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Influenza virus inoculum volume is critical to elucidate age-dependent mortality in mice

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Summary

The elderly exhibit increased mortality to influenza viral infection for unclear reasons. Mice are frequently used to model how aging impacts disease. Several studies have shown that aged mice exhibit an increased mortality to influenza virus, but two recent studies demonstrated the

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opposite. These two studies administered the virus intranasally in 20μL, whereas the other studies used a viral inoculum in at least 30μL. To determine if the volume of the inoculum could explain the conflicting reports, we infected young and aged mice via intranasal instillation of 40μL or 20μL containing $1x10^4$ plaque forming units (PFU) of H1N1 influenza virus. We found that intranasal administration of 40μL but not 20μL of inoculum resulted in age-dependent mortality in mice. Compared to aged mice infected with 40μL inoculum, those infected with 20μL inoculum showed reduced levels of live virus and IFN-β in the lung 3 days post-infection. Furthermore, aged mice administered 40μL of Evans blue intranasally displayed increased dye retention in their bronchoalveolar lavage fluid compared to those administered 20μl of Evans blue. Our data demonstrate that the inoculating volume of virus is critical for adequate delivery of influenza virus to the lung and thus for efficient infection of aged mice. These findings shed light on discrepant results in the literature regarding aged mice and influenza infection, and establish that mice can be used to examine how aging impacts the response to this biomedically important infection.

Introduction, Results and Discussion

People over age 65 account for 90% of influenza-related deaths (Pebody et al., 2010). Mice have been employed in hundreds of studies to examine how aging impacts the immune system (reviewed in Haynes L and SL, 2006; Nikolich-Žugich, 2018) and in aging research in general (reviewed in Vanhooren and Libert, 2013). Discerning the mechanisms by which aging compromises the immune response to influenza in mice and translating the findings to humans might inform on the development of novel therapies to reduce the suffering from influenza infections in the elderly.

The utility of the mice to study how aging affects influenza viral lung infection is somewhat controversial. At least five studies of either C57BL/6 mice or BALB/c mice report that aged mice (16-24 months old) display enhanced morbidity or mortality during influenza A viral lung infection (IAV) compared to young mice (2-4 months old), similar to humans (Steeg et al., 2016; Stout-Delgado et al., 2012; Toapanta and Ross, 2009; Wong et al., 2017; Zhao et al., 2011). In

contrast, two studies of aged C57BL/6 mice (16-30 months old) report that aged mice exhibit reduced mortality during influenza lung infection compared to young mice (2-4 months old) (Lu et al., 2018; Pillai et al., 2016). Interestingly, one distinguishing feature of these two studies was the use of viral inoculum in 20μL, whereas the studies showing that aged mice succumb faster to influenza infection employed a viral inoculum in at least 30μL. Additionally, a prior report in young mice showed that inoculating doses of virus less than 35μL led to reduced mortality to the same dose of influenza virus (Miller et al., 2013). Given these differences, we reasoned that the volume for the viral inoculum could explain the discrepancy between the studies of aged mice.

To test this hypothesis, we infected young (2-4 months) and aged (18 months) male and female C57BL/6 mice via intranasal (i.n.) administration of $1x10^4$ PFU of influenza virus (A/PR/8/34, H1N1) in either 20μL or 40μL of PBS and monitored survival. In mice infected using 20μL inoculum, we did not observe age-dependent alterations in survival in either sex (Figure 1A,1C). In contrast, both sexes of mice infected using 40 μL showed a significant, age-dependent decrease in survival (Figure 1B,1D). As an alternative comparison, both sexes of aged mice infected with 40μL showed significantly reduced survival relative to the same cohort infected with 20μL (aged male $p = 0.04$; aged female $p = 0.01$, Gehan-Breslow-Wilcoxon Test), whereas the volume of inoculum did not significantly alter the outcome for young mice (Figure 1). Thus, the inoculating volume influences the outcome of influenza viral infection (IAV) in aged mice more than in younger mice. Furthermore, an inoculum of 40 μL effectively induced mortality in aged mice and revealed an age-dependent mortality during IAV that was not observed with the 20μL inoculum.

Next, we used a plaque assay to measure the amount of live virus within the lungs. Again, we examined young and aged female mice infected with viral inoculum containing $1x10^4$ PFU of virus in either 20μL or 40μL. At 3 days post-infection, aged female mice administered viral inoculum in 40μL displayed significantly more live virus in the lungs compared to aged female mice that received the 20μL inoculum (Figure 2A). Furthermore, only 4 / 8 young female mice

and 3 / 8 aged female mice administered viral inoculum in 20μL displayed live virus in the lung, whereas all mice except one aged and young mouse administered viral inoculum in 40μL exhibited live virus in the lung (Figure 2A). Together, these data imply a reduced efficacy of infection with 20μL relative to 40μL inoculum volumes especially for aged mice, which would likely contribute to their increased survival upon infection with 20μL vs. 40μL inoculum.

Type I interferons (IFN) are cytokines that are secreted early in response to viral infections including influenza (Asselin-Paturel and Trinchieri, 2005). We measured the levels of IFN-β, a key type I IFN cytokine, within the lung lysate of infected mice at day 3 post-infection. We found that the IFN-β levels in lungs were almost 2-fold higher in aged mice infected using 40μL compared to 20μL viral inoculum (Figure 2B), even though both volumes contained $1x10^4$ PFU virus. These data further suggest that a 40μL inoculum volume is more effective than a 20μL inoculum volume for establishing a robust influenza infection, particularly in aged mice.

Prior work has shown that the volume of administration is critical for effective delivery of agents, including pathogens, to the lungs (Southam et al., 2002). The azo dye Evans blue has been used to measure the immediate efficacy of i.n. delivery in rodents (Visweswaraiah et al., 2002). We used Evans blue to determine whether age and volume of administration impacts the quantity of dye retained in non-infected mouse lungs. Non-infected aged and young mice were i.n., administered 63 picomoles Evans blue, in either 20μL or 40μL. Dye retention in the lungs was examined by harvesting bronchoalveolar lavage fluid (BAL) within five minutes and measuring Evans blue concentration by colorimetry. We found that aged female mice administered 40μL Evans blue displayed a higher retention of dye in the lungs than those administered 20μl (Figure 2C). Thus, using a 40μL rather than 20μl volume for i.n. administration leads to a significant increase in the delivery of dye and virus to the lungs in aged female mice (Figure 2A-C).

Overall, our study demonstrates that mice display an age-dependent acceleration of mortality to IAV infection when the viral inoculum is in 40μL. We found that using viral inoculum in a

20μL volume will inadequately infect lung tissue, particularly for aged mice, confounding analyses. Although factors such as strain of virus, mouse strain, method of anesthesia, route of administration of virus (i.n., or intratracheal) could also contribute to the variation across studies that use aged mice to study influenza viral infection, our study highlights the importance of the volume of viral inoculum. Upon administration of IAV in 40 μL, mice exhibit an age-dependent increase in mortality similar to the clinical phenotype in humans. Therefore, our study confirms that mice are useful to model human aging and the outcomes to influenza viral infection.

Author contributions

CAS, UK and DRG conceptualized the study. CS, UK and JC performed experiments. CS and UK acquired and analyzed the data. DRG reviewed the data and wrote the manuscript. CS and UK edited the manuscript. JC reviewed the manuscript. DRG procured funding.

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Conflict of Interest

None

Figure Legends

Figure 1 *Volume of viral inoculum is critical for eliciting an age-dependent mortality during influenza viral infection*.

Young (2-4 months of age) and aged (18 months of age) C57BL/6 male (**A-B**) or female (**C-D**) mice were intranasally infected with either 20 μL or 40 μL inoculum of virus containing $1x10^4$

PFU of PR8 strain influenza virus and mortality was monitored. In both sexes, an age-dependent increase in mortality was only noted with the 40μL inoculum. * $p < 0.05$, ** $p < 0.01$ (Gehan-Breslow-Wilcoxon Test). n = 10-12 / group (males), 10 / group (females).

Figure 2 *Increase volume of viral inoculum in aged mice via intranasal route leads to higher viral load, and increased IFN-β levels. Increase inoculating volume of dye in non-infected aged mice leads to increase dye detection within bronchoalveolar lavage.*

Female young (2-4 months of age) and aged (18-month old) C57BL/6 mice were intranasally infected with either 20 μL or 40 μL inoculum of influenza virus containing $1x10^4$ PFU virus and live virus within lung tissue was measured 3 days post infection by plaque assay (**A**) and concentration of IFN-β measured in the lung lysate 3 days post infection via ELISA (**B**). Young and aged non-infected female mice were administered at 63 picomoles of Evans blue in either 20 μL or 40 μL and dye was detected within bronchoalveolar lavage fluid by colorimetry (**C**). * $p < 0.05$, ** $p < 0.01$ (two-way analyses corrected for multiple comparisons, see supplementary methods). Each data point represents a biological replicate and data represented as ± 95% confidence interval.

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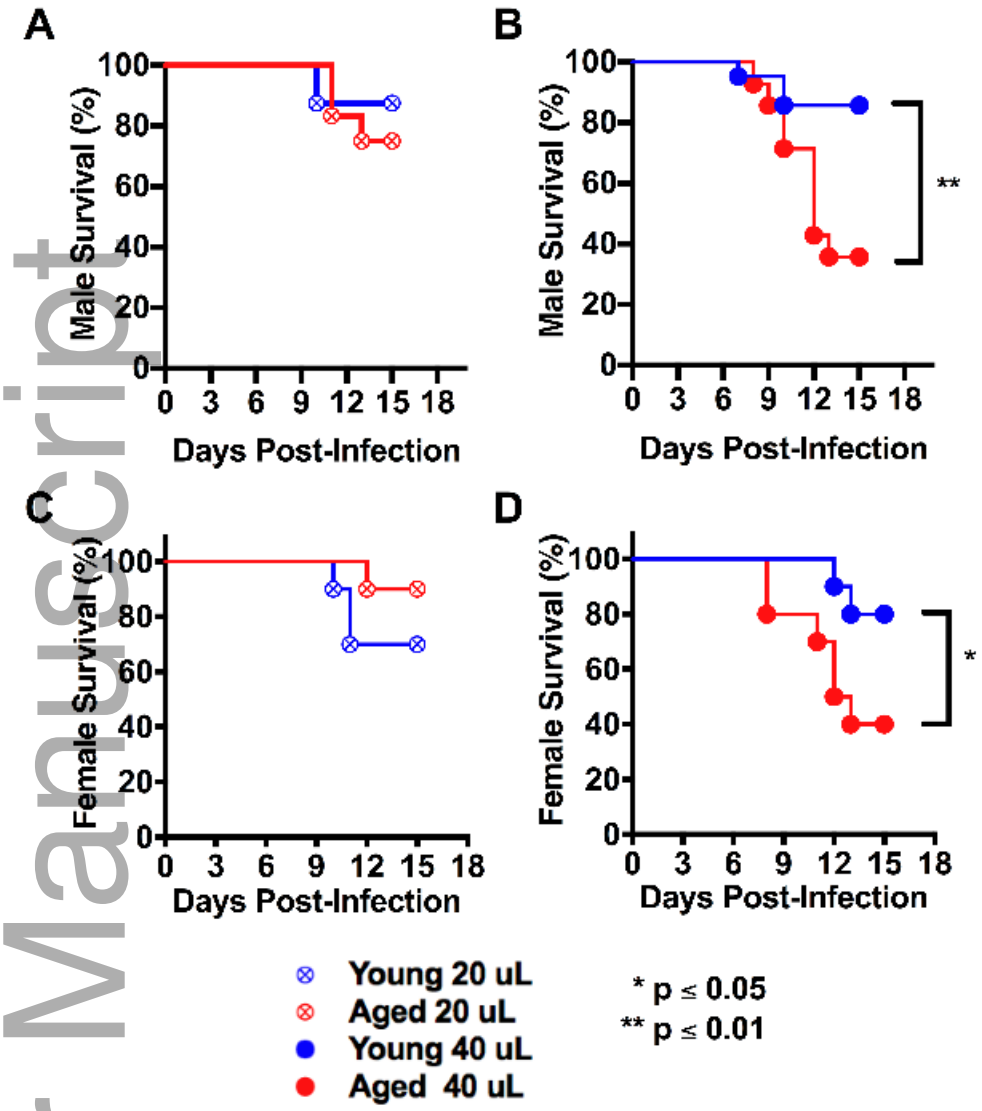

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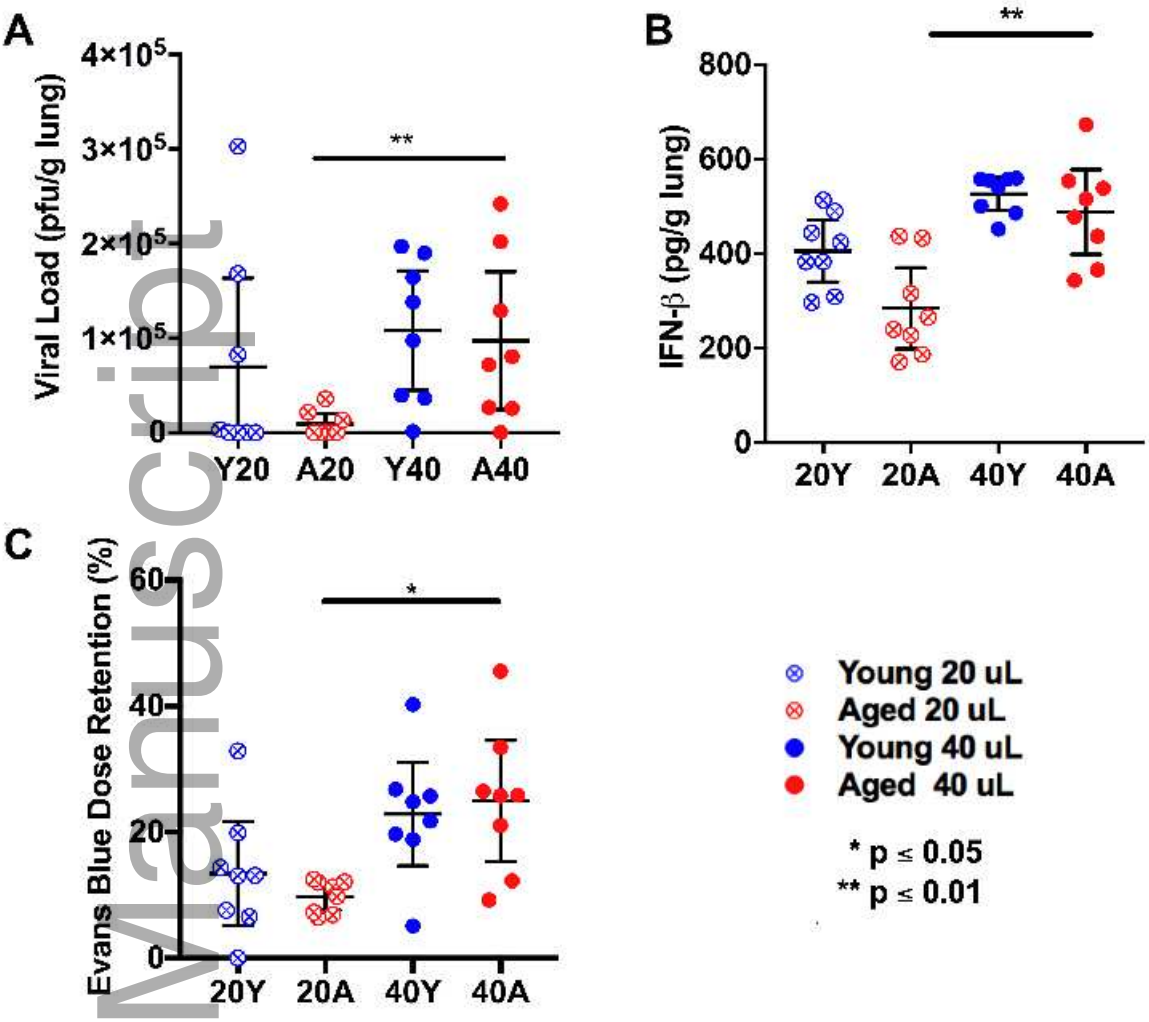

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