

1 **Prophylactic protection against respiratory viruses conferred by a prototype**
2 **live attenuated influenza virus vaccine.**

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20 Running Title – **Attenuated Influenza Virus with Antiviral Properties.**

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24 **Abstract**

25 The influenza A non-structural protein 1 (NS1) is known for its ability to hinder the
26 synthesis of type I interferon (IFN) during viral infection. Influenza viruses lacking
27 NS1 (Δ NS1) are under clinical development as live attenuated human influenza virus
28 vaccines and induce potent influenza virus-specific humoral and cellular adaptive
29 immune responses. Attenuation of Δ NS1 influenza viruses is due to their high IFN
30 inducing properties, that limit their replication in vivo. This study demonstrates that
31 pre-treatment with a Δ NS1 virus results in an immediate antiviral state which
32 prevents subsequent replication of homologous and heterologous viruses,
33 preventing disease from virus respiratory pathogens, including SARS-CoV-2. Our
34 studies suggest that Δ NS1 influenza viruses could be used for the prophylaxis of
35 influenza, SARS-CoV-2 and other human respiratory viral infections, and that an
36 influenza virus vaccine based on Δ NS1 live attenuated viruses would confer broad
37 protection against influenza virus infection from the moment of administration, first by
38 non-specific innate immune induction, followed by specific adaptive immunity.

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41 Key words – Type I IFN, Antiviral Therapy, NS1 protein, Influenza A, Interferon Antagonists, SARS-
42 CoV-2

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48 **Introduction**

49 The type I interferon (IFN) response resulting from invading viral pathogens is
50 considered as one of the first lines of antiviral defence mechanisms in higher
51 organisms. The latter process takes place upon the detection of the pathogen
52 associated molecular patterns (PAMPS) by the host pattern recognition receptors
53 (PRRs). Secretion of interferons takes place in both paracrine and autocrine
54 signalling mechanisms, mediated by the canonical JAK/STAT signal transduction
55 pathway along with the transcriptional activation of a particular set of host genes as
56 well as their corresponding promoters defined as IFN-stimulated response elements
57 (ISREs)¹. Subsequent activation of the downstream interferon stimulated genes
58 (ISGs) lead to the transcriptional induction of a plethora of antiviral proteins,
59 including dsRNA-activated protein kinase (PKR) leading to a halt of protein
60 translation, dsRNA-activated oligoadenylate synthetases (OAS) which facilitate the
61 degradation of RNA by activating RNase L and Mx proteins which essentially
62 sequester incoming viral components such as nucleocapsids^{2, 3}. Many studies have
63 demonstrated that viruses have evolved to encode numerous mechanisms to
64 prevent the host IFN-mediated antiviral response at different stages⁴. Viral non-
65 structural proteins such as those of Toscana virus, dengue and HPV can sequester
66 host factors to inhibit type I IFN response^{5,6,7}, while viruses such as vaccinia, adeno
67 and Ebola viruses secrete soluble ligands^{7,8}, or encode miRNAs^{9, 10} and other
68 proteins to confer immune-evasion.

69 The influenza A virus (IAV) non-structural protein 1 (NS1) facilitates several functions
70 ranging from inhibition of host mRNA polyadenylation and subsequent inhibition of
71 their nuclear export as well as inhibition of pre-mRNA splicing^{11, 12}. A growing body of
72 evidence to date has indicated that influenza NS1 protein has IFN antagonistic

73 activity. It was initially shown that a recombinant influenza A virus that lacks the NS1
74 protein(Δ NS1) grew to a titer similar to that of WT virus in IFN deficient systems,
75 albeit being markedly attenuated in IFN competent hosts¹³. This attenuated
76 phenotype can be explained by the inability of the virus to prevent NS1 mediated IFN
77 inhibition. The NS1 protein has been shown to bind to TRIM25 whereby the
78 ubiquitination of the viral RNA sensor RIG-I is inhibited, which eventually results in
79 the inhibition of IFN induction^{14,15}. NS1 has also been shown to prevent IFN
80 production by sequestering the cellular cleavage and polyadenylation specificity
81 factor 30 (CPSF30) in order to halt the processing of host pre-mRNAs, resulting in
82 accumulation of pre-mRNAs in the nucleus as well as the halt of cellular mRNA
83 export to the cytoplasm¹⁶. This subsequently results in the inhibition of host protein
84 production, including IFNs and proteins encoded by IFN inducible genes^{17,18} NS1
85 has also been shown to inhibit the antiviral activity of several IFN-stimulated genes,
86 such as the 2'-5'- oligo A synthase (OAS)¹⁹.

87 Consistent with its function, deletion of NS1 in recombinant IAV results in a live
88 attenuated and highly immunogenic IAV. As a result, IAV with impaired NS1 function
89 are currently used as vaccines against swine influenza in pigs²⁰ and they are under
90 clinical consideration as live attenuated human influenza virus vaccines²¹⁻²³.

91 Based on the growing body of evidence showing the IFN antagonistic properties of
92 IAV NS1, we investigated the ability of the Δ NS1 viruses to induce an immediate IFN
93 response *in vivo* along with the biological antiviral consequences mediated by the
94 type I IFN induction. Our results demonstrate that the Δ NS1 virus is an efficient
95 inducer of IFN with antiviral properties in both mice and embryonated eggs. Our data
96 indicates the suitability of Δ NS1 virus as a prophylactic agent to induce immediate
97 mucosal antiviral responses with the aim of preventing acute respiratory infections

98 caused by IFN sensitive viruses. Δ NS1 influenza viruses can provide first innate
99 antiviral protection, followed by adaptive specific IAV protection.

100 **Results**

101 **Recombinant influenza A virus lacking the NS1 gene (Δ NS1) induces higher** 102 **levels of interferon than wild type viruses in embryonated chicken eggs.**

103 Previously, we demonstrated that tissue culture-based infections by Δ NS1 viruses
104 induced the transactivation of an ISRE-containing reporter gene¹³, indicating that
105 infection by Δ NS1 viruses induces higher levels of IFN in comparison to its wild type
106 counterparts. To test whether Δ NS1 induces IFN in 10-day old embryonated-chicken
107 eggs, eggs were treated with 10^3 PFU of Δ NS1 or PR8-WT influenza viruses.
108 Subsequently, the allantoic fluids were harvested 18 hours post treatment to
109 measure the levels of IFN by determining the highest dilution that inhibited the
110 cytopathic effect mediated by vesicular stomatitis virus (VSV) in chicken embryo
111 fibroblast (CEF) cells. As indicated in the Supplementary table 1, four hundred Uml⁻¹
112 of IFN were detected in the allantoic fluid of eggs infected by Δ NS1 virus. However,
113 allantoic fluids derived from WT-PR8 or mock infections indicated undetectable
114 levels of IFN (<16 Uml⁻¹).

115 **Pre-treatment with Δ NS1 influenza virus inhibits wild-type viral replication in** 116 **embryonated chicken eggs.**

117 We speculated that the ability of the Δ NS1 virus on inducing high titers of IFN in
118 eggs facilitates an antiviral state that may prevent the replication of wild-type IAV. To
119 evaluate this, increasing amounts of Δ NS1 virus were inoculated into eggs and eight
120 hours post-treatment, the eggs were challenged with wild-type A/WSN/33 (WSN-WT)
121 virus with a dose of 10^3 PFU. Two days post incubation extracted allantoic fluids

122 were titrated via plaque assays. WSN viral titers decreased with Δ NS1 in a dose
123 dependent manner. While the untreated allantoic fluids supported the growth of WSN
124 virus to an approximate titer of 10^8 PFUml⁻¹, administration of a dose as little as
125 2×10^4 PFUml⁻¹ of Δ NS1 prevented the replication of WSN virus (less than 10^2
126 PFUml⁻¹ of WSN were obtained in eggs). The titer of WSN virus was reduced by one
127 log, by pre-treating allantoic fluids with as little as 2 PFU of Δ NS1 (Figure 1A).

128 Interestingly, treatment using Δ NS1 virus further inhibited the replication of other
129 viruses, as depicted in figure 1B. Relative HA titers were obtained from eggs treated
130 with 2×10^4 PFUml⁻¹ of Δ NS1 virus followed by subsequent infection with wild-type
131 Influenza A H1N1 strains WSN and PR8, H3N2 strain X-31, influenza B virus or
132 Sendai virus (SeV; a paramyxovirus). In all cases, pre-treatment with Δ NS1 resulted
133 in a two-log reduction of wild-type viral HA titers.

134 **Severe disease and death caused by infection with the highly virulent PR8**
135 **virus (hvPR8) in A2G mice can be alleviated by Δ NS1 pre-treatment.**

136 In order to assess whether or not the administration of Δ NS1 virus inhibits replication
137 of influenza viruses in mice, an inbred mouse strain that is homozygous for the gene
138 which codes for the IFN induced full-length *Mx1* protein, defined as C57BL/6-A2G
139 (abbreviated as A2G) mice were used for this part of the study^{24, 25}. Previous studies
140 have concluded that IFN administration was ineffective in preventing IAV replication
141 in laboratory mice lacking a functional *Mx1* gene²⁶. In contrast, A2G mice which were
142 administered IFN remained alive upon infection with the highly virulent hvPR8 IAV
143 strain²⁷. The presence of a functional *Mx1* gene in A2G mice better mirrors the
144 human situation, as *Mx1* gene deficiencies in humans are rare. Here, A2G mice
145 were intranasally infected with a dose of 5×10^5 PFUml⁻¹ of Δ NS1 virus or PBS at -24,

146 -8, +3, +24 and +48 hours. Mice were challenged at time 0 intranasally with 5×10^6
147 PFU of hvPR8 virus. Mice treated with Δ NS1 virus were protected from hvPR8 virus
148 as measured by weight loss and death while the PBS treated mice succumbed to
149 death (Figure 2A).

150 Subsequently, we examined whether all five Δ NS1 treatments were essential for the
151 protective effect against hvPR8 infection in mice. Hence, a single dose of 5×10^6 PFU
152 of Δ NS1 virus was given at various time points relative to the infection with hvPR8.
153 Data indicated (Figure 2B) that pre-treatment (hours 24 or 8 before hvPR8
154 challenge) but not post treatment (even 3 hours post hvPR8 challenge) of Δ NS1
155 resulted in the prevention of weight loss disease and subsequent death. Additionally,
156 Δ NS1 virus administered two or four days prior to hvPR8 challenge completely
157 protected mice from disease (Figure 2C).

158 Next, to obtain the effective dose 50 (ED_{50}) of Δ NS1 virus to mediate protection
159 against disease from hvPR8 infection, 2×10^5 , 2×10^4 , 2×10^3 or 2×10^2 doses of Δ NS1
160 virus were intranasally administered to A2G mice 24 hours prior to hvPR8 challenge.
161 As shown in Figure 2D, the ED_{50} of the Δ NS1 virus which conferred protection in
162 A2G mice against hvPR8-induced death was approximately 10^3 PFU.

163 **Induction of *Mx1* specific mRNA in mice treated with Δ NS1 virus.**

164 To investigate whether Δ NS1 infection in mice resulted in induction of the *Mx1* gene,
165 an RT-PCR assay for *Mx1* specific mRNA in infected animal lungs infected was
166 developed. In parallel, infections were performed in BALB/c mice which have a non-
167 functional *Mx1* gene due to a large frameshift deletion²⁶. As seen in figure 3A,
168 treatment with Δ NS1 resulted in the early induction (24 hours post infection) of *Mx1*
169 specific mRNA in both A2G and BALB/c mice. In contrast a very faint band was

170 present in A2G mice infected with hvPR8 virus at the same time post infection and
171 no specific mRNA was detected in mock infected mRNA.

172 **Δ NS1 mediated protection from hvPR8 is *Mx1*-mediated.**

173 As the *Mx1* protein is one of the most potent IFN inducible gene products with anti-
174 influenza virus activity in mice, it is quite possible that the Δ NS1-mediated protection
175 seen in A2G mice is *Mx1*-mediated. To test this hypothesis, we compared the
176 antiviral activity of Δ NS1 in A2G mice and in C57BL/6 mice. C57BL/6 mice harbour
177 a non-functional *Mx1* gene due to a known deletion²⁶ and were used as a back-cross
178 genetic platform for the original A2G strain to generate the *Mx1* positive A2G mice
179 used in our experiments. A dose of PR8- Δ NS1 containing 5×10^6 PFU given 12H
180 before a lethal hvPR8 challenge protected all A2G-*Mx1* mice (n=5) in both morbidity
181 and mortality in comparison to the PBS pre-treated group (n=5). However, all five
182 *Mx1*-deficient mice in the wild-type C57BL/6 group that were given the same dose of
183 PR8- Δ NS1 succumbed to death by a lethal hvPR8 challenge. The morbidity data for
184 these mice based on body weight was also consistent with lack of protection after
185 Δ NS1 treatment from hvPR8 challenge, indicating that the antiviral effect on IAV
186 induced in mice by Δ NS1 treatment is dependent on the IFN-inducible gene *Mx1* w
187 (Figure 3D and 3E).

188 **Δ NS1 viral treatment inhibits the replication of hvPR8 virus in A2G mice lungs.**

189 To better understand the ability of the Δ NS1 virus to inhibit replication of the hvPR8
190 virus in the lungs, A2G mice were intranasally treated with 2×10^5 PFU of Δ NS1 virus
191 alone, 2×10^4 PFU of hvPR8 alone or treatment of 2×10^5 PFU of Δ NS1 virus 24 hours
192 before infecting them with 2×10^4 PFU of hvPR8 virus. Mice were sacrificed at three-
193 and six-days post infection and the lung homogenates were titrated in MDCK or Vero

194 cells (Supplementary table.2). A reduction of hvPR8 titers in lungs by fourfold was
195 observed when mice were pre-treated with Δ NS1 virus. Furthermore, mice solely
196 infected with Δ NS1 virus had titers below the detection limit (<10 PFU/ml¹), while not
197 showing any significant reduction of bodyweight. It was apparent that infection by
198 hvPR8 virus without Δ NS1 administration resulted in the increase of lung weight by a
199 factor of two or three in comparison to mice that were pre-treated with Δ NS1 virus. In
200 the context of this study, increased lung weights are suggestive of lymphocytic
201 infiltration and pulmonary disease during Influenza virus infection^{28, 29}.

202 **Attenuated influenza viruses via a mutation in the Neuraminidase (NA) gene**
203 **does not confer Δ NS1-like antiviral properties.**

204 Antiviral properties observed thus far in this study is from an attenuated influenza
205 virus lacking the NS1 gene (Δ NS1). To confirm that the protective effects observed
206 here are not due to the attenuation caused by the lack of a gene but specifically due
207 to the lack of NS1, the antiviral property of Δ NS1 virus was compared to that of a the
208 recombinant D2 influenza virus. The D2 virus contains a base-pair mutation in the
209 dsRNA region formed by the non-coding sequences of its NA gene. This mutation is
210 responsible for a 10-fold reduction in the NA protein levels as well as a one-log
211 reduction in viral titers within a multicycle growth curve³⁰. The latter D2 strain has
212 also been shown to be highly attenuated in mice with a LD₅₀ of more than 10^6 PFU
213 upon intranasal administration³¹. Identical doses (2.5×10^5 PFU) of D2 or Δ NS1
214 viruses were intranasally administered to A2G mice four hours prior to challenge with
215 5×10^6 PFU of hvPR8. Although a prolonged survival was seen in one of the animals
216 who received D2, pre-treatment with D2 was ineffective in protecting A2G mice from
217 hvPR8 virus-induced disease and death (Figure 4).

218 **Δ NS1 viral treatment prevents death by Sendai virus (SeV) in C57BL/6 mice**

219 Given the fact, that the antiviral effects against hvPR8 mediated by Δ NS1 viral are
220 facilitated by an IFN mediated mechanism (*Mx1* gene induction), we speculated that
221 Δ NS1 treatment should protect mice from infections by other IFN sensitive viruses.
222 Sendai virus was used in this study due to its pneumotropic nature and sensitivity to
223 IFN in *Mx1* deficient mice^{32, 33}. As seen in Figure 1B, treatment with Δ NS1 inhibited
224 Sendai viral replication in embryonated chicken eggs. Moreover, upon two intranasal
225 administrations of 2.5×10^5 PFU of Δ NS1 virus to C57BL/6 mice at times -24 and +24
226 hours or -8 and +72 hours, mice infected with 5×10^5 PFU of Sendai virus were
227 protected from death (Figure 5A). The C57BL/6 mice used here are *Mx1*^{-/-} and it is
228 indicative that the mouse nuclear Mx1 protein does not have any antiviral activities
229 against cytoplasmic viruses such as Sendai virus³⁴. The efficacy of Δ NS1 treatment
230 was compared against three doses of IFN- β using the Sendai virus challenge model.
231 Treatment with the highest dose of IFN- β (2×10^5 U) protected mice from death
232 induced by Sendai virus comparable to treatment with 2.5×10^5 PFU of Δ NS1 virus
233 (Figure 5B).

234 **Δ NS1 virus treatment inhibits viral replication of SARS-CoV-2 virus in K18-
235 hACE2-C57Bl/6 murine lungs.**

236 Given the emergence of the devastating COVID-19 pandemic, we assessed
237 whether prophylactic treatment with Δ NS1 would hinder the replication of SARS-CoV-
238 2. We used the transgenic mouse model that supports the replication of SARS-
239 CoV2. As controls, we used universal IFN, and SeV defective RNA (SDI) which were
240 previously shown to have an IFN inducing effect. Weight determination in all the
241 treated groups showed no major loss in bodyweight, only one mouse each from the

242 SDI treated group (day 8) and the uFN treated group (day 12) reached below 75%
243 bodyweight (Figure 6A). Deaths (4 out of 5) in the mock treated group occurred
244 between days 6-8 post infection. The SDI-RNA treated group lost 2 out of 5 animals
245 on day 8 and 9 while the uFN group lost one animal out of 5 at a later time point
246 (D12; Figure 6B). While both treatments resulted in reduction of viral titers day 3 and
247 5 post infection, mice that received Δ NS1 showed significant inhibition of SARS-
248 CoV2 titers in lung homogenates and no detectable infectious viruses at day 5 post
249 infection (Figure 6C).

250 **Discussion**

251 The NS1 protein of the influenza A virus has been shown to possess IFN antagonist
252 activity whereby it is able to dampen the host innate immune response to provide a
253 favourable environment for the virus to replicate. It has been demonstrated to be
254 highly expressed in the host cytoplasm and nucleus upon viral infection, interacting
255 with a plethora of host factors to inhibit the interferon response³⁵. Data show the
256 ability of NS1 to compete with innate immune sensors such as RLR to bind to
257 dsRNA to avoid innate immune detection³⁶. Additionally, NS1 has been shown to
258 interact with other innate immune signalling components such as PKR³⁷, TRIM25³⁸
259 and CPSF¹⁶, resulting in lowering of the IFN mediated innate immunity³⁹. For these
260 reasons, influenza viruses with impaired NS1 function (and an increased innate
261 immune response) have been under consideration for live attenuated influenza
262 vaccines. There is an existing swine influenza vaccine based on NS1-deficient live
263 attenuated viruses⁴⁰, and clinical trials in humans using an intranasally administered
264 live attenuated Δ NS1 virus have demonstrated potent immunogenicity and good
265 safety profiles. Experimental evidence in mice indicates that the high IFN-inducing
266 properties of Δ NS1 viruses are responsible for their superior immunogenicity as live

267 vaccines^{41, 42}.

268 As Δ NS1 viruses are great IFN inducers, we reasoned that they might provide with
269 innate protection against respiratory virus infection even before the development for
270 an influenza virus specific adaptive immune response. Treatment with Δ NS1 virus
271 inhibited the replication of both homologous and heterologous viruses in eggs
272 (Figure.1). Using the A2G-Mx1 mouse model, we demonstrated that the intranasal
273 administration of the Δ NS1 virus induced an antiviral state, which prevented disease
274 and death by a highly pathogenic influenza A virus (hvPR8) which is otherwise
275 lethal⁴³. Infection with Δ NS1 virus but not WT viruses yielded detectable levels of
276 *Mx1*-specific mRNA levels in lungs 24 hours post infection (Figure 2). A large body of
277 evidence has indicated that the protective impact of IFN against IAV infection in mice
278 is mainly mediated by the IFN inducible antiviral *Mx1* gene⁴⁴⁻⁴⁶. Consistently, we
279 found that *Mx1* was required for the Δ NS1 mediated protection against lethal hvPR8
280 challenge by comparing *Mx1* competent A2G--C57BL/6 mice with *Mx1* deficient WT-
281 C57BL/6 mice.

282 Data depicted in Figure.2C show that pre-treatment of A2G mice with Δ NS1 virus up
283 to four days before the challenge with hvPR8 virus was effective in preventing
284 disease. The *Mx1* protein in mice is known to be stable for several days upon its
285 induction and our observations are consistent with the half-life of the *Mx1* protein
286 described in mice^{47, 48}.

287 Given the inherently attenuated state of the Δ NS1 viruses, it was necessary to
288 confirm that the antiviral state seen here is due to the specific attenuation of the
289 Δ NS1 segment. We used a virus that is known to be attenuated due to its defective
290 neuraminidase segment (D2 virus expressing a full-length NS1)³¹ to demonstrate

291 that protection is not just mediated by any attenuated IAV (Figure.4). Δ NS1 treated
292 mice were also protected from lethal infection with an influenza-unrelated
293 pneumotropic Sendai virus, suggesting that the IFN-mediated innate immune
294 response induced by Δ NS1 has broad-antiviral effects, rather than being a pathogen-
295 specific immune response. As anticipated for Sendai virus, the abovementioned
296 protection was not *Mx1* mediated and is most likely due to the activation of other
297 ISGs such as OAS or PKR upon the Δ NS1-mediated IFN production⁴⁹.

298 The feasibility of Δ NS1 virus as a prophylactic treatment to induce a type I interferon
299 response to prevent acute respiratory infections from IFN sensitive viruses was
300 demonstrated in the current study. Type I interferon administration has been used to
301 treat a range of human diseases ranging from infections such as hepatitis B and C⁵⁰,
302 ⁵¹ to other non-communicable diseases such as melanomas⁵² and hairy-cell
303 leukaemia⁵³. Although IFN has been promoted as a therapeutic agent, administration
304 of exogenous interferon comes with a set of undesirable side effects^{54, 55}, arguably
305 due to its causing major endocrine and metabolic changes in the host⁵⁶. Therefore,
306 various groups have attempted alternative ways to induce local type I IFN responses
307 using different strategies. Some of these strategies were topical administration of
308 plasmid DNA coding for IFN α 1 in the mouse eye to protect against HSV-1
309 encephalitis⁵⁷, liposomic intranasal treatment using dsRNA to induce IFN⁵⁸ as well
310 as recombinant viral vectors such as adenoviruses⁵⁹ and hepatitis B viruses to
311 express type I IFN to protect against infection and tumor regression⁵⁹. Despite these
312 experimental attempts to study the efficacy of IFN, it is still unclear whether virally
313 induced IFN is more or less toxic efficient than IFN itself. This indicates that further
314 work is needed to be done to ascertain the suitability of recombinant viruses as IFN
315 inducers for therapeutic purposes. The physiological half-lives and binding affinities

316 of different types of interferons are well studied and their half-lives can range from
317 minutes to several hours, depending on the type of IFN⁶⁰. Our data showed antiviral
318 properties of Δ NS1 virus for up to four days before the viral challenge. While it is
319 known that therapeutic properties and doses of different types of IFNs are highly
320 variable due to their differential effects contributed by the ISGs, most therapeutic
321 properties of type I interferons are yet to be completely understood^{61, 62}. In this
322 instance, comparable prophylactic responses were obtained by the administration of
323 either 2×10^5 U of IFN- β or 2×10^5 PFU of Δ NS1 virus (Figure.5B). However, it is
324 acknowledged that different subsets of IFN-regulated genes may differ in their
325 relative transcriptional induction between treatments.

326 We also demonstrated that prophylactic treatment using Δ NS1 significantly inhibited
327 viral replication in a relevant mouse model that can be infected with WT SARS-CoV-
328 2 and is known to result in lethal infection⁶³(Figure 6). This agrees with reports that
329 state that SARS-CoV-2 is sensitive to IFN⁶⁴. Interestingly, a similar level of reduction
330 in viral titers was not seen upon intranasal inoculation of universal-IFN nor defective
331 interfering RNA derived from SeV (SDE-RNA; a RIG-I agonist with known
332 adjuvanting properties)⁶⁵. While these treatments resulted in a better outcome in
333 comparison to PBS pre-treatment, high amounts of viral titers were still observed day
334 three and five post infection. Although weight loss and survival were best in the
335 Δ NS1 group, the uIFN treated group showed a protective phenotype indicating that
336 uIFN treatment was better than that provided by SDI-RNA. The difference observed
337 here is likely due to the stimulation of multiple innate immune mechanisms by Δ NS1
338 which potentially primes cells to confer a broad antiviral phenotype. However,
339 analysis of differentially expressed genes (particularly ISGs) via a technique such as
340 bulk RNAseq would provide more insights in explaining the observed protective

341 effects against COVID-19 in the K18 mouse model.

342 In conclusion, we report that prophylactic treatment with an attenuated influenza A
343 virus lacking the NS1 gene induces an innate antiviral response which provides
344 protection against IFN-sensitive viruses in both embryonated chicken eggs and mice.
345 These *in vivo* data further validate previous observations showing the IFN-
346 antagonistic properties of the NS1 protein of influenza A viruses^{13, 66-68}, while
347 highlighting the role of NS1 in inhibiting IFN induction during influenza A virus
348 infections. We also provide evidence for its therapeutic potential as a prophylactic to
349 protect against acute respiratory infections caused by IFN-sensitive viruses including
350 the causative agent of COVID-19 pandemic. Δ NS1 viruses are being clinically
351 developed as live attenuated influenza virus vaccines and in clinical trials they have
352 shown to induce protective antibodies and no adverse responses in human
353 volunteers²¹⁻²³. Here we show that Δ NS1 viruses have the potential to induce
354 immediate protection against viral infection prior to the induction of specific long-
355 lasting protective adaptive immune responses^{69, 70}. Our results should encourage
356 further research on the use of IFN-inducing, live attenuated virus vaccines, to confer
357 innate and adaptive protection against virus pathogens.

358 **Methods**

359 **Cells and viruses**

360 Recombinant influenza A viruses were generated using reverse genetics as
361 previously described^{13, 30}. A derivative of the A/PR/8/34 (PR8) defined as highly
362 virulent PR8 (hvPR8) was kindly provided by O. Haller and J.L. Schulman. Strain 52
363 of Sendai virus was obtained from the ATCC. Vero cells, Madin-Darby bovine kidney
364 (MDBK) cells, baby hamster kidney (BHK) cells or embryonated chicken eggs were

365 used to propagate the following viruses as per standard protocols; Influenza A
366 Δ NS1, hvPR8, PR8, A/WSN/33, A/X-31/H3N2, Influenza B/Lee/40, Sendai virus and
367 vesicular stomatitis virus (VSV). Madin-Darby canine kidney (MDCK) cells or Vero
368 cells were plated to obtain confluent monolayers and plaque assays were performed
369 as previously described and an agar overlay in DMEM-F12 including 1 μ gml⁻¹ of
370 trypsin was used. MDCK, cVero and BHK cells were cultured in DMEM in the
371 presence of 10% FBS and penicillin-streptomycin. The chicken embryo fibroblasts
372 (CEF) purchased from ATCC was maintained in MEM as suggested by ATCC. Vero-
373 E6 cells (ATCC® CRL-1586™, clone E6) were grown in DMEM containing 10%
374 FBS, non-essential amino acids, HEPES and penicillin-streptomycin. SARS-
375 CoV-2, isolate USA-WA1/2020 (BEI resources; NR-52281) was handled under BSL-
376 3 containment in accordance with the biosafety protocols validated by the Icahn
377 School of Medicine at Mount Sinai. Viral stocks were amplified in Vero-E6 cells in the
378 above media containing 2% FBS for three days and were validated by whole-
379 genome sequencing using the Oxford-MinION platform.

380 **Animal studies**

381 All animals used in the study were used at 6-10 weeks of age. A2G mice were kindly
382 provided by Dr. Heinz Arnheiter while the BALB/c and C56BL/6 mice were
383 purchased from Taconic Farms. Hemizygous female K18-hACE2 mice on the
384 C57BL/6J genetic background (Jax strain 034860), were used to conduct studies
385 with SARS-CoV-2 in BSL3 conditions. Anesthetized animals were intranasally
386 infected using 30 to 50 μ l of appropriately diluted viruses or PBS containing the
387 indicated amounts of recombinant murine IFN- β (Calbiochem), universal-IFN (PBL
388 assay science) SDI-RNA⁶⁵. Afterwards, the animals were monitored daily for
389 changes in body weight. All animal studies were done in accordance with the NIH

390 guidelines as well as the guidelines devised by the Icahn School of Medicine with
391 regards to the care and use of laboratory animals.

392 **Measurement of Interferon**

393 Ten day old embryonated eggs were infected with 10^3 PFU in 100 μ l containing either
394 Δ NS1, PR8 viruses or PBS as mock. Next, the eggs were incubated at 37°C and the
395 allantoic fluids were extracted 18 hours post infection. Viral inactivation of the
396 allantoic fluids were conducted by dialysis against 0.1 M KCL-HCL buffer at pH 2 for
397 two days at 4°C. Later, the pH of the samples was adjusted to pH 7 by subsequent
398 dialysis against Hank's balanced sodium salt solution with 20 mM Na_3PO_4 for two
399 more days as described previously⁷¹. The amount of IFN was titrated according to its
400 ability to inhibit the growth of VSV⁷². In summary, CEF cells in 96wells were treated
401 with 100 μ l of different dilutions of the respective samples in tissue culture media.
402 Upon incubating for an hour at 37°C, 200 TCID₅₀ of VSV in 10 μ l were added to the
403 wells before incubating at 37°C until complete lysis of untreated control cells was
404 observed (approximately two days). As a standard control, recombinant chicken IFN
405 donated by Drs. Peter Staeheli and Bernd Kaspers was used⁷³.

406 **Lung Titration**

407 Four A2G mice were intranasally challenged with 2×10^5 PFU of Δ NS1 at day -1.
408 During day 0 mice were intranasally challenged with 2×10^4 PFU of hvPR8 virus.
409 Alternatively, two other groups of four A2G mice were challenged with 2×10^5 PFU
410 Δ NS1 or 2×10^4 PFU of hvPR8. Three days post infection, two animals from each
411 group were humanely sacrificed while the rest of the animals were humanely
412 sacrificed six days post infection. Lungs were weighed and homogenized in 2 ml of
413 PBS. Resulting homogenates were clarified via centrifugation at 3000 rpm for 15

414 minutes at 4°C and the acquired supernatants were tittered by plaque assays using
415 MDCK or Vero cells. Lung homogenates derived from SARS-CoV-2 infected K18
416 mice were handled and titered in Vero-E6 cells as described previously⁷⁴.

417 **Detection of *MX1* Specific mRNA in infected cells**

418 A2G and BALB/c mice were intranasally challenged with 10⁵ PFU of either ΔNS1 or
419 hvPR8 or PBS. Afterwards, lungs were extracted 24 hours post infection, snap
420 frozen, homogenized, total RNA was extracted using TRIreagent (Sigma-Alderich).
421 One microgram of total lung RNA was used to perform a RT reaction in a total
422 volume of 20 µl using *Mx1* specific primer. Two µl of the resulting RT product was
423 used for PCR amplification using *Mx1* specific primers under the following conditions
424 (20 seconds at 95°C, 30 seconds at 55°C, 30 seconds at 72°C for a total of 25
425 cycles). The sense and antisense primer sequences are as follows; 5'-
426 CAGGACATCCAAGAGCAGCTGAGCCTCACT-3' and 5'-
427 GCAGTAGACAATCTGTTCCATCTGGAAGTG-3'. The PCR products were analysed
428 using a 1.2% agarose gel. Correct size for the PCR products in A2G mice was 756
429 bp while it was 333 bp in BALB/c mice due to a deletion in the *Mx1* gene between
430 nucleotides 1120-1543³¹.

431 **Acknowledgements**

432 The authors acknowledge members of AG-S and PP laboratories for their critical
433 discussions. Technical assistance for the study was provided by Louis Ngyenvu and
434 Richard Cadagan. We thank R. Albert for support with the BSL3 facility and
435 procedures at the Icahn School of Medicine at Mount Sinai, New York. A2G mice
436 were kindly provided by Dr. Heinz Arnheiter (NIH). hvPR8 virus was a generous gift
437 from Drs. Otto Haller (University of Freiburg) and Jerome L. Schulman (Icahn School

438 of Medicine). Recombinant Chicken IFN was a gift from Drs. Peter Staeheli
439 (University of Freiburg) and Bernd Kaspers (University of Munich). This work was
440 partly supported by the Center for Research for Influenza Pathogenesis, a Center of
441 Excellence for Influenza Research and Surveillance supported by the National
442 Institute of Allergy and Infectious Diseases (contract number
443 HHSN272201400008C), by the NIAID funded Collaborative Influenza Vaccine
444 Innovation Centers (contract number 75N93019C00051), by NIAID grants
445 R01AI141226, R01AI145870 and P01AI097092, by DARPA grant HR0011-19-
446 2-0020, and by the generous support of the JPB Foundation, the Open Philanthropy
447 Project (research grant 2020-215611 (5384)), and anonymous donors to AG-S and
448 PP.

449

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References

- 451 1. Taft, J. & Bogunovic, D. The Goldilocks Zone of Type I IFNs: Lessons from
452 Human Genetics. *The Journal of Immunology* **201**, 3479-3485 (2018).
- 453 2. Ivashkiv, L. B. & Donlin, L. T. Regulation of type I interferon responses. *Nature*
454 *reviews Immunology* **14**, 36 (2014).
- 455 3. Schneider, W. M., Chevillotte, M. D. & Rice, C. M. Interferon-stimulated genes: a
456 complex web of host defenses. *Annu. Rev. Immunol.* **32**, 513-545 (2014).
- 457 4. García-Sastre, A. Ten strategies of interferon evasion by viruses. *Cell host &*
458 *microbe* **22**, 176-184 (2017).
- 459 5. Gori-Savellini, G., Valentini, M. & Cusi, M. G. Toscana virus NSs protein inhibits
460 the induction of type I interferon by interacting with RIG-I. *J. Virol.* **87**, 6660-6667
461 (2013).
- 462 6. Chan, Y. K. & Gack, M. U. A phosphomimetic-based mechanism of dengue virus
463 to antagonize innate immunity. *Nat. Immunol.* **17**, 523 (2016).
- 464 7. Ronco, L. V., Karpova, A. Y., Vidal, M. & Howley, P. M. Human papillomavirus 16
465 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional
466 activity. *Genes Dev.* **12**, 2061-2072 (1998).

- 467 8. Smith, G. L., Talbot-Cooper, C. & Lu, Y. in *Advances in virus research* 355-378
468 (Elsevier, 2018).
- 469 9. Edwards, M. R. *et al.* Differential regulation of interferon responses by Ebola and
470 Marburg virus VP35 proteins. *Cell reports* **14**, 1632-1640 (2016).
- 471 10. Nidetz, N. F., Gallagher, T. M. & Wiethoff, C. M. Inhibition of type I interferon
472 responses by adenovirus serotype-dependent Gas6 binding. *Virology* **515**, 150-157
473 (2018).
- 474 11. Fortes, P., Beloso, A. & Ortin, J. Influenza virus NS1 protein inhibits pre-mRNA
475 splicing and blocks mRNA nucleocytoplasmic transport. *EMBO J.* **13**, 704-712
476 (1994).
- 477 12. Qiu, Y. & Krug, R. M. The influenza virus NS1 protein is a poly(A)-binding protein
478 that inhibits nuclear export of mRNAs containing poly(A). *J. Virol.* **68**, 2425-2432
479 (1994).
- 480 13. García-Sastre, A. *et al.* Influenza A virus lacking the NS1 gene replicates in
481 interferon-deficient systems. *Virology* **252**, 324-330 (1998).
- 482 14. Castanier, C. *et al.* MAVS ubiquitination by the E3 ligase TRIM25 and
483 degradation by the proteasome is involved in type I interferon production after
484 activation of the antiviral RIG-I-like receptors. *BMC biology* **10**, 44 (2012).
- 485 15. Koliopoulos, M. G. *et al.* Molecular mechanism of influenza A NS1-mediated
486 TRIM25 recognition and inhibition. *Nature communications* **9**, 1820 (2018).
- 487 16. Nemeroff, M. E., Barabino, S. M., Li, Y., Keller, W. & Krug, R. M. Influenza virus
488 NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 3' end
489 formation of cellular pre-mRNAs. *Mol. Cell* **1**, 991-1000 (1998).
- 490 17. Kuo, R. L. *et al.* Role of N Terminus-Truncated NS1 Proteins of Influenza A Virus
491 in Inhibiting IRF3 Activation. *J. Virol.* **90**, 4696-4705 (2016).
- 492 18. Krug, R. M. Functions of the influenza A virus NS1 protein in antiviral defense.
493 *Current opinion in virology* **12**, 1-6 (2015).
- 494 19. Mibayashi, M. *et al.* Inhibition of retinoic acid-inducible gene I-mediated induction
495 of beta interferon by the NS1 protein of influenza A virus. *J. Virol.* **81**, 514-524
496 (2007).
- 497 20. Richt, J. A. *et al.* Vaccination of pigs against swine influenza viruses by using an
498 NS1-truncated modified live-virus vaccine. *J. Virol.* **80**, 11009-11018 (2006).
- 499 21. Nicolodi, C. *et al.* Safety and immunogenicity of a replication-deficient H5N1
500 influenza virus vaccine lacking NS1. *Vaccine* **37**, 3722-3729 (2019).

- 501 22. Morokutti, A., Muster, T. & Ferko, B. Intranasal vaccination with a replication-
502 deficient influenza virus induces heterosubtypic neutralising mucosal IgA antibodies
503 in humans. *Vaccine* **32**, 1897-1900 (2014).
- 504 23. Mössler, C. *et al.* Phase I/II trial of a replication-deficient trivalent influenza virus
505 vaccine lacking NS1. *Vaccine* **31**, 6194-6200 (2013).
- 506 24. Verhelst, J. *et al.* Functional Comparison of Mx1 from Two Different Mouse
507 Species Reveals the Involvement of Loop L4 in the Antiviral Activity against
508 Influenza A Viruses. *J. Virol.* **89**, 10879-10890 (2015).
- 509 25. Haller, O., Arnheiter, H., Lindenmann, J. & Gresser, I. Host gene influences
510 sensitivity to interferon action selectively for influenza virus. *Nature* **283**, 660 (1980).
- 511 26. Staeheli, P., Grob, R., Meier, E., Sutcliffe, J. G. & Haller, O. Influenza virus-
512 susceptible mice carry Mx genes with a large deletion or a nonsense mutation. *Mol.*
513 *Cell. Biol.* **8**, 4518-4523 (1988).
- 514 27. HALLER, O., ARNHEITER, H., HORISBERGER, M., GRESSER, I. &
515 LINDENMANN, J. *GENETIC-CONTROL OF ANTIVIRAL ACTIVITIES OF*
516 *INTERFERON IN MICE* (EXPERIENTIA Ser. 37, BIRKHAUSER VERLAG AG PO
517 BOX 133 KLOSTERBERG 23, CH-4010 BASEL, SWITZERLAND, 1981).
- 518 28. Guo, L. *et al.* Pulmonary immune cells and inflammatory cytokine dysregulation
519 are associated with mortality of IL-1R1 (-/-)mice infected with influenza virus (H1N1).
520 *Zool. Res.* **38**, 146-154 (2017).
- 521 29. Mizuta, T. *et al.* Antisense oligonucleotides directed against the viral RNA
522 polymerase gene enhance survival of mice infected with influenza A. *Nat.*
523 *Biotechnol.* **17**, 583 (1999).
- 524 30. Fodor, E., Palese, P., Brownlee, G. G. & Garcia-Sastre, A. Attenuation of
525 influenza A virus mRNA levels by promoter mutations. *J. Virol.* **72**, 6283-6290
526 (1998).
- 527 31. Solorzano, A. *et al.* Reduced levels of neuraminidase of influenza A viruses
528 correlate with attenuated phenotypes in mice. *J. Gen. Virol.* **81**, 737-742 (2000).
- 529 32. Tashiro, M. & Homma, M. Pneumotropism of Sendai virus in relation to protease-
530 mediated activation in mouse lungs. *Infect. Immun.* **39**, 879-888 (1983).
- 531 33. Perler, L., Pfister, H., Schweizer, M., Peterhans, E. & Jungi, T. W. A bioassay for
532 interferon type I based on inhibition of Sendai virus growth. *J. Immunol. Methods*
533 **222**, 189-196 (1999).
- 534 34. Pavlovic, J., Zurcher, T., Haller, O. & Staeheli, P. Resistance to influenza virus
535 and vesicular stomatitis virus conferred by expression of human MxA protein. *J.*
536 *Virol.* **64**, 3370-3375 (1990).

- 537 35. Hale, B. G., Albrecht, R. A. & García-Sastre, A. Innate immune evasion
538 strategies of influenza viruses. *Future microbiology* **5**, 23-41 (2010).
- 539 36. Min, J. & Krug, R. M. The primary function of RNA binding by the influenza A
540 virus NS1 protein in infected cells: inhibiting the 2'-5' oligo (A) synthetase/RNase L
541 pathway. *Proceedings of the National Academy of Sciences* **103**, 7100-7105 (2006).
- 542 37. Li, S., Min, J., Krug, R. M. & Sen, G. C. Binding of the influenza A virus NS1
543 protein to PKR mediates the inhibition of its activation by either PACT or double-
544 stranded RNA. *Virology* **349**, 13-21 (2006).
- 545 38. Gack, M. U. *et al.* Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to
546 evade recognition by the host viral RNA sensor RIG-I. *Cell host & microbe* **5**, 439-
547 449 (2009).
- 548 39. Das, K., Aramini, J. M., Ma, L., Krug, R. M. & Arnold, E. Structures of influenza A
549 proteins and insights into antiviral drug targets. *Nature structural & molecular biology*
550 **17**, 530 (2010).
- 551 40. Vincent, A. L. *et al.* Efficacy of intranasal administration of a truncated NS1
552 modified live influenza virus vaccine in swine. *Vaccine* **25**, 7999-8009 (2007).
- 553 41. Mueller, S. N., Langley, W. A., Carnero, E., Garcia-Sastre, A. & Ahmed, R.
554 Immunization with live attenuated influenza viruses that express altered NS1
555 proteins results in potent and protective memory CD8+ T-cell responses. *J. Virol.* **84**,
556 1847-1855 (2010).
- 557 42. Kochs, G. *et al.* Strong interferon-inducing capacity of a highly virulent variant of
558 influenza A virus strain PR8 with deletions in the NS1 gene. *J. Gen. Virol.* **90**, 2990-
559 2994 (2009).
- 560 43. Coch, C. *et al.* RIG-I activation protects and rescues from lethal influenza virus
561 infection and bacterial superinfection. *Molecular Therapy* **25**, 2093-2103 (2017).
- 562 44. Ciancanelli, M. J., Abel, L., Zhang, S. & Casanova, J. Host genetics of severe
563 influenza: from mouse Mx1 to human IRF7. *Curr. Opin. Immunol.* **38**, 109-120
564 (2016).
- 565 45. Horisberger, M. A., Staeheli, P. & Haller, O. Interferon induces a unique protein
566 in mouse cells bearing a gene for resistance to influenza virus. *Proc. Natl. Acad. Sci.*
567 *U. S. A.* **80**, 1910-1914 (1983).
- 568 46. Staeheli, P., Haller, O., Boll, W., Lindenmann, J. & Weissmann, C. Mx protein:
569 constitutive expression in 3T3 cells transformed with cloned Mx cDNA confers
570 selective resistance to influenza virus. *Cell* **44**, 147-158 (1986).
- 571 47. Horisberger, M. & STARITZKY, K. D. Expression and stability of the Mx protein
572 in different tissues of mice, in response to interferon inducers or to influenza virus
573 infection. *J. Interferon Res.* **9**, 583-590 (1989).

- 574 48. Pavlovic, J., Haller, O. & Staeheli, P. Human and mouse Mx proteins inhibit
575 different steps of the influenza virus multiplication cycle. *J. Virol.* **66**, 2564-2569
576 (1992).
- 577 49. Basler, C. F. & Garcia-Sastre, A. Viruses and the type I interferon antiviral
578 system: induction and evasion. *Int. Rev. Immunol.* **21**, 305-337 (2002).
- 579 50. Di Bisceglie, A. M. *et al.* Recombinant interferon alfa therapy for chronic hepatitis
580 C. *N. Engl. J. Med.* **321**, 1506-1510 (1989).
- 581 51. Kao, J., Wu, N., Chen, P., Lai, M. & Chen, D. Hepatitis B genotypes and the
582 response to interferon therapy. *J. Hepatol.* **33**, 998-1002 (2000).
- 583 52. Mocellin, S., Pasquali, S., Rossi, C. R. & Nitti, D. Interferon alpha adjuvant
584 therapy in patients with high-risk melanoma: a systematic review and meta-analysis.
585 *J. Natl. Cancer Inst.* **102**, 493-501 (2010).
- 586 53. Bohn, J., Gastl, G. & Steurer, M. Long-term treatment of hairy cell leukemia with
587 interferon- α : still a viable therapeutic option. *memo-Magazine of European Medical*
588 *Oncology* **9**, 63-65 (2016).
- 589 54. Valentine, A. D., Meyers, C. A., Kling, M. A., Richelson, E. & Hauser, P. Mood
590 and cognitive side effects of interferon-alpha therapy. *Semin. Oncol.* **25**, 39-47
591 (1998).
- 592 55. Sleijfer, S., Bannink, M., Van Gool, A. R., Kruit, W. H. & Stoter, G. Side effects of
593 interferon- α therapy. *Pharmacy world and science* **27**, 423 (2005).
- 594 56. Corssmit, E. *et al.* Endocrine and metabolic effects of interferon-alpha in
595 humans. *The Journal of Clinical Endocrinology & Metabolism* **81**, 3265-3269 (1996).
- 596 57. Noisakran, S., Campbell, I. L. & Carr, D. J. Ectopic expression of DNA encoding
597 IFN-alpha 1 in the cornea protects mice from herpes simplex virus type 1-induced
598 encephalitis. *J. Immunol.* **162**, 4184-4190 (1999).
- 599 58. Wong, J. P. *et al.* Liposome-mediated immunotherapy against respiratory
600 influenza virus infection using double-stranded RNA poly ICLC. *Vaccine* **17**, 1788-
601 1795 (1999).
- 602 59. Protzer, U., Nassal, M., Chiang, P. W., Kirschfink, M. & Schaller, H. Interferon
603 gene transfer by a hepatitis B virus vector efficiently suppresses wild-type virus
604 infection. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 10818-10823 (1999).
- 605 60. Arnaud, P. The interferons: pharmacology, mechanism of action, tolerance and
606 side effects. *Rev. Med. Interne* **23 Suppl 4**, 449s-458s (2002).
- 607 61. Pfeffer, L. M. *et al.* Biological properties of recombinant alpha-interferons: 40th
608 anniversary of the discovery of interferons. *Cancer Res.* **58**, 2489-2499 (1998).

- 609 62. Vilcek, J. Fifty years of interferon research: aiming at a moving target. *Immunity*
610 **25**, 343-348 (2006).
- 611 63. Oladunni, F. S. *et al.* Lethality of SARS-CoV-2 infection in K18 human
612 angiotensin-converting enzyme 2 transgenic mice. *Nature communications* **11**, 1-17
613 (2020).
- 614 64. Miorin, L. *et al.* SARS-CoV-2 Orf6 hijacks Nup98 to block STAT nuclear import
615 and antagonize interferon signaling. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 28344-
616 28354 (2020).
- 617 65. Martinez-Gil, L. *et al.* A Sendai virus-derived RNA agonist of RIG-I as a virus
618 vaccine adjuvant. *J. Virol.* **87**, 1290-1300 (2013).
- 619 66. Wang, X. *et al.* Influenza A virus NS1 protein prevents activation of NF-kappaB
620 and induction of alpha/beta interferon. *J. Virol.* **74**, 11566-11573 (2000).
- 621 67. Bergmann, M. *et al.* Influenza virus NS1 protein counteracts PKR-mediated
622 inhibition of replication. *J. Virol.* **74**, 6203-6206 (2000).
- 623 68. Talon, J. *et al.* Activation of interferon regulatory factor 3 is inhibited by the
624 influenza A virus NS1 protein. *J. Virol.* **74**, 7989-7996 (2000).
- 625 69. Romanova, J. *et al.* Preclinical evaluation of a replication-deficient intranasal
626 Δ NS1 H5N1 influenza vaccine. *PloS one* **4**, e5984 (2009).
- 627 70. Wressnigg, N. *et al.* Development of a live-attenuated influenza B Δ NS1
628 intranasal vaccine candidate. *Vaccine* **27**, 2851-2857 (2009).
- 629 71. Morahan, P. S. & Grossberg, S. E. Age-related cellular resistance of the chicken
630 embryo to viral infections. I. Interferon and natural resistance to myxoviruses and
631 vesicular stomatitis virus. *J. Infect. Dis.*, 615-623 (1970).
- 632 72. Lampson, G. P., Tytell, A. A., Field, A. K., Nemes, M. M. & Hilleman, M. R.
633 Inducers of interferon and host resistance. I. Double-stranded RNA from extracts of
634 *Penicillium funiculosum*. *Proc. Natl. Acad. Sci. U. S. A.* **58**, 782-789 (1967).
- 635 73. Plachý, J. *et al.* Protective effects of type I and type II interferons toward Rous
636 sarcoma virus-induced tumors in chickens. *Virology* **256**, 85-91 (1999).
- 637 74. Rathnasinghe, R. *et al.* Comparison of transgenic and adenovirus hACE2 mouse
638 models for SARS-CoV-2 infection. *Emerging microbes & infections* **9**, 2433-2445
639 (2020).

640 **Author contributions**

641 AG-S, PP, RR, MS and TM conceived the project. RR, MS, HZ, TK and SJ
642 conducted experiments while MS, RR analysed the data and wrote the manuscript.

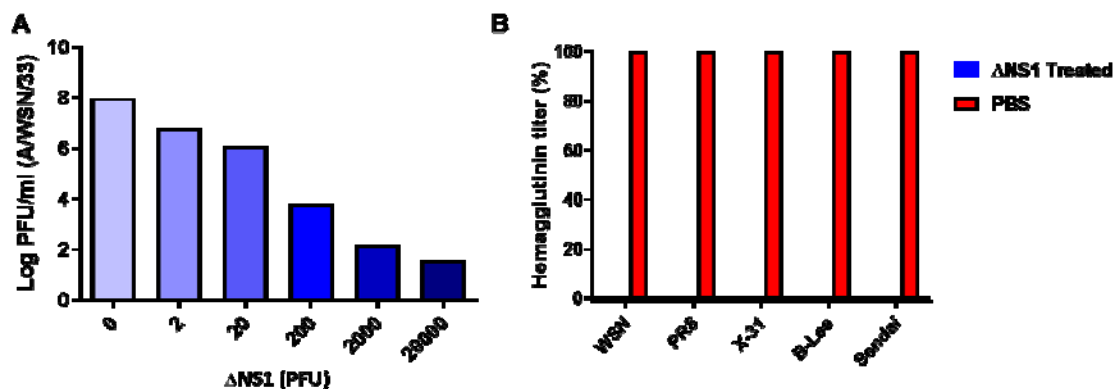
643

644 **Conflicts of interest.**

645 AG-S and PP are inventors in patents owned by the Icahn School of Medicine and
646 licensed to Vivaldi Biosciences concerning the use of NS1 deficient viruses as
647 human vaccines and to BI Vetmedica on the use of NS1 deficient viruses as
648 veterinarian vaccines. The García-Sastre Laboratory has received research support
649 from Pfizer, Senhwa Biosciences, 7Hills Pharma, Pharmamar, Blade Therapeutics,
650 Avimex, Accurius, Dynavax, Kenall Manufacturing, ImmunityBio and Nanocomposix;
651 and A.G.-S. has consulting agreements for the following companies involving cash
652 and/or stock: Vivaldi Biosciences, Pagoda, Contrafect, Vaxalto, Accurius, 7Hills
653 Pharma, Avimex, Esperovax and Farmak.

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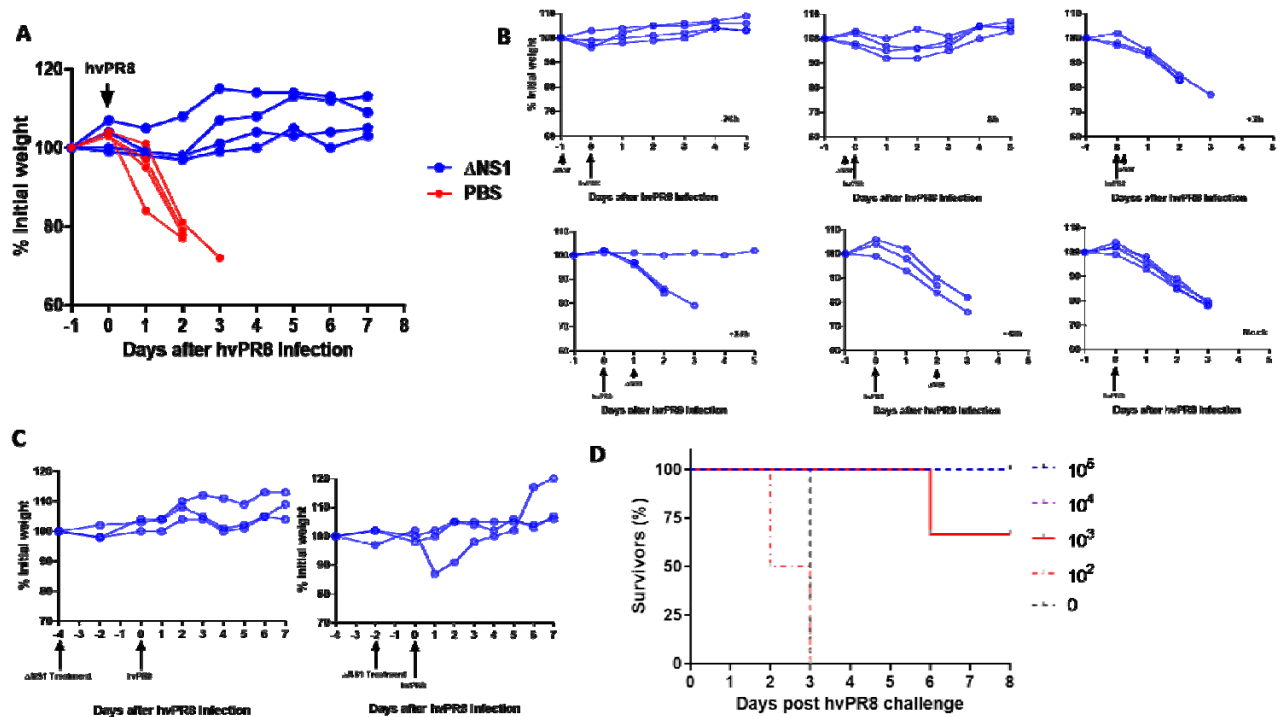
655 **Figures and figure legends**



656

657 **Figure 1. Pre-incubation with Δ NS1 virus inhibits viral replication in**
658 **embryonated chicken eggs. (A)** 10-day-old embryonated chicken eggs (n=2 per
659 group) were inoculated with varying amounts of (PFU) of Δ NS1 virus in the allantoic
660 cavity. Eight hours post infection at 37°C, eggs were re-infected with 10^4 PFU of WT

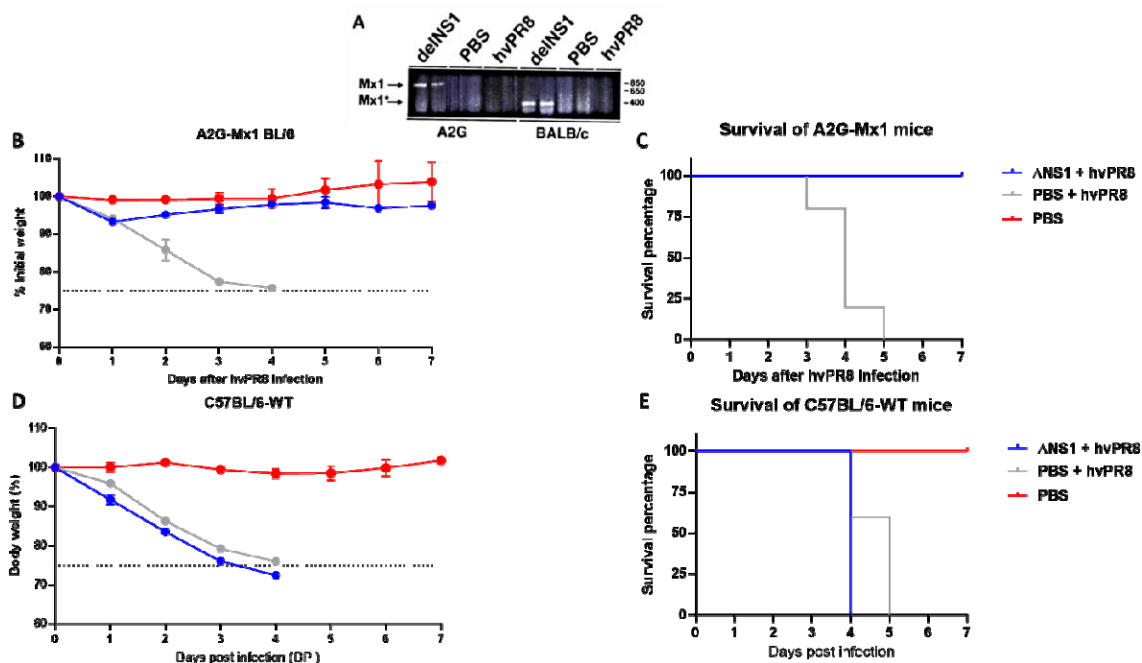
661 A/WSN/33 influenza virus and incubated at 37°C for 40 hours. Allantoic fluids were
662 then titrated by plaque assay MDBK cells. **(B)** 10-day-old embryonated chicken eggs
663 (n=2 per group) were inoculated with 2×10^4 PFU of Δ NS1 virus or PBS (Untreated). 8
664 hours post inoculation at 37°C, the eggs were re-infected with 10^3 PFU of A/WSN/33
665 (WSN/H1N1), A/PR/8 (PR8/H1N1), A/X-31 (X-31/H3N2), B/Lee/40 (B-Lee influenza
666 B) or Sendai Virus (Sendai). B-Lee infected eggs were incubated at 35°C for
667 additional 40 h. All other eggs were incubated at 37°C for additional 40 h. Virus
668 present in the allantoic fluid was titrated by hemagglutination assays. Maximum
669 hemagglutination titers (100%) for each individual virus were 2048 (PR8), 1024 (X-
670 31), 256 (B-Lee), 512 (Sendai)



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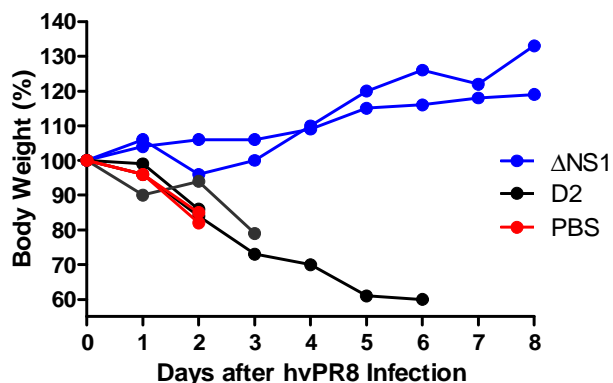
672 **Figure 2. A single dose of Δ NS1 virus protects A2G mice against lethal**
673 **infection by highly virulent hvPR8 influenza virus when given prior to virus**
674 **challenge. (A) Treatment with Δ NS1 virus protects A2G mice against lethal**
675 **infection by highly virulent hvPR8 influenza virus. Eight 6-week old A2G mice**

676 were intranasally infected with 5×10^6 PFU of highly virulent A/PR/8/34 (hvPR8)
677 influenza virus. Half of the mice received a total of five intranasal treatments with
678 5×10^5 PFU of Δ NS1 virus at the following times with respect to the hvPR8 infection: -
679 24 h, -8 h, +3 h, +24 h ad 48 h. The remaining four mice were treated with PBS and
680 the bodyweight changes and survival was monitored. **(B) A single dose of Δ NS1**
681 **virus protects A2G mice against lethal infection by highly virulent hvPR8**
682 **influenza virus when given prior to hvPR8 virus challenge.** Groups of three A2G
683 mice each were mock-treated or treated intranasally with 5×10^5 PFU of Δ NS1 at time
684 points -24 h, -8 h, +3h, +24h, +48h relative to the intranasal infection by 5×10^6 hvPR8
685 influenza virus. **(C) A single dose of Δ NS1 virus protects A2G mice against**
686 **lethal infection by highly virulent hvPR8 influenza virus when given two and**
687 **four days prior to hvPR8 virus administration** Groups of three A2G mice were
688 intranasally treated with 5×10^5 PFU of Δ NS1 virus four days or two days before
689 infection by 5×10^6 hvPR8 influenza virus. Bodyweight changes and survival was
690 monitored. All data points are from individual mice. **(D) Determination of the**
691 **minimal effective therapeutic dose of Δ NS1 to prevent lethal hvPR8 virus**
692 **infection in A2G mice.** Groups of three A2G mice were intranasally infected with
693 10^5 , 10^4 or 10^3 PFU Δ NS1 influenza virus. Additionally, groups of two A2G mice
694 were intranasally challenged with 10^2 of Δ NS1 virus or PBS. 24 hours post
695 inoculation, mice were challenged with by 5×10^6 hvPR8 influenza virus. The
696 percentage of mice surviving the challenge is represented.



697

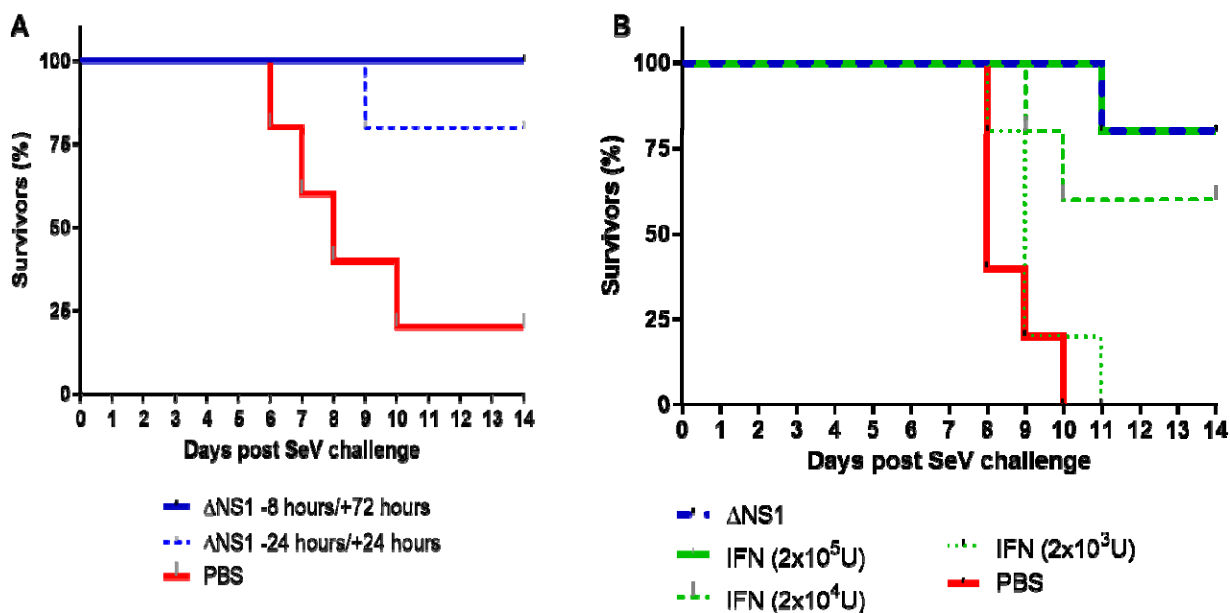
698 **Figure 3. Dose dependent pre-treatment of ΔNS1 protects A2G-Mx1 mice but**
 699 **not wild-type C57BL/6 from a lethal hvPR8 virus challenge. (A) Induction of *Mx1***
 700 **specific mRNA expression in ΔNS1 virus infected mice. Groups of two A2G or**
 701 **BALB/c mice were intranasally treated with PBS or 2.5×10^5 PFU of ΔNS1 hvPR8**
 702 **influenza viruses. 24 hours post challenge, total RNA present in lung tissues were**
 703 **extracted and were used for RT-PCR reactions using *Mx1* specific primers. PCR**
 704 **products were run in an agarose gel; the arrows indicate the predicted size of**
 705 **amplified cDNA from *Mx1* genes of A2G mice (*Mx1*) and BALB/c mice**
 706 **(*Mx1**). (B,C,D,E) Sex matched 6 weeks old groups C57BL/6-A2G-Mx1 mice or**
 707 **C57BL/6-wild-type mice were either intranasally pre-treated with PR8-ΔNS1 (5×10^6**
 708 **PFU; n=5 per group), sterile PBS (n=5) 12 hours before a lethal challenge of hvPR8**
 709 **(5×10^5 PFU; n=5) or treated with only sterile PBS (n=2). (B) Morbidity of C57BL/6-**
 710 **A2G-Mx1 mice. (C). Survival of C57BL/6-A2G-Mx1 mice. (D). Morbidity of C57BL/6-**
 711 **wild-type mice. (E). Survival of C57BL/6-wild-type mice.**



712

713 **Figure 4. Comparison of the antiviral properties in A2G mice of recombinant**
714 **influenza A viruses ΔNS1 and D2.** A2G mice were intranasally treated with PBS or
715 2.5×10^5 PFU of ΔNS1 or D2 viruses for 24 hours before infection with 5×10^6 PFU of
716 hvPR8 influenza virus. Bodyweight changes and survival were monitored. Data
717 shown are from individual mice.

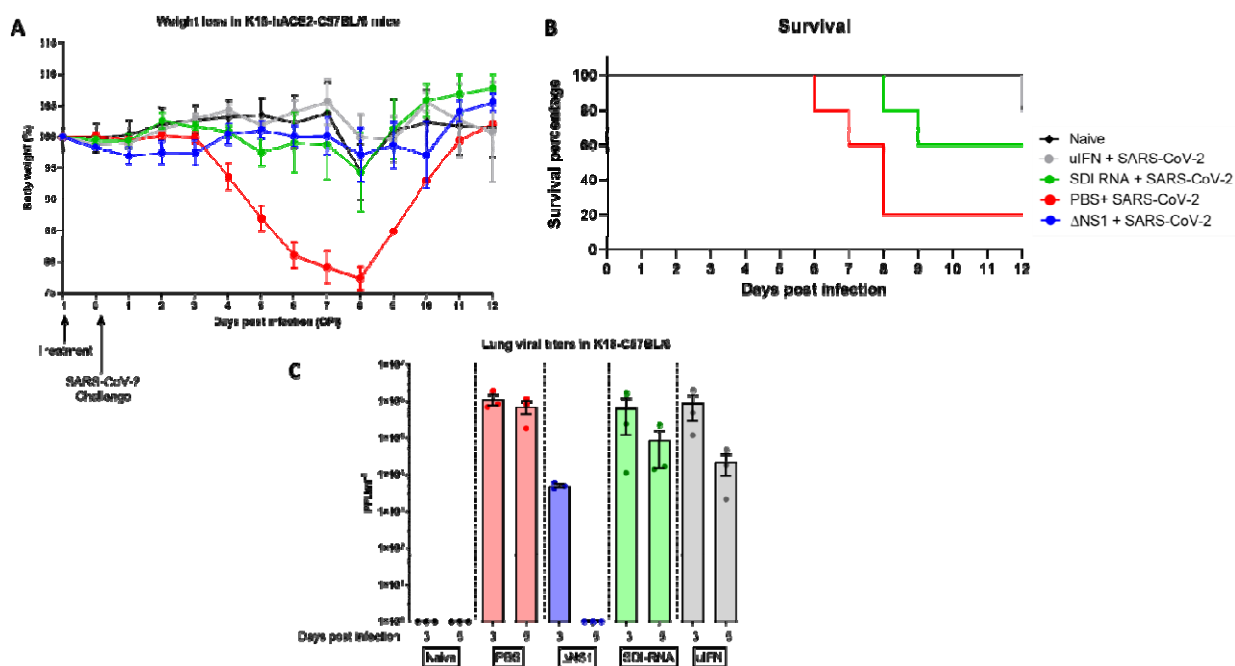
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719

720 **Figure 5. Treatment with ΔNS1 influenza virus protects C57BL/6 mice against**
721 **lethal infection with Sendai virus.** All mice were challenged intranasally with a

722 lethal dose of Sendai virus corresponding to **(A)** 5×10^5 PFU or **(B)** 1.5×10^5 PFU. The
723 percentage of mice surviving the challenge is represented. **(A)** Groups of five mice
724 were treated intranasally with 2.5×10^5 PFU of Δ NS1 virus at the indicated times. **(B)**
725 Groups of five mice were intranasally treated at -24h and +24h with respect to the
726 infection with Sendai virus with 2.5×10^5 PFU of Δ NS1 or with the indicated amounts
727 of IFN- β .



728

729 **Figure 6. Treatment with Δ NS1 influenza virus inhibits viral replication in the**
730 **lungs of K18-hACE2 mice challenged with SARS-CoV-2.** Mice were intranasally
731 treated with 30 μ l containing PBS, 2.5×10^6 PFU of Δ NS1, 1 μ g defective interfering
732 RNA from Sendai virus (SDI-RNA), 2.5×10^5 U of universal-interferon (uIFN) 24 hours
733 before intranasal challenge with 10^4 PFU of SARS-CoV-2/USA/WA1 isolate. **(A)**
734 weight-loss was monitored in mice ($n=11$ for treated groups and $n=6$ naive) and **(B)**
735 survival was monitored for 12 days. **(C)** Lungs were harvested at days three and five
736 post infection ($n=3$ per group per day) were homogenized and were titered in Vero-

737 E6 cells using standard plaque assays.

738 **Supplementary Table 1.** Units of IFN present in the allantoic fluid of 10-days
 739 embryonated chicken eggs which were inoculated with WT A/PR8/34 or ΔNS1
 740 influenza A viruses.

Virus ^a	Egg number	IFN (Uml ⁻¹) ^b
rWT PR8	1	<16
	2	<16
ΔNS1	3	400
	4	400
Mock	5	<16
	6	<16

741 ^aEggs were inoculated with 10³ PFU of rWT-PR8 or ΔNS1 virus

742 ^bAmount of IFN in the allantoic fluid was measured 18 hours post inoculation

743

744 **Supplementary Table 2.** Viral titers, bodyweight changes and lung weights in A2G

745 mice infected with ΔNS1 and hvPR8 viruses.

Virus	Day 3				Day 6			
	Mouse	PFU/ml ^a	Bodyweight changes ^b	Lung weight	Mouse	PFU/ml ^a	Bodyweight changes ^b	Lung weight
ΔNS1^c	1	<10	+0.73 g	0.13 g	7	<10	+1.59 g	0.14 g
	2	<10	+1.19 g	0.13 g	8	<10	+2.89 g	0.15 g
hvPR8^e	3	3 x 10 ⁸	-3.05 g	0.32 g	9	3 x 10 ⁶	-6.65 g	0.34 g
	4	3 x 10 ⁷	-3.09 g	0.27 g	10	7 x 10 ⁶	-4.69 g	0.24 g
ΔNS1+ hvPR8^d	5	2 x 10 ⁴	+1.58 g	0.15 g	11	<10	-0.03 g	0.12 g
	6	6 x 10 ²	-0.42 g	0.11 g	12	1 x 10 ⁴	+0.37 g	0.11 g

746 ^a Lungs were homogenized in 2 ml of PBS and viral titers were determined by standard plaque assay

747 ^b Numbers represent differences with respect to the bodyweight at the time of infection

748 ^c Animals were treated intranasally on day 0 with 2×10^5 PFU of Δ NS1 virus

749 ^d Animals were treated intranasally on day -1 with 2×10^5 PFU of Δ NS1 virus followed by intranasal
750 challenge on day 0 with 2×10^4 PFU of hvPR8 virus

751 ^e Animals were infected intranasally on day 0 with 2×10^4 PFU of hvPR8

752