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Swine influenza virus strains that induce interferon β in SJPL cells but are insensitive to exogenous recombinant swine interferon β

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Abstract

Background: Viral infections induce expression of type I interferons (IFNα/β) which result in an antiviral state within cells or animals. However, some viruses, notably influenza viruses (IV), have evolved mechanisms of evasion and blocking of IFN to survive in nature. Swine influenza virus (SIV) can inhibit IFN and as with other IV does it through its NS1 protein, identified as a virulence factor in pigs.

Methods: To determine whether different SIV strains varied in their ability to induce IFNβ in cell culture and the relative sensitivity of these strains to exogenous IFNβ, three strains were tested in SJPL cells originally deposited as epithelial-like cell line of swine lung origin (ATCC, Richmond, MD) but later characterized as cells of monkey origin.

Results: The SIV strains tested here induced significant synthesis of IFNβ in SJPL cells, with levels that were higher to those induced by poly I:C. The induction of IFNβ by SIV peaked at three days post-infection and was not dependent on the infectious virus dose. Interestingly, priming the cells with exogenous recombinant swine IFNβ had no significant inhibitory effect on the replication of these SIV strains. NS1-antagonistic compounds did not change IFNβ induction and had variable and unexpected effects on virus replication of the three SIV strains tested.

Conclusions: Our study showed that while the SIV strains tested here were capable to induce IFNβ in SJPL cells these were relatively resistant to the antiviral effects of exogenous IFNβ. NS1-antagonistic compounds did not affect IFNβ induction and had variable effects on the replication of the SIV strains tested. These seemingly contradicting results suggest virus evasion by mechanisms other than NS1.

Keywords: Swine influenza virus, interferon β, SJPL cells, NS1 protein

Introduction

Influenza, a respiratory disease affecting humans and many mammals and birds, is caused by Influenza viruses (IV). Influenza viruses are enveloped, negative-sense, single-stranded, segmented RNA viruses of the family *Orthomyxoviridae* [1,2]. In humans, Influenza virus has been responsible for several pandemics that resulted in high mortality in 1918 (A/H1N1), 1957(A/H2N2), 1968 (A/H3N2) and 1977 (A/H1N1) [3]. The emergence of a new highly pathogenic avian influenza (HPAI), strain, A/H5N1, in 1997 in Hong Kong increased the awareness of the possibility of future Influenza pandemics. However, the most recent influenza pandemic was not caused by the HPAI (H5N1), but by a new influenza strain known as A/H1N1-SOIV (swine-origin influenza virus) which emerged in Mexico in 2009 [4,5]. In 2013, avian to human transmission of a new H7N9 resulting in fatalities was recorded in China [6].

Pigs are thought to have an important role in the epidemiology of influenza. Pig respiratory epithelial cells have receptors for both human as well as avian influenza viruses [9,10]. There is evidence that both humans and avian species can transmit influenza virus to pigs [11-13]. Thus, pigs are considered "mixing vessels" that facilitate re-assortment [7,8] from which new influenza strains responsible for pandemics may arise. Secondly, influenza type strains cause swine influenza, a constituent of

the porcine respiratory disease complex. Swine influenza has a high morbidity rate in pigs, although the mortality rate may be low [14]. Thirdly, swine influenza viruses (SIV)s can cause disease in humans, especially in those who have close contact with sick pigs [9,15].

The innate immune response, as first line of host defense against viral infection involves the induction of type I interferons (IFNα/β) and pro-inflammatory cytokines, which prevent the rapid replication and spread of viruses [16]. In addition to antiviral functions, type I IFNs has an important role in bridging innate and adaptive immunity [17]. While most viruses including influenza can induce the synthesis of type I IFN both in cell culture and in animals [18-21], the rate of induction may vary between strains and the type of host cells or animal species employed [22]. Besides viruses, many natural and synthetic molecules such as synthetic double stranded polyribonucleotide (poly I:C) can also induce IFN expression [23].

The induction of type I IFN by human influenza viruses [24-26] and several animal viruses including swine influenza virus (SIV) in cell culture and in pigs has been reported [27-29] Recently, induction of both IFNα and Mx protein in lungs of pigs experimentally inoculated with a strain of SIV has been demonstrated [30]. The production of Mx protein was speculated to be induced by IFNα. Mx1 protein is involved in protection of swine from SIV as shown by the increased susceptibility to SIV in swine with deletions in their Mx1 genes [31]. Mx may control SIV by blocking endocytic trafficking of the virus [32]. It has also been reported that the presence of pro- inflammatory cytokines including type I IFNs, TNFα, IL6 in the lungs of pigs inoculated with SIV correlates with severity of disease [28,33-35], There is also evidence that viruses have also evolved mechanisms to subvert innate immunity IFN [42] and NS1 was identified in influenza viruses as one of the viral proteins responsible for such 93 effects [19,41,44,45]. The experimental use of NS-1 antagonists resulted in rescue of 94 IFN induction in influenza virus infected cells confirming NS1 inhibitory role [38].

Given the crucial role of type I IFN in innate antiviral immunity and in activation of adaptive immunity, it is important to determine the effects on IFNβ synthesis in the context of influenza virus infection. This study was undertaken to determine whether different SIV strains varied in their ability to induce or suppress IFNβ in cell culture and examine the relative sensitivity of these strains to exogenous IFNβ.

Materials and methods
Cells
SJPL cells (ATCC, Richmond, MD), originally reported as epithelial-like cell line of swine lung origin were used in the study which was completed before the publication characterizing SJPL cells as of monkey origin [36]. The cells were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate, 2mM L-glutamine, 10 mM non-essential amino acids, 2.5 μg/ml fungizone, 100 U/ml 108 penicillin and 100 μg/ml streptomycin. SJPL cells were incubated in a humidified incubator with 5% CO_2 at 37⁰C. All cell culture procedures were performed in a sterile class II Biosafety cabinet.

Virus strains and virus titration
Three strains of SIV, A/Sw/Iowa/73 (H1N1), A/Sw/NC/18893/01 (rH1N1), and A/Sw/Tx/4199/98 (H3N2), obtained from the National Veterinary Services Laboratory (Ames, Iowa) were evaluated. The viruses were propagated in 11 day-old embryonated chicken eggs by inoculation in the allantoic cavity. Allantoic fluids were harvested 3-4 days after infection. Virus growth was monitored by the hemagglutination test Identification of the viruses was performed using hemagglutination inhibition tests. The viruses were then aliquoted and stored at -80⁰C until used.

Infectious virus particles were enumerated by conventional plaque assays in SJPL cells. Briefly, SJPL cells were seeded in 6-well plates, maintenance medium was removed and the cells were rinsed twice with serum-free media. Test viruses were diluted in serum-free media containing 1 μg/ml TPCK-trypsin and incubated in cell monolayers for 1 h at 37°C in a 5% CO_2 atmosphere. The inoculum was removed and cells were overlaid with agarose mixed with DMEM supplemented with 2x:penicillin-streptomycin, fungizone, L-glutamine, glucose, non-essential amino acids, sodium pyruvate and 2x TPCK-trypsin. The plates were incubated at 37°C, in a 5% CO_2 atmosphere. The cells were fixed with 95% cold methanol for 20 min and plaques stained with crystal violet.

Induction of IFNβ in cell culture
SJPL cells seeded in 24-well plates were inoculated with each SIV strain (MOI 0.005) and incubated for 1 h at 37°C in a 5% CO_2 atmosphere in serum-free DMEM containing 1 μg/ml TPCK-trypsin. The inoculum was removed, the cells were washed, and serum- free medium was added. The infected cells were incubated up to 72 h at 37°C, in a humidified 5% CO_2 atmosphere. Culture fluids were collected at 0, 24, 48, 72 h post- infection and were acid-treated for 24 h and neutralized to pH 7.2. IFNβ was measured by an indirect ELISA test as described below.

IFNβ ELISA
An ELISA to detect IFNβ in culture fluids was developed. Briefly, culture fluids from infected or control cells acidified to pH 2.5 and 18 hours later neutralized back to pH 7.2 were diluted in carbonate-bicarbonate buffer pH 9.6 and coated onto 96-well ELISA plates (Immulon®) overnight at 4°C. The next day, plates were washed once with wash buffer, PBS containing 0.05% Tween-20 (PBS-T), and blocked for 3 h at room temperature (RT) with 5% skim milk made in PBS-Tween-20 (PBS-T). After 5 washes, a monoclonal Ab (mAb) specific against swIFNβ, developed in our laboratory (10E9), was added at an optimized

concentration, and incubated at RT for 1 h. Plates were then washed 5 times with PBS-T and a diluted secondary antibody conjugate goat anti- mouse IgG HRPO (KPL, Gaithersburg, Maryland, USA) was added and incubated for 1 h at RT. Plates were washed 5 times, ABTS peroxidase substrate was added and the plate was read in an ELISA reader (Synergy HT, BioTek, Vermont, USA) at a wavelength of 405 nm. Affinity purified swIFNβ and normal culture medium were used as a positive and negative controls for comparative purposes and to ensure the soundness of the test.

Swine IFNβ bioassay

Recombinant replication-defective adenoviruses expressing recombinant swine IFNβ (Ad5-swIFNβ) or His-tagged Ad5-swIFNβ constructed previously [37] were utilized as a source of recombinant swine IFNβ (swIFNβ) in the study. The recombinant adenoviruses were propagated in HEK 293 cells for production of swIFNβ. The swIFNβ thus produced was used in assays either as crude cell culture fluids or fractionated through affinity Sepharose-anti-swIFNβ or nickel columns.

To test the sensitivity of the SIV strains to swIFNβ, SJPL cells cultured in 6-well tissue culture plates were pre-treated for 24 h with varying concentrations of swIFNβ. The cells were then inoculated, with each of the three SIV strains being tested, at an MOI of 0.005, and incubated for 72 h in serum-free DMEM containing 1 μg/ml TPCK- trypsin. Culture fluids were collected at 0, 24, 48, 72 h after infection. Virus titers were determined by plaque assays as described elsewhere.

Testing effects of NS1-antagonistic compounds

The effects of two NS1 antagonistic compounds, NSC10934 AND NSC128I164 kindly provided by the National Cancer Institute, on IFNβ induction and virus replication were tested *in vitro* essentially as described previously [38]. A working concentration of the compounds was determined experimentally based on this report [38]. Culture fluids were collected 72 hours post-infection and plaque assays were performed as described above to determine the virus titers.

Results

Poly I:C induces IFNβ production in SJPL cells

Polyriboinosinic-polyribocytidylic acid (poly I:C) which was previously shown to induce IFNβ in PK-15 cells [39], was used to ascertain and validate IFNβ induction in SJPL cells. Briefly, SJPL cells were treated with poly I:C (InvivoGen, San Diego, CA, USA) at different concentrations; 10, 25, and 50 μg/ml. Culture supernatants were collected at different time points after poly I:C stimulation and acidified to pH 2.0 overnight. The next day, supernatants were adjusted to pH 7.2 for use in ELISA which. Showed that poly I:C treatment induced increasing synthesis of IFNβ in SJPL cells over time of stimulation (**Figure 1**). There were significantly higher levels of IFNβ in poly I:C stimulated cells at the lowest dose (10 μg/ml) compared to unstimulated control cells (p<0.05). Nevertheless, increasing the dose of
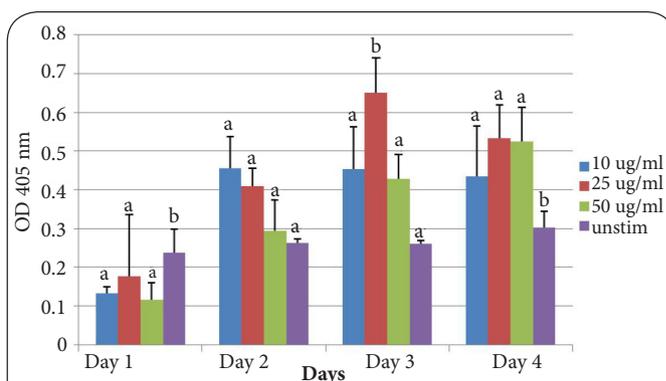

Figure 1. IFNβ induction in SJPL cells by treatment with poly I:C. The expression of IFNβ was measured in the culture media of SJPL cells collected at different time points after stimulation with different concentrations of poly I:C. Bars represent the mean of IFNβ levels produced and standard deviations of two independent experiments. Different letters mean statistically significant differences (p<0.05).

poly I:C did not increase the amount of IFNβ induced. The demonstration that poly I:C treatment induced expression of IFNβ in SJPL cells, was an important step to select these cells for testing IFNβ induction and sensitivity with influenza viruses. Poly I:C-stimulated SJPL cells were utilized as a positive IFNβ inducible control in the study.

The three SIV strains tested induce IFNβ in SJPL cells

The induction of IFNβ by poly I:C in SJPL cells is important since it validated the use of these cells as a system to evaluate the activation of type I IFN pathway by respiratory viruses such as influenza virus. SIV (A/Sw/Iowa/73 (H1N1), A/Sw/NC/18893/01 (rH1N1), A/Sw/Tx/4199/98(H3N2) were examined for their ability to induce IFNβ in SJPL cells. ELISA results showed that the three SIV strains tested induced the production of IFNβ by SJPL cells which indicated a cross-reactivity with the mAb 10E9 used in the test which was raised against recombinant swIFNβ. The levels of IFNβ induced were similar among the three strains tested (**Figure 2**). The levels of IFNβ detected at 72 h p.i. were significantly higher than those in uninfected control cells (p<0.05). Strains A/Sw/Iowa/73 and A/Sw/NC/18893/01 induced higher levels of IFNβ than A/Sw/Tx/4199/98 but the differences were not statistically significant. ELISAs were repeated several times with consistent results.

To determine the effect of virus dose on induction of IFNβ, SJPL cells were infected at various MOI's with A/Sw/Iowa/73 (H1N1) and infected cell supernatants were treated as previously described and assayed by indirect ELISA. The results indicate that the induction of IFNβ was not dependent on the infectious virus dose (**Figure 3**).

The SIV strains tested were insensitive to exogenous swIFNβ

The IFNβ sensitivity tests showed that treatment with 100, 250, or 500 U of swIFNβ had no effect on virus replication as
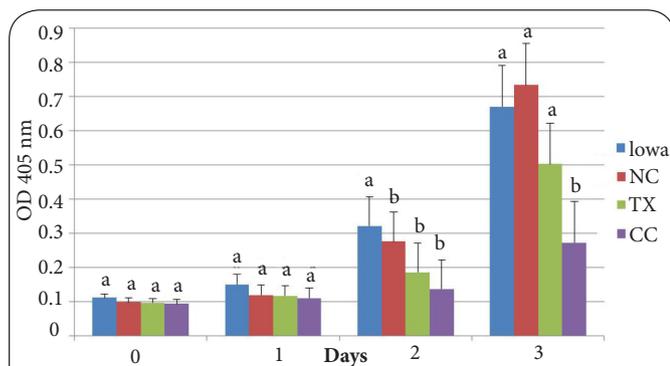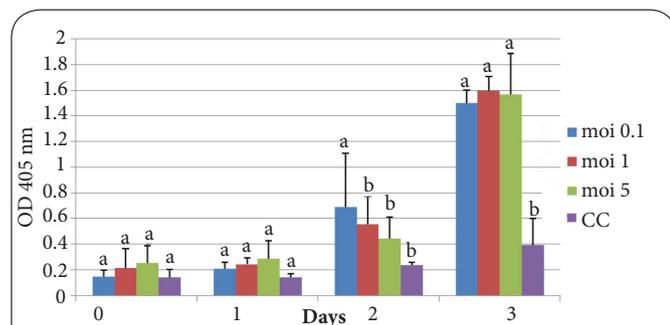
Figure 2. IFNβ induction in SJPL cells infected with different SIV strains. Expression of IFNβ was measured in culture media collected at the indicated times after infection of SJPL cells with one of three swine influenza virus strains in the presence of TPCK-trypsin. Bars represent the mean of swIFNβ levels produced and standard deviations of two independent experiments. Different letters mean statistically significant differences (p<0.05). cc uninfected control cells.

Figure 3. Induction of IFNβ in SJPL cells infected with different doses of SIV. The effect of virus dose on IFNβ expression was assessed by measuring IFNβ in the culture medium of SJPL cells after infection with A/sw/Iowa/73. Bars represent the mean of IFNβ levels produced and standard deviations of two independent experiments. Different letters mean statistically significant differences (p<0.05). cc uninfected control cells.

observed after 72 h of infection (data not shown). Thus, the three SIV strains tested here, A/Sw/Iowa/73 (H1N1), A/Sw/NC/18893/01 (H1N1), and A/Sw/Tx/4199/98 (H3N2), were not sensitive to exogenous swIFNβ under the conditions used as their 72 h titers were comparable to those of each of the corresponding viruses that were not exposed to IFNβ. The lowest concentration of recombinant swIFNβ used (i.e., 100 U) totally inhibited the growth of a swIFNβ-sensitive porcine reproductive and respiratory syndrome virus (PRRSV) run in parallel in each bioassay. The bioassays were repeated with consistent results.

Variable effects of NS1-inhibitors
Based on preliminary runs, NS1-inhibition experiments per-

formed in this study utilized at least 5 times the concentrations of inhibitor reported previously which reduced significantly the replication of three human influenza virus strains [38] and were repeated with consistent results. Neither NSC10934 nor NSC128164 had significant effects on SIV-mediated induction of IFNβ (data not shown) and these had partial or no effect on viral replication. The addition of NS1-inhibitor NSC10934 resulted in a reduction in virus titer of strain A/Sw/Iowa/73 (H1N1) of over 1 log10, while no visible effects on titer were detected with NSC128164 with this strain (**Figure 4**). In contrast, none of the two NS1 inhibitors tested here had effects on the replication of A/Sw/Tx/4199/98 (H3N2) while increases in the titers of A/Sw/NC/18893/01 (rH1N1) were detected in the presence of both compounds (**Figure 4**).
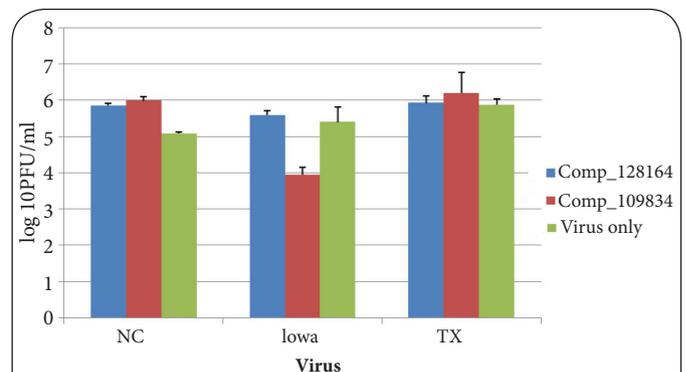

Figure 4. Replication of SIV strains in the presence of NS1 antagonistic compounds. SJPL cells were infected with a moi of 0.005 of the three swine influenza strains in the presence or absence of 50 μM NS1 antagonistic compounds (NSC 109834 and NSC 128164). Plaque assays were performed with supernatants collected at 72 h post-infection. Bars are mean virus titers and standard deviation, and are representative of two independent experiments.

Discussion

Type I IFNs are induced within hours upon virus infection [20]. Influenza virus, as many other RNA viruses, has the ability to induce interferon both *in vitro* and *in vivo* [24,27,28,30,34,40]. Hayman et al., using a panel of human influenza viruses in a human alveolar cell line [A cells] demonstrated that several, but not all, human influenza viruses like A/Sydney/5/97, A/England/41/96 both A/H3N2) induced IFNβ. The three strains of swine influenza in our study induced expression of IFNβ in SJPL cells and it is possible that other SIV strains exist that may be non-inducers or poor inducers of IFNβ as reported for the human influenza virus [24]. We also observed, with at least one of the strains, that the induction of IFNβ was not dependent on the infectious virus dose. Interestingly, the three study SIV strains induced IFNβ but were not susceptible to the antiviral effects of exogenous recombinant swFNβ supplied under the conditions used in these cell culture bioassays. These results are not entirely surprising as influenza viruses have

been shown to evade the effects of interferon [24,41,42]. Our results parallel, to an extent, those reported earlier in which the strain A/Sydney/5/97, an IFNβ- inducing virus, was shown to be insensitive to exogenous IFNα [24]. Also the results with SIV here appear to parallel those of a previous study in our laboratory with PRRSV, a swine arterivirus, where marked differences in sensitivity to recombinant swine IFNβ were observed in different cell types [43]. In that study, several PRRSV strains were relatively resistant to recombinant swIFNβ in MARC 145 cells, an African green monkey cell line, while proving fully sensitive in porcine alveolar macrophages. Thus, the lack of sensitivity to exogenous swIFNβ of these SIV strains in SJPL cells, may reflect lesser or absent effects of such treatment in cells of different species.

Alternatively, it could be speculated that the SIV infection induced blockage of swIFNβ-mediated signaling events leading to poor antiviral state as reported earlier [46] as a potential mechanism of resistance. Correlating resistance to swIFNβ of these SIV strains as a marker of higher virulence compared to swIFNβ-sensitive strains, as reported with Venezuelan equine encephalitis viruses [47] would be at best speculative at this point. Studies in animals would be required to establish whether swIFNβ-inducing, swIFNβ-resistant SIV strains can override the induced antiviral state, as recently reported with non-IFN inhibitory, highly pathogenic avian influenza viruses [48], and demonstrate higher pathogenicity than that of swIFNβ-sensitive strains.

The NS1 protein of influenza virus has been shown to down-modulate IFNα/β induction as a mechanism of evasion of host response [44]. Accordingly, NS1 deletion mutants of SIV have a decreased ability to block IFNα/β production *in vitro* and are attenuated in pigs [45]. NS1 is a natural antagonist of IFNα/β and its effects can be inhibited by IFNα/β certain compounds [38]. In our study, NS1 antagonistic compounds had no significant effect on the SIV-mediated induction of IFNβ and had partial or no effect on viral replication. The later results were unexpected. When tested with or without NS1 inhibitors, the three SIV strains induced significant levels of IFNβ as compared to uninfected cells. On the other hand, the three SIV strains were resistant to exogenous IFNβ supplied in cell culture. These two biological abilities, may have masked subtle changes in IFNβ levels, if there were any in cells treated with NS1 inhibitors. The disparity of the effects of NS1 inhibitors observed on replication of the strains tested is more difficult to interpret. SJPL cells, were considered adequate for testing the effects of NS1 inhibition on SIV infection since the cells were shown to be IFNβ-competent both by poly I:C induction assays as well as SIV infection. Moreover, the NS1-inhibition experiments utilized at least 5 times the concentrations of inhibitor reported previously to reduce significantly the replication of three human influenza virus strains [38]. NS1-independent mechanisms of evasion from type I IFN should also be considered [26,42] especially for the two SIV strains that showed resistance to both inhibitors. In light of data on

human or avian influenza NS1s negative effects on human IFNα/β expression [24,41], closer analysis of the NS1 biology for IFNβ-inducing and non-inducing strains would be necessary to better understand the results observed with the SIV strains examined here. The SJPL cells, used here without prior knowledge of host origin, proved useful to assay SIV in the context of IFNβ.

Conclusions

SJPL produce IFNβ upon stimulation with poly I:C thus making them appropriate to test for IFNβ induction post viral infection. Three strains of SIV tested here, were shown to induce significant synthesis of IFNβ in SJPL cells. However, it was also shown that exogenous recombinant swine IFNβ had no significant inhibitory effect on the replication of these SIV strains. Moreover, NS1-antagonistic compounds did not affect IFNβ induction and had variable and unexpected effects on replication of the SIV strains tested. These seemingly contradicting results suggest virus evasion by mechanisms other than NS1.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	KNAP	MJG	AEG
Research concept and design	✓	✓	✓
Collection and/or assembly of data	✓	--	--
Data analysis and interpretation	✓	✓	✓
Writing the article	✓	--	✓
Critical revision of the article	✓	✓	✓
Final approval of article	✓	✓	✓
Statistical analysis	✓	--	--

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