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Use of plethysmography in assessing the efficacy of antivirals in a mouse model of pandemic influenza A virus

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ABSTRACT

The recently emerged swine-origin H1N1 influenza A virus (IAV) caused a pandemic outbreak in 2009 with higher risk of severe disease among children and pregnant women in their third trimester (Van Kerkhove et al., 2011), and is continuing to be important seasonal IAV strain. Mice are commonly used in antiviral studies as models of influenza disease, which utilize morbidity and mortality to assess the efficacy of a test compound. Here, we investigated the utility of unrestrained plethysomography to quantify the lung function of IAV-infected BALB/c mice. Administration of a lethal dose (~30X LD_{50}) of pandemic H1N1 IAV resulted in a rapid decline in breath volume, as determined by a significant ($P < 0.001$) decrease in the pressure associated with inspiration and expiration detected as early as 2 days after virus challenge. Severe disease was also accompanied by a significant ($P < 0.05$) increase in breath time on 8 dpi. Plethysmography parameters correlated with weight loss and other parameters of disease such as gross pathology and the weight of the lung. Breath time was reduced in surviving mice challenged with a sublethal dose of virus as compared with normal controls, and is a predictive indicator of outcome in these mice. In antiviral studies, the use of plethysmography resulted in the detection of a clear and rapid treatment response, which was similar to other non-invasive parameters, such as weight change. Oseltamivir and ribavirin significantly ($P < 0.001$) improved parameters of lung function, particularly mean breath volume, as early as 2 dpi and in a dose-dependent manner. Moreover, a combination of these two drugs further improved these parameters. Plethysmography provides a sensitive evaluation of lung function in IAV-infected mice in response to antiviral therapy.

1. Introduction

Influenza viruses infect millions of people annually to cause subclinical and mild to fulminant and lethal disease. The severity of disease is dependent on many host and viral factors. Host factors such as age and health status contribute to individual outcome, although pathogenic determinants associated with the virus lead to severe outbreaks (Bogs et al., 2010; Maines et al., 2011; Van Kerkhove et al., 2011; Zhou et al., 2011). Immune-mediated enhancement of disease is a recently discovered mode of influenza virus pathogenesis and explains the increased morbidity in certain age groups that do not normally experience higher mortality rates during outbreaks (Monsalvo et al., 2011). An understanding of mechanisms of pathogenesis aids in the development of appropriate strategies to inhibit viral replication and disease progression.

Damage to the lung is a critical consequence of influenza infection. This is due in large part to the immunopathology that is associated with an immune response to virus infection, which includes the infiltration of immune cells into lung tissue, cytokine signaling as a result of activated immune cells, and fluid accumulation in damaged tissue (Kim et al., 2011; McGill et al., 2009). Inhibition of the TNF-alpha signaling pathway, which is an important mediator of immunopathology during respiratory virus infection, results in a decrease of lung injury after influenza infection (Srikiatkhachorn et al., 2010), which underscores the importance of immunopathology and potential usefulness of targeting these pathways as an approach to controlling disease. Similarly, inhibiting the production of reactive oxygen species (ROS) was also shown to reduce disease severity and virus replication in the lungs of infected mice (Vlahos et al., 2011). Reduction or limitation of virus replication through treatment with antiviral compounds such as T-705 also reduces virus titers in the lung, which correlates with improved lung pathology (Sidwell et al., 2007), likely through the indirect amelioration of the immune response to reduce the viral load in the lungs. An important consideration of antiviral therapy is to determine how treatment will affect lung disease that is associated with influenza infection, which may occur directly through inhibition of host

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mediators of immunopathology or indirectly through the inhibition of virus replication.

Since lung pathology is an important determinant for the outcome of influenza disease in mouse models, it is very important to monitor lung function during experimental infection studies especially when characterizing a therapeutic agent designed to improve disease outcome when administered after viral infection. A benefit of plethysmography is that lung function can be longitudinally assessed *ante mortem*. Plethysmography has utility in assessing mechanical lung function (Bates et al., 2008). For example, it has been utilized in different virus infection studies in mice, including bacterial co-infection studies (de Vrankrijker et al., 2009), airway inflammation studies (Marsland et al., 2004; Stowell et al., 2009), and basic virus–host interaction studies (Snelgrove et al., 2006) in intact and conscious hosts. The purpose of the present study was to characterize a method of lung function analysis for antiviral treatment experiments of influenza virus infection in a mouse model, and to correlate non-invasive plethysmograph parameters with other parameters typically employed in mouse influenza models. Severe pneumonia can be a consequence of severe influenza disease in humans, especially those infected with the recent pandemic H1N1 influenza (Higuera Iglesias et al., 2011). Therefore, it is important to identify the effect of potential antiviral drugs on this disease phenotype.

Herein, we describe the characterization of a plethysmograph developed in-house and the utility of this instrument in assessing the efficacy of known antiviral compounds in a mouse model of pandemic H1N1 influenza A virus infection and disease.

2. Materials and methods

2.1. Mice

Specific pathogen-free BALB/c mice weighing approximately 17–19 g were obtained from Charles River Laboratories (Wilmington, MA). The mice were fed standard rodent chow and had *ad libitum* access to water. Animals were quarantined at least 48 h prior to experimental manipulation. This study was conducted in accordance with the approval of the Institutional Animal Care and Use Committee of Utah State University. The work was conducted in the AAALAC-accredited Laboratory Animal Research Center of Utah State University.

2.2. Virus

Influenza A/California/04/2009 (H1N1) (IAV), strain designation 175190, was received from Dr. Elena Govorkova, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN (Govorkova et al., 2011). The virus was adapted at St. Jude to replicate in the lungs of BALB/c mice by 9 sequential passages through mouse lungs. Virus was plaque-purified in MDCK cells and a virus stock was prepared by growth in embryonated chicken eggs followed by MDCK cells. The stock virus had a titer of $10^{6.24}$ 50% cell culture infectious doses ($CCID_{50}$)/ml.

2.3. Plethysmography chamber

The plethysmograph chambers (MottFab Designs, Wellsville, Utah) were made of 1.5″ acrylic tubes, which were approximately 5″ long. One tightly stoppered end contained a small valve that acted as a barometric-shock absorber, while the other stoppered end was connected to the transducer through superthane pneumatic tubing (Small Part, Inc., Lexington, KY). The plethysmograph utilized a Grass Industries PT5 volumetric transducer (Warwick, RI) linked to a laptop computer running the LabView programming (National Instruments, Austin, TX) via a National Instruments Analog to Digital Acquisition card (USB-6212) and a shielded signal conditioning connector box (SCB 68). LabView Software (National Instruments, Austin Texas) was used to construct a program that exported data in MS Excel (Microsoft, Redmond Washington) format.

For measurement of plethysmography parameters, mice were injected intraperitoneally (i.p.) with 1 mg/kg of acepromazine to limit the body movement other than that associated with breathing. Breathing was recorded with data points taken every millisecond for a total duration of 3 s and were exported as an Excel file. Mice were kept in the plethysmograph chamber for 3 min or less, and were removed after a suitable recording was obtained.

Plethysmography data were analyzed using MacPlethy software written by Utah State University Computer Science student Mike Moss. A single representative breath curve from each animal was selected for measurement of the inspiratory and expiratory pressure and time. The inspiratory and expiratory pressure measurements (the change in pressure within the chamber due to respiration) were combined to give mean breath volume (MBV). Similarly, the mean breath time (MBT) was a combination of the time of inspiration with the time of expiration. The inspiratory and expiratory pressure and time values were combined as breath volume and breath time, respectively. A single breath was measured from the beginning of inspiration through the end of expiration. Data were plotted and analyzed using Prism 5 (GraphPad Software, Inc. La Jolla, CA).

2.4. Antiviral compounds

Oseltamivir phosphate (Tamiflu®) was purchased from a local pharmacy. Ribavirin was provided by ICN Pharmaceuticals, Inc (Costa Mesa, CA) as a powder. Compounds were prepared in sterile saline. Because of the presence of filler material in Tamiflu capsules, the contents of whole capsules were used in drug preparation. Further dilutions in saline were performed as needed. Calculation of mg/kg/day doses of oseltamivir were based upon each capsule containing 75 mg of oseltamivir (after conversion of the prodrug, oseltamivir phosphate, in vivo). Compounds were prepared less than 18 h prior to use and stored for the duration of the treatment period at 4 °C.

2.5. Experimental design

Animals were randomly assigned to experimental groups containing 10 mice/group. Mice were individually identified with ear tags. Initial studies to characterize the use of the plethysmograph chambers used the following two virus challenges: a lethal dose of $10^{4.1}$ $CCID_{50}$ (~30X 50% mouse lethal dose [MLD_{50}]), which is known to cause 100% mortality in BALB/c mice, and less lethal doses of $10^{3.5}$ or $10^{3.2}$ $CCID_{50}$ (~7.5 or 3.8 X MLD_{50}, respectively). Virus was administered through intranasal (i.n.) instillation under ketamine + xylazine anathesia. Lung function was measured using the plethysmograph in individual mice over the course of the disease through 21 d post-virus instillation (dpi).

Studies to correlate disease with plethysmography output were conducted using 5 mice/group with 2 infection groups (high and low virus challenge) and 3 mice/group of sham-infected control mice. Breath volume, breath time, oxygen saturation, and body weight were measured, followed by harvesting the lung tissue of infected mice to determine the virus titer and pathology of the lungs. Lung samples were also fixed in paraformaldehyde and evaluated histologically by Dr. Ramona Skirpstunas (Utah State Veterinary Diagnostics Laboratory, Logan, UT) after samples were sectioned and stained with hemotoxylin and eosin. Mice were

harvested every other day beginning on the day of challenge through day 18 post-virus infection.

For antiviral studies, mice were challenged with the aforementioned virus challenge of $10^{4.1}$ $CCID_{50}$. Mice were treated with oseltamivir or ribavirin twice daily for 5 days beginning 4 h prior to virus challenge. Plethysmography parameters, survival, and weight change were recorded daily for 5 animals in each group to determine the effect of antiviral treatment on lung function of mice infected with IAV. Mice were euthanized if they were found to be non-responsive to stimulus or were unable to right themselves.

An additional 5 animals per group were necropsied at 5 dpi to obtain samples to quantify weight, score, and virus titer of the lung. Lungs were harvested from sacrificed mice and lung weight was obtained. Dissected lungs were assigned a consolidation score from 0 (normal appearance) to 4 (100% of the lung exhibiting a plum color) and then were frozen at −80 °C until titrated for virus. Thawed lungs were homogenized in 1 ml of cell culture medium and refrozen for convenience in plating many samples. Homogenized lung samples were thawed and centrifuged at 2,000*g* for 10 min. Samples were serially diluted in 10-fold increments and titrated by end-point dilution in 96-well microplates. The $CCID_{50}$ titers were calculated per gram of lung tissue.

2.6. Histopathology

In order to assess the pathology of the lung associated with influenza infection and to correlate such observations with data obtained by plethysmography, lung tissues were sent (blinded to the pathologist) for histological evaluation (Veterinary Diagnostics Laboratory, Utah State University, Logan, UT). Fixed sections of lung tissue were stained with hematoxylin and eosin and observed for pathological changes and evidence of virus infection.

2.7. Statistical analysis

Kaplan-Meier survival curves were generated and compared by the Log-rank (Mantel-Cox) test followed by pairwise comparison using the Gehan-Breslow-Wilcoxon test in Prism 5.0b (GraphPad Software Inc.). Mean body weights were analyzed by one-way ANOVA followed by Tukey's multiple comparison tests using Prism 5.0b. Timecourse plots were analyzed using a 2-way ANOVA until initial mortality was observed.

3. Results

Lung function was impaired during the course of IAV infection (Fig. 1). Inspiratory and expiratory volumes, as inferred by pressure changes within the plethysmograph, were significantly ($P < 0.001$) reduced as early as 2 dpi and continued to decrease until the mice succumbed to disease. Pressure of inspiration (P(i)) and expiration (P(e)) are shown in healthy (Fig. 1A) and moribund (Fig. 1B) animals. When measured with a plethysmograph, the pressure of inspiration (P(i)) and expiration (P(e)) were very similar, while the time of inspiration and expiration were fairly distinct and variable, especially in severely ill mice. To better identify trends in infected animals and to reduce the variability of the data, the P(i) and P(e) were combined to represent the total mean breath volume (MBV) of a single breath (Fig. 1C) and the T(i) and T(e) were combined to evaluate the mean breath time (MBT) (Fig. 1D). The MBT of infected mice just prior to death was significantly ($P < 0.05$) lengthened compared to the normal breath rate, while the MBV was decreased and resulted in long, shallow breaths (Fig. 1B). The breath time lengthened in severe disease cases on days 7–9, while animals that would eventually recover often had a significant ($P < 0.001$) decrease in breath time as compared to sham-infected animals even 3–4 weeks after virus challenge (Fig. 1D).

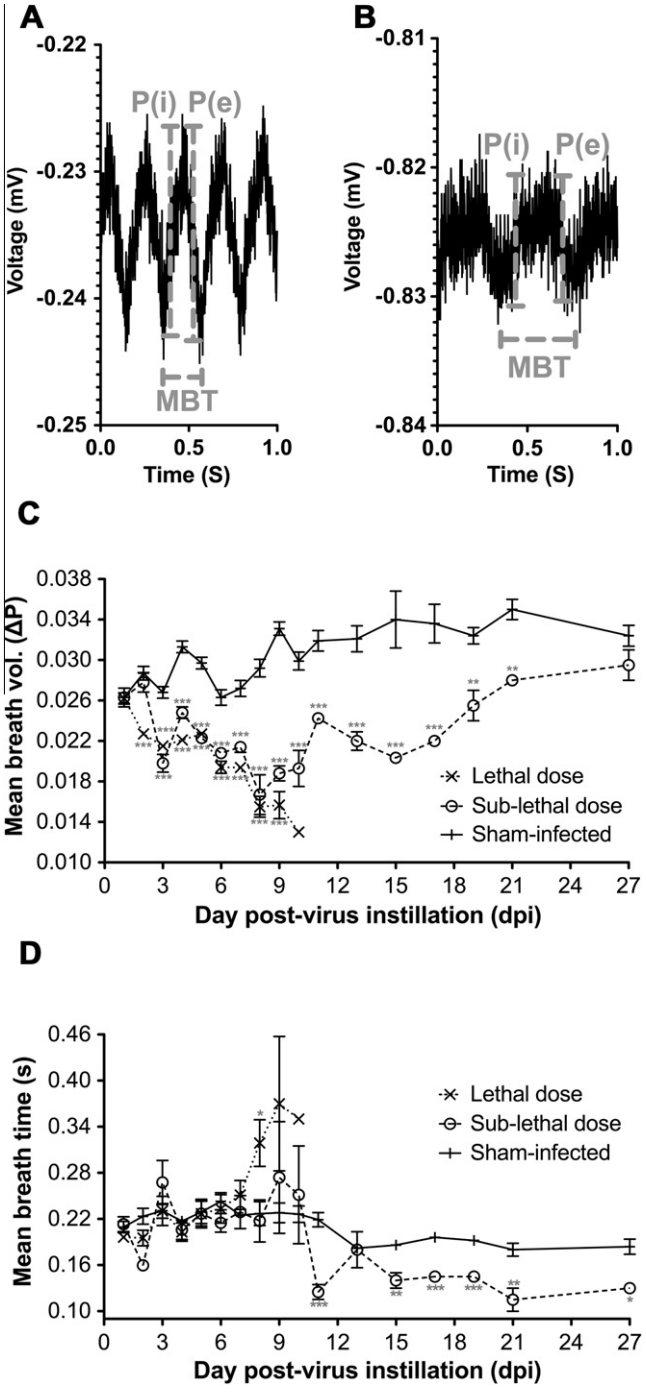

Fig. 1. Output and analysis of plethysmography data obtained from mice infected with influenza virus. Representative breathing curves are shown for (A) healthy and (B) influenza-infected mice. These curves were analyzed to determine the pressure of inspiration (P(i)) and expiration (P(e)) as well as the mean breath time (MBT) of a representative breath from each mouse within a group. MacPlethy analysis software was used to measure the time course of (C) MBV (P(i) + P(e)) and (D) the MBT of mice infected with lethal, sub-lethal, and sham virus challenge ($^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, as compared with sham infection).

Plethysmography parameters, including MBV and MBT, generally followed a similar course as other commonly measured parameters of infected mice as well as more commonly used parameters of weight change, oxygen saturation, lung titers, weights and scores, and survival. Lung samples taken from infected mice after assessing the lung function by plethysmography were

Table 1
The mean breath volume (MBV) associated with histopathological changes in five BALB/c mice challenged with a sub-lethal dose of influenza A virus (H1N1).

Day	MBV ± SD	Significant histological findings
0	0.0244 ± 0.001	No significant findings
2	0.0240 ± 0.002	Lower airways lined with necrotic cells, luminal debris and neutrophils
4	0.0202 ± 0.001[a,c]	As day 2. Edema fluid and neutrophils surround scattered arterioles. Neutrophils and macrophages present in alveoli
6	0.0194 ± 0.002[b]	As day 4. Lymphocytes also surround scattered arterioles
8	0.0204 ± 0.002	Lower airways with luminal necrotic debris, edema fluid. Neutrophils and macrophages present in alveoli
10	0.0210 ± 0.005	Same as day 8
12	0.0190 ± 0.002[b,d]	Same as day 10
14	0.0190 ± 0.002[b,c]	Same as day 12
16	0.0198 ± 0.004[a,c]	Neutrophils, histiocytes and lymphocytes surround scattered arterioles and widen alveolar septae
18	0.0242 ± 0.004	Areas of atelectasis with peribronchiolar and perivascular lymphoid aggregates

[a] $P < 0.05$ as compared to day 0.
[b] $P < 0.01$ as compared to day 0.
[c] $P < 0.05$ as compared to sham-infected controls ($n = 3$).
[d] $P < 0.001$ as compared to sham-infected controls ($n = 3$).

stained for histopathological evaluation. Table 1 shows the major histopathological observations in relation to the change in MBV of mice infected with a dose of virus that resulted in 80% mortality. Immune cell infiltration and associated fluid accumulation occurred as MBV decreased. The surviving 20% of infected mice showed an improvement over time of MBV, although histopathological evidence of virus infection persisted through 18 dpi, which mainly consisted of infiltrating cells of the immune system. As necrosis of lung cells, infiltration of immune cells, and edema were observed, concomitant changes in breath volume were also noted.

Changes in the time-course of various commonly used disease parameters of IAV-infected mice are shown in Fig. 2. Lethal and sublethal virus infection resulted in rapid and prolonged mortality curves, respectively (Fig. 2A). Weight change was also reduced, with weight loss in excess of 30% (Fig. 2B). Saturated oxygen levels were observed to be reduced on 4 dpi in animals infected with a lethal virus infection, while a delay in the curve was observed in animals infected with the sub-lethal dose (Fig. 2C). Lung score (Fig. 2D) and lung weight (Fig. 2E) increased over time, with higher values for these parameters in animals infected with a lethal challenge as compared with those from sub-lethal infection groups. Virus titers peaked early during the course of virus infection on 2 dpi, followed by a steady decline to lower titers just prior to the demise of the animal (Fig. 2F). The MBV was significantly ($P < 0.001$) reduced shortly after virus challenge (day 2, Fig. 1C). The rapid decline in MBV from 0 to 4 dpi correlated with corresponding virus titers ($R^2 = 0.610$) and weight change ($R^2 = 0.530$), but not with lung weights ($R^2 = 0.072$). A better correlation was observed between lung weight correlated and MBT ($R^2 = 0.153$), although this was still not a very strong correlation.

Plethysmography parameters were used to further characterize the antiviral effect of oseltamivir and ribavirin on the lung function of mice compared to placebo treatment. Treatment with effective doses of oseltamivir and ribavirin resulted in an improvement in MBV as compared to placebo treatment (Fig. 3A). Lower doses of these drugs showed a dose-dependent decrease in improvement of MBV. A similar improvement in MBT was also observed (Fig. 3B). Treated animals generally had shorter MBT than normal control animals that were untreated and uninfected, which was noted in animals recovering from virus challenge starting about day 10 of the infection (Fig. 1D). An ineffective dose of 5 mg/kg/d of ribavirin resulted in mean MBVs and MBTs that were similar to those in infected controls, while a higher dose improved these parameters. The higher effective doses of 100 mg/kg/d of oseltamivir and 75 mg/kg/d of ribavirin were completely protective, while lower doses were less protective and were consistent with the plethysmography data (Fig. 3C). The mean percent weight change curve of treated animals showed a trend towards improvement as compared to sham-treated controls, although severe weight loss was also observed in treated animals as compared to uninfected, untreated controls (Fig. 3D).

Lung samples were taken at 6 dpi to determine the effect of antiviral treatment on tissue weight, gross pathology (lung score), and virus titer in lung homogenate as compared to the placebo treatment. Oxygen saturation (SaO_2) levels were also assessed at 6 dpi prior to necropsy. As anticipated, a significant improvement in lung weight, lung score, and SaO_2 were observed in drug-treated animals as compared to placebo treatment in a dose-dependent manner (Table 2). The dose-dependent improvement in these parameters supports the results obtained by plethysmography and validates the MBV and MBT data obtained using our in-house designed and constructed plethysmograph. Moreover, these results underscore the observation that treatment improves lung health and function. No improvement in virus titer measured from lung homogenate samples was observed in any of the treatment groups when compared with placebo, which is consistent with previous results (Sidwell et al., 2005, 1992).

After demonstrating that antiviral therapy improved lung function, we next wished to verify this observation and determine if combination therapy would further improve outcome. As anticipated, a combination of oseltamivir and ribavirin at doses of 100 and 75 mg/kg/d, respectively, administered 4 h after virus challenge was highly effective at reducing the disease burden in influenza-treated mice (Fig. 4). The combination therapy improved MBV to a level that was similar to uninfected, untreated control animals, while monotherapy treatment with either ribavirin or oseltamivir alone showed a less robust improvement (Fig. 4A). Furthermore, the MBV values from animals treated with combination therapy were not significantly ($P > 0.05$) different from those of uninfected, untreated control animals, while monotherapy treatment resulted in significant ($P < 0.01$) differences in MBV when compared with these controls. Placebo treatment resulted in a precipitous drop in MBV beginning at 2 dpi and continuing through 8 dpi. A concomitant increase in MBT occurred, which became apparent beginning later during infection on 6 dpi, and continued through 8 dpi. Animals treated with combination therapy had a reduction in MBT during the first two weeks of the experiment compared with normal controls, but the curves eventually reached similar levels and were sustained through the rest of the experiment. In contrast, the MBT of animals treated with either monotherapy remained reduced for the duration of the experiment (Fig. 4B).

As expected, all animals treated with the combination therapy survived ($P < 0.001$), with 80% and 100% survival in the oseltamivir and ribavirin treatment groups, respectively, while all of the placebo-treated mice succumbed to disease by 8 dpi (Fig. 4C). An initial weight loss was observed in animals treated with the oseltamivir and ribavirin combination, although after 5 dpi animals

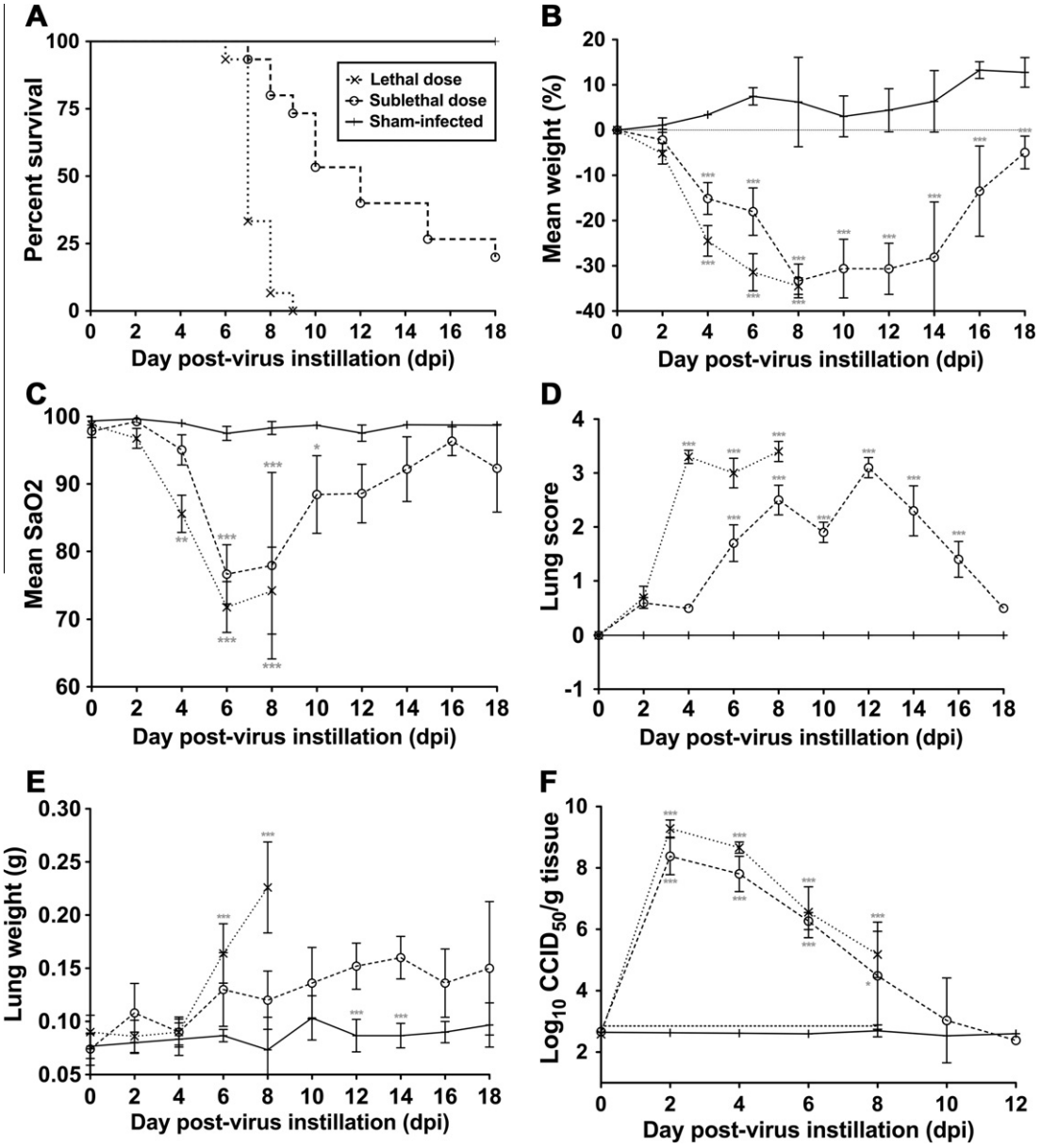

Fig. 2. A time course of various disease parameters of mice infected with a lethal, sublethal or sham influenza virus challenge. Disease parameters include (A) mortality, (B) mean weight change, (C) mean oxygen saturation, (D) visual assessment of lung disease, (E) lung weight, and (F) virus titer in the lung (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, as compared with sham infection).

in this group began to regain weight and reached a mean weight that was similar to normal controls by the end of the experiment (Fig. 4D). Ribavirin treatment resulted in a similar decline and rebound, although the% weight loss in this group was more pronounced through 17 dpi, at which point these animals had similar weights to those receiving the combination treatment. Oseltamivir-treated animals had a slight delay in their weight loss as compared to the mice in other drug treatment groups, although the weights continued to decline through 12 dpi, after which a weight gain was observed. However, the weight of these animals did not return to the level of the other drug-treated animals (Fig. 4D). Placebo treatment resulted in a severe decline in weight that was consistent with other disease parameters.

4. Discussion

The use of lung function analysis in antiviral studies will increase the utility of animal models. Various animal models, including various laboratory mouse strains as well as ferrets, are used to identify potential candidate compounds for clinical evaluation. Common measurements of disease used in these types of studies include mortality, weight change, measurement of saturated oxygen levels, and a myriad of other virus and host parameters that can be used to identify effective treatments (Bantia et al., 2011; Sidwell et al., 2007; Smee et al., 2010a,b). The addition of plethysmography to antiviral studies will provide an additional relevant parameter to more fully characterize potential therapies.

In this study, we have characterized a method of lung function analysis for use in antiviral studies in a mouse influenza model. Previous studies have used plethysmography to obtain information on airway obstruction in the form of the enhanced pause parameter (Penh), which is defined as:

$$\text{Penh} = (\text{PEF}/\text{PIF}) \times (\text{Te}/\text{Rt} - 1)$$

where PEF = peak expiratory height, PIF = peak inspiratory height, Te = expiratory time, and Rt = time to expire (Taken from Buxco

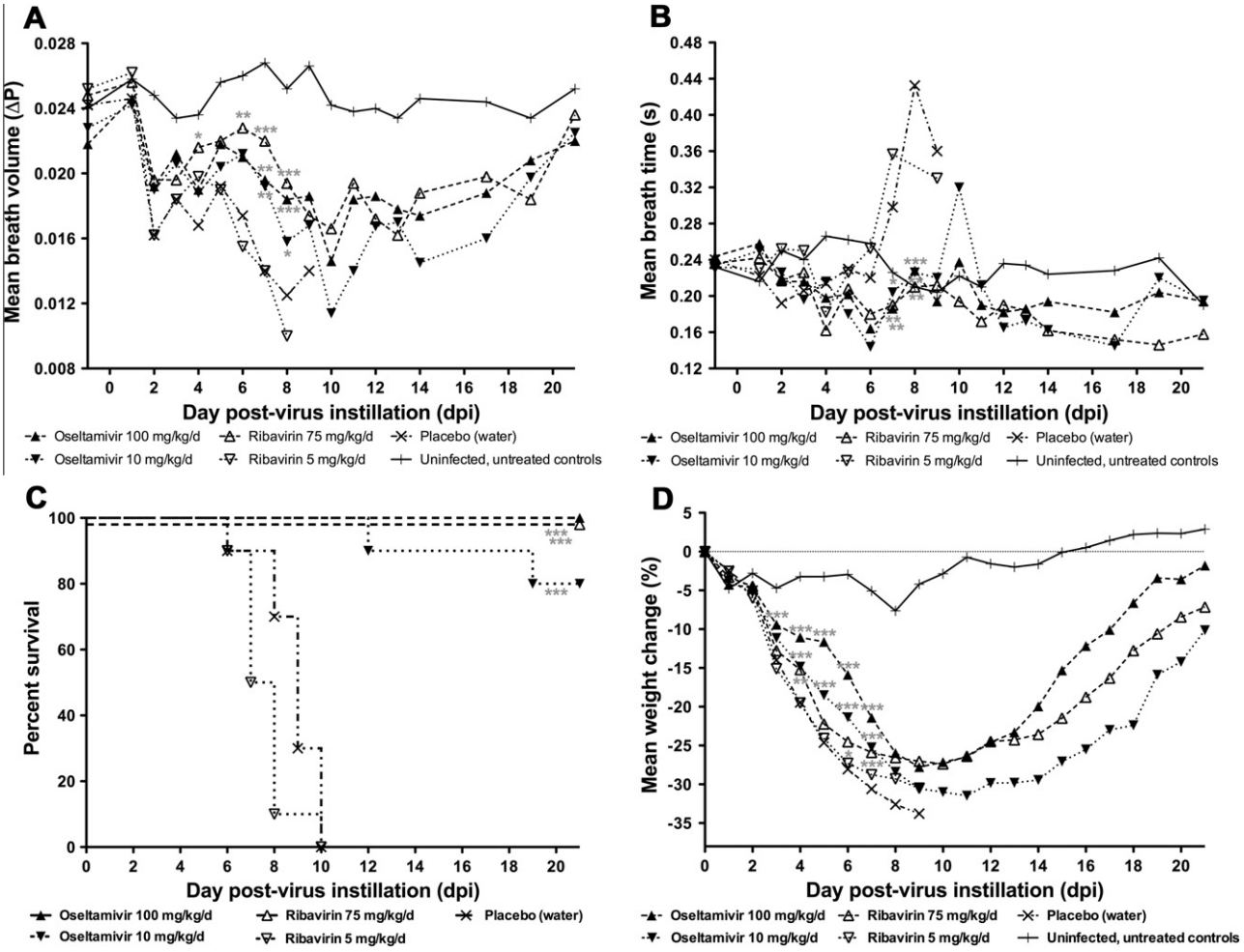

Fig. 3. The effect of treatment with oseltamivir or ribavirin on (A) MBV, (B) MBT, (C) survival, or (D) the mean weight change of mice infected with influenza virus (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, as compared with placebo treatment).

Table 2
The effect of antiviral therapy on various disease parameters of mice infected with influenza A virus. Parameters include survival, percent saturated oxygen concentration and weight, disease score, and titer of the lungs.

Virus: Pandemic influenza A virus (H1N1) *Virus route*: intranasal instillation			*Duration of experiment*: 21 days *Treatment regimen*: 0.1 ml p.o., bid X 5 beg + 4 h			
Treatment	Dose (mg/kg/d)	Survival (l/t)[a]	Lung wt. (g)[b]	Lung score[c]	Lung titers (log_{10} $CCID_{50}$)[d]	SaO_2 (%)[e]
Oseltamivir	100	10/10***	0.22 ± 0.05***	1.4 ± 0.5***	6.2 ± 0.3	98.5 ± 1.0***
Oseltamivir	10	8/10***	0.27 ± 0.02***	2.3 ± 0.3**	6.0 ± 0.5	98.2 ± 0.7***
Ribavirin	75	10/10***	0.19 ± 0.03***	0.2 ± 0.3***	5.2 ± 0.3	97.1 ± 1.7***
Ribavirin	5	0/10	0.29 ± 0.05**	2.7 ± 0.3*	6.0 ± 0.7	91.8 ± 2.9**
Placebo	NA	0/20	0.37 ± 0.05	3.4 ± 0.2	5.7 ± 0.6	83.3 ± 10.7
Normal Cont.	NA	5/5	0.12 ± 0.01***	0.0 ± 0.0***	–	98.4 ± 0.7

[a] Survival (live per total) of animals from the treatment groups through 21 days post-virus instillation (dpi).
[b] Lung weights.
[c] Visual lung score based on gross appearance and assigned a value from 0 to 4 from least affected to most affected, respectively.
[d] Lung titers were measured by cell culture infectious assay and are recorded as 50% cell culture infectious doses.
[e] Percent saturated oxygen concentration.
[c–e] Measured on 6 dpi.
* $P < 0.05$ as compared with placebo treatment.
** $P < 0.01$.
*** $P < 0.001$.

website, buxco.com). This parameter is commonly used, although there is some debate on the validity of this measurement for assessing airway restriction (Adler et al., 2004; Lundblad et al., 2002).

The instrumentation utilized in the present study was not sophisticated enough to provide Penh data. Data obtained using plethysmography varied according to several external influences due to the sensitivity of the instrument, observed in the graph as increase or decrease in pressure. This required the incorporation of appropriate control groups into the study in order to characterize the effect of virus challenge or antiviral treatment on lung

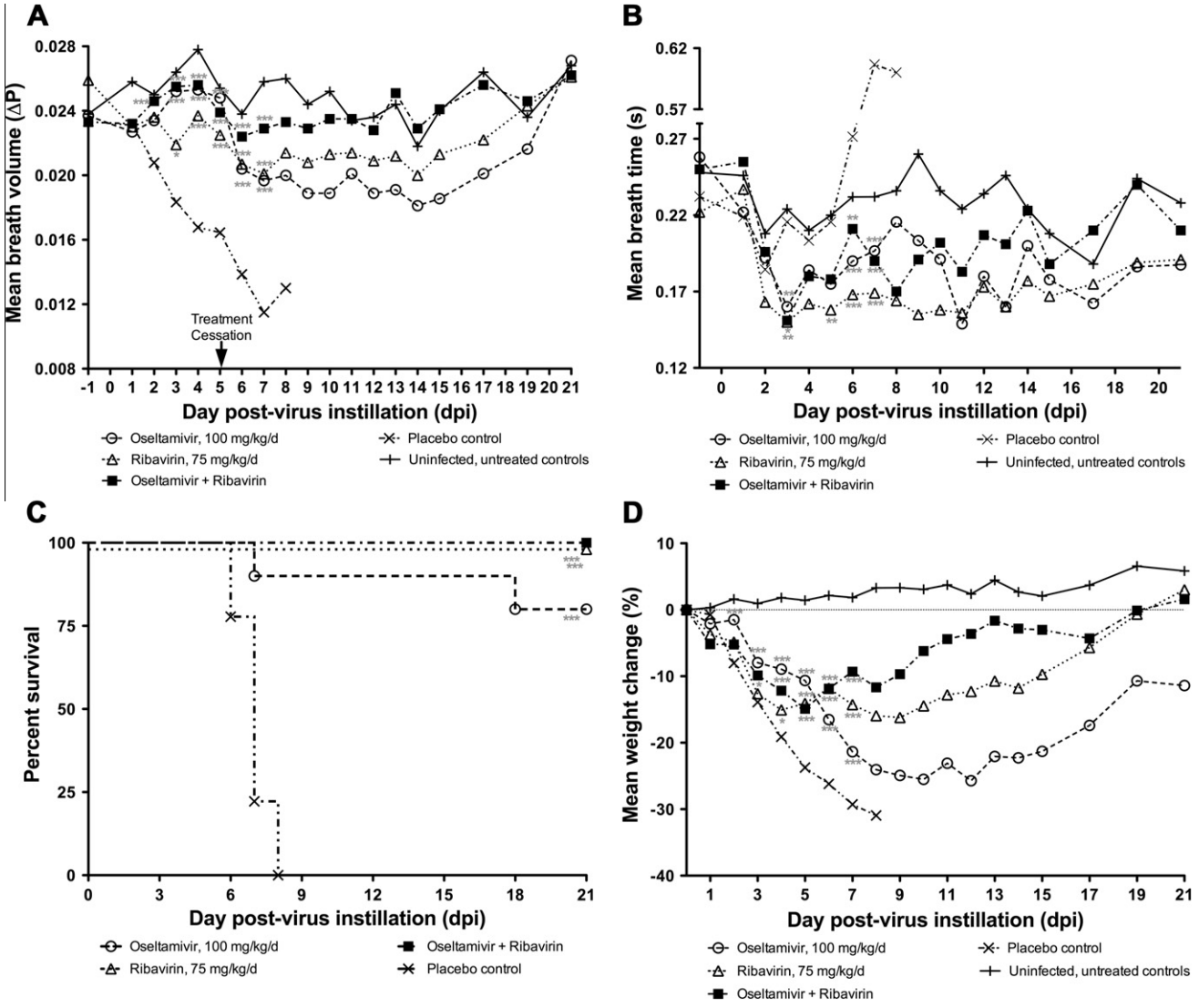

Fig. 4. The effect of combination therapy with oseltamivir and ribavirin as compared to monotherapy and placebo treatment on (A) MBV, (B) MBT, (C) survival, or (D) the mean weight change of mice infected with influenza virus (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, as compared with placebo treatment).

function. Despite the variability, trends in time-course curves could be seen and statistical analyses could be conducted with resulting data. Our plethysmograph allowed us to characterize lung function by measuring the mean volume and time of a single breath. Several factors, including primarily the humidity and temperature of the air within the chamber, were ignored and the data generated from our simple plethysmograph represent the mean relative change in pressure within the chamber. The inspiratory and expiratory changes in relative pressure due to breathing within the chamber were then plotted and each breath identified. The inspiratory and expiratory pressures of a single representative breath were combined to represent the net pressure change for one breath, which was represented as MBV. A similar combination of inspiratory and expiratory times was also performed to yield MBT. While interesting observations were made in regard to MBT, this parameter is quite variable and does not appear to be as useful a parameter as MBV in antiviral studies for assessing lung function. The measurement of MBT, however, may have some utility in identifying severely ill animals that are close to death. Interestingly, it was observed that treated and surviving animals had a significantly lower MBT than uninfected animals. Over time, influenza infection resulted in decreased MBV and increased MBT, which coincided with infiltrating cells of the immune system as well as cellular necrosis and fluid accumulation in the lungs. While this disease phenotype of deep lung infection in mice is rarely seen in human cases, it represents a worst-case scenario.

The use of this simple plethysmography unit provides a functional analysis method to assess the effect of antiviral therapy on lung function. Despite the use of this less sophisticated system compared to other commercially available instruments, the data obtained using our plethysmograph correlated with the disease burden observed in the lungs of infected mice and improved after the administration of antiviral treatment. These results are similar to a study investigating the effect of monoclonal antibody treatment directed against the viral fusion protein of metapneumovirus, which was shown to cause a significant reduction of Penh in a mouse model of disease (Hamelin et al., 2010).

Pulse oximeters have been utilized to obtain blood oxygen saturation levels as an indirect measurement of the effects of influenza infection on lung function in rodents (Sidwell et al., 1992; Verhoeven et al., 2009). In the present study, saturated oxygen levels reached peak reductions between 25% and 30% at 6 dpi, just prior to death. At this time, the mice are generally very sick. With a lethal infection, significant ($P < 0.01$) reductions in saturated

oxygen levels can be observed as early as 4 dpi, while with a sublethal infection the levels are significantly ($P < 0.001$) reduced later on 6 dpi. The use of plethysmography, however, can measure a direct and significant ($P < 0.001$, as compared to uninfected animals) reduction in lung function as soon as 2 dpi and provides a more robust quantification of lung function than measuring saturated oxygen levels.

Lung virus titer on 5 dpi was not affected by treatment. It is unknown why virus titer is not affected, but in a similar study, virus titers on 3 dpi were improved with amantadine and ribavirin treatment, although generally only modest reductions (0.5–1.0 $\log_{10}$ - $CCID_{50}$/g lung tissue) in virus titers were reported for monotherapy treatment (Smee et al., 2009). A high virus titer ($\sim 10^4$ $CCID_{50}$/mouse) is required for 100% mortality, which may increase the difficulty of reducing virus titers by antiviral therapy. A single sample time for the assessment of lung virus titer does not seem to give adequate data to completely demonstrate the utility of lung virus titer assessment in regard to the benefit of antiviral therapy. Survival, weight change, and plethysmography more fully reflect the benefit of treatment and may be assessed ante mortem, which allows the longitudinal tracking of individual animals within treatment groups to more fully identify the effect of treatment on outcome.

Treatment data from this study suggest that the protective effects of antiviral therapy with effective doses of oseltamivir and ribavirin administered 4 h after virus challenge provide protection from death and also reduce the severity of other disease parameters such as weight loss and lung function. One must be careful to include suitable controls during antiviral studies that use plethysmography, since other studies have demonstrated an increase in Penh of animals treated with the immunomodulatory agent Poly IC/LC, which has also been used to treat influenza virus infection in animal models (Boukhvalova et al., 2010; Wong et al., 2009, 1995). Sham-infected controls treated with monotherapy or a combination therapy with ribavirin and oseltamivir were included in our studies and showed MBV and MBT values that were similar to uninfected, untreated controls (data not shown). These results suggested that the treatment itself did not affect lung function.

One interesting observation in this study was the decrease in MBT of infected animals that survived infection through 27 dpi. Decreased MBT occurred regardless of eventual outcome in all animals that survived past 11 dpi (data not shown). This phenomenon was also observed during the rebound stage in recovering animals treated with single antiviral treatment regimens, while the combination therapy restored MBT to levels observed in the uninfected controls. It is unclear why this occurred, although a shorter MBT may be the result of a compensatory increase in respiratory rate in response to the continued pathology of the lung. Indeed, lung samples from mice that were recovering from virus infection had persistent lung disease, including atelectasis on 18 dpi, despite the absence of virus. These observations suggest an extended impairment of the lung well after virus clearance (around 10 dpi). It is likely that the residual effects of influenza infection can last for several weeks after the virus is no longer present in the lungs, and future studies will use plethysmography to characterize the duration of lung impairment in mice after influenza virus infection.

Using the in-house developed plethysmograph and the MacPlethy analysis software, we were able to track the lung function of influenza virus-infected mice over time. This instrument could also be used to demonstrate the utility of lung function analysis as a part of antiviral studies. The use of plethysmography is complementary to commonly used parameters of survival, weight change, and saturated oxygen levels and represents a further functional assay for use in the preclinical evaluation of antiviral therapy in mouse models. The use of plethysmography allows for the direct evaluation of the effect of antiviral treatment on lung pathology as it influences respiration in infected mice, which can not be tracked with mortality, weight change, or other commonly used disease parameters. Overall, plethysmography is a useful tool for influenza studies in a mouse model and should be more fully exploited for this purpose.

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References

Adler, A., Cieslewicz, G., Irvin, C.G., 2004. Unrestrained plethysmography is an unreliable measure of airway responsiveness in BALB/c and C57BL/6 mice. J. Appl. Physiol. 97, 286–292.

Bantia, S., Kellogg, D., Parker, C., Upshaw, R., Ilyushina, N.A., Babu, Y.S., 2011. A single intramuscular injection of neuraminidase inhibitor peramivir demonstrates antiviral activity against novel pandemic A/California/04/2009 (H1N1) influenza virus infection in mice. Antiviral Res. 90, 17–21.

Bates, J.H., Thompson-Figueroa, J., Lundblad, L.K., Irvin, C.G., 2008. Unrestrained video-assisted plethysmography: a noninvasive method for assessment of lung mechanical function in small animals. J. Appl. Physiol. 104, 253–261.

Bogs, J., Veits, J., Gohrbandt, S., Hundt, J., Stech, O., Breithaupt, A., Teifke, J.P., Mettenleiter, T.C., Stech, J., 2010. Highly pathogenic H5N1 influenza viruses carry virulence determinants beyond the polybasic hemagglutinin cleavage site. PLoS ONE 5, e11826.

Boukhvalova, M.S., Sotomayor, T.B., Point, R.C., Pletneva, L.M., Prince, G.A., Blanco, J.C., 2010. Activation of interferon response through toll-like receptor 3 impacts viral pathogenesis and pulmonary toll-like receptor expression during respiratory syncytial virus and influenza infections in the cotton rat Sigmodon hispidus model. Journal of Interferon & Cytokine Research: The Official Journal of the International Society for Interferon and Cytokine Research 30, 229–242.

de Vrankrijker, A.M., Wolfs, T.F., Ciofu, O., Hoiby, N., van der Ent, C.K., Poulsen, S.S., Johansen, H.K., 2009. Respiratory syncytial virus infection facilitates acute colonization of Pseudomonas aeruginosa in mice. J. Med. Virol. 81, 2096–2103.

Govorkova, E.A., Marathe, B.M., Prevost, A., Rehg, J.E., Webster, R.G., 2011. Assessment of the efficacy of the neuraminidase inhibitor oseltamivir against 2009 pandemic H1N1 influenza virus in ferrets. Antiviral Res. 91, 81–88.

Hamelin, M.E., Gagnon, C., Prince, G.A., Kiener, P., Suzich, J., Ulbrandt, N., Boivin, G., 2010. Prophylactic and therapeutic benefits of a monoclonal antibody against the fusion protein of human metapneumovirus in a mouse model. Antiviral Res. 88, 31–37.

Higuera Iglesias, A.L., Kudo, K., Manabe, T., Corcho Berdugo, A.E., Baeza, A.C., Ramos, L.A., Gutierrez, R.G., Manjarrez Zavala, M.E., Takasaki, J., Izumi, S., Bautista, E., Perez Padilla, J.R., 2011. Reducing occurrence and severity of Pneumonia due to pandemic H1N1 2009 by early Oseltamivir administration: a retrospective study in Mexico. PLoS ONE 6, e21838.

Kim, T.S., Sun, J., Braciale, T.J., 2011. T cell responses during influenza infection: getting and keeping control. Trends Immunol..

Lundblad, L.K., Irvin, C.G., Adler, A., Bates, J.H., 2002. A reevaluation of the validity of unrestrained plethysmography in mice. J. Appl. Physiol. 93, 1198–1207.

Maines, T.R., Chen, L.M., Belser, J.A., Van Hoeven, N., Smith, E., Donis, R.O., Tumpey, T.M., Katz, J.M., 2011. Multiple genes contribute to the virulent phenotype observed in ferrets of an H5N1 influenza virus isolated from Thailand in 2004. Virology 413, 226–230.

Marsland, B.J., Scanga, C.B., Kopf, M., Le Gros, G., 2004. Allergic airway inflammation is exacerbated during acute influenza infection and correlates with increased allergen presentation and recruitment of allergen-specific T-helper type 2 cells. Clin. Exp. Allergy 34, 1299–1306.

McGill, J., Heusel, J.W., Legge, K.L., 2009. Innate immune control and regulation of influenza virus infections. J. Leukoc. Biol. 86, 803–812.

Monsalvo, A.C., Batalle, J.P., Lopez, M.F., Krause, J.C., Klemenc, J., Hernandez, J.Z., Maskin, B., Bugna, J., Rubinstein, C., Aguilar, L., Dalurzo, L., Libster, R., Savy, V., Baumeister, E., Cabral, G., Font, J., Solari, L., Weller, K.P., Johnson, J., Echavarria, M., Edwards, K.M., Chappell, J.D., Crowe Jr., J.E., Williams, J.V., Melendi, G.A., Polack, F.P., 2011. Severe pandemic 2009 H1N1 influenza disease due to pathogenic immune complexes. Nat. Med. 17, 195–199.

Sidwell, R.W., Huffman, J.H., Gilbert, J., Moscon, B., Pedersen, G., Burger, R., Warren, R.P., 1992. Utilization of pulse oximetry for the study of the inhibitory effects of antiviral agents on influenza virus in mice. Antimicrob. Agents Chemother. 36, 473–476.

Sidwell, R.W., Bailey, K.W., Wong, M.H., Barnard, D.L., Smee, D.F., 2005. In vitro and in vivo influenza virus-inhibitory effects of viramidine. Antiviral Res. 68, 10–17.

Sidwell, R.W., Barnard, D.L., Day, C.W., Smee, D.F., Bailey, K.W., Wong, M.H., Morrey, J.D., Furuta, Y., 2007. Efficacy of orally administered T-705 on lethal avian

influenza A (H5N1) virus infections in mice. Antimicrob. Agents Chemother. 51, 845–851.
Smee, D.F., Hurst, B.L., Wong, M.H., Bailey, K.W., Morrey, J.D., 2009. Effects of double combinations of Amantadine, Oseltamivir, and Ribavirin on influenza A (H5N1) virus infections in cell culture and in mice. Antimicrob. Agents Chemother. 53, 2120–2128.
Smee, D.F., Hurst, B.L., Wong, M.H., Bailey, K.W., Tarbet, E.B., Morrey, J.D., Furuta, Y., 2010a. Effects of the combination of Favipiravir (T-705) and Oseltamivir on influenza A virus infections in mice. Antimicrob. Agents Chemother. 54, 126–133.
Smee, D.F., Hurst, B.L., Wong, M.H., Tarbet, E.B., Babu, Y.S., Klumpp, K., Morrey, J.D., 2010b. Combinations of Oseltamivir and Peramivir for the treatment of influenza A (H1N1) virus infections in cell culture and in mice. Antiviral Res. 88, 38–44.
Snelgrove, R.J., Edwards, L., Rae, A.J., Hussell, T., 2006. An absence of reactive oxygen species improves the resolution of lung influenza infection. Eur. J. Immunol. 36, 1364–1373.
Srikiatkhachorn, A., Chintapalli, J., Liu, J., Jamaluddin, M., Harrod, K.S., Whitsett, J.A., Enelow, R.I., Ramana, C.V., 2010. Interference with intraepithelial TNF-alpha signaling inhibits CD8(+) T-cell-mediated lung injury in influenza infection. Viral Immunol. 23, 639–645.
Stowell, N.C., Seideman, J., Raymond, H.A., Smalley, K.A., Lamb, R.J., Egenolf, D.D., Bugelski, P.J., Murray, L.A., Marsters, P.A., Bunting, R.A., Flavell, R.A., Alexopoulou, L., San Mateo, L.R., Griswold, D.E., Sarisky, R.T., Mbow, M.L., Das, A.M., 2009. Long-term activation of TLR3 by poly(I:C) induces inflammation and impairs lung function in mice. Respir. Res. 10, 43.
Van Kerkhove, M.D., Vandemaele, K.A., Shinde, V., Jaramillo-Gutierrez, G., Koukounari, A., Donnelly, C.A., Carlino, L.O., Owen, R., Paterson, B., Pelletier, L., Vachon, J., Gonzalez, C., Hongjie, Y., Zijian, F., Chuang, S.K., Au, A., Buda, S., Krause, G., Haas, W., Bonmarin, I., Taniguichi, K., Nakajima, K., Shobayashi, T., Takayama, Y., Sunagawa, T., Heraud, J.M., Orelle, A., Palacios, E., van der Sande, M.A., Wielders, C.C., Hunt, D., Cutter, J., Lee, V.J., Thomas, J., Santa-Olalla, P., Sierra-Moros, M.J., Hanshaoworakul, W., Ungchusak, K., Pebody, R., Jain, S., Mounts, A.W., 2011. Risk factors for severe outcomes following 2009 influenza A (H1N1) infection: A global pooled analysis. PLoS Med. 8, e1001053.
Verhoeven, D., Teijaro, J.R., Farber, D.L., 2009. Pulse-oximetry accurately predicts lung pathology and the immune response during influenza infection. Virology 390, 151–156.
Vlahos, R., Stambas, J., Bozinovski, S., Broughton, B.R., Drummond, G.R., Selemidis, S., 2011. Inhibition of Nox2 oxidase activity ameliorates influenza A virus-induced lung inflammation. PLoS Pathog. 7, e1001271.
Wong, J.P., Saravolac, E.G., Sabuda, D., Levy, H.B., Kende, M., 1995. Prophylactic and therapeutic efficacies of poly(IC.LC) against respiratory influenza A virus infection in mice. Antimicrob. Agents Chemother. 39, 2574–2576.
Wong, J.P., Christopher, M.E., Viswanathan, S., Karpoff, N., Dai, X., Das, D., Sun, L.Q., Wang, M., Salazar, A.M., 2009. Activation of toll-like receptor signaling pathway for protection against influenza virus infection. Vaccine 27, 3481–3483.
Zhou, B., Li, Y., Halpin, R., Hine, E., Spiro, D.J., Wentworth, D.E., 2011. PB2 residue 158 is a pathogenic determinant of pandemic H1N1 and H5 influenza a viruses in mice. J. Virol. 85, 357–365.