

1074 **Figure 13. Depletion of pDCs during IAV infections in vivo results in significant**
1075 **reduction of both Type I and Type III IFNs**

1076 (A) Representative flow cytometry plots show pDC populations in the spleen and blood
1077 of IAV-infected mice (10^6 PFUs WSN strain; 24 hrs post-infection) that were treated with
1078 the pDC-depleting antibody (anti-PDCA1) or the Rat IgG isotype 24 hours and 48 hours
1079 prior to IAV infection. Graphs on the right show mean percentages, and SEM, of pDCs
1080 in the spleen and blood of each indicated cohort. (B) BALs were harvested 24 hours
1081 post-IAV infection for IFN- λ protein measurements. Graphs represent data from two
1082 independent experiments

1083

1084 **Figure 14. IFN- λ induction by NDV is MAVS-dependent**

1085 (A) IFN- λ and (B) IFN- α protein levels were measured by ELISA in BALs of mock-
1086 infected or NDV-infected (24 hrs, 10^7 PFUs) WT and MAVS^{-/-} mice on the C57BL/6
1087 strain background. Differences in IFN- λ levels between WT and MAVS^{-/-} mice were
1088 statistically significant as determined by student t test (P = 0.0474). No IFN- α protein
1089 was detectable in mock-infected or NDV-infected MAVS^{-/-} animals. Data were pooled
1090 from two independent experiments

1091

1092 **Figure 15. Contribution of MAVS and MyD88 signaling in IFN- λ induction during**
1093 **IAV infections is dependent on IAV strain**

1094 (A) IFN- λ protein levels were measured by ELISA from BALs of IAV-infected (10^6 PFUs
1095 PR8) wildtype C57BL/6J mice or MAVS^{-/-} mice at the indicated time points. (B) IFN- λ
1096 protein levels measured by ELISA from BALs of IAV-infected (10^5 PFUs WSN) wildtype
1097 C57BL/6J mice, MyD88^{-/-}, or MAVS^{-/-} mice at 48 hours post-infection. Graphs represent
1098 the mean and SEM of IFN- λ protein measured from the BALs of each cohort. Data from
1099 each figure were pooled from two independent experiments.

1100

1101 **Figure 16. Murine tracheal epithelial cells produce IFN- λ in response to WSN but**
1102 **not to PR8 infection**

1103 Murine tracheal epithelial cultures were infected apically, at an MOI of 1, with NDV,
1104 WSN, PR8 or UV-inactivated PR8 for 24 hours. Supernatants were then assayed by
1105 ELISA for IFN- λ protein.

1106

1107 **Figure 17. IFN- λ production by pDCs during IAV infection is dependent on type I**
1108 **IFN signaling**

1109 (A) Flow cytometry plots showing GFP expression in pDCs (DAPI-
1110 /CD45⁺/CD11c⁺/CD11b⁻/B220⁺/PDCA1⁺) from the lungs of IFNAR^{+/+} or IFNAR^{-/-} IFN- λ
1111 reporter mice, or 129 SvEv mice, mock-infected or IAV-infected (PR8 10⁶ PFUs, for 72
1112 hrs). The graph on the right represents mean and SEM of %GFP⁺ cells from each
1113 cohort. (B) Supernatants from mock-infected or IAV-infected (WSN; MOI=1) WT and
1114 IFNAR^{-/-} FLT3L-cultured bone-marrow derived pDCs were collected 24 hours post-
1115 infection, and assayed for IFN- λ protein by ELISA. (C) qPCR measurements comparing
1116 basal levels of IRF7 mRNA expression between WT and IFNAR^{-/-} bone-marrow derived
1117 pDCs. Data were pooled from two independent experiments.

1118

1119 **Figure 18. IFN- λ production during NDV infection is type I IFN-independent**

1120 GFP expression was assayed by flow cytometry of cells obtained from collagenase-
1121 digested lungs of 129 SvEv WT and IFNAR^{-/-} IFN- λ reporter mice which were mock-
1122 infected, or infected intranasally with NDV for 24 hrs. Epithelial cells were defined as
1123 CD45⁻, EPCAM⁺. WT 129SvEV mice were included as controls for GFP expression,
1124 gating in the same manner. No significant differences in GFP expression were seen
1125 between IFN- λ reporter animals on the WT or IFNAR^{-/-} backgrounds.

1126

1127 **Supplemental Figure 1. Viral loads are equivalent in WT and reporter mice**

1128 Cohorts of 6-10 week old WT 129 SvEv mice or strain-matched IFN- λ reporter mice
1129 were infected with 10² PFUs of IAV (WSN). Viral titers from the lungs were assayed by

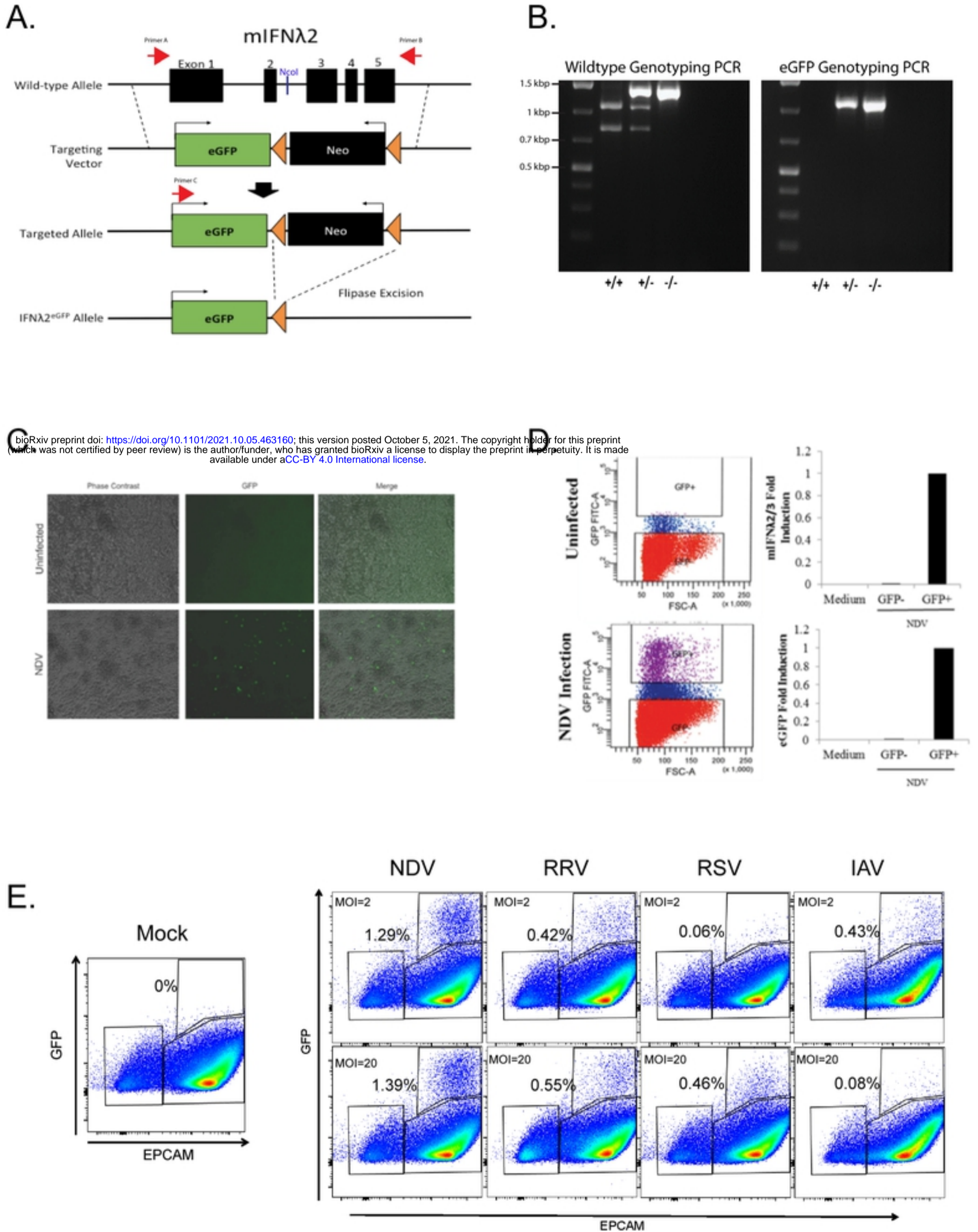
1130 immunofocus assay from lung homogenates collected at 72 hrs post-infection. The
1131 graph represents SEM of PFUs quantified for each cohort. This is experiment was
1132 performed twice.

1133

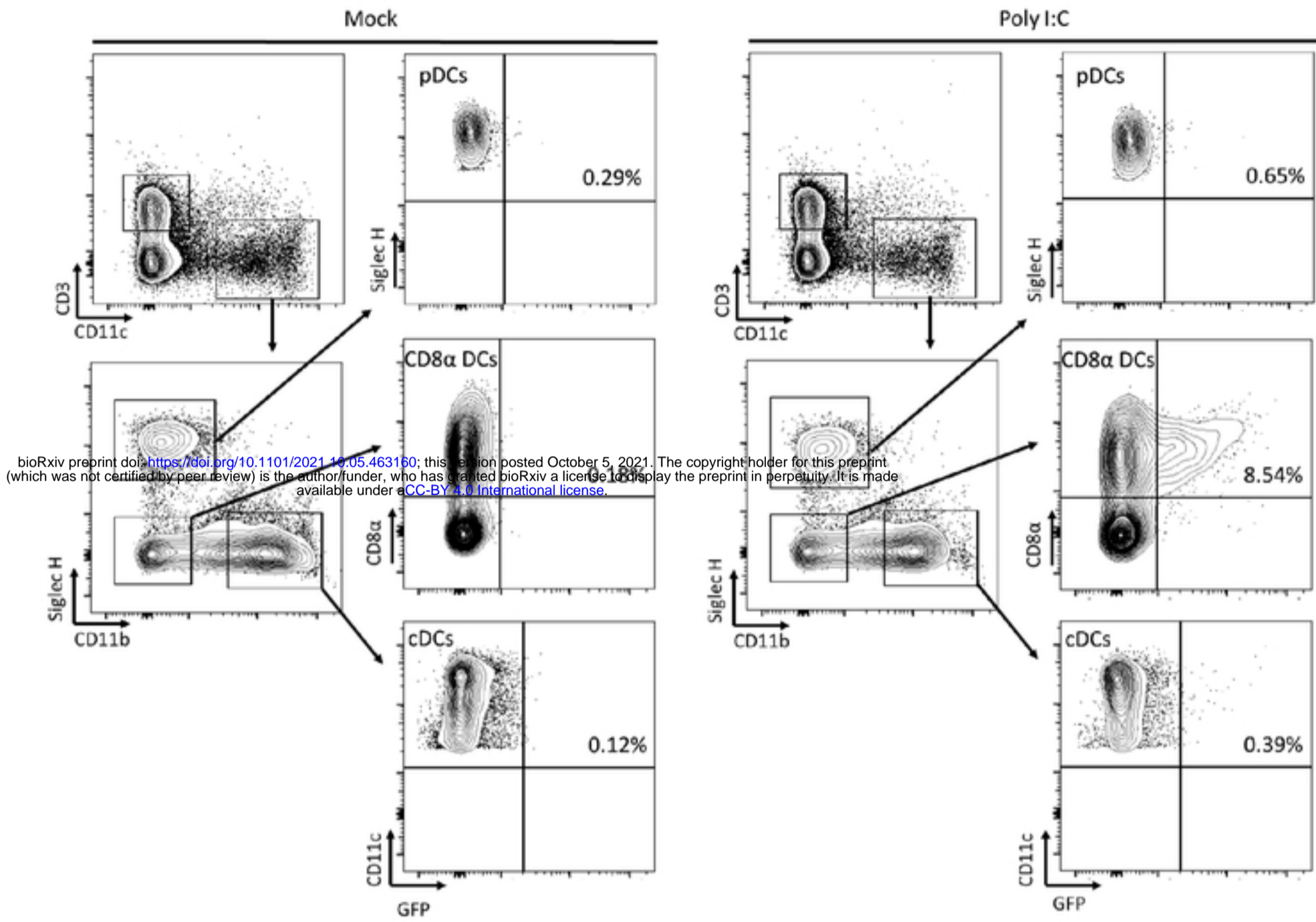
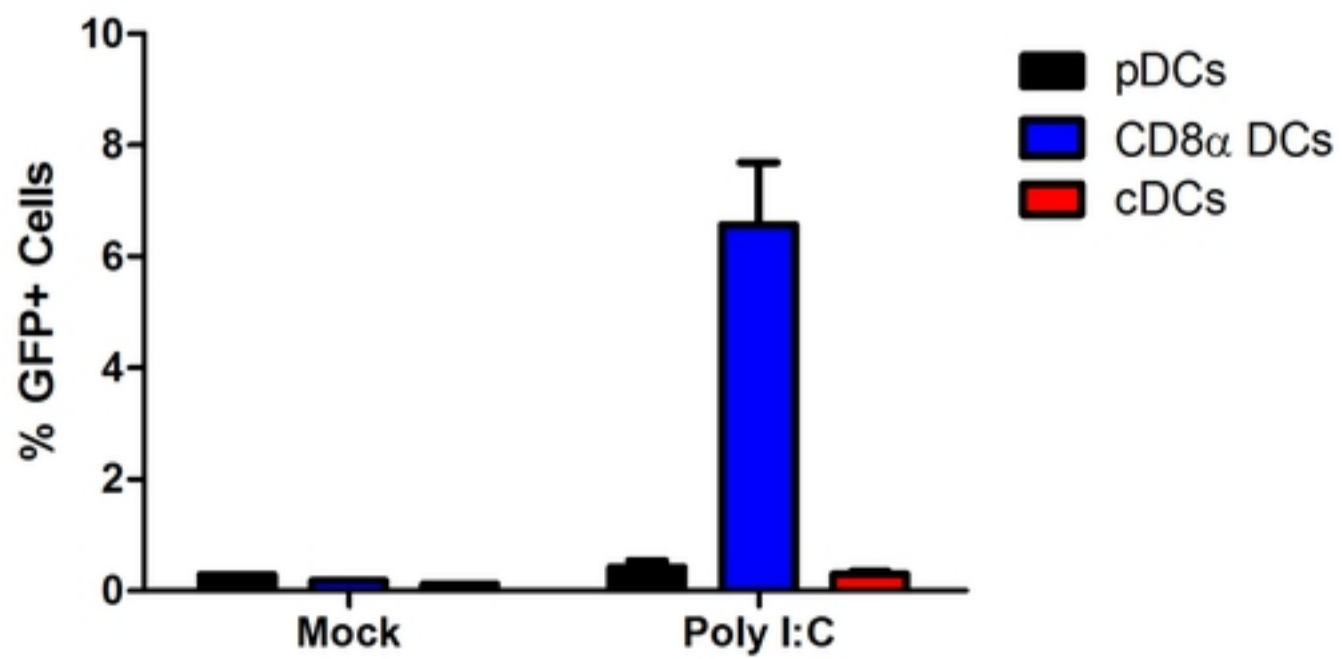
1134 **Supplemental Figure 2. Generation of *ifnl*^{-/-} mice**

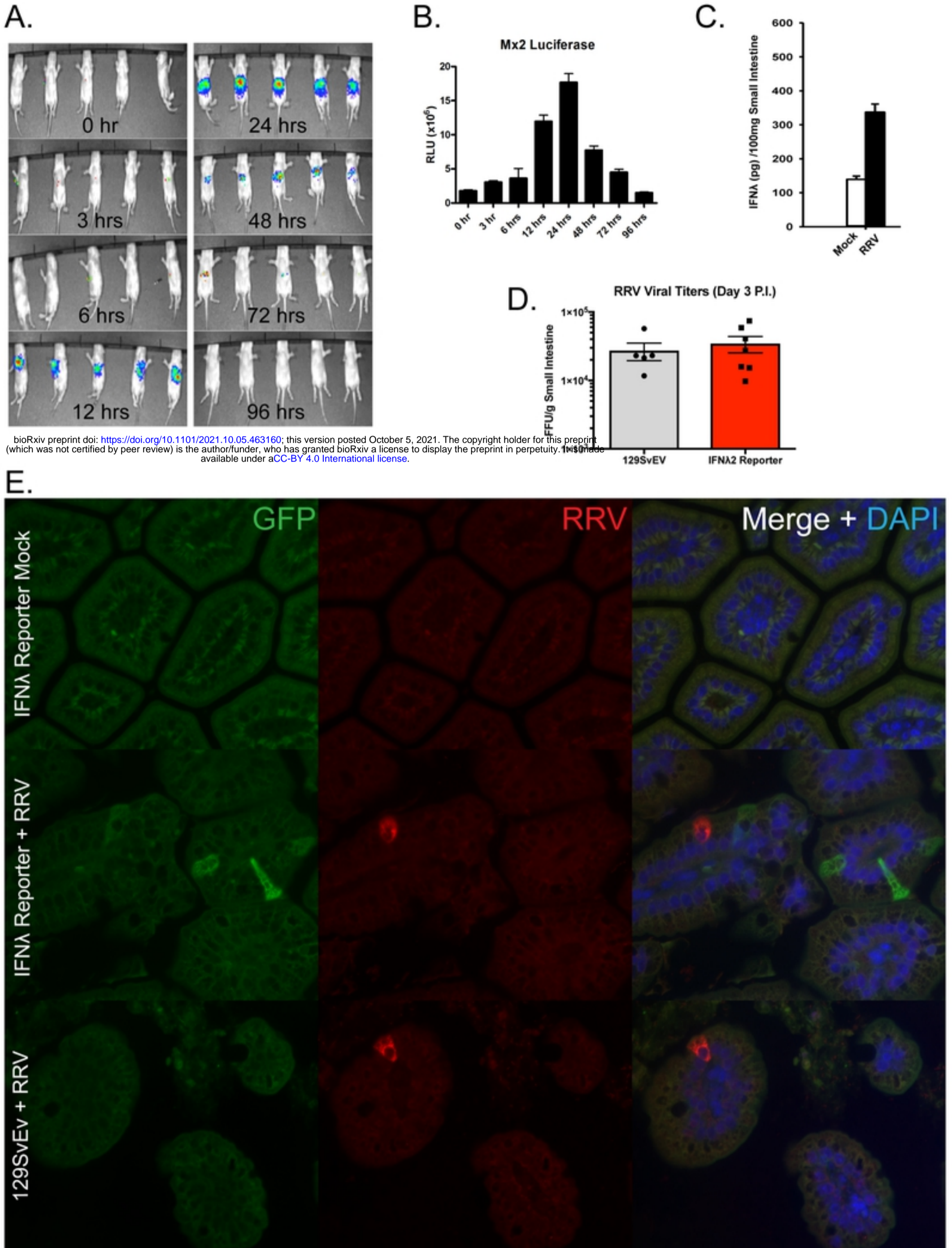
1135 The mouse genome contains two functional IFN- λ genes, the IFN- λ 2 and IFN- λ 3 genes,
1136 which are juxtaposed in a head-to-head orientation on chromosome 7. Guidance RNAs
1137 (gRNAs) were designed to target sequences flanking these genes, and the
1138 CRISPR/Cas9 technology was used to generate mice lacking both IFN- λ genes, to
1139 create an IFN- λ 2/3 knock-out (KO) strain. The selected IFN- λ 2/3 KO strain contains
1140 19,803 base pair deletion (from 28,506,772 to 28,526,525 nucleotide positions; NCBI
1141 GRCm38.p4) that removed the entire IFN- λ 2 and IFN- λ 3 genes including their
1142 promoters and 3'UTR, and replaced these with ATAACTTCGTATAGCATA sequence.

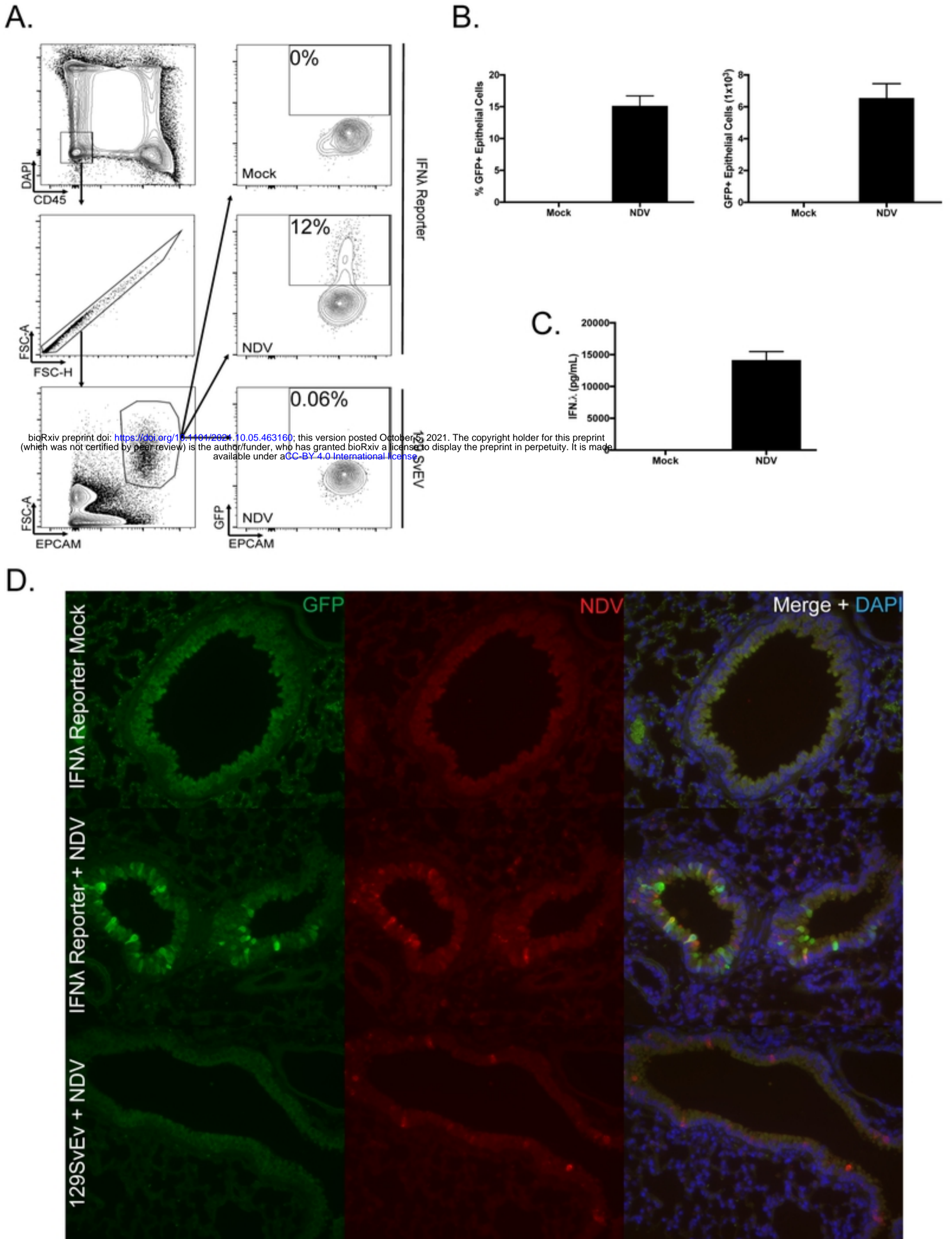
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Figure

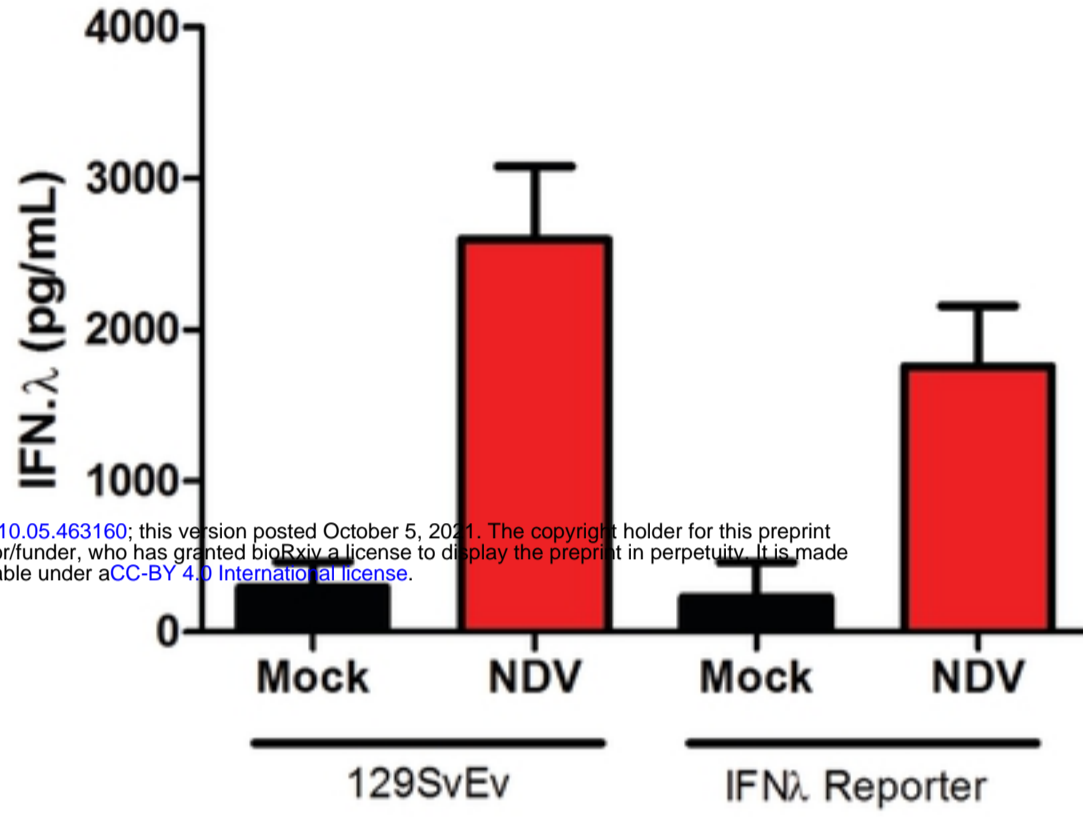
A.**B.**



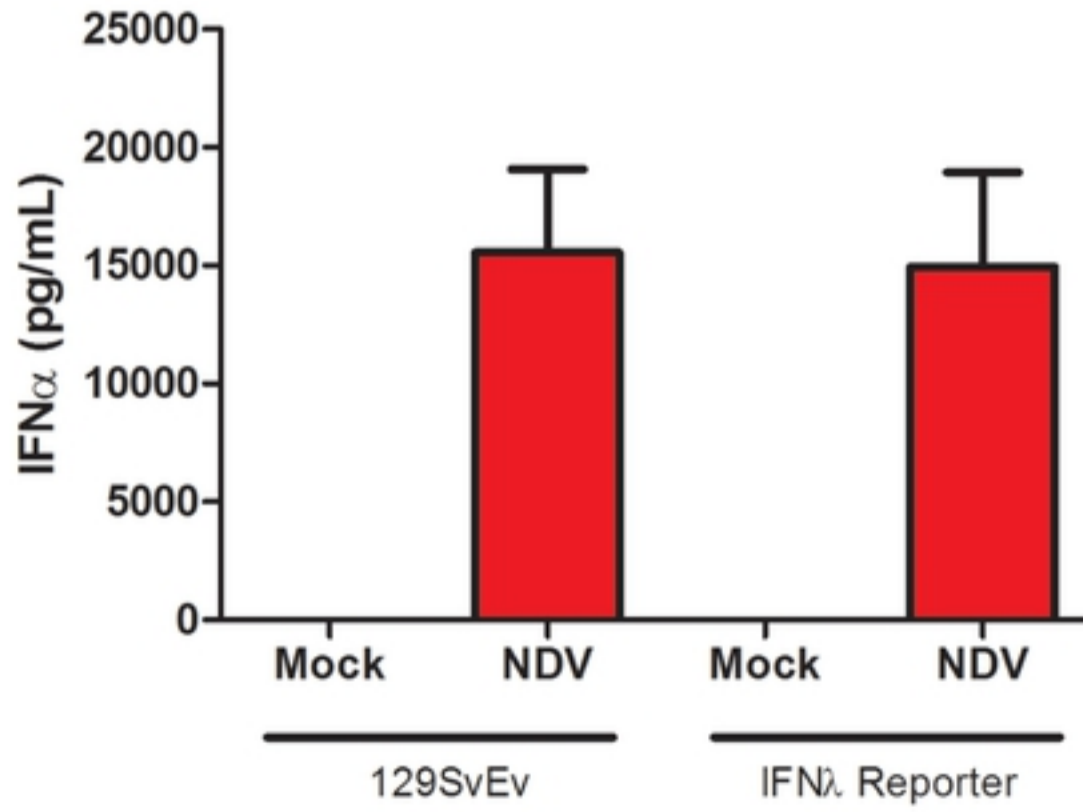


Figure

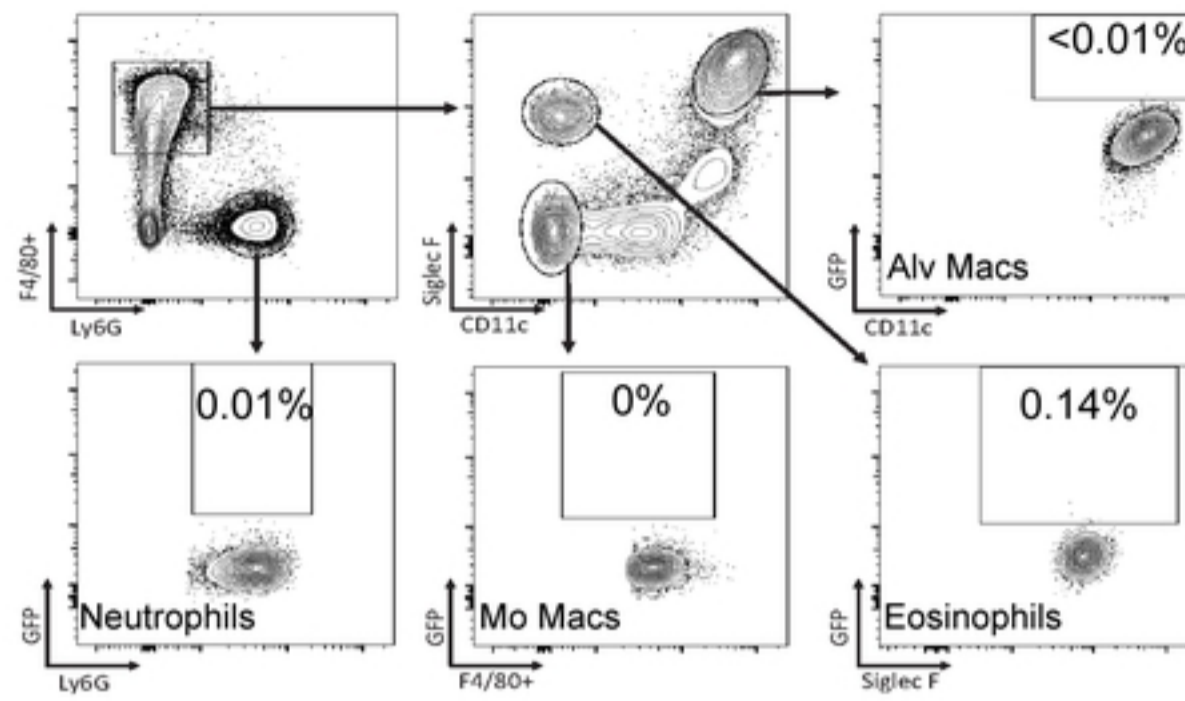
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B.

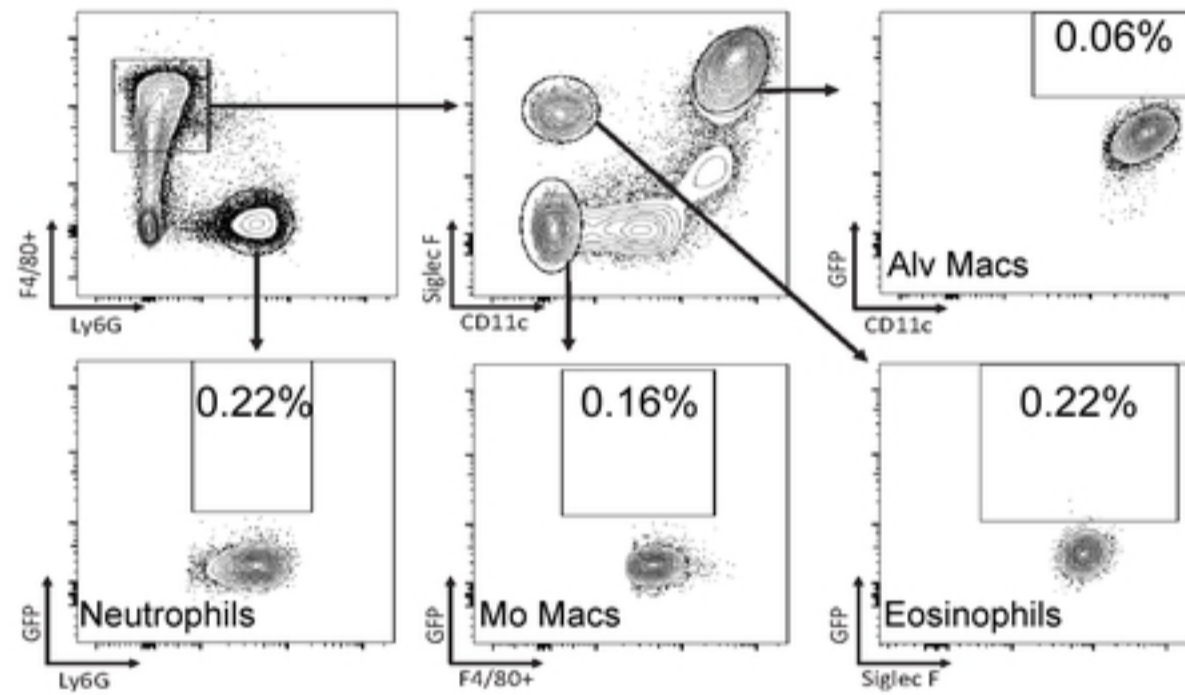


IFN λ Reporter Mock

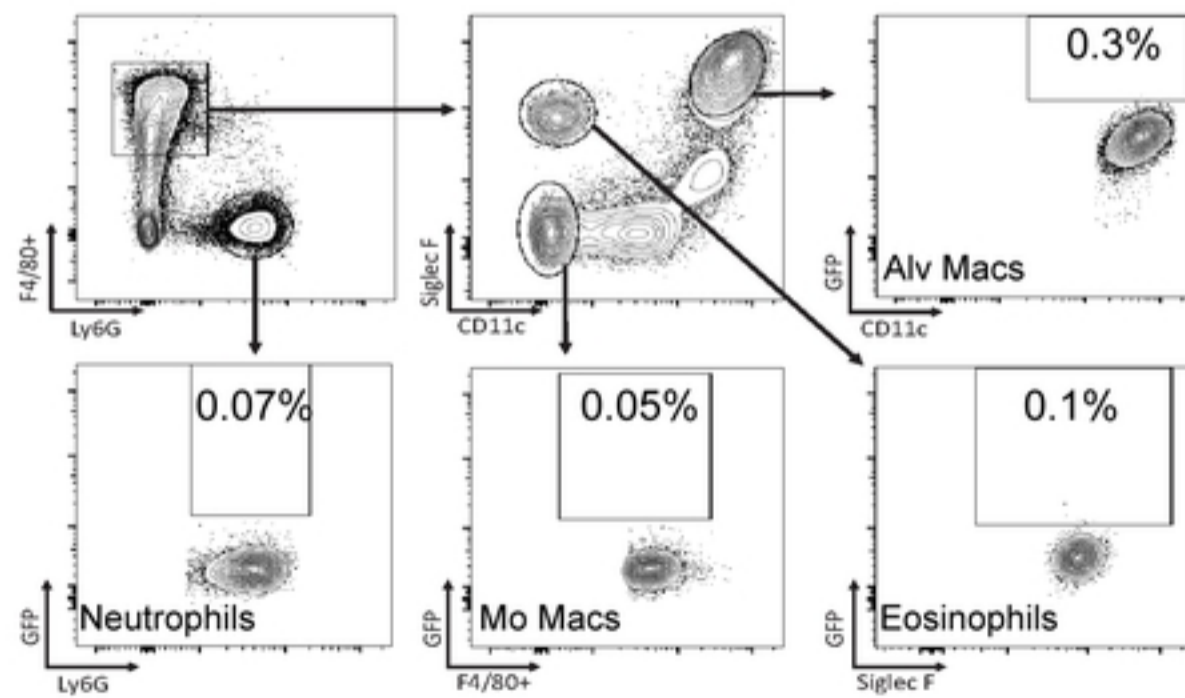


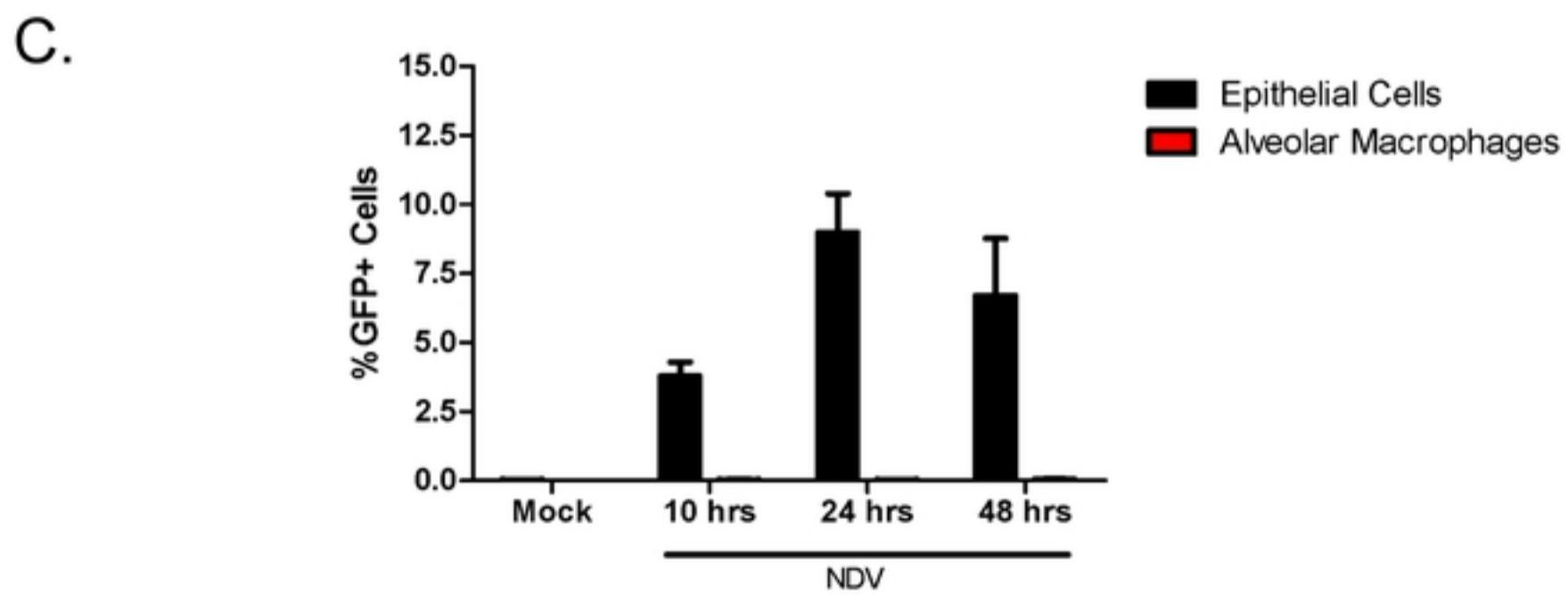
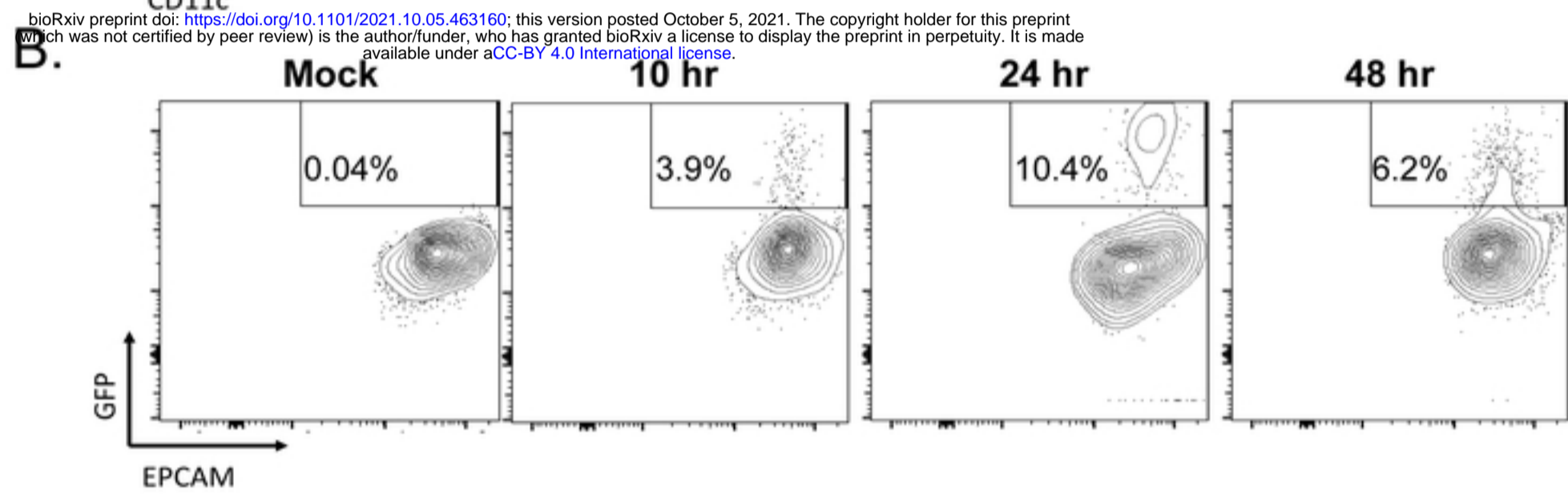
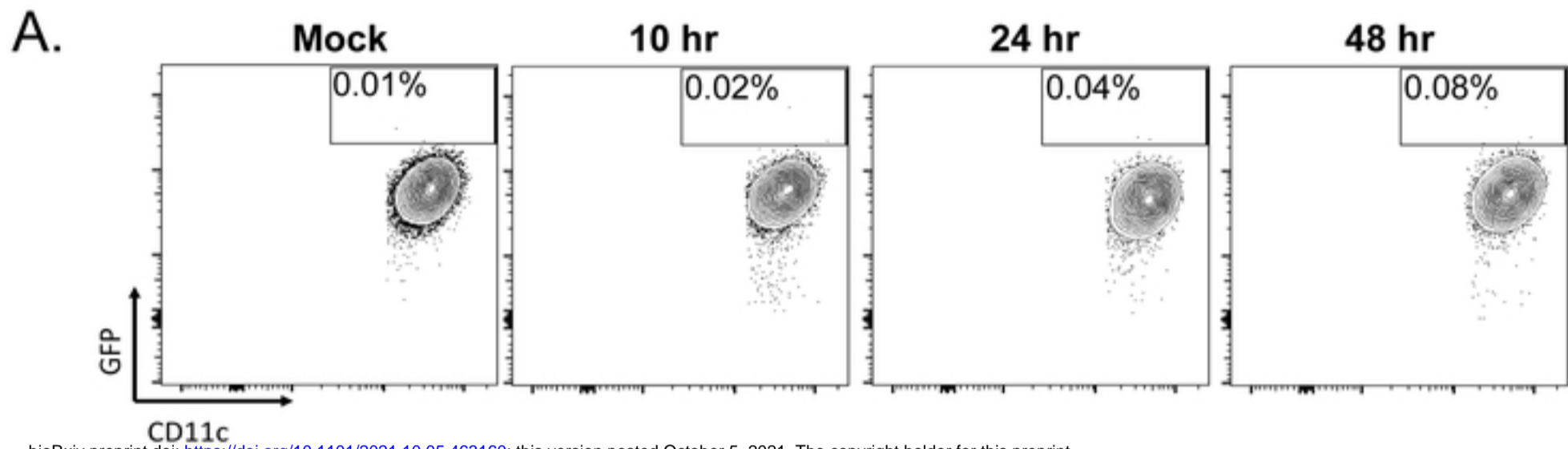
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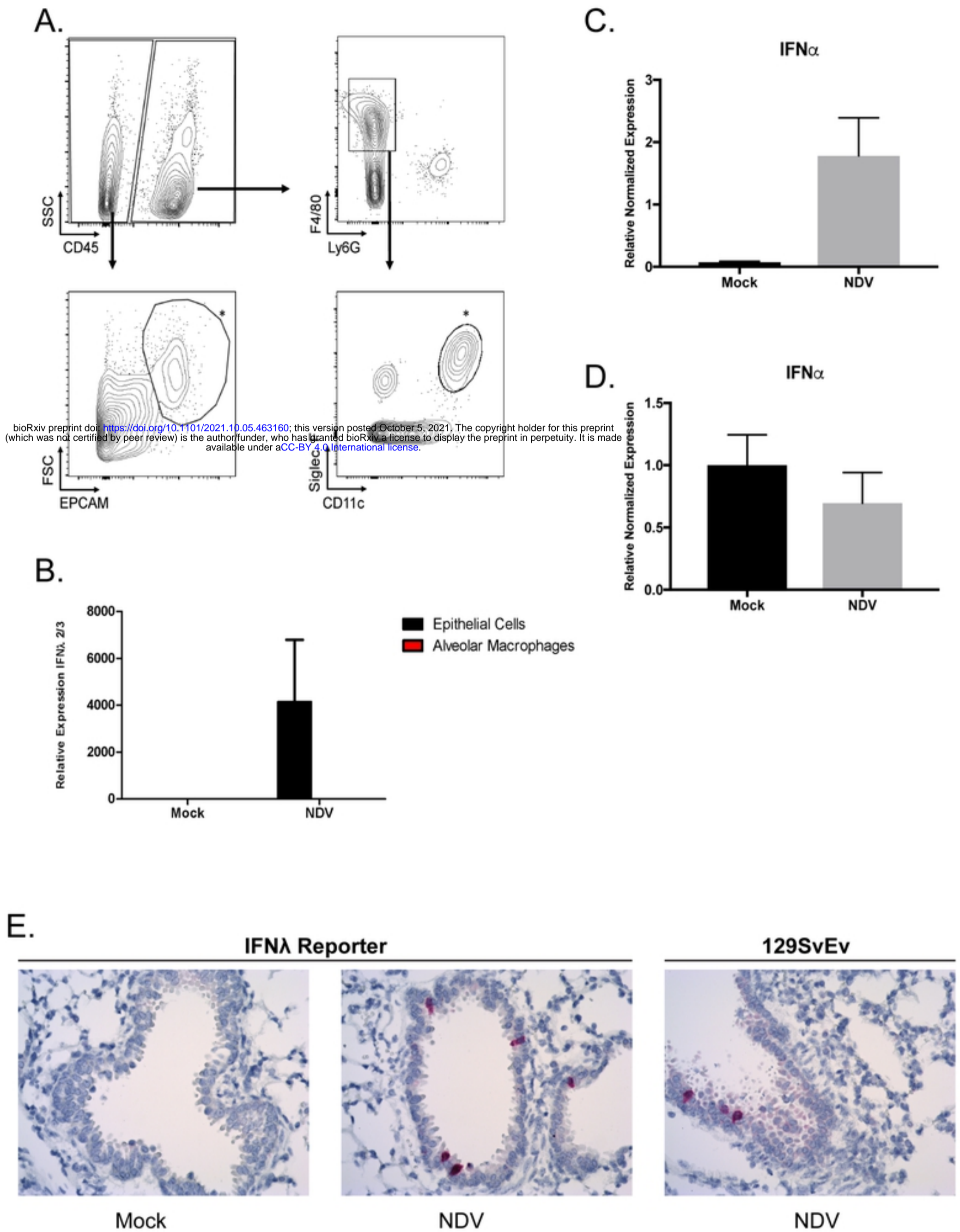
IFN λ Reporter + NDV



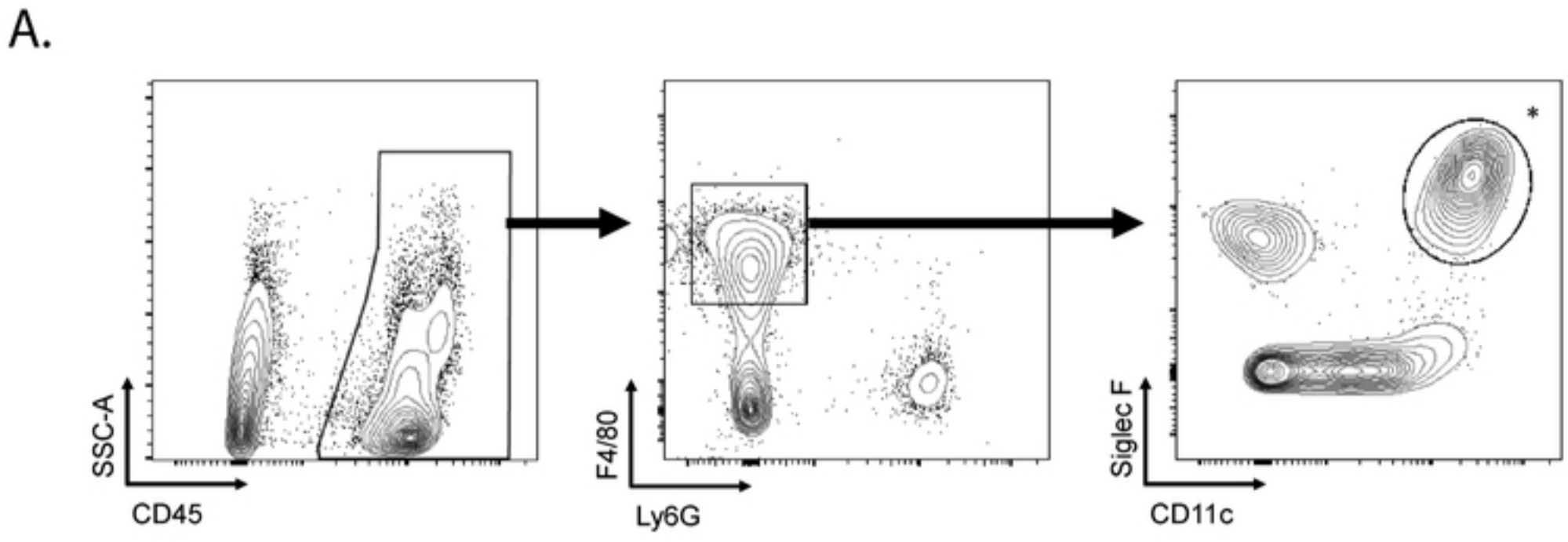
129SvEv + NDV



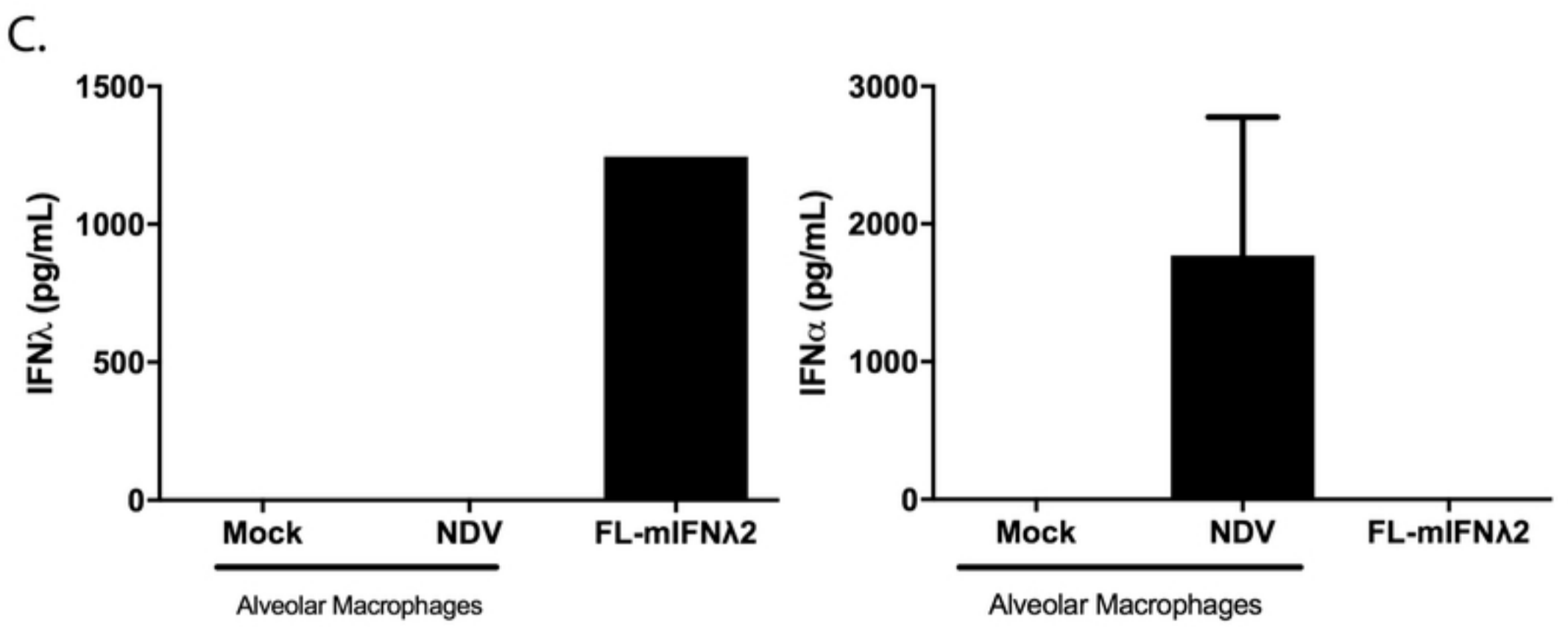
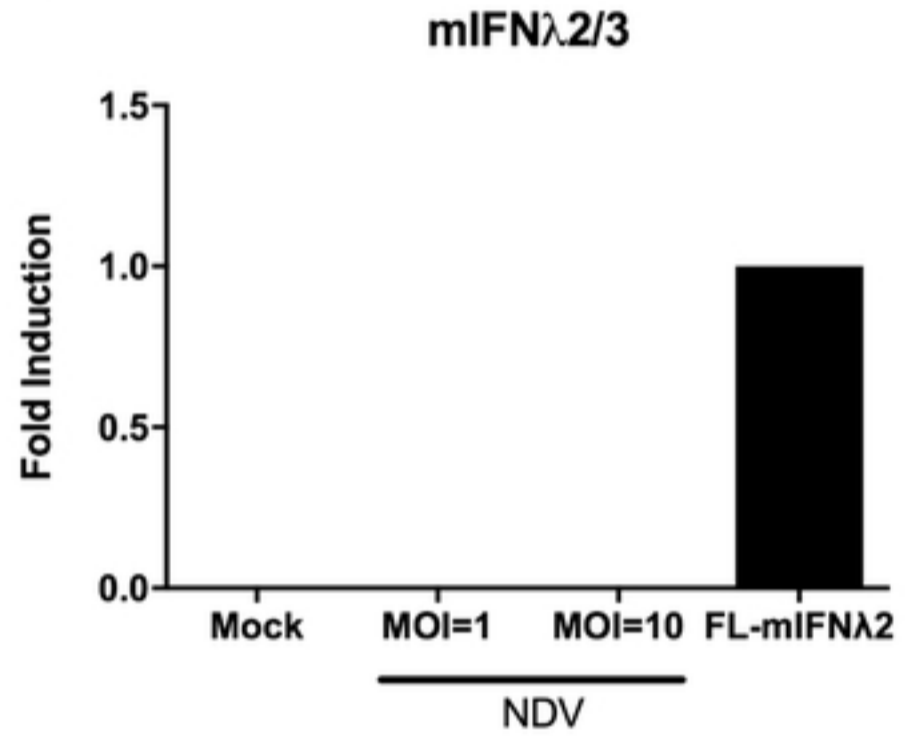




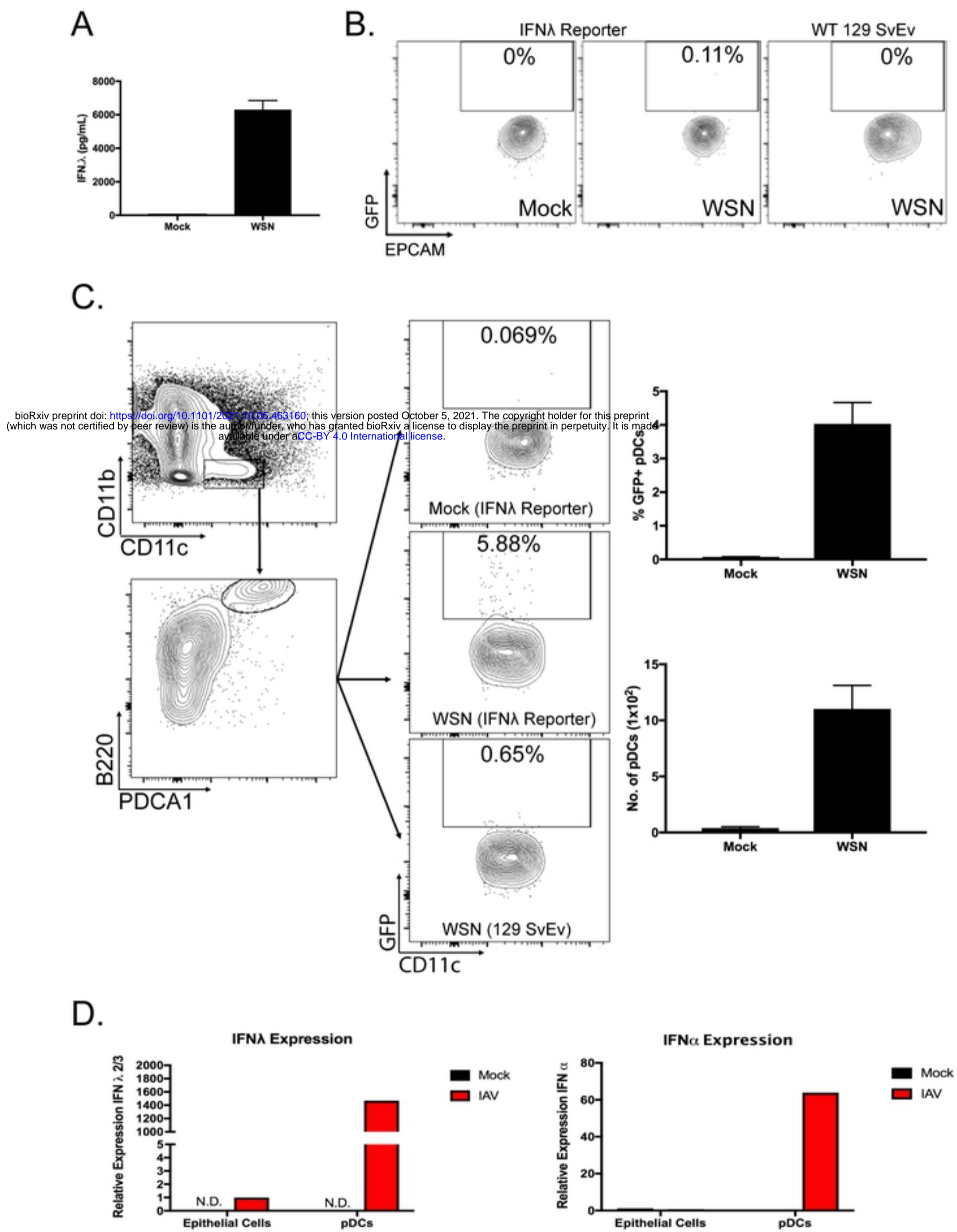
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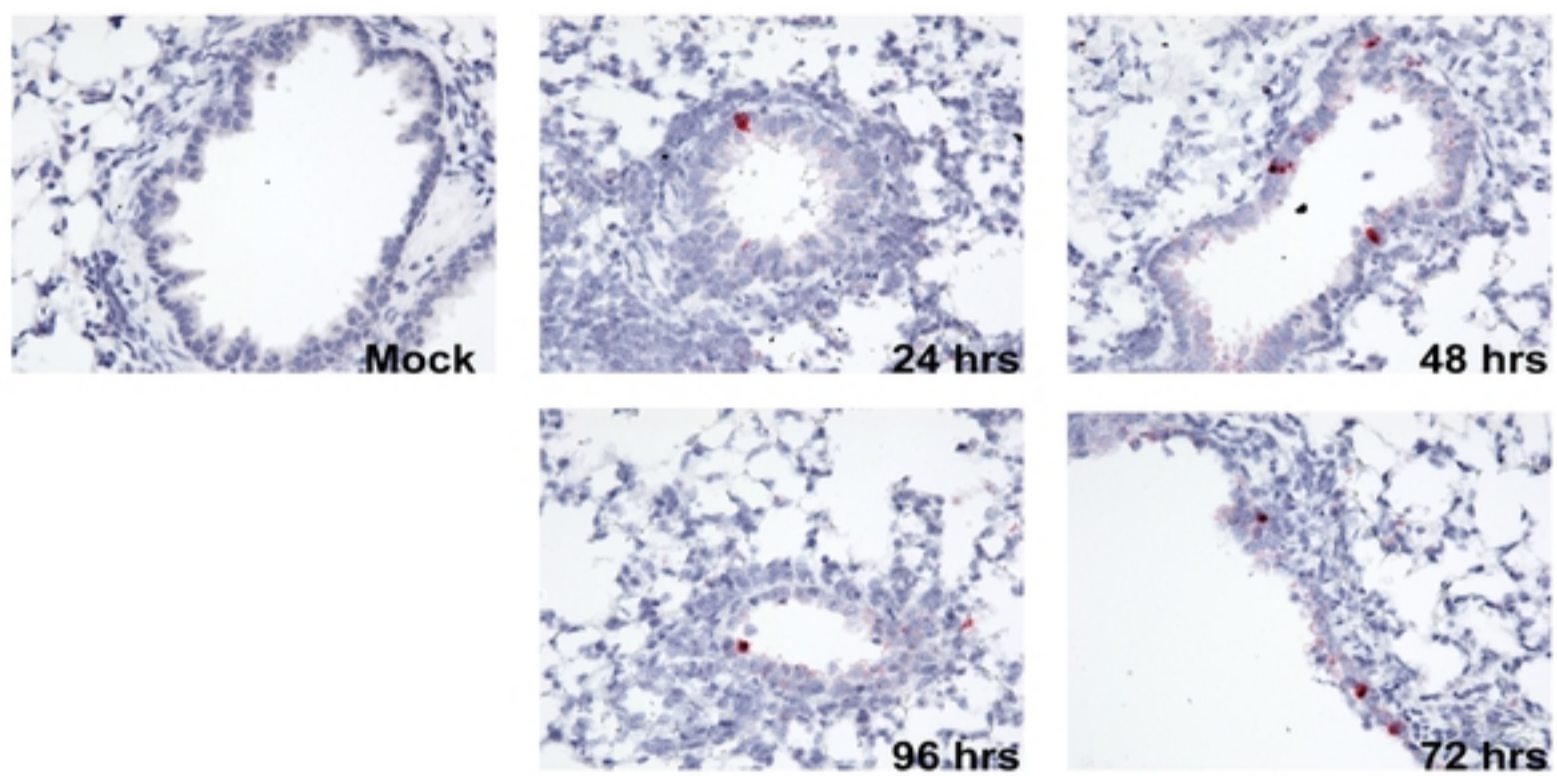


Figure

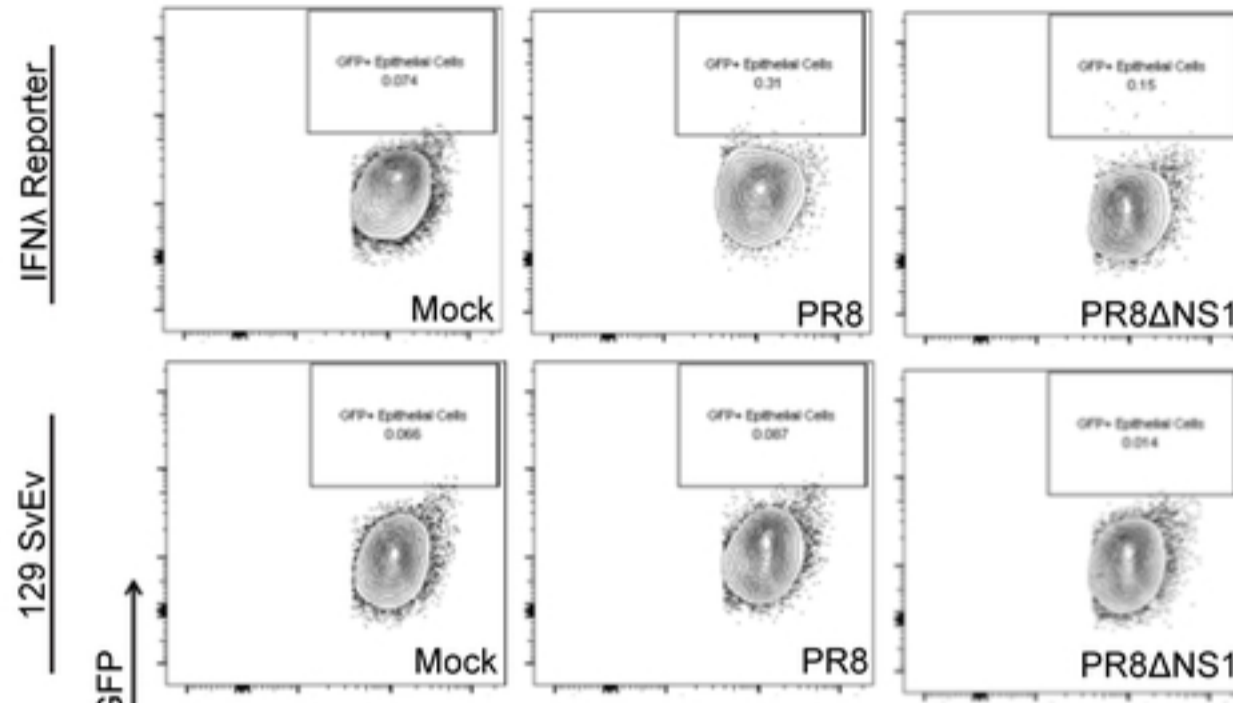


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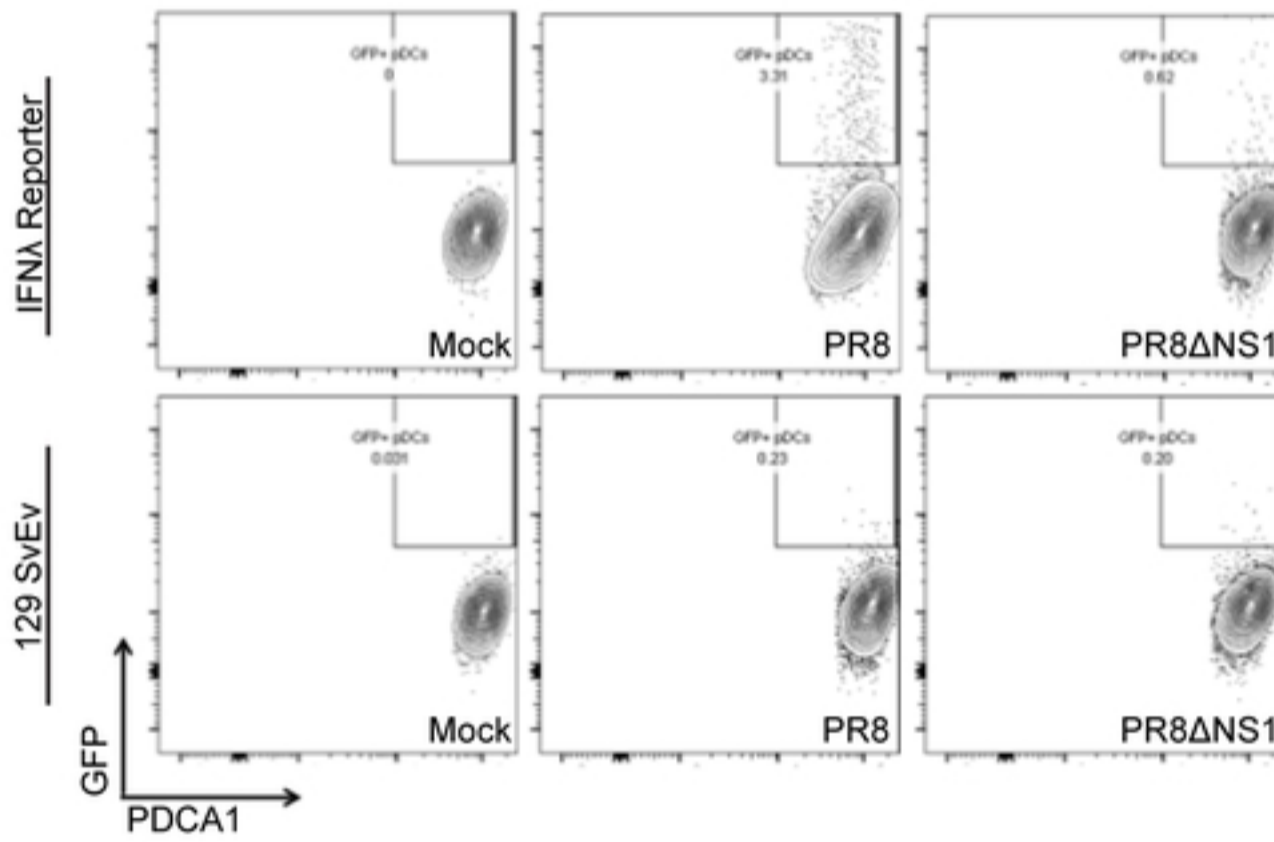


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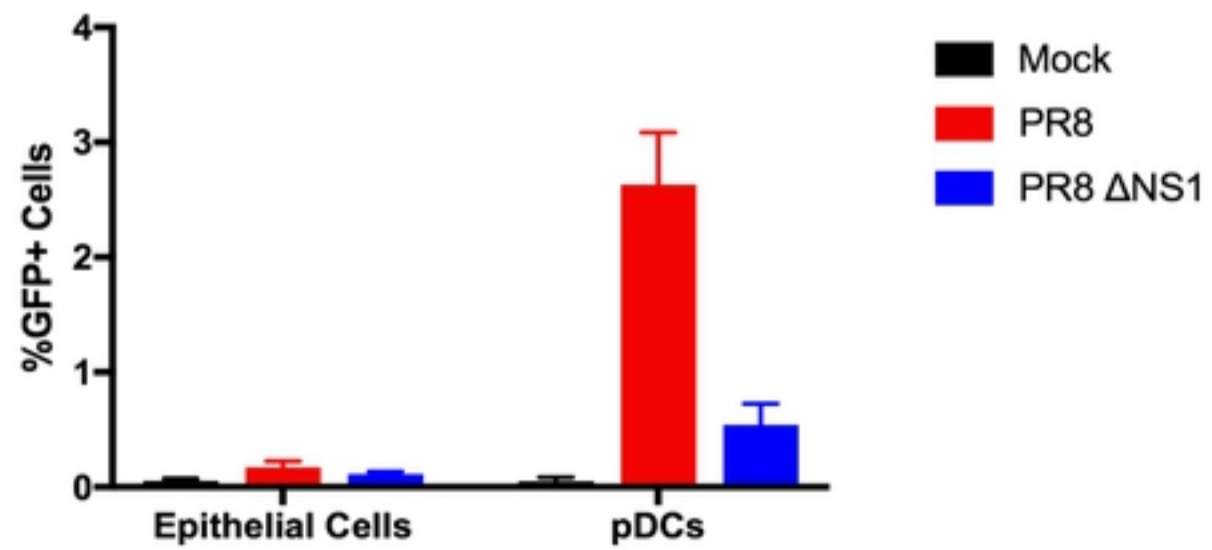


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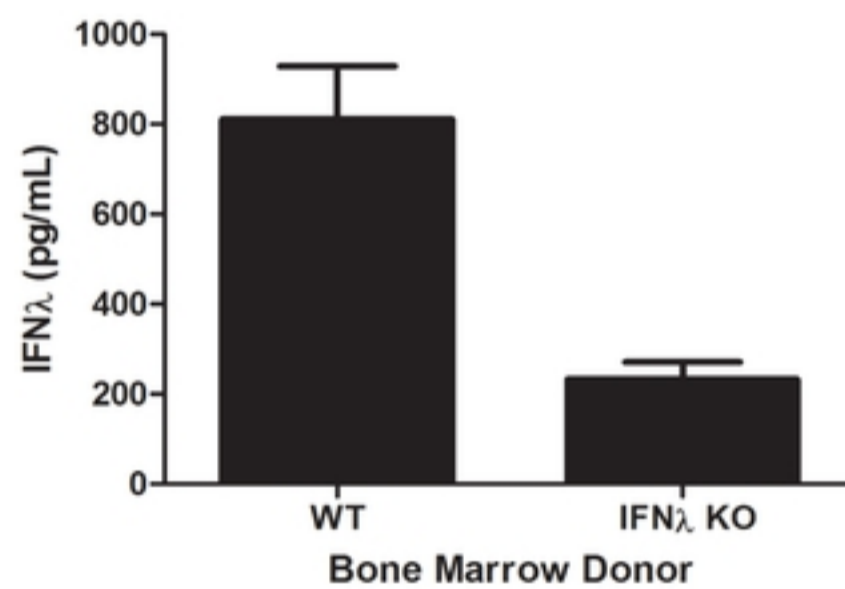
B.



C.

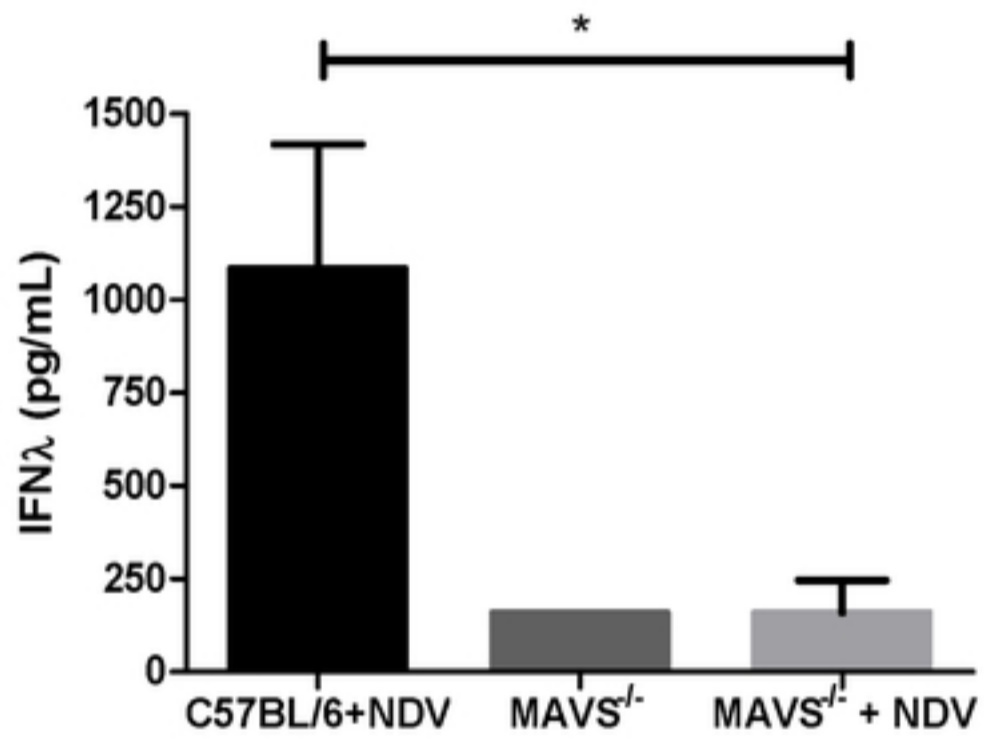


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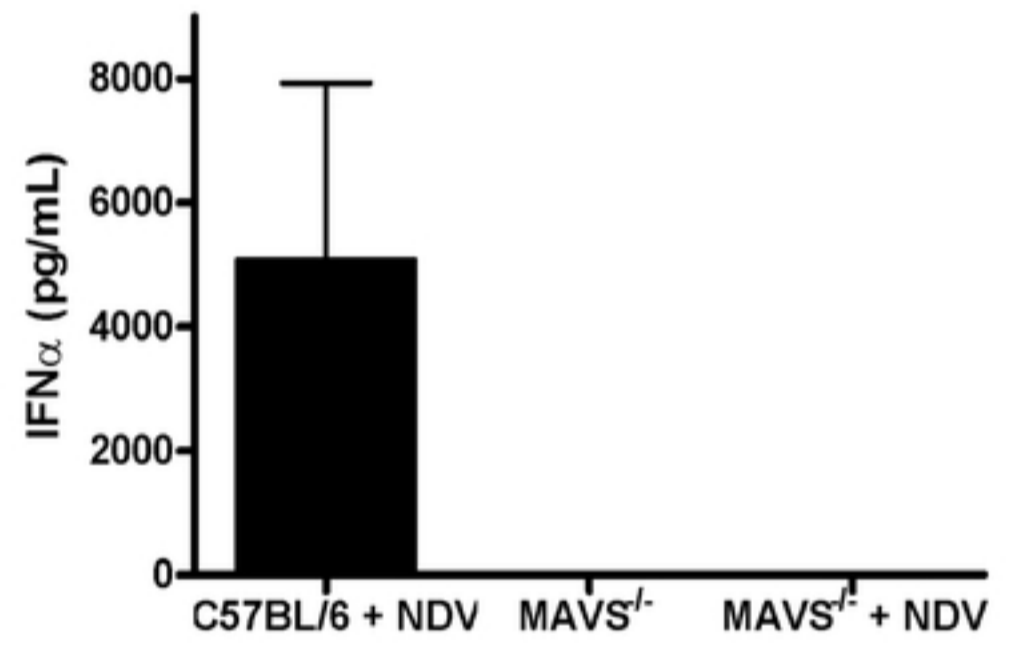


A.

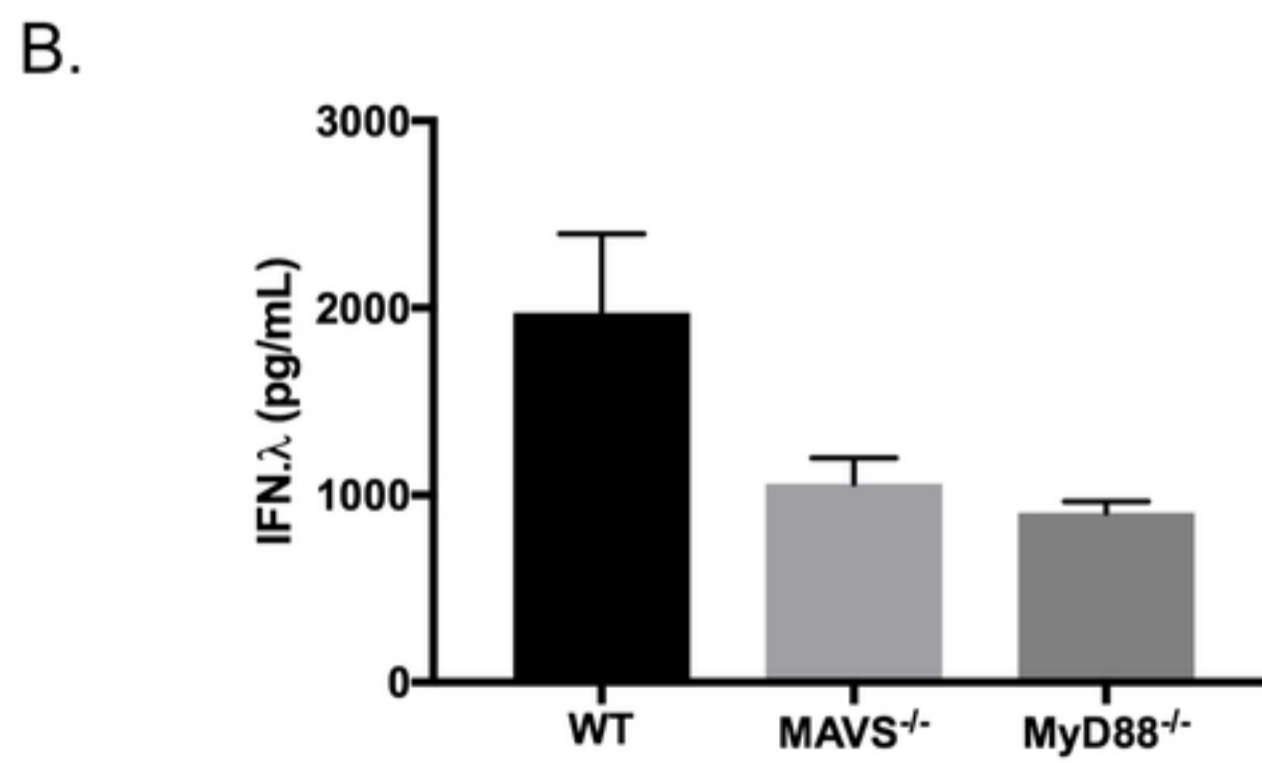
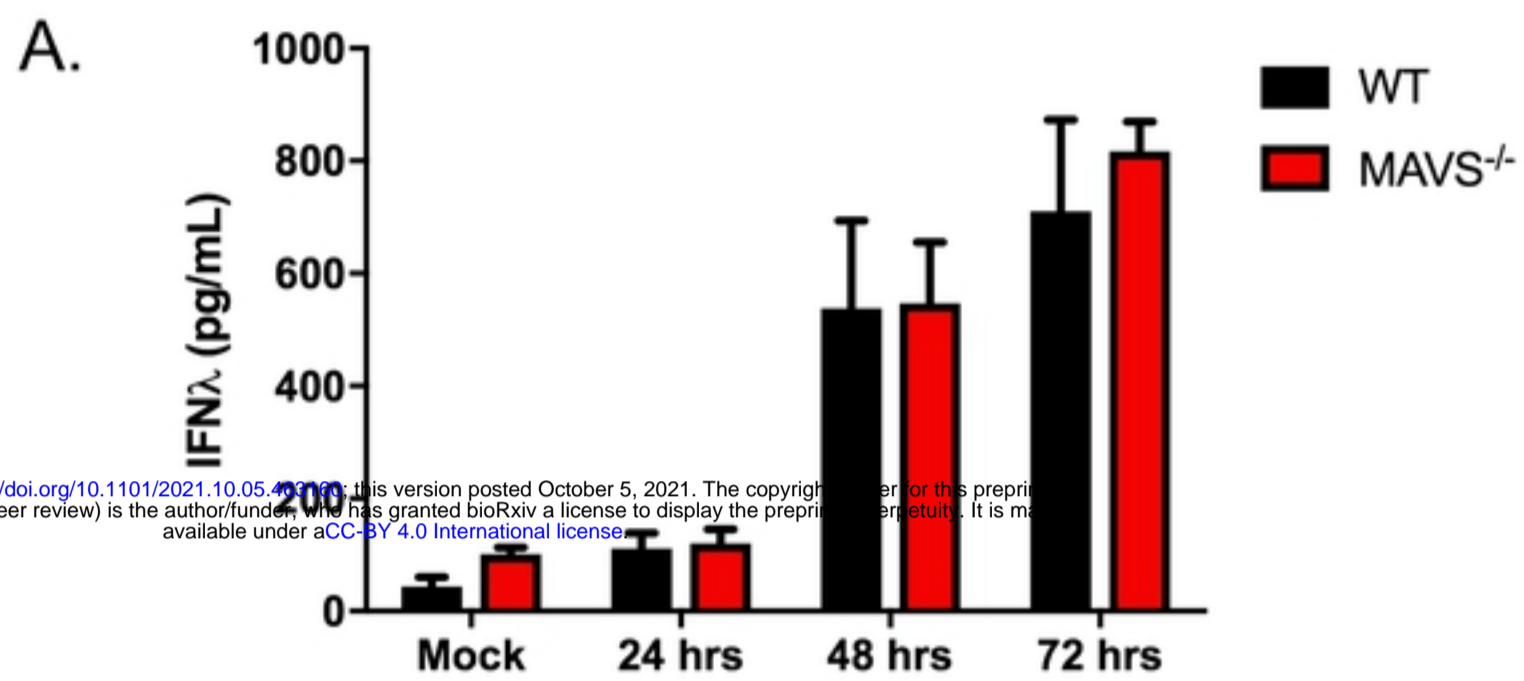
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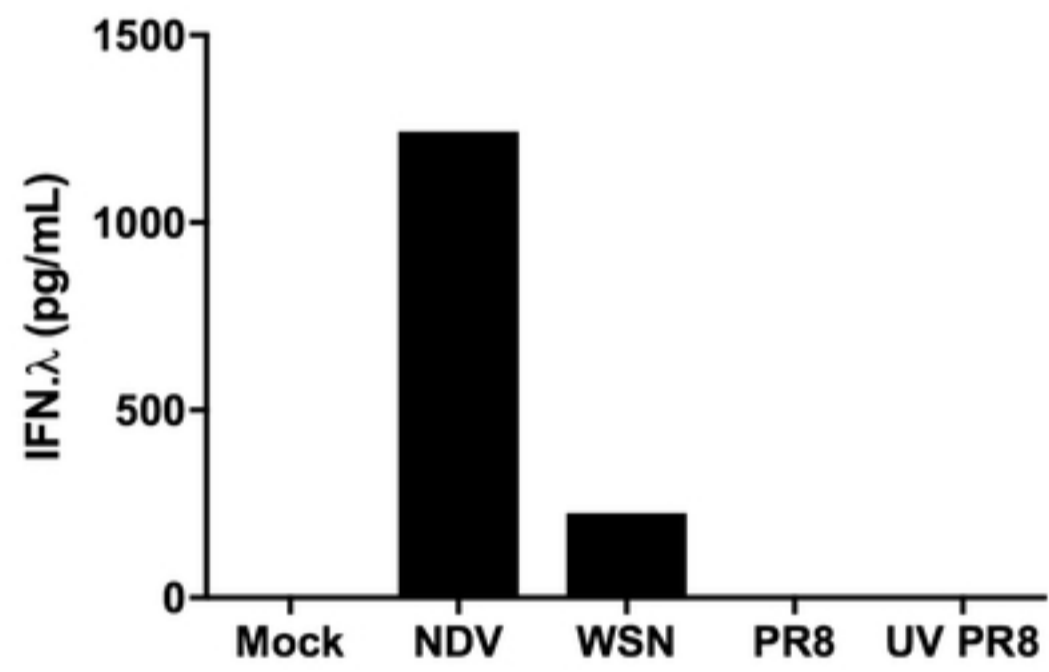
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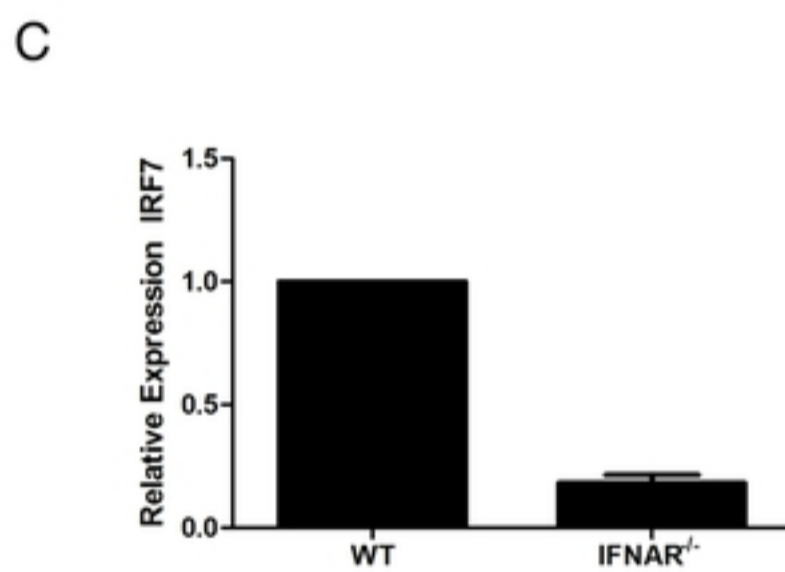
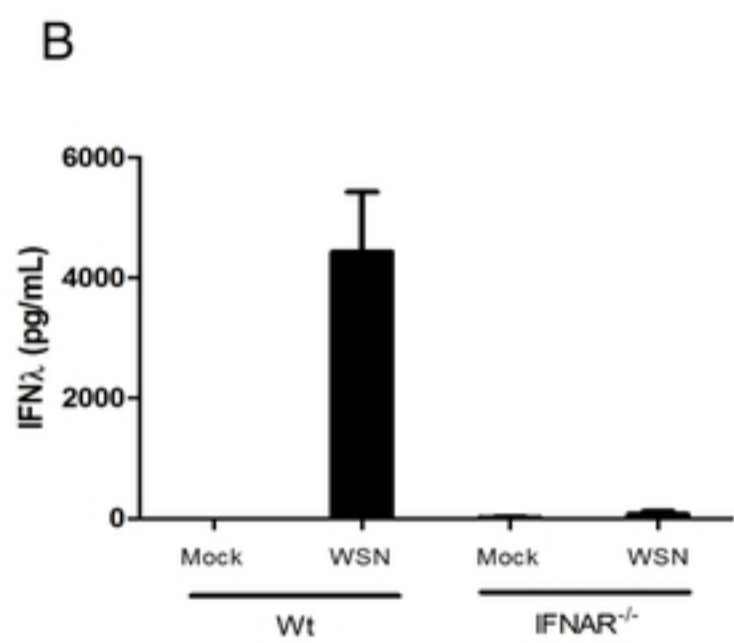
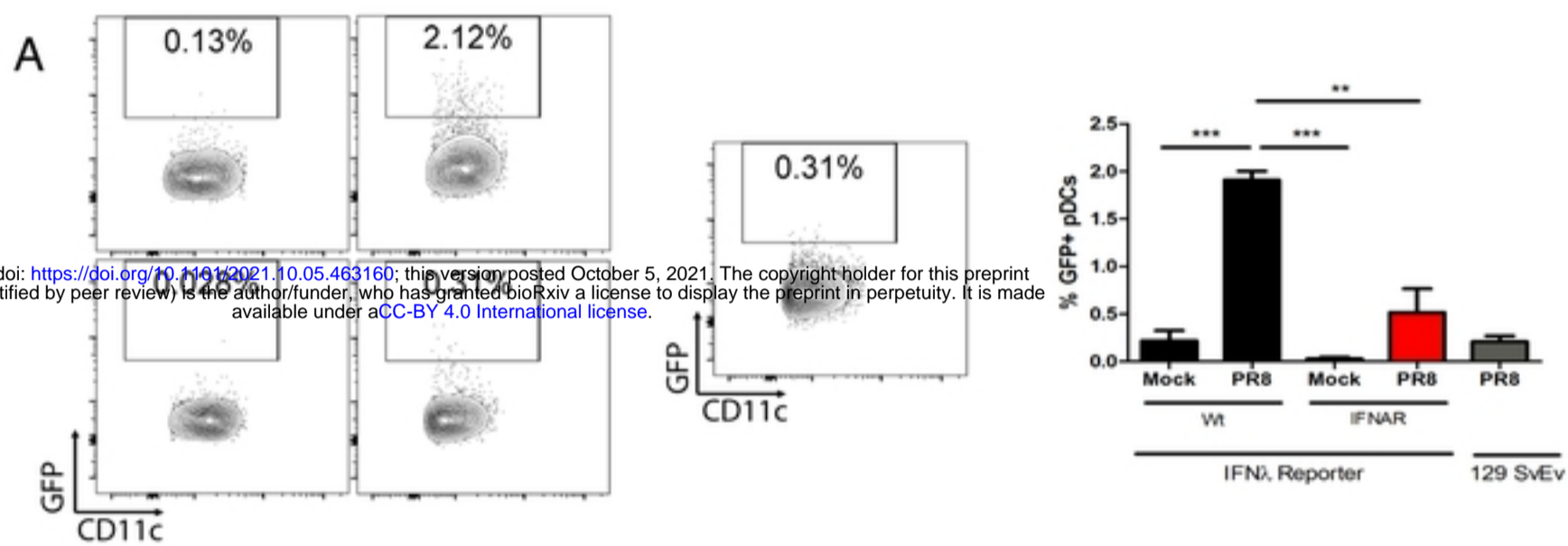
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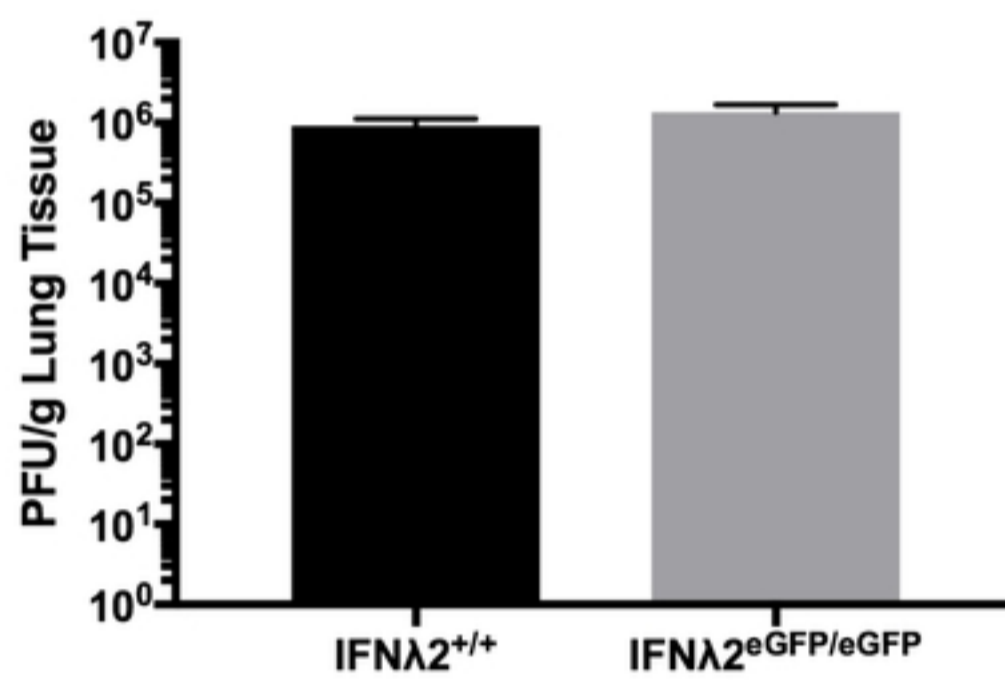
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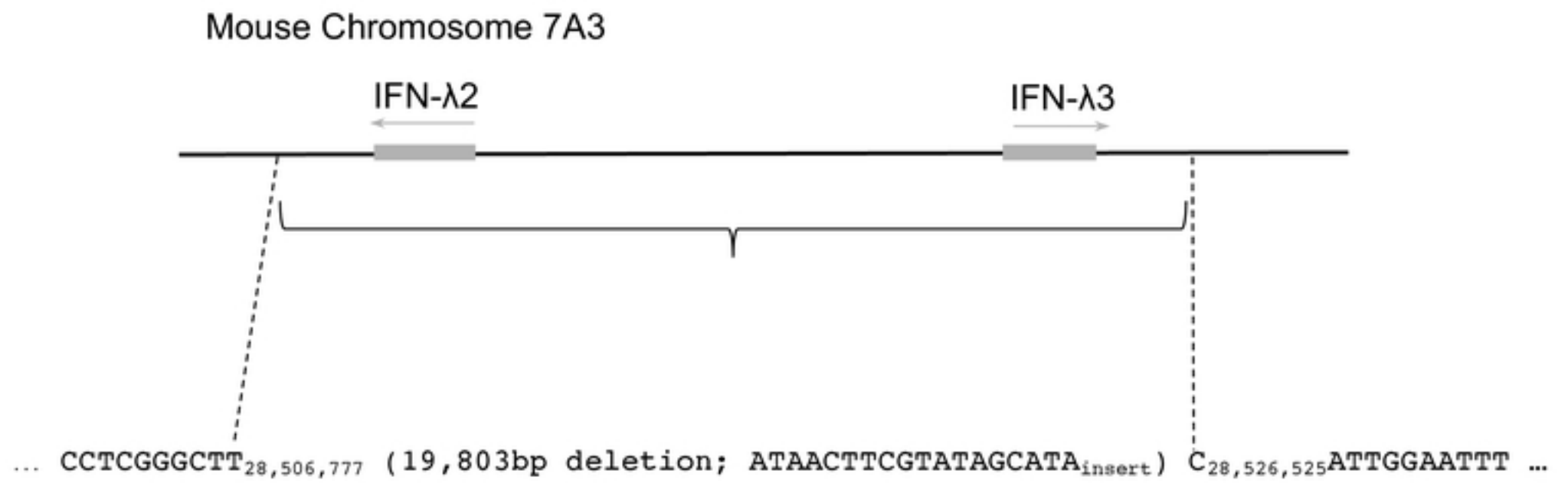


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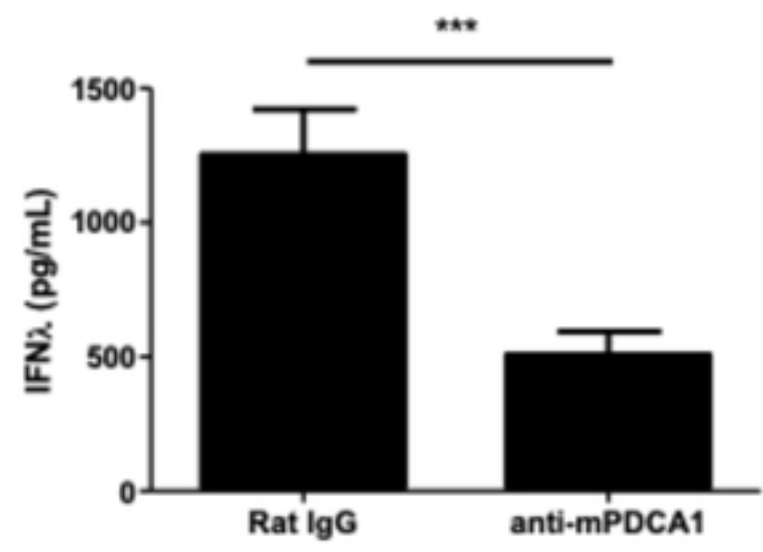
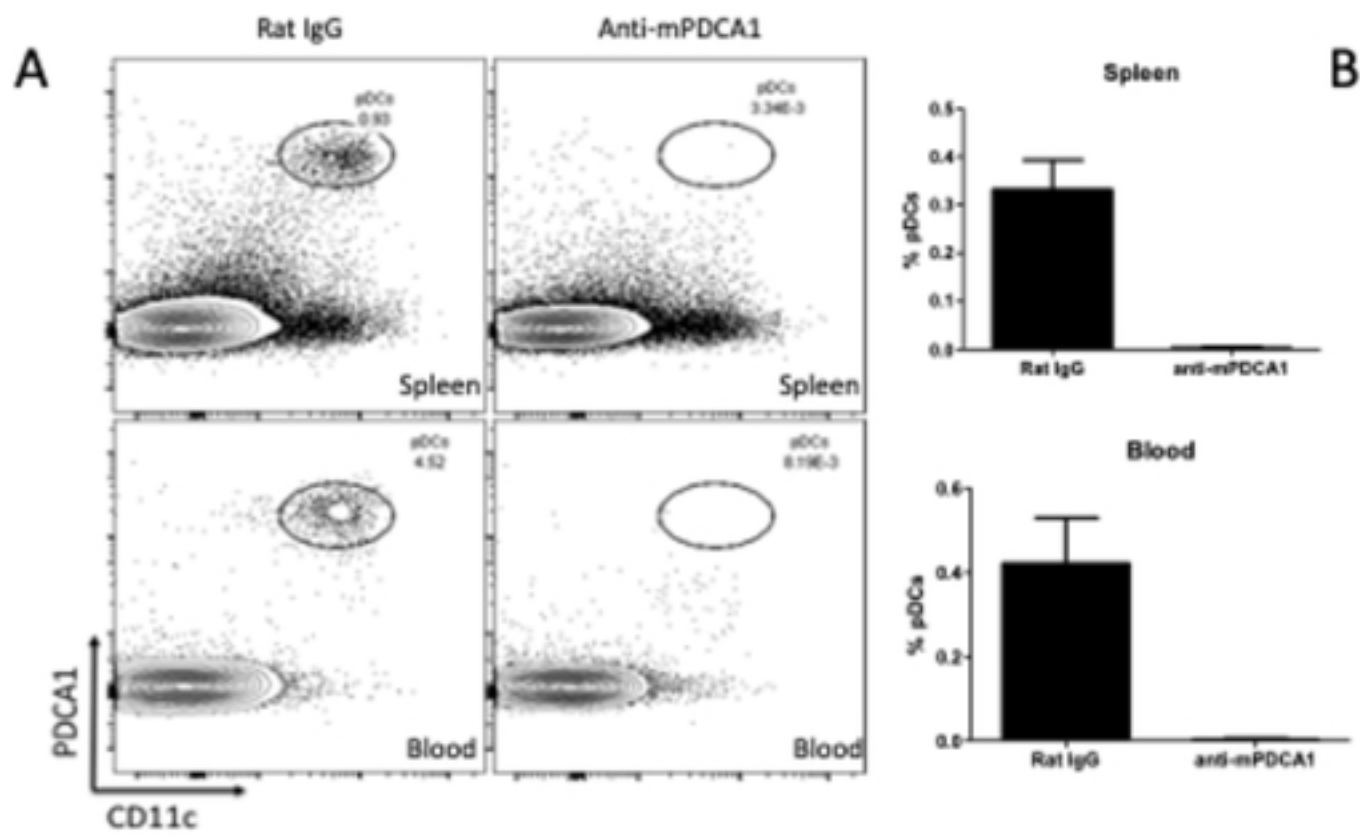


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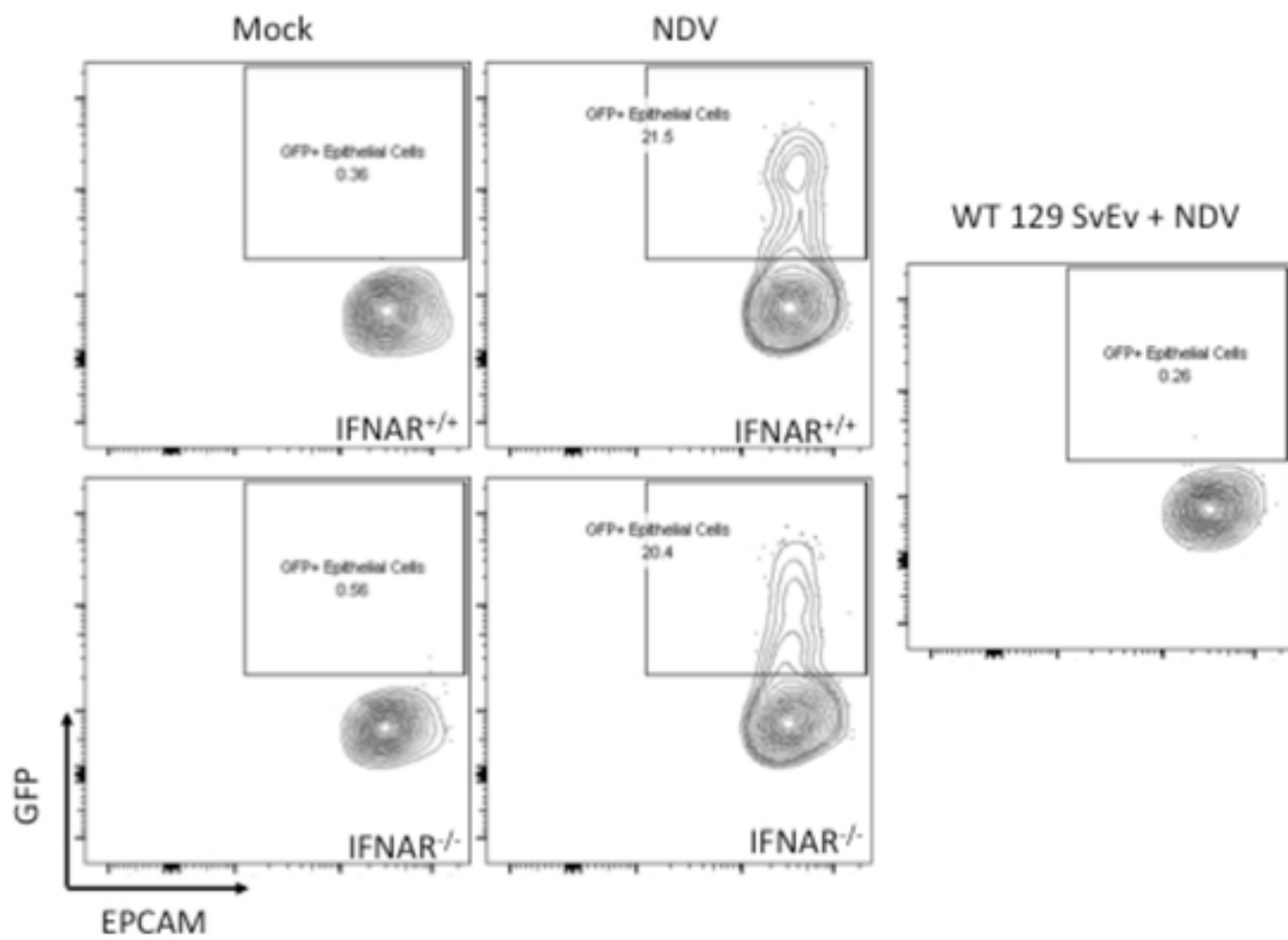




The region encompassing the IFN- λ 2 and IFN- λ 3 genes on murine chromosome 7, that was deleted by the Crispr/Cas9 Technology, is schematically shown.



Figure



Figure