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Protection from pulmonary tissue damage associated with infection of cynomolgus macaques by highly pathogenic avian influenza virus (H5N1) by low dose natural human IFN-α administered to the buccal mucosa

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ABSTRACT

Using an established nonhuman primate model for H5N1 highly pathogenic influenza virus infection in humans, we have been able to demonstrate the prophylactic mitigation of the pulmonary damage characteristic of human fatal cases from primary influenza virus pneumonia with a low dose oral formulation of a commercially available parenteral natural human interferon alpha (Alferon N Injection®). At the highest oral dose (62.5 IU/kg body weight) used there was a marked reduction in the alveolar inflammatory response with minor evidence of alveolar and interstitial edema in contrast to the hemorrhage and inflammatory response observed in the alveoli of control animals. The mitigation of severe damage to the lower pulmonary airway was observed without a parallel reduction in viral titers. Clinical trial data will be necessary to establish its prophylactic human efficacy for highly pathogenic influenza viruses.

1. Introduction

Highly pathogenic avian influenza A (H5N1) virus (HPAI-H5N1) is endemic in wild birds in Asia and the Middle East, causing disease with high lethality and transmissibility in domestic poultry. Infection of humans is relatively rare but is associated with a high mortality rate (~60%) due to a highly alveolar destructive primary viral pneumonia frequently expressed clinically as acute respiratory distress syndrome (ARDS) (Abdel-Ghafar et al., 2008). The histopathology parallels the viral pneumonia associated with the H1N1 influenza pandemic of 1918 (Taubenberger and Morens, 2008). High viral load and intense inflammatory responses associated with hypercytokinemia are central elements in H5N1 pathogenesis in humans (de Jong et al., 2006).

The observed high mortality in humans with no prior immunological memory for the various current avian H5N1 clades of highly pathogenic influenza virus in humans (HPIVh) remains as a potential pandemic threat. Low pathogenic influenza A (H7N9) virus (LPAI-H7N9) is a more recent emerging avian influenza virus with low pathogenicity for poultry (Gao et al., 2013) with close parallels to HPAI-H5N1 and similar human pandemic potential (Morens et al., 2013). Based on a report of efficacy following oral administration of Alferon N (Cummins et al., 2005) as well as initial microchip evidence in humans of gene modulation (unpublished), a study was initiated to determine whether an experimental low dose oral formulation (Alferon® LDO) of the commercially available natural interferon-α (IFN-α/Alferon N Injection®) could modulate the pathogenesis of HPIVh in an established nonhuman primate H5N1 model (Kuiken et al., 2003; Rimmelzwaan et al., 2003). Alferon N has a significantly higher specific activity against HIV and VSV than commercial recombinant IFNs (Fan et al., 1993). It is composed of at least 6 of the 14 alleles that encode for human IFN-α (GeneCard, 2014). The data from this study demonstrates

Abbreviations: HPAI-, highly pathogenic avian influenza; LPAI-, low pathogenic avian influenza; HPIVh, highly pathogenic influenza virus in humans; TLR, toll-like receptor; LDO, low dose oral; IFN, interferon.

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that Alferon LDO provides protection from the pulmonary damage observed in lethal cases of primary HPIVh pneumonia although no direct effect on pulmonary HPAI-H5N1 titers was observed at the doses administered.

2. Materials and methods

2.1. Cynomolgus macaque experimental

All biohazard experiments were conducted at Viroclinics Biosciences BV, Rotterdam, Netherlands, under contract with Hemispherx Biopharma, Inc. (Philadelphia, PA, USA).

2.1.1. Treatment protocol

Based on initial microarray assays of mRNA peripheral blood responses in humans, three Alferon LDO treatment groups (10, 25, and 62.5 IU/kg body weight) and a vehicle citrate buffer control group were randomly established in groups of 3 cynomolgus macaques (*Macaca fascicularis*: age ca. 26–38 months, bodyweights 2.1–3.2 kg on the initial day of dosing). All animals were obtained from Hartelust BV, Tilburg, The Netherlands and originated from Guangxi, China. All animals were visually inspected for signs of ill health by a veterinarian and were considered healthy for use in the study. Animals were housed by treatment group for the duration of the study in isolator cages and identified by tattoo. Bodyweights were recorded daily. All animals were offered food and water ad libitum. Lighting was controlled to give a 12 h light/12 h dark cycle. Room temperature and humidity levels were maintained at 21 ± 1 °C and 50 ± 10%, respectively. Doses of Alferon LDO were prepared from a 1000 IU/ml buffered citrate solution of commercial Alferon N in which titers were established against an NIH standard (Gxa23-902-530) within one hour of administration. Alferon LDO at 62.5, 25, and 10 IU/kg in 0.25 ml was applied to the oral mucosa of anesthetized animals (ketamine, 10 mg/kg and dormitor, 0.1 mg/kg) to inhibit immediate ingestion and insure at least a 2 min exposure to the buccal mucosal target site daily from −8 to +5 days. Infection was established on day 0 with the H5N1 strain A/Vietnam/1194/2004 by intratracheal administration of 2.5×10^4 $TCID_{50}$ in a 5 ml volume. All use of viable H5N1 virus was conducted under strict BSL-III governmental guidelines and oversight. Preparation and administration of H5N1 were performed in a BSL-III laboratory and BSL-III (negatively pressurized glove box) isolator cages, respectively.

2.1.2. HPAI virus quantitation

Viral titers were established from homogenized tissue samples of the right lung frozen at necropsy at −80° by an H5N1-specific quantitative reverse transcription qRT-PCR with primers derived from conserved regions in the matrix gene segment as described previously (Fouchier et al., 2000) and infectivity assays by virus titration on Madin Darby Canine Kidney (MDCK) cells ($TCID_{50}$) at day 5 of infection following euthanasia under ketamine anesthesia.

2.1.3. Pathological examination

A macroscopic post-mortem examination was performed on all animals. This included examination of the external surfaces, the thoracic, abdominal and pelvic cavities with their associated organs and tissues and the neck with its associated organs and tissues. All lung lobes (left cranial lobe with cranial and caudal segments, left caudal lobe, right cranial, middle and caudal lobes and accessory lobe) were inspected and lesions described. The left lung was collected during autopsy, inflated intra-bronchially with 10% neutral-buffered formalin and stored for fixation/histology and microscopic examination.

2.2. Statistical analysis

In order to minimize a type II statistical error due to the small numbers of macaques in each of the four treatment group ($n = 3$), the two-tailed, nonparametric rank order Jonckheere–Terpstra test (T_{JT}) (Bewick et al., 2004) was used to test the hypothesis that the population means at each Alferon dose were ordered as a response to drug as a function of dose. The degree of severity of the histopathological lesions were ranked as follows: marginal = 1, slight = 2, moderate = 3, marked = 4, and severe = 5. The gross number of observed lesions was used to establish an independent rank order analysis of the significance of the observed anatomic abnormalities as a function of Alferon LDO dose. The calculated T_{JT} statistic was evaluated for its associated probability value using a standard Normal distribution table.

3. Results

3.1. Effect of Alferon LDO on H5N1 infection in a cynomolgus macaque model

Based on the initial observation of gene modulation in humans, Alferon LDO was prophylactically administered to the buccal mucosa of 3 dosage groups of cynomolgus macaques plus a placebo control for 8 days prior to the intratracheal administration of 2.5×10^4 $TCID_{50}$ H5N1 (strain A/Vietnam/1194/2004). On day 5 of infection all animals were sacrificed under ketamine anesthesia and a post-mortem examination and viral titers were performed as well as weights and WBC analyses. All treatment and placebo groups lost weight (~5%) during the course of the experimental protocol (Fig. S1); WBC responses to infection and treatment were highly variable with no observable trend (data not shown). Table 1 summarizes the pulmonary H5N1 viral titers on day 5 following infection. There was no inhibition of viral replication as a function of Alferon LDO as determined by qRT-PCR and viral infectivity assays. Gross and microscopic examination of the lungs, however, revealed compelling morphologic evidence of a dose dependent mitigation of the primary atypical pneumonia tissue damage associated with human morbidity and mortality from avian HPAIV.

The major site of HPAI-H5N1 HPIVh replication in the nonhuman primate model used in this study is the lung that results in a severe viral primary atypical pneumonia. Fig. 1A and B illustrates a representative severe histopathological lesion from a placebo control animal at day 5 of infection. The HPAI-H5N1 induced lesion is characterized by hemorrhage and a marked inflammatory response (neutrophils and lymphocytes), alveolar edema, interstitial edema, and emphysema with destruction of alveolar septae. Administration of the higher doses of Alferon LDO results in a reduction in the severity of gross and histopathological lesions from HPAI-H5N1 challenge on day 5 of infection. Fig. 1C–F are representative of HPAI-H5N1 induced histopathological lesions of decreasing severity that are Alferon LDO dose dependent. An area of pneumonia of marginal severity seen at the 62.5 IU/kg dose level is illustrated in Fig. 1F. There is a marked reduction in the inflammatory response with minor elements of alveolar interstitial edema and inflammation. The alveoli are intact with minimal evidence of an inflammatory response. The gross pathology was consistent with the microscopic analysis. The lungs from the placebo animals at 5 days post HPAI-H5N1 virus challenge exhibited multiple areas of punctate and diffuse dark red areas on the pulmonary surface consistent with the microscopic inflammatory response and alveolar hemorrhage observed in this study and consistent with the anatomic lesions observed in the original description of the model (Kuiken et al., 2003; Rimmelzwaan et al., 2003). No

Table 1
Pulmonary H5N1 viral loads at 5 days following daily Alferon-LDO administration to the buccal mucosa.

Alferon-LDO dose	Primate ID	Quantitative pulmonary viral loads[a]		
		$TCID_{50}$		qPCR (CDU[b]/kg tissue)
		Total lung	Biopsy sample	Biopsy sample
62.5 IU/Kg	2059	4.25	2.80	4.09
	9047	2.50	4.22	4.39
	5577	5.00	4.51	4.73
	Mean (±SD)	3.92 ± 1.28	3.84 ± 0.92	4.40 ± 0.32
25 IU/Kg	7531	5.00	5.26	5.73
	2445	1.25	1.70	4.89
	6424	3.00	3.76	3.92
	Mean (±SD)	3.08 ± 1.88	3.57 ± 1.79	4.85 ± 0.91
10 IU/Kg	5641	3.75	2.93	3.94
	5585	3.50	4.74	4.30
	7437	4.00	3.97	5.47
	Mean (±SD)	3.75 ± 0.25	3.88 ± 0.91	4.57 ± 0.80
Placebo	3413	3.50	3.47	4.78
	7671	2.00	1.90	3.92
	1003	4.25	5.70	5.54
	Mean (±SD)	3.25 ± 1.15	3.69 ± 1.91	4.75 ± 0.81
p-Value[c]		>0.5	>0.5	>0.5

[a] log 10 per gram lung tissue.
[b] Control dilution units.
[c] 1-Factor ANOVA.

macroscopic lesions of this type were found in the 62.5 IU/kg dose group. Gross evidence of an adhesive fibrinous pleuritis was observed in the placebo and 10 IU/kg treatment groups. Microscopic evidence of a fibrinous pleuritis was observed in all treatment groups. The elements characteristic of a primary viral atypical pneumonia in each group were ranked based on the aggregate gross (Table 2) and histopathological severity (Table 3) and evaluated as differences in severity between the 4 groups by a two-sided Jonckheere–Terpstra test. The gross pathology as a function of Alferon LDO dose was consistent with the microscopic evaluation. Two of the 3 animals in the 62.5 IU/kg group demonstrated no evidence of infection by gross anatomic examination. Similarly, there was a dose dependent reduction in the quantitative severity of microscopic lesions, which was statistically significant in the caudal lobes ($p = 0.002$). The primary viral pneumonia was less severe in the cranial lobes yielding less discrimination with treatment. The highest dose level of Alferon LDO (62.5 IU/kg) demonstrated the greatest protection against the macroscopic and histopathological lesions associated with H5N1 infection.

4. Discussion

We have evaluated an oral natural human IFN-α as a prophylactic inhibitor of the pulmonary damage from primary H5N1 pneumonia in an established nonhuman primate model for HPAI-H5N1 infection in humans using a standardized evaluation protocol for gross and microscopic evaluation. This morphological evidence of mitigation of the primary lesion associated with human mortality from infection by HPAI-H5N1was observed in the face of continued viral replication at day 5 of infection by the prophylactic daily short-term administration to the buccal mucosa of a low dose form of an approved parenteral human IFN-α (Alferon N Injection).

A study of H1N1 influenza virus infection in mice demonstrated that the viral induced cytokine storm is associated with protease activation with damage to endothelial tight junctions resulting in a lethal pulmonary edema (Wang et al., 2010). This association is supported by the observation in mice that the hypercytokinemia from severe influenza is linked to a marked upregulation of cellular trypsins and matrix metalloproteinase-9 in various organs and cells, particularly endothelial cells, resulting in vascular hyperpermeability and multi-organ failure (Kido et al., 2012).

Among the potential mechanisms for the mitigation of pulmonary injury in our primate model in the face of an active pulmonary HPAI-H5N1 replication is a differential regulation of IFN-α inducible genes as a function of IFN-α buccal mucosa administration and concentration. Type I IFNs induce approximately 400 IFN-stimulated genes (ISGs) with a wide variety of antiviral, antigrowth, and immunomodulatory activities (Sadler and Williams, 2008). The disassociation of antiviral activity and pulmonary protection from HPAI-H5N1 infection achieved with low doses of Alferon LDO administration via the buccal mucosal route remains unexplained. One possible mechanism is differential induction of ISGs providing protection against pulmonary tissue destruction while failing to induce sufficient antiviral related ISGs to inhibit HPAI-H5N1 replication. Several recent studies provide a framework for future investigation of this hypothesis. Schmeisser et al. (2010) have demonstrated differential antiviral and antiproliferative activities of the ISG with 25 ISGs restricted to antiviral effects. Moreover, unique sets of ISGs target specific viruses while some ISGs paradoxically promote viral proliferation (Schoggins et al., 2011). Viruses have evolved a variety of mechanisms to evade the ISGs. The NS1 protein controls a number of cellular functions during infection and has been implicated in the abrogation of antiviral gene expression including NF-κB activation, IFN-α/β induction, and binding to dsRNA (Wang et al., 2000). The C-terminal EP/SEV sequence of H5N1 NS1 has been postulated as a potential virulence factor by binding to PDZ domain proteins (Obenauer et al., 2006). PDZ domains function as scaffold proteins that coordinate supramolecular complexes involved in intracellular signaling functions (Sheng and Sala, 2001). PDZ proteins are common targets for many pathogenic viruses interfering with tight junction formation, establishment of cell polarity, and apoptosis (Javier and Rice, 2011). One or more ISGs may function as a PDZ domain protein and serve as a molecular decoy for NS1 of H5N1. Similarly, cytokine storms are postulated to be responsible in part for the lethality of HPAI-H5N1 as well as responsible for the more serious outcomes from

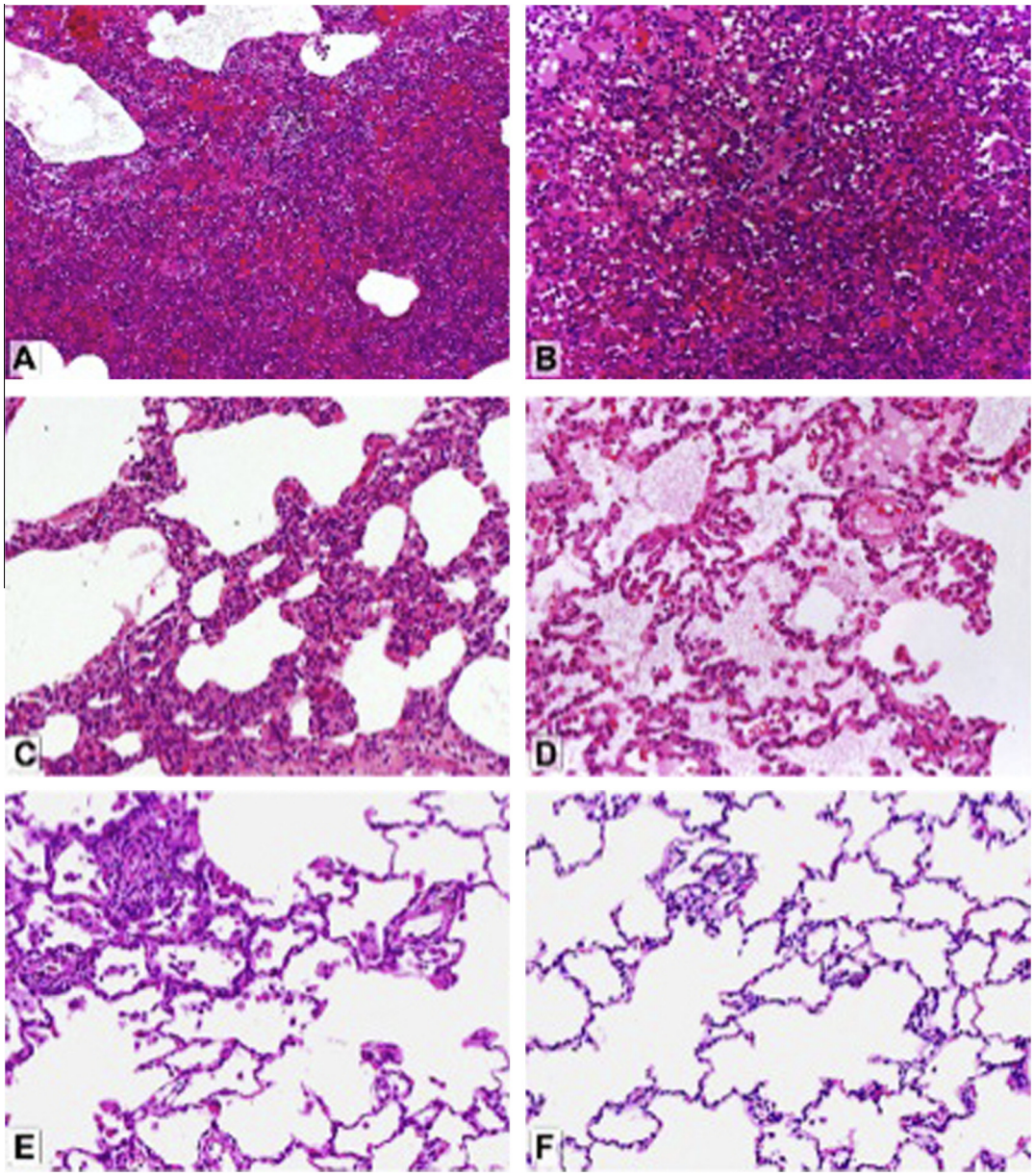

Fig. 1. Pulmonary photomicrographs of animals infected with HPAI-H5N1 at day 5 post infection. (A) Example of severe primary atypical pneumonia in an infected animal from the placebo treatment group. Note the alveolar consolidation of pulmonary tissue with alveolar epithelial necrosis, formation of hyaline membranes, interstitial and alveolar edema with alveoli containing fibrin-rich edema fluid sometimes hemorrhagic with macrophage rich exudate, pulmonary emphysema and infiltration of mixed inflammatory cells (original 100×). (B) Higher power illustration of severe lesion (original 200×). (C) Typical example of marked histopathological response to HPIV-H5N1 infection with marked interstitial inflammation (original 200×). (D) Moderate interstitial inflammatory response (original 200×). (E) Slight interstitial inflammatory response (original 200×), and (F) example of marginal primary atypical (interstitial) pneumonia in an animal in the 62.5 IU/kg treatment group). There is a dramatic sparring of the tissue damage observed in this treated animal cohort (original 200×).

Table 2
Gross pathology observation of Alferon-LDO efficacy against H5N1 in cynomolgus macaques.

Macroscopic observation	Alferon® LDO dose (IU/mL)	Number of animals (n)	Mean	Median	Standard deviation	*p*-Value[c]
Lung count[a]	0 (placebo)	3	7.3	8.0	1.2	
	10	3	7.3	8.0	1.2	$p = 0.016$
	25	3	4.7	4.0	1.2	
	62.5	3	0.7	0.0[d]	1.2	
Lung severity[b]	0 (placebo)	3	13.0	13.0	1.0	
	10	3	16.3	16.0	1.5	$p = 0.010$
	25	3	6.7	6.0	3.1	
	62.5	3	0.7	0.0[d]	1.2	

[a] Number of observed macroscopic lesions.
[b] Macroscopic abnormalities were assessed as 1 = no categorization; 2 = marginal; 3 = slight; 4 = moderate; 5 = marked; and 6 = severe.
[c] Two-sided exact Jonckheere–Terpstra test.
[d] Two of three animals did not show any macroscopic abnormalities; hence, the median is zero.

Table 3
Microscopic severity of primary viral pneumonia (H5N1) related to Alferon®LDO treatment dose.

Primary Atypical Pneumonia		Alferon® LDO dosage level (IU/mL)				*p*-Value[a]
Lobe	**Severity**	**0**	**10**	**25**	**62.5**	
Cranial	Marginal	0	0	0	2	
	Slight	2	2	1	0	$p < 0.5$
	Moderate	1	1	2	1	
Caudal	Slight	0	0	0	3	
	Moderate	1	2	3	0	$p = 0.002$
	Marked	1	1	0	0	
	Severe	1	0	0	0	

[a] Two-sided Jonckheere–Terpstra test.

infection by seasonal influenza virus in the elderly and immunocompromised populations (Butler, 2007). Differential down regulation of ISG proteins such as one or more members of the TNFα family provides an alternative mechanism for the observed disassociation of antiviral activity and pulmonary protection observed for Alferon LDO in this HPAI-H5N1 virus nonhuman primate animal model of infection.

Although the IFNs are physiologically active in the picomolar range, IFN has traditionally been administered in massive concentrations by subcutaneous, intramuscular, or intralesional injection. Matzinger et al. (2011) demonstrated the induction of ISGs following systemic and mucosal administration of a recombinant pegylated IFN-α in *Rhesus macaques* associated with a reduction in tracheal viral titers and systemic responses (fever, body weight) following infection with A(H1N1). There is a substantial body of literature supporting antiviral activity of oral IFN that has been comprehensively reviewed by Cummins et al. (2005). The bulk of reports in a variety of animal species including humans have indicated efficacious responses from the oral administration of low dose IFN against a wide variety of indications. The initial regulatory approval for LDO-IFN-α was granted in Japan for the treatment of rotavirus disease in calves <30 days of age. The intranasal administration of ferret IFN-α resulted in reduced morbidity from A(H1N1) infection. Survival against H5N1 challenge, however, was not affected (Kugel et al., 2009). Controlled studies of human low dose IFN-α as an oral or nasal agent in seasonal influenza support clinical trials of Alferon LDO as a prophylactic agent for HPAI-H5N1 virus infection. Solov'ev (1969) reported highly efficacious responses against seasonal influenza virus infection from the intranasal administration of human LDO-IFN-α in both adults ($p < 0.001$) and children ($p < 0.001$) in the Soviet Union. Further studies using a nasal spray (500 U) or an oral form (600 U) reduced the length of symptoms (Jordan et al., 1973). Subsequent studies in children from Bulgaria reported an 85% prevention rate of seasonal upper respiratory infections with a nasal IFN-α formulation (Arnaoudova et al., 1977). Dai et al. (1987) using a combined nasal/oral administration of a very low dose of IFN-α for 4 days (b.i.d.) reduced the length of symptoms from seasonal upper respiratory infections.

The economic and human consequences of an influenza pandemic with high lethality in humans without effective counter measures is significant. Recent structural studies have demonstrated that the avian sialic receptor is cone shaped denying access to the human configuration for both HPAI-H5N1 (Tharakaraman et al., 2013a) and LPAI-H7N9 (Tharakaraman et al., 2013b). The human receptor is more open (umbrella v. cone) allowing access to the α2-6 sialiated glycan. Some H5N1 clades (dkEgy10) are a single amino acid mutation (Q226L) from high affinity binding ($kD' \sim 100$ pM) similar to the 2009 pandemic H1N1. The current LPAI-H7N9 is only one mutation (G226S) from allowing this more open confirmation and high affinity binding. The development of vaccines directed against H5N1 has been relatively rapid although the high protein dosage levels and apparent lack of cross clade protection has been a problem. LPAI-H7N9 has been reported to have similar low antigenicity. Moreover, Tharakaraman et al. (2013b) have identified several coevolving amino acid sites that appear to be in the antigenic regions of H7. Prepandrix, a low dose inactivated split A/Vietnam/1194/2004 vaccine adjuvanted with a proprietary Glaxo Smith Kline oil-in-water preparation, has been reported to have achieved significant cross clade protection in ferrets (Baras et al., 2008). Alternatively, a nasal preparation of a seasonal vaccine in Japan that was adjuvanted with a toll-like receptor (TLR) 3 agonist provided cross-clade protection in mice against H5N1 (Ichinohe et al., 2007). Absent a broadly protective vaccine currently, antiviral control is dependent on agents approved for use (neuraminidase inhibitors) or agents derived from currently approved uses in which regulatory approval can be more quickly navigated. The LDO-IFN-α used in the studies reported here is a drug approved for parenteral use in humans at much higher concentrations. It is a multispecies glycosylated natural IFN-α product with broad antiviral activity including oseltamivir resistant LPAI-H7N9 (Liu et al., 2014) that has not experienced the development of anti-IFN antibodies associated with recombinant, nonglycosylated IFNs in clinical use (Strayer and Carter, 2012).

The precise mechanism for the observed protection against the pulmonary tissue damage of virus-induced primary atypical pneumonia in an established nonhuman primate model of HPAI-H5N1 highly pathogenic in humans (HPIVh) is unknown. A study in aged macaques suggests that the differential response between aged and young adult macaques to infection with the coronavirus responsible for severe acute respiratory syndrome (SARS-CoV) is related to a more zealous inflammatory gene activation response to viral infection in the aged animals associated with a reduction in expression of type-1 IFN (Smits et al., 2010). Similar to this study treatment with type-1 IFN reduced pulmonary pathology without reducing SARS-CoV replication in the lungs.

Further development of LDO-IFN-α in animals and humans may provide a readily available, orally administered, and economic source for an effective prophylactic against HPIVh pulmonary tissue damage as well as other indications such as SARS and/or MERS with similar pulmonary pathologies (Gu and Korteweg, 2007).

Competing interests

WMM and WAC are members of the Board of Directors of Hemispherx Biopharma Inc. (a public company). WAC and DRS are employed by Hemispherx. WMM, WAC, and DRS are shareholders in the company.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.07.010.

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