Prophylactic Protection Against Respiratory Viruses Conferred by a Prototype Live Attenuated Influenza Virus Vaccine

Raveen Rathnasinghe
Icahn School of Medicine at Mount Sinai
Mirella Salvatore
Icahn School of Medicine at Mount Sinai
Hongyong Zheng
Icahn School of Medicine at Mount Sinai
Sonia Jangra
Icahn School of Medicine at Mount Sinai
Thomas Kehrer
Icahn School of Medicine at Mount Sinai
Ignacio Mena
Icahn School of Medicine at Mount Sinai
Michael Schotsaert
Icahn School of Medicine at Mount Sinai
Thomas Muster
Medical University of Vienna
Peter Palese
Icahn School of Medicine at Mount Sinai
Adolfo Garcia-Sastre (Adolfo.Garcia-Sastre@mssm.edu)
Icahn School of Medicine at Mount Sinai

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

Raveen Rathnasinghe[1, 2, 3], Mirella Salvatore[4], Hongyong Zheng[1], Sonia Jangra[1, 3], Thomas Kehrer[1, 2, 3], Ignacio Mena[1,3], Michael Schotsaert[1, 3], Thomas Muster[5], Peter Palese[1, 6], Adolfo García-Sastre[1, 3, 6, 7*].

Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA[1]

Graduate School of Biomedical Sciences, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA[2]

Global Health and Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA[3]

Department of Medicine, Weill Cornell Medical College, New York, NY, USA[4]

Department of Dermatology, University of Vienna Medical School, 1090 Wien, Austria[5]

Department of Medicine, Division of Infectious Diseases, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA[6]

The Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA[7]

*Corresponding author: Mailing address – Department of Microbiology, Icahn School of Medicine at Mount Sinai, Box 1124, One Gustave L. Levy place, New York, NY 10029, USA. Phone (212) 241-7769. Fax (212) 534-1684. E-mail – adolfo.gracia-sastre@mssm.edu

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

Abstract

The influenza A non-structural protein 1 (NS1) is known for its ability to hinder the synthesis of type I interferon (IFN) during viral infection. Influenza viruses lacking NS1 (ΔNS1) are under clinical development as live attenuated human influenza virus vaccines and induce potent influenza virus-specific humoral and cellular adaptive immune responses. Attenuation of ΔNS1 influenza viruses is due to their high IFN inducing properties, that limit their replication in vivo. This study demonstrates that pre-treatment with a ΔNS1 virus results in an immediate antiviral state which prevents subsequent replication of homologous and heterologous viruses, preventing disease from virus respiratory pathogens, including SARS-CoV-2. Our studies suggest that ΔNS1 influenza viruses could be used for the prophylaxis of influenza, SARS-CoV-2 and other human respiratory viral infections, and that an influenza virus vaccine based on ΔNS1 live attenuated viruses would confer broad protection against influenza virus infection from the moment of administration, first by non-specific innate immune induction, followed by specific adaptive immunity.

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

Introduction

The type I interferon (IFN) response resulting from invading viral pathogens is considered as one of the first lines of antiviral defence mechanisms in higher organisms. The latter process takes place upon the detection of the pathogen associated molecular patterns (PAMPS) by the host pattern recognition receptors (PRRs). Secretion of interferons takes place in both paracrine and autocrine signalling mechanisms, mediated by the canonical JAK/STAT signal transduction pathway along with the transcriptional activation of a particular set of host genes as well as their corresponding promotors defined as IFN-stimulated response elements (ISREs)[1]. Subsequent activation of the downstream interferon stimulated genes (ISGs) lead to the transcriptional induction of a plethora of antiviral proteins, including dsRNA-activated protein kinase (PKR) leading to a halt of protein translation, dsRNA-activated oligoadenylate synthetases (OAS) which facilitate the degradation of RNA by activating RNAse L and Mx proteins which essentially sequester incoming viral components such as nucleocapsids[2, 3]. Many studies have demonstrated that viruses have evolved to encode numerous mechanisms to prevent the host IFN-mediated antiviral response at different stages[4]. Viral non-structural proteins such as those of Toscana virus, dengue and HPV can sequester host factors to inhibit type I IFN response[5,6,7], while viruses such as vaccinia, adeno and Ebola viruses secrete soluble ligands[7,8], or encode miRNAs[9, 10] and other proteins to confer immune-evasion.

The influenza A virus (IAV) non-structural protein 1 (NS1) facilitates several functions ranging from inhibition of host mRNA polyadenylation and subsequent inhibition of their nuclear export as well as inhibition of pre-mRNA splicing[11, 12]. A growing body of evidence to date has indicated that influenza NS1 protein has IFN antagonistic activity. It was initially shown that a recombinant influenza A virus that lacks the NS1 protein

(ΔNS1) grew to a titer similar to that of WT virus in IFN deficient systems, albeit being markedly attenuated in IFN competent hosts[13]. This attenuated phenotype can be explained by the inability of the virus to prevent NS1 mediated IFN inhibition. The NS1 protein has been shown to bind to TRIM25 whereby the ubiquitination of the viral RNA sensor RIG-I is inhibited, which eventually results in the inhibition of IFN induction[14,15]. NS1 has also been shown to prevent IFN production by sequestering the cellular cleavage and polyadenylation specificity factor 30 (CPSF30) in order to halt the processing of host pre-mRNAs, resulting in accumulation of pre-mRNAs in the nucleus as well as the halt of cellular mRNA export to the cytoplasm[16]. This subsequently results in the inhibition of host protein production, including IFNs and proteins encoded by IFN inducible genes [17,18] NS1 has also been shown to inhibit the antiviral activity of several IFN-stimulated genes, such as the 2'-5'- oligo A synthase (OAS)[19].

Consistent with its function, deletion of NS1 in recombinant IAV results in a live attenuated and highly immunogenic IAV. As a result, IAV with impaired NS1 function are currently used as vaccines against swine influenza in pigs[20] and they are under clinical consideration as live attenuated human influenza virus vaccines[21-23].

Based on the growing body of evidence showing the IFN antagonistic properties of IAV NS1, we investigated the ability of the ΔNS1 viruses to induce an immediate IFN response *in vivo* along with the biological antiviral consequences mediated by the type I IFN induction. Our results demonstrate that the ΔNS1 virus is an efficient inducer of IFN with antiviral properties in both mice and embryonated eggs. Our data indicates the suitability of ΔNS1 virus as a prophylactic agent to induce immediate mucosal antiviral responses with the aim of preventing acute respiratory infections caused by IFN sensitive viruses. ΔNS1 influenza viruses can provide first innate antiviral protection, followed by adaptive specific IAV protection.

Results

Recombinant influenza A virus lacking the NS1 gene (ΔNS1) induces higher levels of interferon than wild type viruses in embryonated chicken eggs.

Previously, we demonstrated that tissue culture-based infections by ΔNS1 viruses induced the transactivation of an ISRE-containing reporter gene[13], indicating that infection by ΔNS1 viruses induces higher levels of IFN in comparison to its wild type counterparts. To test whether ΔNS1induces IFN in 10-day old embryonated-chicken eggs, eggs were treated with 10^3 PFU of ΔNS1 or PR8-WT influenza viruses. Subsequently, the allantoic fluids were harvested 18 hours post treatment to measure the levels of IFN by determining the highest dilution that inhibited the cytopathic effect mediated by vesicular stomatitis virus (VSV) in chicken embryo fibroblast (CEF) cells. As indicated in the Supplementary table 1, four hundred Uml^{-1} of IFN were detected in the allantoic fluid of eggs infected by ΔNS1 virus. However, allantoic fluids derived from WT-PR8 or mock infections indicated undetectable levels of IFN (<16 Uml^{-1}).

Pre-treatment with ΔNS1 influenza virus inhibits wild-type viral replication in embryonated chicken eggs.

We speculated that the ability of the ΔNS1 virus on inducing high titers of IFN in eggs facilitates an antiviral state that may prevent the replication of wild-type IAV. To evaluate this, increasing amounts of ΔNS1 virus were inoculated into eggs and eight hours post-treatment, the eggs were challenged with wild-type A/WSN/33 (WSN-WT) virus with a dose of 10^3 PFU. Two days post incubation extracted allantoic fluids were titrated via plaque assays. WSN viral titers decreased with ΔNS1 in a dose dependent manner. While the untreated allantoic fluids supported the growth of WSN virus to an approximate titer of 10^8 $PFUml^{-1}$, administration of a dose as little as $2x10^4$ $PFUml^{-1}$ of Δ

NS1 prevented the replication of WSN virus (less than 10^2 PFUml^{-1} of WSN were obtained in eggs). The titer of WSN virus was reduced by one log, by pre-treating allantoic fluids with as little as 2 PFU of ΔNS1 (Figure 1A).

Interestingly, treatment using ΔNS1 virus further inhibited the replication of other viruses, as depicted in figure 1B. Relative HA titers were obtained from eggs treated with $2x10^4$ PFUml^{-1} of ΔNS1 virus followed by subsequent infection with wild-type Influenza A H1N1 strains WSN and PR8, H3N2 strain X-31, influenza B virus or Sendai virus (SeV; a paramyxovirus). In all cases, pre-treatment with ΔNS1 resulted in a two-log reduction of wild-type viral HA titers.

Severe disease and death caused by infection with the highly virulent PR8 virus (hvPR8) in A2G mice can be alleviated by ΔNS1 pre-treatment.

In order to assess whether or not the administration of ΔNS1 virus inhibits replication of influenza viruses in mice, an inbred mouse strain that is homozygous for the gene which codes for the IFN induced full-length *Mx1* protein, defined as C57BL/6-A2G (abbreviated as A2G) mice were used for this part of the study[24, 25]. Previous studies have concluded that IFN administration was ineffective in preventing IAV replication in laboratory mice lacking a functional *Mx1* gene[26]. In contrast, A2G mice which were administered IFN remained alive upon infection with the highly virulent hvPR8 IAV strain[27]. The presence of a functional *Mx1* gene in A2G mice better mirrors the human situation, as *Mx1* gene deficiencies in humans are rare. Here, A2G mice were intranasally infected with a dose of $5x10^5$ PFUml^{-1} of ΔNS1 virus or PBS at -24, -8, +3, +24 and +48 hours. Mice were challenged at time 0 intranasally with $5x10^6$ PFU of hvPR8 virus. Mice treated with ΔNS1 virus were protected from hvPR8 virus as measured by weight loss and death while the PBS treated mice succumbed to death (Figure 2A).

Subsequently, we examined whether all five ΔNS1 treatments were essential for the protective effect against hvPR8 infection in mice. Hence, a single dose of $5x10^6$ PFU of ΔNS1 virus was given at various time points relative to the infection with hvPR8. D ata indicated (Figure 2B) that pre-treatment (hours 24 or 8 before hvPR8 challenge) b ut not post treatment (even 3 hours post hvPR8 challenge) of ΔNS1 resulted in the pr evention of weight loss disease and subsequent death. Additionally, ΔNS1 virus adm inistered two or four days prior to hvPR8 challenge completely protected mice from di sease (Figure 2C).

Next, to obtain the effective dose 50 (ED_{50}) of ΔNS1 virus to mediate protection again st disease from hvPR8 infection, $2x10^5$, $2x10^4$, $2x10^3$or $2x10^2$ doses of ΔNS1 virus w ere intranasally administered to A2G mice 24 hours prior to hvPR8 challenge. As sho wn in Figure 2D, the ED_{50} of the ΔNS1 virus which conferred protection in A2G mice against hvPR8-induced death was approximately 10^3 PFU.

Induction of *Mx1* specific mRNA in mice treated with ΔNS1 virus.

To investigate whether ΔNS1 infection in mice resulted in induction of the *Mx1* gene, an RT-PCR assay for *Mx1* specific mRNA in infected animal lungs infected was deve loped. In parallel, infections were performed in BALB/c mice which have a non-functi onal *Mx1* gene due to a large frameshift deletion[26]. As seen in figure 3A, treatment wi th ΔNS1 resulted in the early induction (24 hours post infection) of *Mx1* specific mRN A in both A2G and BALB/c mice. In contrast a very faint band was present in A2G mi ce infected with hvPR8 virus at the same time post infection and no specific mRNA w as detected in mock infected mRNA.

ΔNS1 mediated protection from hvPR8 is *Mx1*-mediated.

As the Mx1 protein is one of the most potent IFN inducible gene products with anti-inf

luenza virus activity in mice, it is quite possible that the ΔNS1-mediated protection se en in A2G mice is Mx1-mediated. To test this hypothesis, we compared the antiviral a ctivity of ΔNS1 in A2G mice and in C57BL/6 mice. C57BL/6 mice harbour a non-funct ional *Mx1* gene due to a known deletion[26] and were used as a back-cross genetic pla tform for the original A2G strain to generate the Mx1 positive A2G mice used in our e xperiments. A dose of PR8-ΔNS1 containing $5x10^6$ PFU given 12H before a lethal hv PR8 challenge protected all A2G-Mx1 mice (n=5) in both morbidity and mortality in co mparison to the PBS pre-treated group (n=5) (Figures 3B and 3C). However, all five MX1-deficient mice in the wild-type C57BL/6 group that were given the same dose of PR8-ΔNS1 succumbed to death by a lethal hvPR8 challenge. The morbidity data for t hese mice based on body weight was also consistent with lack of protection after ΔN S1 treatment from hvPR8 challenge, indicating that the antiviral effect on IAV induced in mice by ΔNS1 treatment is dependent on the IFN-inducible gene *Mx1* w (Figure 3D and 3E).

ΔNS1 viral treatment inhibits the replication of hvPR8 virus in A2G mice lungs.

To better understand the ability of the ΔNS1 virus to inhibit replication of the hvPR8 v irus in the lungs, A2G mice were intranasally treated with $2x10^5$ PFU of ΔNS1 virus a lone, $2x10^4$ PFU of hvPR8 alone or treatment of $2x10^5$ PFU of ΔNS1 virus 24 hours b efore infecting them with $2x10^4$ PFU of hvPR8 virus. Mice were sacrificed at three- an d six-days post infection and the lung homogenates were titrated in MDCK or Vero ce lls (Supplementary table.2). A reduction of hvPR8 titers in lungs by fourfold was obse rved when mice were pre-treated with ΔNS1 virus. Furthermore, mice solely infected with ΔNS1 virus had titers below the detection limit (<10 $PFUml^{-1}$), while not showing any significant reduction of bodyweight. It was apparent that infection by hvPR8 virus without ΔNS1 administration resulted in the increase of lung weight by a factor of two

or three in comparison to mice that were pre-treated with ΔNS1 virus. In the context o f this study, increased lung weights are suggestive of lymphocytic infiltration and pulm onary disease during Influenza virus infection[28, 29].

Attenuated influenza viruses via a mutation in the Neuraminidase (NA) gene do es not confer ΔNS1-like antiviral properties.

Antiviral properties observed thus far in this study is from an attenuated influenza viru s lacking the NS1 gene (ΔNS1). To confirm that the protective effects observed here are not due to the attenuation caused by the lack of a gene but specifically due to the lack of NS1, the antiviral property of ΔNS1 virus was compared to that of a the recom binant D2 influenza virus. The D2 virus contains a base-pair mutation in the dsRNA r egion formed by the non-coding sequences of its NA gene. This mutation is responsi ble for a 10-fold reduction in the NA protein levels as well as a one-log reduction in vi ral titers within a multicycle growth curve[30]. The latter D2 strain has also been shown to be highly attenuated in mice with a LD_{50} of more than 10^6 PFU upon intranasal ad ministration[31]. Identical doses ($2.5x10^5$ PFU) of D2 or ΔNS1 viruses were intranasally administered to A2G mice four hours prior to challenge with $5x10^6$ PFU of hvPR8. Alt hough a prolonged survival was seen in one of the animals who received D2, pre-tre atment with D2 was ineffective in protecting A2G mice from hvPR98 virus-induced di sease and death (Figure 4).

ΔNS1 viral treatment prevents death by Sendai virus (SeV) in C57BL/6 mice

Given the fact, that the antiviral effects against hvPR8 mediated by ΔNS1 viral are fa cilitated by an IFN mediated mechanism (*Mx1* gene induction), we speculated that Δ NS1 treatment should protect mice from infections by other IFN sensitive viruses. Se ndai virus was used in this study due to its pneumotropic nature and sensitivity to IFN

in *Mx1* deficient mice[32, 33]. As seen in Figure 1B, treatment with ΔNS1 inhibited Send ai viral replication in embryonated chicken eggs. Moreover, upon two intranasal admi nistrations of $2.5x10^5$ PFU of ΔNS1 virus to C57BL/6 mice at times -24 and +24 hour s or -8 and +72 hours, mice infected with $5x10^5$ PFU of Sendai virus were protected f rom death (Figure 5A). The C57BL/6 mice used here are $Mx1^{-/-}$ and it is indicative tha t the mouse nuclear Mx1 protein does not have any antiviral activities against cytopla smic viruses such as Sendai virus[34]. The efficacy of ΔNS1 treatment was compared a gainst three doses of IFN-β using the Sendai virus challenge model. Treatment with t he highest dose of IFN-β ($2x10^5$ U) protected mice from death induced by Sendai viru s comparable to treatment with $2.5x10^5$ PFU of ΔNS1 virus (Figure 5B).

ΔNS1 virus treatment inhibits viral replication of SARS-CoV-2 virus in K18-hAC E2-C57Bl/6 murine lungs.

Given the emergence of the devastating COVID-19 pandemic, we assessed whether prophylactic treatment with ΔNS1 would hinder the replication of SARS-CoV-2. We u sed the transgenic mouse model that supports the replication of SARS-CoV2. As con trols, we used universal IFN, and SeV defective RNA (SDI) which were previously sh own to have an IFN inducing effect. Weight determination in all the treated groups sh owed no major loss in bodyweight, only one mouse each from the SDI treated group (day 8) and the uIFN treated group (day 12) reached below 75% bodyweight (Figure 6 A). Deaths (4 out of 5) in the mock treated group occurred between days 6-8 post inf ection. The SDI-RNA treated group lost 2 out of 5 animals on day 8 and 9 while the u IFN group lost one animal out of 5 at a later time point (D12; Figure 6B). While both tr eatments resulted in reduction of viral titers day 3 and 5 post infection, mice that rece ived ΔNS1 showed significant inhibition of SARS-CoV2 titers in lung homogenates an d no detectable infectious viruses at day 5 post infection (Figure 6C).

Discussion

The NS1 protein of the influenza A virus has been shown to possess IFN antagonist activity whereby it is able to dampen the host innate immune response to provide a favourable environment for the virus to replicate. It has been demonstrated to be highly expressed in the host cytoplasm and nucleus upon viral infection, interacting with a plethora of host factors to inhibit the interferon response[35]. Data show the ability of NS1 to compete with innate immune sensors such as RLR to bind to dsRNA to avoid innate immune detection[36]. Additionally, NS1 has been shown to interact with other innate immune signalling components such as PKR[37], TRIM25[38] and CPSF[16], resulting in lowering of the IFN mediated innate immunity[39]. For these reasons, influenza viruses with impaired NS1 function (and an increased innate immune response) have been under consideration for live attenuated influenza vaccines. There is an existing swine influenza vaccine based on NS1-deficient live attenuated viruses[40], and clinical trials in humans using an intranasally administered live attenuated ΔNS1 virus have demonstrated potent immunogenicity and good safety profiles. Experimental evidence in mice indicates that the high IFN-inducing properties of ΔNS1 viruses are responsible for their superior immunogenicity as live vaccines[41, 42].

As ΔNS1 viruses are great IFN inducers, we reasoned that they might provide with innate protection against respiratory virus infection even before the development for an influenza virus specific adaptive immune response. Treatment with ΔNS1 virus inhibited the replication of both homologous and heterologous viruses in eggs (Figure.1). Using the A2G-Mx1 mouse model, we demonstrated that the intranasal administration of the ΔNS1 virus induced an antiviral state, which prevented disease and death by a highly pathogenic influenza A virus (hvPR8) which is otherwise lethal[43]. Infection with ΔNS1 virus but not WT viruses yielded detectable levels of *Mx1*-specific mRNA level

s in lungs 24 hours post infection (Figure 2). A large body of evidence has indicated t hat the protective impact of IFN against IAV infection in mice is mainly mediated by th e IFN inducible antiviral *Mx1* gene[44-46]. Consistently, we found that Mx1 was required for the ΔNS1 mediated protection against lethal hvPR8 challenge by comparing Mx1 competent A2G--C57BL/6 mice with Mx1 deficient WT-C57BL/6 mice.

Data depicted in Figure.2C show that pre-treatment of A2G mice with ΔNS1 virus up t o four days before the challenge with hvPR8 virus was effective in preventing diseas e. The Mx1 protein in mice is known to be stable for several days upon its induction a nd our observations are consistent with the half-life of the Mx1 protein described in m ice[47, 48].

Given the inherently attenuated state of the ΔNS1 viruses, it was necessary to confir m that the antiviral state seen here is due to the specific attenuation of the ΔNS1 seg ment. We used a virus that is known to be attenuated due to its defective neuraminid ase segment (D2 virus expressing a full-length NS1) [31] to demonstrate that protection is not just mediated by any attenuated IAV (Figure.4). ΔNS1 treated mice were also p rotected from lethal infection with an influenza-unrelated pneumotropic Sendai virus, suggesting that the IFN-mediated innate immune response induced by ΔNS1 has bro ad-antiviral effects, rather than being a pathogen-specific immune response. As antic ipated for Sendai virus, the abovementioned protection was not *Mx1* mediated and is most likely due to the activation of other ISGs such as OAS or PKR upon the ΔNS1-mediated IFN production[49].

The feasibility of ΔNS1 virus as a prophylactic treatment to induce a type I interferon r esponse to prevent acute respiratory infections from IFN sensitive viruses was demo nstrated in the current study. Type I interferon administration has been used to treat a

range of human diseases ranging from infections such as hepatitis B and C[50, 51] to ot her non-communicable diseases such as melanomas[52] and hairy-cell leukaemia[53]. Al though IFN has been promoted as a therapeutic agent, administration of exogenous i nterferon comes with a set of undesirable side effects[54, 55], arguably due to its causin g major endocrine and metabolic changes in the host[56]. Therefore, various groups ha ve attempted alternative ways to induce local type I IFN responses using different str ategies. Some of these strategies were topical administration of plasmid DNA coding for IFNα1 in the mouse eye to protect against HSV-1 encephalitis[57], liposomic intrana sal treatment using dsRNA to induce IFN[58] as well as recombinant viral vectors such as adenoviruses[59] and hepatitis B viruses to express type I IFN to protect against infe ction and tumor regression[59]. Despite these experimental attempts to study the effica cy of IFN, it is still unclear whether virally induced IFN is more or less toxic efficient th at IFN itself. This indicates that further work is needed to be done to ascertain the sui tability of recombinant viruses as IFN inducers for therapeutic purposes. The physiolo gical half-lives and binding affinities of different types of interferons are well studied a nd their half-lives can range from minutes to several hours, depending on the type of IFN[60]. Our data showed antiviral properties of ΔNS1 virus for up to four days before t he viral challenge. While it is known that therapeutic properties and doses of different types of IFNs are highly variable due to their differential effects contributed by the IS Gs, most therapeutic properties of type I interferons are yet to be completely underst ood[61, 62]. In this instance, comparable prophylactic responses were obtained by the a dministration of either $2x10^5$ U of IFN-β or $2x10^5$ PFU of ΔNS1 virus (Figure.5B). How ever, it is acknowledged that different subsets of IFN-regulated genes may differ in th eir relative transcriptional induction between treatments.

We also demonstrated that prophylactic treatment using ΔNS1 significantly inhibited v

318 iral replication in a relevant mouse model that can be infected with WT SARS-CoV-2
319 and is known to result in lethal infection[63](Figure 6). This agrees with reports that stat
320 e that SARS-CoV-2 is sensitive to IFN[64]. Interestingly, a similar level of reduction in v
321 iral titers was not seen upon intranasal inoculation of universal-IFN nor defective inter
322 fering RNA derived from SeV (SDE-RNA; a RIG-I agonist with known adjuvanting pro
323 perties)[65]. While these treatments resulted in a better outcome in comparison to PBS
324 pre-treatment, high amounts of viral titers were still observed day three and five post i
325 nfection. Although weight loss and survival were best in the ΔNS1 group, the uIFN tre
326 ated group showed a protective phenotype indicating that uIFN treatment was better t
327 han that provided by SDI-RNA. The difference observed here is likely due to the stim
328 ulation of multiple innate immune mechanisms by ΔNS1 which potentially primes cell
329 s to confer a broad antiviral phenotype. However, analysis of differentially expressed
330 genes (particularly ISGs) via a technique such as bulk RNAseq would provide more i
331 nsights in explaining the observed protective effects against COVID-19 in the K18 mo
332 use model.

333 In conclusion, we report that prophylactic treatment with an attenuated influenza A vir
334 us lacking the NS1 gene induces an innate antiviral response which provides protecti
335 on against IFN-sensitive viruses in both embryonated chicken eggs and mice. These
336 *in vivo* data further validate previous observations showing the IFN-antagonistic prop
337 erties of the NS1 protein of influenza A viruses[13, 66-68], while highlighting the role of N
338 S1 in inhibiting IFN induction during influenza A virus infections. We also provide evid
339 ence for its therapeutic potential as a prophylactic to protect against acute respiratory
340 infections caused by IFN-sensitive viruses including the causative agent of COVID-1
341 9 pandemic. ΔNS1 viruses are being clinically developed as live attenuated influenza
342 virus vaccines and in clinical trials they have shown to induce protective antibodies a

nd no adverse responses in human volunteers[21-23]. Here we show that ΔNS1 viruses have the potential to induce immediate protection against viral infection prior to the induction of specific long-lasting protective adaptive immune responses[69, 70]. Our results should encourage further research on the use of IFN-inducing, live attenuated virus vaccines, to confer innate and adaptive protection against virus pathogens.

Methods

Cells and viruses

Recombinant influenza A viruses were generated using reverse genetics as previously described[13, 30] A derivative of the A/PR/8/34 (PR8) defined as highly virulent PR8 (hvPR8) was kindly provided by O. Haller and J.L. Schulman. Strain 52 of Sendai virus was obtained from the ATCC. Vero cells, Madin-Darby bovine kidney (MDBK) cells, baby hamster kidney (BHK) cells or embryonated chicken eggs were used to propagate the following viruses as per standard protocols; Influenza A ΔNS1, hvPR8, PR8, A/WSN/33, A/X-31/H3N2, Influenza B/Lee/40, Sendai virus and vesicular stomatitis virus (VSV). Madin-Darby canine kidney (MDCK) cells or Vero cells were plated to obtain confluent monolayers and plaque assays were performed as previously described and an agar overlay in DMEM-F12 including 1 µgml^{-1} of trypsin was used. MDCK, cVero and BHK cells were cultured in DMEM in the presence of 10% FBS and penicillin-streptomycin. The chicken embryo fibroblasts (CEF) purchased from ATCC was maintained in MEM as suggested by ATCC. Vero-E6 cells (ATCC® CRL-1586™, clone E6) were grown in DMEM containing 10% FBS, non-essential amino acids, HEPES and penicillin-streptomycin. SARS-CoV-2, isolate USA-WA1/2020 (BEI resources; NR-52281) was handled under BSL-3 containment in accordance with the biosafety protocols validated by the Icahn School of Medicine at Mount Sinai. Viral stocks were am

plified in Vero-E6 cells in the above media containing 2% FBS for three days and were validated by whole-genome sequencing using the Oxford-MinION platform.

Animal studies

All animals used in the study were used at 6-10 weeks of age. The Institutional Animal Care and Use Committee (IACUC) of the Icahn School of Medicine at Mount Sinai (ISMMS) reviewed and approved the *in vivo* protocols included in this study. The animal work of this study is in accordance with the ARRIVE guidelines. A2G mice were kindly provided by Dr. Heinz Arnheiter while the BALB/c and C56BL/6 mice were purchased from Taconic Farms. Hemizygous female K18-hACE2 mice on the C57BL/6J genetic background (Jax strain 034860), were used to conduct studies with SARS-CoV-2 in BSL3 conditions. Anesthetized animals (Ketamine and Xylazine diluted in PBS administered via intraperitoneal injection) were intranasally infected using 30 to 50 µl of appropriately diluted viruses or PBS containing the indicated amounts of recombinant murine IFN-β (Calbiochem), universal-IFN (PBL assay science) SDI-RNA[65]. Afterwards, the animals were monitored daily for changes in body weight. All animal studies were done in accordance with the NIH guidelines as well as the guidelines devised by the Icahn School of Medicine with regards to the care and use of laboratory animals.

Measurement of Interferon

Ten day old embryonated eggs were infected with 10^3 PFU in100 µl containing either ΔNS1, PR8 viruses or PBS as mock. Next, the eggs were incubated at 37°C and the allantoic fluids were extracted 18 hours post infection. Viral inactivation of the allantoic fluids were conducted by dialysis against 0.1 M KCL-HCL buffer at pH 2 for two days at 4°C. Later, the pH of the samples was adjusted to pH 7 by subsequent dialysis a

gainst Hank's balanced sodium salt solution with 20 mM NA_3PO_4 for two more days a s described previously[71]. The amount of IFN was titrated according to its ability to inh ibit the growth of VSV[72]. In summary, CEF cells in 96wells were treated with 100 µl of different dilutions of the respective samples in tissue culture media. Upon incubating f or an hour at 37°C, 200 $TCID_{50}$ of VSV in 10 µl were added to the wells before incuba ting at 37°C until complete lysis of untreated control cells was observed (approximate ly two days). As a standard control, recombinant chicken IFN donated by Drs. Peter S taeheli and Bernd Kaspers was used[73].

Lung Titration

Four A2G mice were intranasally challenged with $2x10^5$ PFU of ΔNS1 at day -1. Duri ng day 0 mice were intranasally challenged with $2x10^4$ PFU of hvPR8 virus. Alternativ ely, two other groups of four A2G mice were challenged with $2x10^5$ PFU ΔNS1 or 2x1 0^4 PFU of hvPR8. Three days post infection, two animals from each group were hum anely sacrificed while the rest of the animals were humanely sacrificed six days post i nfection. Lungs were weighed and homogenized in 2 ml of PBS. Resulting homogen ates were clarified via centrifugation at 3000 rpm for 15 minutes at 4°C and the acqui red supernatants were tittered by plaque assays using MDCK or Vero cells. Lung ho mogenates derived from SARS-CoV-2 infected K18 mice were handled and titered in Vero-E6 cells as described previously[74] .

Detection of *MX1* Specific mRNA in infected cells

A2G and BALB/c mice were intranasally challenged with 10^5 PFU of either ΔNS1 or h vPR8 or PBS. Afterwards, lungs were extracted 24 hours post infection, snap frozen, homogenized, total RNA was extracted using TRIreagent (Sigma-Alderich). One micr ogram of total lung RNA was used to perform a RT reaction in a total volume of 20 µl

using *Mx1* specific primer. Two µl of the resulting RT product was used for PCR amplification using *Mx1* specific primers under the following conditions (20 seconds at 95°C, 30 seconds at 55°C, 30 seconds at 72°C for a total of 25 cycles). The sense and antisense primer sequences are as follows; 5'-CAGGACATCCAAGAGCAGCTGAGCCTCACT-3' and 5'-GCAGTAGACAATCTGTTCCATCTGGAAGTG-3'. The PCR products were analysed using a 1.2% agarose gel. Correct size for the PCR products in A2G mice was 756 bp while it was 333 bp in BALB/c mice due to a deletion in the *Mx1* gene between nucleotides 1120-1543[31].

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Author contributions

AG-S, PP, RR, MS and TM conceived the project. RR, MS, HZ, TK , IM and SJ cond ucted experiments while MS, RR analysed the data and wrote the manuscript.

Conflicts of interest.

AG-S and PP are inventors in patents owned by the Icahn School of Medicine and lic ensed to Vivaldi Biosciences concerning the use of NS1 deficient viruses as human v accines and to BI Vetmedica on the use of NS1 deficient viruses as veterinarian vacc ines. The García-Sastre Laboratory has received research support from Pfizer, Senh wa Biosciences, 7Hills Pharma, Pharmamar, Blade Therapeutics, Avimex, Accurius, Dynavax, Kenall Manufacturing, ImmunityBio and Nanocomposix; and A.G.-S. has co nsulting agreements for the following companies involving cash and/or stock: Vivaldi Biosciences, Pagoda, Contrafect, Vaxalto, Accurius, 7Hills. The rest of the authors h ave no conflicts to declare.

Pharma, Avimex, Esperovax and Farmak.

Figures and figure legends

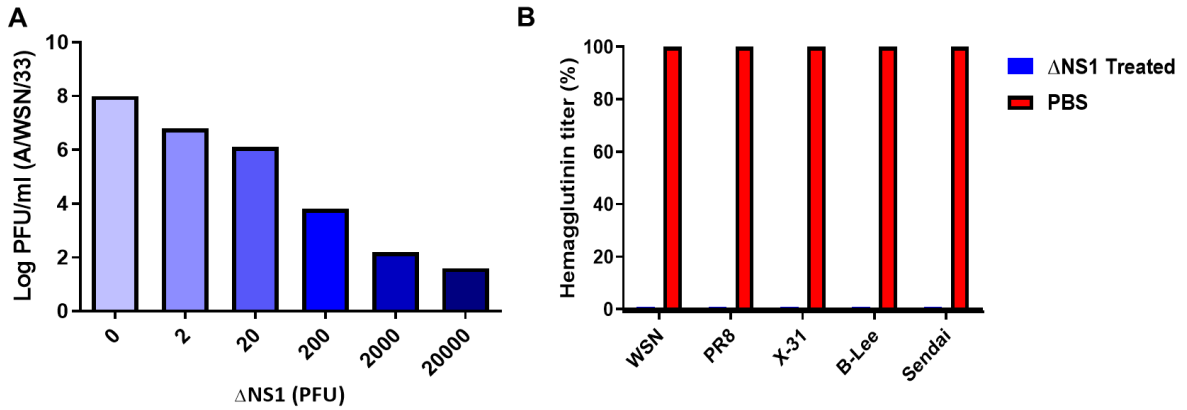
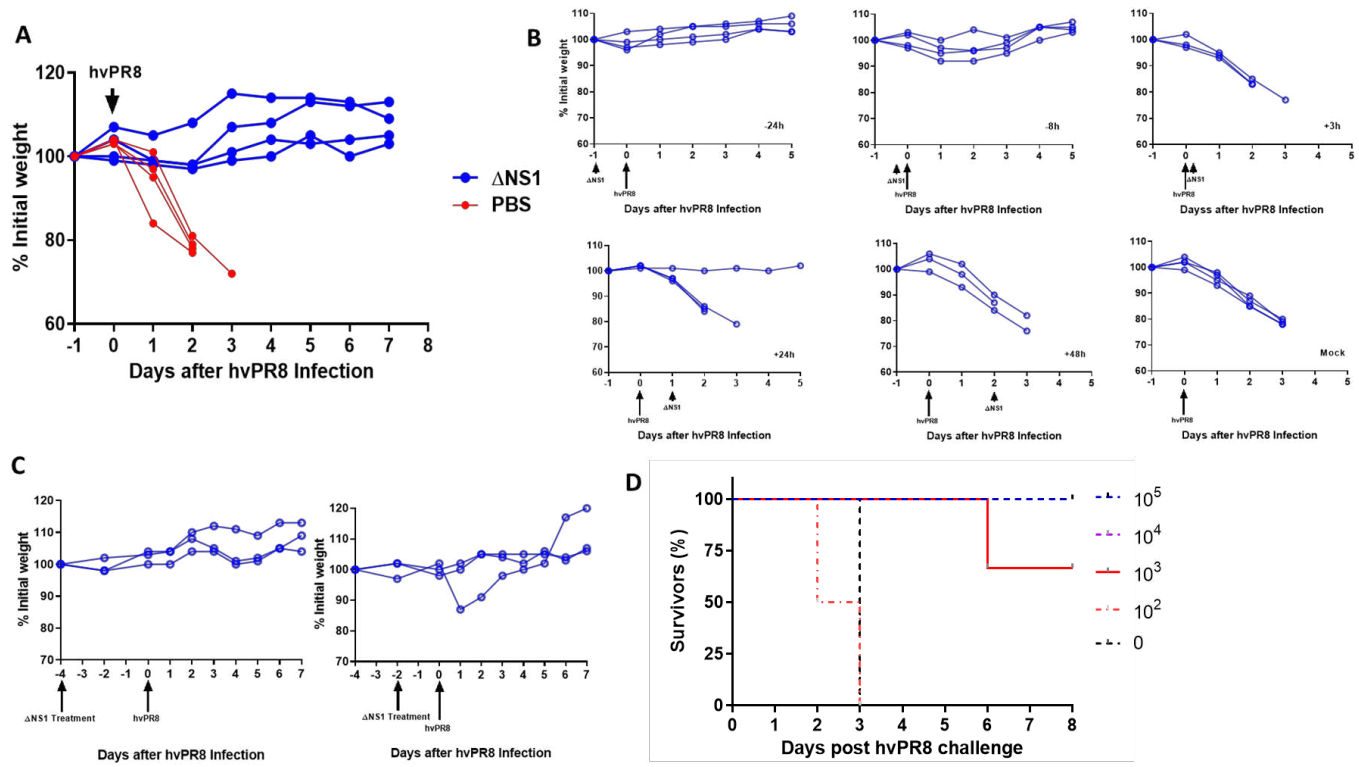


Figure 1. Pre-incubation with ΔNS1 virus inhibits viral replication in embryonated chicken eggs. (A) 10-day-old embryonated chicken eggs (n=2 per group) were inoculated with varying amounts of (PFU) of ΔNS1 virus in the allantoic cavity. Eight hours post infection at 37°C, eggs were re-infected with 10^4 PFU of WT A/WSN/33 influenza virus and incubated at 37°C for 40 hours. Allantoic fluids were then titrated by plaque assay MDBK cells. **(B)** 10-day-old embryonated chicken eggs (n=2 per group) were inoculated with $2x10^4$ PFU of ΔNS1 virus or PBS (Untreated). 8 hours post inoculation at 37°C, the eggs were re-infected with 10^3 PFU of A/WSN/33 (WSN/H1N1), A/PR/8 (PR8/H1N1), A/X-31 (X-31/H3N2), B/Lee/40 (B-Lee influenza B) or Sendai Virus (Sendai). B-Lee infected eggs were incubated at 35°C for additional 40 h. All other eggs were incubated at 37°C for additional 40 h. Virus present in the allantoic fluid was titrated by hemagglutination assays. Maximum hemagglutination titers (100%) for each individual virus were 2048 (PR8), 1024 (X-31), 256 (B-Lee), 512 (Sendai)



662

663 **Figure 2. A single dose of ΔNS1 virus protects A2G mice against lethal infectio**
664 **n by highly virulent hvPR8 influenza virus when given prior to virus challenge. (**
665 **A) Treatment with ΔNS1 virus protects A2G mice against lethal infection by hig**
666 **hly virulent hvPR8 influenza virus.** Eight 6-week old A2G mice were intranasally in
667 fected with 5x10^6 PFU of highly virulent A/PR/8/34 (hvPR8) influenza virus. Half of th
668 e mice received a total of five intranasal treatments with 5x10^5 PFU of ΔNS1 virus at t
669 he following times with respect to the hvPR8 infection: -24 h, -8 h, +3 h, +24 h ad 48
670 h. The remaining four mice were treated with PBS and the bodyweight changes and s
671 urvival was monitored. **(B) A single dose of ΔNS1 virus protects A2G mice agains**
672 **t lethal infection by highly virulent hvPR8 influenza virus when given prior to h**
673 **vPR8 virus challenge.** Groups of three A2G mice each were mock-treated or treate
674 d intranasally with 5x10^5 PFU of ΔNS1 at time points -24 h, -8 h, +3h, +24h, +48h rel
675 ative to the intranasal infection by 5x10^6 hvPR8 influenza virus. **(C) A single dose of**
676 **ΔNS1 virus protects A2G mice against lethal infection by highly virulent hvPR8**

influenza virus when given two and four days prior to hvPR8 virus administrati on Groups of three A2G mice were intranasally treated with $5x10^5$ PFU of ΔNS1 viru s four days or two days before infection by $5x10^6$ hvPR8 influenza virus. Bodyweight changes and survival was monitored. All data points are from individual mice. **(D) Det ermination of the minimal effective therapeutic dose of ΔNS1 to prevent lethal h vPR8 virus infection in A2G mice.** Groups of three A2G mice were intranasally infe cted with 10^5, 10^4 or 10^3 PFU ΔNS1 influenza virus. Additionally, groups of two A2G mice were intranasally challenged with 10^2 of ΔNS1 virus or PBS. 24 hours post inoc ulation, mice were challenged with by $5x10^6$ hvPR8 influenza virus. The percentage o f mice surviving the challenge is represented.

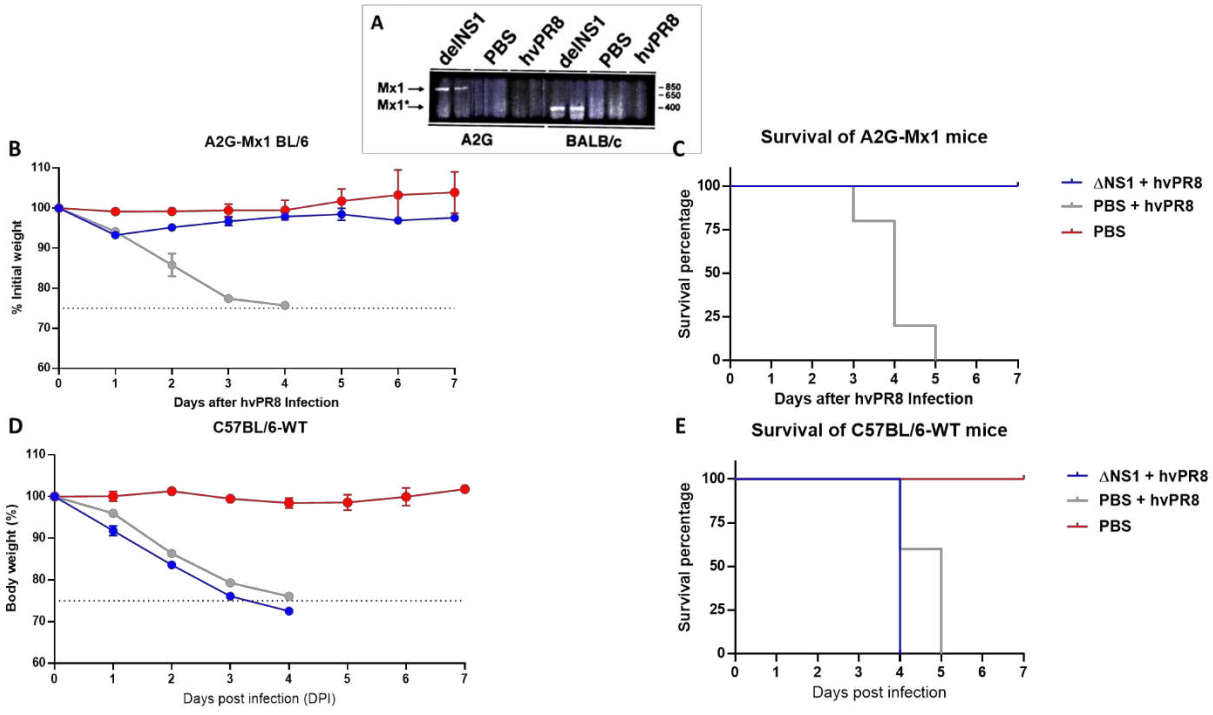
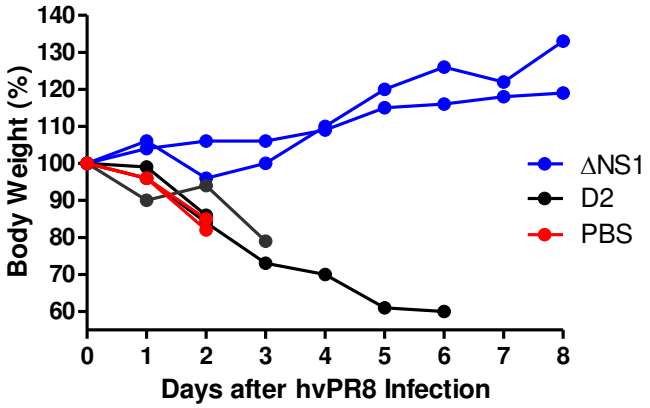


Figure 3. Dose dependent pre-treatment of ΔNS1 protects A2G-Mx1 mice but n ot wild-type C57BL/6 from a lethal hvPR8 virus challenge. (A)Induction of *Mx1* s pecific mRNA expression in ΔNS1 virus infected mice. Groups of two A2G or BA LB/c mice were intranasally treated with PBS or $2.5x10^5$ PFU of ΔNS1 hvPR8 influen za viruses. 24 hours post challenge, total RNA present in lung tissues were extracted

693 and were used for RT-PCR reactions using *Mx1* specific primers. PCR products were
694 run in an agarose gel; the arrows indicate the predicted size of amplified cDNA from
695 *Mx1* genes pf A2G mice (Mx1) and BALB/c mice (Mx1*).**(B,C,D,E)** Sex matched 6 we
696 eks old groups C57BL/6-A2G-Mx1 mice or C57BL/6-wild-type mice were either intran
697 asally pre-treated with PR8-ΔNS1 (5x10^6 PFU; n=5 per group), sterile PBS (n=5) 12 h
698 ours before a lethal challenge of hvPR8 (5x10^5 PFU; n=5) or treated with only sterile
699 PBS (n=2). **(B)** Morbidity of C57Bl/6-A2G-Mx1 mice. **(C)**. Survival of C57Bl/6-A2G-M
700 x1 mice. **(D)**. Morbidity of C57Bl/6-wild-type mice. **(E)**. Survival of C57Bl/6-6-wild-type
701 mice.



702

703 **Figure 4. Comparison of the antiviral properties in A2G mice of recombinant inf**
704 **luenza A viruses ΔNS1 and D2.** A2G mice were intranasally treated with PBS or 2.5
705 x10^5 PFU of ΔNS1 or D2 viruses for 24 hours before infection with 5x10^6 PFU of hvP
706 R8 influenza virus. Bodyweight changes and survival were monitored. Data shown ar
707 e from individual mice.

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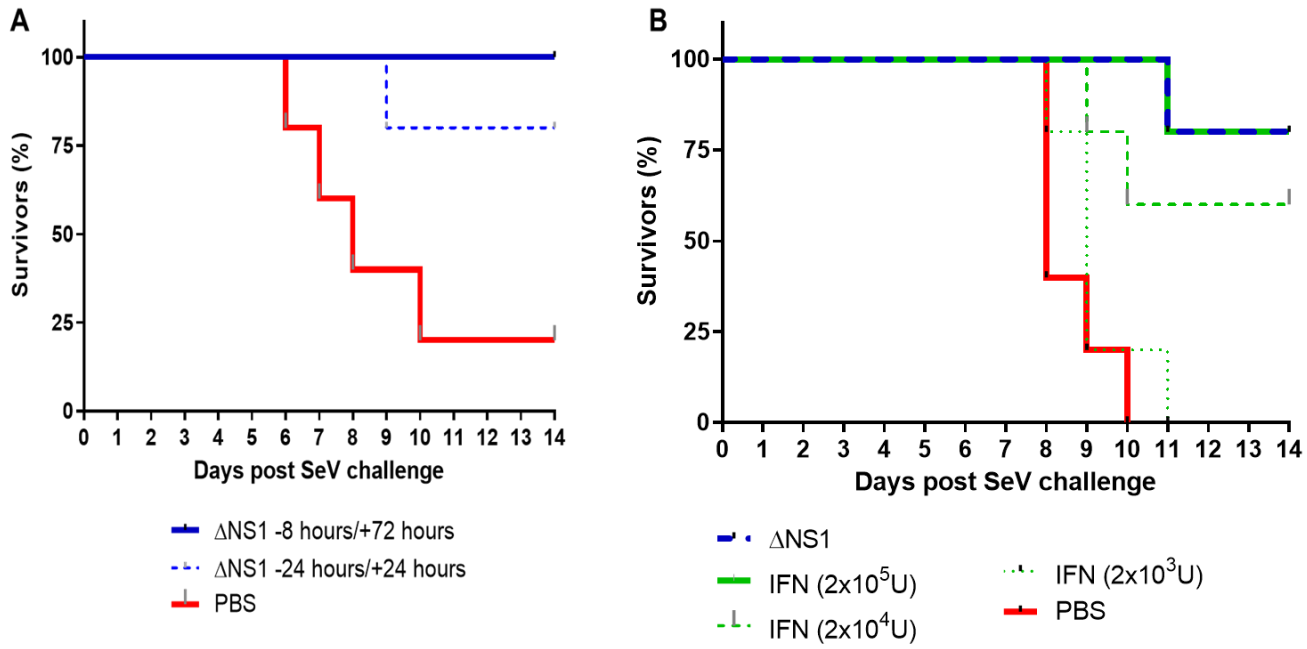
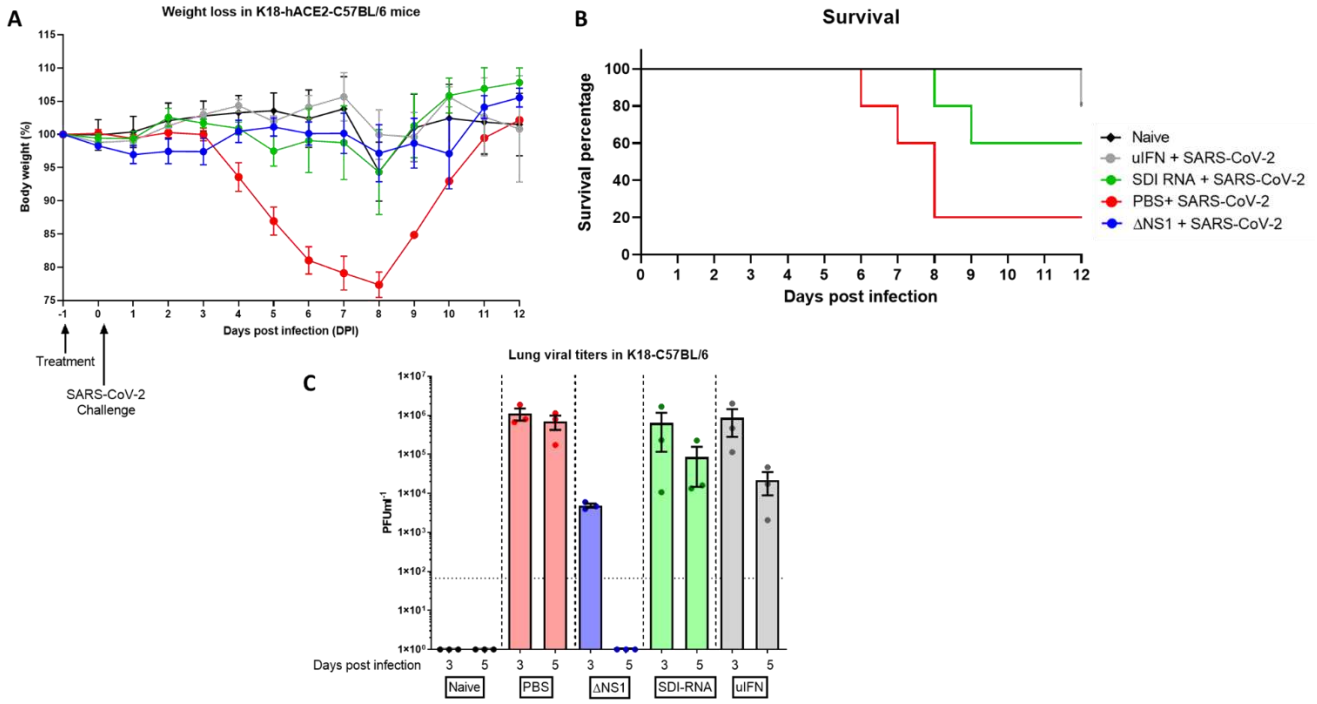


709

710 **Figure 5. Treatment with ΔNS1 influenza virus protects C57BL/6 mice against le**
711 **thal infection with Sendai virus.** All mice were challenged intranasally with a lethal
712 dose of Sendai virus corresponding to **(A)** 5x10^5 PFU or **(B)**1.5x10^5 PFU. The percen
713 tage of mice surviving the challenge is represented. **(A)** Groups of five mice were tre
714 ated intranasally with 2.5x10^5 PFU of ΔNS1 virus at the indicated times**. (B)** Groups o
715 f five mice were intranasally treated at -24h and +24h with respect to the infection wit
716 h Sendai virus with 2.5x10^5 PFU of ΔNS1 or with the indicated amounts of IFN-β.



717

718 **Figure 6. Treatment with ΔNS1 influenza virus inhibits viral replication in the lu**
719 **ngs of K18-hACE2 mice challenged with SARS-CoV-2.** Mice were intranasally trea
720 ted with 30 ul containing PBS, 2.5x10^6 PFU of ΔNS1, 1 μg defective interfering RNA f
721 rom Sendai virus (SDI-RNA), 2.5x10^5 U of universal-interferon (uIFN) 24 hours before
722 intranasal challenge with 10^4 PFU of SARS-CoV-2/USA/WA1 isolate. **(A)** weight-loss
723 was monitored in mice (n=11 for treated groups and n=6 naïve) and **(B)** survival was
724 monitored for 12 days. **(C)** Lungs were harvested at days three and five post infection
725 (n=3 per group per day) were homogenized and were titered in Vero-E6 cells using s
726 tandard plaque assays.

727

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