Impaired heterologous immunity in aged ferrets during sequential influenza A H1N1 infection

Stéphane G. Paquette a,b,1, Stephen S.H. Huang a,c,†, David Banner a, Luoling Xu a, Alberto León a, Alyson A. Kelvin d,*, David J. Kelvin a,b,c,e,f,g

a Division of Experimental Therapeutics, Toronto General Hospital Research Institute, University Health Network, Toronto, Ontario, Canada
b Institute of Medical Science, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada
c Department of Immunology, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada
d Immune Diagnostics & Research, Toronto Medical Discovery Tower, 101 College Street 3-913, Toronto, Ontario, Canada M5G 1L7
International Institute of Infection and Immunity, Shantou University Medical College, Guangdong, Shantou, China
f Sezione di Microbiologia Sperimentale e Clinica, Dipartimento di Scienze Biomediche, Università degli Studi di Sassari, Sassari, Italy

ARTICLE INFO

Article history:
Received 7 May 2014
Returned to author for revisions 23 May 2014
Accepted 7 July 2014
Available online 1 August 2014

Keywords:
Influenza A H1N1 Pandemic influenza Influenza Heterologous immunity Aging Immunological senescence Ferret model Humoral response T-cells

ABSTRACT

The major burden of influenza morbidity resides within the elderly population. The challenge managing influenza-associated illness in the elderly is the decline of immune function, where mechanisms leading to immunological senescence have not been elucidated. To better represent the immune environment, we investigated clinical morbidity and immune function during sequential homologous and heterologous H1N1 influenza infection in an aged ferret model. Our findings demonstrated experimentally that aged ferrets had significant morbidity during monosubtypic heterologous 2 challenge with significant weight loss and respiratory symptoms. Furthermore, increased clinical morbidity was associated with slower and shorter hemagglutinin antibody generation and attenuated type 1 T-cell gene responses in peripheral blood. These results revealed dampened immune activation during sequential influenza infection in aged ferrets. With the presence of an aged model, dissecting clinical morbidity, viral dynamics and immune response during influenza infection will aid the development of future prophylactics such as age specific influenza vaccines.

© 2014 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/3.0/).

Introduction

The elderly influenza disease rate in humans carries a heavy burden on healthcare systems and perpetuates virus circulation in the general population. During the 2012–2013 season, persons aged ≥ 65 years accounted for ~ 50% of all influenza-related hospitalizations in the US (Centers for Disease Control and Prevention (CDC), 2013). Since the host immune system deteriorates significantly during aging, the inability to generate effective, broad-spectrum immune memory following infection or vaccination contributes to increased influenza burden among the elderly (Bridges et al., 2000; Castle, 2000; Dao et al., 2010; Thompson et al., 2003). Aging influences various facets of the immune response, but T-cell populations are prominently affected due to thymus involution limiting naïve T-cell production (Aw and Palmer, 2011; Buchholz et al., 2011; Castle, 2000). Post-thymic homeostatic T-cell proliferation compensates for production deficit (Aw and Palmer, 2011; Buchholz et al., 2011), but long-term T-cell replication leads to cell-intrinsic dysfunction highlighted by progressive loss of repertoire diversity and weakened responses (Buchholz et al., 2011). This directly influences cell-mediated T-cell responses, most evident during viral infection (Buchholz et al., 2011; Deng et al., 2004; Effros et al., 2003), while indirectly affecting humoral responses (Eaton et al., 2004). Together with high rates of antigenic change of circulating virus population, this process puts the elderly at greater risk of recurring influenza infection (Bridges et al., 2000).

Age-related immune dysregulation modestly impacts disease severity in animal models of primary influenza infection (Guo et al., 2012; Josset et al., 2012; Muto et al., 2012; Pica et al., 2012). Heterosubtypic influenza A immune memory is severely impaired in aged animals (Bender and Small, 1993; Decman et al., 2010), although the elderly’s sensitivity to monosubtypic antigenic change is unclear. To address this question, we investigated clinical morbidity, viral dynamics, and subsequent immune responses to sequential influenza A H1N1 infection for the first time in an aged ferret model. Here we report dampened immunity in aged ferrets...
upon monosubtypic heterologous 2\(^{\text{nd}}\) challenge associated with diminished antibody production and altered T-cell responses in peripheral blood. These findings help elucidate the immune dynamics which contribute to elderly influenza susceptibility and put forth the aged ferret model for further study of aging immunity and influenza.

Results

Aged ferrets develop more severe disease than adults during heterologous monosubtypic 2\(^{\text{nd}}\) challenge

Ferrets closely mimic the clinical manifestations of influenza infection in humans as shown previously (Banner and Kelvin, 2012; Belser et al., 2011; Huang et al., 2011, 2013; Rowe et al., 2010). Naive adult (4–6 months old) and aged (≥4 years old) male ferrets were placed into groups for either homologous or heterologous H1N1 sequential infection studies. The homologous sequentially infected group was first infected intranasally with pandemic 2009 H1N1 strain A/Mexico/4108/2009 (Mex/4108) as the 1\(^{\text{st}}\) infection after 46 days the animals were then infected with pandemic 2009 H1N1 A/California/07/2009 (Cal/07) as 2\(^{\text{nd}}\) challenge. The heterologous 1\(^{\text{st}}\)–2\(^{\text{nd}}\) challenge group was infected with seasonal H1N1 A/Brisbane/59/2007 (Bris/59) and then subsequently infected with Mex/4108 Day 39 post 1\(^{\text{st}}\) infection. Animals were intranasally infected with the indicated virus strain at 10\(^{6}\) EID\(_{50}\) and clinical signs were monitored for 14 days post-infection/challenge (temperature, weight change, nasal discharge (clear discharge or color dry mucus/exudate), sneezing, and lethargy).

During both 1\(^{\text{st}}\) strain infections aged animals exhibited clinical morbidity that was modestly more pronounced than in adults, with greater weight loss (7–8% peak loss) and more frequent production of mucus/exudate. Sneezing was also observed more frequently in our aged cohorts, but sneezing incidence rates in aged ferrets (50%) were consistent with previously reported rates in adults during shH1N1 or H1N1pdm infection (Huang et al., 2011). Upon homologous 1\(^{\text{st}}\) challenge with Cal/07, neither age group exhibited clinical symptoms except for sporadic detection of clear nasal discharge and sneezing (Fig. 1A). In contrast, during heterologous 2\(^{\text{nd}}\) challenge with Mex/4108, adult ferrets showed mild clinical morbidity whereas the aged animals developed greater illness with >6% peak weight loss, sneezing, prominent nasal discharge (yellow–brown color exudate or dry mucus), and lethargy.

Having detected disease differences between aged and adult ferrets during sequential influenza infection, we next examined if morbidity was associated with viral burden in nasal washes. Live virus titers were measured at Days 3 and 7. No significant differences in viral burden/clearance were detected between the age groups at any time-point tested. Virus was undetectable by Day 3 following homologous 2\(^{\text{nd}}\) challenge in both age groups. Mex/4108 virus titers were dramatically reduced at Day 3 post-heterologous 2\(^{\text{nd}}\) challenge when compared to 1\(^{\text{st}}\) infection (~100-fold), consistent with previous reports (Fang et al., 2012) (Fig. 1C). Interestingly, aged animals still developed severe clinical morbidity during heterologous 2\(^{\text{nd}}\) challenge (Fig. 1B) despite reduced viral titers.

Antibody production in aged ferrets is delayed and not sustained at levels equivalent to adults

The increased disease severity in aged ferrets during heterologous 2\(^{\text{nd}}\) challenge prompted us to investigate possible causes of the disease disparity between aged and adult animals. Roles for humoral (Fang et al., 2012) and cell-mediated immunity (Guo et al., 2011; Tu et al., 2010) have been identified in heterologous influenza A H1N1 rechallenge models. First we investigated humoral responses in our cohort by assaying sera taken from the ferrets at designated time points for haemagglutination inhibition (HI) against Bris/59 or Mex/4108 viruses. Aged ferrets failed to maintain antibody titers at the same levels as adults following either 1\(^{\text{st}}\) Bris/59 or Mex/4108 infection. By Day 28 post Mex/4108 1\(^{\text{st}}\) infection, aged ferrets had significantly reduced haemagglutinin antibody levels compared to adults which remained significantly lower through the 2\(^{\text{nd}}\) infection with Cal/07 (Fig. 2A). Aged ferrets were also slower to mount an initial humoral response to the Bris/59 virus during 1\(^{\text{st}}\) infection, as adults had generated antibodies toward Bris/59 by Day 7 post-infection whereas aged animals had undetectable levels (Fig. 2B). Similar antibody responses were seen between adult and aged ferrets during the 2\(^{\text{nd}}\) challenge with the Mex/4108 virus. Together, these results show that aged ferrets respond differently (slower and less) compared to adult ferrets in respect to protective antibody generation which may be dependent on the virus strain and the sequence of insult.

Peripheral type 1 T-cell gene responses to heterologous monosubtypic 2\(^{\text{nd}}\) challenge are attenuated in aged ferrets

We next investigated other peripheral immune responses to complement the humoral immunity evaluation. Host gene expression analysis for the molecular dissection of circulating immune cell regulation (Fig. 3) was performed on in-life peripheral blood samples taken from animals throughout the infection time course. In our analysis we included gene sets for cell mediated immunity, inflammatory cytokines and T cell regulation. Strikingly, we detected rapid increases in CD4 and CD8 mRNA expression during heterologous 2\(^{\text{nd}}\) challenge in adult but not aged ferrets, with peak differences in CD4 (2-fold) and CD8 (3-fold) expression detected at Day 1 post-2\(^{\text{nd}}\) challenge (p2\(^{\text{nd}}\)) (Fig. 3B (i and ii)). A similar expression profile was detected for CD28 (T-cell activation cell surface marker) with significantly higher expression in adults. (Fig. 3B (iii)). Together, these profiles suggested rapid mobilization of adult T-cells to peripheral blood during heterologous challenge which was diminished in the aged. Moreover, we also detected a trend of reduced CD19 (B-lymphocyte maker) expression in aged ferrets (Fig. 3B (iv)), consistent with impaired humoral responses as suggested by our HI data. In contrast, similar innate response gene profiles (CXCL8, CXCL10, and TLR3) were detected in both groups with mostly no changes in gene regulation throughout the time course except for acute upregulation of CXCL8 and CXCL10 during heterologous 2\(^{\text{nd}}\) challenge at Day 1 p2\(^{\text{nd}}\) (Fig. 3 (v–vii)) (2-fold and 10–20-fold increase, respectively).

Given the role of type 1 T-cell responses in heterologous immunity (Guo et al., 2011; Tu et al., 2010), we further investigated circulating T-cell population effector status by measuring TBX21 (transcription factor expressed in Th1-committed CD4\(^{+}\) T-cells and CD8\(^{+}\) T-cells) (Sullivan et al., 2003; Szabo et al., 2000), GZMA (cytotoxic T-cell effector molecule) (Anthony et al., 2010), as well as IFN\(\gamma\) and TNF\(\alpha\) (type 1 cytokines) (Grivennikov et al., 2005; Xu et al., 2004) levels (Fig. 3 (viii–xi)). As above, TBX21 and TNF\(\alpha\) mRNA expression declined early (Day 1 p2\(^{\text{nd}}\)) during heterologous 2\(^{\text{nd}}\) challenge in aged ferrets while remaining stable in adult ferrets (TBX21: 2-fold) (TNF\(\alpha\): 2-fold) (Fig. 3B (viii and x)). Furthermore, peak GZMA mRNA expression was reduced in aged ferrets (Fig. 3 (ix)). Together, these findings suggest a potential age-related decrease in type 1 T-cell responses specific to heterologous 2\(^{\text{nd}}\) challenge which may have contributed to disease (Bender and Small, 1993; Decman et al., 2010).
A  

**Homologous H1N1 2° Challenge**

**i. Temperature**

**ii. Weight**

<table>
<thead>
<tr>
<th>Clinical Sign</th>
<th>Adult</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary challenge (Mex/4108)</td>
<td>Clear Discharge</td>
<td>100.0% (4/4)</td>
</tr>
<tr>
<td></td>
<td>Color dry mucus &amp; exudate</td>
<td>0.0% (0/4)</td>
</tr>
<tr>
<td></td>
<td>Sneezing</td>
<td>0.0% (0/4)</td>
</tr>
<tr>
<td></td>
<td>Inactivity Index</td>
<td>1.000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clinical Sign</th>
<th>Adult</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secondary challenge (Cal/07)</td>
<td>Clear Discharge</td>
<td>75.0% (3/4)</td>
</tr>
<tr>
<td></td>
<td>Color dry mucus &amp; exudate</td>
<td>0.0% (0/4)</td>
</tr>
<tr>
<td></td>
<td>Sneezing</td>
<td>25.0% (1/4)</td>
</tr>
<tr>
<td></td>
<td>Inactivity Index</td>
<td>1.000</td>
</tr>
</tbody>
</table>

B  

**Heterologous H1N1 2° Challenge**

**i. Temperature**

**ii. Weight**

<table>
<thead>
<tr>
<th>Clinical Sign</th>
<th>Adult</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary challenge (Bris/59)</td>
<td>Clear Discharge</td>
<td>50.0% (2/4)</td>
</tr>
<tr>
<td></td>
<td>Color dry mucus &amp; exudate</td>
<td>0.0% (0/4)</td>
</tr>
<tr>
<td></td>
<td>Sneezing</td>
<td>0.0% (0/4)</td>
</tr>
<tr>
<td></td>
<td>Inactivity Index</td>
<td>1.000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clinical Sign</th>
<th>Adult</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secondary challenge (Mex/4108)</td>
<td>Clear Discharge</td>
<td>75.0% (3/4)</td>
</tr>
<tr>
<td></td>
<td>Color dry mucus &amp; exudate</td>
<td>25.0% (1/4)</td>
</tr>
<tr>
<td></td>
<td>Sneezing</td>
<td>0.0% (0/4)</td>
</tr>
<tr>
<td></td>
<td>Inactivity Index</td>
<td>1.000</td>
</tr>
</tbody>
</table>

C  

**Nasal Wash Viral Load**

**i. Homologous H1N1 2° Challenge**

**ii. Heterologous H1N1 2° Challenge**

Fig. 1. Aged ferrets exhibited increased clinical morbidity during heterologous 2° challenge. Clinical sign monitoring of aged (> 4 years old) and adult (4–6 months old) ferrets during homologous [1: A/Mexico/4108/2009 (H1N1); 2: A/California/07/2009 (H1N1)] (A) or heterologous [1: A/Brisbane/59/2007 (H1N1); 2: A/Mexico/4108/2009 (H1N1)] monosubtypic sequential infections (B). All infections at 10⁶ EID₅₀. Temperature and weight were recorded daily following 1° infection until Day 5 post-infection (pi), then at Days 7, 10, and 14 pi. Temperature and weight were recorded daily after 2° challenge for 14 days. Both are reported as a percentage relative to the average preinfection level calculated from Days 0 and –1. Animals were also evaluated daily for nasal discharge, sneezing, and inactivity level, and the highest percentages (and fractions [number of ferrets displaying symptoms/total number of ferrets tested]) of infected ferrets displaying symptoms are shown. The physical inactivity index measures the degree to which ferrets respond to environmental stimuli, with the basal level being 1.000. Viral titers were measured in nasal washes collected from aged and adult ferrets at Days 3 and 7 after 1° infection and 2° challenge and determined by titration on MDCK cells and reported as TCID₅₀/mL (C).
Discussion

Our study revealed increased disease severity in aged ferrets during sequential heterologous H1N1 influenza infection which was associated with altered aged T-cell responses and antibody production (Fig. 4). Our data suggested modulation of T-cell function during vaccination as a potential target for improving elderly immune memory against influenza, and recommends the aged ferret influenza model for the development of immunomodulatory influenza therapeutics and vaccines. Moreover, antibody titers in aged ferrets were not sustained at the same levels observed in adults which may also be a consequence of dampened T-cell responses and should be further explored. The elderly have the highest influenza-related hospitalization rates placing stress on healthcare systems and increasing virus exposure to other members of the community such as hospital workers and other hospitalized patients. Better management of influenza infection in the older population is urgently needed. Boosting elderly T-cell responses may be a valuable therapeutic target for broadening of immune memory.

The analysis of clinical symptoms in aged versus adult ferrets was highly insightful and recapitulated the clinical disease course observed in elderly humans as well as other animal studies. Here we found that aged ferrets did not recover to their original weight following sequential heterologous H1N1 influenza infection (Fig. 4), a finding consistent with observations of long-term disability in the elderly following repeated influenza exposure (McElhaney, 2005). Clinical signs in our aged ferret model were also in agreement with previous animal studies where the disease severity of aged animal was modestly increased during 1st infection (Guo et al., 2012; Josset et al., 2012; Muto et al., 2012; Pica et al., 2012) but importantly, heterologous monosubtypic 1st infection–2nd challenge caused severe morbidity in aged ferrets similar to previous rechallenge mouse studies (Bender and Small, 1993; Decman et al., 2010). Our work together with previously published studies has begun to suggest a profile of the aged clinical following subsequent influenza infection.

Along with a more severe clinical profile, aged ferrets had an attenuated peripheral humoral and T cell immune response compared to adults and previously modeled immune responses following influenza infection (Cheng et al., 2013; Huang et al., 2011; Jang et al., 2013; Kelvin et al., 2014; O’Donnell et al., 2012; Paquette et al., 2014). We found aged ferrets to have a significant decrease in sustained HA antibody production following 1st and continued into secondary 2nd pandemic H1N1 influenza infection which may indicate a factor in the increased susceptibility to sequential influenza infections in the elderly. Also, aged ferrets were markedly slower to mount an initial humoral response to the Bris/59 virus during 1st infection where antibody levels remained undetected 7 days following infection which may play a role in prolonged disease in our aged ferrets. Conversely, we found that the 1st Mex/4108 infection did not show differential HA antibody generation. It is possible that higher viral titers detected during Mex/4108 versus Bris/59 1st infection (~100-fold at Day 3) may have masked humoral deficiencies in the aged cohort. Alternatively, these disparate data sets may indicate influenza strain-specific immune responses in aged ferrets which could be further investigated. A protective role for cross-reactive antibodies has been identified during sequential influenza A H1N1 infection (Fang et al., 2012) and it is possible in aged animals that lower antibody titers may have contributed to the increased disease severity observed during heterologous 2nd challenge.

Interestingly, peripheral T cell responses in the aged ferrets also appeared to be dampened as determined by real-time RT-PCR. Specifically, CD4, CD8, and CD28 were significantly more robust in the adults compared to the aged during 1st Bris/59–2nd Mex/4108 infection combination. Previous reports in humans and other mammals have suggested T-cell responses are impaired in the elderly (Aw and Palmer, 2011; Buchholz et al., 2011). Similarly, we detected attenuated type 1 T-cell gene responses in aged ferret peripheral blood which may indicate intrinsic differences in the aged ferret T-cell population. Reduced antibody titers in aged ferrets may also have been a consequence of impaired T-cell function (Eaton et al., 2004; Haynes et al., 2005). Together, these observations suggest altered T-cell populations and/or activity in aged ferrets may have limited the breadth of immune memory following 1st infection, recommending additional investigation of the aged ferret T-cell population. Reduced T-cell proliferation and interleukin-2 production in response to antigen have been linked to immune decline in aged mice (Decman et al., 2010; Deng et al., 2004;
Aging has also been associated with declines in T-cell repertoire diversity (Buchholz et al., 2011; Naylor et al., 2005; Yager et al., 2008) which may be attributable in part to reduced naive T-cell production (Aw and Palmer, 2011; Buchholz et al., 2011; Castle, 2000) and gradual deletion of low-avidity T-cell clones as peripheral T-cell expansion is increasingly biased towards high-avidity clones with age (Rudd et al., 2011). Loss of clonal diversity in aged mice has been shown to impair heterosubtypic immunity (Yager et al., 2008) and may have similarly contributed to the weaker memory responses detected in aged ferrets during heterologous 2 infection. Accordingly, changes in the T-cell repertoire during ferret aging should also be the focus of future work. Meanwhile, our data provides indication that boosting T-cell responses to antigen during vaccination of the elderly may help limit susceptibility to severe influenza infection. Studies in human cells ex vivo and in mice have shown that increasing inflammatory cytokine production by antigen presenting cells improves aged T-cell responses to antigen (Behzad et al., 2012; Jones et al., 2010). Accordingly, the aged ferret model described here may serve as a valuable tool for the future development of such immune boosting therapies. Further study of aged T-cell function during sequential influenza infection in ferrets may also reveal more direct therapeutic targets.

Influenza disease rates are increased among the elderly due in part to a failure in generating broad, long-lasting immunity following influenza exposure (Bridges et al., 2000; Castle, 2000; Centers for Disease Control and Prevention (CDC), 2013; Dao et al., 2010; Thompson et al., 2003). New approaches to improve elderly immune responses and immune memory are needed, yet aged animal models for the study of influenza infection and immunity are limited (Bender and Small, 1993). Our data puts forth the aged ferret influenza model and showed that aged ferrets failed to mount an equivalent immune response as adults to monosubtypic heterologous 2 challenge which was associated with altered peripheral T-cell responses, decreased antibody production, and increased morbidity.

**Materials and methods**

**Ethics statement**

Animal work was performed in strict accordance with the Canadian Council of Animal Care (CCAC) guidelines. The animal use protocol was approved by the Animal Care Committee (ACC) of the University Health Network (UHN) where UHN has certification with the Animals for Research Act (Permit Number: #0045 and #0085 of the Ontario Ministry of Agriculture, Food and Rural Affairs) and follows NIH guidelines (OLAW #A5408-01). Infections and subsequent sample collection were performed under 5% isoflurane anesthesia in an effort to minimize suffering.

**Animal infections and clinical monitoring**

Ferrets were bred and housed in an on-site specific-pathogen-free facility (UHN, Toronto, ON, Canada). Adult ferrets were defined as 4–6 months of age, while aged ferrets were defined as > 4 years of age (all male). All ferrets were tested for the presence of antibodies against circulating influenza A and B virus strains by HI and shown to be seronegative prior to infection.

**Fig. 3.** Type 1 T-cell gene responses detected in peripheral blood of adult but not aged ferrets upon heterologous 2 challenge. Gene expression analysis from peripheral blood total RNA collected from aged and adult ferrets at Days 0, 3, 7, and 14 during 1 infection and Days 0, 1, 3, and 7 during 2 challenge. Expression levels of CD4 (i), CD8 (ii), CD28 (iii), CD19 (iv), CXCL8 (v), CXCL10 (vi), TLR3 (vii), TBX21 (viii), GZMA (ix), TNFα (x), IFNγ (xi), and CD44 (xii) mRNA were determined by qRT-PCR, normalized to house-keeping gene β-Actin and expressed as fold change difference in expression relative to Day 0 adult basal levels. Three samples from each time point were collected per group. Error bars represent standard errors of the means. *p < 0.05 by Student's t test comparing infected adult and aged ferrets.
Infection experiments were conducted with H1N1pdm strains A/Mexico/4108/2009 (H1N1) and A/California/07/2009 (H1N1) or sH1N1 strain A/Brisbane/59/2007 (H1N1). All viruses were provided by the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA). Viral stocks were propagated in embryonated eggs for no more than one passage, stored in liquid nitrogen, and thawed prior to use. All infections and 2^nd challenges were performed at 10^6 EID50. Infections and daily clinical sign monitoring were performed as previously described (Huang et al., 2012).

Viral and humoral kinetics

Nasal washes were collected at Days 3 and 7 post-infection/challenge and viral titers were determined by titration over Madin-Darby Canine Kidney (MDCK) cells and TCID50/mL calculation using the Reed–Muench method (Huang et al., 2012). In-life bleeds were performed at designated time-points for isolation of sera and determination of antibody titers against Bris/59 and Mex/4108 haemagglutinin by HI assay, as previously described (Huang et al., 2012).

Peripheral blood gene expression analysis

In-life bleeds were performed at designated time-points for isolation of peripheral blood total cellular RNA. Blood was collected in PAXgene Blood RNA tubes and stored at −80 °C until extraction by PAXgene Blood RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland), as per manufacturer’s instructions. Purified RNA was reverse transcribed using ImProm-II Reverse Transcription System (Promega, Madison, WI, USA) and host gene expression was investigated by qRT-PCR using ABI-Prism 7900HT Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA). Each sample was run in quadruplicate at 5 ng cDNA/reaction well. Host gene expression was normalized to β-actin, and quantified by the relative standard curve method. Primer sequences are listed in Table S1.

Statistical methods

The Student’s t-test (α=0.05) was used to ascertain significance for two group comparisons, assuming two-tailed distributions and unequal variances.

Acknowledgments

This study was supported by Li Ka-Shing Foundation of Canada, Immune Diagnostics & Research, Shantou University Medical College and NIH U01AI111598 Subaward no. 0038951(123721-3). We would also like to thank the UHN Animal Resources Centre (ARC) staff for help with animal care and aging of the animals. All influenza strains (A/California/07/2009 (H1N1), A/Mexico/4108/2009 (H1N1), and A/Brisbane/59/2007 (H1N1)) were obtained through the Influenza Reagent Resource, Influenza Division,
Appendix A: Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.07.013.