Investigating Influenza A Virus Infection: Tools To Track Infection and Limit Tropism

Jessica K. Fiege, Ryan A. Langlois
Department of Microbiology and Immunology and Center for Immunology, University of Minnesota, Minneapolis, Minnesota, USA

Influenza A viruses display a broad cellular tropism within the respiratory tracts of mammalian hosts. Uncovering the relationship between tropism and virus immunity, pathogenesis, and transmission will be critical for the development of therapeutic interventions. Here we discuss recent developments of several recombinant strains of influenza A virus. These viruses have inserted reporters to track tropism, microRNA target sites to restrict tropism, or barcodes to assess transmission dynamics, expanding our understanding of pathogen-host interactions.

Influenza A viruses (IAVs) are negative-sense, segmented RNA viruses that drive seasonal infections and have the capacity to cause devastating pandemics. Cellular tropism is a critical determinant of the abilities of IAV to transmit across species, spread within hosts, cause pathology, and elicit antiviral immune responses. The reverse genetics system has allowed the construction of a diverse range of recombinant viruses that can be used as tools to enhance the study of IAV. This Gem will discuss the application of viruses capable of identifying infected cells, noninvasive tracking of virus spread, treatment efficacy, and controlling tropism through microRNAs. These powerful tools have facilitated new insights into the biology of IAV in vivo.

INFLUENZA A VIRUS TROPISM IN VIVO

Previous research using protein- and RNA-mediated detection methods has identified a plethora of cell populations infected by IAV. Pulmonary epithelial cells, including ciliated and nonciliated cells, as well as type I and II alveolar cells, can be infected by IAV (1–3). In addition, multiple immune cell subsets, including macrophages and dendritic cells, are positive for virus antigen, which could occur through exogenous uptake and/or direct infection (4). While in vitro models have provided a wealth of information about IAV tropism, the use of IAV reporter strains has increased our understanding of virus-host interactions and long-term consequences of IAV infection in vivo. Numerous reporter IAVs have been engineered over the past few years that allow enhanced determination of virus tropism in vivo; a selected number of them are highlighted in Table 1. The first viable, replication-competent reporter virus utilized a recombinant nonstructural (NS) segment that split the coding sequence of NS1 and the nuclear export protein (NEP) (5). This group generated the NS segment as a single polyprotein expressing NS1-green fluorescent protein (GFP) with a viral 2A cleavage site upstream of NEP. Subsequent reporter strains placed luciferase or Cre recombinase at the C terminus of one of the polymerase segments separated by a cleavage site (Table 1) (6–9). This strategy was somewhat counterintuitive, given that these are the largest IAV segments and therefore were generally thought not to be able to tolerate additional sequence length. However, despite the increased size of the largest segments, viable viruses are still generated. While these reporter IAVs may demonstrate attenuation in mice compared to their parental strains, these viruses are replication competent and can assist in the rapid quantification of virus levels, identification and tracking of cell types infected in real time, and determination of the fate of infected cells.

Luciferase genes are ideal candidates for IAV reporter strains because of their small coding size and ease of detection with little manipulation of the culture system or host animal. Upon introduction of the substrate, the luciferase enzyme quickly generates a robust and easily detectable bioluminescent signal. One of the most exciting applications of this system is the noninvasive imaging of infected tissues in live mice and ferrets over time (7–10). Additionally, in the ferret system, where both direct and respiratory contact infections can be tracked, the IAV-derived luciferase was used to visualize transmission to new hosts (9). Importantly, this technique was able to detect transmission events that would otherwise have been missed by standard approaches (9). Another important attribute is the ability to use virus-driven luminescence to rapidly assess virus replication levels (7–11). Multiple groups have published positive, linear correlations of virus titers in vitro, ex vivo, and in vivo, providing a more rapid and high-throughput approach than standard plaque assays (Table 1) (7–11). This technique has been utilized for rapid, noninvasive determination of therapies, including amantadine, oseltamivir, zanamivir, antiviral serum, and novel antibodies or vaccinations, by detecting levels of fluorescent/luminescent signal after treatment in vitro, ex vivo, or in vivo (Table 1) (5, 7–13). These studies have added to our understanding of virus replication and spread in live animals. Future studies could utilize reporter viruses both in vitro and in vivo to assess properties and treatment options for emerging strains.

Reporter gene IAVs have also been used to identify infected cell types in real time and to determine the fate of infected cells. In three instances, these viruses were used for the identification of infected cells on the single cell level (5, 6). In the first report, Manicassamy et al. generated IAV with GFP inserted into the split NS gene segment and used this tool to identify GFP+ infected
<table>
<thead>
<tr>
<th>Reference</th>
<th>Parental virus</th>
<th>Tag and location</th>
<th>Attenuation in mice</th>
<th>Virus titer correlation via luminescence</th>
<th>Imaging</th>
<th>Evaluation of antiviral therapies</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 PR8</td>
<td>PR8</td>
<td>GFP; NS1 C terminus</td>
<td>Yes, 2 log units</td>
<td>NA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Ex vivo, whole-lung and single-cell suspensions, in vitro</td>
<td>Yes, reduced signal with amantadine or oseltamivir treatment</td>
</tr>
<tr>
<td>12 A/Guinea fowl/Hong Kong/WF10/99 (H9N2)</td>
<td>A/Guinea fowl/Hong Kong/WF10/99 (H9N2)</td>
<td>Gaussia luciferase, GFP, or H5; NS1 C terminus, NEP moved to segment 2</td>
<td>Yes, but not quantified</td>
<td>No</td>
<td>In vitro</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>7 PR8</td>
<td>PR8</td>
<td>Gaussia luciferase; PB2 C terminus</td>
<td>Yes, 2 log units</td>
<td>Yes</td>
<td>Live noninvasive</td>
<td></td>
</tr>
<tr>
<td>10 PR8</td>
<td>PR8</td>
<td>Gaussia luciferase; NA C terminus</td>
<td>Yes, 3 log units</td>
<td>Yes</td>
<td>Live noninvasive, ex vivo, in vitro</td>
<td>Yes, reduced signal in vitro and in vivo after treatment with antiviral serum</td>
</tr>
<tr>
<td>8 WSN</td>
<td>WSN</td>
<td>NanoLuc; PA C terminus</td>
<td>No</td>
<td>Yes</td>
<td>Live noninvasive</td>
<td>Yes, reduced signal due to virus infection with mutated polymerase</td>
</tr>
<tr>
<td>13 A/California/04/2009 (H1N1)</td>
<td>A/California/04/2009 (H1N1)</td>
<td>Gaussia luciferase or GFP; NS1 C terminus, NEP moved to segment 2</td>
<td>Yes, but not quantified</td>
<td>ND</td>
<td>In vitro, GFP</td>
<td>Yes, reduced signal in vitro and in vivo after amantadine or oseltamivir treatment</td>
</tr>
<tr>
<td>11 PR8</td>
<td>PR8</td>
<td>Gaussia luciferase, GFP or tuRFP; NS1 C terminus</td>
<td>ND</td>
<td>Yes</td>
<td>In vitro, GFP, tuRFP</td>
<td>Yes, reduced signal in vitro after zanamivir or U18666A treatment</td>
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<tr>
<td>6 PR8</td>
<td>PR8</td>
<td>Cre recombinase; PB2 C terminus</td>
<td>Yes, 1 log unit</td>
<td>NA</td>
<td>Ex vivo, single-cell suspensions, in vitro</td>
<td>ND</td>
</tr>
<tr>
<td>9 A/California/04/2009 (H1N1)</td>
<td>A/California/04/2009 (H1N1)</td>
<td>NanoLuc; PA C terminus</td>
<td>No attenuation in ferrets</td>
<td>Yes</td>
<td>Live noninvasive</td>
<td>Yes, can be used to verify vaccination efficiency</td>
</tr>
<tr>
<td>14 PR8, H5N1 VN1203</td>
<td>PR8, H5N1 VN1203</td>
<td>eCFP&lt;sup&gt;d&lt;/sup&gt;, eGFP&lt;sup&gt;e&lt;/sup&gt; Venus, or mCherry; NS1 C terminus</td>
<td>Yes, &lt;1 log unit</td>
<td>NA</td>
<td>Ex vivo, whole-lung, two-photon, and single-cell suspensions</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup>NA, not applicable.
<sup>b</sup>ND, not determined.
<sup>c</sup>tuRFP, turbo red fluorescent protein.
<sup>d</sup>eCFP, enhanced cyan fluorescent protein.
<sup>e</sup>eGFP, enhanced GFP.
epithelial cells and hematopoietic cells in the lung (5). Multiple immune cell types were found to contain GFP: macrophages, dendritic cells, neutrophils, B cells, CD4 T cells, and CD8 T cells (5). Additionally, as macrophages and dendritic cells are known to internalize apoptotic cells and debris, it is unclear whether these cells are truly infected, acquire exogenous virus material, or a combination of the two (5). Subsequently, Kawaoka and colleagues generated several fluorescent reporter viruses by using the NS split segment (14). These viruses were passaged through mice, reducing their attenuation in vivo (14). They then compared PR8 (H1N1) and H5N1 tropism in nonhematopoietic cells and macrophages and found that a greater percentage of macrophages were infected with the H5N1 virus (14). Additionally, mice were infected with a cocktail of all four distinct fluorescent viruses, allowing the visualization of coinfected epithelial cells. While these viruses can be used to identify cells that are infected with IAV, GFP and endogenous virus products have transient expression and therefore cannot define infected cells after replication has ceased. The prevailing theory in the field was that all infected cells would allow attenuation in mammals but would not affect the growth of the vaccine. This led to the discovery of species-restricted miRNA-93, which is expressed in both mice and humans but not in chickens (16). Incorporation of two near-perfect miRNA-93 target sites into the nucleoprotein-coding region was achieved with minimal changes in the physical properties of the corresponding amino acids (16). Infection with miRNA-93-targeted IAV resulted in virus attenuation in human cell lines and mouse lungs but not during growth in embryonated eggs, which lack the cognate miRNA. Vaccination of mice induced a robust immune response with the generation of neutralizing antibodies and provided 100% protection from a lethal challenge (16). Thus, the use of miRNA-mediated gene silencing in a species-specific manner can aid in the creation of IAV vaccines. An advantage of miRNA-based attenuation over traditional temperature-mediated attenuation is the ability to modulate virus fitness by the number and location of target sites or the complementarity to the miRNA. Additionally, this system could be applied to other IAV strains and tailored to specific tissues or cell types.

Exploitation of miRNAs can be extended beyond vaccines to provide tools to better understand the relationship between tropism and immunity in vivo. Both immune and nonimmune cells are infected and can participate in immune responses. However, the contribution of each of these disparate compartments to the generation of immunity in vivo was unknown. In order to better understand how the host gains viral immunity after infection, tenOever and colleagues selectively prevented replication in immune cells by using hematopoietic-cell-specific miRNA-142 (17). Interestingly, normal activation of CD8 T cells occurred even in the absence of virus replication in dendritic cells (17). As dendritic cells can acquire antigens via direct infection or internalization of exogenous particles, this finding suggests that virus replication in these cells is dispensable for antigen presentation to CD8 T cells (17). In contrast, virus replication in dendritic cells is required for the optimal production of type I interferon and was necessary for virus sensing by retinoic-acid-inducible gene receptors (17). This study highlights two separate functions of innate immune cells during IAV infection: antigen presentation and virus sensing. Previous studies, which altered the host environment, were unable to separate these two events in vivo, but the use of miRNAs to alter virus tropism allowed for this discovery. This technique can be applied to additional cell- and/or tissue-specific miRNAs to further elucidate mechanisms of tropism-dependent immunity.

In 2012, concerns over the generation of mammalian aerosol-transmissible strains of H5N1 led to a voluntary yearlong moratorium on gain-of-function work with H5N1 (18). After the moratorium was lifted, a broader suspension of federal funding was announced and is currently in place (18). In an effort to clarify which gain-of-function studies are dual-use research of concern, the acronym aTRIP (alter transmission, range and resistance, in vivo) was developed to allow the work the field deems important to continue. The acronym aTRIP offers an enticing way to mitigate potential safety concerns involved in the study of pathogenic, transmissible IAVs (19). Small RNA deep sequencing was again employed to discover a species-specific miRNA, miRNA-192, expressed in primary human respiratory tract epithelial cells and mouse lungs but absent from the ferret respiratory tract, the model system used

**MIRNA-MEDIATED CONTROL OF INFLUENZA A VIRUS TROPSM**

Live attenuated vaccines effectively immunize the host by activating both the innate and adaptive arms of the immune system (15). Traditional techniques of creating live attenuated vaccines have involved the passage of a virus in cell culture or in nonhuman hosts to alter virus tropism. In 2008, Andino and colleagues pioneered a new approach for generating protective live attenuated vaccines by utilizing microRNA (miRNA)-based gene silencing to control poxvirus replication (15). This technique opened up new avenues for the study of IAV allowing vaccine development and the analysis of cellular tropism, immune activation, and biological containment.

In 2009, miRNA-mediated gene silencing was used to generate a live attenuated influenza virus (16). Given that IAV vaccines are traditionally grown in eggs, tenOever and colleagues used deep sequencing to identify a miRNA present in mammals but absent from chickens. Harnessing of a divergently expressed miRNA...
to study virus transmission (19). Mice infected with miR-192-targeted IAV demonstrated less morbidity and mortality than those infected with the control virus. Importantly, infection of ferrets resulted in normal virus replication and aerosol transmission to secondary hosts (19). The inclusion of this method, in addition to standard physical safety measures, could allow researchers to study potential aTRIP IAV strains in the ferret model with less risk to human health.

TRACKING VIRUS TRANSMISSION

Despite our expanding knowledge of IAV tropism, little is known about the population dynamics of transmissible infection. Tracking of the transmission of IAV to new individuals could aid in our understanding of the global migration of the virus. To monitor virus spread, Varble et al. added neutral barcodes to identify transmission bottlenecks (20). Ferrets were inoculated with the bar-coded library to assess virus diversity in recipient hosts infected via direct contact versus aerosol transmission. Animals infected via direct contact received only a small number of viruses from the library, while those infected via airborne contact faced an even more severe bottleneck, often comprising only two or three bar-coded viruses (20). Transmission between ferrets correlated with the starting barcode frequency in the inoculum and was limited to viruses replicating in the upper respiratory tract (20). Viruses that were found only in the lower respiratory tract were not transmitted (20). These data demonstrate that despite a potential for rich virus diversity within the host, aerosol transmission is a severe bottleneck. These data should be incorporated into models defining the potential emergence and spread of new pathogenic strains.

CONCLUSIONS

The reverse genetics system for IAV has allowed the engineering of innovative virus tools with which to track infected cells and experimentally control tropism. Together, these complementary approaches have facilitated greater insights into virus tropism and the relationship between tropism and immune response. These powerful new tools have also garnered insights into transmission dynamics, and while this review has highlighted some of the recent accomplishments, we still have much to learn. A more complete understanding of the cells that drive virus spread, both within the lung and to new hosts, and the specific contributions of cell type infected in driving immune responses and pathology, all need to be further elucidated. In the future, these tools will also allow rapid and high-throughput testing and validation of new antiviral drugs and vaccination regimens.

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REFERENCES