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Replication-competent influenza A viruses expressing a red fluorescent protein

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ABSTRACT

Like most animal viruses, studying influenza A in model systems requires secondary methodologies to identify infected cells. To circumvent this requirement, we describe the generation of replication-competent influenza A red fluorescent viruses. These influenza A viruses encode mCherry fused to the viral non-structural 1 (NS1) protein and display comparable growth kinetics to wild-type viruses *in vitro*. Infection of cells with influenza A mCherry viruses was neutralized with monoclonal antibodies and inhibited with antivirals to levels similar to wild-type virus. Influenza A mCherry viruses were also able to lethally infect mice, and strikingly, dose- and time-dependent kinetics of viral replication were monitored in whole excised mouse lungs using an *in vivo* imaging system (IVIS). By eliminating the need for secondary labeling of infected cells, influenza A mCherry viruses provide an ideal tool in the ongoing struggle to better characterize the virus and identify new therapeutics against influenza A viral infections.

Introduction

Influenza A virus (IAV), a member of the family *Orthomyxoviridae*, is a human respiratory pathogen that causes annual epidemics and occasional pandemics of considerable public health and economic impact (Molinari et al., 2007). The 20th century was marred by three documented influenza A pandemics: the Spanish flu (H1N1) of 1918, the Asian flu (H2N2) of 1957 and the Hong Kong flu (H3N2) of 1968, which together resulted in upwards of 60 million deaths (Kilbourne, 2006). The first influenza pandemic of the 21st century was declared in 2009 after the emergence of a quadruple-reassortant swine-origin H1N1 IAV that in less than one year infected more than 600,000 people worldwide (Louie et al., 2011; Smith et al., 2009). Additionally, the emergence of avian IAV strains that infect humans sporadically, such as H5N1, H7N9, and H10N8 have heightened worldwide concerns for pandemic preparedness, and together with the 2009 pandemic H1N1 IAV, provide credence for the improvement in monitoring, preventing and treating IAV infections (Ali et al., 2000; Herfst et al., 2012; Lopez-Martinez et al., 2013; Uyeki and Cox, 2013).

The IAV genome contains eight single-stranded RNA molecules of negative polarity, which are encapsidated into distinct viral ribonucleoprotein (vRNP) particles that contain the viral polymerase subunits PB2, PB1 and PA and multiple copies of the viral nucleoprotein (NP) (Palese, 2007). Genome replication and transcription occurs in the nucleus of infected cells and requires the tripartite polymerase complex, NP, and viral RNA (vRNA) (Wright et al., 2007). Reverse genetics, or the ability to rescue recombinant viruses from plasmid DNA, requires vRNA expression of all eight genome segments and protein expression of the aforementioned PB2, PB1, PA and NP (Fodor et al., 1999; Neumann et al., 1999). This approach has provided an excellent tool to study IAV biology (Ozawa and Kawaoka, 2011) and to develop a new generation of IAV vaccines (Martinez-Sobrido and Garcia-Sastre, 2010). Furthermore, modification of the IAV genome has resulted in replication-incompetent (Baker et al., 2013; Martinez-Sobrido et al., 2010) or -competent viruses that express foreign genes, including several reporter genes, which are expressed as fusion products to viral proteins (Avilov et al., 2012; Heaton et al., 2013; Kittel et al., 2004; Lakdawala et al., 2014; Li et al., 2010; Manicassamy et al., 2010; Martinez-Sobrido et al., 2010; Ozawa et al., 2011; Pan et al., 2013; Pena et al., 2013; Rimmelzwaan et al., 2007; Tran et al., 2013). An IAV protein commonly targeted for such genetic manipulation is

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the non-structural 1 (NS1) protein because of its short length, high copy number in infected cells, and redundant functions in antagonizing innate immunity (Hale et al., 2008; Palese, 2007). The NS1 protein is encoded on a collinear mRNA derived from the smallest vRNA segment eight, which is alternatively spliced to produce the nuclear export protein (NEP) mRNA, and is expressed to 10% the levels of NS1 (Lamb and Lai, 1980; Paterson and Fodor, 2012). Virus assembly requires NEP, thus genetic modifications that yield differences in levels of expression can hinder successful virus rescue or attenuate the virus (Paterson and Fodor, 2012).

To evaluate viral infection *in vitro* and *ex* or *in vivo*, fluorescent viruses are advantageous because secondary steps such as immunofluorescence staining or addition of bioluminescence substrates are not required to detect infected cells (Kayali et al., 2008; Kumar and Henrickson, 2012). A green fluorescent protein (GFP)-expressing IAV has been described (Kittel et al., 2004; Manicassamy et al., 2010), but its use is limited due to spectral overlap of GFP and tissue autofluorescence (Vintersten et al., 2004) or when GFP-expressing animals or cell lines serve as hosts of infection (Miller, 2011). As an alternative reporter, red fluorescent proteins have an excitation and emission spectra that is more conducive to *in vivo* or *ex vivo* imaging, offering less autofluorescence and deeper tissue penetrance (Shaner et al., 2005, 2007). Furthermore, laboratory evolution of red fluorescent proteins has led to a molecule, mCherry, that possesses higher quantum yield, a longer half-life, and that does not aggregate, as opposed to its ancestral protein derived from *Discosoma sp.* (Shaner et al., 2005).

Here, we describe for the first time the generation of replication-competent red fluorescent IAVs, where the NS1 protein of A/Puerto Rico/08/1934 (H1N1; PR8) is fused to mCherry, and the remaining seven segments are derived from PR8 or A/California_NYICE_E3/04/2009 (pH1N1). *In vitro*, mCherry IAVs are neutralized by monoclonal antibodies or inhibited by antivirals similarly to wild-type (WT) recombinant viruses, representing an excellent option for the rapid identification of neutralizing antibodies or antivirals using high-throughput screening. In mice, PR8 mCherry replication can be directly visualized and quantified from whole excised lungs using an *in vivo* imaging system (IVIS). These results offer a promising option to directly study the biology of influenza virus and to evaluate experimental countermeasures to treat influenza viral infections *in vitro* and *ex vivo.*

Results

Generation of a recombinant influenza A PR8 virus expressing the mCherry fluorescent protein

Generation of replication-competent reporter-expressing IAV requires that the inserted reporter gene neither impair critical functions of the protein(s) encoded by the native gene segment to which it is appended nor disrupt the packaging signals therein. The NS1 protein has previously been shown to tolerate fusion to foreign proteins including GFP (Kittel et al., 2004; Manicassamy et al., 2010; Perez et al., 2013), and thus is an ideal viral protein for reporter gene conjugation. However, red fluorescent proteins are preferred for *in vivo* studies (Shaner et al., 2005, 2007). Because the NS segment is alternatively spliced to produce NEP, two silent mutations were introduced in the splice acceptor site to avoid splicing (Hale et al., 2008; Kochs et al., 2007). To produce NEP, the porcine teschovirus-1 (PTV-1) 2A autoproteolytic cleavage site was inserted between NS1 and NEP so that both proteins (NS1 and NEP) would be translated individually (Fig. 1), like previously described (Manicassamy et al., 2010). Importantly, the NS1 and NEP N-terminal overlapping open reading frame was duplicated downstream of the PTV-1 2A site to assure NEP synthesis (Paterson and Fodor, 2012). Using two unique BsmBI restriction sites, mCherry was cloned and fused to NS1 and used to generate a recombinant PR8 NS1-mCherry virus (hereafter referred to as PR8 mCherry) using plasmid-based reverse genetics (Martinez-Sobrido and Garcia-Sastre, 2010).

Characterization of PR8 mCherry virus

To evaluate if PR8 encoding NS1 fused to mCherry could be directly visualized *in vitro* and to evaluate the subcellular localization of NS1 during PR8 WT and mCherry infection, fluorescence (mCherry) and indirect immunofluorescence microscopy were used (Fig. 2A and B). As expected, only cells infected with PR8 mCherry were fluorescent upon examination with a red filter. In cells infected with PR8 mCherry, the nuclear localization of NP (Fig. 2A) was similar to that of PR8 WT. Importantly, NS1 was similarly distributed in PR8 WT and mCherry infected cells (Fig. 2B).

PR8 WT and mCherry virus identity was then confirmed by RT-PCR and Western blotting (Fig. 2C and D). Expected band sizes of approximately 890 and 1891 nucleotides were amplified and resolved, corresponding to the NS vRNA from PR8 WT or mCherry, respectively (Fig. 2C). Additionally, primers amplifying the NS1-mCherry fusion only amplified an accurately sized band (1433 nt) from PR8 mCherry infected cells. As expected, NP mRNA levels were detected similarly from both PR8 WT and mCherry infected cells. We next evaluated protein expression by Western blotting using antibodies specific for NS1, mCherry, or NP as a control of viral infection (Fig. 2D). The amount of NS1 was slightly decreased in cells infected with PR8 mCherry as compared with PR8 WT, although NS1-mCherry was easily detected using the mCherry PAb. Differences between NS1 and NS1-mCherry signal intensities observed with the 1A7 monoclonal antibody correlate with a lower level of NP in PR8 mCherry infection, but may additionally be due to lower affinity of 1A7 when NS1 is fused to mCherry (Fig. 2D).

Growth properties of PR8 mCherry

Virus fitness in cell culture was next assessed by examining the multicycle growth properties and plaque formation of PR8 mCherry, as compared to PR8 WT (Fig. 3). PR8 mCherry viral kinetics were similar, albeit the total virus yield was lower after 24 h, with respect to PR8 WT (Fig. 3A). When evaluating the plaque phenotype, only PR8 mCherry formed fluorescent plaques (Fig. 3B), but in agreement with virus kinetics, the plaque size was slightly reduced compared to PR8 WT by immunostaining with an anti-NP monoclonal antibody (Fig. 3C). Importantly, all plaques detected using the anti-NP monoclonal antibody expressed mCherry (white arrows), indicating that all infectious viruses express mCherry.

Ability of NS1-mCherry fusion protein to inhibit IFNβ promoter activation

NS1 is a multifunctional protein that uses multiple mechanisms to counteract the type I interferon (IFN) response during viral infection (Hale et al., 2008). In order to evaluate if NS1-mCherry retained the ability to antagonize IFNβ activation, MDCK cells expressing GFP and FFluc under the control of the IFNβ promoter (Hai et al., 2008) were infected with PR8 WT and mCherry viruses (Fig. 4). As an internal control, cells were similarly infected with PR8 ΔNS1 (Garcia-Sastre et al., 1998), which potently induces IFNβ promoter activation (Geiss et al., 2002). GFP expression in infected cells indicated that PR8 mCherry infection inhibited IFNβ promoter activation to levels comparable to PR8 WT and, as expected,