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THE EFFECT OF INFLAMMATORY COMPONENTS ON INFLUENZA A VIRULENCE EVOLUTION

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ABSTRACT

The methodology for passaging influenza A virus in the mouse model has widely been established. However, traditional infection methods involving the inoculation of lung homogenates fail to account for the possible effects of host-associated factors on measures of virulence and in the virulence evolution of the virus.

To test these effects, we performed a series of experiments utilizing single-origin virus to compare lung homogenate inoculations to infections with bronchoalveolar lavage fluid or with homogenate purified through a custom tissue culture-based technique. To assess whether host-associated factors cause allogeneic effects, virus adapted to a specific mouse genotype was used to infect both the familiar and novel host genotypes, either via lung homogenate or purified virus infections. Finally, to test whether host-associated factors affect the virulence evolution of influenza A virus, we also compare the virulence of virus serially passaged via lung homogenates to virus passaged via bronchoalveolar lavages.

Our results indicate that there are no major differences in virulence measurements when influenza virus contained in homogenate versus bronchoalveolar lavage or cell culture supernatant is used to infect same-genotype hosts. There is also no major effect on viral virulence evolution. However, there is a strong allogeneic effect and associated increase in virulence when virus in lung homogenates is used to infect novel host genotypes. Infection assays and sequencing of pre- and post-purification viral populations demonstrate that our novel cell-based purification method is effective at eliminating allogeneic effects caused by host-associated factors in lung homogenate with negligible effects on the genetic composition of the viral populations.

INTRODUCTION

Influenza A virus (IAV) is a single stranded RNA virus that is able to quickly adapt to its host due to high mutation rates. IAV circulates through the population naturally and infects the respiratory tracts of 3-5 million people annually, but transmission and mortality rates increase exponentially during pandemics (Iuliano et al., 2019; Saunders-Hastings & Krewski, 2016). The high virulence of pandemic IAV is usually attributed to antigenic shift, however the mechanisms of IAV evolution and spread are still not well understood (Taubenberger & Morens, 2006; Tscherne et al., 2011).

Mice are the most commonly used animal model in the study of influenza A virus, due to the low cost and husbandry requirements compared to other species such as ferrets or guinea pigs. However, unlike these other animal models, mice do not transmit influenza naturally to uninfected cage mates (Bouvier & Lowen, 2010; Schulman & Kilbourne, 1963). This is likely due to both reduced shedding and efficient neutralization of virus by salivary components (Gilbertson et al., 2017). Due to the lack of natural transmission methods in mouse models, traditional methodologies for passaging IAV in mouse models resort to the inoculation of 20-50μL of clarified lung homogenate on the nostrils of anesthetized mice. This method introduces the viral containing medium directly into the lungs on mice and allows for the maximization of viral titers and thus an increase in the probability of productive infection. However, this method does not correctly simulate the natural conditions through which the virus is transmitted between hosts. There are two major considerations that traditional passage methods do not consider, bottleneck size and host associated factors.

Natural transmission of IAV occurs via direct contact, aerosols, respiratory droplets, and fomites. These infection routes allow for short and long range transmission (Mubareka et al., 2009) and result in smaller amounts of transmitted virus compared with experimental infections, with transmission bottlenecks calculated to be of about 2 genomes per successful infection (McCrone et al., 2018). The smaller doses of virus likely reduce the evolvability of the virus (the potential for the virus to evolve), as there is less genetic material available for selection to act upon.

The other major difference between natural and experimental transmission is the presence of host-associated factors in the lung homogenate samples. The innate immune response is the first line of defense against IAV infection and is comprised of physical barriers (mucus), resident phagocytes, and inflammatory cytokines such as interferons (IFNs) from infected cells. After initial barriers are breached, the innate immune system recognizes viral pathogen associated molecular patterns (PAMPs) that are recognized by the host as foreign (Cao, 2016). A cellular cascade is triggered and transcription factors, interferon regulatory factor 3 (IRF 3) and IRF 7 are made along with NF-κB. These cause a variety of IFNs and proinflammatory cytokines to be produced such as tumor necrosis factor (TNFα) and interleukin 6 (IL6) (Hiscott et al., 2006). Immune cells are then recruited into the inflamed tissue, including macrophages, dendritic cells (DCs), T-cells and NK cells (Chen et al., 2018). These go on to educate members of the adaptive immune system such as $CD4^+$ T cells and $CD8^+$ T cells. Infected host cells are then destroyed by the $CD8^+$ T cells and uninfected cells start producing antiviral factors that protect them from infection such as IFN-α, IFN-β and IFN-γ(Chen et al., 2018). Virulent IAV infections can result in an overreactive immune response, characterized by excessive immune cell recruitment and cytokine release that result in damage to the host's own tissues (immunopathology), also known as 'cytokine storm' (Liu et al., 2016).

Due to the inflammatory immune response to viral infection, the inoculation of long homogenate supernatants likely introduces many proinflammatory cytokines and other damage signals that were being produced during the infection of the previous host. It is possible that

these cytokines induce the inflammatory response early in the newly infected mouse. The homogenization of lung tissue also introduces host-specific antigens, which may cause an allogeneic immune response in hosts of different genotypes, further inducing immunopathological effects.

With both of these effects in mind, it can be assumed that virulence measurements may be altered when mice are infected with viral-containing homogenates. The host environment that IAV encounters in a naïve mouse inoculated with lung homogenate supernatant may not be representative of what would usually be encountered outside of a laboratory setting. Thus, it is also possible that such experimental infection regimens alter the selective pressures and therefore the virulence trajectory of the virus.

In this project, we tested how lung homogenate infections affect the virulence output of IAV by comparison with infections of virus contained in bronchoalveolar lavage fluid (contains only extracted virions from the respiratory tract instead of whole lung), which more closely resembles the natural medium (mucus) that IAV is usually transmitted in, or with virus purified by an overnight passage in cell culture, which does not contain any host-associated factors. We also tested how different serial passage methods (bronchoalveolar lavage or lung homogenization) affect the virulence evolution of the virus. Our hypothesis is that any adaptations and changes in virulence obtained from lung homogenate virus may not represent those acquired naturally, as the virus adapts to an inflammatory environment instead of a typical host environment. In addition, bronchoalveolar lavages only collect a portion of the infectious virus in the whole lung (about 10%) so passages with the lavage fluid may limit the amount of genetic material available for viral evolution, which may limit virulence increases but more accurately represent the natural evolution of IAV in a naïve population.

METHODS

Infection and serial passage methods

Influenza strain A/Hong Kong/1/1968 (H3N2) was passaged using a modified standard protocol (Shevach, 2011). Mice under isoflurane anesthesia were inoculated intranasally with 20 µL of virus-containing fluid. After three days, mice were euthanized and bronchoalveolar lavage (BAL) fluid and/or lung homogenates (HOM) were collected. For BAL fluid collection, after tracheostomy, 1 mL of PBS was pipetted 10 times in and out of the lungs and collected into a 2 mL tube. The fluid was then centrifuged, and supernatant was collected, mixed, aliquoted, and stored at -80 °C. For HOM collection, lungs were extracted into a 2 mL microtube and homogenized with a rotor-stator homogenizer. Samples were brought up to a volume of 1 mL using PBS (about 900 µL). Homogenates were centrifuged, and supernatant was collected, mixed, aliquoted, and stored at -80 °C.

To adapt virus to mice before test phase experiments, serial passages were conducted by pooling virus from four infected mice every passage round for either 9 rounds of passage or for 5 rounds of passage, according to the experiment (Fig. 1). Passages were done in both female BALB/c and C57BL/6 mice. After the final passages, test phases were conducted, in which infection doses were standardized to 4000 $TCID_{50}$ per inoculate.

Viral quantification

To measure virulence, mouse weights were recorded daily. Mice that dropped below 75% of their initial weights or showed visible signs of distress were euthanized to maintain humane treatment of the animals. All passages had animals balanced by litter, age, and origin, mice were ordered from the Jackson Laboratory or bred in our facility.

Viral titers were measured using $TCID_{50}$ assays in Madin-Darby canine kidney (MDCK) cells and calculated using the Reed-Muench method (Reed & Muench, 1938). For intermediate passages, presence or absence of virus in samples was determined using RT-qPCR with the

Verso 1-step RT-qPCR kit (Thermo Fisher) using primers specific for the M segment of IAV (Elden et al., 2001).

Purification methods

Traditional purification methods (sucrose gradient) were unreliable for low titer viral extracts. Instead, non-viral components of viral extracts were removed via a custom cell-culture purification method. Confluent MDCK cells in 6-well plates were inoculated with 400 μL of viral growth medium containing the viral extracts at a multiplicity of infection (MOI) of 0.1. After a 60-minute adsorption, viral extract containing medium was washed off and replaced with new viral growth medium and incubated overnight (20 hours) at 37 °C. After incubation, the medium was aspirated into a 2 mL microtube and centrifuged for 5 minutes at 300 x g, 4 c. Supernatant was then collected, aliquoted and stored at -80 °C.

Sequencing

Samples of post-passage virus collected from lung homogenates, both pre- and post-purification, along with unpassaged Hong Kong 1968 virus were sent to a collaborator for Illumina NextSeq sequencing.

Statistical methods

Virulence as measured by weight loss was assessed with a linear mixed model (LMM) predicting test phase body weight (% of initial), based on the fixed effect of time (day post-infection), and its interaction with multiple infection treatment. Mouse ID was modeled as a random slope (with time) to control for repeated measures and nested within random slopes estimated for mouse type (strain/sex). As LMMs assume linearity, only the initial 7 days post-infection were assessed as recovery from infection leads to the restoration of body weights. Separate LMMs were used to assess the influence of viral populations from single unfamiliar and mixed viral lines on body weight loss. All LMMs were fit in R using the lme4 package and degrees of freedom were estimated using a Satterthwaite approximation in LmerTest (Bates et al., 2015).

EXPERIMENTS AND RESULTS

Host associated factors have no clear effect on overall virulence

To test whether host-associated factors (such as cytokines) present in lung homogenates caused alterations in virulence measurements, we infected mice with bronchoalveolar lavage (BAL), lung homogenate supernatant (HOM) and with purified lung homogenate (PUR) originating from a single mouse, in duplicate. To eliminate dose effects, doses were also standardized (Fig. 1).

Statistical analysis showed a marginally significant reduction in the virulence of mice infected with BAL, when compared to HOM (LMM; $p = 0.0612$) (Fig. 2). This suggests that there either is a weak effect of lung-associated factors that are present in lower quantities in the lavage vs the whole lung, or that the viral populations in the washable portion of the lungs may be different from those deep in the lung tissue. There were no significant differences in virulence between PUR and BAL (LMM; $p = 0.1853$) or PUR and HOM (LMM; $p = 0.0612$). It is possible that any reduction in virulence due to the absence of inflammatory factors in PUR may be offset by the elimination of defective interfering particles (DIPs) during the purification process due to the low MOI. These viral particles have defective genomes that reduce the overall infectivity of the virus (Diefenbacher et al., 2018). Another possibility is that because there are no inflammatory signals being introduced in the new host along with the purified virus, the immune response is delayed, and the virus is allowed to replicate to higher titers, eventually causing similar levels of virulence. Overall, these results indicate that there is no significant increase in virulence due to the presence of host-associated factors.

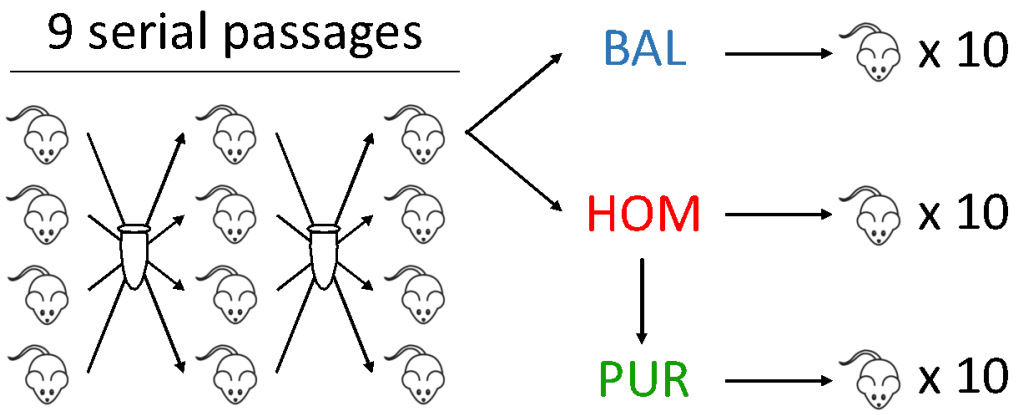

Figure 1. Diagram schematic of passage methods. Serial passages were performed for 9 rounds using HOM. In the final round, bronchoalveolar lavage (BAL) was collected from infected mice, followed by lung homogenate (HOM) collection. A cell-based purification step was then performed on aliquots of the homogenates to remove host-associated factors. Mice were then infected with a standardized dose of 4000 $TCID_{50}$ of each viral treatment. n = 30.

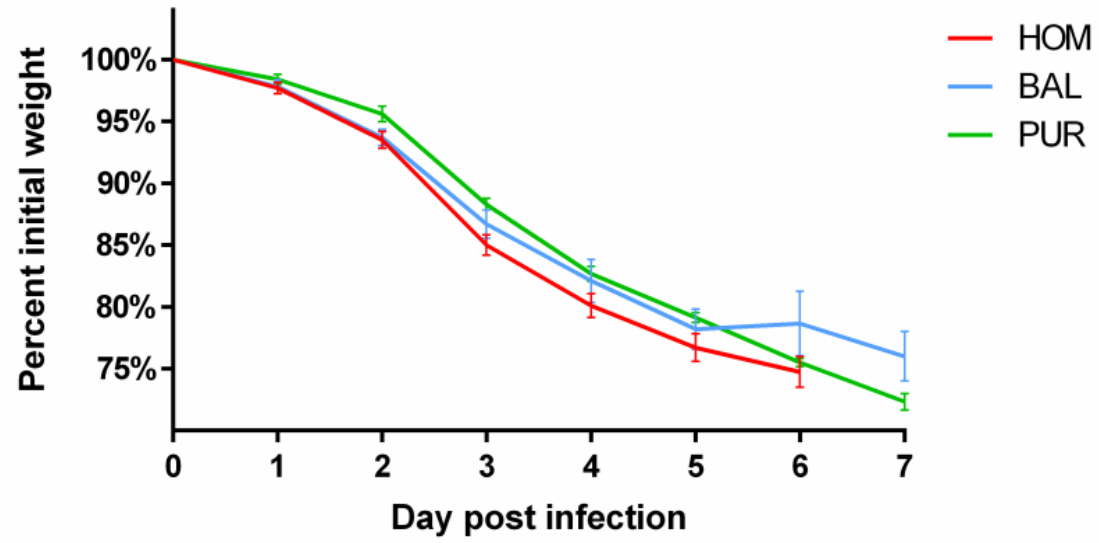

Figure 2. Virulence of three different treatments with virus originated from the same host. HOM = lung homogenate; BAL = bronchoalveolar lavage fluid; PUR = purified lung homogenate. n = 30. LMM for first 7 days post infection only. Error bars are SEM.

Serial passage method does not alter the virulence evolution of IAV

To assess whether the introduction of host-associated factors can affect the evolutionary trajectory of IAV, we compared the overall virulence of virus adapted to mice via HOM passages to virus passaged using BAL infections. To do this, we serially passaged virus for 5 rounds using either passage treatment separately (Fig. 3). After the passages, in order to eliminate effects associated with the viral containing medium, including host factors, we purified the viruses using our custom cell-culture purification method, standardized dose and infected groups of mice with each treatment.

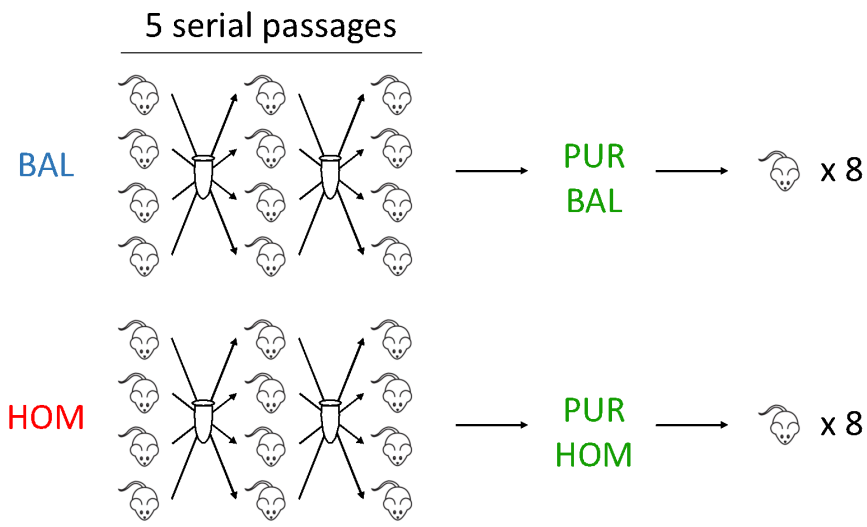

Figure 3. Experimental design to test evolutionary effects of passage methodology. After 5 serial passages with each treatment, virus was purified, dose standardized and used to infect groups of mice. n = 16.

There were no observable differences between virus passaged through BAL or HOM passages (LMM; p = 0.3531) (Fig. 4). This indicates that although there may be slight effects in virulence measurements when mice are infected with viral media containing host-associated factors, these putative inflammatory signals do not alter the evolutionary trajectory of the virus, as measured by induced weight loss.

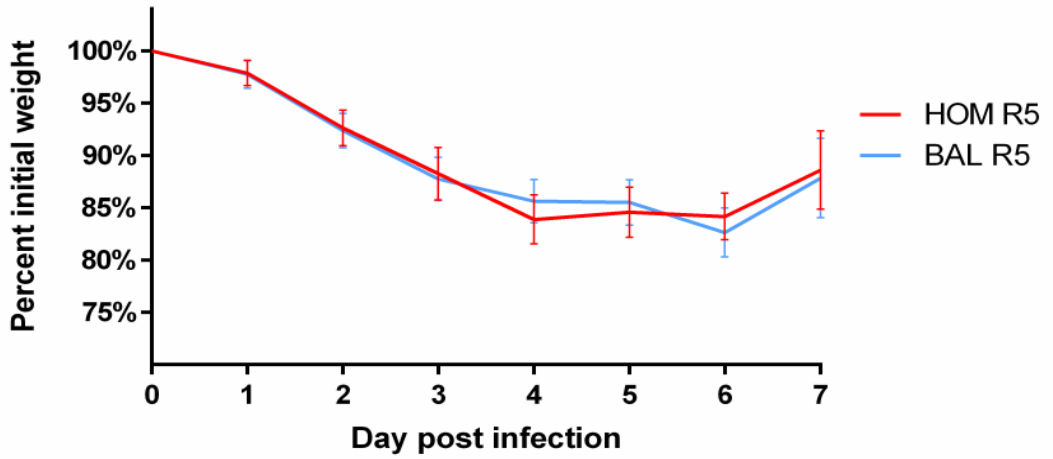

Figure 4. Comparison between virus serially passaged via BAL infections vs HOM infections. Both lines were purified before the test phase and infection dose was 4000 $TCID_{50}$. n = 16. Error bars are SEM.

To test whether our cell-based purification method is selecting for adaptations related to cell-culture growth, we sequenced passage 10 virus (BALB/c-adapted) before and after purification, along with stock Hong Kong 1968. Our results show that while there were several new mutations compared to stock after 10 rounds of adaptation, all mutations were present after purification, and the frequency of these mutations was similar, despite the possible bottleneck induced by the low MOI *in vitro* infection (Table 1). This demonstrates that this purification

method is effective at removing the host-associated factors while maintaining the quality of the viral populations.

MUTATION	FREQUENCY	
	HOM	PUR
HA G234W	0.999	0.999
HA N499D	0.732	0.700
NP T146A	0.979	0.985
NR L108I	0.698	0.544
NS1 G168E	0.635	0.620
PB2 D740N	0.510	0.597

Table 1. Sequencing results for homogenate (HOM) vs purified homogenate (PUR) after 10 rounds of passage. All mutations detected are deviations from the reference sequence of the stock Hong Kong 1968 virus and are present in both HOM and PUR.

Host associated factors increase virulence in unfamiliar host genotypes

To assess whether host-associated factors can affect virulence output when introduced into novel hosts, HOM or PUR virus was used to infect mice of the same genotype of passage (i.e. BALB/c-adapted virus into BALB/c mice and C57BL/6-adapted virus into C57BL/6 mice) or in a novel (unfamiliar) host genotype (i.e. BALB/c-adapted virus into C57BL/6 mice and C57BL/6-adapted virus into BALB/c mice). All treatments were dose standardized to 4000 $TCID_{50}$ per inoculate and data for both strains were combined.

As observed in the first experiment, infections of the familiar host have similar virulence regardless of treatment (HOM fam vs PUR fam, LMM; p= 0.910) (Fig. 5, solid lines). However, when virus in lung homogenate (HOM) is introduced into a new host genotype, there is a significant increase in virulence (LMM; p = 0.0159) (Fig. 5, red lines). If the virus is purified prior to infection of the unfamiliar host genotype, overall virulence is not significantly affected (LMM; p = 0.8766) (Fig. 5, green lines). This indicates that there are host-associated factors in the lung homogenate that likely induce an allogeneic immune response in unfamiliar hosts. Additionally, these results demonstrate that cell-based viral purification effectively removes these host associated inflammatory factors and is an adequate method for purifying virus prior to virulence measurement infections.

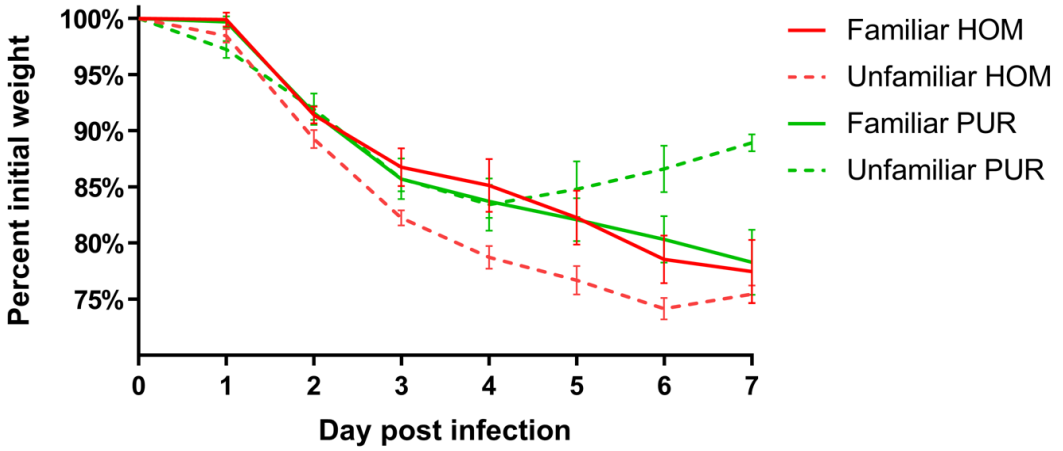

Figure 5. Comparison between round 10 homogenate (HOM) and purified homogenate (PUR) infections in familiar (solid lines) and unfamiliar hosts (dashed lines). Infections were performed in BALB/c (n=12) and C57BL/6 (n=12) female mice. Error bars are SEM.

DISCUSSION

This study experimentally tests the effect of inflammatory components, such as host cytokines, on virulence evolution. We show that there are very subtle differences overall between the three different treatment groups (PUR, HOM, and BAL). This is an interesting result, as the inoculate contents differed between PUR, BAL, and HOM from having the least to the most cytokine presence respectively. All treatment groups were standardized according to titer; thus, the viral dose was the same. It is possible that the dilution of homogenate for the dose standardization reduced the host-associated factors enough to minimize virulence differences. The BAL may still have cytokines present in the wash, though fewer than homogenate. Furthermore, viral populations in the upper respiratory tract extracted from bronchoalveolar lavage may differ from those present in the lower respiratory tract (deep lung tissue) used in whole lung homogenate. This could explain why it is slightly less virulent, but not significantly. The purified virus (PUR), although lacking inflammatory components, may show similar virulence to HOM and BAL due to the elimination of DIPs during the purification process. As previously mentioned, these particles reduce the overall infectivity of the virus (Lakkis & Lechler, 2013). Moreover, the lack of inflammatory components present in PUR may allow the virus to replicate to higher titers because of delayed immune response, causing similar levels of virulence.

In the second experiment, we did not observe a significant difference between the BAL and HOM serial passages. This indicates that although there may be differences when mice are infected with inoculate containing inflammatory cytokines, those inflammatory signals do not alter the evolutionary trajectory of the virus. Thus, the bottleneck potentially introduced during passages with BAL (10 x less virus recovered compared to HOM) does not affect virulence evolution. It should also be noted that the immediately inflammatory environment created by cytokines present in the inoculate seem to play an insignificant role when comparing BAL and HOM passages. This may indicate that BAL is not significantly different from HOM in terms of cytokine content. Additionally, the lack of significant differences confirms that either passage is sufficient for serial passages within familiar hosts (similar genotypes) to induce similar virulence evolution (Shevach, 2011).

In the final experiment, we demonstrate that despite no difference between PUR vs HOM in familiar hosts, there is a significant difference when inoculating unfamiliar hosts with genotype-adapted HOM virus, as these mice lose weight much faster than any other treatment

group (Fig. 5). This is most likely due to the host cytokines present in the inoculate that is not recognized by host MHC molecules and causes inflammation (Lakkis & Lechler, 2013). Additionally, we observe that purification removes the non-viral virulence effects of cytokines. Combined with the sequencing data that demonstrates that there are no differences in the genetic composition of viral populations when homogenate is purified via an overnight passage in cell culture, this novel result demonstrates that purification of homogenate prior to inoculation of uninfected mice could be a proper method for passaging and more importantly testing virulence of influenza A virus in mouse models.

Overall, we demonstrated that there were subtle differences between treatment groups (PUR, HOM and BAL) that may be due to cytokine content. Additionally, within adapted lineages the use of HOM over BAL or PUR does not significantly affect the virulence evolution of the virus within familiar hosts. Conversely, inoculating an unfamiliar strain with virus adapted HOM could show a dramatic increase in virulence that does not accurately represent natural transmission processes. Perhaps, the methodology should be modified when adapting unfamiliar strains of mice with host adapted virus. In conclusion, purification of viral samples is an essential step in order to compare virulence of samples with different titers, eliminating the effect of diluting homogenate when standardizing dose, or different origins, to prevent allogeneic effects.

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