miRNAs are short RNAs (20–24 nt) that regulate gene expression at a post-transcriptional level by binding to the 3’ untranslated region (UTR) of target sequences, resulting in translational repression or mRNA cleavage (Olsen & Ambros, 1999; Zeng et al., 2003) and are important gene regulators in many biological functions, including immune function (Lindsay, 2008).

A fundamental hurdle in the real world application of miRNA technology is the ability to deliver miRNAs efficiently to target cells. Numerous delivery strategies are currently being investigated including: (i) plasmid-based systems, (ii) lentiviral systems (Stegmeier et al., 2005; Tiscornia et al., 2004) and (iii) replication-competent avian sarcoma-leukosis (RCAS) retrovirus vector systems. While these systems have demonstrated varying degrees of efficacy, they have also been associated with deleterious sequelae. For example, plasmid-based delivery systems work well in vitro but are not feasible for use in vivo as transfection reagents required for plasmid entry are not available. Moreover, although lentivirus and RCAS systems can be used in vivo, they are known to integrate miRNAs permanently into the host genome. This uncontrolled expression of exogenous miRNA has been shown to have undesirable side-effects and has often been associated with myeloproliferative disorders (O’Connell et al., 2008).

We took advantage of the splicing mechanism associated with the expression of the non-structural (NS) gene segment of influenza virus to generate an artificial intron between the NS1 and NS2 gene products for the delivery of an immunomodulatory miRNA. Upon translation, the intron was excised, allowing expression of functional NS1 and NS2 proteins alongside the expressed miRNA. The tenOever lab originally designed the influenza A-based miRNA delivery technology and have published a number of articles showing both in vitro and in vivo use of this system (Langlois et al., 2013; Perez et al., 2009; Varble et al., 2010; Varble & tenOever, 2011). It is important to note, however, that our experiments demonstrate the first use, to our knowledge, of this delivery system to modulate immunity. We inserted the immunomodulatory miRNA pri-miR-155 within the NS segment to inhibit protein translation of the suppressor of cytokine signalling 1 (SOCS1), a known target of miR-155, and a negative regulator of cytokine production (Lu et al., 2009). Previous studies have demonstrated that increased expression of SOCS1 results in decreased expression of cytokines, including IFN-β and IL-6. Moreover, SOCS1 inhibition has been shown to enhance type I interferon antiviral responses directly (Fenner et al., 2006; Pothlichet et al., 2008; Sun et al., 2012). We therefore hypothesized that our miRNA delivery system would increase antiviral activity and decrease damaging proinflammatory responses. By using miRNA specific quantitative PCR (qPCR), a SOCS1 linked luciferase reporter assay and qPCR for cytokine expression we were able to confirm our hypothesis.

The design of our modified A/Puerto Rico/8/1934 (PR8) influenza A NS gene segment was similar to that described by Varble et al. (2010). Briefly, the NS1 splice acceptor site was altered using site-directed mutagenesis to prevent splicing. The NS1 fragment, including the 3’ non-coding region and NS1 ORF, was amplified from the splice acceptor mutant using Bm-NS-1 (Hoffmann et al., 2001) and a reverse primer described previously (Varble et al., 2010). The NS2 fragment, including the NS2 ORF and 5’ non-coding region, was amplified using a novel forward primer (NS2 EcoR I F: CTCTACCTCGAGATCTCCTCGAAA-TTCCACCATGGCCCTCTTCCTCCAGG) and Bm-NS-890r (Hoffmann et al., 2001). The murine pri-miR155 loop and flanking sequence was amplified from genomic DNA using novel primers (mmu-miR-155 forward: CGCTACCTC-GAGAAGTGTGTGGTTTAAGTTGC and mmu-miR-155 reverse: CTAATTGAGACATGACTGACG) and cloned into the pCAGGS expression vector (Uozumi et al., 2002). The murine pri-miR155 gene was then cloned into the A/Puerto Rico/8/1934 (PR8) influenza A virus plasmid. The virus was then used to transfect 293T cells and the resulting virus was then used to transfect BHK cells in order to assess the delivery of the miRNA.
reverse: CTCTACGAATTCCACGGTGGCCATTTGTTC) (Fig. 1a). It is important to note that the miR-155 seed region and the corresponding Socs1 target sequence are highly conserved in mammalian species (Fig. 1b). A scramble control miRNA was produced by replacing the mature miR155 sequence in the pri-miR155 hairpin with a scramble sequence. We then ligated the modified NS1, NS2 and pri-miRNA together to form our manipulated NS gene segment, which was subsequently ligated into the pHW2000 expression plasmid for later use in reverse genetics experiments.

Reverse genetics was performed using a modified version of the eight plasmid system described previously (Hoffmann et al., 2000). Briefly, eight pHW2000 plasmids, seven encoding gene segments of the influenza PR8 H1N1 virus (NA, HA, NP, PB1, PB2, PA and NP) and one encoding the modified NS construct, were transfected into a co-culture of HEK-293T and MDCK cells using FuGENE 6 transfection reagent (Promega). This mix was incubated at 37 °C, 5% CO2 for 6 h before the transfection medium was replaced with Opti-MEM (Life Technologies) supplemented with 100 U ml\(^{-1}\) penicillin and streptomycin. Eighteen hours later, an additional 1 ml of Opti-MEM supplemented with 100 U ml\(^{-1}\) penicillin and streptomycin as well as 0.5 mg ml\(^{-1}\) TPCK trypsin (final concentration) was added per well. The co-culture was then incubated at 37 °C, 5% CO2 for an additional 72 h before the supernatant was collected and the presence of virus determined using a haemagglutination assay. Virus stocks were then amplified in 10 day old embryonated eggs and insertion of miRNA was confirmed by sequencing.

Sequencing of the NS gene was conducted on all rescued modified viruses. Viral RNA was extracted using the RNeasy Mini kit (Qiagen) as per the manufacturer’s instructions. RT-PCRs were performed with AMV Reverse Transcriptase (Promega) using the UNI12 specific primer, which binds to the 5' promoter region of all eight segments (Hoffmann et al., 2001). The NS segment was amplified using a method described previously (Hoffmann et al., 2001) before sequencing with the BigDye Terminator v3.1 Sequencing kit (Applied Biosystems). Sequencing products were analysed using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).

Expression of miR155 from infected MDCK cells was determined using a previously described method (Shi & Chiang, 2005). Confluent MDCK cells were infected with a PR8 virus expressing miR-155 (PR8-155) or a control PR8 virus expressing a scramble sequence (PR8-scramble) at an m.o.i. of 0.01. At 72 h post-infection, cells were analysed for miR-155 levels by qPCR. Total RNA was extracted from infected cells using the miRNeasy Mini kit (Qiagen) as per the manufacturer’s instructions and treated to remove

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**Fig. 1.** (a) A diagram illustrating the construction of the modified NS gene. (b) Alignment of the Canis sp. SOCS1 gene containing the miR-155 target with miR-155 of both Canis sp. and Mus sp. The box defines the miR-155 seed region and its corresponding target. The sequence logo illustrates conservation of this region for human, monkey, canine and murine SOCS1 genes. (c) Mean fold changes in the expression of miR-155 and an unrelated miRNA (miR-107) 24 h post-infection in MDCK cells infected with PR8-155 and PR8-scramble at an m.o.i. of 0.1. Data represent the mean ± sd from three individual experiments. *P<0.05 compared with PR8-scramble, Student’s t-Test.
genomic DNA using RQ1 DNase (Promega). The RNA was then polyadenylated using Yeast Poly(A) Polymerase (PAP) (Affymetrix) and cDNA synthesis was performed using the Superscript III First-Strand Synthesis kit (Life Technologies). Expression of mir-155 was measured by qPCR using SYBR Green PCR Master Mix (Life Technologies) with specific forward primers (mmu-miR-155 SYBR F: GGCGGCTTAATGCTAATTGTGATAG and mmu-mir-107 SYBR F: GCAGCAGCATTGTACAGGGCT) and a universal reverse primer that binds to the miR-PTA sequence (Shi & Chiang, 2005). Levels were normalized to 5S rRNA (a stably expressed small RNA) and compared with an irrelevant miRNA, miR-107. The △△Ct method was used for relative expression analysis and melt curves were analysed using StepOnePlus software (v2.0, Applied Biosystems). Presence of a single product was confirmed by 1.5 % agarose gel electrophoresis. Results demonstrate a statistically significant 65.5±15.3-fold increase in mature miR-155 levels in cells infected with PR8-155 compared with PR8-scramble or uninfected controls (Fig. 1c). There was no significant change in expression of an endogenous unrelated miRNA, miR-107, in our culture systems following infection with either virus.

SOCS1 is a known target of miR-155 (Lu et al., 2009). The ability of PR8-155 to inhibit expression of SOCS1 was determined using a dual luciferase reporter assay system (Promega). Briefly, a 578 bp fragment of the murine Socs1 gene, including the miR-155 seed region, was amplified from genomic DNA using specific primers (mmu-Socs1 F: GTTCCACTCGAGCACCTTCTTGGTGCGCGACAG and mmu-Socs1 R: GTTCCACTCGAGCACACACAAAGTGCGTCATACACC). The amplified fragment was ligated into the 3’ end of the Renilla luciferase gene within the psiCHECK-2 vector (Promega) as per the manufacturer’s instructions. The Socs1 psiCHECK-2 plasmid was transfected into confluent Vero cells, which were infected 24 h later with PR8-155 or PR8-scramble at a m.o.i. of 1. At 48 h post-infection, cells were processed using a Dual-Luciferase Reporter Assay (Promega) as per the manufacturer’s instructions and fluorescence was measured using a Synergy H4 luminometer (BioTek). A mirIDIAN murine miR-155 mimic (Thermo Scientific) was used as a positive control in these assays. At 48 h post-infection, there was a statistically significant decrease in SOCS1 linked Renilla luciferase activity in cells infected with PR8-155 (P<0.05) and cells transfected with a positive control murine miR-155 mimic (P<0.01) when compared with uninfected controls (Fig. 2a). There was no decrease in activity in cells infected with a PR8-scramble control (Fig. 2a).

To confirm inhibition of SOCS1, Western blot analysis of infected A549 cells was performed. WT PR8 or PR8-155 virus was pre-treated for 30 min with a 0.5 % TPCK trypsin solution to pre-cleave the HA protein. Confluent A549 cells were then infected at a m.o.i. of 2, and 24 h post-infection cells were lysed with RPMI lysis buffer supplemented with protease inhibitors (cComplete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets; Roche Applied Science) for 30 min at 4 °C. Cell lysates were centrifuged and the supernatant added to NuPAGE LDS Sample Buffer (Life Technologies) and incubated for 10 min at 70 ºC. The samples were run on a 4–15 % SDS-PAGE gradient gel (Bio-Rad) and then transferred to a PVDF membrane. The SOCS1 protein was visualized by Western blotting using an anti-mouse SOCS1 (MBL International) primary antibody (cross-reacts with human SOCS1), and a sheep anti-mouse HRP-conjugated secondary antibody (Merck Millipore). Human β-actin was detected by washing blots with TBST (Tris buffered saline containing Tween 20) followed by incubation in stripping buffer [1.5 % (w/v) glycine, 0.1 % (w/v) SDS, 1 % Tween 20, pH 2.2] for 10 min. After further washing in PBS and TBST, blots were blocked for 1 h with skimmed milk before incubation with rabbit anti-human β-actin antibody (Cell Signaling) and HRP-conjugated sheep anti-rabbit IgG secondary antibody (Life Technologies). Following this, influenza A nucleoprotein (NP) was detected by stripping
the blot as above and incubating with a mouse anti-NP antibody (Bio-Rad). Increased knockdown of SOCS1 protein was observed in cells infected with PR8-155 compared with PR8-WT and uninfected controls (Fig. 2b).

Previous studies have also shown that progesterone-induced decreases in miR-155 expression can lead to inhibition of IL-6, and IFN-\(\beta\) cytokine production facilitated increases in SOCS1 expression (Sun et al., 2012). Moreover, SOCS1 inhibition has been shown to directly enhance IL-6 production (Fenner et al., 2006). We therefore hypothesized that an increase in miR-155 expression using our modified influenza viruses would lead to an increase in IL-6 and IFN-\(\beta\) production in our system. To determine IL-6 and IFN-\(\beta\) cytokine expression levels following in vitro infection studies, TaqMan qPCR primer-probe sets were used. Confluent MDCK cells were infected with PR8 or PR8-155 at an m.o.i. of 0.1. At 6 and 24 h post-infection, total RNA was extracted from infected cells using an miRNeasy Mini kit (Qiagen) as per the manufacturer’s instructions and treated to remove genomic DNA using RQ1 DNase (Promega). cDNA synthesis was subsequently performed using a Superscript III First-Strand Synthesis kit (Life Technologies). Expression levels of IL-6, IFN-\(\beta\) and influenza M gene were detected using commercially available TaqMan primer-probe sets (Applied Biosystems) in combination with TaqMan Universal Master Mx (Life Technologies). A statistically significant increase in IL-6 (738.5 \(\pm\) 432.7-fold) and IFN-\(\beta\) expression levels (56.1 \(\pm\) 12.3-fold) was observed (Fig. 3a, b). qPCR of the influenza M gene was used to determine the level of influenza virus in our cultures. Virus titres were equivalent at all time points as indicated by M gene expression for PR8-155 and PR8-WT infected cultures, indicating no overall difference in viral fitness (Fig. 3c).

Viral titres were confirmed using plaque assay analysis. MDCK cells were washed with RPMI medium supplemented with 100 U ml\(^{-1}\) of both penicillin and streptomycin (RPMI-anti) and then incubated at 37 °C, 5 % CO\(_2\) for 1 h. Log dilutions of viral supernatant were prepared and were added to the wells. The plates were incubated at 37 °C, 5 % CO\(_2\) for 45 min, with gentle mixing. Finally, cells were overlaid with 3 ml Leibovitz’s L15/agarose solution, containing: 1 \(\times\) Leibovitz’s L15 medium (Life Technologies), 0.9 % agarose and 2 \(\mu\)g ml\(^{-1}\) TPCK trypsin. They were then incubated at 37 °C, 5 % CO\(_2\) for 72 h, after which plaques were counted. Plaque assay analysis confirmed that there was no overall difference in viral titres/fitness (Fig. 3d). Similar observations were made using A549 cells (data not shown).

A critical aspect in the development of miRNA therapeutics is the safe and efficient delivery of miRNAs to target cells. Herein we describe the use of an influenza A virus-based miRNA delivery system to modulate the immune system via inhibition of the SOCS1 pathway (Pothlichet et al., 2008). Previous studies have shown that induction of miR-155 can

**Fig. 3.** Cytokine production and viral growth kinetics in MDCK cells. Mean relative fold changes in IL-6 (a), IFN-\(\beta\) (b) and M gene (c) expression in MDCK cells infected with PR8-155 and WT PR8 at an m.o.i. of 0.1 at 6 and 24 h post-infection. Data represent the mean \(\pm\) SD from three individual experiments. *P<0.05 compared with WT PR8 infected cells, Student’s \(t\)-test. (d) Viral titres of PR8-155 and WT PR8 in MDCK cells infected at an m.o.i. of 0.01 at 6 and 24 h post-infection as determined by plaque assay analysis. Data represent the mean \(\pm\) SD from two individual experiments.
enhance host innate antiviral immune responses by targeting SOCS1, a negative regulator of type I IFN signalling (Wang et al., 2010). We hypothesized that expression of miR-155 in infected cells following infection with our recombinant influenza virus would alter the levels of two cytokines, IL-6 and IFN-β, both of which are regulated by SOCS1. IL-6 and IFN-β were significantly increased in cells infected with PR8-155. These results clearly suggest that our delivery system is able to inhibit SOCS1 expression in infected cells, leading to enhanced cytokine production. This inhibition was confirmed using a SOCS1-conjugated luciferase reporter assay system.

The use of a live delivery system could lead to the production of self-adjuvanting influenza virus vaccines through expression of miRNAs that directly or indirectly influence the function of the immune system, encouraging proliferation and/or differentiation of appropriate immune cell populations. A similar approach was recently published by Schmid et al. (2014), who used an influenza A-based non-replicating virus-like vector to show successful in vivo delivery of miRNAs. Currently known miRNA candidates include miR-155, miR-146a, miR-150 and miR-181a, all of which influence proliferation and/or differentiation of B and T cells (Baltimore et al., 2008). Moreover, it is anticipated that the expected increase in immune cell effector function associated with this delivery system could enhance immune responses of current influenza vaccines in susceptible populations such as the elderly, where immunosenescence has been shown to reduce current vaccine efficacy to as low as 17%, compared with up to 90% in young adults (Goodwin et al., 2006). Also, it could provide a mechanism for viable dose-sparing strategies in times of vaccine shortage in the event of new emerging pandemics.

Influenza virus infection is a self-limiting disease, and so concerns surrounding persistent overexpression of miRNAs (present in the other systems) are moderated. Moreover, a key advantage of our delivery system is that the expression of miRNAs could also be controlled through the use of antiviral agents such as oseltamivir. This would allow delivery of miRNAs at the time when it is most beneficial and allow termination of expression before deleterious sequelae emerge. It is important to note that the influenza NS gene segment is not the only segment with splicing characteristics, with the M gene also being able to utilize this mechanism to produce two proteins, M1 and M2 (Lamb et al., 1981). This technology could therefore be extended to enable expression of multiple miRNAs from the one virus.

In conclusion, this study reports the first use, to our knowledge, of a recombinant influenza A virus expressing an miRNA that directly targets a protein associated with regulation of immune responses. This delivery system is directly applicable to current real world vaccine technology (i.e. live attenuated influenza vaccines) and would allow miRNAs to fine tune or regulate immunity at the cellular level during respiratory infection. It is important to note that use of live vaccines has been associated with increased risk of adverse events in certain susceptible populations. Having said this, currently commercially available live influenza vaccines e.g. Flumist, have been safely administered to immunocompromised individuals (HIV + patients and those undergoing chemotherapy) without additional sequelae. Moreover, we expect this platform technology to have broad-reaching implications in the field of respiratory vaccine development, as it could enable tailoring or biasing of innate and adaptive immune responses to achieve appropriate long-term efficacious immunity.

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