A System for Production of Pure Influenza A Virus Defective Interfering Particles (DIPs) and Assessment of their Antiviral Activity

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no materials previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree of the university or other institute of higher education, except where due acknowledgment has been made in the text.

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1. Abstract

Influenza A virus (IAV) epidemics and pandemics constitute a major threat to human health. The ability of IAV to rapidly acquire mutations can render antivirals and vaccines ineffective and novel antiviral strategies are urgently needed. IAV particles harbor eight genomic RNA segments and defective interfering RNAs (DI RNAs) are naturally occurring byproducts of viral genome replication. These RNAs interfere with replication of the WT RNAs and, upon packaging into defective interfering particles (DIPs), can inhibit spread of WT IAV. It has been postulated that DIPs interfere with IAV infection by competing for cellular resources and inducing the interferon (IFN) response. However, the relative contribution of these processes to DIP antiviral activity has been unclear and it was unknown whether DIPs harboring more than one DI RNA exert increased antiviral activity. Further, development of DIPs for IAV treatment was hampered by the need to use WT IAV as helper virus for DIP amplification, which raises safety concerns. The goals of this thesis were to remove these roadblocks as a step forward towards the development of DIPs as novel antivirals.

Studies with a prototypic DI RNA, DI-244, revealed that providing the open reading frame in trans that had been destroyed upon DI RNA generation allows to generate DIPs in the absence of helper virus. The DIPs produced under those conditions suppressed IAV infection and antiviral activity was not increased when particles were engineered to harbor more than one DI RNA. Further, it was revealed that any central deletion in the genomic IAV segments 1, 2 and 3 is sufficient to convert these RNAs into DI RNAs. Antiviral activity of these DI RNAs was inversely correlated to DI RNA length in the absence of a functional IFN response. However, in the presence of a functional IFN response, antiviral activity was independent from DI RNA length and was associated with the expression of interferon (IFN) stimulated genes (ISGs) but not IFN. In conclusion, this thesis reports a system to safely produce DIPs in the absence of WT virus and demonstrates that DIP antiviral activity might be mainly due to induction of ISG expression which may rely on a previously uncharacterized pathway since DIPs did not induce IFN expression.

Key words: Influenza A virus (IAV), defective interfering particles (DIPs), DI244, IFN induction, ISG induction, replication interference.

2. Introduction

Influenza A virus is a highly infectious respiratory pathogen and annual influenza epidemics are associated with significant morbidity and mortality. In addition, the virus has a wide host range and a segmented genome, which allows the exchange of genomic information between human and animal strains (reassortment), which may give rise to new viruses with pandemic potential. Antiviral drugs are available but their efficacy can be compromised by resistance mutations. Moreover, vaccines have to be reformulated on an annual basis to protect against seasonally circulating viruses and offer little or no protection against pandemic influenza. This highlights the need for new approaches to influenza therapy and prophylaxis (Bailey et al., 2018; Clark & Lynch, 2011).

2.1 Influenza- the disease

Influenza is an acute illness of the upper and lower respiratory tract and a common cause for morbidity and mortality (Bailey et al., 2018; Clark & Lynch, 2011). The causative agent of this disease are the influenza viruses which were first discovered by Smith and colleagues in 1933 (W. Smith et al., 1933). The spread of influenza viruses predominantly occurs through contact of respiratory mucosa with aerosol particles produced by coughing and sneezing. Influenza viruses can also be transmitted via contact with contaminated surfaces (e.g. shaking hands), followed by hand to mouth or hand to nose contact. Following infection, influenza viruses replicate in the nasal and pharyngeal mucosa (Clark & Lynch, 2011). Clinical manifestations appear after an incubation period of approximately 3-5 days and include fever, headaches, myalgia, sneezing and a dry cough. However, roughly 20-60% of the infections are asympotomatic (Bartosch et al., 2003; Halloran et al., 2008; Milne et al., 2008). Individuals with underlying health conditions are at increased risk for more serious disease outcomes. Thus, influenza virus infection of patients who suffer from a preexisting condition such as chronic heart disease, diabetes, or lung disease can result in complications including bacterial superinfection, pneumonia (Laing et al., 2001; Oliveira et al., 2001) and myocardial infarction (Warren-Gash et al., 2012). Additionally, age is a risk factor. Young children infected with influenza virus are at risk of primary influenza pneumonia, encephalitis, myocarditis, and death (Aykac et al., 2018; Surtees & DeSousa, 2006; Thomas et al., 2003). Moreover, the elderly are particularly prone to viral and bacterial pneumonia as a result of

their weak immune systems and are at high risk to develop exasperation of previously existing health issues such as chronic lung disease, heart disease, and endocrine disorders (Ciabattini et al., 2018). Hence, influenza virus is a prominent health threat with significant morbidity and mortality.

2.2 Classification

Influenza viruses are enveloped negative strand RNA viruses belonging to the family *Orthomyxoviridae*. The six genera within this family *include Influenzavirus A, Influenzavirus B, Influenzavirus C, Thogo virus, Isavirus,* and the more recently identified *influenzavirus D* (Bailey et al., 2018; Foni et al., 2017; Shaw & Palese, 2013). The name "influenza" is the Italian form of the latin term for "influential", and was coined in the mid 17th century on the notion that the illness caused by this virus emerged under the "influence" of celestial and occult origins (*Insight into Influenza Viruses of Animals and Humans | Sanjay Kapoor | Springer*, n.d.). Influenza viruses have segmented genomes, which allow for the exchange of intact segments through a process known as re-assortment. The four genera of influenza virus differ in terms of viral structure, epidemiology, pathogenicity and host range (Lamb, 2008). Sequencing analysis has revealed that these genera share common genetic ancestry but have diverged such that re-assortment events only occur within the same genus or type but not across the distinct influenza types (Bouvier & Palese, 2008b; Szewczyk et al., 2014). From an epidemiological perspective, viruses within the genus *Influenzavirus A* have the most important impact on human health and can be further classified into subtypes based on surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). There have been 16 HA and 9 NA subtypes identified in circulating influenza A viruses. However, only viruses of the H1N1, H2N2 and H3N2 subtypes have been found to cause pandemics (Baigent & McCauley, 2003; Bouvier & Palese, 2008a). In addition, bat influenza viruses bearing H17, H18 and N10, N11 subtypes have recently been identified. Interspecies transmission of these IAV has not been documented owing to the failure of these viruses to re-assort with conventional IAVs; due to an incompatibility between the vRNA packaging signals and NP (Ciminski et al., 2017).

Influenza A viruses (IAV) have a wide spectrum of hosts including humans, swine, poultry and horses. Influenza B viruses (IBV) are mostly restricted to humans. However, reports have indicated the presence of these viruses in seals. Both IAVs and IBVs have an 8-segment genome

and surface glycoproteins HA and NA (Foni et al., 2017; Muraki & Hongo, 2010). Influenza C viruses (ICV) can infect both humans and swine but do not pose as a serious threat to either species (Brown et al., 1995; Yamaoka et al., 1991). The more recently discovered influenza D viruses (IDV) have thus far been detected in swine and cattle (Chiapponi et al., 2016; Hause et al., 2014). Both ICVs and IDVs possess a 7-segment genome. Moreover, viruses within these 2 genera have only one surface glycoprotein, the hemagglutinin-esterase fusion protein (HEF), which has the combined functions of receptor binding, receptor cleaving and membrane fusion (Foni et al., 2017; Muraki & Hongo, 2010). The nomenclature of influenza viruses is standardized and includes the virus type, species of isolation (if not human), location of isolation, isolate number, and isolate year. Additionally and only for influenza A viruses, the subtype of HA and NA glycoproteins is also included in the nomenclature (Bouvier & Palese, 2008b; Shaw & Palese, 2013).

2.3 Epidemiology of Influenza A viruses

Influenza A viruses are responsible for the majority of seasonal outbreaks and all so far recorded influenza pandemics. The unique ability of IAV to accumulate point mutations in HA and NA segments results in progressive antigenic variation known as antigenic drift, which allows it to evade recognition by the host immune response leading to seasonal epidemics. Within in the northern hemisphere, seasonal outbreaks generally peak during the winter and fall seasons when humidity and temperature conditions are optimal for transmission (Clark & Lynch, 2011; Widdowson & Monto, 2013). Approximately 10% of the world population gets infected with influenza viruses every year. The World Health Organization (WHO) estimates an annual rate of 290,000 to 650,000 deaths globally due to seasonal influenza (*WHO | Burden of Disease*, n.d.). Additionally, the economic burden of influenza is estimated at $5.8 billion annually in the US alone (Ozawa et al., 2016). Within Germany, the Robert Koch Institute (RKI) recorded approximately 3.8 million cases of acute respiratory illness associated with the influenza virus for the year 2018. The strains responsible were influenza A(H1N1)pdm09 and influenza A(H3N2) (RKI - Infectious Diseases in Germany - Report on the Epidemiology of Influenza in Germany 2018/2019). Moreover, the annual influenza-associated economic burden in Germany ranges between €53 million as in 2012 and €120 million as in 2015 (Scholz et al., 2019).

In addition to antigenic shift, IAVs can undergo a form of re-assortment termed "antigenic shift" leading to pandemics. This refers to the event when IAVs from human and animal origins re-assort giving rise to a new virus against which no one in the population has preexisting immune responses (MacKellar, 2007). In the past century, there have been four recorded influenza A virus pandemics. The most deadly pandemic recorded was the H1N1 Spanish influenza, with an estimated 500 million apparent cases of infection. The pandemic encompassed three waves between the years 1918-1919 and has been called 'the greatest medical holocaust in history' where it killed 40-50 million people worldwide (Saunders-Hastings & Krewski, 2016). Additional influenza pandemics include the Asian flu (1957-1958), the Hong Kong flu (1968-1970), and the more recent swine flu (2009-2010) (MacKellar, 2007)(Figure 2). In the case of the 2009 swine flu pandemic, the extent of commercial travel and global trade allowed the virus to spread to 122 countries within a matter of weeks (G. J. D. Smith et al., 2009). This is in stark contrast to previous influenza pandemics, which required an average of 6 months to spread globally. The resulting overall economic burden was estimated to range between 0.5% and 1.5% GDP loss across countries (R. D. Smith et al., 2009). This estimate, however, fails to take into consideration the economic effects resulting from the reduction in tourism, school foreclosures, changes in global trade, and the overall loss of productivity at the work place. Since the 2009 pandemic, H1N1 and H3N2 strains have been the predominating strains in circulation (Saunders-Hastings & Krewski, 2016). Additionally, there have been several documented human infections with zoonotic avian influenza A viruses. However they have not been associated with human-to-human transmission (WHO | Human infection with avian influenza A (H5N8) – the Russian Federation). It remains unclear and unpredictable whether the next pandemic will be the result of an antigenic shift of a currently circulating virus or whether it may result from direct transmission of a new influenza A virus from animals to humans, potentially to a mutation in a lethal avian influenza A virus strain that allows human-human transmission (Saunders-Hastings & Krewski, 2016).

Pandemic Name	Year	Strain	Suspected Origin of Outbreak	Approximate Number of Deaths
Spanish flu	1918–1920	H1N1	China	40–50 million
Asian flu	1957–1958	H2N2	China	1–2 million
Hong Kong flu	1968–1970	H3N2	China	500,000–2 million
Swine flu	2009–2010	H1N1	Mexico	Up to 575,000

Figure 2: Recorded influenza A virus pandemics. Table taken from (Saunders-Hastings & Krewski, 2016)

2.4 Virion architecture

Influenza virions are pleomorphic and can either be filamentous in shape reaching a length of 300 nm or spherical with a diameter between 80 to 120 nm (Bouvier & Palese, 2008a; Couch, 1996). All influenza viruses have a viral envelope derived from the plasma membrane of the host cells in which the virus was grown (Lamb, 2008). Influenza A and B virus are virtually indistinguishable by electron microscopy, while influenza C viruses can be distinguished through the hexagonal arrangement of their HA proteins in the viral envelope (Lamb, 2008; Su et al., 2017). IAV and IBV viruses have a viral envelope studded with HA and NA proteins at a ratio of four to one (Shaw & Palese, 2013). The HA is a rod shaped homotrimer facilitating receptor binding and membrane fusion, while the NA is a mushroom shaped homotetramer responsible for destroying receptors by hydrolyzing sialic acid groups from glycoproteins (Fujiyoshi et al., 1994; Lamb, 2008). ICV and IDV are studded with HEF glycoproteins, which share an overall similarity to the structure and folding of HA (Rosenthal et al., 1998). Additionally, influenza viruses harbor ion channels in their envelope: M2 for IAV, BM2 for IBV, and CM2 for ICV, and DM2 for IDV (Su et al. 2017; Betáková and Kollerová 2006; Fischer and Sansom 2002).

In the case of Influenza A virus, M2 ion channels are tetramers present at a ratio of roughly one M2 channel to 10 or 100 HA glycoproteins and allow the acidification of the viral interior space during infection. The M1 matrix protein underlays the viral lipid membrane and encapsulates the viral core containing the eight viral RNA segments. The gene assignment for IAV are as follow: segment 1 encodes PB2, segment 2 encodes PB1, segment 3 encodes PA, segment 4 encodes HA, segment 5 encodes NA, segment 7 encodes for both M1 and M2, and finally segment 8 encodes for NS1 and NS2/NEP (Figure 1A). The RNA polymerase complex is composed of the subunits PB1, PB2, and PA, which are present as 300 to 60 copies per virion. NP proteins bind all eight vRNA segments and the viral polymerase complex to form the viral ribonucleoprotein complex (vRNP) (Figure 1). Each NP monomer interacts with approximately 20 nucleotides of vRNA through association with its phosphate sugar backbone. The NS2/NEP proteins associate

with the M1 protein in order to transport vRNPs outside the cell nucleus. The NS1 protein is multifunctional which mainly inhibits host immune responses through interferon (IFN) production (Hale et al., 2008; Rosário-Ferreira et al., 2020).

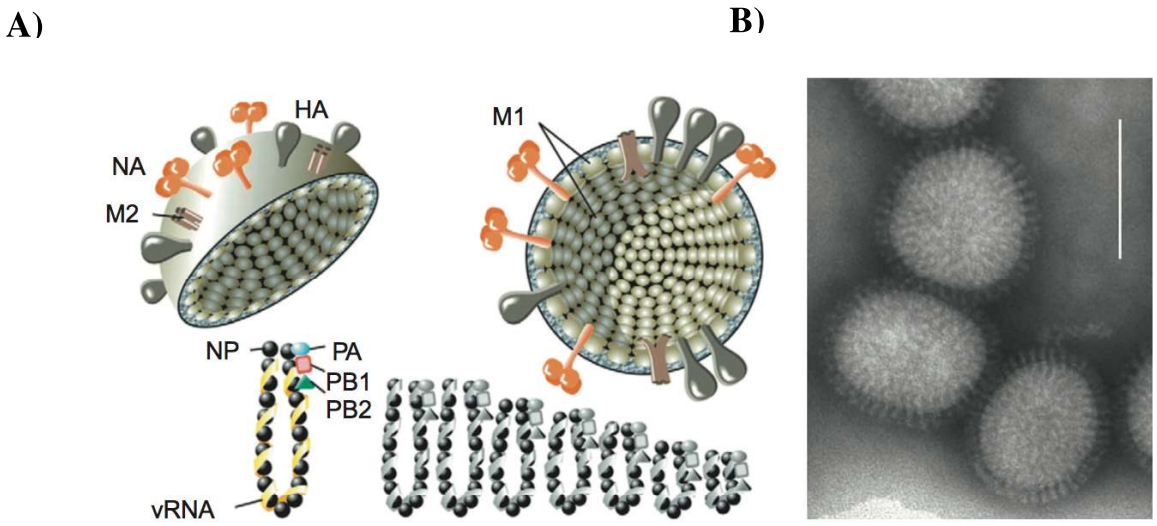

Figure 1: Structure of the influenza A virus. A) The influenza A virus envelope is contains the glycoproteins HA and NA and the ion channel M2. The IAV genome consists of 8 vRNA segments associated with the polymerase complex (PB2, PB1, PA) and the NP protein to form the vRNP. The vRNPs are connected to the viral envelope through the matrix protein M2. **B)** Electron micrograph of IAV virions, the bar represents 100 nm. Figures were taken from Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses, p. 681.

2.5 Replication of Influenza A virus

2.5.1 Receptor binding and fusion

The infection cycle of IAV begins upon the binding of HA to terminal sialyic acid residues on glycoproteins or glycolipids in the cellular membrane (Dou et al., 2018). Subsequently, virus particles enter the cell either via macropinocytosis or a clathrin-dependent endocytosis (Dou et al., 2018; Rosenthal et al., 1998). Once inside the cell, the virus is trafficked into the early endosome

where low pH triggers the membrane fusion activity of HA. For this, the N- terminal fusion peptide in HA2 inserts into the endosomal membrane. Next, HA2 collapses and forms a six-helix-bundle leading to the ultimately fusion of the viral and cellular membrane. As a result of membrane fusion the vRNPs inside the viral particles can be released into the host cytoplasm (Dou et al., 2018; Lazarowitz & Choppin, 1975), a process that relies on M2-dependent influx of protons into the interior of the virus (Dou et al., 2018).

2.5.2 Transcription of vRNAs into viral mRNA

Unlike other RNA viruses, which replicate in the cytoplasm, members of the family Orthomyxoviredea replicate in the nucleus. This is because these viruses are dependent on "snatching" host cellular RNA to prime the transcription of their mRNA by a process known as "cap-snatching". In order to initiate transcription, the IAV PB2 subunit snatches short nascent host RNA which is used to cap the 5' end of viral mRNA (Newcomb et al., 2009; Plotch et al., 1981). Subsequently, the capped PB2 domain is repositioned into the PB1 active site where elongation of the viral mRNA occurs using vRNA as a template. The cap-snatching mechanism makes the viral mRNA transcription process significantly more efficient than that of vRNA and cRNA transcription (Reich et al., 2014). The mRNA transcripts produced are polyadenylated and exported into the cytoplasm to be translated by cytoplasmic ribosomes (Jorba et al., 2009).

2.5.3 Replication of vRNAs

Replication of the IAV genome takes place in the nucleus and is mediated by the heterotrimeric viral polymerase. The negative sense vRNA is transcribed into positive sense complimentary RNA (cRNA) and used as a template for further production of positive sense vRNA (E. Fodor, 2013; Velthuis & Fodor, 2016). The process of vRNA transcription into cRNA is an unprimed event and is mediated by the association of free rNTPs (ATP and GTP) with the 3' end of the vRNA (Newcomb et al., 2009; York et al., 2013) . Elongation of the cRNA occurs from an A-G dinucleotide bond formed within the PB1 segment of the viral polymerase (Robb et al., 2016). Newly transcribed cRNA associates with NP and a copy of the viral polymerase complex

in order to form cRNP, which can be used as a template for production of more negative sense vRNPs (York et al., 2013) .

2.5.4 Assembly and packaging of newly formed virions

Once viral mRNA is exported into the cytoplasm via the NS2/NEP protein, cytosolic ribosomes translate the PB1, PB2, PA,NP, NS1, NS2, and M1 mRNAs, while mRNAs encoding for HA, NA, and M2 are translated by ribosomes of the endoplasmic reticulum (York & Fodor, 2013). HA is trafficked to the endoplasmic reticulum as HA0, which is the un-cleaved nonfunctional precursor. In order for HA to gain its membrane function activity, it is cleaved into HA1 and HA2 by a cellular protease. Human and low pathogenic avian viruses contain a monobasic cleavage site, which is processed by TMPRSS2 and potentially other type II transmembrane serine proteases (Dou et al., 2018). Hatesuer and colleagues demonstrated that TMPRSS2 knock out mice were protected from pathogenic H1N1 (Hatesuer et al., 2013). These results were also confirmed in separate studies examining the activation of HA in H1N1,H7N9 and H3N2 (Sakai et al., 2014; Tarnow et al., 2014).

Progeny influenza virus particles are assembled at the plasma membrane and are released from infected cells by budding. For this, the viral structural proteins, i.e. proteins contained within the influenza virion, are associated with the plasma membrane. HA is localized within lipids rafts of the plasma membrane due to the fatty acid modifications of its C-terminus in the Golgi apparatus (Dou et al., 2018; Rossman & Lamb, 2011; Zurcher et al., 1994). Similarly, NA is enriched at the plasma membrane due a property of its C-terminus transmembrane domain (Barman et al., 2004) and it has been proposed that M1 localizes to the budding site through associating with the short cytoplasmic tails of HA and NA (Dou et al., 2018; Rossman & Lamb, 2011). Next, Rab11 delivers vRNPs to the budding site where they bind to M1 (Dou et al., 2018; Rossman & Lamb, 2011). Once the IAV components are assembled at the budding site, HA/NA and M1 initiate the curvature of the cell plasma membrane. The scission of plasma membrane and viral envelope is achieved possibly via the viral M2 protein (Rossman & Lamb, 2011). Subsequent to budding, NA glycoproteins hydrolyze the glycocidic linkages between cell sialic acid residues and viral HA allowing release of progeny particles from infected cells (Dou et al., 2018; Rossman & Lamb, 2011).

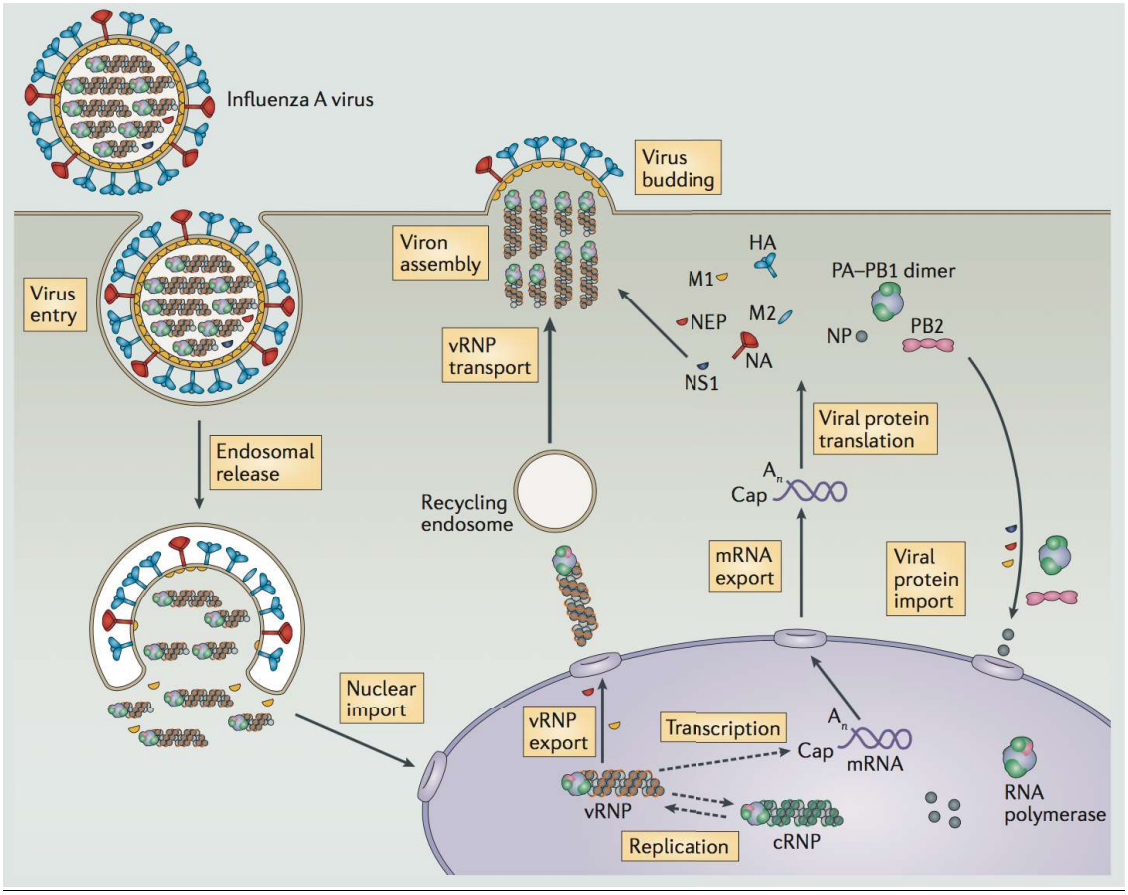

Figure 3: The replication cycle of IAV. The replication cycle of IAV is initiated upon the binding of the spike glycoprotein HA to sialic acid residues on the surface of respiratory epithelial cells. Subsequently, the virus enters the cell through endocytosis. Once inside the cell, the acidification of the endosomal environment triggers HA-driven fusion of the viral and endosomal membrane and promotes M2-dependent particle disassembly, allowing the release of vRNPs into the cytoplasm. vRNPs are imported into the nucleus where replication and transcription takes place (Dimmock et al. 2008; Dimmock and Easton 2015). Transcribed viral mRNA is exported into the cytoplasm where it is translated by host ribosomes. The newly produced viral components are assembled at the plasma membrane and progeny virions exit the host cell via budding. Finally, NA removes sialic acids from the cell surface, thereby preventing tethering of progeny virions to infected cells by binding of HA on the virion surface to sialic acids on infected cells. Image taken from (Velthuis & Fodor, 2016).

2.6 Limitations in available influenza therapy and prophylaxis

At present there are six FDA approved drugs for the treatment of influenza (FDA, 2020), which belong to three classes. The first class of inhibitors includes the amantadanes (amantadine and rimantadine), which block IAV M2 ion channels, but have no effect on the M2 ion channel of IBV. Moreover, drugs belonging to this class have a reduced efficacy owing to the emergence and global circulation of resistant viruses, whereby a single amino acid substitution in M2 is sufficient for resistance (Bright et al., 2005; Hayden et al., 1989). Notably, the CDC has reported a prevalence of 92 % amantadane resistance in H3N2 viruses during the 2005-2006 influenza seasons. (*CDC,2006*) Additionally, amantadane resistance was demonstrated against for most 2009 H1N1 viruses tested. Therefore, the use of amantadanes for influenza therapy is discouraged (*WHO | WHO Guidelines for Pharmacological Management of Pandemic (H1N1) 2009 Influenza and Other Influenza Viruses*, n.d.).

The second class of inhibitors includes the neuraminidase inhibitors (oseltamivir, and zanamivir, and peramivir), which inhibit the activity of NA by mimicking its sialic acid substrate (CDC, 2020). These inhibitors act on both influenza A and B viruses, but have also been associated with resistant strains (Gubareva et al., 2000). This was especially pronounced between the years 2002-2009 where global frequency of resistance to oseltamivir increased from 12% to 96% (Ujike et al., 2010).

The newest available influenza therapy is baloxavir marboxil (trade name Xofluza), which blocks viral transcription by inhibiting the endonuclease activity of the PA subunit in both influenza A and B viruses (O'Hanlon & Shaw, 2019). Phase two trials have demonstrated the presence of resistant IAV strains to baloxivir marboxil in at least 5 adult patients with IAV H1N1 and 15 pediatric patients with IAV H3N2 (Omoto et al., 2018). Additionally, *in vitro* studies have shown that A/WSN/33 serially passaged in the presence acquired mutations that reduced susceptibility to inhibition by 20% to 40% (O'Hanlon & Shaw, 2019).

In terms of prophylaxis, there are currently three types of licensed influenza vaccines. All of which are multivalent, meaning they are effective against multiple strains of both influenza A and B virus (Clark and Lynch 2011). The first type of influenza vaccines are inactivated split virion vaccines (IIVs). These contain purified HA protein, which can induce the production of

neutralizing antibodies and can be administered to individuals 6 months and older. The second type are live attenuated vaccines (LAIVs), which contain temperature sensitive influenza viruses that can only replicate in the nasal cavities. This vaccine type can induce the production of both serum and mucosal anti influenza virus antibodies, and additionally prompts cytotoxic T cell responses (Coelingh et al., 2014). The third type of influenza vaccines are recombinant HA vaccines, which contain HA expressed from baculovirus vectors. Unlike IIVs and LAIVs which are produced in hen eggs, these vaccines are produced from insect cells and can be safely administered to individuals allergic to eggs (Grohskopf et al., 2014). At present, influenza vaccines require annual reformulation to keep pace with circulating strains, owing to constant antigenic drift in HA and NA (Grohskopf et al., 2014). Moreover, these vaccines harbor multiple other shortcomings, which include dependence of IIVs and LAIVs on chicken eggs, lengthy timeline for vaccine production, little to no protection against pandemic influenza strains (Sridhar et al., 2015), limited immunogenicity in the elderly (Haq & McElhaney, 2014), and the need to revaccinate every year. Efforts are currently underway to develop universal influenza vaccines, which may have the ability to protect against a broad spectrum of IAV strains. Strategies for the development of such vaccines centers on targeting conserved regions of the HA stalk (Corder et al., 2020). One such strategy involves recombinant stalk specific HA whereby the globular HA is deleted so that antibodies are created targeting stalk epitopes. To date, vaccines employing this strategy remain in clinical trials (Corder et al., 2020). Another strategy involves chimeric recombinant HA, whereby the stalk from the H1 clade IAV is fused with the globular head of non-human influenza strains (Nachbagauer et al., 2021). This strategy posits that antibodies targeting both a non-human IAV and the H1 stalk are produced and can protect against pandemic influenza originating from a non human IAV strain. A collaborative vaccine between GlAxoSmithKlein (GSK) and the Icahn school of medicine at Mount Sinai has recently completed phase I clinical trials in May 2020 and has shown promise (Nachbagauer et al., 2021), however its fate remains unknown as it is not listed in GSK's development pipeline (*Pipeline | GSK*, n.d.).

In conclusion, the current strategies for influenza prophylaxis and therapy demonstrate substantial limitations owing to the high mutation rate of the virus. This highlights the urgent need to develop new broadly active strategies to combat influenza.

2.7 Defective interfering particles as an alternative for influenza therapy

Defective interfering (DI) genomic IAV segments can be produced in IAV infected cells as a result of errors committed by the viral polymerase during genome replication (Dimmock & Easton, 2014, 2015). These DI genomes often harbor a large central deletion, which usually inactivates the ORF encoded by the segment, but leaves terminal sequences required for replication, transcription and packaging intact (Dimmock & Easton, 2014, 2015). DI genomes can be packaged into progeny virions called defective interfering particles (DIPs). Studies have demonstrated the ability of DI-244, a prototypic DIP derived from A/PR/8/34 H1N1, to interfere with the replication of IAV; potentially by competing for viral and cellular resources through a process known as replication interference (Dimmock et al., 2008; Frensing et al., 2013; Pelz et al., 2021). In addition, DI-244 can induce an IFN response and subsequent ISG induction (Scott et al., 2011b). However, the relative contribution of replication interference and induction of the IFN system to DIP antiviral activity are currently unclear. Moreover, DI-244 exerts potent antiviral activity against infectious influenza in both prophylactic and therapeutic settings *invivo* (Dimmock et al., 2008; Dimmock & Easton, 2015).

2.7.1 Discovery of defective interfering particles derived from influenza viruses

The first observation of DIPs was probably documented by Friedewald and Pickels in 1944. They revealed that influenza A viruses harvested from the allantoic fluid of infected chicken eggs were associated with a sedimentation constant of about 700S. In addition, the authors observed a less homogenous group of influenza particles with an average sedimentation constant of about 460S. . The latter group of sedimenting was inoculated onto cells and found to be none infectious. It was suggested that these particles were the result of viral disintegration. Subsequent studies by Gard et al demonstrated that inactivation of influenza viruses does not alter the sedimentation constant. Consequently, the identity of these particles was not properly explored until 1954 when Preben Von Magnus reported results obtained upon passaging influenza A virus in embryonated chicken eggs at high multiplicities of infection (von Magnus, 1954). Von Magnus showed that under certain experimental conditions a high amount of non-infectious influenza A virus particles are generated, as judged by a marked discrepancy between the titer of total influenza A virus

particles as measured by the hemagglutination assay in comparison to titers of infectious particles determined by focus formation assay. Von Magnus called these non-infectious influenza A virus particles “incomplete viruses” and demonstrated their capacity to inhibit the propagation of infectious virus. Subsequenetly, Nayak et al renamed these particles as “defective interfering” and showed that they are the result of defective RNA synthesis (Nayak, 1972).

2.7.2 Molecular features of defective interfering particles

DIP formation has been observed for both DNA and RNA viruses containing single stranded and double stranded genomes (Yang et al., 2019b). Defective interfering (DI) genomes typically contain a large internal deletion, but retain terminal sequences required for genome packaging into particles and for genome amplification by viral polymerases (Dimmock & Easton, 2014). Because of these deletions, DIPs are none infectious and can only replicate in the presence of an infectious “helper” virus (Dimmock & Easton, 2014; Yang et al., 2019b). The generation of these DI genomes is the result of errors made by the viral polymerase during replication. This phenomenon is referred to as the “copy choice” mechanism whereby the viral polymerase erroneously translocates from one template to another or dissociates during transcription and reattaches downstream leading to the generation of a truncated protein (Yang et al., 2019b). To date, five types of DI genomes have been identified (Yang et al., 2019b) (Figure 4). These are: internal deletion DI genomes, copy back DI genomes, hairpin DI genomes, mosaic DI genomes, and mutation DI genomes (Yang et al., 2019a). Internal deletion DI genomes are produced when the viral polymerase skips a fragment of the template, hence generating a truncated nascent strand (Jaworski & Routh, 2017). Copy back DI genomes, also known as panhandle genomes, are generated when the viral replicase partially transcribes the template strand and then loops back to the 5’ end again, creating a panhandle shaped genome (Mura et al., 2017; Yang et al., 2019b). Hairpin DI genomes, also known as snapback genomes, are the result of the polymerase partially transcribing the template strand and then switching to the daughter nascent strand as a template (Schubert & Lazzarini, 1981; Yang et al., 2019b). Mosaic DI genomes are generated when the virus incorporates fragments from the host genome or from helper virus (Beauclair et al., 2018). Finally, the most recently identified are “mutation” DI genomes, which were discovered for

influenza A virus. Thus, a DIP harboring 37 mutations in segment seven was discovered and the mutations altered promoter regions, packaging signals, and protein sequences (Kupke et al., 2019).

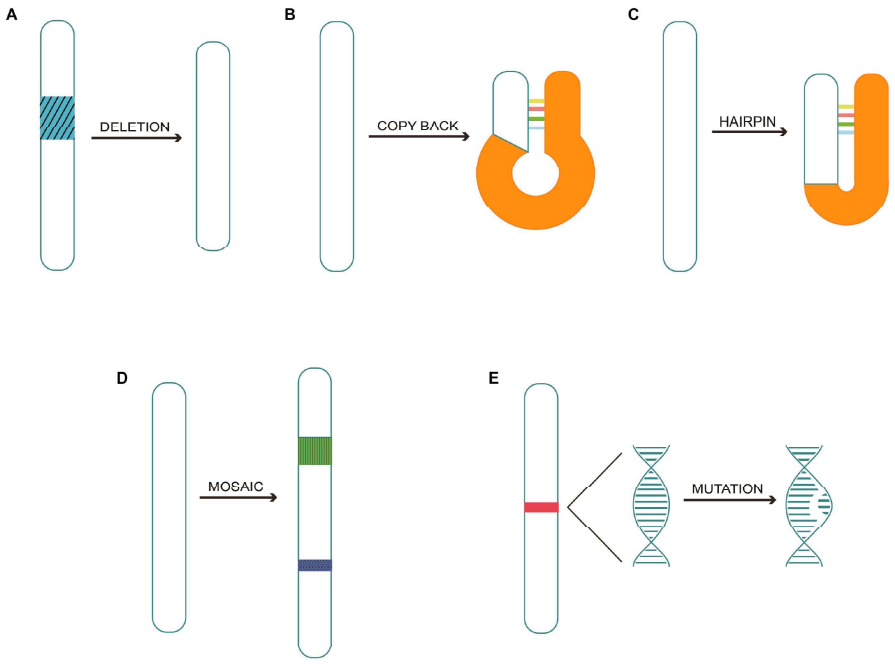

Figure 4: Types of DI genomes. A) Deletion DI genomes: created when a fragment of template strand is skipped. B) Copy-back DI genomes: created when the polymerase partially transcribes the template and switches back to transcription of the 5' end. C) Hairpin DI genomes: created when the polymerase partially transcribes the template and the loops over to transcribe the daughter nascent strand. D) Mosaic DI genomes: created by insertion of genome fragments from the helper virus or from the host. E) Mutation genomes: Novel type, generated by mutations affecting promoter regions and packaging signals (Yang et al., 2019b).

2.7.3 The current approach of influenza DIP production

Subsequent to studies demonstrating inhibition of influenza A virus propagation by DIPs in cell culture, Dimmock and colleagues showed that DIPs also inhibit viral spread in the infected host (Dimmock et al., 2008; Dimmock & Easton, 2015). Their work revolves around DI-244, which is the most potent influenza DI RNA described to date. DI-244 is a naturally occurring 395 nucleotide DI RNA derived from segment 1 of A/PR/8/34 (H1N1). DI-244 harbors a large internal deletion of 1,946 nucleotides (Dimmock et al., 2008; Dimmock & Easton, 2015). The deletion

removes most of the PB2 open reading frame (ORF) but spares 244 nucleotides at the 3' end and 151 nucleotides at the 5' end of segment 1, which are sufficient for replication and packaging (Dimmock et al., 2008) (Figure 5).

Figure 5: A schematic representation of DI244. Taken from (Dimmock & Easton, 2014).

Numerous studies have also described DI RNAs derived from genomic influenza A virus segments other than segment 1 (Dimmock & Easton, 2014; Frensing et al., 2013). However, DIPs arise most frequently from segments 1, 2, and 3, which encode subunits of the viral polymerase (Dimmock & Easton, 2014; Frensing et al., 2013). Moreover, the position of the deletion within a segment is highly variable. Studies have indicated that more than 50 DI RNAs derived from segments 1, 2, and 3 can be detected in a single DI preparation in cell culture, with deletions removing on average 80% of the ORF (Susan D. Duhaut & Dimmock, 1998).

At present, the production of DIPs requires the presence of infectious helper virus. In this approach, plasmids encoding the eight influenza A virus genomic segments (Ervin Fodor et al., 1999; Neumann et al., 1999) jointly with a DI RNA encoding plasmid are transfected into cells (Dimmock et al., 2008). This results in the "rescue" of DIPs, which refers to the successful replication of DI RNAs and their packaging into DIPs. DIP production under those conditions is possible because the helper virus provides the viral protein in trans that cannot be produced by the DI RNA (S. D. Duhaut & Dimmock, 2003). For instance, the PB2 ORF is destroyed in the context of DI-244 and DI-244 particles can be propagated in cells coinfected with helper virus that harbors the genetic information for PB2. Since DIP preparations obtained under conditions described above contain a mixture of WT and DI viruses, the infectious helper virus is inactivated by UV irradiation at a 254 nm wave length for 20 seconds (Dimmock et al., 2008; Wasik et al., 2018). This procedure is suitable for selective inactivation of helper virus since the frequency of UV-induced nucleotide lesions is proportional to segment size, meaning that that larger WT segment

will receive more lesions as compared to the corresponding DI RNA. Moreover, and more importantly, UV light induced lesions anywhere within the 13,600-nucleotide genome might abrogate viral infectivity while only lesions within the DI RNAs of DIPs will compromise DIP production in cells coinfected with helper virus and DIP (Dimmock et al., 2008; Wasik et al., 2018).

If- DI244 is to be developed for antiviral therapy, the production process described above is not suitable since the need for UV irradiation to eliminate contaminating helper virus raises safety concerns (Dimmock & Easton, 2014; Wasik et al., 2018). In addition, it is not possible to physically separate DIPs from infectious helper virus, since the presence of DI RNA instead of cognate full length RNA does not affect viral density or size (Dimmock & Easton, 2014). Hence, a novel approach is required for the production of DIPs in the absence of helper virus.

2.8 The antiviral activity of DI-244 in vivo

The potential of DI-244 as a therapeutic and prophylactic agent has been widely demonstrated within the literature (Dimmock et al., 2008; Dimmock, Dove, Meng, et al., 2012). For instance, preclinical mouse models have revealed that DI2-44 exerts both homologous and heterologous interference (Easton et al., 2011; Ulfert Rand et al., 2021; Scott et al., 2011a). Homologous interference refers to the phenomenon whereby DI-244 inhibits the propagation of the Influenza A virus from which is derived or of other closely related viruses. In contrast, heterologous interference refers to the antiviral activity of DI-244 against unrelated viruses such as Influenza B or vesicular stomatitis virus (Ziegler & Botten, 2020).

2.8.1 Homologous interference

The infection of mice with virulent strains of influenza virus results in acute respiratory disease, which progresses into pneumonia and ultimately death (Dimmock & Easton, 2014). Dimmock and colleagues readily demonstrated that a single dose of DI244 given intranasally to mice at the same time as infection with A/WSN H1N1 or one day later was enough to provide complete protection and prevent all clinical signs of infection (Dimmock et al., 2008). These studies were also confirmed in ferrets, which are better models to study human influenza, since

they do not develop pneumonia (Dimmock, Dove, Scott, et al., 2012). Moreover, it was revealed that DI-244 in ferrets exerted protection that was statistically more significant than that achieved by oseltmavir, the major influenza antiviral at present (Dimmock, Dove, Meng, et al., 2012).

2.8.2 Heterologous interference

In addition to inhibition of homologous viruses, DI-244 can also inhibit unrelated viruses such as Influenza B virus (Scott et al., 2011a), pneumovirus (member of the family Paramyxoviridae) (Easton et al., 2011), and the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (U. Rand et al., 2021). Cell culture studies analyzing the interference of D-I244 with SARS-CoV-2 and influenza B virus infection revealed that DI-244 induced interferon (IFN) responses that abrogated the replication these viruses (U. Rand et al., 2021; Scott et al., 2011a). These data highlight that DI-244 holds potential not only for the treatment of IAV infection but also for treatment of other IFN-sensitive respiratory viruses (U. Rand et al., 2021)

2.8.3 DI-244 mode of antiviral activity

It has been proposed that DI-244 exerts antiviral activity via two mechanisms. The shorter size of DI-244 RNA allows it to outcompete WT viral RNAs for resources essential for replication, such as amino acids, nucleotides, ATP, and polymerase proteins (Dimmock & Easton, 2014; Manzoni & López, 2018). This mechanism, termed replication interference, may contribute to homologous interference. Additionally, DI-244 may inhibit viral spread by activation of innate responses (Dimmock & Easton, 2015; Yang et al., 2019b), which may account for heterologous interference, as discussed above. In addition, it remains unknown so far whether DIPs may inhibit the replication of heterologous viruses by outcompeting for the consumption of replicative resources such as ATP and NTP pools.

2.8.3.1 Replication interference

In the absence of WT virus, DIPs are unable to replicate (Dimmock & Easton, 2014; Laske et al., 2016). However, upon coinfection with WT virus, DIPs can replicate due to trans-complementation of genetic defects of the DI RNAs by the corresponding WT RNAs (Figure 6)

(Dimmock & Easton, 2014; Pathak & Nagy, 2009). Importantly, amplification of DIPs results in inhibition of WT virus propagation. The underlying molecular mechanisms are incompletely defined but preferential amplification of DI RNA over full-length viral RNAs might be at the center. Thus, most DI RNAs are smaller than their WT counterparts and the viral polymerase thus synthesizes more copies of the DI RNAs per unit time as compared to the WT RNAs (Dimmock & Easton, 2014; Yang et al., 2019b; Ziegler & Botten, 2020). In turn, this leads to the reduction of essential replication resources such as viral polymerase, nucleoproteins, nucleotides, and amino acids; and ultimately prevents amplification of WT viral RNAs (Dimmock & Easton, 2014; Frensing et al., 2013; Laske et al., 2016).

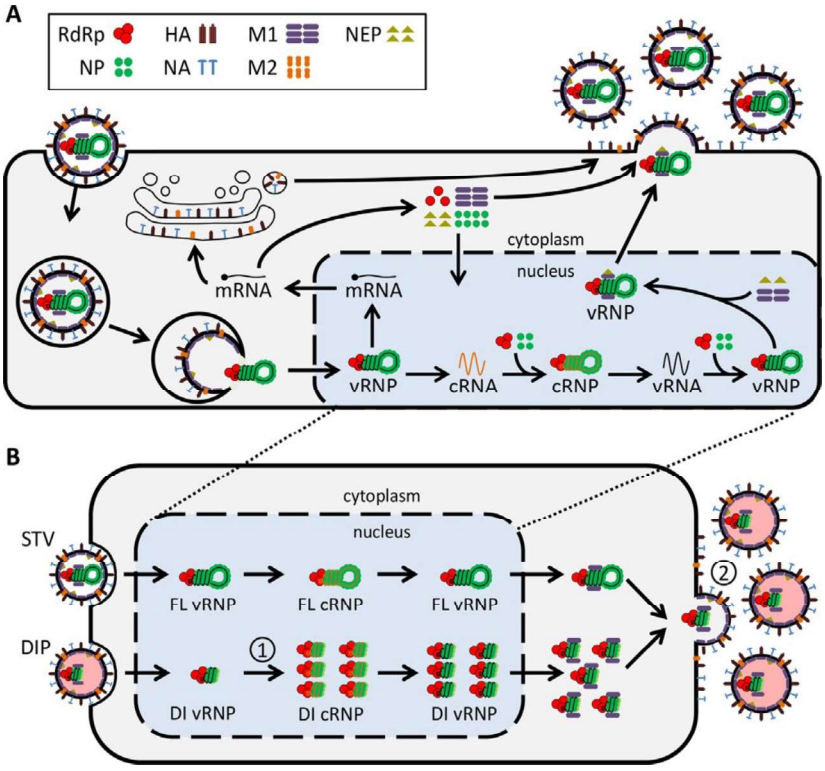

Figure 6: DI RNA-mediated interference with influenza A virus replication (Laske et al., 2016). A) The replication cycle of IAV in the absence of DI RNA. B) Coinfection of DIP and WT virus (standard virus, STV). DI cRNA has a replication advantage over full length cRNA (1), leading to the preferential amplification and packaging of DI RNAs into particles (2). RdRP: RNA

dependent-RNA polymerase, vRNP: viral ribonucleoprotein, cRNP: complimentary ribonucleoprotein.

2.8.3.2 Activation of innate immunity

DIPs can exert antiviral activity by the activation of innate immunity, in particular the IFN response (Laske et al., 2016). Influenza virus-mediated induction of the IFN response is initiated upon the binding of pathogen recognition receptors (PRRs) RIG-I and MDA-5 to pathogen associated molecular patterns (PAMPs) of the viral RNA (Chen et al., 2018; Yang et al., 2019b). Studies in mice have demonstrated that the critical PRR required for IFN stimulation upon IAV infection is RIG-I (Kato et al., 2006; Killip et al., 2015; Yang et al., 2019b). Short nucleotide sequences (12-13 nucleotides) at the 5' and 3' termini of genomic segments present in both influenza virus RNAs and DI-244 form a panhandle structure that interacts with RIG-I to expose its caspase activation and recruitment domains (CARDs). Subsequently, CARD dependent association of RIG-I with the mitochondrial antiviral signaling protein (MAVS) leads to the activation of the NF-κB and IFN regulatory factors (IRF-3 and IRF-7) to stimulate the production of IFNs and ultimately IFN-stimulated genes (ISGs) (Killip et al., 2015; Yang et al., 2019b). Several ISGs exert anti-influenza activity. A prominent example is the Mx family of GTPases, which forms oligomeric rings around the viral nucleocaspid limiting nuclear import. Other examples are viperin, which limits the release of progeny virions from infected cells, and the IFN-induced transmembrane proteins (IFITIMs), which limit viral entry (Chen et al., 2018; Killip et al., 2015).

3. Aims

Annual influenza epidemics and occasional pandemics result in significant morbidity and mortality (CDC, 2020). Due to the high mutation rate of the influenza viruses, currently available antivirals have limited efficacy and novel therapeutics are urgently needed. Defective interfering particles (DIPs) are naturally occurring byproducts of influenza virus replication and can interfere with the spread of wild type (WT) virus. At present, the generation of DIPs requires use WT virus (termed "helper virus" in this context), which raises biosafety concerns (Dimmock & Easton, 2014). Therefore, one goal of this thesis was to establish cell culture systems allowing the generation of influenza A virus (IAV)-derived DIPs in the absence of helper virus.

It is posited that DIPs interfere with the spread of WT virus by competing for cellular resources required for genome replication (termed replication interference) and by induction of the interferon (IFN) response (Dimmock & Easton, 2015). However, the relative contributions of these processes to DIP antiviral activity are poorly understood. Moreover, it is unclear whether the presence of more than one DI RNA segment in DIPs can augment antiviral activity and such particles have so far not been generated under controlled conditions. Hence, as second aim of this thesis was to elucidate the contribution of replication interference and IFN induction to DIP antiviral activity and to assess the interfering potential of DIPs harboring more than DI RNA. In summary the aims of this thesis were to:

i) Establish a system for the production of IAV DIPs in the absence of WT virus.

ii) Generate DIPs harboring more than one DI RNA and assess their antiviral activity in comparison to isogenic counterparts harboring a single DI RNA.

iii) To examine the relative contribution of replication interference and IFN induction to DIP antiviral activity.

4. Manuscripts

A system for production of defective interfering particles in the absence of infectious influenza A virus

PLoS One, 2019 Mar 1;14(3):e0212757. doi: 10.1371/journal.pone.0212757

Interferon induction and not replication interference is the major determinant of anti-influenza virus activity of defective interfering particles (DIP)

Submitted to mBio: Control number is mBio01329-21

Evidence that two instead of one defective interfering RNA in influenza A virus-derived defective interfering particles (DIPs) does not enhance antiviral activity

Submitted to Scientific Reports,
Ref: Submission ID 9283a75a-359f-47f3-8bfa-3b90a425a402

First Manuscript

A system for production of defective interfering particles in the absence of infectious influenza A virus

Najat bdeir, Prerna Arora , Sabine Gärtner , Markus Hoffmann , Udo Reichl, Stefan Pöhlmann , Michael Winkler

PLoS One, 2019 Mar 1;14(3):e0212757. doi: 10.1371/journal.pone.0212757

Contributions: I performed experiments and repeats resulting in the data presented by Figure 1 (panel A and B), Figure 3 (panel A, B, and C), Figure 4, Figure 5 (A, B, and C), Figure 6 (A and B). I analyzed and plotted data, and contributed to the writing of the manuscript (Materials and methods).

PLOS ONE

RESEARCH ARTICLE

A system for production of defective interfering particles in the absence of infectious influenza A virus

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Abstract

Influenza A virus (IAV) infection poses a serious health threat and novel antiviral strategies are needed. Defective interfering particles (DIPs) can be generated in IAV infected cells due to errors of the viral polymerase and may suppress spread of wild type (wt) virus. The antiviral activity of DIPs is exerted by a DI genomic RNA segment that usually contains a large deletion and suppresses amplification of wt segments, potentially by competing for cellular and viral resources. DI-244 is a naturally occurring prototypic segment 1-derived DI RNA in which most of the PB2 open reading frame has been deleted and which is currently developed for antiviral therapy. At present, coinfection with wt virus is required for production of DI-244 particles which raises concerns regarding biosafety and may complicate interpretation of research results. Here, we show that cocultures of 293T and MDCK cell lines stably expressing codon optimized PB2 allow production of DI-244 particles solely from plasmids and in the absence of helper virus. Moreover, we demonstrate that infectivity of these particles can be quantified using MDCK-PB2 cells. Finally, we report that the DI-244 particles produced in this novel system exert potent antiviral activity against H1N1 and H3N2 IAV but not against the unrelated vesicular stomatitis virus. This is the first report of DIP production in the absence of infectious IAV and may spur efforts to develop DIPs for antiviral therapy.

Introduction

Influenza A virus infection is responsible for annual influenza epidemics and intermittent pandemics that are associated with significant morbidity and mortality [1]. The ability of IAV to constantly change in response to immune pressure or antiviral treatment limits the effectiveness of currently used antiviral interventions. Thus, vaccines against seasonal influenza need to be annually reformulated and will provide little if any protection against pandemic influenza [1]. Moreover, the effectiveness of antivirals targeting the viral proteins M2 and neuraminidase

is compromised by the frequent emergence and transmission of resistance mutations [1, 2]. Therefore, novel approaches to combat influenza are urgently needed.

IAVs are enveloped and harbor eight segments of genomic viral RNA. Defective interfering (DI) genomic segments can be generated in IAV infected cells due to errors of the viral polymerase [3, 4]. DI segments usually harbor a large deletion which inactivates the open reading frame encoded by the segment [3, 4]. The DI segments can interfere with amplification of wild type (wt) segments, potentially by competing for viral and cellular resources required for segment replication. Moreover, DI RNAs can be packaged into progeny virions, termed defective interfering particles (DIPs), and coinfection of target cells with DIPs and IAV will result in preferential amplification of DIPs and suppression of IAV spread [3, 4]. This effect has been observed in cell culture [5–8] and in experimentally infected animals [5, 9–15] and may extend to unrelated viruses [14, 16], due to the activation of the interferon system [15, 16]. Moreover, DIP application in a therapeutic or preventive setting prevents or ameliorates influenza in animal models [3–5, 10–16]. In sum, DIPs can be considered natural antivirals produced in the context of infection with IAV and many other viruses and may provide a basis for the development of new strategies for antiviral intervention.

At present, amplification of DIPs requires coinfection of cells with DIPs and wt virus, termed standard or helper virus, which subsequently needs to be inactivated by UV light [3, 4, 17, 18]. The presence of standard virus poses a safety concern when products for animal and human use are generated and complicates the interpretation of experimental data. Plasmid systems encoding for wt and DI segments along with cell lines expressing the IAV proteins for which the genomic information has been lost upon DI RNA formation might circumvent this issue [4, 19]. However, expression of the viral polymerase subunit PB2 in trans was found to be insufficient for robust amplification of IAV variants harboring temperature sensitive mutations [20, 21] and it has been speculated that similar limitations might apply to the production of DIPs [4]. Moreover, it has been suggested that PB2 expression might be toxic to cells [4]. Therefore, it is currently unknown whether the strategy outlined above might allow for production of segment 1-derived DIPs and at present no system for generation of DIPs in the absence of standard virus has been reported.

DI-244 is a naturally occurring DI-RNA found in hen's eggs [22]. DI-244 is derived from segment 1, which encodes PB2, and harbors a 1,946 nucleotides comprising deletion [4, 22]. This deletion removes most of the PB2 ORF but leaves the 3' 244 nucleotides and 5' 151 nucleotides of segment 1 intact which are sufficient for segment replication and packaging [4, 22]. Here, we investigated whether coexpression of wt segments 2–8, PB2 protein and DI-244 RNA allows for production of DIPs. Employing a novel DI-244 variant encoding mScarlet-i, we show that DI-244-based DIPs are efficiently produced in cells expressing a codon optimized version of PB2 and that these DIPs exert potent antiviral activity.

Material and methods

Plasmids and oligonucleotides

Plasmids for rescue of the A/PR/8/34 (H1N1) strain, pHW191-pHW198, were used throughout this study and have been previously described [23]. To generate a retroviral vector encoding PB2, the PB2 open reading frame was amplified from pHW191 using primers PB2-QCX IP-5N (5– CCGCGGCCGCACCATGGAAAGAATAAAAGAACTAC–3) and PB2-3XBgl (5–GG AGATCTCGAGCTAATTGATGGCCATCCGAAT–3) and cloned into the retroviral vector pQCXIP-mcs using NotI and XhoI [24]. This self-inactivating vector allows constitutive expression of PB2 and puromycin resistance genes coupled by an internal ribosome entry site (IRES). An optimized sequence of PB2 was generated by hand to maximize sequence deviation

from PB2 and optimizing codon usage for influenza A virus and humans (S1 Fig). This sequence was synthesized and cloned by GeneArt (Regensburg, Germany) and subcloned using NotI and XhoI sites into pQCXIP-mcs. A plasmid for DI-244 rescue was generated by splice overlap PCR, using pHW191 as template and primer pairs fluA AarI-PB2-1G (5- CGA TCACCTGCTCGAGGGAGCGAAAGCAGGTC-3)/IAVseg1-DI244rep-rev (5- AATGAGGAA TCCCCTCAGTTAAGCGGCCGCTGCGGTACCAGATCTCTTCTCCTGTCTTCCTGA-3) and IAVseg1-DI244rep-for (5- TCAGGAAGACAGGAGAAGAGATCTGGTACCGCAGCGGCCGCT TAACTGAGGGGATTCCTCATT-3)/fluA AarI-PB1-2341R (5- CGATCACCTGC TCTCTAT TAGTAGAAACAAGGCATTT-3). The product of the splice overlap PCR was then purified and amplified with the segment specific primer pair fluA AarI-PB2-1G/fluA AarI-PB1-2341R and cloned into pHW2000-GGAarI, using golden gate cloning, generating pHW2000-DI244-mcs [25]. In addition, a construct containing a multiple cloning site (mcs) was generated for later insertion of reporter genes. For this, the PCR fragments were amplified using pHW191 as template and primer pairs fluA AarI-PB2-1G/IAVseg1-DI244rep-rev (5- AATGAGGAATCCCCT CAGTTAAGCGGCCGCTGCGGTACCAGATCTCTTCTCCTGTCTTCC TGA-3) and IAVseg1-DI244rep-for (5- TCAGGAAGACAGGAGAAGAGATCTGGTACCGCA GCGGCCGCTTAACTG AGGGGATTCCTCATT-3)/ fluA AarI-PB1-2341R followed by splice overlap joining and golden gate cloning. As reporter gene, mScarlet-i without internal SalI and NotI sites and fused to the porcine teschovirus-1 (PTV1) 2A sequence (GATNFSLLKQAGDVEENPGP) was cloned into the mcs as a BglII/NotI fragment. In this way, a PB2 (aa 1–41)-2A-mScarlet-i ORF was generated, which allows the detection of the presence of DI-244 via mScarlet-i fluorescence. The template for mScarlet-i, pmScarlet-i_C1, was a gift from Dorus Gadella (Addgene plasmid # 85044) [26]. The integrity of PCR-amplified, cloned sequences was verified by sequence analysis.

Cells and viruses

All cells were cultured at 37˚C and 5% CO_2. 293T human embryonic kidney cells and Vero cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS, Gibco), penicillin (Pen, 100 IU/ml) and streptomycin (Strep, 100 μg/ml). 293T cell lines stably expressing PB2 were grown in the presence of 1 μg/ml puromycin. Madin-Darby canine kidney cells (MDCK) were cultured in Glasgow's MEM (GMEM) with 10% fetal bovine serum (FBS, Gibco) and Pen/Strep. All cell lines were obtained from collaborators and were regularly checked for mycoplasma contamination. MDCK cells stably expressing PB2 or PB2opt were cultivated in the presence of 1.5 μg/ml puromycin. Influenza A viruses A/Panama/2007/99 (H3N2) [24] and A/PR/8/34 (H1N1) produced in embryonated chicken eggs were used to assess the antiviral activity of DIPs. We further employed a recombinant vesicular stomatitis virus (VSV) that expresses a dual reporter consisting of eGFP and firefly luciferase from an additional transcription unit located between the open reading frames for the viral glycoprotein and polymerase [27].

Production of retroviral vectors

The production of MLV particles for transduction of cells followed an established protocol [25, 28]. Briefly, 293T cells seeded in T25 flasks were transfected with 6 μg of retroviral vector (e.g. pQCXIP-PB2), 3 μg MLV-gag-pol plasmid and 3 μg VSV-G expression plasmid, employing the calcium phosphate transfection method. The culture medium was exchanged at 8 h after transfection. After 48 h, MLV particle-containing supernatant was harvested, cleared by passing through a 0.45 μm filter, aliquoted and then stored at -80˚C.

Transduction and selection of cell lines

For retroviral transduction, cells were seeded in 96-well plates at 5,000 (MDCK) or 10,000 (293T) cells/well in 50 μl cell culture medium. On the next day, 50 μl of supernatant containing MLV particles was added per well followed by spinoculation at 4,000 × g for 30 min for enhancement of transduction [29]. Two days after transduction, the cells were detached and transferred into 24-well plates containing cell culture medium supplemented with 1 μg/ml (293T) and 1.5 μg/ml (MDCK) puromycin. In parallel, non-transduced cells were treated similarly to control for effective cell killing by the antibiotics.

Mini-replicon assay

293T were seeded at a cell density of 2×10^5 cells/well in 12-well plates. The following day, the cells were transfected using the calcium phosphate method. The concentrations of plasmids to be transfected were largely adapted from published work [30]: 10 ng of pCAGGS plasmids encoding viral RNA polymerase proteins (PB2, PB, PA) and 100 ng of plasmid encoding NP were cotransfected with 50 ng of plasmid pPolI-Luc, which encodes the firefly luciferase reporter gene flanked by the noncoding regions of segment 8 of A/WSN/33. Empty plasmid was used to ensure that all transfections were conducted with the same total amount of plasmid DNA. For analysis of functionality of PB2 in 293T cells stably expressing this protein, transfection was carried out as described above but the plasmid encoding PB2 was omitted. As control, the plasmid encoding PB1 was omitted. The cells were washed at 6–8 h after transfection and harvested at 24 h post transfection. Luciferase activities in cell lysates were measured using the Plate Chameleon V plate reader (Hidex) and Microwin 2000 software.

Immunoblot

For analysis of PB2 expression in 293T and MDCK cells, the cells were seeded in 6-well plates, incubated for 24 h, harvested and lysed in 200 μL of Laemmli SDS-PAGE sample buffer (5% glycerine, 1% SDS, 2.5% ß-mercaptoethanol, 0.5% Bromophenol blue, 0.5 mM EDTA,0.5M Tris pH 6.8). Samples were heated to 95˚C for 10 min and separated via SDS-PAGE using 12.5% polyacrylamide gels. Proteins were then transferred onto a nitrocellulose membrane (GE health care) using a Mini-PROTEAN Tetra Cell (BioRad) powered at 110 V for 90 minutes. Membranes were blocked with 5% skimmed milk diluted in PBS-Tween and incubated with primary rabbit polyclonal antibodies against PB2 (1:1,000, GenTex, Irvine, USA) overnight at 4˚C. Subsequently, membranes were washed and incubated with anti-rabbit HRP (horseradish peroxidase)-conjugated secondary antibodies (1:10,000, Dianova) for one hour. Finally, chemiluminescent substrate HRP juice plus (P.J.K.) was added onto the membrane and bands were visualized using a ChemoCam imager (INTAS). In order to detect ß-actin, the membrane was subsequently stripped using stripping buffer (62.5 mM Tris HCl pH 6.8, 2% SDS, 100 mM ß-mercaptoethanol) for 30 min at 5˚C, washed three times with PBS-Tween, and incubated with anti ß-actin mouse (1:500 Sigma-Aldrich) overnight. The membrane was then washed and incubated with anti-mouse HRP-conjugated secondary antibody (1:10,000, Dianova) for one hour. HRP juice plus was added and bands were visualized as previously described. Quantification of PB2 and PB2opt expression was carried out using the program ImageJ (FIJI distribution) [31]. In order to normalize data, signals measured for PB2/PB2opt were divided by those measured for beta-actin.

Production of defective interfering particles

For DIP production, a coculture of 200,000 MDCK cells and 700,000 293T cells stably expressing PB2 was seeded in T25 flasks. The next day, cells were cotransfected via the calcium

phosphate method with 1 μg each of plasmids encoding DI-244-mScarlet-i and wt IAV genomic segments 2–8. Culture medium was changed at 8 h post transfection. At 48 h post transfection, cells were washed with phosphate buffered saline (PBS) without calcium and magnesium and DMEM medium supplemented with 0.2% BSA (MACS BSA), 0.5 μg/ml tosyl-phenylalany-chloromethyl-ketone (TPCK)-trypsin (Sigma), penicillin (100 IU/ml) and streptomycin (100 μg/ml) was added. As negative control, transfection of parental MDCK and 293T cells was analyzed. Supernatants were harvested from all cultures at 4, 6, 8 and 10 days post transfection, cleared by centrifugation at 4,000 rpm for 10 min to remove debris, aliquoted and stored at -80˚C. Infectivity of supernatants was analyzed by focus formation assay as described [25, 32] but using MDCK cells expressing PB2 or PB2opt as targets. In brief, MDCK-PB2/PB2opt cells seeded in 96-well plates were washed and incubated for 1 h with serial dilutions of DIP-containing supernatants. Thereafter, supernatants were removed and infection medium (GMEM with 0.2% BSA and Pen/Strep) supplemented with 0.5% methylcellulose and 0.5 μg/ml TPCK-trypsin was added. Plates were incubated for 72 h and then stained using anti IAV polyclonal antibody (Millipore).

Immunofluorescence

Images were taken on a Zeiss LSM800 equipped with a 10x/0.45 plan-apochromat objective, 488 nm and 561 nm diode lasers and ZEN imaging software (Zeiss). Fluorescent signals (red channel, 561 nm laser) were detected with GaAsP detector employing the same sensitivity for all images of a series, while bright field signals were recorded with an ESID detector (photodiode) with individually adjusted sensitivity.

Analysis of antiviral activity of DIPs

To test antiviral activity of DIPs against IAV and unrelated VSV, we performed infection experiments in the presence of DIP-containing or DIP-free supernatants and subsequently compared viral titers in the culture supernatants. For this, MDCK cells were seeded in 96-well plates at a density of 10,000 cells/well. On the next day, DIP-containing supernatants or DIP-free control supernatants were 10-fold serially diluted. Subsequently, MDCK cells were washed twice with PBS and 50 μl of the respective supernatants were mixed with 50 μl of virus and the mixture inoculated onto the MDCK cells. After a 1 h incubation, 100 μl of fresh infection medium supplemented with 0.5 μg/ml TPCK-trypsin was added and the cells were further incubated for 24 h (VSV) or 72 h (IAV) before viral titers in the culture supernatants were determined. Virus titration was performed on confluent monolayers of MDCK (IAV) or Vero (VSV) cells that were grown in 96-well plates. After aspiration of the culture medium, cells were washed twice with PBS and inoculated with 50 μl of 10-fold serial dilutions of the culture supernatants of IAV or VSV infected MDCK cells. After 1 h of incubation with IAV containing supernatants, the medium was removed and 100 μl infection medium supplemented with 1% Avicel and 0.5 μg/ml TPCK-trypsin (IAV/MDCK) was added per well. After 1h incubation with VSV-containing supernatants, 200 μl infection medium supplemented with 0.5% methylcellulose (VSV/Vero) were added on top, and the cells were further incubated for 24 h. IAV titers were quantified by antibody staining, using the focus formation assay as previously described [25, 32]. In order to quantify VSV titers, eGFP-positive foci were counted under the fluorescence microscope. All titers are given as focus forming units per ml (ffu/ml).

Results

Generation of 293T and MDCK cells stably expressing functional PB2

We sought to determine whether DI-244 particles can be amplified in the absence of standard virus if producer cells are engineered to express PB2. For this, we first used retroviral

transduction and selection antibiotics to generate 293T and MDCK cell lines stably expressing PB2. Immunoblot revealed that the cell lines obtained by selection expressed robust levels of PB2 (Fig 1A and Fig 1B). In order to analyze whether PB2 is functional in these cells, we employed a mini-replicon system, which measures the amplification of a firefly luciferase encoding IAV reporter segment upon coexpression of PB2, PB1, PA and NP [30]. We found that transfection of 293T-PB2 cells with a plasmid encoding the reporter segment alone yielded luciferase activity in the background range while cotransfection of PB2, PB1, PA and NP expression plasmids increased luciferase activity more than 1,000-fold (Fig 1C). Importantly, this increase was not observed when the PB1 plasmid was omitted while omission of the PB2 plasmid had no impact on reporter activity (Fig 1C). Thus, the PB2 protein stably expressed in 293T cells was functional. Unfortunately, similar studies in MDCK cells were not feasible due to the low transfectability of these cells.

PB2 expression allows production of infectious DI-244 in the absence of standard virus

We next investigated whether the 293T-PB2 and MDCK-PB2 cells allowed the generation of DI-244 particles, using the experimental setup depicted in Fig 2A. In order to be able to visually inspect DI-244 production and spread, we generated a DI-244 variant that encodes for mScarlet-i, a red fluorescent protein [26]. Transfection of a mixture of 293T/MDCK cells with plasmids encoding IAV wt segments 2–8 jointly with a plasmid encoding DI-244-mScarlet-i resulted in occasional and moderate red fluorescence (Fig 3A). In contrast, frequent and prominent red fluorescence was observed in 293T-PB2/MDCK-PB2 cocultures (Fig 3A), indicating that the stably expressed PB2 promoted amplification of the DI-244-mScarlet-i DI RNA.

In order to examine whether amplification of the DI-244-mScarlet-i DI RNA resulted in the production of infectious DIPs, the supernatants of the transfected 293T-PB2/MDCK-PB2 cells were inoculated onto MDCK-PB2 cells (Fig 2B). As controls, the supernatants were also added to MDCK wt cells. Inoculation of MDCK-PB2 cells with supernatants from 293T-PB2/MDCK-PB2 cells resulted in infection of the target cells, as determined by expression of mScarlet-i (Fig 3B). The number of mScarlet-i-positive cells was concentration dependent and supernatants taken at 6 days post transfection from DIP producing cells contained the highest amount of infectivity (Fig 3B). Finally, no cells with prominent red fluorescence were detected under control conditions, indicating that DIPs were only infectious for MDCK-PB2 but not MDCK wt cells.

We next asked whether DI-244 production could be quantified by focus formation assay, which is based on detection of IAV antigens by antibody staining and is frequently employed to measure IAV infectivity. Moreover, we examined whether results obtained in the focus formation assay would match those obtained upon counting of foci based upon red fluorescence. Foci were observed in MDCK-PB2 but not in MDCK control cells, confirming that DIP infectivity requires PB2 expression in target cells. Quantification of DIP infectivity by focus formation assay revealed that maximum titers of roughly 1 x 10^3 DIPs per ml were obtained and counting red fluorescent foci yielded roughly comparable results (Fig 3B and Fig 3C). Thus, expression of PB2 is sufficient for DI-244 production in the absence of helper virus but production efficiency is moderate.

Codon optimization of PB2 allows increased PB2 expression and DIP production

DIP titers of 1 x 10^3 particles per ml are low and may limit experimentation. Therefore, we next asked whether alteration of codon usage for PB2 expression might increase PB2

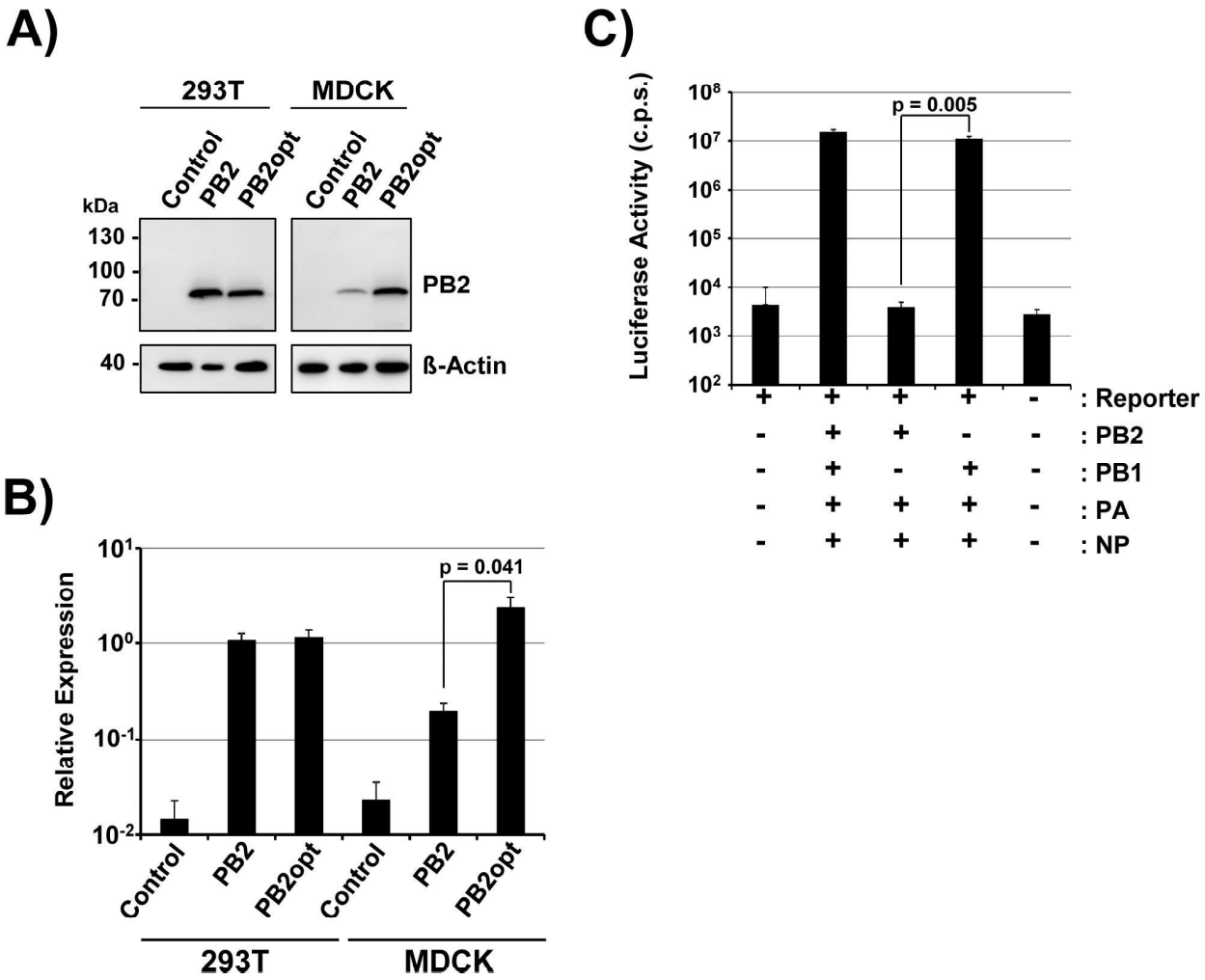

Fig 1. Stable expression of active PB2 protein in 293T and MDCK cells. (A) 293T and MDCK cells engineered to stably express PB2 or codon-optimized PB2 (PB2opt) were analyzed for PB2 expression by immunoblot using anti-PB2 antibody. Detection of beta-actin expression served as loading control. Similar results were obtained in four separate experiments. (B) The average of five experiments conducted as described for panel A and quantified via the ImageJ program is shown. Signals measured for PB2 or PB2opt were normalized against those measured for beta-actin. Error bars indicate standard error of the mean (SEM). Two tailed paired students t-test was used to assess statistical significance. (C) 293T cells stably expressing PB2 were cotransfected with plasmids encoding an IAV luciferase reporter segment and the indicated IAV proteins. Luciferase activities in cell lysates were determined at 24 h post transfection. The results of a representative experiment carried out with triplicate samples are shown. Error bars indicate standard deviation. Two tailed paired students t-test was used to assess statistical significance. Similar results were obtained in three separate experiments. C.p.s., counts per second.

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expression efficiency and DIP production. For this, we modified the codons in the PB2 expression plasmid (S1 Fig) to reflect codon preferences of human genes and IAV. As a second criterion for codon choice, we opted for maximal sequence difference between the A/PR/8/34-based sequence previously used for PB2 expression and the newly generated, optimized PB2 sequence (PB2opt), in order to prevent potential recombination events. 293T and MDCK cells were engineered to stably express PB2opt and immunoblot revealed that expression levels of PB2opt in MDCK but not 293T cells were higher than those obtained upon expression of non-codon-optimized PB2 (Fig 1A and Fig 1B). Moreover, growth of PB2opt cells was comparable to that of control cells and PB2opt expression was readily detectable after multiple passages, suggesting that expression was not associated with overt cytotoxicity. Finally, analysis of

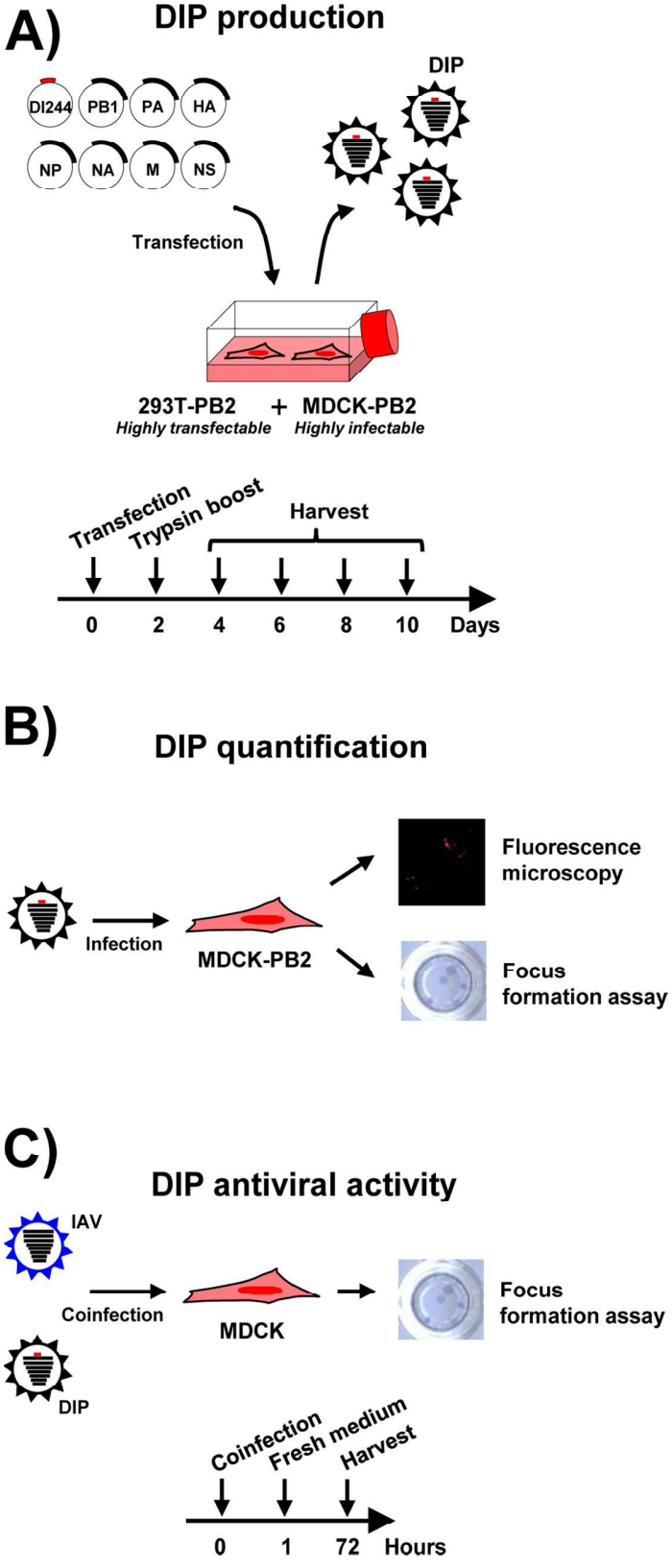
A)
DIP production
DIP
DI244
PB1
PA
HA
NP
NA
M
NS
Transfection
293T-PB2 + MDCK-PB2
Highly transfectable
Highly infectable
Transfection
Trypsin boost
Harvest
0
2
4
6
8
10
Days
B)
DIP quantification
Infection
MDCK-PB2
Fluorescence microscopy
Focus formation assay
C)
DIP antiviral activity
IAV
Coinfection
MDCK
DIP
Focus formation assay
Coinfection
Fresh medium
Harvest
0
1
72
Hours

Fig 2. Schematic representation of the work-flow employed for DIP production and characterization. (A) For production of DIPs (DI-244-mScarlet-i), a coculture of 293T-PB2 and MDCK-PB2 cells was cotransfected with plasmids harboring DI-244-mScarlet-i and the wt IAV genomic segments two to eight. Subsequently, trypsin was added for HA activation and supernatants were harvested at the indicated time points. (B) For quantification of DIP production, MDCK-PB2 cells were inoculated with DIP containing supernatants and the number of red cells was counted or the number of foci was determined using focus formation assay. (C) For analysis of antiviral activity of DIPs, MDCK cells were coinfected with IAV wt and DIPs followed by focus formation assay.

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293T-PB2opt cells in the mini-replicon assay showed that PB2opt supported IAV segment replication (Fig 4).

Next, we examined whether PB2opt supports DIP production with higher efficiency than unmodified PB2. Efficient DI-244-mScarlet-i DI RNA amplification was observed in transfected PB2opt cells (not shown) and supernatants obtained from these cells were highly infectious for MDCK-PB2opt cells even when diluted 1:1,000 (Fig 5A). In contrast, the supernatants were not infectious for MDCK cells (Fig 5A). Moreover, a direct comparison of 293T-PB2/MDCK-PB2 and 293T-PB2opt/MDCK-PB2opt cells for production of infectious DIPs and for DIP amplification upon infection revealed that the PB2opt cells were more efficient. Thus, more red fluorescent cells were observed when supernatants from PB2 expressing cells were added to MDCK-PB2opt as compared to MDCK-PB2 cells (Fig 5B). Similarly, supernatants from PB2opt cells were more infectious for target MDCK-PB2opt cells as compared to MDCK-PB2 cells. In keeping with this observation, quantification of production of infectious DIPs by focus formation assay and counting of red fluorescent cells revealed that at least 80% of foci (identified by antibody staining) were positive for mScarlet-i, as expected, and that PB2opt cells produced up to 4 x 10^6 infectious DIPs per ml and thereby exceeded titers obtained with PB2 cells (2,5 x 10^3) by ~1,500-fold (Fig 5C and Fig 5D).

DI-244 produced in the absence of standard virus exerts antiviral activity

DI-244 can inhibit spread of diverse IAVs and, likely via induction of interferon (IFN), may also inhibit spread of unrelated viruses [3, 4]. In order to investigate the antiviral activity of DI-244-mScarlet-i, we first analyzed whether DI-244-mScarlet-i produced in PB2opt cells interfered with the spread of a homologous IAV, A/PR/8/34, in MDCK cells (Fig 2C). For this, MDCK cells were coinfected with the indicated dilutions of DI-244 containing supernatants and A/PR/8/34 at an MOI of 0.1, 0.01 and 0.001 (Fig 6A). This resulted in IAV/DIP ratios of approximately 1:10 (undiluted DIP containing supernatants, IAV at MOI 0.1), 1:100 (undiluted DIP containing supernatants, IAV at MOI 0.01) and 1:1,000 (undiluted DIP containing supernatants, IAV at MOI 0.001), respectively. The supernatants from 293T/MDCK wt cells transfected with plasmids for DI-244 production were used as negative control. The control supernatants did not appreciably interfere with A/PR/8/34 infection while supernatants from PB2opt cells efficiently blocked IAV infection in a concentration dependent manner, with highest antiviral activity observed at an IAV/DIP ratio of 1:1,000 (Fig 6A). Specifically, infection efficiency relative to untreated virus (set as 100%) was 1 ± 0.5% in the presence of DIP containing supernatants at a dilution of 10^0 and 93 ± 13% in the presence of control supernatants (average of six independent experiments). Moreover, DI-244 containing supernatants also inhibited infection by A/Panama/2007/99 (H3N2) in a concentration dependent manner (Fig 6B), in keeping with the concept that DI-244 exerts broad anti-IAV activity [3, 4]. Finally, DI-244 containing supernatants did not inhibit VSV infection (Fig 6C), indicating that DI-244 neither interfered with VSV genome replication nor altered viral control by a potential IFN response in MDCK cells. These results show that DI-244 produced in PB2opt expressing cells exerts potent anti-IAV activity.

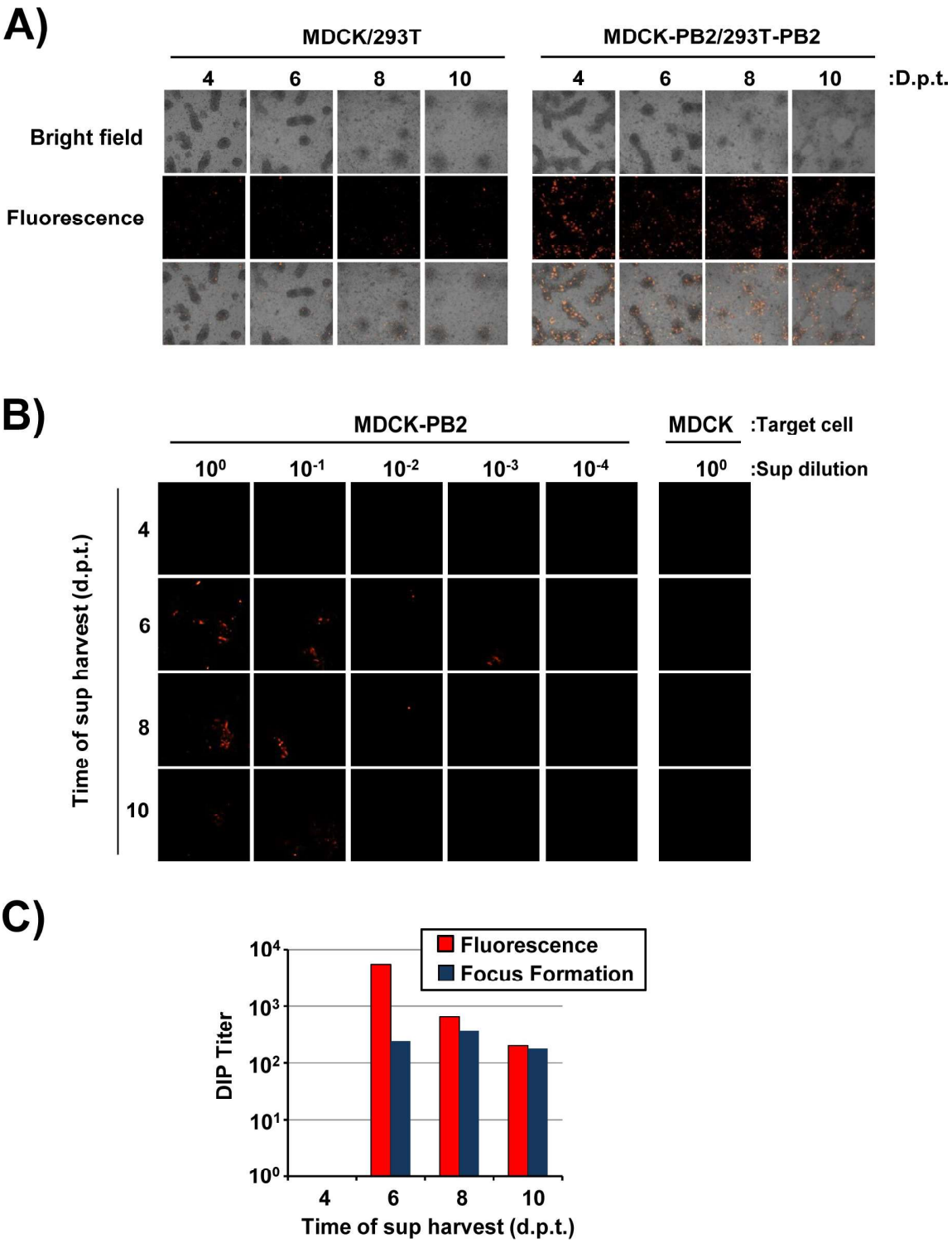

Fig 3. Production of DI-244 particles in PB2 expressing cell lines. (A) Cocultures of 293T-PB2/MDCK-PB2 cells were transfected with plasmids encoding wt IAV segments 2–8 and DI-244-mScarlet-i. The presence of red fluorescence at 4, 6, 8 and 10 days post transfection was analyzed using confocal microscopy. (B) MDCK control and MDCK-PB2 cells were infected with serially diluted DI-244 containing supernatants harvested at the indicated time points and produced as described in panel A followed by removal of supernatants and addition of methyl cellulose overlay. Thereafter, the presence of red fluorescent cells was analyzed at 72 h post infection using confocal

microscopy. (C) The number of infected cells (as determined by red fluorescence) in panel B was quantified. In parallel, infection of cells was analyzed by focus formation assay and the number of foci quantified. The results of a representative experiment are shown in panels A-C and were confirmed in two separate experiments.

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Discussion

The generation of DIPs in IAV infected cells has been recognized by von Magnus several decades ago [33] and DIPs hold promise as novel antiviral agents [3, 4]. However, exploitation of DIPs for antiviral therapy requires efficient production systems that do not depend on the presence of standard virus. Here, we report a DI-244 variant encoding a fluorescent protein that permits monitoring of DIP production. Moreover, we demonstrate that cells expressing

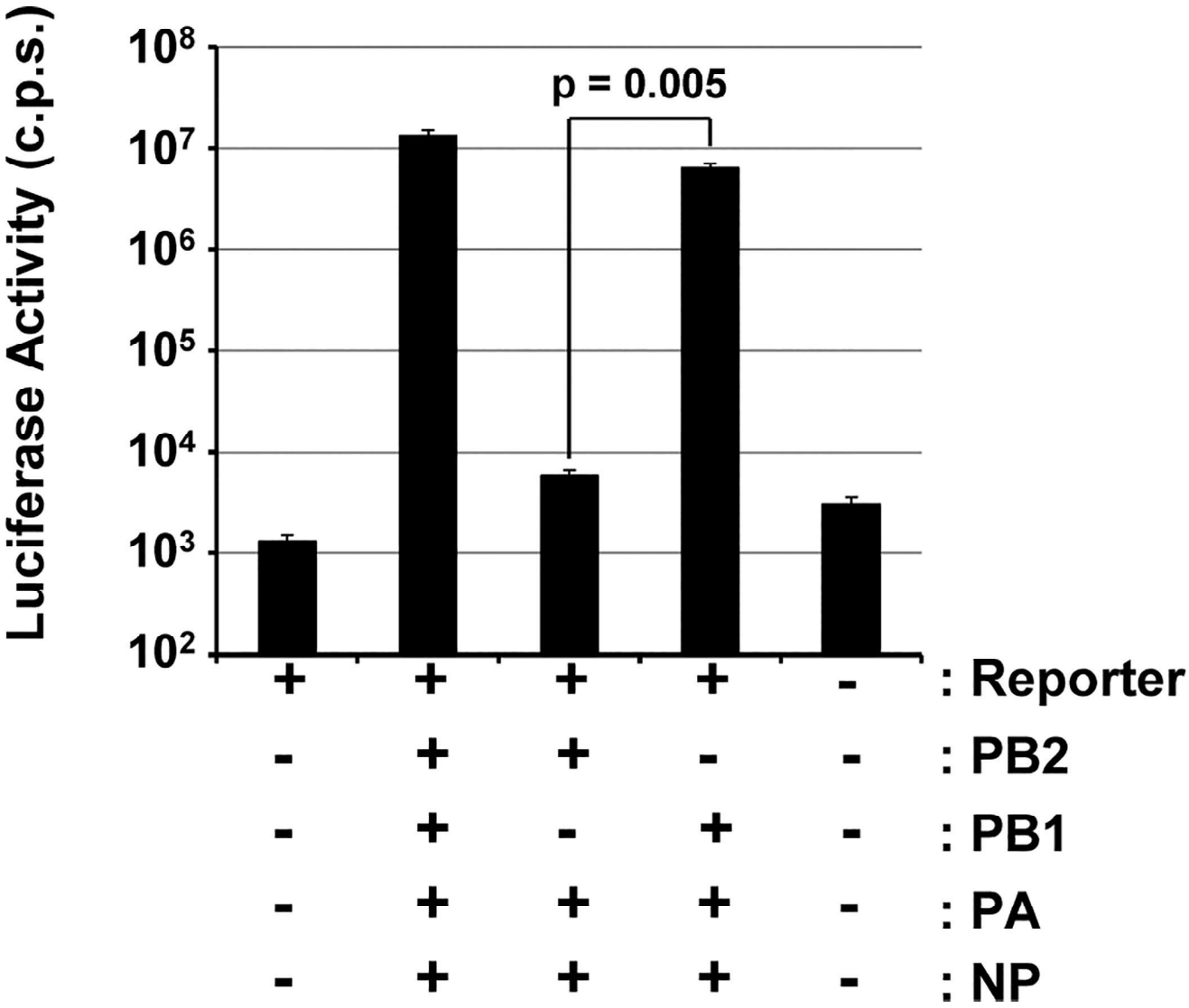

Fig 4. PB2opt stably expressed in 293T cells is active. 293T cells stably expressing PB2opt were cotransfected with plasmids encoding an IAV luciferase reporter segment and the indicated IAV proteins. Luciferase activities in cell lysates were determined at 24 h post transfection. The results of a representative experiment carried out with triplicate samples are shown. Error bars indicate standard deviation. Similar results were obtained in three separate experiments. Two tailed paired students t-test was used to assess statistical significance. C.p.s., counts per second.

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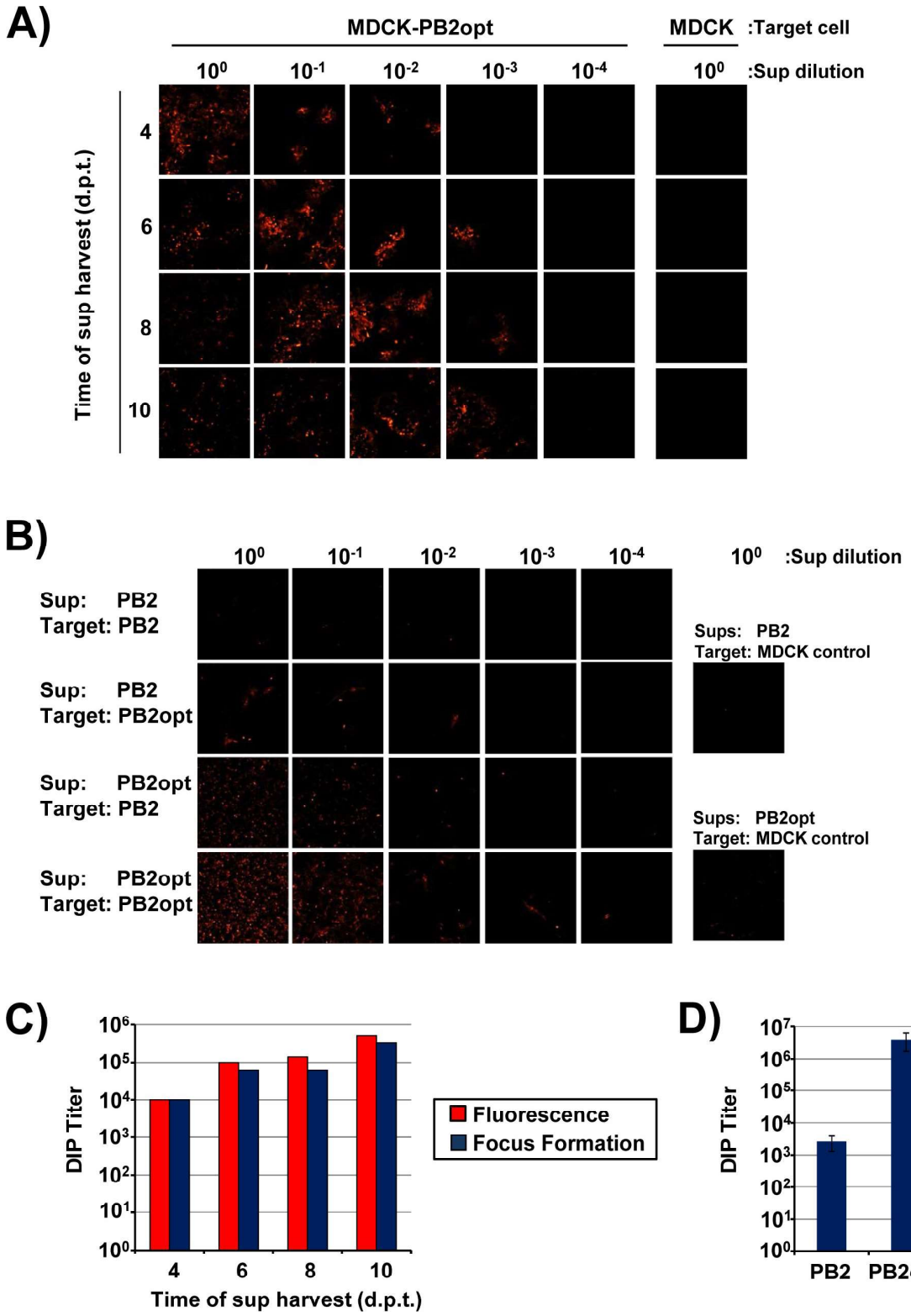
A)
MDCK-PB2opt
MDCK :Target cell
10^0 10^-1 10^-2 10^-3 10^-4
10^0 :Sup dilution
Time of sup harvest (d.p.t.)
4
6
8
10
B)
10^0 10^-1 10^-2 10^-3 10^-4
10^0 :Sup dilution
Sup: PB2
Target: PB2
Sup: PB2
Target: PB2opt
Sup: PB2opt
Target: PB2
Sup: PB2opt
Target: PB2opt
Sups: PB2
Target: MDCK control
Sups: PB2opt
Target: MDCK control
C)
DIP Titer
Fluorescence
Focus Formation
Time of sup harvest (d.p.t.)
D)
DIP Titer
PB2
PB2opt

Fig 5. Codon optimization of PB2 results in increased DIP production. (A) DI-244 containing supernatants were produced in 293T-PB2opt/MDCK-PB2opt cells and harvested at the indicated time points as described for panel A of Fig 2. Subsequently, MDCK control and MDCK-PB2opt cells were infected with serially diluted DI-244 containing supernatants followed by removal of supernatants and addition of methyl cellulose overlay. Thereafter, the presence of red fluorescent cells was analyzed at 72 h post infection using confocal microscopy. (B) DI-244 supernatants produced in cells expressing PB2 or PB2opt were inoculated onto the indicated MDCK target cells as described for panel B and the presence of red fluorescent cells was analyzed at 72 h post infection using confocal microscopy. (C) The number of infected cells (as determined by red fluorescence) in panel A was quantified. In parallel, infection of cells was analyzed by focus formation assay and the number of foci was quantified. The results of a representative experiment are shown in panels A-C and were confirmed in two separate experiments. (D) The average of three (PB2) and six (PB2opt) independent experiments conducted as described for panel A is shown. Supernatants obtained at six days post transfection were analyzed, infection of cells was quantified by focus formation assay. Error bars indicate SEM.

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PB2 allow generation of infectious DI-244 particles solely from plasmids and in the absence of standard virus. Finally, our study shows that DIPs produced in this system suppress spread of different IAV subtypes but not VSV in cell culture.

DI-244 particles and other DIPs have so far been amplified in cell culture or hen's eggs in the presence of standard virus [3, 4, 17]. In addition, production of DI-244 particles from a plasmid system has been described [34, 35]. This approach relies on the transfection of plasmids for production of infectious IAV in conjunction with a plasmid containing the DI-244 segment and results in the co-production of DIPs and standard virus [34, 35]. Before DIP preparations produced in these systems can be used for experimentation, the remaining standard virus needs to be inactivated by UV light [18]. This approach builds on the preferential inactivation of standard virus relative to DIPs. Thus, a mutation in a gene essential for viral spread will abrogate infectivity of standard virus but may have no effect on DIP infectivity since the missing proteins will be complemented in trans in cells coinfected with DIPs and standard virus. However, controlling the efficiency of UV inactivation of standard virus is technically challenging. Moreover, the effect of UV light on DIP infectivity is difficult to determine and both issues may complicate large scale production of DIPs as well as interpretation of experimental data and animal trials. Thus, establishment of novel cell culture systems for DIP production in the absence of standard virus is an important task.

Our results show that cell lines expressing PB2 allow production and quantification of DI-244 particles solely from plasmids and in the absence of standard virus. This finding was not expected given that several reports indicate that PB2 expression alone is insufficient to allow robust spread of IAV variants with temperature sensitive mutations in the PB2 gene at non-permissive temperatures [20, 21]. Moreover, it has been suggested that PB2 expression might be associated with unwanted cytotoxic effects [4]. The present study suggests that up to 4 x 10^6 DI-244 particles/ml can be produced in cells expressing codon optimized PB2, which roughly translates into production of 10 infectious DIPs per cell, and it can be speculated that efficiency of DIP production can be further increased by employing cell lines stably coexpressing PB1, PB2 and PA. Occasionally, weak fluorescence has been observed in DIP inoculated control cells. This is most likely attributable to low levels of DI-244 mRNA expression facilitated by PB2 protein associated with DI-244 vRNA present in the infecting DIPs. In contrast, no evidence for production of infectious IAV due to recombination between the DI-244 RNA and the RNA encoding for PB2 was obtained, as judged by bright field microscopy, immunofluorescence, focus formation assay and RT-PCR analysis, indicating that the DIP production system reported here is safe.

Quantification of DIP production so far relied on quantitative RT-PCR and hemagglutination assay [4, 8, 17], which do not provide information on particle infectivity. This limitation has been overcome by the present study which demonstrates that infectivity of DI-244 particles can be quantified using a standard technique, focus formation assay. The availability of this

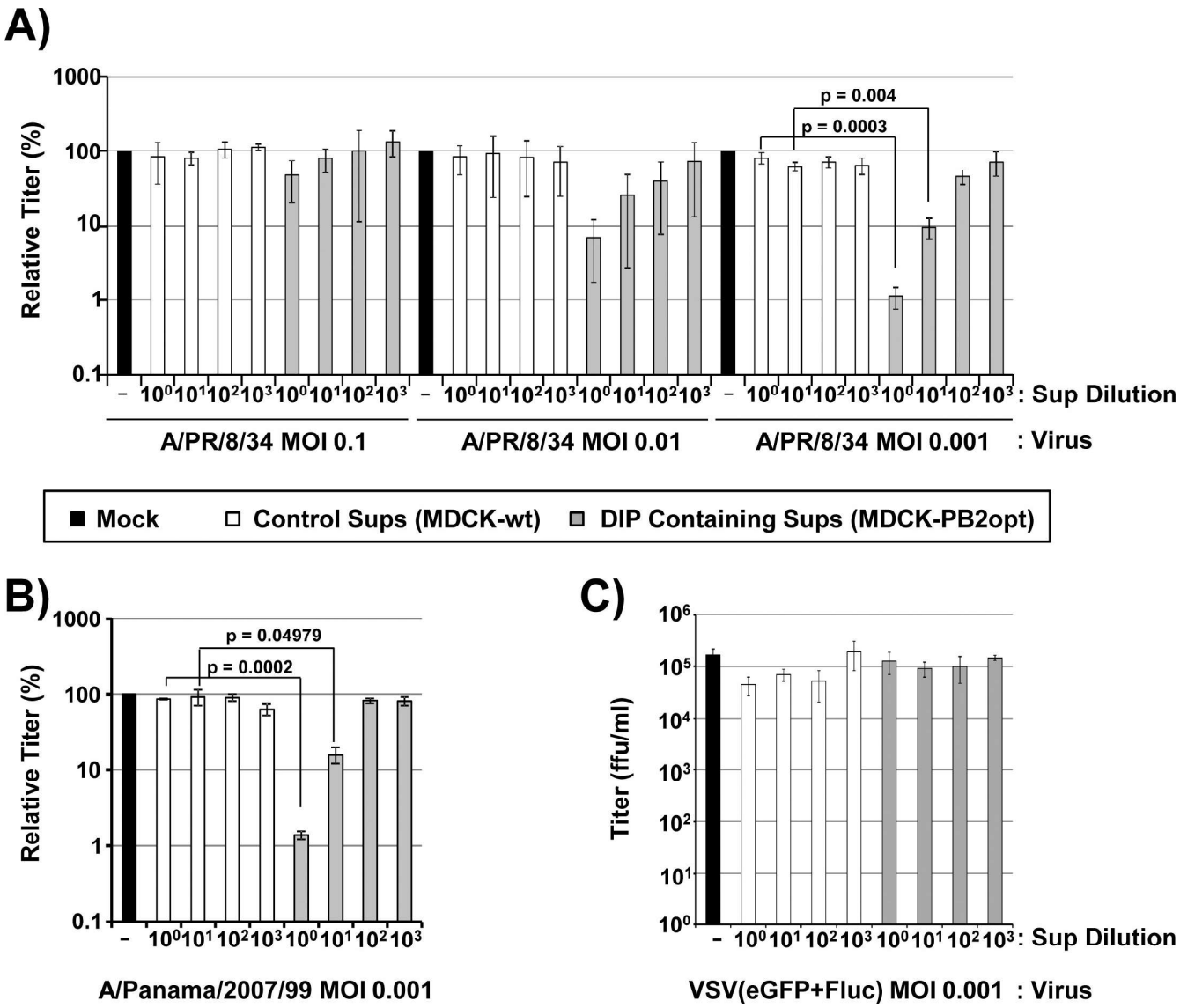

Fig 6. DI-244 produced in PB2opt expressing cell lines exerts anti-IAV activity. (A) Undiluted or 10-fold serially diluted DIP supernatants harvested from transfected 293T-PB2opt/MDCK-PB2opt cells or supernatants from transfected 293T/MDCK control cells were co-inoculated with A/PR/8/34 (H1N1) onto MDCK cells. Fresh medium was added at 1 h post infection and infectivity present in supernatants harvested at 72 h post infection was analyzed by focus formation assay. The average of three (MOI 0.1, MOI 0.01) and six (MOI 0.001), respectively, independent experiments is shown. Infection in the absence of supernatants was set as 100%. Error bars indicate standard error of the mean (SEM). Two tailed paired students t-test was used to assess statistical significance. (B) The experiment was carried out as described for panel A but A/Panama/2007/99 (H3N2) was used for infection. The average of three independent experiments is shown. Infection in the absence of supernatants was set as 100%. Error bars indicate SEM. Two tailed paired students t-test was used to assess statistical significance. (C) The experiment was carried out as for panel A but cells were infected with GFP-encoding VSV and supernatants were harvested for titration at 24 h post infection. The results of a single representative experiment conducted with triplicate samples are shown and were confirmed in two separate experiments.

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method should help comparing results obtained with different DI-244 preparations or other segment 1 DIPs and should thus advance the development of DIPs as antiviral agents. In this context, it is noteworthy that a IAV/DIP ratio of 1:1,000 resulted in the most prominent antiviral activity in our hands and a very similar ratio, 1:3,400 (as determined by estimations based on quantitative RT-PCR (DIP) and infectious units (IAV)), was previously reported to be

minimally required to protect mice from severe influenza [4]. Thus, our study confirms and extends published work indicating that DIPs have to be provided in vast excess to exert antiviral activity. Whether sufficient numbers of DIPs can be delivered to the human respiratory tract and remain stable to provide protection against influenza for a prolonged time remains to be determined. In this context, one can speculate that an IAV:DIP ratio of less than 1:1,000 might be sufficient for antiviral activity in humans, since DIPs might exert direct antiviral activity by inhibiting IAV genome replication and induce the IFN system. Moreover, DIPs were reported to have a long residence time in the respiratory tract of mice and DIP-treated animals were found to still be protected at one week after treatment [4, 35]. Thus, DIP stability in the respiratory tract might not pose a major hurdle to the use of DIPs for influenza prevention and therapy in humans. Finally, it should be stated that reassortment of DIPs with IAV in coinfected cells is likely to occur. However, if DIPs based on the low pathogenic A/PR/8/34 or related viruses are used (like in the present study), such reassortment events should not result in viruses with increased transmissibility or virulence as compared to the wt virus.

It is believed that DI-244 can interfere with spread of diverse IAV in cell culture due to genome competition [3, 4]. Indeed, DI-244 produced in PB2opt cells exerted comparable antiviral activity against H1N1 and H3N2 IAV (no statistically significant differences), in keeping with H3N2 polymerase complexes being fully functional on H1N1 genomic segments [36]. This matches data published for DI-244 generated by use of standard virus [35] and demonstrates that DIPs produced in PB2 expressing cells are fully functional, although the activity of purified DIPs remains to be examined. DI-244 can also interfere with the spread of influenza B virus (IBV) and unrelated respiratory viruses in the infected host and this is thought to be due to induction of innate immune responses, particularly the IFN response [14, 16]. In contrast, DIP-mediated inhibition of IBV infection in cell culture is not observed, due to absence of genome competition [13, 14]. The absence of antiviral activity of DIPs against VSV confirms lack of genome competition. Moreover, it suggests that DIPs might not have modulated a potential IFN response in MDCK cells, although it should be noted that such a response might have been impeded due to the presence of trypsin in the culture medium [37].

Collectively, we report, to our knowledge, the first experimental system for production of DIPs without standard virus and for quantification of DIP infectivity, which should promote efforts to develop DIPs for antiviral therapy.

Supporting information

S1 Fig. Alignment of PB2 and codon optimized PB2. The nucleotide sequences of PB2-wt (PB2) and codon optimized PB2 (PB2opt) were aligned using the Clustal W algorithm of AlignX (Vector NTI). Divergent nucleotides are marked in black.
(PDF)

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Writing – original draft: Stefan Pöhlmann.

Writing – review & editing: Najat Bdeir, Prerna Arora, Markus Hoffmann, Udo Reichl, Michael Winkler.

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Second Manuscript

Interferon induction and not replication interference is the major determinant of anti-influenza virus activity of defective interfering particles (DIP)

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Individual contributions: I performed experiments and repeats resulting in the data presented by Figure 1 (panel A, C, F, and D), and in Figure 2 (panel E). I analyzed and plotted data, and contributed to the writing of the manuscript (Materials and methods).

Interferon induction and not replication interference mainly determines anti-influenza virus activity of defective interfering particles

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Abstract

Defective interfering (DI) RNAs arise during influenza virus replication, can be packaged into particles (DIPs) and suppress spread of wildtype (WT) virus. However, the molecular signatures of DI RNAs and the mechanism underlying antiviral activity are incompletely understood. Here, we show that any central deletion is sufficient to convert a viral RNA into a DI RNA and that antiviral activity of DIPs is inversely correlated with DI RNA length when induction of the interferon (IFN) system is disfavored. When induction of the IFN system was allowed, it was found to be the major contributor to DIP antiviral activity. Finally, while both DIPs and influenza virus triggered expression of IFN-stimulated genes (ISG) only virus stimulated robust expression of IFN. These results suggest a key role of innate immune activation in DIP antiviral activity and point towards previously unappreciated differences in DIP- and influenza virus-mediated activation of the effector functions of the IFN system.

Importance

Defective interfering (DI) RNAs naturally arise during RNA virus infection. They can be packaged into defective interfering particles (DIPs) and exert antiviral activity by suppressing viral genome replication and inducing the interferon (IFN) system. However, inhibition of influenza virus infection by DI RNAs has been incompletely understood. Here, we show that induction of the IFN system and not suppression of genome replication is the major determinant of DIP antiviral activity. Moreover, we demonstrate that DIPs induce IFN-stimulated genes (ISG) but not IFN with high efficiency. Our results reveal unexpected major differences in influenza virus and DIP activation of the IFN system, a key barrier against viral infection, and provide insights into how to design DIPs for antiviral therapy.

Introduction

The annually recurring influenza epidemics are a major source of global morbidity and mortality and intermittent pandemics can have even more severe consequences (1). Influenza therapy and vaccination are available but suffer from serious shortcomings (1). The success of influenza therapy with currently licensed drugs, which target the viral proteins neuraminidase (NA), matrix protein 2 (M2) or polymerase acidic protein (PA), can be compromised by resistance development (2). Moreover, vaccines against epidemic influenza need to be annually adjusted to the viruses expected to circulate during the next influenza season and offer little or no protection against emerging pandemic viruses (1). Thus, the identification of novel targets and strategies for antiviral intervention is an important task.

Influenza viruses contain a segmented, negative sense RNA genome. The genomic segments are replicated by the viral polymerase, which consists of the subunits polymerase basic proteins 1 (PB1) and 2 (PB2) as well as PA (3). The error rate of the viral polymerase is high and can result in the synthesis of genomic segments that harbor deletions (4-10). These defective segments may interfere with the amplification of wt segments and are thus termed defective interfering (DI) RNAs (4-6, 8-10). Packaging of DI RNAs into viral particles results in the formation of DI particles (DIPs), which suppress wt influenza virus spread (4, 5). It has been proposed that DIPs suppress influenza virus infection by interfering with genome replication (a process subsequently termed replication interference) and by inducing an interferon (IFN) response (4, 5, 11-17). However, this concept has not been systematically investigated and the relative contribution of replication interference and IFN induction to DIP antiviral activity is unknown.

We recently developed a cell culture system that allows production of genetically defined DIPs based on reverse genetics and a cell line complementing defects in influenza A virus (IAV)

genomic segment 1 (18). Here, we used this system as well as a mini-replicon assay (19) to analyze the contribution of replication interference and IFN induction to the antiviral activity of DIPs. We report that in the mini-replicon assay any central deletion in segment 1, 2 or 3 converts these segments into DI RNAs, which suppress replication of diverse target segments. Inhibitory activity of these DI RNAs was inversely correlated with segment length and a similar correlation was seen in the context of DIP and IAV infection under conditions which disfavored IAV inhibition by DIP-dependent induction of the IFN system. If induction of the IFN system was allowed before IAV infection, it largely accounted for DIP antiviral activity. Finally, DIPs robustly induced IFN-stimulated gene (ISG) but not IFN expression, indicating that IAV and DIPs may differ in the activation of the effector functions of the IFN system. Our results suggest that although interference with genome replication contributes to DIP antiviral activity, the induction of the IFN system is the major determinant of suppression of virus infection by DIPs.

120 # Results

121

122 **DI-244 inhibits segment replication in a mini-replicon assay and inhibition is independent**
123 **of the truncated PB2 open reading frame**

124 We first investigated whether a previously described IAV mini-replicon assay (19) is suitable to
125 detect inhibition of IAV genome replication by a prototypic segment 1-derived DI RNA, DI-244
126 (20). This assay is based on a firefly luciferase open reading frame flanked by the 5' and 3' ends
127 of IAV segment 8, which is amplified in cells upon coexpression of the constituents of the viral
128 polymerase complex PB1, PB2, and PA, and the viral nucleoprotein (NP) (19). Transfection of
129 293T cells with plasmids encoding the mini-genome reporter segment and the IAV proteins
130 mentioned above resulted in luciferase activities in cell lysates that were approximately 1,000-
131 fold higher than those measured in cells transfected with the reporter alone or transfected with the
132 full set of plasmids except the PB2 encoding plasmid (Figure 1A). Moreover, cotransfection of
133 two different amounts of DI-244 encoding plasmid resulted in a concentration dependent
134 decrease in luciferase activity, indicating that DI-244 inhibited replication of the reporter segment
135 (Figure 1A). This inhibitory activity was also observed when the PB2 start codon in DI-244 and
136 two subsequent ATGs (positions 11 and 28) were mutated (Figure 1B). In contrast, transfection
137 of expression plasmid pCAGGS containing the truncated PB2 ORF of DI-244 or empty pCAGGS
138 did not reduce luciferase signals (Figure 1B). These results indicate that inhibition of segment
139 replication by DI-244 can be visualized in the mini-replicon assay and does not require
140 expression of truncated PB2.

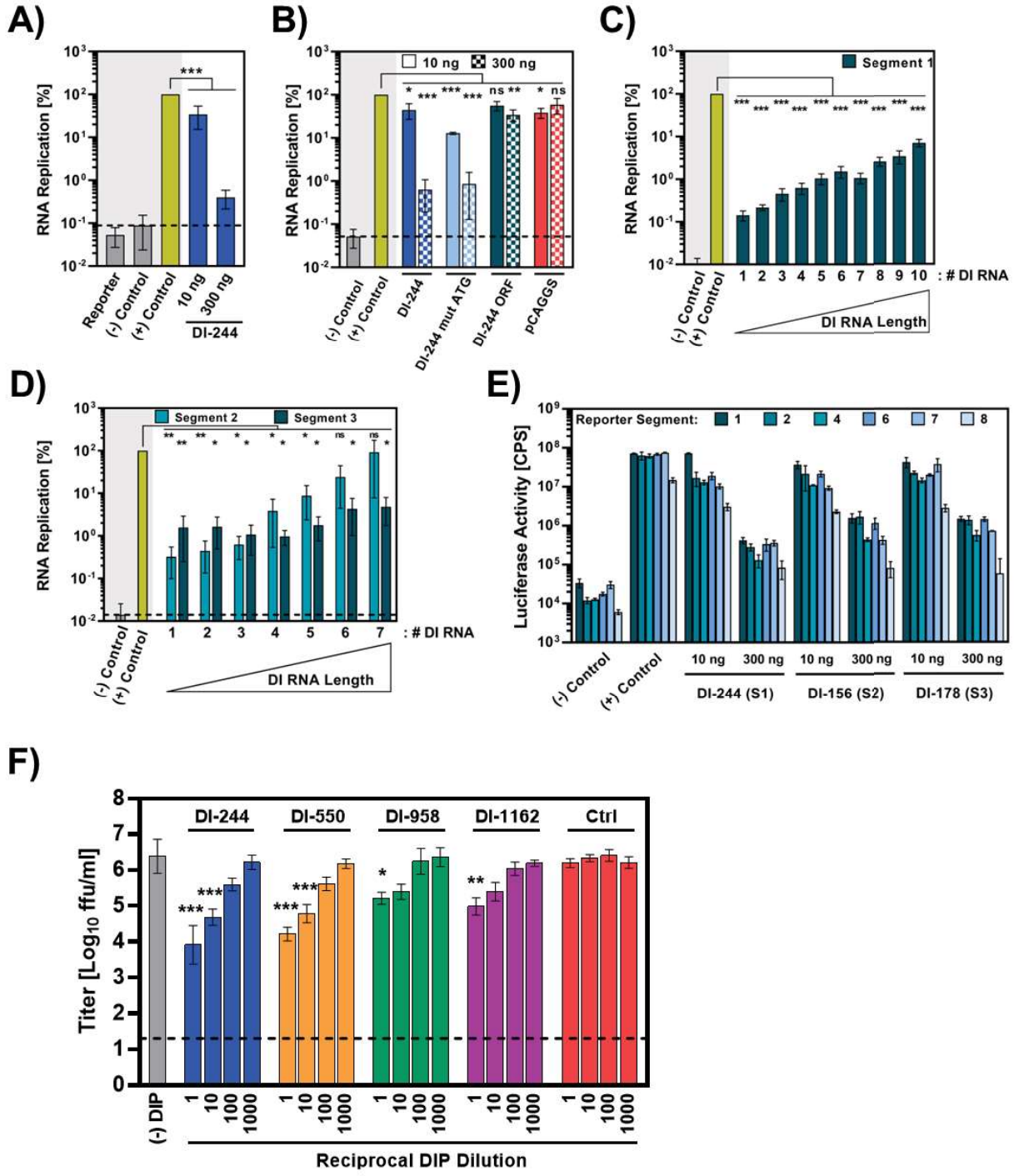


Figure 1 Antiviral activity of DI RNAs inversely correlates with DI RNA length

(*A*) DI-244 inhibits genome replication in the mini-replicon assay. 293T cells were transfected with plasmids encoding the viral polymerase proteins, NP, a segment 8-based luciferase reporter

145 (mini-replicon system) and either empty plasmid or plasmid for expression of DI-244 mRNA (10
146 and 300 ng) and vRNA. Removing the plasmid encoding PB2 from the transfection mix served as
147 negative control. Cotransfection of all support plasmids and empty plasmid instead of DI-244
148 encoding plasmid served as positive control. The average of five independent experiments is
149 shown, for which the positive control was set as 100%. Error bars indicate standard error of the
150 mean (SEM).
151 (*B*) The truncated open reading frame of DI-244 does not contribute to inhibition of genome
152 replication in the mini-replicon assay. The experiment was carried out as described for panel A
153 but the cells were cotransfected with a plasmid for expression of DI-244 mRNA and vRNA with
154 or without the first three ATGs of the PB2 ORF being intact (DI-244, DI-244 mut ATG), a
155 plasmid for expression of DI-244 mRNA (DI-244 ORF) or empty plasmid pCAGGS. The
156 average of three independent experiments is shown, for which the positive control was set as
157 100%. Error bars indicate SEM.
158 (*C*) The inhibitory activity of segment 1-derived DI RNAs in the mini-replicon assays is
159 inversely correlated with DI RNA length. The experiment was carried out as described for panel
160 A but 300 ng of plasmids harboring the indicated segment 1-derived DI RNAs were
161 cotransfected. The DI RNAs tested were numbered as shown in table S1. The average of five
162 independent experiments is shown, for which the positive control was set as 100%. Error bars
163 indicate SEM.
164 (*D*) The inhibitory activity of segment 2- and 3-derived DI RNAs in the mini-replicon assays is
165 inversely correlated with DI RNA length. The experiment was conducted as described for panel
166 A but 300 ng of plasmids harboring the indicated segment 2 and 3-derived DI-RNAs were
167 cotransfected. The DI RNAs tested were numbered as shown in table S1. The average of three

168 independent experiments is shown, for which the positive control was set as 100%. Error bars
169 indicate SEM.
170 (*E*) The inhibitory activity of DI RNAs in the mini-replicon assays is independent from the origin
171 of the reporter segment. The experiment was carried out as described for panel A but the
172 indicated reporter segments and segment 1, 2 and 3-derived DI RNAs were used. The results of a
173 single representative experiment are shown and were confirmed in an independent experiment.
174 Error bars indicate standard deviation (SD).
175 (*F*) Antiviral activity of segment 1-derived DIPs is inversely correlated with DI RNA lengths in
176 the presence of trypsin. MDCK cells were coinfected with the indicated DIPs (MOI 1) and
177 A/PR/8/34 (MOI 0.001) in the presence of trypsin, washed, and cultured in medium with trypsin.
178 DIP-negative supernatants served as controls. At 72 h post infection, viral titers in culture
179 supernatants were determined by focus formation assay. The average of four independent
180 experiments is shown; error bars indicate SEM.
181 In panels A-D statistical significance of differences between values measured for cells
182 cotransfected with support plasmids and either empty plasmid (+ control) or DI RNA encoding
183 plasmid was determined using one-way ANOVA with Sidak´s (panel A) or with Dunnett´s
184 posttest (panel B-D). In panel F statistical significance of differences between values measured
185 for cells with virus and DIPs at reciprocal DIP dilution was determined using one-way ANOVA
186 with Dunnett´s posttest. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$
187
188
189 **Inhibitory activity of segment 1, 2 and 3-derived DI RNAs is inversely correlated with RNA**
190 **length and is independent of the target segment**

191 It is believed that the short length of DI-244, as compared to wt segment 1, results in faster
192 amplification of DI-244 and ultimately in suppression of amplification of the wt segment (4, 5). If
193 correct, one would assume that DI RNA length is a major determinant of antiviral activity. We
194 explored this possibility by investigating the capacity of a set of ten segment 1-derived RNAs
195 with nested central deletions to inhibit segment amplification in the mini-replicon assay. All
196 RNAs tested exerted inhibitory activity and an inverse correlation between RNA length and
197 inhibitory activity was observed (Figure 1C, Table S1). Moreover, further shortening of DI-244
198 did not augment inhibitory activity (not shown), suggesting that DI-244 length may be optimal
199 for inhibition of wt segment replication. In sum, our results show that the ability of segment 1-
200 derived DI RNAs to block replication of a wt segment is dependent on the DI RNA length.
201 We next explored whether the inverse correlation between length and inhibitory activity is
202 also observed for segment 2- and 3-derived DI RNAs. For this, we introduced central, nested
203 deletions in segment 2 and 3 and investigated inhibitory activity in the mini-replicon system. As
204 for segment 1-derived RNAs, all segment 2- and 3-based RNAs with deletions exerted inhibitory
205 activity and inhibition inversely correlated with RNA length, although this correlation was more
206 pronounced for segment 2 as compared to segment 3 (Figure 1D, Table S1).
207 Next, we examined whether the segment 1-, 2- and 3-derived DI RNAs with the largest
208 deletion (constructs DI-244 (segment 1, S1), DI-156 (segment 2, S2), DI-178 (segment 3, S3),
209 Table S1) were able to efficiently suppress replication of different IAV segments or were mainly
210 active against segment 8, which was so far employed in the mini-replicon assay. For this, we
211 added the 5' and 3' ends of segments 1, 2, 4, 6, and 7 to the firefly luciferase sequence and tested
212 the amplification of these reporter segments in the mini-replicon assay. In the absence of DI
213 RNAs, all segments were efficiently amplified, as demonstrated by high luciferase activity in
214 lysates of cells coexpressing PB2, PB1, PA and NP (Figure 1E). Cotransfection of two different

amounts of segment 1-, 2- or 3-derived DI RNAs reduced replication of all reporter segments efficiently and in a concentration dependent manner (Figure 1E). Thus, in the mini-replicon assay, introduction of a deletion into an IAV genomic segment is sufficient to convert it into a DI RNA and length and inhibitory activity of these DI RNAs are inversely correlated.

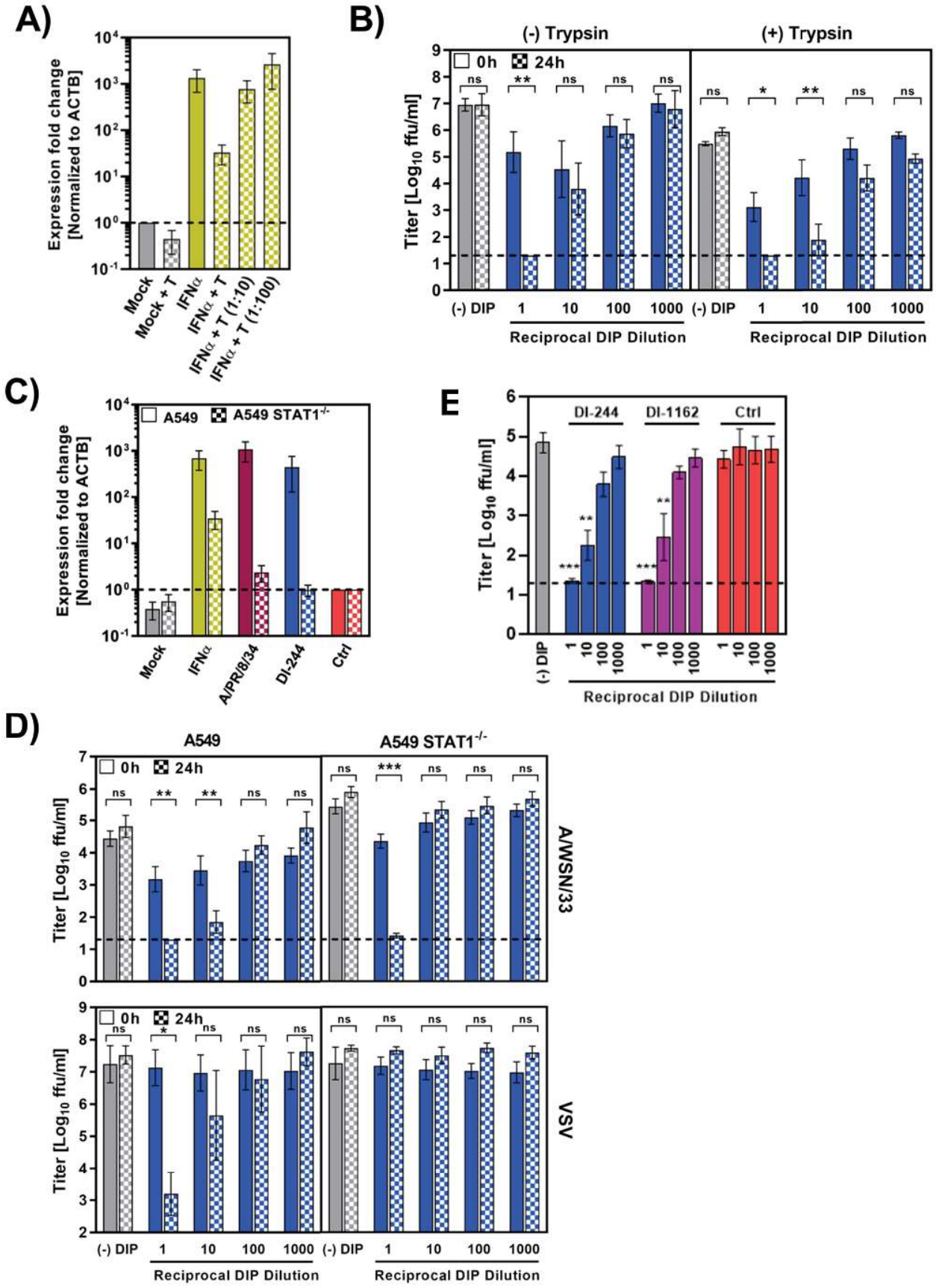
Inverse correlation between anti-IAV activity of DIPs and DI RNA length

We recently reported a cell culture system for production of DIPs in the absence of helper virus, which relies on IAV reverse genetics and DIP producer cell lines stably expressing the PB2 protein (18). We employed this system to generate DIPs with nested deletions in segment 1 and assessed their ability to inhibit infection of MDCK cells with A/PR/8/34 (PR8). We found that DI-244, which contains the smallest DI RNA, inhibited PR8 infection with the highest efficiency and that inhibitory activity of DIPs decreased as DI RNA length increased (Figure 1F). Thus, an inverse correlation between DI RNA length and inhibitory activity observed in the mini-replicon assay could be confirmed in the context of DIPs, at least under the conditions tested.

Preincubation of target cells with DI-244 increases antiviral activity

It has been reported that DIPs can block viral infection by stimulating the IFN system (11, 12). Therefore, we sought to clarify whether induction of the IFN system could contribute to DI-244 antiviral activity in MDCK cells. Trypsin is used for A/PR8/34 activation but can inactivate IFNα (Figure 2A) (21) and can thus cofound analyses of IAV inhibition by the IFN system. Therefore, we switched to A/WSN/33 (WSN) as challenge virus and WSN-derived DIPs, since WSN can replicate trypsin-independently in cell cultures containing fetal bovine serum (FBS) (22). To obtain first insights into a potential role of the IFN system in DIP antiviral activity, we reasoned that if induction of the IFN system was a major determinant of DIP antiviral activity, then time-

239 of-DIP addition to target cells should have a major impact on the efficiency of IAV inhibition by
240 DIPs. Thus, addition of DIPs and virus to target cells at the same time should preclude the
241 establishment of a robust DIP-induced antiviral state prior to IAV infection. In contrast, addition
242 of DIPs at 24 h before virus should allow for establishment of such an antiviral state and might
243 thereby boost DIP antiviral activity. Preincubation of target MDCK cells with DI-244 for 24 h
244 indeed increased DIP antiviral activity as compared to simultaneous addition of DI-244 and IAV,
245 especially when high doses of DI-244 were analyzed (Figure 2B, left panel). Unexpectedly,
246 similar results were obtained in the presence of trypsin (Figure 2B, right panel), indicating that
247 the enhanced antiviral activity of DI-244 upon 24 h preincubation with target cells was likely not
248 due to induction of IFNα or another trypsin-sensitive antiviral host cell protein.
249


A)
Expression fold change [Normalized to ACTB]
Mock
Mock + T
IFNα
IFNα + T
IFNα + T (1:10)
IFNα + T (1:100)
B)
(-) Trypsin
(+) Trypsin
0h
24h
Titer [Log10 ffu/ml]
(-) DIP
1
10
100
1000
Reciprocal DIP Dilution
C)
A549
A549 STAT1-/-
Mock
IFNα
A/PR/8/34
DI-244
Ctrl
E
DI-244
DI-1162
Ctrl
D)
A549
A549 STAT1-/-
A/WSN/33
VSV

250

Figure 2 Induction of the IFN system is a major contributor to DIP antiviral activity

(*A*) Trypsin inactivates IFNα. A549 wt cells were exposed to recombinant IFNα (100 U/ml) in the presence and absence of serially diluted trypsin (T). Undiluted trypsin (IFNα + T) was added at a concentration of 0.5 µg/ml. After 24 h, cells were harvested, RNA isolated and *MX1* expression analyzed by quantitative RT-PCR. *MX1* transcripts levels were normalized against *ACTB (β-actin)* transcript levels. The average of three independent experiments is shown. Error bars indicate SEM.

(*B*) Pre-exposure of target cells to DIPs boosts DIP antiviral activity independent of trypsin. Left panel, - Trypsin condition: MDCK cells were either coinfected with DI-244 (MOI 10) and A/WSN/33 (MOI 0.1) in the absence of trypsin or DI-244 was added to cells at 24 h before virus. Cells were washed 1 h after addition of virus and maintained in growth medium without trypsin. At 72 h post infection, viral titers in culture supernatants were determined by focus formation assay. Right panel, + trypsin condition: The experiment was carried out as described for the left panel, but A/WSN/33-derived DIPs (MOI 1) and A/WSN/33 (MOI 0.001) were used and maintained in infection medium supplemented with trypsin. The average of three independent experiments is shown in both panels; error bars indicate SEM.

(*C*) STAT1 is required for *MX1* induction by IAV and DIP. A549 cells and A549 STAT1$^{-/-}$ cells were exposed to IFNα (100 U/ml), A/PR/8/34 or DI-244 (all MOI 1, in the presence of trypsin) for 1 h, washed, incubated for 24 h in the absence of trypsin and *MX1* mRNA expression quantified using qRT-PCR. The average of five independent experiments is shown. Error bars indicate SEM.

(*D*) Anti-IAV activity of DI-244 is partially and anti-VSV activity of DIP is fully dependent on STAT1. Antiviral activity of DI-244 was analyzed as described for the left panel of figure 2B but A549 wt and A549 STAT1$^{-/-}$ cells were used. At 96 h post infection, viral titers in culture

supernatants were determined by focus formation assay. The average of six (A/WSN/33) and three independent experiments (VSV) is shown. Error bars indicate SEM.

(*E*) DI RNA length does not modulate DIP antiviral activity in the context of a functional IFN system. Antiviral activity of the indicated DIPs was analyzed as described for panel D adding DIPs 24 h before virus. The average of five independent experiments is shown. Error bars indicate SEM.

In panels B and D statistical significance of differences between values measured for cells inoculated with DIPs at 24 h before IAV infection and cells to which IAV and DIPs were added at the same time was determined using two-way ANOVA with Sidak´s posttest. In panel E statistical significance of differences in viral titers obtained on cells treated with different concentrations of DIPs or without (-) DIPs was determined using one-way ANOVA with Dunnett´s posttest. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$

DI-244 induces anti-IAV activity in A549 cells in a STAT1-independent fashion

In order to more directly assess the contribution of the IFN system to DI-244 antiviral activity, we employed A549 wt cells and A549 cells which lack STAT1 (signal transducer and activator of transcription 1, $STAT1^{-/-}$) and are thus defective in IFN-induced signaling. Confirmatory experiments revealed that IFNα, IAV and DI-244 strongly upregulated *MX1* gene expression in A549 wt but not $STAT1^{-/-}$ cells, in keeping with a defective JAK/STAT signaling pathway (Figure 2C). Addition of undiluted and 1:10 diluted DI-244 to A549 cells at 24 h before infection with WSN resulted in 100 -fold higher antiviral activity as compared to DI-244 added at the same time as virus (Figure 2D), confirming and extending the data obtained with MDCK cells.

Unexpectedly, addition of undiluted DIP to A549 $STAT1^{-/-}$ cells still resulted in high antiviral

299 activity (Figure 2D), although 10-fold diluted DI-244 showed markedly reduced antiviral activity
300 in STAT1$^{-/-}$ cells as compared to wt cells. In contrast, inhibition of vesicular stomatitis virus
301 (VSV) infection by DI-244 was completely dependent on STAT1, independent of the DIP dose
302 used (Figure 2D). Finally, we asked whether the antiviral activity of DIPs still depended on the
303 DI RNA length if DIPs were added to cells before virus. In contrast to what was observed with
304 MDCK cells in the presence of trypsin, all DIPs with nested deletions in segment 1 inhibited
305 WSN infection of A549 wt cells with similar efficiency (Figure 2E and data not shown),
306 indicating that the contribution of replication interference to DIP antiviral activity was minor or
307 absent under those conditions. Collectively, our findings indicate that DIPs can induce robust,
308 partially STAT1-independent anti-IAV activity that is not determined by DI RNA length and
309 markedly more potent than DIP-mediated inhibition of IAV genome replication.
310
311 **DI-244 induces robust expression of ISGs but not IFN**
312 In order to understand how DIPs activate the IFN system, we compared DIP- and IAV-mediated
313 stimulation of IFN expression. For this, an IFN bioassay was employed that was based on VSV, a
314 highly IFN-sensitive virus (23). A549 or A549 STAT1$^{-/-}$ effector cells were incubated with IAV,
315 VSV or DI-244 for 24 h, the supernatants collected and heat and acid treated to inactivate viral
316 particles but not IFN, which is known to display a certain heat and acid stability. Subsequently,
317 the supernatants were added to sentinel cells (A549) for 24 h followed by inoculation of the
318 sentinel cells with a single-cycle reporter VSV replicon and quantification of infection. For
319 standardization, A549 cells were incubated with recombinant IFNα, infected with the single-
320 cycle VSV and infection efficiency quantified. Supernatants from IAV exposed A549 wt cells but
321 not A549 STAT1$^{-/-}$ cells potently inhibited subsequent VSV infection (Figure 3A), indicating that
322 IAV induced production of IFN in a STAT1-dependent fashion, as expected. Similar findings

were made with supernatants from VSV exposed cells but antiviral activity was independent of STAT1 expression (Figure 3A), again in agreement with published data (24). Finally, and unexpectedly, supernatants from A549 wt cells exposed to DI-244 were not inhibitory and the same finding was made for supernatant from DI-244 treated A549 STAT1$^{-/-}$ cells, indicating that IFN induction by DI-244 was low or absent.

The ability of DI-244 to inhibit IAV and VSV infection without inducing IFN, as determined in the bioassay, posed the question how DI-244 alters gene expression in target cells to block infection. To address this question, A549 cells and A549 STAT1$^{-/-}$ were either incubated with control supernatants or supernatants containing DI-244 or IAV and subjected to RNAseq analysis. PR8 was employed for these studies, in order to limit viral replication to a single cycle (since no trypsin was present after virus inoculation). Neither PR8 nor DI-244 induced the expression of IFN receptors (Figure 3B). In contrast, PR8 but not DI-244 induced expression of IFNβ and IFNλ (Figure 3B), in agreement with our results obtained in the bioassay. Despite the differential upregulation of IFNs by PR8 and DI-244 both induced the robust expression of antiviral ISGs, including MX1, IFITM1 and ISG15 in A549 wt cells, although induction by PR8 was more efficient than that observed for DI-244 (Figure 3B). Moreover, no ISG induction was observed in PR8 or DIP treated A549 STAT1$^{-/-}$ cells, with the exception of ISG15 and RSAD2 (Viperin), the expression of which was induced by PR8 but not DI-244 (Figure 3B). Finally, results with A549 wt cells were confirmed by qRT-PCR analyses. Induction of IFNβ and IFNλ by DI-244 was detectable but at least 100-fold less efficient as compared to PR8 while differences in ISG induction were frequently less than 10-fold (Figure 3C). In sum, these results suggest that DI-244 inhibits viral infection by STAT1-dependent induction of ISG expression without inducing appreciable expression of IFN.

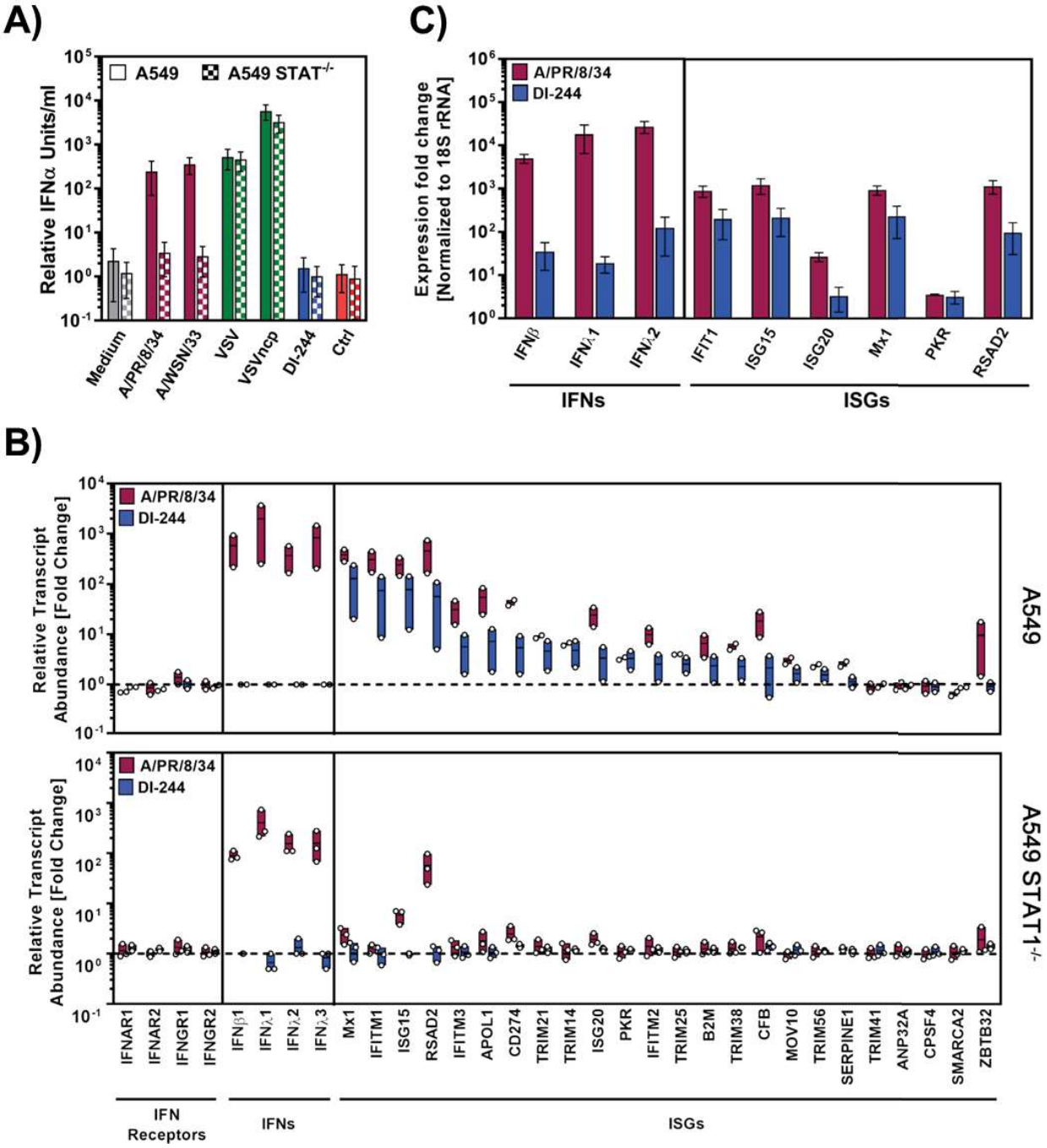


Figure 3 DI-244 robustly induces ISG but not IFN expression

(*A*) DI-244 does not induce IFN expression as determined in a VSV-replicon-based bioassay. A549 and A549 STAT1$^{-/-}$ effector cells were exposed to IAV, VSV or DI-244 and supernatants

collected, heat inactivated, acid treated and added onto A549 sentinel cells followed by infection with VSV. For calibration, A549 cells were incubated with recombinant IFNα, VSV infected and infection efficiency was quantified. The average of three independent experiments is shown. Error bars indicate SEM.

(*B*) DI-244 induces robust ISG but not IFN expression as determined by RNAseq. A549 cells (top panel) and A549 STAT1$^{-/-}$ cells (bottom panel) were incubated with IAV (A/PR/8/34), DI-244 and control supernatants at an MOI of 1 in the absence of trypsin and subjected to RNAseq analysis. Expression of selected ISGs is shown. The average of two independent experiments (A549) and three experiments (A549 STAT1$^{-/-}$) is presented. Error bars indicate SEM and black lines within the floating bars indicate the mean.

(*C*) DI-244 induces robust ISG but not IFN expression as determined by qRT-PCR analysis. The A549 wt cells described in panel B were subjected to qRT-PCR analysis of ISG expression. The average of three independent experiments is shown.

Discussion

DI RNAs arise in IAV infected cell cultures, eggs, animals and patients (4, 5, 20, 25-28). They inhibit IAV infection and might modulate IAV intra- and interpatient spread and pathogenesis. However, the mechanism underlying DI RNA antiviral activity and the determinants controlling whether a defective viral genomic RNA is also interfering are incompletely understood. Here, we show that any central deletion in segments 1, 2 and 3 of IAV is sufficient to convert these RNAs into DI RNAs and that inhibitory activity of the respective DI RNAs extends to all tested IAV genomic RNAs. Moreover, we provide evidence that the contribution of replication interference to DIP antiviral activity in cell culture is minor as compared to induction of the IFN system.

IAV and influenza B virus DI RNAs usually contain deletions relative to the genomic RNAs they arose from (4, 5), although an exception has recently been reported (29). Moreover, DI RNAs derived from IAV segments 1-3, which encode the subunits of the viral polymerase, arise more frequently than those derived from other segments (4-6, 8, 9, 30, 31) and were thus in the focus of the present study. The almost universal presence of a deletion in DI RNAs suggests that their shorter length might allow them to out-compete their parental RNAs for resources required for RNA replication. Although this hypothesis is frequently posited (4, 5), direct experimental proof is largely lacking. Here we provide this proof by demonstrating that deleting any internal sequence from segments 1, 2 and 3 is sufficient to generate a DI RNA. Furthermore, we demonstrate that the inhibitory activity of these DI RNAs is determined by their length, at least in the absence of an IFN response, and extends to all target segments tested. The latter observation fits with the finding that DI-244 interferes with replication of several genomic RNAs in IAV infected cells (32). In sum, deleting the sequences between the conserved 5' and 3' ends of any IAV RNA, which are required for transcription and translation, should generate potent DI RNAs. In some cases, the truncated open reading frame encoded by such DI-RNAs might

391 contribute to antiviral activity (33) but this was not observed for DI-244, in keeping with
392 previous results (32).

393 Type I IFNs trigger the expression of about 400 genes, many of which encode proteins
394 with antiviral activity, including MX1 (34). The present study shows that when conditions are
395 chosen that allow DIPs to robustly activate the IFN system, DIPs are potent inducers of ISG
396 expression and the contribution of replication interference to DIP antiviral activity is minor.
397 Notably, RNAseq analysis revealed that IAV but not DIPs induced type I and type III IFN
398 expression although both triggered ISG expression in a STAT1-dependent fashion. A potential
399 explanation for this discrepancy is that DIPs induced IFN expression at levels too low to be
400 detected by RNAseq but still sufficient to induce ISGs. Indeed, qRT-PCR analysis revealed
401 modest upregulation of type I and III IFN upon DIP treatment. Alternatively, DIPs may induce
402 ISGs via an unidentified IFN-independent, STAT1-dependent pathway. Interestingly, Wang and
403 colleagues also reported that DIPs induce robust levels of ISGs but only moderate levels of IFN
404 (35) and further research is required to explore why IFN and ISG induction are not correlated in
405 the context of DIPs. Moreover, it is unclear how undiluted DIPs exerted anti-IAV but not anti-
406 VSV activity in STAT1$^{-/-}$ cells without inducing ISGs or other cellular genes. Competition of
407 DIPs with IAV for engagement of entry receptors is one possibility. Collectively, our results
408 underline previous findings that DIPs are potent inducers of antiviral responses (13-17) and show
409 that DIP antiviral activity due to induction of ISG expression outweighs that due to replication
410 interference.

411 What are the major implications of our findings for DIP development as antivirals and for
412 elucidating the role of naturally occurring DIPs in IAV infection? First, it is essential that
413 antiviral activity of DIPs is examined in IFN competent animal models which express ISGs with
414 potent anti-IAV activity, particularly MX1. Second, antiviral activity due to replication

415 interference can be attained only if DIPs are added in 100 to 1,000 fold excess relative to virus
416 (18) and it remains to be examined whether the strong IFN induction under those conditions
417 exerts unwanted toxic effects in animals and humans. Third, DIP treatment should be more
418 effective in the prophylactic as compared to the therapeutic setting, since only in the former DIP-
419 induced IFN can fully contribute to antiviral activity. Fourth, design of DI RNA and analysis of
420 DI RNAs emerging in patients should focus on the smallest RNAs, since they can be expected to
421 exert the highest antiviral activity.
422
423

Material and Methods

Plasmids and oligonucleotides

Plasmids for rescue of A/PR/8/34, pHW191-pHW198, and A/WSN/33, pHW181-pHW188, were previously described (36). Plasmids encoding DI RNAs were generated by splice overlap PCR, joining 5' and 3'-end sequences of desired length, following a strategy previously described for DI-244 (18). A multiple cloning site (mcs) for later insertion of a reporter gene was included in the respective oligonucleotide sequences (Table S2). The PCR products were cloned into pHW2000-GGAarI by golden gate cloning (37). Start codons in DI-244 were mutated using splice overlap PCR primer pairs mutIAV-seg1-ATG-for (5'-TCAATTATATTCAATTTGGAAAGAATAAAAG -3')/mutIAV-seg1-ATG-rev (5'-CTTTTATTCTTTCCAAATTGAATATAATTGA-3') and DImut2+3ATG-for (5'-ACTACGAAATCTAATCTCGCAGTCTCGCACCCGCGAGATACTCACAAAAACCACCGTGGACCATATCGCCATAATCAAGAAG-3')/DImut2+3ATG-rev (5'-CTTCTTGATTATGGCGATATGGTCCACGGTGGTTTTTGTGAGTATCTCGCGGGTGCGAGACTGCGAGATTAGATTTCGTAGT-3'). PCR constructs were cloned into pHW2000-GGAarI as described above.

For expression of the truncated PB2 ORF from DI-244, the ORF was amplified from pHW2000GG-DI244-rep using primers PB2-QCXIP-5N (5'-CCGCGGCCGCACCATGGAAAGAATAAAAGAACTAC-3')/PB2-3XBgl (5'-GGAGATCTCGAGCTAATTGATGGCCATCCGAAT-3') digested with NotI/XhoI and cloned into NotI/SalI digested pCAGGS-mcs bearing an altered multiple cloning site (XhoI-SacI-Asp718I-NotI-EcoRV-ClaI-EcoRI-SmaI-SalI-SphI-NheI-BglII).

447 For generation of empty vector p19polI-GGAarI the insert was amplified from pHW2000-
448 GGAarI by splice overlap PCR using primers HW2-GG-5Bgl, CCdelE-rev (5'-
449 CGTCTTTCATTGCCATACGAAACTCCGGATGAGCATTCATCAG-3'), CCdelE-for (5'-
450 CTGATGAATGCTCATCCGGAGTTTCGTATGGCAATGAAAGACG-3')/ rRNA-Pr(GG)-
451 3Eco (5'-GCGAATTCTATAGAATAGGGCCAGGTC-3') and cut with BglII and EcoRI for
452 insertion into BamHI and EcoRI digested p19luc (38).
453 Reporter plasmids for mini-replicon assay have been described (pPolI-Luc (vRNA/FLUAV/NS1
454 Seg8-NCR) (19) or were newly generated. First, the reporter with segment 8 ends was amplified
455 with primers fluA AarI-NS-1 and fluA AarI-NS-890R (Table S3) and inserted into vector
456 p19polI-GGAarI by Golden Gate cloning. To generate reporters with ends derived from other
457 segments of IAV, the luciferase reporter gene was amplified with primers encoding the respective
458 untranslated regions (Table S3) and cloned into vector p19polI-GGAarI as described before. All
459 PCR amplified sequences were confirmed by automated sequence analysis.
460
461 **Cells and viruses**
462 293T, A549 wt and A549 STAT1$^{-/-}$ cells were maintained in Dulbecco's Modified Eagle Medium
463 (DMEM; Gibco) containing 10% fetal bovine serum (FBS, Gibco), penicillin (Pen, 100 IU/mL)
464 and streptomycin (Strep, 100 µg/ml). BHK-21 cells were cultivated in Dulbecco's modified
465 Eagle medium (DMEM, Pan Biotech) supplemented with 5% fetal bovine serum and pen/strep.
466 293T cell lines stably expressing codon optimized PB2 (293T-PB2opt) were cultured in the
467 presence of 1µg/ml puromycin. Madin-Darby canine kidney cells (MDCK) were cultured in
468 Glasgow's Modified Eagle Medium (GMEM; Gibco) supplemented with 10% fetal bovine serum
469 (FBS, Gibco) and pen/strep. MDCK cells stably expressing PB2opt were maintained in the
470 presence of 1.5µg/ml puromycin. For generation of A549 STAT1$^{-/-}$ cells, A549 wt cells were

transduced with pLentiCRISPR v2 (Addgene, plasmid 52961), a lentivirus expressing Cas9, puromycin resistance, and a guide RNA targeting human *STAT1* (TTCAAGACCAGCGGCCTCTGAGG). Transduced cells were puromycin selected for seven days and surviving cells were plated in 96-well dishes as single cells and expanded. Clonal populations were then lysed and whole cell extract was examined for STAT1 expression by immunoblot. These efforts identified a single clone that demonstrated a complete loss of STAT1 expression, which we refer herein as $STAT1^{-/-}$ cells. All cells lines were regularly tested for mycoplasma contamination.

A/PR/8/34 (H1N1) and A/WSN/33 (H1N1) (36, 39) were produced in embryonated chicken eggs as described previously (40) while A/WSN/33 adapted to growth in A549 cells was obtained from the strain repository of the IVM Münster and was amplified in A549 cells by continuous passaging. IAV titers were determined using focus formation assay as described (18, 37, 41). Replication-competent vesicular stomatitis virus (VSV) expressing eGFP and either wildtype VSV matrix protein (VSV*) or a matrix protein variant harboring four amino acid substitutions associated with increased induction of type-I interferon response (VSV*M_Q) have been described elsewhere (42) and were amplified using BHK-21 cells. Further, a VSV glycoprotein trans-complemented, single-cycle VSV replicon that lacks the genetic information for VSV-G but instead codes for eGFP and firefly luciferase genes (VSV*ΔG-FLuc) (43) was employed and propagated on BHK-G43 cells (44). All VSV variants were titrated on BHK-21 cells and eGFP-positive foci (replication-competent VSV) or eGFP-positive single cells (single-cycle VSV) were counted as described previously (45).

Mini-replicon assay

494 The mini-replicon assay was performed as described (19). In brief, 293T cells seeded in 12-well
495 plates at a cell density of 2 x 10^5 cells per well were cotransfected with plasmids encoding PB1
496 (10 ng), PB2 (10 ng), NP (100 ng), reporter segment encoding firefly luciferase (50 ng) and
497 plasmid encoding a DI RNA or empty plasmid (amounts indicated in figures or figure legends).
498 Cells were washed at 6-8 h and harvested at 24 h post transfection. Firefly luciferase activity in
499 cell lysates was measured using a commercial kit (PJK) and the Plate Chameleon V reader
500 (Hidex, Turku, Finland) jointly with Microwin 2000 software.
501
502 **Production of DIPs**
503 A coculture of 1.4 ×10^6 293T cells and 0.4 ×10^6 MDCK cells each stably expressing PB2opt and
504 seeded in T-25 flask was cotransfected with plasmids encoding IAV genomic segments 2-8 of
505 either PR8 or WSN origin and a plasmid encoding a segment 1-derived DI-RNA. After overnight
506 incubation, cells were washed once with PBS and, for production of A/PR/8/34-derived DIPs,
507 DMEM infection medium (0.2% MACS BSA, 1% pen/strep) supplemented with TPCK trypsin
508 (0.5 µg/ml) was added. For production of A/WSN/33-derived DIPs, DMEM growth medium (2%
509 FCS, 1% pen/strep) was added. As a negative control, parental MDCK and 293T cells were
510 transfected. Supernatants containing A/PR/8/34-derived DIPs were harvested at 4, 6, 8 and 10
511 days post transfection while supernatants containing A/WSN/33-derived DIPs were harvested at
512 3, 5, 7 and 9 days post transfection. Supernatants were cleared from debris by centrifugation,
513 aliquoted and stored at -80 °C for further use. For some experiments, DIPs were further amplified
514 in MDCK-PB2opt cells. For this, a total of 3 ×10^6 cells were seeded in T-75 flasks and infected at
515 an MOI of 0.01 or lower. Upon detection of CPE, supernatants were cleared from debris by
516 centrifugation and sterile-filtration (0.45 µm filter), aliquoted and stored at -80 °C for further use.
517 Integrity of selected DIP preparations was controlled with segments specific PCR. Infectious

518 titers of supernatants were determined by focus formation assay using MDCK-PB2opt cells as
519 targets, as described (18, 37, 41).
520
521 **Analysis of antiviral activity of DIPs**
522 For testing the antiviral activity of DIPs in MDCK cells in the presence of trypsin, cells were
523 seeded at 10,000 cells/well in 96-well plates and coinfected with DIP (MOI 1, and 10-fold
524 dilutions) and IAV (A/PR/8/34, MOI 0.001) for 1 h in Glasgow's MEM (GMEM) infection
525 medium containing trypsin (0.5 µg/ml). Alternatively, DIPs were added 24 h prior to the virus.
526 For analysis of DIP antiviral activity in MDCK cells, A549 wt and A549 STAT1$^{-/-}$ cells in the
527 absence of trypsin, cells were again seeded at 10,000 cells/well in 96-well plates and either
528 coinfected with DIP (MOI 5 or 10, and 10-fold dilutions) and IAV (A/WSN/33, MOI 0.1) in
529 DMEM medium without trypsin or DIPs added 24 h prior to the virus. After 1 h, cells coexposed
530 to DIPs and virus were washed and culture medium with or without trypsin was added.
531 Supernatants were harvested after 72 h (MDCK) and 96 h (A549 wt and A549 STAT1$^{-/-}$). Viral
532 titers in culture supernatants were quantified using focus formation assay and MDCK cells, as
533 described (37, 41).
534
535 **Quantitative RT-PCR analysis**
536 In order to investigate modulation of *MX1* mRNA expression by IAV, DIPs and IFN, a
537 quantitative RT-PCR assay was performed. For this, A549 cells were seeded at a cell density of 2
538 $\times 10^5$ cells/well in 12-well plates and inoculated with IAV (MOI 1), DIPs (MOI 1) or pan-IFNα
539 (100 U/ml, PBL Assay Science) using DMEM infection medium for 1 h (DMEM infection
540 medium without trypsin was added to cells exposed to IFNα). Then cells were washed once with
541 PBS and cultured in DMEM infection medium without trypsin for 24 h. To assess the effect of

542 trypsin on *MX1* induction by IFNα, cells were incubated for 24 h with IFNα in the presence of 0,
543 0.5, 0.05 and 0.005 µg/ml trypsin. At 24 h post treatment, total cellular RNA was extracted using
544 the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. After determining the
545 RNA content, 1 µg RNA was used as template for cDNA synthesis employing the SuperScript III
546 First-Strand Synthesis System (ThermoFisher Scientific), following the protocol for random
547 hexamers. Subsequently, 1 µl of cDNA (total volume after cDNA synthesis: 20 µl) was analyzed
548 by quantitative PCR on a Rotorgene Q device (Qiagen) employing the QuantiTect SYBR Green
549 PCR Kit (Qiagen). Each sample was analyzed in triplicates for transcript levels - given as cycle
550 threshold (Ct) values - of ß-actin (ACTB, internal transcript control) and myxovirus resistance
551 protein 1 (*MX1*, indicator for IFN induction, target transcript) with primer previously reported by
552 Biesold and colleagues (46). In order to analyze gene expression, the $2^{-\Delta\Delta Ct}$ method was used
553 (47).
554 The impact of IAV or DIP- on expression of cellular mRNAs was measured by RNAseq
555 and results for certain mRNAs were confirmed by quantitative RT-PCR. Expression of these
556 mRNAs was assayed using TB Green™ Premix Ex Taq™ II (Tli RNase H Plus; Takara)
557 according to manufacturer's instructions with QuantiTect primer assays (Qiagen). For this, RNA
558 was isolated as described above and reverse transcribed into cDNA with the prime Script RT
559 reagent kit (Takara). 10 ng of cDNA was used in a 1X reaction consisting of 12.5 µl TB Green
560 Premix Ex Taq II (Tli RNaseH plus) (2X), 2 µl 10X QuantiTect primer assay, and 0.5 µl 50X
561 ROX reference dye in a final reaction volume of 25 µl. PCR reactions were performed in a
562 StepOne Plus Instrument (Thermo Fisher). The following QuantiTect primer assays were used.
563 Hs_IFIT1_1_SG (QT00201012), Hs_ISG20_1_SG (QT00225372), Hs_IFNB1_1_SG
564 (QT00203763). Hs_MX1_1_SG (QT00090895), Hs_IFNL1_2_SG (QT01033564),
565 Hs_EIF2AK2_1_SG (QT00022960), Hs_IFNL2_1_SG (QT00222488), Hs_RR18s

(QT00199367), Hs_ISG15_1_SG (QT00072814), Hs_RSAD2_1_SG (QT00005271). 18S RNA is used as housekeeping gene. Fold gene induction over mock treated control is calculated by the ΔΔCT method.

Vesicular Stomatitis Virus Replicon-Based Bioassay

To analyze the relative contribution of IFN induction to antiviral activity, a VSV replicon-based bioassay (43) was performed. This assay is based on the principle that inoculation of effector cells with virus or DIPs leads to the induction of the innate immune system, resulting in the release of type-I IFN into the culture supernatant. These supernatants are then used to inoculate sentinel cells. Here, the type-I IFN will bind to the IFNα/β receptors and trigger a signaling cascade leading to the induction of an antiviral state. Subsequent inoculation of the sentinel cells with a highly IFN-sensitive VSV replicon containing a luciferase reporter will yield luciferase activities that inversely correlate with the extent of the induced antiviral state. A549 and A549 STAT1$^{-/-}$ cells (= effector cells) were seeded in 12-well plates (200,000 cells/well) and inoculated with IAV, VSV*, VSV*M_Q, or DIPs (all at MOI of 1) using DMEM infection medium containing trypsin for 1h. The cells were washed once with PBS and cultured in DMEM infection medium without trypsin (used for all further steps) for 16-18 hours. Next, supernatant was harvested and infectious virus was inactivated by addition of 0.1 M HCl and heating the samples for 30 min to 56 °C. After the samples cooled down to room temperature, alkaline treatment was performed using 0.1 M NaOH to neutralize the acidic pH. Subsequently, two-fold serial dilutions of the samples were prepared. In addition, medium containing two-fold serial dilutions of recombinant pan IFNα (starting at a concentration of 400 U/ml) were treated in the same fashion. These samples served as reference and were later used to calculate the relative antiviral activity present in the different supernatants (given as relative IFNα units per ml). The diluted

590 supernatants and IFNα reference samples were added in quadruplicates to a confluent layer of
591 A549 cells grown in 96-well plates (= sentinel cells) and incubated for 24 h. Thereafter, the cells
592 were inoculated with VSV*ΔG-FLuc reporter virus (MOI of 3) and further incubated for 6 h.
593 Then, the medium was aspirated and 50 µl/well of 1x luciferase lysis buffer was added.
594 Following an incubation period of 30 min the lysates were transferred into white, opaque-walled
595 96-well plates and firefly luciferase activity in cell lysates was measured as described above for
596 the mini-replicon assay.
597 For normalization, luciferase activity was set as 100 % for cells that received regular culture
598 medium instead of diluted culture supernatant/IFNα prior to inoculation with VSV*ΔG-FLuc.
599 Using the normalized luciferase values of cells treated with the IFNα reference samples and a
600 non-linear regression model we then calculated the relative IFNα content (given as units per ml)
601 for the effector cell supernatants.
602
603 **RNA-seq analysis**
604 For analysis of IAV and DIP mediated modulation of cellular gene expression, A549 wt and
605 A549 STAT1$^{-/-}$ cells were exposed for 1h to DMEM infection medium supplemented with TPCK
606 trypsin (0.5 µg/ml) and containing A/PR/8/34 or DI-244 at an MOI of 1 or were exposed to
607 control supernatants. Subsequently, cells were washed and cultured with DMEM infection
608 medium without trypsin. At 24 h post treatment, total cellular RNA was extracted using the
609 RNeasy Mini kit (Qiagen) following the manufacturer's instructions and subsequently sent for
610 RNAseq analysis at the Integrative Genomics Core Unit (NIG), Department of Human Genetics,
611 University Medical Center Göttingen.
612 RNA-seq libraries were performed using the non-stranded mRNA Kit (Illumina). Quality and
613 integrity of RNA was assessed with the Fragment Analyzer using the standard sensitivity RNA

614 Analysis Kit (Advanced Analytical). All samples selected for sequencing exhibited an RNA
615 integrity number of >8. After library generation, we used the QuantiFluor™dsDNA System
616 (Promega) for accurate quantitation of cDNA libraries. The size of final cDNA libraries was
617 determined by using the dsDNA 905 Reagent Kit (Advanced Analytical) exhibiting a sizing of
618 300 bp in average. Libraries were pooled and sequenced on an Illumina HiSeq 4000 (Illumina)
619 generating 50 bp single-end reads (28-35 Mio reads/sample). The raw read & quality check were
620 done by transforming sequence images the BaseCaller software (Illumina) to BCL files,
621 whichwere demultiplexed to fastq files with bcl2fastq v2.20. The sequencing quality was asserted
622 using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).
623 For subsequent data analysis, ISGs with anti-IAV activity were selected based on work by
624 Schoggins and colleagues (34). ISG expression in IAV- or DIP-treated cells was further
625 normalized to ISG expression in control-treated cells.
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653 **Competing interests**

654 The authors declare no competing interests.

655

656 **Author Contributions:**

657 Prerna Arora, Investigation, Methodology, Writing—review and editing; Najat Bdeir,

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Additional files

Table 1: Names of Segment 1-2-3-derived DI-RNAs

Table 2: Oligonucleotides for cloning of DI RNAs

Table 3: Oligonucleotides used for cloning of replicon reporter constructs

Data availability

All data in this study are included in the manuscript and supporting files.

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Supplemental Table 1

Segment 1-derived DI-RNAs				
Nr.	**Name**	**Total (b)**	**5' end (b)**	**3' end (b)**
1	DI-244	421	151	244
2	DI-346	623	252	346
3	DI-448	829	354	448
4	DI-550	1032	456	550
5	DI-662	1236	558	652
6	DI-754	1440	660	754
7	DI-856	1645	762	856
8	DI-958	1849	864	958
9	DI-1060	2051	966	1060
10	DI-1162	2256	1068	1162
Segment 2-derived DI-RNAs				
Nr.	**Name**	**Total (b)**	**5' end (b)**	**3' end (b)**
1	DI-156	334	151	156
2	DI-258	537	252	258
3	DI-360	741	354	360
4	DI-462	945	456	462
5	DI-666	1353	660	666
6	DI-870	1761	864	870
7	DI-1074	2169	1068	1074
Segment 3-derived DI-RNAs				
Nr.	**Name**	**Total (b)**	**5' end (b)**	**3' end (b)**
1	DI-178	346	141	178
2	DI-280	550	243	280
3	DI-382	754	345	382
4	DI-484	958	447	484
5	DI-688	1366	651	688
6	DI-892	1774	855	892
7	DI-1096	2182	1059	1096
All constructs contain an mcs located between the viral sequences				

818 **Table S2**

Oligonucleotides for cloning of DI RNAs	
Name	*Sequence*
IAVseg1-DI244-for	CAGGAAGACAGGAGAAGACTGAGGGGATTCCTCATTC
IAVseg1-DI244-rev	GAATGAGGAATCCCCTCAGTCTTCTCCTGTCTTCCTG
IAVseg1-DI244rep-for	TCAGGAAGACAGGAGAAGAGATCTGGTACCGCAGCGGCCGCTTAACTGAGGGGATTCCTCATT
IAVseg1-DI244rep-rev	AATGAGGAATCCCCTCAGTTAAGCGGCCGCTGCGGTACCAGATCTCTTCTCCTGTCTTCCTGA
DIP-346-for	GAGAAATGAGCAAGGACAAGGATCCGGTACCGCAGCGGCCGCTTAACTATAACAAGGCCACG
DIP-346-rev	CGTGGCCTTGTTATAGTTAAGCGGCCGCTGCGGTACCGGATCCTTGTCCTTGCTCATTTCTC
DIP-448-for	CCAATAACAAATACAGTTGGATCCGGTACCGCAGCGGCCGCTTAAGCCGCTCCACCAAAGCAA
DIP-448-rev	TTGCTTTGGTGGAGCGGCTTAAGCGGCCGCTGCGGTACCGGATCCAACTGTATTTGTTATTGG
DIP-550-for	CAAGTCAAAATACGTCGGGGATCCGGTACCGCAGCGGCCGCTTAAGAGGCCAATACAGTGGG
DIP-550-rev	CCCACTGTATTGGCCTCTTAAGCGGCCGCTGCGGTACCGGATCCCCGACGTATTTTGACTTG
DIP-652-for	GTGGGAGCCAGGATACTAGGATCCGGTACCGCAGCGGCCGCTTAAGAAACTGGGAAACTGTT
DIP-652-rev	AACAGTTTCCCAGTTTCTTAAGCGGCCGCTGCGGTACCGGATCCTAGTATCCTGGCTCCCAC
DIP-754-for	CATGTTGGAGAGAGAACTGGGATCCGGTACCGCAGCGGCCGCTTAACACAGGGAACAGAGAAAC
DIP-754-rev	GTTTCTCTGTTCCCTGTGTTAAGCGGCCGCTGCGGTACCGGATCCCAGTTCTCTCTCCAACATG
DIP-856-for	CTGGGAACAGATGTATACTGGATCCGGTACCGCAGCGGCCGCTTAAGAGTACTCCAGCACGGAGA
DIP-856-rev	TCTCCGTGCTGGAGTACTCTTAAGCGGCCGCTGCGGTACCGGATCCAGTATACATCTGTTCCCAG
DIP-958-for	AGCAGATCCACTAGCATCTGGATCCGGTACCGCAGCGGCCGCTTAACCTATCGACAATGTGATGG
DIP-958-rev	CCATCACATTGTCGATAGGTTAAGCGGCCGCTGCGGTACCGGATCCAGATGCTAGTGGATCTGCT
DIP-1060-for	CGTGGATATATGCAAGGCTGGATCCGGTACCGCAGCGGCCGCTTAATTTCGTCAATAGGGCGA
DIP-1060-rev	TCGCCCTATTGACGAAATTAAGCGGCCGCTGCGGTACCGGATCCAGCCTTGCATATATCCACG
DIP-1162-for	GGAAGAGGTGCTTACGGGCGGATCCGGTACCGCAGCGGCCGCTTAATAGTGAGTGGGAGAGACG

DIP-1162-rev	CGTCTCTCCCACTCACTATTAAGCGGCCGCTGCGGTACCGGATCCGC CCGTAAGCACCTCTTCC
DIP164-for	ATCAGGAAGACAGGAGAAGAGAAAGGAGAGAAGGCTAATG
DIP164-rev	CATTAGCCTTCTCTCCTTTCTCTTCTCCTGTCTTCCTGAT
DIP164P-for	AGACAGGAGAAGAACCCAGCGAAAGGAGAGAAGGCTAATG
DIP164P-rev	CATTAGCCTTCTCTCCTTTCGCTGGGTTCTTCTCCTGTCT
DIP164-80-for	GGATAACGGAAATGATTCCTGAAAGGAGAGAAGGCTAATG
DIP164-80-rev	CATTAGCCTTCTCTCCTTTCAGGAATCATTTCCGTTATCC
DIP204-for	ATCAGGAAGACAGGAGAAGAATGGGCCAGCACTAAGCATC
DIP204-rev	GATGCTTAGTGCTGGCCCATTCTTCTCCTGTCTTCCTGAT
fluA AarI-PB2-1G	CGATCACCTGCTCGAGGGAGCGAAAGCAGGTC
fluA AarI-PB2-2341R	CGATCACCTGCTCTCTATTAGTAGAAACAAGGTCGTTT
DIPS2-P-for	GGATACACCATGGATACTGAGCCCGAATTGATGCAC
DIPS2-P-rev	GTGCATCAATTCGGGCTCAGTATCCATGGTGTATCC
DIPS2-P-mcs-for	GGATACACCATGGATACTGGTACCGCAGCGGCCGCTTAAGAGCCCG AATTGATGCAC
DIPS2-P-mcs-rev	GTGCATCAATTCGGGCTCTTAAGCGGCCGCTGCGGTACCAGTATCCA TGGTGTATCC
DIPS2-252mcs-for	GGGCCACTGCCAGAAGACGGTACCGCAGCGGCCGCTTAAAAATGTA CCAAAGGTGCT
DIPS2-252mcs-rev	AGCACCTTTGGTACATTTTTAAGCGGCCGCTGCGGTACCGTCTTCTG GCAGTGGCCC
DIPS2-354mcs-for	AACTCGTGTATTGAAACGGGTACCGCAGCGGCCGCTTAAAAAACAT GGAGTATGATG
DIPS2-354mcs-rev	CATCATACTCCATGTTTTTTAAGCGGCCGCTGCGGTACCCGTTTCAA TACACGAGTT
DIPS2-456mcs-for	GCTGCAACAGCATTGGCCGGTACCGCAGCGGCCGCTTAAATTACCA GGGGCGTTTAT
DIPS2-456mcs-rev	ATAAACGCCCCTGGTAATTTAAGCGGCCGCTGCGGTACCGGCCAAT GCTGTTGCAGC
DIPS2-660mcs-for	AAAAAGAAGCAGAGATTGGGTACCGCAGCGGCCGCTTAATCATCAA AGATTACAGGT
DIPS2-660mcs-rev	ACCTGTAATCTTTGATGATTAAGCGGCCGCTGCGGTACCCAATCTCT GCTTCTTTTT
DIPS2-864mcs-for	GGCAATGAGAAGAAAGCAGGTACCGCAGCGGCCGCTTAAACATAA ACAGAACAGGTA
DIPS2-864mcs-rev	TACCTGTTCTGTTTATGTTTAAGCGGCCGCTGCGGTACCTGCTTTCTT CTCATTGCC
DIPS2-1068mcs-for	ATGTTCTCAAACAAAATGGGTACCGCAGCGGCCGCTTAATAAGCAC TGTATTAGGCG
DIPS2-1068mcs-rev	CGCCTAATACAGTGCTTATTAAGCGGCCGCTGCGGTACCCATTTTGT TTGAGAACAT
fluA AarI-	CGATCACCTGCTCGAGGGAGCGAAAGCAGGCA

PB1-1-4G	
fluA AarI-PB1-2341R	CGATCACCTGCTCTCTATTAGTAGAAACAAGGCATTT
DIPS3-P-for	AAATTTGCAGCAATATGCCCTGGGACCTTTGATCTT
DIPS3-P-rev	AAGATCAAAGGTCCCAGGGCATATTGCTGCAAATTT
DIPS3-P-mcs-for	AAATTTGCAGCAATATGCAGATCTGGTACCGCAGCGGCCGCTTAACCTGGGACCTTTGATCTT
DIPS3-P-mcs-rev	AAGATCAAAGGTCCCAGGTTAAGCGGCCGCTGCGGTACCAGATCTGCATATTGCTGCAAATTT
DIPS3-243mcs-for	CCAAATGCACTTTTGAAGAGATCTGGTACCGCAGCGGCCGCTTAATCGGTATTCAACAGCTTG
DIPS3-243mcs-rev	CAAGCTGTTGAATACCGATTAAGCGGCCGCTGCGGTACCAGATCTCTTCAAAAGTGCATTTGG
DIPS3-345mcs-for	AAACCAAAGTTTCTACCAAGATCTGGTACCGCAGCGGCCGCTTAAGAGTTCTTTGAGAACAAA
DIPS3-345mcs-rev	TTTGTTCTCAAAGAACTCTTAAGCGGCCGCTGCGGTACCAGATCTTGGTAGAAACTTTGGTTT
DIPS3-447mcs-for	AATAAAATTAAATCTGAGAGATCTGGTACCGCAGCGGCCGCTTAAAAATGGGGAATGGAGATG
DIPS3-447mcs-rev	CATCTCCATTCCCCATTTTTAAGCGGCCGCTGCGGTACCAGATCTCTCAGATTTAATTTTATT
DIPS3-855rev-for	AATGGGCCTCCCTGTTCTAGATCTGGTACCGCAGCGGCCGCTTAAAGCATGAGAAGGAATTAT
DIPS3-855mcs-rev	ATAATTCCTTCTCATGCTTTAAGCGGCCGCTGCGGTACCAGATCTAGAACAGGGAGGCCCATT
DIPS3-651mcs-for	AGGTTTGAAATCACAGGAAGATCTGGTACCGCAGCGGCCGCTTAAAGATCCCACTTAAGGAAT
DIPS3-651mcs-rev	ATTCCTTAAGTGGGATCTTTAAGCGGCCGCTGCGGTACCAGATCTTCCTGTGATTTCAAACCT
DIPS3-1059mcs-for	CAAGTACTGGCAGAACTGAGATCTGGTACCGCAGCGGCCGCTTAAGAGAACATGGCACCAGAA
DIPS3-1059mcs-rev	TTCTGGTGCCATGTTCTCTTAAGCGGCCGCTGCGGTACCAGATCTCAGTTCTGCCAGTACTTG
fluA AarI-PA1-1-4G	CGATCACCTGCTCGAGGGAGCGAAAGCAGGTAC
fluA AarI-PA1-2233R	CGATCACCTGCTCTCTATTAGTAGAAACAAGGTACTT

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821 **Table S3**

Oligonucleotides used for cloning of replicon reporter constructs	
Name	*Sequence*
fluA AarI-NS-1	CGATCACCTGCTCGAGGGAGCAAAAGCAGGGTG
fluA AarI-NS-890R	CGATCACCTGCTCTCTATTAGTAGAAACAAGGGTGTTTT
fluA AarI-seg2rep-5	CGATCACCTGCTCGAGGGAGCGAAAGCAGGCAAACCATTTGAATGGAAGACGCCAAAAACATAAAG
fluA AarI-seg2rep-3	CGATCACCTGCTCTCTATTAGTAGAAACAAGGCATTTTTTCATGAAGGACAAGCTAAATTCATTACACGGCGATCTTTCCG
fluA AarI-seg4rep-5	CGATCACCTGCTCGAGGGAGCAAAAGCAGGGGAAAATAAAAACAACCAAAATGGAAGACGCCAAAAACATAAAG
fluA AarI-seg4rep-3	CGATCACCTGCTCTCTATTAGTAGAAACAAGGGTGTTTTTCCTCATATCTCTGAAATTCTAATCTTACACGGCGATCTTTCCG
fluA AarI-seg6rep-5	CGATCACCTGCTCGAGGGAGCAAAAGCAGGAGTTTAAAATGGAAGACGCCAAAAACATAAAG
fluA AarI-seg6rep-3	CGATCACCTGCTCTCTATTAGTAGAAACAAGGAGTTTTTTGAACAGATTACACGGCGATCTTTCCG
fluA AarI-seg7rep-5	CGATCACCTGCTCGAGGGAGCAAAAGCAGGTAGATATTGAAAGATGGAAGACGCCAAAAACATAAAG
fluA AarI-seg7rep-3	CGATCACCTGCTCTCTATTAGTAGAAACAAGGTAGTTTTTTACACGGCGATCTTTCCG
fluA AarI-repPB2-5	CGATCACCTGCTCGAGGGAGCGAAAGCAGGTCAATTATATTCAATATGGAAGACGCCAAAAACATAAAG
fluA AarI-repPB2-3	CGATCACCTGCTCTCTATTAGTAGAAACAAGGTCGTTTTTAAACTATTCGACATTACACGGCGATCTTTCCG

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Third Manuscript

Evidence that two instead of one defective interfering RNA in influenza A virus-derived defective interfering particles (DIPs) does not enhance antiviral activity

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Individual Contributions: I performed experiments resulting in the data shown in all figures. I analyzed and plotted data. Additionally, I contributed to the writing and correction of the manuscript.

Evidence that two instead of one defective interfering RNA in influenza A virus-derived defective interfering particles (DIPs) does not enhance antiviral activity

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Abstract

Influenza A virus (IAV) infection constitutes a significant health threat. Defective interfering particles (DIPs) can arise during IAV infection and inhibit spread of wild type (WT) IAV. DIPs harbor defective RNA segments, termed DI RNAs that usually contain internal deletions and interfere with replication of WT viral RNA segments. Here, we asked whether DIPs harboring two instead of one DI RNA exert increased antiviral activity. For this, we focused on DI RNAs derived from segments 1 and 3, which encode the polymerase subunits PB2 and PA, respectively. We demonstrate the successful production of DIPs harboring deletions in segments 1 and/or 3, using cell lines that co-express PB2 and PA. Further, we demonstrate that DIPs harboring two instead of one DI RNA do not exhibit increased ability to inhibit replication of a WT genomic RNA segment. Similarly, the presence of two DI RNAs did not augment the induction of the interferon-stimulated gene MxA and the inhibition of IAV infection. Collectively, our findings suggest that the presence of multiple DI RNAs derived from genomic segments encoding polymerase subunits might not result in increased antiviral activity.

48 # Introduction

49 Influenza A viruses (IAVs) are a global health threat responsible for annual epidemics and
50 occasional pandemics [1,2] . Currently available influenza therapy includes M2 ion channel
51 inhibitors (Rimantadine and Amantadine) [3], neuraminidase inhibitors (Zanamivir and
52 Oseltamivir)[4,5], and an inhibitor of the viral polymerase (Baloxavir marboxil)[4-6]. However,
53 resistance mutations can render these drugs ineffective[7]. Similarly, vaccines against epidemic
54 influenza have to be reformulated on an annual basis due to antigenic drift of the circulating
55 influenza virus strains and offer little or no protection against newly emerging, pandemic
56 strains[8,9]. Hence, there is an urgent need for the development of novel prophylactic and
57 therapeutic strategies.

58 Influenza viruses are enveloped and harbor a negative-sense, segmented RNA genome. The
59 viral nucleoprotein (NP) and the trimeric viral polymerase consisting of the subunits PB1, PB2
60 and PA are required for genome replication[9,10]. Errors made by the viral polymerase during
61 genome replication may result in the production of defective genomic RNAs, which frequently
62 harbor deletions [11]. Some of these defective RNAs interfere with replication of WT RNAs and
63 the packaging of these defective interfering (DI) RNAs into particles yields DI particles, DIPs
64 [11,12]. DIPs inhibit infection with WT influenza viruses by interfering with genome replication and
65 by inducing the expression of interferon stimulated genes (ISGs), including the MxA gene. DIPs
66 can modulate influenza virus spread in the host and could be developed for antiviral therapy [13,14].
67 However, it is at present unclear whether DIPs harboring more than one DI RNA will exert
68 increased antiviral activity as compared to otherwise isogenic DIPs harboring a single DI RNA.

69 We have reported the establishment of a cell culture system which allows the production of
70 DIPs bearing a segment 1-derived DI RNA in the absence of infectious virus[15]. Here, we
71 modified this system in order to produce DIPs harboring DI RNAs derived from segments 1 and

3. We found that DIPs harboring DI RNAs derived from segments 1 and 3 can be readily generated in this system but do not show augmented antiviral activity as compared to DIPs harboring a single DI RNA.

RESULTS

Generation of 293T and MDCK cells stably expressing functional PA and PB2

We have previously generated a cell line that stably expresses PB2 (which is encoded by segment 1) and allows amplification of DIPs harboring segment 1-derived DI RNAs[15]. For production of DIPs harboring segment 1- and/or 3-derived DI RNAs we employed the same strategy. Thus, we engineered 293T and MDCK cells to co-express PB2 and PA (encoded by segment 3). Immunoblot analysis revealed that these cells indeed expressed robust levels of the desired proteins (Fig 1B). In order to analyze whether the PB2, PB1 and PA stably expressed in 293T cells are functional, we used a mini replicon system. This assay measures amplification of a reporter segment (segment 8) encoding luciferase upon co-expression of PB1, PB2, PA and NP[16]. We found that transfection of this 293T cell line with a plasmid encoding the IAV reporter segment yielded background levels of luciferase activity, while co-transfection of these cells with plasmids encoding the reporter segment and PB2, PB1, PA and NP increased luciferase activity more than 1000-fold (Fig 1C). Notably, the omission of plasmids encoding PB2, PB1 and PA did not appreciably reduce reporter activity (Fig 1C); demonstrating that our 293T cell line expresses functional PB2, PB1 and PA. Comparable functionality could not be analyzed for the MDCK cell line expressing PA and PB2 due to low transfectability (not shown).

PB2 and PA co-expressing cell lines allow production of DIPs harboring segment 1- and/or segment 3-derived DI RNAs

We next explored whether the generated stable cell lines allowed production of DIPs harboring a segment 1-derived DI RNA (S1 DIP) or a segment 3-derived DI RNA (S3 DIP) or a segment 1- and a segment 3-derived DI RNA (S1S3 DIPs), employing the experimental setup depicted in Fig 1A. For DIP production, segment 1- and 3-derived derived DI RNAs with an internal deletion of

120 1451 nts (S1) and 1193 nts (S3) were chosen based on our unpublished findings indicating that
121 these DI RNAs exert antiviral activity and are compatible with robust DIP production.
122 Quantification of DIP infectivity by focus formation assay revealed titers of roughly 10^6 ffu/mL
123 for S1 and S3 DIPs and 10^4 ffu/mL for S1S3 DIPs. In contrast, no DIPs were produced when
124 293T WT and MDCK WT cells were used for production (Fig 2A). Next, we sought to confirm
125 the incorporation of S1 and S3 DI RNAs into DIPs via segment specific RT-PCR. The RT-PCR
126 yielded bands of the expected sizes, 516 bp for S1 DI RNA, 1.3 kb for WT segment 1, 530 bp for
127 S3 DI RNA and 1.2 kb for segment 3 WT. Importantly, S1 DI RNA but not WT segment 1 was
128 detected in S1 DIPs and the corresponding observation was made for WT segment 3 and S3
129 DIPs. Similarly, S1 DI RNA and S3 DI RNA but not the corresponding WT segments were
130 detected in S1S3 DIPs, confirming the purity of our DIP preparations (Fig 2B). Thus, the newly
131 established cell lines allowed production of S1, S3 and S1S3 DIPs harboring the desired DI
132 RNAs.
133
134 **Co-expression of S1 and S3 DI RNAs does not augment inhibition of segment replication**
135 DI RNAs suppress replication of WT segments and we investigated whether combining S1 DI
136 RNA and S3 DI RNA increases antiviral activity as compared to the single DI RNAs. For this
137 purpose, we employed the mini replicon assay as described above but used WT 293T cells.
138 Transfection of 293T cells with the IAV reporter segment alone yielded background levels of
139 luciferase activity, while cotransfection of these cells with plasmids encoding the reporter, IAV
140 polymerase proteins and NP yielded luciferase levels a 1000-fold over background. Additional
141 cotransfection of plasmids encoding S1 DI RNA or S3 DI RNA at two concentrations resulted in
142 a dose-dependent decrease of luciferase activity, with S1 DI RNA showing a stronger inhibitory
143 effect as compared to S3 DI RNA. Further, cotransfection of plasmids encoding both DI RNAs

did not result in further decrease of luciferase activity as compared to that measured for S1 DI RNA alone, indicating that the presence of two DI RNAs did not increase replication interference in the mini replicon assay.

S1S3 DIPs do not induce MxA expression with increased efficiency as compared to S1 or S3 DIPs

The induction of the interferon system is believed to contribute to DIP antiviral activity. Therefore, we assessed whether the presence of two DI RNAs as compared to one modulated induction of MxA expression. Interferon α or infection with A/WSN/33 induced MxA expression at least 100-fold, as determined by quantitative RT-PCR (Fig 4). S1 and S3 DIPs induced MxA with similar efficiency as A/WSN/33 and MxA induction was not augmented when S1S3 DIPs harboring two DI RNAs were studied (Fig 4). Thus, combining S1 and S3 DI RNA in DIPs did not enhance induction of MxA expression.

S1S3 DIPs do not show increased antiviral activity as compared to S1 or S3 DIPs

We finally investigated whether the presence of two DI RNAs increases DIP antiviral activity. For this, we used MDCK cells, a dog cell line frequently used to propagate IAV. Furthermore, we employed the human lung cell line Calu-3 as a mimic of human respiratory epithelium. In order to assess DIP antiviral activity in these cell lines, different DIP dilutions were either added 24 h prior to virus (24 h setting) or DIP and virus were added at the same time (0 h setting). Addition of DIPs at 24 h prior to virus resulted in increased antiviral activity in both MDCK and Calu-3 cells as compared to co-inoculation of cells with virus and DIP (Fig 5). Further, S1 DIP showed increased antiviral activity as compared to S3 DIP in keeping with the increased inhibitory activity of S1 relative to S3 DI RNA in the minireplicon assay. Finally, the antiviral

activity of S1S3 DIPs was comparable to that of the S1 DIP, demonstrating that under the conditions chosen the presence of two DI RNAs instead of one did not augment antiviral activity.

Discussion

DIPs are naturally occurring byproducts of IAV replication. They contain DI RNA that inhibit IAV infection and could be developed for antiviral intervention[11,13,17]. However, it is unclear whether the presence of two DI RNAs within DIPs can augment antiviral activity as compared to otherwise isogenic counterparts. Here we demonstrate that cell lines expressing PB2 and PA allow the generation of DIPs harboring segment 1- and 3-derived DI RNAs in the absence of WT virus. Moreover, we provide evidence that the presence of two DI RNAs does not enhance interferon induction and antiviral activity.

In our current study, we chose to produce IAV DIPs with deletions in segments 1 and 3, since DI RNAs preferentially arise from segments 1, 2 and 3 [18,19] . Further, we chose DI RNAs with medium sized internal deletions that result in readily detectable but not maximal antiviral activity, which could have been obtained upon using DI RNAs with larger deletions. However, we have previously observed that increasing the deletion size does not only increase antiviral activity but also reduces the efficiency of DIP production (not shown), most likely due to DIP auto-inhibition, as discussed below. In contrast, we used a system in which potentiation of DIP antiviral activity due to the presence of two DI RNAs should have been readily detectable since DIPs harboring a single DI RNA exerted measurable but not maximal antiviral activity.

DIPs may interfere with IAV infection in two ways. They are robust activators of the IFN response and potent inducers of ISGs and the antiviral activity of ISG products is believed to contribute to DIP antiviral activity [20]. Moreover, the smaller size of DIP RNAs allows them to replicate faster than the corresponding WT DI RNAs, hence allowing DIPs to outcompete WT virus for resources that limit genome replication [13]. We would have expected that the presence of two DI RNAs might increase sensing of DIP by RIG1 and MDA5 and/or might augment the DIP induced limitation of resource for genome replication, which both should result in increased

213 antiviral activity. However, the presence of two DI RNAs did augment neither inhibition of
214 genome replication nor induction of MxA or antiviral activity.
215 At present, we can only speculate why the presence of two DI RNAs did not increase antiviral
216 activity. One potential explanation could be the above-mentioned auto-inhibition. The rescue of
217 S1S3 DIPs yielded 100-fold lower titers as compared to S1 or S3 DIPs, in keeping with
218 potentially increased self-inhibiting capacity of S1S3 DIPs relative to S1 or S3 DIPs. It is thus
219 conceivable that this auto-inhibitory activity might have limited S1S3 DIP amplification in IAV
220 co-infected cultures and might have thereby limited DIP antiviral activity. In fact, previous
221 studies have documented DIP auto-inhibition and have proposed a role for NP levels in this
222 process. Finally, it is noteworthy that our findings are consistent with work from Zhao and
223 colleagues who showed that simultaneous expression of S1-, S2- and S3-derived DI RNAs did
224 not enhance antiviral activity relative to the single DI RNAs both in cell culture and in animals [21].
225 In conclusion, our study provides evidence that the presence of two DI RNAs in IAV
226 DIPs does not enhance antiviral activity. This finding should be useful to current efforts to
227 develop DIPs for antiviral activity and might promote our understanding of the role of DIP in
228 IAV spread and pathogenesis.
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235

Materials and Methods

Plasmids and oligonucleotides. Sequences of PB1 and PA were optimized for efficient human and influenza A virus codon usage and for maximal divergence from original sequences to reduce the potential for recombination with WT viral sequences [22] . Optimized sequences were synthesized (GeneArt, Germany) and subcloned via NotI and XhoI into pQCXIP-mcs [23]. For insertion of alternative selections markers we used a derivative vector, pQCXIP-Cherry-mcs, where a puromycin resistance-mCherry fusion gene had been inserted into pQCXIP-mcs using an EcoRV site in the vector and introducing a MunI site upstream of the puromycin resistance gene. The puromycin resistance-mCherry fusion was then replaced by the neomycin (from pcDNA3), hygromycin (from pGL4.32) and blasticidin (pcDNA6/TR) resistance genes, to give pQCXIN-mcs, pQCXIHy-mcs and pQCXIBL-mcs, respectively. Using these vectors the retroviral plasmids pQCXIN-PB2opt, pQCXIBL-PAopt and pQCXIHy-PB1opt were generated by subcloning the respective genes via NotI and XhoI.

Plasmids to rescue influenza virus strains PR8wt [22,24] and WSN [24] have been published. To generate segment 1 (S1) derived DIPs with intermediate size regions encompassing the packaging regions (829 bp, 354 bp 5', 448 bp 3'), we used splice overlap PCR using primers fluA AarI-PB2-1G (5- CGATCACCTGCTCGAGGGAGCGAAAGCAGGTC)/ DIP-448-rev (5-TTGCTTTGGTGGAGCGGCTTAAGCGGCCGCTGCGGTACCGGATCCAACTGTATTTGTTATTGG) and DIP-448-for (5-CCAATAACAAATACAGTTGGATCCGGTACCGCAGCGGCCGCTTAAGCCGCTCCACCAAAGCAA)/ fluA AarI-PB2-2341R (5-CGATCACCTGCTCTCTATTAGTAGAAACAAGGTCGTTT). The generation of segment 3 (S3) derived DIPs with intermediate size regions encompassing the packaging regions of the respective segments (958 bp, 447 bp 5', 484 bp 3'), also employed splice-overlap PCR using

260 primers fluA AarI-PA1-1 (5- CGATCACCTGCTCGAGGGAGCAAAAGCAGGTAC-3)/
261 DIPS3-447mcs-rev (5-
262 CATCTCCATTCCCCATTTTTAAGCGGCCGCTGCGGTACCAGATCTCTCAGATTTAATT
263 TTATT-3) and DIPS3-447mcs-for (5-
264 AATAAAATTAAATCTGAGAGATCTGGTACCGCAGCGGCCGCTTAAAAATGGGGAAT
265 GGAGATG-3)/fluA AarI-PA1-2233R (5-
266 CGATCACCTGCTCTCTATTAGTAGAAACAAGGTACTT-3). Both assembled fragments
267 were cloned into pHW2000GGAar by Golden Gate cloning as described earlier [25].
268
269 **Cells and viruses.** All cells were cultured at 37°C and 5% CO_2. Madin-Darby Canine Kidney
270 cells (MDCK) were incubated in Glasgow Minimum Essential Medium (GMEM; Gibco) with
271 10% fetal bovine serum (FBS; PAN Biotech), penicillin (100 IU/ml) and streptomycin (100
272 µg/ml) (pen/strep; PAN Biotech). MDCK PB2opt/PAopt cells were cultured in the presence of
273 1.5 µg/ml puromycin and 500 µg/ml neomycin respectively. 293T human embryonic kidney cells
274 were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco) containing 10% FBS
275 and pen/strep. 293T PB2opt/PB1opt/PAopt were grown in presence of 1.5 µg/ml puromycin, 500
276 µg/ml neomycin and 5 µg/ml blasticidin. Calu-3 cells were maintained in Minimum Essential
277 Medium (MEM) with 10% FBS, pen/strep, 1x non-essential amino acid solution (10x stock,
278 PAA) and 10 mM sodium pyruvate (Thermo Fisher Scientific). Identity of human cells lines was
279 confirmed by STR typing [26]. Species specificity of MDCK cells was confirmed by cytB
280 sequencing [27].
281 Influenza A virus A/WSN/33 produced in embryonated chicken eggs and adapted by propagation
282 on A549 cells through multiple passaging was used to assess antiviral activity of DIPs. We also
283 used a recombinant vesicular stomatitis virus (VSV) that expresses a dual reporter consisting of

284 eGFP and firefly luciferase from an additional transcription unit located between the open-
285 reading frames for the viral glycoprotein and polymerase [28].
286
287 **Production of retroviral vectors.** For the production of MLV particles, 293T cells were seeded
288 in T25 flasks at a concentration of 2 x 10^5 cells/ml in DMEM. The next day, cells were
289 transfected using the calcium phosphate transfection method with 6 µg of retroviral vector
290 pQCXIP-PB2opt, pQCXIN-PB2opt, pQCXIBL-PAopt, or pQCXIHy-PB1opt along with 3 µg
291 MLV-gag-pol plasmid and 3 µg VSV-G expression plasmid [29,30]. At 48 hours post transfection,
292 supernatant containing the MLV particles was harvested and cleared by passaging through a 0.45
293 µm filter and stored at -80°C for further use.
294
295 **Transduction and selection of cell lines.** 96 well plates were seeded with 5,000 (MDCK) or
296 10,000 (293T) cells/well in 50 µl of cell culture medium. MLV transduction particles (100µl per
297 well) were added the next day followed by spinoculation at 4,000 x g for 30 minutes to enhance
298 transduction efficiency. At 48 hours post transduction, transduced cells were detached and seeded
299 in 24 well plates in culture media supplemented with antibiotics. Selection was continued until
300 untransduced control cells had died.
301 To generate double transduced cells, transductions were performed in sequential order by first
302 transducing PB2 gene followed by selection. Expression of PB2 was confirmed by western blot.
303 Subsequently PB2 expressing cells were transduced for further expression of PB1 and PA
304 proteins. For selection of stable cell lines, 1 µg/ml and 1.5 µg/ml of puromycin was used for
305 293T cells and MDCK cells respectively , and 500 µg/ml neomycin, and 5 µg/ml blasticidin were
306 used for both 293T cells and MDCKs.
307

Immunoblot. MDCK and 293T cells stably expressing the IAV polymerase proteins were seeded in 6 well plates at a cell density of 2 x 10^5 cells/well. The next day, cells were harvested, lysed in 200 µL of Laemmli SDS-PAGE sample buffer (5% glycerin, 1% SDS, 2.5% ß-mercaptoethanol, 0.5% Bromophenol blue, 0.5 mM EDTA, 0.5 M Tris pH 6.8) and heated at 95 °C for 10 min. For each sample, 10 µL were loaded on 12.5% polyacrylamide gels and separated via SDS-PAGE. Proteins were blotted onto a nitrocellulose membrane (GE health care) using a Mini-PROTEAN Tetra Cell (BioRad). Membranes were blocked by incubation in 5% skim milk diluted in PBS-0.1% Tween (PBS-T) for 1 hour. Subsequently, membranes were incubated with primary antibodies against PB2 (1:1,000, GeneTex, Irvine, USA), PB1 (1:1,000, GeneTex), PA (1:500, GeneTex), and ß-actin (1:1,000, Sigma-Aldrich) overnight at 4°C. The next day, membranes were washed in PBS-T and incubated with anti-rabbit HRP (horseradish peroxidase)-conjugated secondary antibodies (1:10,000, Dianova) for one hour at room temperature. In order to visualize protein bands, chemiluminescent substrate HRP juice plus (PJK) was added to the membranes and signals recorded with a ChemoCam imager (Intas).

Minireplicon assay. 293T cells were seeded in 12 well plates at a concentration of 2 × 10^5 cells/well. Cells were transfected following an established protocol [16]. Briefly, cells were cotransfected with PB1 (10 ng), PB2 (10 ng), NP (100 ng), reporter segment encoding firefly luciferase (50 ng) and plasmid encoding a DI RNA or an empty plasmid (concentrations indicated in figures). Cells were washed 6 hours post transfection and fresh DMEM was added. After 24 hours post transfection, cells were harvested and firefly luciferase activity was measured by using a Beetle-Juice Luciferase substrate (PJK). The values were recorded on a Plate Chameleon V reader (Hidex) using Microwin 2000 software.

Production of DIPs. T25 flasks were seeded with a coculture of 293T cells stably expressing PB1, PB2, PA (1.4×10^6 cells) and MDCK cells stably expressing PB2 and PA (0.4×10^6 cells) in DMEM growth medium (Gibco). For the production of DIPs encoding two DI segments derived from IAV genomic segments 1 and 3 (S1S3 DIPs), cells were cotransfected with plasmids encoding DI RNA derived from segments 1 and 3 of PR8 origin, jointly with plasmids encoding IAV genomic segments 2 and 4 to 8 of WSN origin using the calcium phosphate method. Similarly, for the production of DIPs expressing single DI segments with a deletion in segment 1 (S1 DIPs) or segment 3 (S3 DIPs), cells were cotransfected with 7 IAV genomic segments and either one DI segment derived from S1 (for S1 DIPs) or one derived from S3 (for S3 DIPs). After overnight incubation cells were washed once with PBS and fresh DMEM infection medium (2% FBS, 1% pen/strep) was added. As negative control, parental MDCK and 293T cells were also transfected. Supernatants from rescue were harvested at 3, 5 and 7 days post transfection, cleared by centrifugation at 1500 x g for 10 minutes and stored at -80 °C for further use. Titers for DIP supernatants were determined by focus formation assay on PB2, PA expressing MDCK cells, as described [15].

Characterization of DIP integrity. Integrity of DIPs was controlled by segment specific RT-PCR. For the isolation of viral RNA from DIPs, 1.5 mL of DIP supernatant were centrifuged at 13,300 rpm for 2 hours and total viral RNA from the resulting pellet was extracted using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. 0.1 µg RNA was used as template for cDNA synthesis using the SuperScript III First-Strand Synthesis System (ThermoFisher Scientific). cDNAs corresponding to S1, S3 or S1S3 DIPs was amplified using segment specific PCR employing published primers [31] fluA PB2-1 (5-AGCRAAAGCAGGTCAATTATATTCA)/ fluA PB2-2341R (5-

356 AGTAGAAACAAGGTCGTTTTTAAACTA)for S1 and fluA PA-1 (5-
357 AGCRAAAGCAGGTACTGATYCGAAATG)/ fluA PA-2233R(5-
358 AGTAGAAACAAGGTACTTTTTTGGACA) for S3
359
360 **Antiviral activity of DIPs.** In order to assess antiviral activity of DIPs, MDCK cells were seeded
361 at a concentration of 10,000 cells/well in 96 well plates and co-infected with A/WSN/33 (MOI
362 0.1) or VSV (MOI 0.01) jointly with DIPs (MOI 1 and 10 fold dilutions) for one hour in GMEM
363 growth medium. Alternatively, cells were incubated with the DIPs 24 hours prior to infection
364 with A/WSN/33 or VSV. Co-infected cells were washed one hour post coinfection and GMEM
365 growth medium was added. Supernatants were harvested 72 hours post coinfection and infectious
366 titer was quantified by focus formation assay on MDCK cells, as described [32]. For analysis of
367 DIP antiviral activity in Calu-3 cells, cells were seeded at 20,000 cells/well in 96 well plates in
368 MEM growth medium and either co-infected with A/WSN/33 (MOI 0.001) and DIPs (MOI 0.1
369 and 10 fold dilutions) or preincubated with DIPs 24 hours prior to infection with A/WSN/33.
370 After one hour, co-exposed cells were washed and MEM growth medium was added.
371 Supernatants were harvested 72 hours post infection and infectious titer was quantified by focus
372 formation assay on MDCK cells.
373
374 **Quantitative real time PCR analysis.** The induction of MxA by S1, S3 and S1S3 DIPs was
375 assessed by qRT PCR. For this assay, Calu-3 cells were seeded in 12 well plates at 4 x 10^5
376 cells/well and inoculated with A/WSN/33 (MOI 0.1), DIPs (S1, S3 or S1S3 MOI 0.1) or pan-
377 IFNα (100 U/ml, PBL Assay Science) in MEM. As negative controls, cells were also inoculated
378 with fresh MEM media or supernatants cleared from rescue of DIPs from parental MDCK and
379 293T cells. Following inoculation, cells were incubated for one hour, then washed once with

PBS, and cultured in fresh MEM media. 24 hours post treatment, cells were harvested and total RNA was extracted using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. Extracted RNA was then treated with RNase free DNase (New England BioLabs, NEB) and quantified using nanodrop. For cDNA synthesis, 0.5 µg of RNA was used as template using the SuperScript III First-Strand Synthesis System (ThermoFisher Scientific), following the protocol for random hexamers in a total volume of 20 µl. Subsequently, 1 µl of cDNA was analyzed utilizing the QuantiTect SYBR Green PCR Kit (Qiagen). All samples were tested in triplicates on a Rotorgene Q device (Qiagen) for transcript levels of ß-actin (ACTB, internal transcript control) and myxovirus resistance protein A (MxA, indicator for IFN induction) using primers mentioned [33]. Transcript levels were given as cycle thresholds (Ct values) and data are shown as fold change expression of MxA relative to ACTB. For this, the average CT values for samples with MxA and ACTB were averaged. The differential fold expression of the target gene over the internal control was calculated using the Livak method [34] as $2^{-\Delta\Delta Ct}$ where: ΔCt =average Ct (target gene/MxA) – average Ct (reference gene/ACTB) and $\Delta\Delta Ct = \Delta Ct$ (values for experimental conditions) –ΔCt (values for control conditions).

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Competing interests

The authors declare no competing interests.

Author Contributions:

N.B., Investigation, Methodology, Analysis, Writing - review and editing; P.A., Investigation, Methodology, Analysis, Writing - review and editing; S.G., Investigation, Methodology; M.W., Conceptualization, Methodology, Formal Analysis, Investigation, Writing – review and editing, Supervision; S.P., Conceptualization, Writing – Original Draft Preparation, Supervision, Funding Acquisition.

Data availability

All data in this study are included in the manuscript

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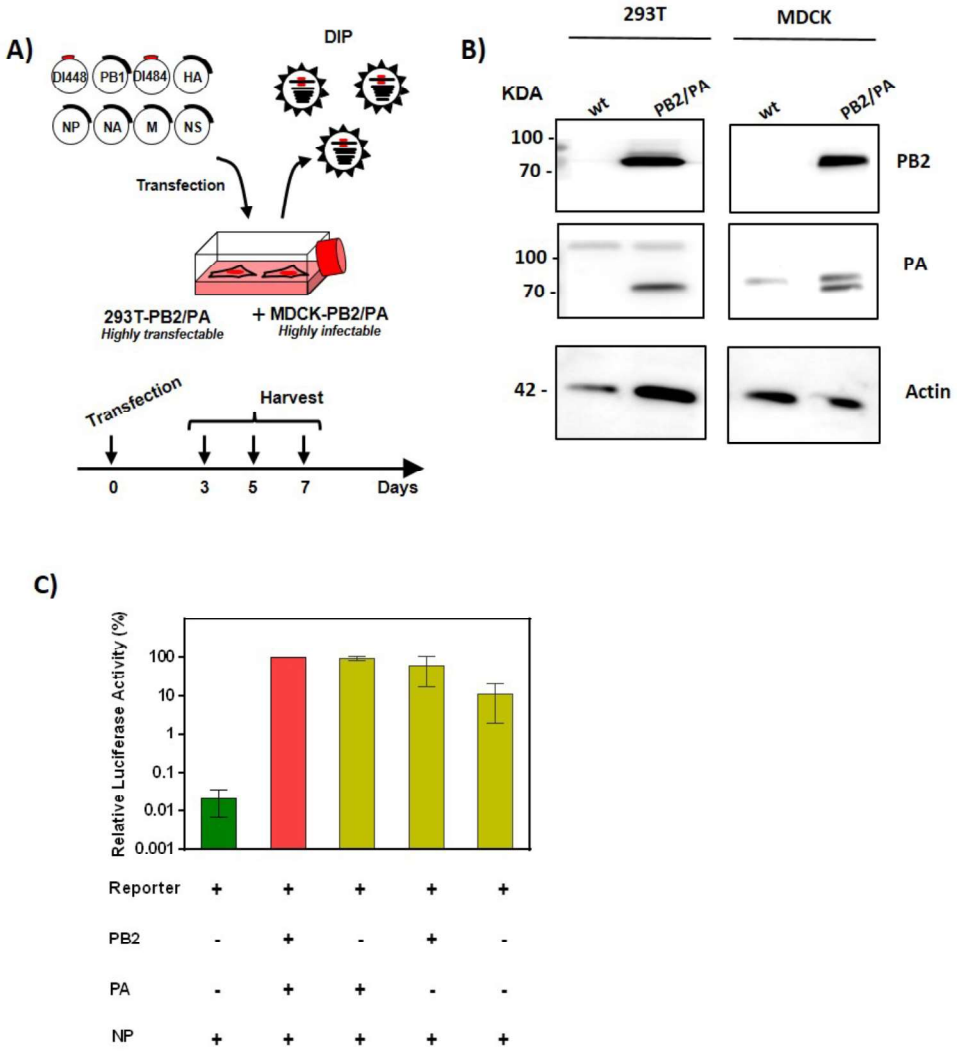

527

528 **Figure 1: Construction of MDCK and 293T cells stably expressing IAV polymerase proteins**
529 **and analysis of DI RNA-mediated inhibition of genome replication. A)** Work flow for
530 production and quantification of DIPs harboring segment 1- and 3-derived DI RNAs. For
531 production of DIPs, a co-culture of 293T and MDCK cells stably expressing at least two IAV
532 polymerase proteins was co-transfected with plasmids encoding WT IAV genomic segments 2
533 and 4-8 as well as segments 1 and 3 containing internal deletions that convert these segments into
534 DI RNAs. Supernatants were harvested at the indicated time points. **B)** Stable expression of PB2
535 and PA proteins in 293T and MDCK cell lines. Expression of PB2, PB1 and PA in 293T and PB2

536 and PA in MDCK cells was analyzed by immunoblot. Detection of β-actin served as a loading
537 control. **C)** Inhibition of genome replication by DI RNAs. 293T cells stably expressing PB2,
538 PB1 and PA were transiently co-transfected with the indicated combinations of plasmids
539 encoding PB2, PB1, PA and NP and an IAV luciferase reporter segment. Luciferase activities in
540 cell lysates were determined at 24 h post transfection. Luciferase activity measured for cells co-
541 transfected with all plasmids was set as 100%. The average of three independent experiments is
542 shown. Error bars indicate SEM.
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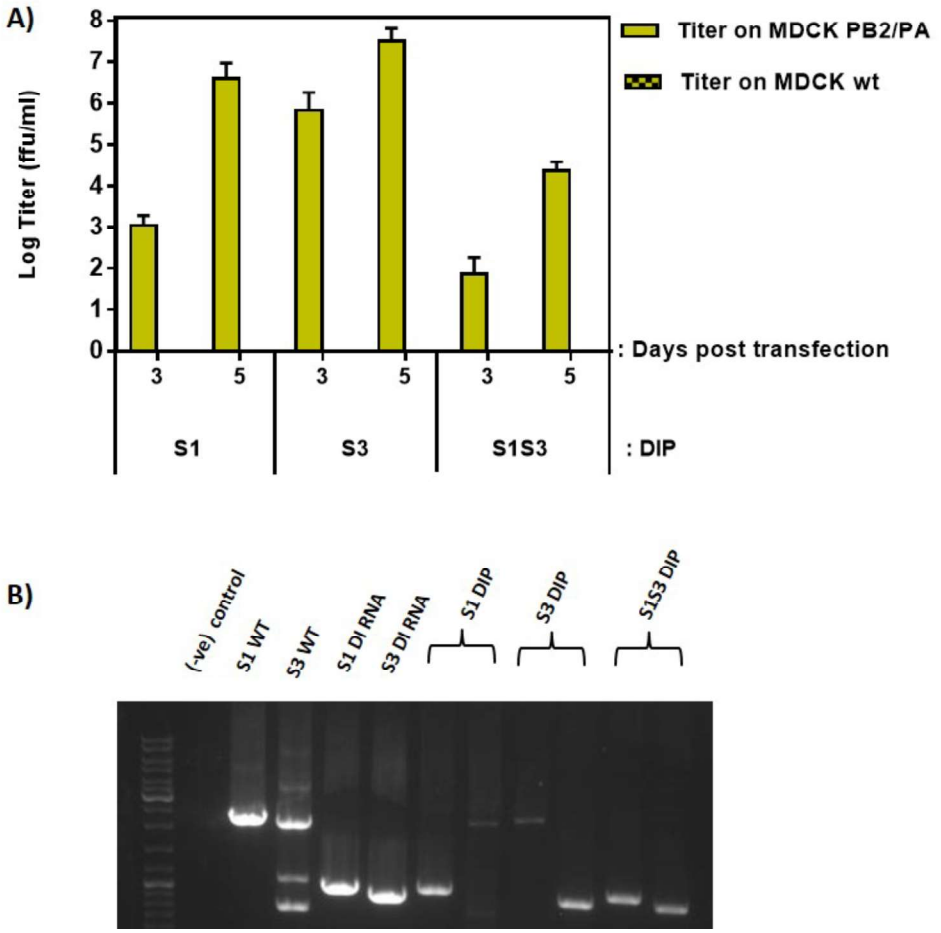

559

560 **Figure 2: Production of DIPs from complementing cell lines. A)** A co-culture of PB1/PB2/PA
561 293T cells and PB2/PA MDCK cells was transfected with plasmids encoding S1, S3 or S1S3 DI
562 RNAs jointly with the remaining IAV WT genomic segments. Supernatants were harvested on
563 days 3, 5 and 7-post transfection. Titers were determined by focus formation assay using PA/PB2
564 MDCK cells. The average of three independent experiments is shown. **B**) DI RNA incorporation
565 into DIPs. Supernatants containing DIPs harboring DI RNAs derived from segment 1 (S1) or 3
566 (S3) or harboring both DI RNAs (S1S3) were analyzed by RT-PCR. Plasmids encoding wt
567 segments S1, S3 or the corresponding DI RNAs served as positive control. A single
568 representative experiment is shown. Results were confirmed in four independent experiments.

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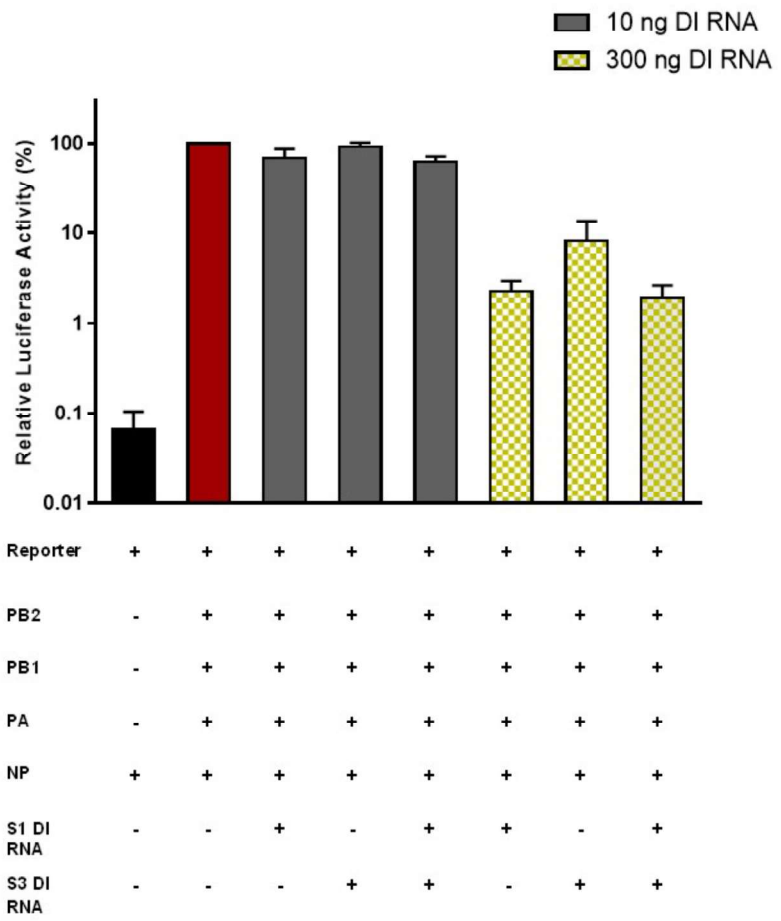

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571 **Figure 3: The presence of two DI RNAs does not increase inhibition of genome replication.**

572 293T cells were cotransfected with the indicated combinations of plasmids encoding the viral

573 polymerase proteins, NP, a segment 8-based luciferase reporter and the indicated DI RNAs at 10

574 and 300 ng. Luciferase activity was determined at 24 h posttransfection. The results of single

575 round DI RNA induced replication inhibition are shown. The average of three independent

576 experiments is shown. Error bars indicate SEM.

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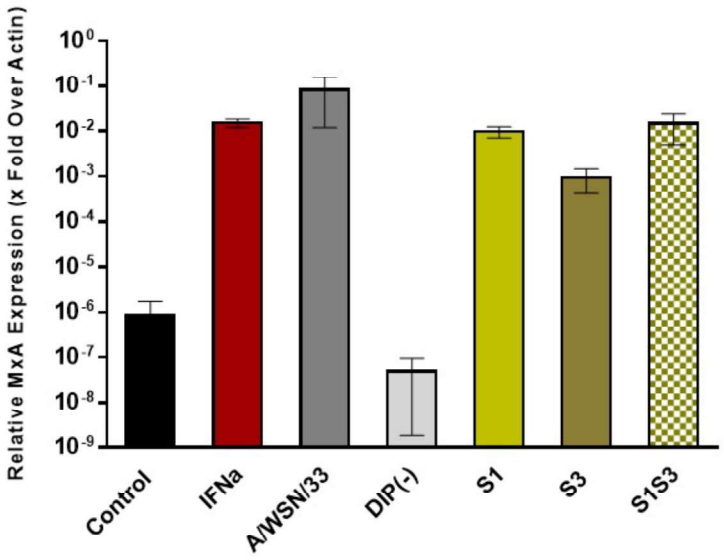

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582 **Figure 4: The presence of two DI RNAs does not increase MxA induction.** Calu-3 cells were
583 either treated with interferon α or inoculated with IAV (A/WSN/33) or DIPs at an MOI of 0.1.
584 Cell lysates were harvested 24 h after treatment or infection, RNA was isolated and MxA
585 expression was analyzed by quantitative real time PCR. MxA transcript levels were normalized
586 against transcript levels of β-actin. The average of three independent experiments is shown as x
587 fold over actin. Error bars indicate SEM.

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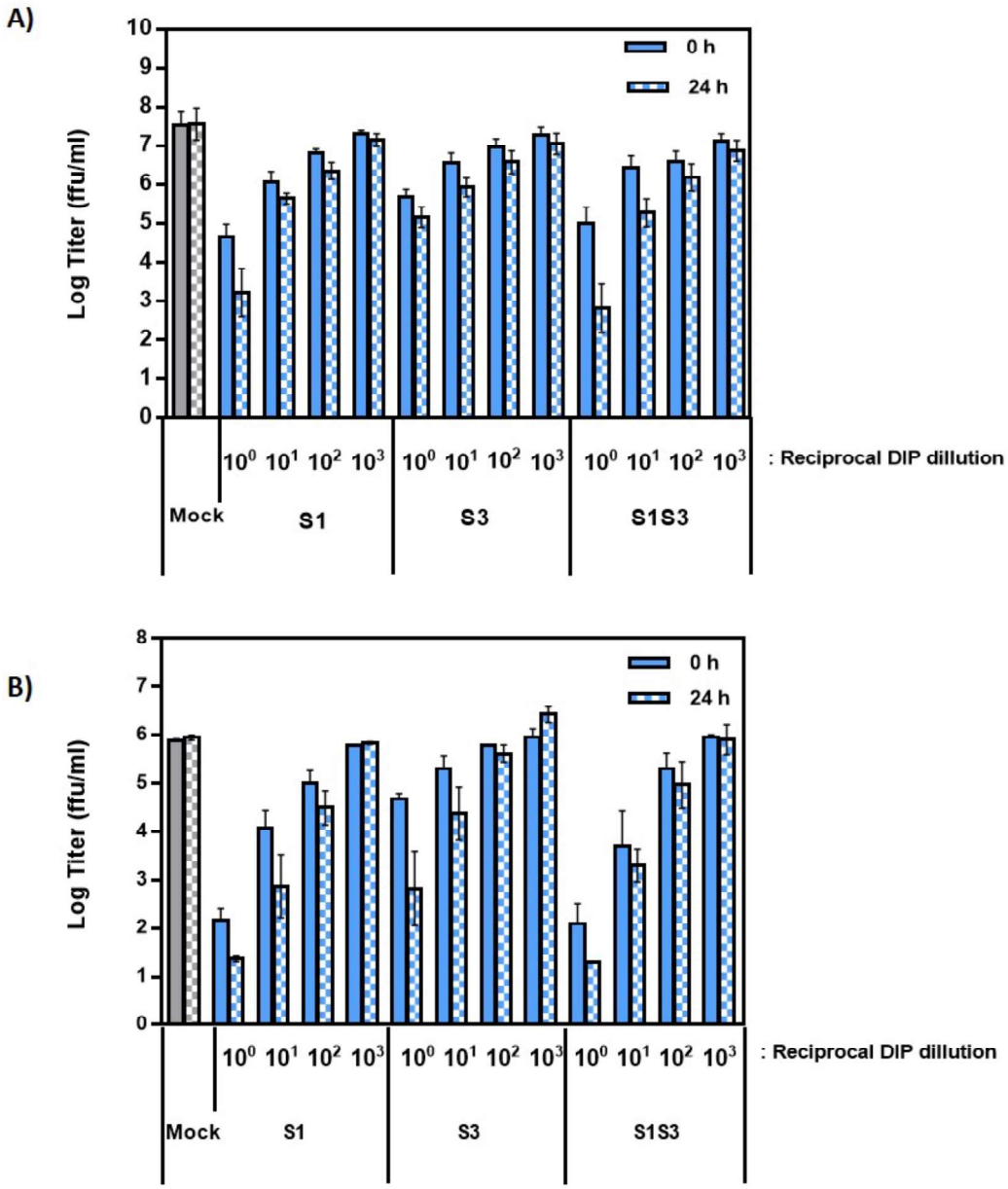

Figure 5: The presence of two DI RNAs does not increase antiviral activity. A) MDCK cells we either co-inoculated with A/WSN/33 and the indicated DIPs at the indicated dilutions or cells were preincubated with DIPs for 24 h before virus was added. Infectivity was measured at 72 h postinfection. The Log transformed average of five independent experiments is shown. Error bars indicate SEM. **B)** The experiment was carried out as described for panel A but Calu-3 cells were

600 used as targets. The log transformed average of three independent experiments is shown. Error
601 bars indicate SEM.
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5. Discussion

The influenza disease is a serious respiratory illness associated with significant morbidity and mortality (CDC, 2020). The ability of IAV to constantly accumulate mutations may render current therapeutic approaches ineffective and vaccines must be reformulated on an annual basis (Coelingh et al., 2014). Hence, the development of novel strategies to combat influenza is an important task. DIPs contain all structural proteins found in WT virus but harbor at least one defective genomic segment, which interferes with the amplification of WT virus. It is posited that DIPs replicate their genome faster than WT virus owing to their shorter defective segments, which allows them to outcompete WT virus for cellular and viral resources (Dimmock & Easton, 2015). Moreover, DIPs can induce the IFN response and hold potential as novel antiviral agents (Laske et al., 2016). However, the relative contributions of these processes to DIP antiviral activity are unclear. It is also unknown whether antiviral activity can be further enhanced by the use of DIPs harboring more than one DI RNA. Finally, DIPs amplification requires coinfection with WT virus, which raises biosafety concerns and has hampered the development of DIPs as potential antivirals. The first goal of my thesis was to establish a system that allows the safe production of DIPs in the absence of infectious virus. Upon the successful establishment of such a system, my thesis focused on assessing the relative contribution of IFN induction and replication interference to DIP antiviral activity. Finally, this thesis explored whether DIPs with more than DI RNA can augment DIP antiviral activity.

5.1. A cell culture system allowing production of DIPs in absence of infectious virus

The production of DI-244 from a plasmid system has been established (Dimmock et al., 2008; Duhaut & Dimmock, 2002). In this system, cells are transfected with the eight IAV genomic segments in combination with a plasmid encoding the DI-244 segment, which results in the generation of both WT virus and DIPs. In order to utilize these preparations, WT virus as known as standard virus must first be inactivated by UV irradiation (Dimmock & Easton, 2015; Nayak et al., 1978). This approach posits that UV irradiation inactivates standard virus but not DIPs, since the lesions induced by UV light anywhere within the 13,600-nucleotide genome of the WT virus will eliminate infectivity, while only lesions within the truncated DI RNA segments of DIPs will

compromise DIP production in cells coinfected with helper virus and DIP (Dimmock et al., 2008; Wasik et al., 2018). However, the need to ensure inactivation of WT virus and batch-to-batch differences in DIP infectivity and may complicate the interpretation of experimental data (Dimmock & Easton, 2015) and renders this approach unrealistic for production of DIPs as therapeutics. My results showed that cell lines engineered to express PB2 in trans allow the rescue and quantification of DI-244 in the absence of WT and only from plasmids. The generation of DI-244 reported in the first publication is based on the IAV strain A/PR/8/1934 (PR8). It is well demonstrated that laboratory strains of PR8 are avirulent in human trials even upon infection with high doses (Beare et al., 1975; Dimmock & Easton, 2015). Hence, re-assortment between PR8-derived DI-244 and WT IAVs should not result in viruses with enhanced virulence or transmissibility compared to WT virus. A fraction of the elderly might have pre-existing immunity, since individuals who were infected with this virus in the course of the influenza epidemics of 1934 are now around 80 years old (Dimmock & Easton, 2015). However, immune responses fade over time and any responses against PR8 acquired in 1934 should be weak now and would not interfere markedly with DI-244 antiviral activity. Thus, PR8-based DI-244 is safe for use in humans and immunity against this virus is not expected.

MDCK cells expressing codon optimized PB2 (PB2opt) yielded titers up to 4 million pfu/ml and no infectious virus was produced due to recombination between DI-244 RNA and PB2 RNA encoded by the cells. The absence of infectious virus was confirmed by RT-PCR analysis, immunofluorescence, and focus formation assay, indicating that DI-244 preparations are infectious and safe. MDCK-PB2opt cells also allowed the quantification of DI-244 infectious particles. This is a significant step ahead since so far it was only possible to quantify total viral particles by hemagglutination assays and RT-PCR but not be assays measuring infectivity (Dimmock & Easton, 2015; Wasik et al., 2018). Moreover, my study demonstrates that rescued DI-244 exerts antiviral activity against both H1N1 and H3N2 viruses, in line with H3N2 replicase recognizing and replicating H1N1 genome segments. Moreover, my data show that a ratio 1000 DI-244 particles to 1 WT infectious particle is required for DI-244-mediated antiviral activity. This is in line with previous work by Dimmock and colleagues, where it was estimated that a ratio of 3400: 1 DIP to virus particles is required to protect mice from a lethal dose of influenza A virus (Dimmock & Easton, 2015).

5.2 ISG induction and not replication interference mainly contributes to DIP antiviral activity

It is commonly held that the smaller size of DI-244 allows it to outcompete WT virus for vital replicative resources. However, direct evidence in support of this concept is largely lacking (Schoggins et al., 2011). Further, it is largely unclear whether replication interference or IFN induction is mainly responsible for DIP antiviral activity. In order to answer these questions, the current study describes the construction of DIP segments with nested deletions derived from segments 1, 2, and 3. These segments where chosen since literature indicates that DI RNAs more frequently arise from these segments as compared to others (Dimmock & Easton, 2014; Frensing et al., 2013). The present study shows that any internal deletion within the open reading frames of segments 1, 2, or 3 is sufficient to convert the genomic RNAs into DI RNAs. Moreover, the replication inhibition mediated by these DI RNAs is inversely correlated to their size, at least in the absence of an intact IFN response.

The induction of a type I IFN response results in the expression of over 400 ISGs, many of which inhibit viral infection (Schoggins et al., 2011). In addition, it is known from the literature and re-confirmed in the current work that trypsin, which is required for HA cleavage, also inactivates IFN. In order to assess the contribution of IFN induction to DIP antiviral activity, DI-244 bearing a A/WSN/33 backbone (A/WSN/33 does not require trypsin for HA cleavage) was inoculated onto A549wt and A549 STAT1$^{-/-}$ cells 24 hours prior to infection with WT virus in the absence of trypsin. We rationalized that the addition of DIPs to A549 cells containing an intact IFN response would induce the IFN response and would thereby enhance antiviral activity. Indeed, inoculation of DI-244 on A549 cells 24 hours prior infection with WT augmented antiviral activity between 100 to 1000-fold. Additionally, in the presence of an intact IFN response, DI RNA length had little to no impact on antiviral activity. Unexpectedly, DI-244 also displayed moderate antiviral activity in A549 STAT1$^{-/-}$ cells and did not lead to the up regulation of MX1 induction or secretion of IFN. These results indicate that DIPs may inhibit WT virus by a partially STAT1-independent manner. It should be noted that STAT1 is dispensable for IRF3-dependent stimulation of ISG expression (Ashley et al., 2019; Majoros et al., 2017; Nan et al., 2017). Studies have demonstrated that STAT2 can form homodimers which interact with IRF9 to form an ISGF3 like complex which activates the transcription of ISGs in response to low levels of IFN α. These

findings reveal that it is important to study DIPs in IFN competent model systems and that DIPs may be more effective in the prophylactic (DIP added before virus) setting in comparison to the therapeutic setting (DIP added after virus), since cells pre-incubated with DI-244 were more efficiently protected against influenza A virus infection.

5.3 Evidence that two instead of one DI RNA does not enhance DIP antiviral activity

At present, DI-244 is the most widely studied influenza A virus DIP owing to its potent antiviral activity *in vivo* and *in vitro* (Dimmock et al., 2008; Dimmock & Easton, 2015). During replication, the influenza virus packages one copy of every genome segment into particles (Lamb, 2008) and it is likely that a subset of virions can carry more than one defective RNA derived from different segments (Dimmock & Easton, 2015). However, the presence of such defective particles has not been documented in the literature and it is unknown whether DIPs containing two DI RNAs have enhanced antiviral activity compared to their isogenic counterparts. Thus, one could speculate that DIPs harboring more than one DI RNA may induce the IFN response or may interfere with genome replication more efficiently than their otherwise isogenic counterparts.

My results demonstrate that cell lines expressing PA and PB2 allow the generation of S1S3 DIPs harboring deletions in both segments 1 and 3. I chose to produce S1S3 DIPs harboring medium sized deletions, which retained 829 bp and 958 bp, encompassing the packaging regions of the S1 and S3 segments, respectively. The production of DIPs harboring two DI RNAs with larger deletions was not possible, probably owing to DIP self-inhibition as discussed below. Contrary to expectation, DIPs with segment 1 and 3 derived DI RNAs (S1S3 DIPs) did not exhibit enhanced Mx1 induction or antiviral activity as compared to DIPs harboring either a segment 1 or a segment 3 derived DI RNA (S1 DIP, S3 DIP). These results are consistent with previous work by Zhao and colleagues which demonstrated that simultaneous transfection of DI RNAs encoding S1, S2, and S3 did not enhance antiviral activity against WT virus in both *in vitro* and *in vivo* settings (Zhao et al., 2018).

Cells co-infected with S1S3 DIPs and WT virus have the capacity to give rise to DIPs harboring deletions in segment 1, 3, and both. It is conceivable that this accumulation of DIPs may have a counterintuitive effect on antiviral activity whereby DIPs inhibit their own replication (Laske et al., 2016; Tapia et al., 2019). Previous studies have proposed that self-inhibition might be the result of NP depletion during the replication process and stipulate that DIPs with a very

high synthesis rate at high MOIs are mediocre competitors since they limit their own growth (Laske et al., 2016). Hence, it is conceivable that S1S3 DIPs harboring two DI RNA segments replicate faster than isogenic S1 and S3 DIPs leading to NP exhaustion and a reduction of encapsidated vRNAs into vRNPs.

In conclusion, my work provides evidence that presence of two DI RNAs does not enhance antiviral activity. This finding may prove useful to efforts attempting to develop and enhance DIP antiviral activity as novel IAV therapeutics.

6. Outlook

During my doctoral thesis I established cell culture systems allowing for the first time the production of IAV DIPs in the absence of WT virus and demonstrated their antiviral activity. The generation of such systems will facilitate the developments of DIPs as antivirals and significantly reduce biosafety issues. A notable finding was that introducing of a deletion of any size within genomic segments S1, S2, and S3 of IAV was enough to generate DIPs. Additionally, it was demonstrated that induction of ISG expression and not replication interference is the main contributing factor in DIP antiviral activity. Remarkably, my thesis provided evidence suggesting that DIPs may induce ISGs through an IFN-independent, partially STAT1-dependent pathway. These findings provide a basis for future attempts to develop DIPs as antivirals. They highlight that the antiviral activity of DIPs must be studied within an interferon competent system and that DIPs are more likely to be efficient in the prophylactic setting (DIPs before virus) rather than the therapeutic setting (DIPs after virus).

Further studies are required to understand why IFN induction and ISG expression are unrelated in the DIP-mediated immune pathway. It also remains unclear why DIPs inhibited IAV in STAT1$^{-/-}$ cells without inducing ISGs. Analyzing a potential competition of DIPs with IAV for cellular receptors is one possibility that needs to be addressed.

My work showed that DIPs harboring two DI RNAs instead of one do not have augmented antiviral activity and one possible explanation is DIP self-inhibition. To this end, further studies are needed to explore the determinants behind the self-inhibiting capacity of DIPs. Individual replication kinetics of S1, S3, and S1S3 DIPs must be analyzed and the availability of vital replicative resources including polymerase, NP, and NTP pools must be quantified.

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8. Appendix

8.1 List of abbreviations

World health organization	WHO
Hemmaglutinin	HA
Neuraminidase	NA
Influenza A virus	IAV
Influenza B virus	IBV
Influenza C virus	ICV
Influenza D virus	IDV
Hemmaglutinin Esterase Fusion Protein	HEF
Viral Ribonucleoprotein Complex	vRNP
Interferon	IFN
complimentary RNA	cRNA
Ribonucleoside tri-phosphate	rNTPs
Robert Koch Institute	RKI
Human airway trypsin like proteases	HATs
Inactivated Influenza vaccines	IIV
Live attenuated Influenza vaccines	LAIV
Defective interfering particles	DIPs
Wild type virus	WT
Defective interfering genomes	DI
IFN stimulated genes	ISGs
Open reading frame	ORF
Human embryonic kidney cells	HEK 293T cells
Madin Darbey canine kidney cells	MDCK cells
Pathogen recognition receptors	PRRs
Pathogen associated molecular patterns	PAMPs
Caspase activation and recruitment domains	CARDs
Mitochondrial antiviral signaling protein	MAVS
IFN induced family of transmembrane proteins	IFITIMs

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