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VACCINES AND ANTIVIRAL AGENTS

Efficient Inhibition of Avian and Seasonal Influenza A Viruses by a Virus-Specific Dicer-Substrate Small Interfering RNA Swarm in Human Monocyte-Derived Macrophages and Dendritic Cells

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ABSTRACT Influenza A viruses (IAVs) are viral pathogens that cause epidemics and occasional pandemics of significant mortality. The generation of efficacious vaccines and antiviral drugs remains a challenge due to the rapid appearance of new influenza virus types and antigenic variants. Consequently, novel strategies for the prevention and treatment of IAV infections are needed, given the limitations of the presently available antivirals. Here, we used enzymatically produced IAV-specific double-stranded RNA (dsRNA) molecules and *Giardia intestinalis* Dicer for the generation of a swarm of small interfering RNA (siRNA) molecules. The siRNAs target multiple conserved genomic regions of the IAVs. In mammalian cells, the produced 25- to 27-nucleotide-long siRNA molecules are processed by endogenous Dicer into 21-nucleotide siRNAs and are thus designated Dicer-substrate siRNAs (DsiRNAs). We evaluated the efficacy of the above DsiRNA swarm at preventing IAV infections in human primary monocyte-derived macrophages and dendritic cells. The replication of different IAV strains, including avian influenza H5N1 and H7N9 viruses, was significantly inhibited by pretransfection of the cells with the IAV-specific DsiRNA swarm. Up to 7 orders of magnitude inhibition of viral RNA expression was observed, which led to a dramatic inhibition of IAV protein synthesis and virus production. The IAV-specific DsiRNA swarm inhibited virus replication directly through the RNA interference pathway although a weak induction of innate interferon responses was detected. Our results provide direct evidence for the feasibility of the siRNA strategy and the potency of DsiRNA swarms in the prevention and treatment of influenza, including the highly pathogenic avian influenza viruses.

IMPORTANCE In spite of the enormous amount of research, influenza virus is still one of the major challenges for medical virology due to its capacity to generate new variants, which potentially lead to severe epidemics and pandemics. We demonstrated here that a swarm of small interfering RNA (siRNA) molecules, including more than 100 different antiviral RNA molecules targeting the most conserved regions of the influenza A virus genome, could efficiently inhibit the replication of all tested avian and seasonal influenza A variants in human primary monocyte-derived macrophages and dendritic cells. The wide antiviral spectrum makes the virus-specific siRNA swarm a potentially efficient treatment modality against both avian and seasonal influenza viruses.

KEYWORDS Dicer-substrate siRNA, DsiRNA, RNA interference, avian influenza virus, gene silencing, human macrophage, human moDC, influenza A virus, IAV, interferon response, IFN, siRNA swarm, viral replication

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Influenza viruses are common and notorious pathogens of humans due to their unique ability to cause diseases in two epidemiologic forms: annual epidemics and occasional pandemics. Among three types of influenza viruses (types A, B, and C), influenza A virus (IAV) is the most pathogenic and the only influenza virus which has been associated with global pandemics. Seasonal IAV epidemics have caused high morbidity and economic losses in the past. During the past century humans have experienced five influenza pandemics by three subtypes of IAVs: H1N1 in 1918 (Spanish flu), H2N2 in 1957 (Asian flu), H3N2 in 1968 (Hong Kong flu), H1N1 in 1977 (Russian flu), and again H1N1 in 2009 (swine flu). Collectively these pandemics led to the death of tens of millions of people (1–4).

After the first report of the highly pathogenic avian influenza H5N1 virus transmission from poultry to humans (1997), avian influenza viruses became a great concern (5, 6). Subsequently, there have been about 900 confirmed H5N1 infections of humans, with over 50% mortality (7). Infections of highly pathogenic H5N1 IAV in poultry resulted in the preventive culling of more than 200 million chickens, which led to considerable economic losses (8). Moreover, in March 2013 another avian influenza virus, H7N9, was reported to be associated with severe disease in humans. The virus had low pathogenicity to birds but was highly pathogenic to humans as it caused severe pneumonia and acute respiratory distress syndrome in infected individuals (9, 10).

Epithelial cells that line the respiratory tract are the primary cells targeted by IAV. From epithelial cells IAV spreads to other tissues and cells, including alveolar macrophages and dendritic cells (DCs). Alveolar macrophages and DCs are innate immune system sentinel cells, and they form the first line of defense against IAV infection together with the epithelial cells. These early events in IAV infection are essential in determining the outcome of the infection (11). Human monocyte-derived DCs (moDCs) express α-2,3- and α-2,6-linked sialic acids and, thus, can be infected with both human and avian IAVs (12).

The constant emergence of new influenza virus strains challenges the generation of concurrent effective vaccines and antiviral drugs. Presently, the main vaccination strategy against IAV is to design vaccines that contain hemagglutinin (HA) and neuraminidase (NA) antigens of recently circulating influenza viruses. The vaccines can stimulate the production of neutralizing antibodies and also probably enhance cellular immunity. However, IAV gene reassortment, called antigenic shift, and the mutations in HA and NA genes, known as antigenic drift, may abrogate the binding of antibodies to the target antigens and enable IAV to evade the neutralizing ability of antibodies that have been generated by vaccination (13). Moreover, there will be only limited amounts of strain-specific vaccines available during the early stages of a pandemic involving newly emerged IAVs. The drug strategy against IAV is also restricted by the expense, availability of the drugs, potential side effects, and the timing of delivery (14). Furthermore, the existing drugs are becoming less efficient due to the emergence of drug-resistant IAV variants (15, 16). Therefore, given the limitations of available antiviral substances, novel strategies or therapies for the prevention and treatment of zoonotic IAV infections are urgently needed.

RNA interference (RNAi) is an evolutionarily conserved mechanism and one of the oldest host defense responses in eukaryotic cells (17). It constitutes a class of processes that result in the formation of small interfering RNA (siRNA) duplexes. The formation of siRNAs is dependent on the activity of a Dicer enzyme, an RNase III endonuclease (18, 19). The Dicer enzyme recognizes and binds to the 5′ end of long double-stranded RNA (dsRNA) substrate and cuts the RNA molecule into small RNA duplexes with 2-nucleotide (nt)-long 3′ terminal overhangs. The size of the produced siRNAs varies depending on the organism in which the fragmentation occurs: 21- to 23-nt-long siRNAs are produced by the human Dicer whereas digestion by the *Giardia intestinalis* Dicer results in the formation of 25- to 27-nt-long siRNAs (20–22). These siRNAs are incorporated in the RNA-induced silencing complexes (RISC) that recognize and cleave

complementary target mRNAs, which leads to the degradation of the target mRNAs followed by gene silencing (23).

siRNA molecules can inhibit viral infections by targeting and degrading viral RNAs (24). The discovery of the potential of siRNA-based prophylaxis opens up the possibility of generating new therapeutic approaches for the treatment of a wide spectrum of viral diseases. The potential of siRNA-based therapies for the treatment of many RNA virus infections, including influenza virus, sever acute respiratory syndrome (SARS) coronavirus, poliovirus, hepatitis C virus, West Nile virus, and dengue virus, have been studied, and siRNA approaches have also been shown to be effective against DNA viruses as well (25–30). siRNA treatment has many advantages compared to treatment with conventional antiviral drugs: (i) viral mRNA is a uniform target, (ii) small amounts of siRNA can dramatically decrease viral mRNA expression, (iii) siRNAs can be used in cells of different animal species, (iv) siRNAs can be used against different targets including new emerging viral diseases, (v) siRNAs are quickly designed and produced, (vi) and antiviral siRNAs can be combined with other antiviral substances.

Previously, it has been shown that chemically synthesized 25- to 27-nt-long siRNAs are substrates for the Dicer enzyme (31). These Dicer-substrate siRNAs (DsiRNAs) can be recognized and processed into shorter 21-nt-long siRNAs by endogenous Dicer when they are introduced into mammalian cells (31). This interaction with Dicer facilitates the loading of the siRNAs into the RISC, and accordingly DsiRNAs have been reported to be more potent inducers of RNAi than canonical 21-nt-long siRNAs (31–33).

Typically, RNAi is activated by a chemically synthetized siRNA that represents a single selected sequence that corresponds to the target. The choice of suitable target sequences in such a strategy plays an important role, especially in RNAi approaches against viruses, for which the problem of viral escape has been recognized as one of the major concerns for the long-term use of antiviral siRNAs (34, 35). Different viral variants also circulate simultaneously, which increases the likelihood of the development of antiviral resistance. As an alternative for the single-site siRNAs, our approach therefore uses a swarm of siRNAs that contains hundreds of different target-specific siRNA molecules. The use of an siRNA swarm should solve the problem of viral escape and also counter the heterogeneity in natural viral populations. Furthermore, the concentration of each individual siRNA type in the swarm is low and, thus, reduces the risk of severe off-target effects.

The feasibility of the siRNA-based therapy of IAV infection has been demonstrated previously using chemically synthetized single-site siRNAs (36), whereby the siRNAs that targeted the IAV RNA polymerase genes reduced mortality in experimental IAV infection and also virus replication in the lungs (37–39). In the present study, we enzymatically generated an siRNA swarm containing DsiRNAs that targeted multiple conserved regions of the IAV genome by using viral RNA polymerases and the Dicer of *Giardia intestinalis* (40).

The aim of the present study was to investigate the inhibition of viral gene expression, protein synthesis, and the production of IAVs for seasonal IAVs and highly pathogenic avian strains in human cells before and after transfection with DsiRNA molecules. We show that a swarm of IAV-specific DsiRNA molecules can efficiently inhibit the replication of human seasonal and avian influenza viruses in primary human macrophages, moDCs, and lung epithelial Calu-3 and A549 cells. This opens up new possibilities for the development of wide-spectrum antiviral strategies against influenza, including highly pathogenic H5N1 and H7N9 avian influenza viruses.

RESULTS

Construction of cIAV-specific cDNA molecules and siRNA production. We aligned genomic sequences of several H5N1 strains of avian/swine IAVs, including A/chicken/Jiangsu/cz1/2002, A/goose/Jilin/hb/2003, A/swine/Henan/wy/2004, A/wild duck/Hunan/211/2005, and A/avian/Hong Kong/0828/2007, and human isolates A/Hong Kong/482/97, A/Viet Nam/1203/2004, and A/Anhui/1/2005, in order to obtain a DsiRNA swarm with a high efficacy of gene silencing against IAV infection and with a minimum

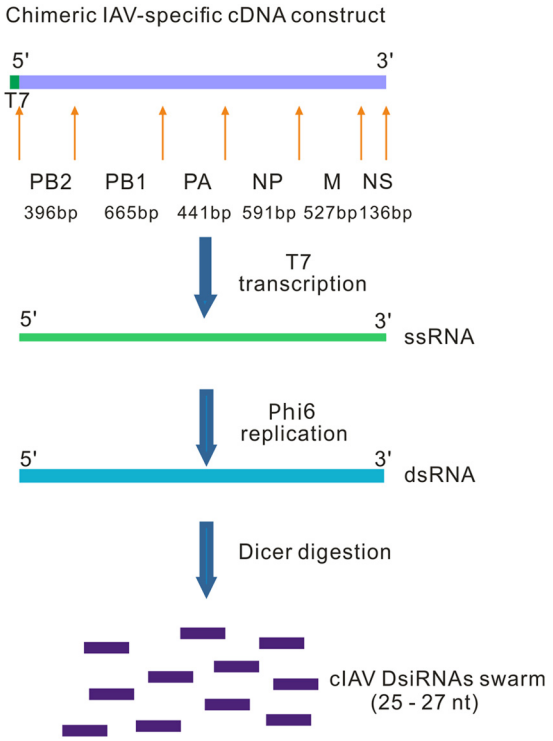

FIG 1 Schematic representation of enzymatic *in vitro* synthesis of DsiRNA molecules of a chimeric IAV construct. A chimeric cDNA construct containing selected conserved regions of the PB1, PB2, PA, NP, M, and NS genes of the A/wild duck/Hunan/211/2005 (H5N1) IAV strain and the promoter sequence of the T7 polymerase is shown. The sizes of the sequences derived from the different IAV genes are indicated. The ssRNA molecules are transcribed from the chimeric cDNA construct using bacteriophage T7 polymerase. The ssRNA molecules are used as templates for the bacteriophage φ6 RNA-dependent RNA polymerase to produce corresponding dsRNA molecules. Produced dsRNA molecules are then digested into a swarm of DsiRNA molecules (cIAV DsiRNA swarm) using recombinant *Giardia intestinalis* Dicer. The chimeric IAV sequence is shown at the top with the T7 promoter indicated in green. Other bars represent the ssRNA and dsRNA synthesis intermediate products and the cIAV siRNA swarm, as indicated.

off-target effects. Several highly conserved sequences were identified within six of the IAV genome segments (data can be provided upon request), and a chimeric IAV (cIAV) construct of 2,756 bp comprising these sequences was generated based on the A/wild duck/Hunan/211/2005 virus sequences (Fig. 1). HA and NA segments were excluded from the construct due to their significant genetic variability.

Full-length dsRNA that corresponded to the sequence of the chimeric IAV construct was produced *in vitro* using T7 RNA polymerase and φ6 RNA-dependent RNA polymerase, followed by dsRNA digestion by *Giardia intestinalis* Dicer to obtain a swarm of 25- to 27-nt siRNAs that corresponded to the most conserved regions of the IAV genome (cIAV DsiRNA) (Fig. 1). An enhanced green fluorescent protein (eGFP)-expressing DsiRNA swarm was generated as a control in a similar fashion using *in vitro*-synthesized eGFP gene-specific dsRNA as a substrate for the Dicer.

cIAV DsiRNA swarm induced significant inhibition of avian IAV replication in human macrophages and moDCs. To assess the antiviral effect of an *in vitro*-produced cIAV DsiRNA swarm, cIAV DsiRNAs and control siRNAs were transfected into human macrophages or moDCs; 21 h later cells were infected with H5N1 (A/Vietnam/1194/04) and H7N9 (A/Anhui/1/13) avian IAVs, which are highly pathogenic in humans, using a multiplicity of infection (MOI) of 1 for an additional 7 h or 24 h. No significant changes of cell viability were noticed after siRNA or DsiRNA transfection (data not shown). An equal amount of UV-inactivated IAV was used as a control representing the incoming virus. The mRNA expression of the viral matrix protein gene (M1) was analyzed by gene-specific quantitative reverse transcriptase PCR (qRT-PCR). Avian H5N1 virus appeared to infect macrophages more efficiently than H7N9 virus; in particular, the mRNA

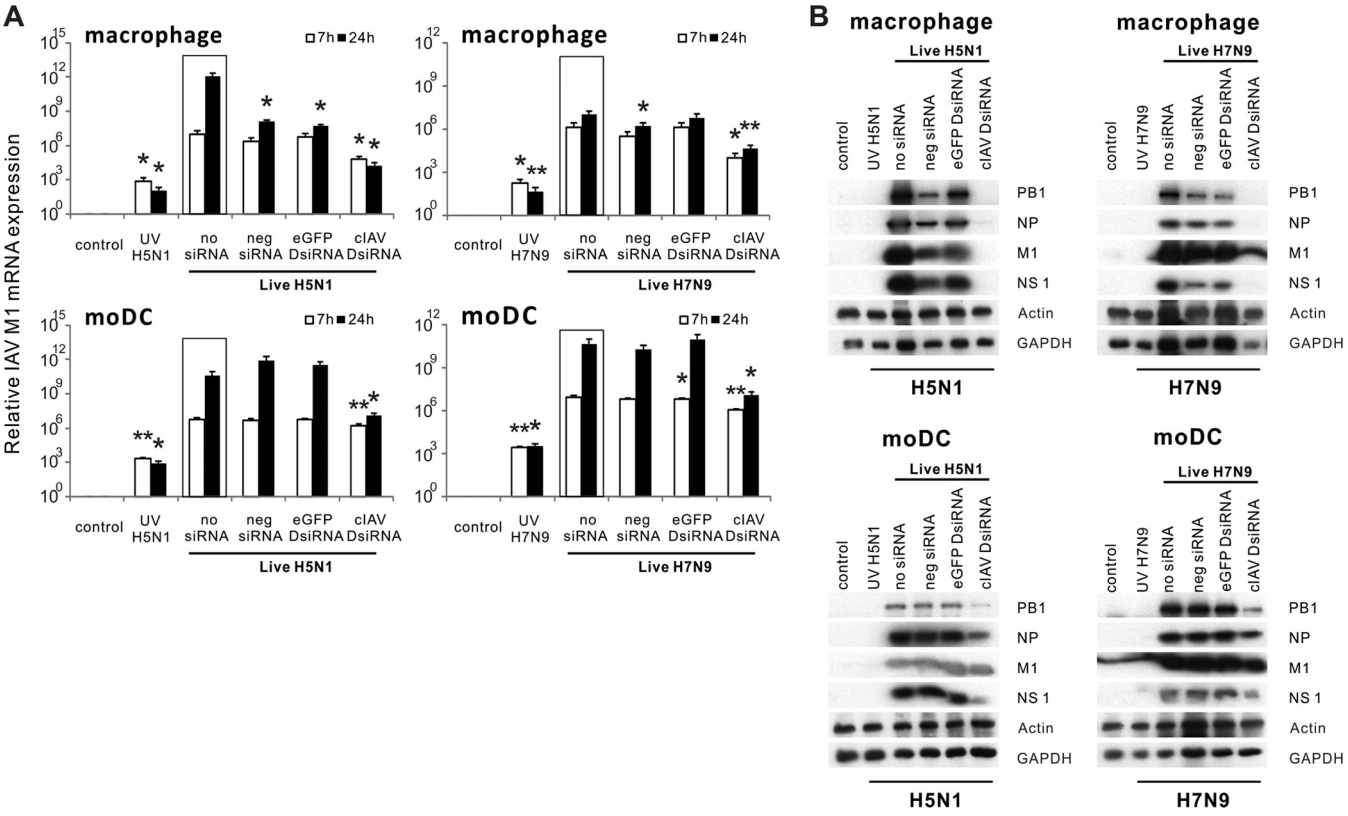

FIG 2 Inhibition of avian IAV replication in human macrophages and moDCs by pretransfection with cIAV siRNAs. Human primary macrophages or moDCs obtained from four different blood donors were separately mock transfected (control, UV IAV, or no-siRNA bars) or pretransfected with the indicated siRNA or DsiRNAs (10 nM) for 21 h. Cells were then infected with live or UV-irradiated H5N1 or H7N9 virus at an MOI of 1. Macrophages were washed twice with PBS at 1 h p.i. and then maintained in macrophage medium. Input virus was retained in moDC cultures (A) Cells from four different blood donors were subsequently collected at 7 and 24 h p.i. and were pooled; then IAV M1 RNA expression was determined by qRT-PCR from isolated total cellular RNA samples. The values were normalized against β-actin gene-specific mRNA, and relative IAV M1 RNA levels were calculated by the $\Delta\Delta C_T$ method using untreated cellular RNA as a calibrator. The means ($\pm$SD) of three parallel analyses are shown. Data are representative of three individual experiments. Statistical significance was determined against results from samples of nontransfected cells (boxed bars). *; $P < 0.05$, **, $P < 0.01$. (B) Western blot analysis for the expression of viral PB1, NP, M1, and NS1 proteins and β-actin and GAPDH proteins in siRNA/DsiRNA transfected human macrophages and moDCs. Cells were collected at 24 h after avian H5N1 or H7N9 IAV infection, and whole-cell lysates were prepared. Cellular proteins (30 μg/lane) were separated by 10% SDS-PAGE, followed by electrophoretic transfer of the proteins onto polyvinylidene difluoride membranes and visualization of the transferred proteins by protein-specific antibodies, as indicated. The data of one representative experiment of three independent experiments is shown.

expression level of the M1 gene of H5N1 virus was much higher in infected macrophages than that with H7N9 virus infection at 24 h postinfection (p.i.) (Fig. 2A, upper panels). However, the relative infection efficiencies of H5N1 and H7N9 viruses were almost equal in moDCs (compare the specific qRT-PCR signals with the signal of the UV-inactivated control sample, shown in the lower panels of Fig. 2A). Pretransfection of cells with a nonspecific chemically synthetized 21-nt siRNA (Fig. 2, neg siRNA) or with an eGFP-specific DsiRNA swarm prior to H5N1 virus infection inhibited viral M1 RNA expression in macrophages at 24 h p.i. but failed to inhibit viral M1 mRNA expression in moDCs (Fig. 2A). Furthermore, viral M1 RNA expression remained practically unchanged in H7N9 virus-infected macrophages or moDCs after transfection with the control siRNA or DsiRNAs (Fig. 2A).

Next, macrophages and moDCs were transfected with the DsiRNA swarm derived from the chimeric IAV construct. The pretransfection of cells with cIAV DsiRNAs dramatically inhibited the viral M1 mRNA expression (by 2 to 7 orders of magnitude) of both H5N1 and H7N9 viruses, and this effect was especially prominent at 24 h p.i. (Fig. 2A).

The antiviral effects of cIAV DsiRNAs on viral protein expression were further analyzed by Western blotting of samples from infected cells. Both H5N1 and H7N9 virus strains expressed high levels of viral PB1, NP, M1, and NS1 proteins in virus-infected

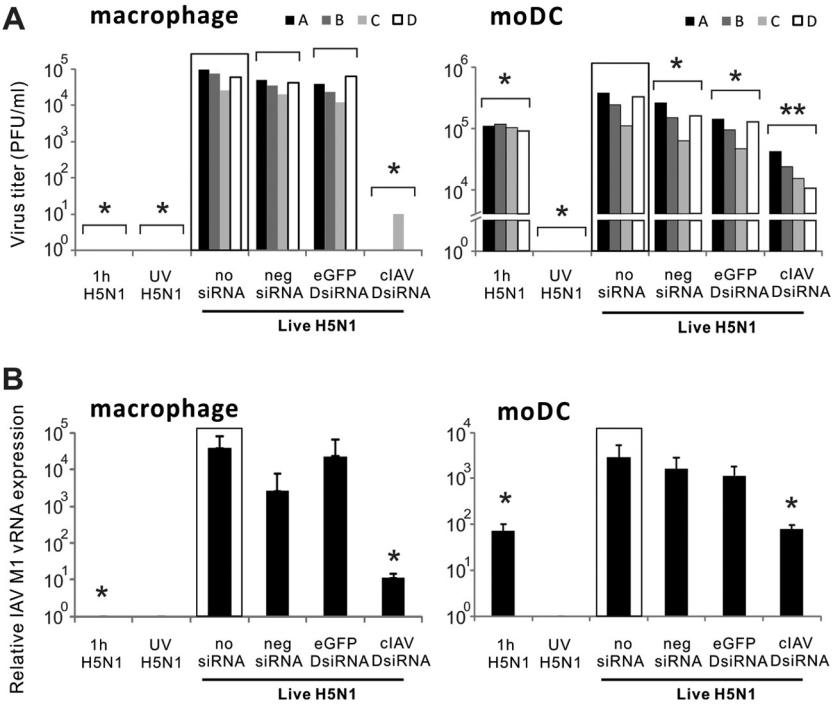

FIG 3 Inhibition of the productivity of H5N1 infection by pretransfection with cIAV DsiRNA swarm in human macrophage and moDCs. Human primary macrophages or moDCs obtained from four different blood donors (A, B, C, and D) were left nontransfected (control, UV IAV, or no siRNA) or separately pretransfected with the indicated siRNA or DsiRNAs (10 nM) for 21 h. Cells were then infected with live or UV-irradiated H5N1 viruses at an MOI of 1. To remove the input virus, macrophages were washed twice with PBS at 1 h p.i. and then maintained in a macrophage medium. MoDC cultures were not washed, and therefore the H5N1 virus titers in supernatant samples at 1 h p.i. represented the input amounts of virus. (A) The infective viral titers produced from macrophages and moDCs were determined by plaque assay in Madin-Darby canine kidney cells. Statistical significance was determined against results from samples of nontransfected H5N1 virus-infected cells (no siRNA; boxed bars). *, $P < 0.05$; **, $P < 0.01$. (B) The RNA was isolated from the supernatant samples from macrophages and moDCs, and the viral M1 gene-specific RNA levels were detected by qRT-PCR. The viral RNA expression was calculated relative to the level in UV-irradiated samples with the $\Delta\Delta C_T$ method. Statistical significance was determined against results from samples of nontransfected H5N1 virus-infected cells (no siRNA; boxed bars). *, $P < 0.05$.

macrophages and moDCs at 24 h p.i. (Fig. 2B). Viral proteins could not be visualized in cells infected with UV-irradiated virus since UV treatment completely inactivated the infectivity of IAVs. Pretransfection with the single-site negative siRNA reduced the expression of PB1 and NS1 in H5N1 or H7N9 virus-infected macrophages to some extent (Fig. 2B, neg siRNA). The nonspecific antiviral activity of the control DsiRNA swarm was weak (Fig. 2B). However, the control siRNA and eGFP DsiRNAs had no antiviral effect in moDCs. Pretransfection with cIAV DsiRNAs completely blocked the expression of viral proteins in H5N1 virus-infected macrophages, and it also completely prevented the expression of PB1, NP, and NS1 proteins and markedly reduced the expression of M1 protein in H7N9 virus-infected cells. Pretransfection of moDCs with cIAV DsiRNA swarm also clearly reduced the expression of H5N1 and H7N9 virus proteins, but the inhibition was not as dramatic as that seen in macrophages (Fig. 2B).

Next, we analyzed whether the treatment of human macrophages or moDCs with cIAV DsiRNA could block the secretion of infectious H5N1 virus. For that, cIAV DsiRNAs and control siRNAs were transfected into human macrophages or moDCs, cells were infected with H5N1 (MOI of 1) at 21 h posttransfection, and plaque assays were performed with supernatant samples at 1 or 24 h p.i. (Fig. 3). An equal amount of UV-inactivated IAV was used as a control. The amount of infectious virus in nontransfected macrophage culture supernatant was significantly increased at 24 h, resulting in approximately a 10^4- to 10^5-fold increase in virus titers (Fig. 3A, left panel). Pretreatment with a nonspecific siRNA or eGFP-specific DsiRNA swarm did not affect the production

of infectious viruses. However, pretreatment of macrophages with a cIAV-specific DsiRNA swarm blocked the secretion of H5N1 virus into cell supernatants (Fig. 3A, left panel). In moDC suspension cultures, it was technically not possible to remove the input virus as was done for macrophages. The H5N1 virus titer in supernatant samples at 1 h p.i. was approximately 10^5 PFU/ml, and this represented the input amount of viruses. The amount of infectious H5N1 virus was slightly higher in the supernatant samples collected from the control cells (no siRNA treatment and treatment with control siRNA) at 24 h p.i., while in cIAV DsiRNA-pretreated cells such an increase was not detected; rather, there was some reduction in the amount of infectious virus in the sample at 24 h p.i. compared to that at 1 h p.i. (Fig. 3A, right panel).

In addition to analyzing the amount of secreted infectious H5N1 virus, we also investigated viral RNA levels at 24 h in the macrophage supernatants of virus-infected cells. This is an alternative to use of cell culture supernatants as a way to quantitate IAV production (41). The total viral RNA level increased almost 10^5-fold in the macrophage supernatant samples without pretreatment with any siRNAs, and pretreatment with a nonspecific siRNA or eGFP-specific DsiRNA swarm did not significantly decrease the viral RNA level in the supernatants (Fig. 3B). However, in cIAV-specific DsiRNA-pretreated cells, viral RNA levels significantly decreased, approximately 10^4-fold (Fig. 3B, left panel). In moDCs, cIAV DsiRNA pretreatment significantly reduced H5N1 virus production as measured by PCR-based viral RNA quantitation in cell culture supernatants (Fig. 3B, right panel). The data indicate that pretreatment with cIAV-specific DsiRNA can efficiently inhibit the productive replication of H5N1 virus in human primary macrophages and moDCs. The production of infectious H7N9 virus in macrophage culture supernatants was low due to impaired activation cleavage of the strain used, LPAI H7N9 (41), but yet pretreatment with cIAV DsiRNA showed some efficacy in inhibition of H7N9 virus secretion compared to results with siRNA-untreated control (data not shown).

cIAV DsiRNA swarm also induced significant inhibition of seasonal IAV replication in human macrophages and moDCs. The chimeric IAV construct showed over 90% sequence homology with genome sequences of seasonal influenza viruses A/Udorn/307/1972 (H3N2/72), A/Beijing/353/1989 (H3N2/89) (Beijing/89), A/Wisconsin/67/2005 (H3N2/05), A/New Caledonia/20/1999 (H1N1/99), A/Brisbane/59/2007 (H1N1/07), and A/Finland/643/2009 (H1N1/09). Thus, it was interesting to determine whether the cIAV DsiRNA swarm would inhibit the replication of seasonal IAVs as well. Human moDCs that had been pretransfected with different siRNAs were infected with the selected seasonal IAVs at an MOI of 1, and virus replication in the infected cells was analyzed at 24 h p.i. by measuring viral RNA by qRT-PCR and protein expression by Western blotting. All seasonal IAVs replicated efficiently in moDCs, as analyzed by viral M1 RNA expression (Fig. 4A), and the pretransfection of cells with either a negative siRNA or eGFP-specific DsiRNA swarm did not reduce viral M1 RNA expression of any of the seasonal IAVs except the Beijing/89 strain (Fig. 4A). Accordingly, no reduction was detected in viral protein expression under these conditions (Fig. 4B). However, pretransfection of moDCs with the cIAV DsiRNA swarm resulted in a reduction by approximately 2 orders of magnitude in viral M1 RNA expression for all of the analyzed seasonal IAVs (Fig. 4A). Interestingly, pretransfection of the cells with cIAV DsiRNAs specifically inhibited the expression of the PB1 protein of H1N1/99 virus and the M1 protein of H3N2/72 virus, and it completely blocked the expression of PB1 and M1 of all the other tested IAVs in the IAV-infected moDCs. Furthermore, cIAV DsiRNA pretransfection resulted in complete inhibition of NP and NS1 protein expression in H3N2/72 virus-infected cells and substantial reduction in the expression of NP and NS1 proteins of all other tested seasonal IAVs in infected moDCs (Fig. 4B).

The cIAV DsiRNA swarm induced significant inhibition of IAV replication but not influenza B virus (IBV) replication in Calu-3 cells. To further evaluate the antiviral potential of siRNA in another cell model system, cIAV DsiRNAs and control siRNAs were transfected in human lung carcinoma epithelial cells, Calu-3 cells, followed by infection with three different strains of IAV at an MOI of 1. Cells were collected at 24 h p.i., and

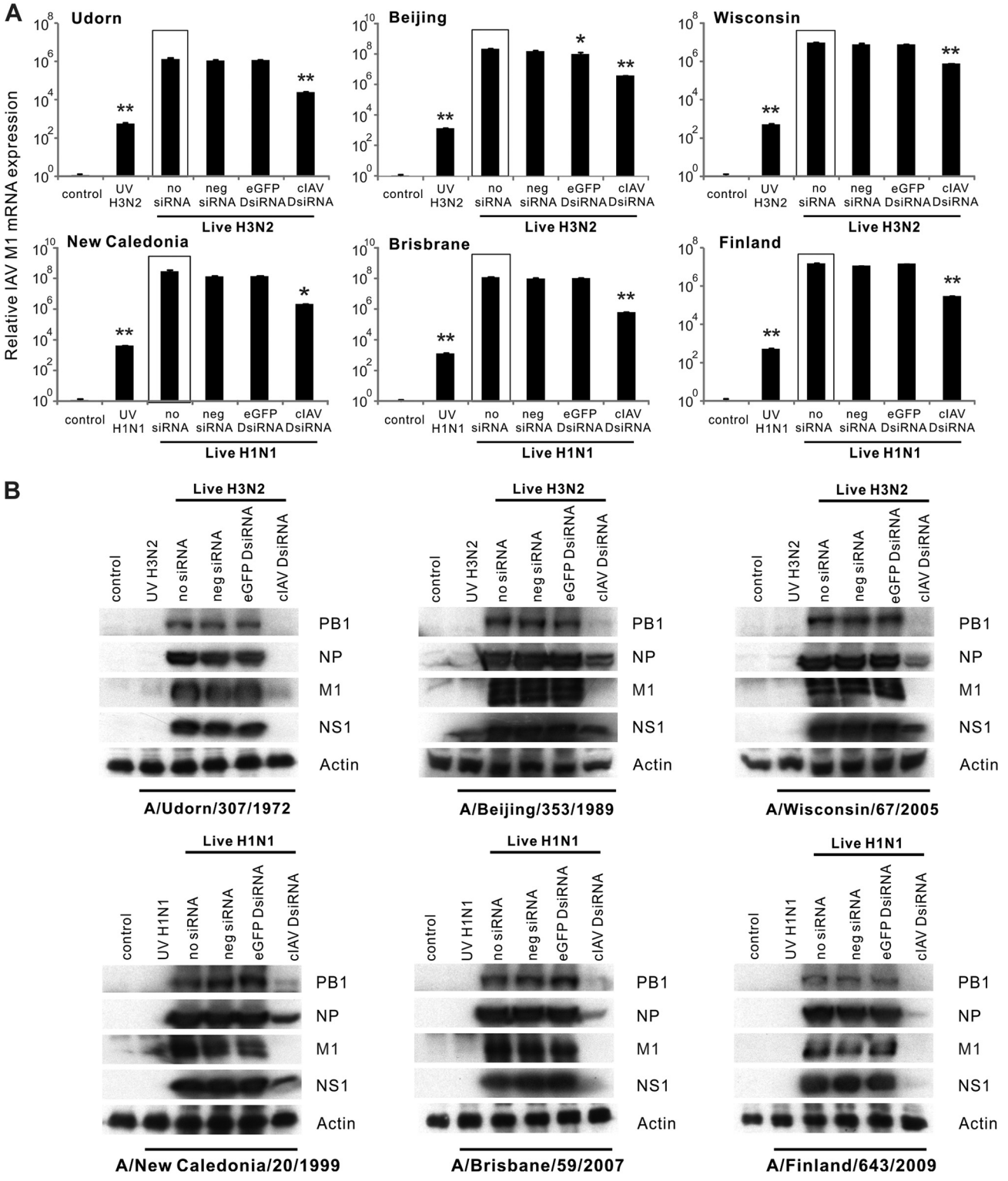

FIG 4 Inhibition of seasonal IAV replication in human moDCs by pretransfection with a cIAV DsiRNA swarm. Human moDCs from four different blood donors were separately mock transfected (control, UV IAV, or no-siRNA bars) or pretransfected with the indicated siRNAs (10 nM) for 21 h. Cells were subsequently infected with the indicated live or with UV-irradiated seasonal IAVs at an MOI of 1. (A) At 24 h p.i. cells from different blood donors were pooled, and the IAV M1 RNA expression was determined by qRT-PCR from isolated total cellular RNA samples. The values were normalized against β-actin gene-specific mRNA, and relative IAV M1 RNA levels were calculated by the $\Delta\Delta C_T$ method using untreated cells as a calibrator. The means ($\pm$SD) of three parallel analyses are shown. Data are representative of three individual experiments. Statistical significance was determined against results from samples of nontransfected cells (no siRNA; boxed bars). *, $P < 0.05$; **, $P < 0.01$. (B) Western blot analysis for the expression of viral PB1, NP, M1, and NS1 proteins and β-actin in siRNA/DsiRNA transfected moDCs infected with the indicated seasonal IAVs. Cells were collected at 24 h p.i., and whole-cell lysates were prepared. Cellular proteins (30 μg/lane) were separated by 10% SDS-PAGE, followed by Western blot analysis with the indicated antibodies. One representative experiment of three independent experiments is shown.

viral RNA and protein expression were analyzed by qRT-PCR and Western blotting, respectively. All strains of IAV replicated with high efficiency in Calu-3 cells. Consistent with our previous results (Fig. 4A), pretransfection with the cIAV DsiRNA swarm significantly reduced viral M1 RNA expression in H3N2/72, H3N2/89, and H1N1/09 virus-infected Calu-3 cells (Fig. 5A). Western blotting data also showed dramatic inhibition of viral protein expression in infected cells under such conditions (Fig. 5B).

The Calu-3 cells were transfected with cIAV DsiRNAs, and 21 h later the cells were infected with B/Shandong/7/97, a vaccine strain of IBV, using an MOI of 1 to investigate the specificity of the antiviral effect of cIAV DsiRNAs. IBV replicated equally as efficiently as IAV in Calu-3 cells (Fig. 5A). However, cIAV DsiRNAs failed to inhibit IBV NP RNA expression in IBV-infected cells (Fig. 5A). Western blot data also confirmed this lack of inhibition (Fig. 5B).

The cIAV DsiRNA swarm induced significant inhibition of IAV replication in A549 cells. To investigate the antiviral effect of the cIAV DsiRNA swarm against IAV infection in another human lung cell model, A549 cells were transfected with cIAV DsiRNAs and control siRNAs, followed by infection with two different strains of IAV at an MOI of 1 (H3N2/89 and H1N1/09 virus). Cells were collected at 24 h p.i., and viral RNA expression was analyzed by qRT-PCR. Consistent with the data above (Fig. 5A), pretransfection with the cIAV DsiRNA swarm significantly reduced viral M1 RNA expression in H3N2/89 and H1N1/09 virus-infected A549 cells (Fig. 6A).

We also assessed the amount of secreted infectious H3N2/89 and H1N1/09 virus by measuring viral RNA levels isolated from the 24-h supernatants of virus-infected A549 cells. We noticed that pretreatment with a nonspecific siRNA or eGFP-specific DsiRNA swarm did not decrease viral RNA levels in the supernatants, whereas viral RNA levels were decreased in the supernatants from cIAV-specific DsiRNA-pretreated cells (Fig. 6B). The data indicate that pretreatment with cIAV-specific DsiRNA can also inhibit the replication of H3N2/89 and H1N1/09 virus in human lung epithelial cells.

cIAV DsiRNAs can inhibit IAV replication in human moDCs only when administered prior to infection. MoDCs were transfected with siRNAs 21 h before or 1 h after the infection with H3N2/72 virus (MOI of 1) to estimate the optimal time of siRNA delivery for antiviral effects. Cells were collected at 24 h p.i., and viral RNA and protein expression was analyzed by qRT-PCR and Western blotting, respectively. Pretransfection with cIAV DsiRNAs led to a 90% to 95% reduction in viral M1 RNA expression in infected cells, whereas transfection with cIAV DsiRNAs after IAV infection (1 h) failed to inhibit viral M1 RNA expression (Fig. 7A). Western blot analysis also showed that pretransfection with cIAV DsiRNAs efficiently inhibited the expression of PB1, NP, NS1, and M1 proteins in IAV-infected moDCs. However, transfection with cIAV DsiRNAs after IAV infection had no effect on the expression of any of the analyzed viral proteins (Fig. 7B).

Pretransfection with cIAV DsiRNAs induced weak IFN gene expression but led to significant inhibition of IAV replication in human macrophages and moDCs. In order to study whether DsiRNA swarms induce interferon (IFN) responses in our cell model systems, we analyzed the expression of IFN-β and IFN-λ1 mRNAs and the expression of MxA protein in siRNA and DsiRNA transfected macrophages and moDCs. Different siRNAs were transfected into moDCs, and IFN-β1 and IFN-λ1 mRNA expression levels were analyzed by qRT-PCR from cell samples collected at 21 h after transfection. An 88-bp dsRNA, which is an efficient RIG-I agonist and IFN inducer (42), was used as a positive control. Transfection with the 88-bp dsRNA induced a significant induction of IFN gene expression in macrophages and also in the moDCs, whereas all the siRNAs (both single-site and DsiRNA swarms) induced only relatively weak IFN-β1 and IFN-λ1 mRNA expression (Fig. 8A). Western blot analysis revealed that siRNAs induced weak (macrophages) or no MxA (moDCs) protein expression in stimulated cells, while the 88-bp dsRNA readily enhanced MxA protein expression (Fig. 8B). In contrast, a reduction in the expression of viral M1 RNA was most pronounced in cells transfected with cIAV DsiRNAs (Fig. 8C). The reduction in M1 RNA expression was smaller in infected cells that had been pretransfected with the 88-bp dsRNA (Fig. 8C) even though the 88-bp dsRNA

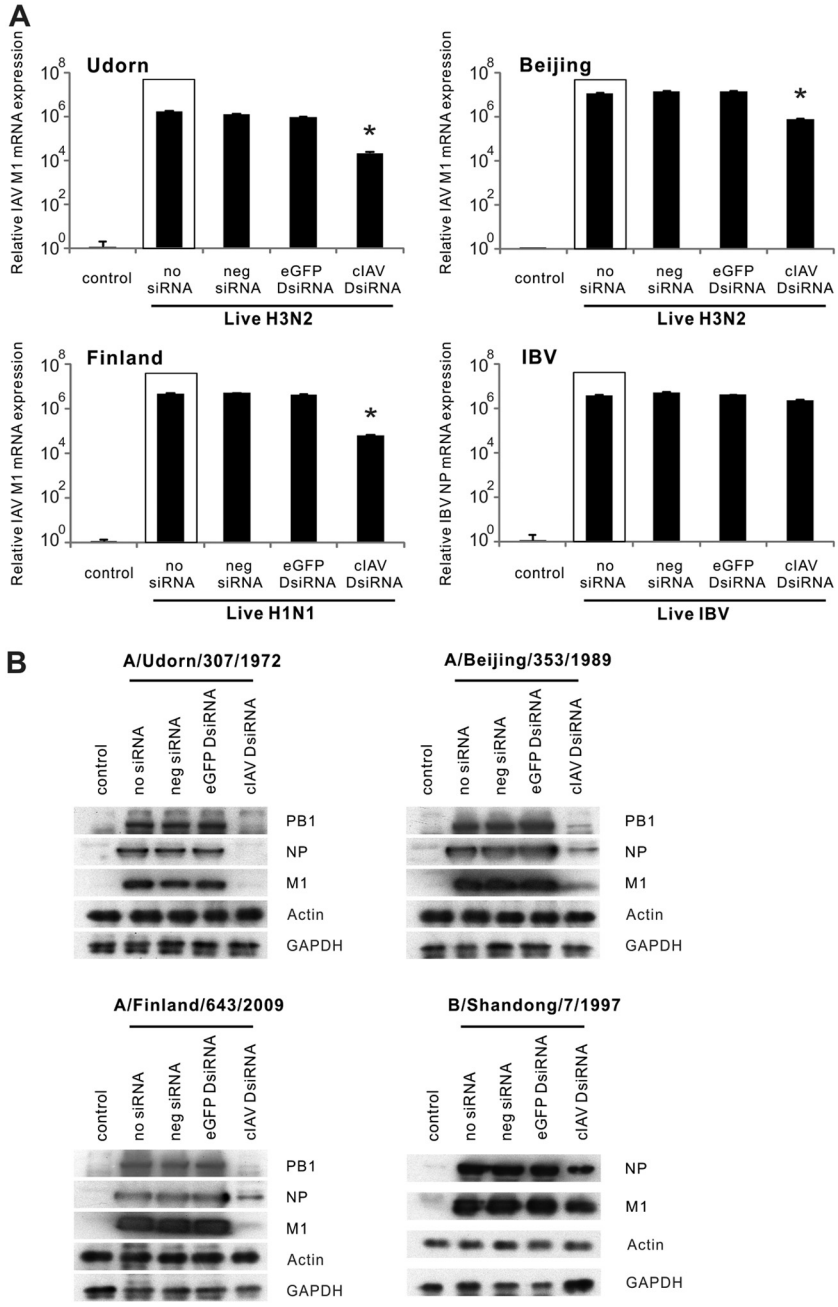

FIG 5 Antiviral specificity of cIAV DsiRNAs in Calu-3 cells. Human lung cancer Calu-3 cells (in 12-well plates; 5×10^5 cells/well) were mock transfected (control or no-siRNA bars) or pretransfected with the indicated control and IAV-specific siRNA/DsiRNAs (10 nM). After 21 h of incubation, cells were infected with the indicated IAVs or influenza B virus (IBV) at an MOI of 1 for an additional 24 h. Cells were then collected for RNA isolation and quantitative RT-PCR analysis and for Western blot analysis. (A) The values of RT-PCR analyses were normalized against β-actin gene-specific mRNA, and relative IAV M1 RNA or IBV NP RNA levels were calculated by the $\Delta\Delta C_T$ method using untreated control cells as a calibrator. The means ($\pm$SD) of three parallel analyses are shown. Data are representative of three individual experiments. Statistical significance was determined against results from samples of nontransfected cells (no siRNA; boxed bars). *, $P < 0.05$. (B) Western blot analysis for the expression of IAV proteins PB1, NP, and M1, IBV proteins NP and M1, and cellular β-actin and GAPDH proteins in siRNA/DsiRNA transfected moDCs after the infection of indicated IAVs or IBV. Cells were collected at 24 h after infection, and whole-cell lysates were prepared. Cellular proteins (30 μg/lane) were separated by 10% SDS-PAGE, followed by Western blot analysis with the indicated antibodies. One representative experiment of three independent experiments is shown.

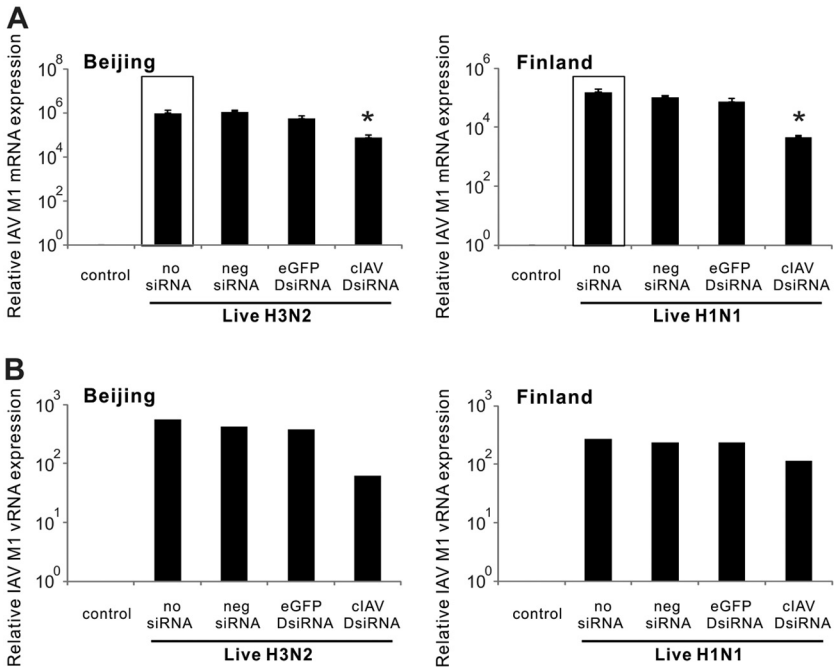

FIG 6 Inhibition of seasonal IAV replication by pretransfection with a cIAV DsiRNA swarm in A549 cells. Human lung epithelial A549 cells (in 12-well plates; 5×10^5 cells/well) were mock transfected (control or no-siRNA bars) or pretransfected with the indicated control and IAV-specific siRNA/DsiRNAs (10 nM). After 21 h of incubation, cells were infected with the indicated IAVs at an MOI of 1 for an additional 24 h. Cellular or the supernatant samples from A549 cells were then collected for RNA isolation and quantitative RT-PCR analysis. (A) The values of RT-PCR analyses were normalized against β-actin gene-specific mRNA, and the relative cellular IAV M1 mRNA level was calculated by the $\Delta\Delta C_T$ method using untreated cells as a calibrator. The means ($\pm$SD) of three parallel analyses are shown. Data are representative of three individual experiments. Statistical significance was determined against results from samples of nontransfected cells (boxed bars). *, $P < 0.05$. (B) Cell culture supernatant viral RNA levels from isolated total RNA were calculated relative to the level in untreated control cell supernatants with the $\Delta\Delta C_T$ method.

induced much higher IFN gene and MxA expression levels than the cIAV DsiRNAs (Fig. 8A and B). Control siRNA and DsiRNA swarm transfections showed no antiviral activity even though they induced low levels of IFN mRNA and MxA protein expression (Fig. 8A to C).

The antiviral effect of a cIAV DsiRNA swarm is independent of a functional IFN system. Pretransfection of siRNAs induced weak IFN responses in macrophages and moDCs (Fig. 8A and B). To further investigate whether the IFN signaling pathway is essential for the antiviral effect of a cIAV-specific DsiRNA swarm, we used cell lines generated from mouse embryonic fibroblast (MEF) cells lacking functional interferon regulatory factor 3 (IRF3) and IRF 7 (IRF3/7 knockout [KO]), IFN-α/β receptor 1 (IFNAR1 KO), or NF-κB RelA, c-Rel, and Nfkb1 (NF-κB KO) genes. Wild-type (wt) and KO cells were first pretransfected with negative siRNA, eGFP DsiRNA, or cIAV-specific DsiRNA swarm for 21 h, followed by an infection with the IAV A/Udorn/307/1972 (H3N2) strain at an MOI of 1 for an additional 24 h. Cells were collected, and viral M1 gene expression was analyzed by qRT-PCR; viral NP and PB1 protein expression was analyzed by Western blotting. Pretransfection of cells with either a negative siRNA or eGFP-specific DsiRNA swarm did not reduce IAV M1 RNA expression in any types of cells compared to the level in the sample with no siRNA pretransfection (Fig. 9A). Accordingly, no reduction in viral protein expression was detected in control siRNA-treated cells (Fig. 9B). However, IAV M1 RNA expression was significantly decreased in cIAV-specific DsiRNA pretransfected wt, IFNAR KO, and NF-κB KO cells (Fig. 9A). Western blot results also showed that pretransfection of the cIAV-specific DsiRNA swarm dramatically inhibited viral PB1 and NP protein expression in all types of cells (Fig. 9B). Collectively (Fig. 8 and

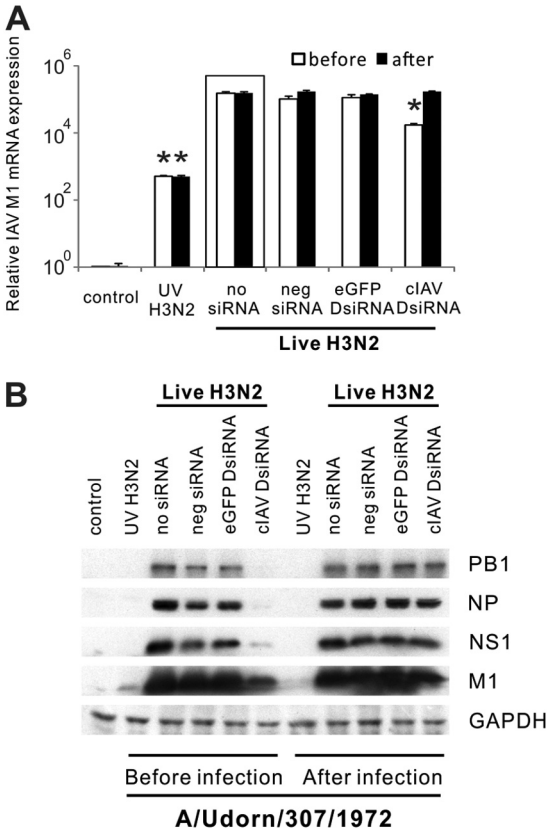

FIG 7 Difference between antiviral effects of cIAV DsiRNAs in moDCs transfected before and after IAV infection. Human moDCs from four different blood donors were separately mock transfected (control, UV IAV, or no-siRNA bars) or pretransfected with the indicated siRNAs (10 nM) either 21 h before or 1 h after A/Udorn/307/72 (H3N2) virus infection. Cells were infected with live or UV-irradiated A/Udorn/307/72 IAV at an MOI of 1. At 24 h p.i. cells from four different blood donors were pooled, and IAV M1 RNA expression was determined by qRT-PCR from isolated total cellular RNA or by Western blotting from cell lysates. (A) Relative IAV M1 RNA expression in cells transfected 21 h before infection or 1 h after infection. The values were normalized against β-actin gene-specific mRNA, and relative IAV M1 RNA levels were calculated by the $\Delta\Delta C_T$ method using untreated cells as a calibrator. The means ($\pm$SD) of three parallel determinations are shown. Data are representative of three individual experiments. Statistical significance was determined against results from samples of nontransfected cells (boxed bars). *, $P < 0.05$. (B) Western blot analysis for the expression of viral PB1, NP, M1, and NS1 proteins and GAPDH protein in siRNA/DsiRNA transfected moDCs after A/Udorn/72 IAV infection. Cells were collected at 24 h p.i., and whole-cell lysates were prepared. Cellular proteins (30 μg/lane) were separated by 10% SDS-PAGE, followed by Western blot analysis with the indicated antibodies. One representative experiment of three independent experiments is shown.

9), these data indicate that the inhibition of IAV replication by pretransfection with cIAV DsiRNAs is largely due to specific IAV gene silencing and that the IFN system has a more minor role in this inhibition.

DISCUSSION

IAVs are responsible for yearly epidemics and occasional pandemics in humans and domestic animals, which lead to significant morbidity and huge economic losses. Moreover, the newly emerged avian influenza viruses constitute a severe global threat to humans. In the present study, we created a DsiRNA swarm, namely, cIAV DsiRNAs that harbor multiple DsiRNAs and target several conserved gene elements in the IAV genome (Fig. 1). We showed that this novel DsiRNA swarm has significant efficacy in inhibiting the replication of several strains of IAVs in primary human macrophages and moDCs as well as lung carcinoma Calu-3 cells and A549 cells (Fig. 2 and 6). This broad-spectrum but IAV-specific DsiRNA swarm protected uninfected cells against IAV infection with maximum efficacy when the siRNAs were delivered before the infection

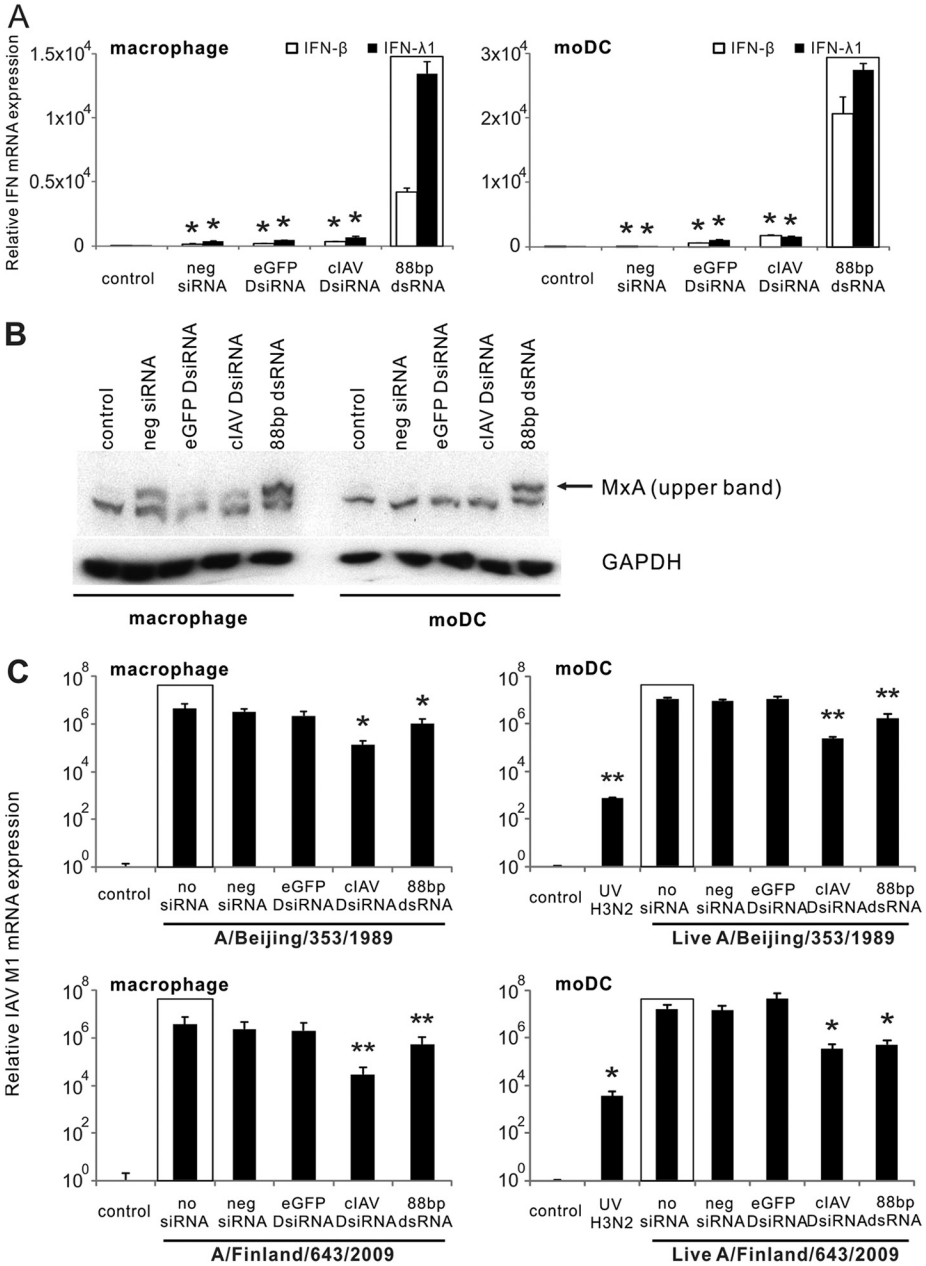

FIG 8 Induction of IFN gene expression and inhibition of IAV replication by siRNA, DsiRNA swarms, and an 88-bp dsRNA in human macrophages and moDCs. Human macrophages or moDCs were left nontransfected (control, UV IAV, or no siRNA) or separately transfected with the indicated siRNA or DsiRNA swarms or 88-bp dsRNA (10 nM) for 21 h. (A) qRT-PCR analysis of IFN gene expression. IFN-β and IFN-λ1 mRNA expression is shown. Values are the means ($\pm$SD) of three parallel analyses. Data are representative of three individual experiments. The statistical significance of results with siRNA/DsiRNA swarm pretransfected cellular samples was determined against results with s 88-bp dsRNA pretransfected cellular samples (positive control, boxed bars). *, $P < 0.05$. (B) Western blot analysis for the expression of IFN-$\alpha/\beta/\lambda$-inducible MxA protein and GAPDH in siRNA/DsiRNA/88-bp dsRNA transfected macrophages or moDCs. Cells were collected at 21 h after transfection, and whole-cell lysates were prepared. Cellular proteins (30 μg/lane) were separated by 10% SDS-PAGE, followed by Western blot analysis with anti-MxA and anti-GAPDH antibodies. One representative experiment of three independent experiments is shown. (C) Transfected macrophages or moDCs were infected with the indicated live or UV-irradiated seasonal IAV at an MOI of 1. Macrophages were washed twice with PBS at 1 h p.i. and subsequently maintained in a macrophage medium, while the input virus remained in moDCs throughout the experiment. At 24 h after infection cells from different blood donors were pooled, and IAV M1 mRNA expression was determined by qRT-PCR from isolated total cellular RNA. The values were normalized against β-actin gene-specific mRNA, and relative IAV M1 mRNA levels were calculated with the $\Delta\Delta C_T$ method using untreated control cells as a calibrator. The means ($\pm$SD) of three parallel analyses are shown. Data are representative of three individual experiments. Statistical significance was determined against results of mock transfected cellular samples (no-siRNA boxed bars). *, $P < 0.05$; **, $P < 0.01$.

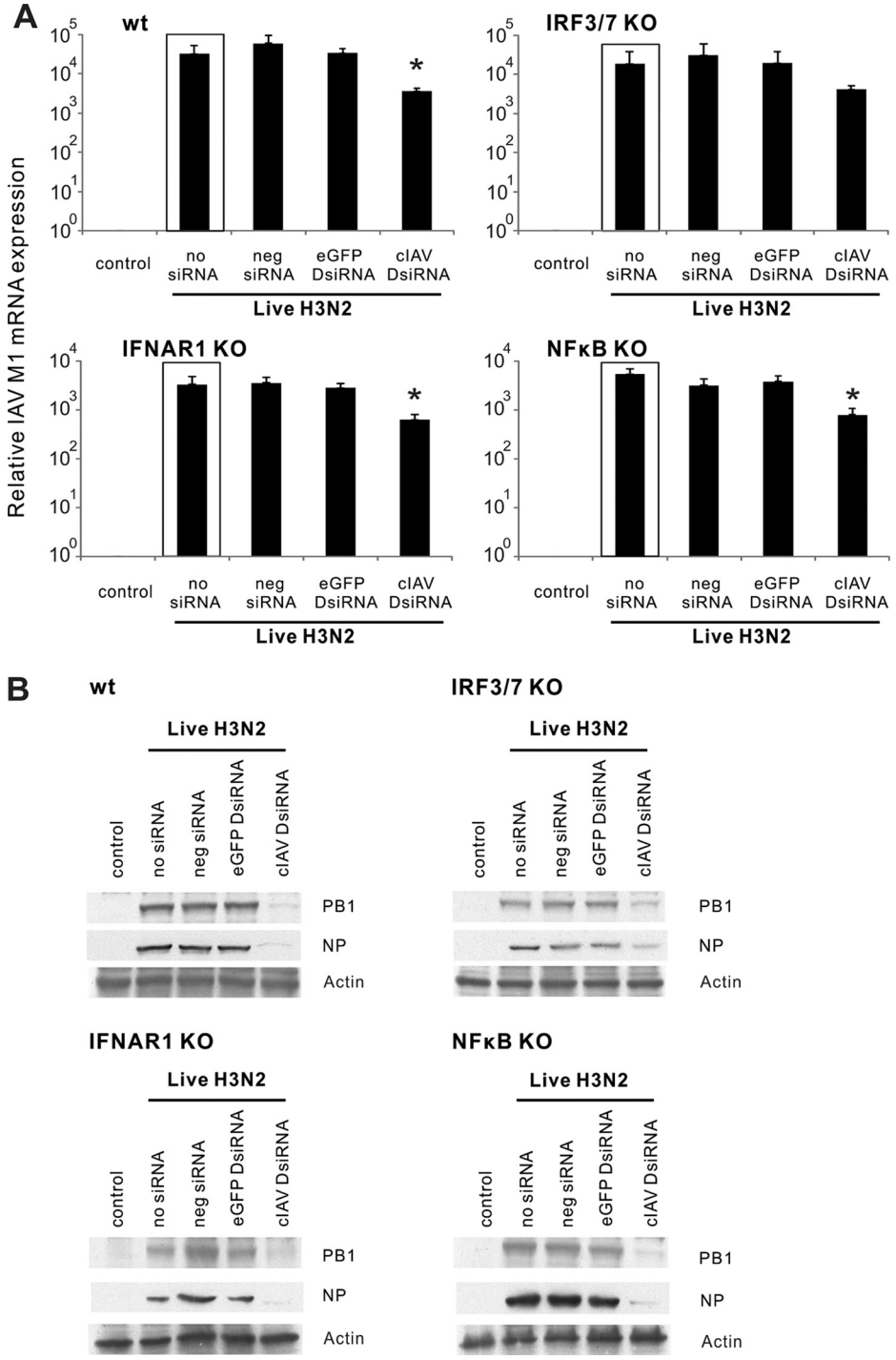

FIG 9 Inhibition of H3N2 IAV replication by pretransfection with cIAV DsiRNA swarm in mouse KO cells. Mouse wt cells, IRF3/IRF7 double-KO cells (IRF3/7 KO cells), NF-κB RelA/c-Rel/Nfkb1 triple-KO cells (NF-κB KO cells), and IFN-α/β receptor 1 KO cells (IFNAR1 KO cells) (in 12-well plates; 5×105 cells/well) were mock transfected (control or no-siRNA bars) or pretransfected with the indicated control and IAV-specific siRNA/DsiRNAs (10 nM). After 21 h of incubation, cells were infected with H3N2 IAV (A/Udorn/307/1972) at an MOI of 1 for an additional 24 h. Cells were then collected for RNA isolation and quantitative RT-PCR analysis and for Western blot analysis. (A) The values of RT-PCR analyses were normalized against β-actin gene-specific mRNA, and the relative IAV M1 mRNA level was calculated by the $\Delta\Delta C_T$ method using untreated control cells as a calibrator. The means (±SD) of three parallel analyses are shown. Data are representative of three individual experiments. Statistical significance was determined against results of samples of nontransfected cells (boxed bars). *, $P < 0.05$. (B) Western blot analysis for the expression of IAV proteins PB1 and NP and cellular β-actin in siRNA/DsiRNA transfected cells after the infection of H3N2 IAV. Cells were collected at 24 h after infection, and whole-cell lysates were prepared. Cellular proteins (30 μg/lane) were separated by 10% SDS-PAGE, followed by Western blot analysis with the indicated antibodies. One representative experiment of three independent experiments is shown.

(Fig. 7). Although DsiRNAs also induced some IFN responses, the inhibition of IAV replication by pretransfection with cIAV DsiRNAs is largely due to a specific IAV gene silencing effect through the RNAi pathway (Fig. 8 and 9).

Presently, one of the major preventive means against IAV infections is vaccination (43). However, the rapid evolution of IAVs and the appearance of new virus variants create problems for the clinical efficacy of IAV vaccines. In future influenza pandemics it will be very difficult to provide sufficient amounts of vaccines in time to protect the general population (44). Thus, the development of various types of siRNA strategies may provide a novel means to prevent and treat, e.g., severe IAV infections. Virus-specific siRNA therapy is not dependent on the adaptive immune system of the host, and it should, thus, be equally effective in all individuals. Different types of RNAs are expressed in virus-infected cells during an IAV life cycle, including mRNA, viral genomic RNA (vRNA), and complementary RNA (cRNA), all of which can be potential targets for degradation mediated by siRNAs. Various types of siRNAs have been reported to be effective in inhibiting the replication of IAV, including siRNAs specific for PB2, PB1, PA, NP, M1, M2, and NS1 viral genes (25, 37, 39, 45–49). However, most of the studies have been based on chemically synthesized single-site siRNAs.

It has been shown that DsiRNAs are more potent inducers of RNAi than the canonical 21-nt siRNAs and that a combination of siRNAs may decrease the off-target effects and increase the virus specificity of gene silencing (31, 50). Furthermore, simultaneous targeting of multiple influenza virus RNA segments and mRNAs by different siRNAs should guarantee that at least one type of siRNA is active against the viral RNA target. In the present study, we designed a chimeric cDNA construct of IAV which harbors several conserved regions derived from the six most conserved segments of the IAV genome. Using this chimeric construct as a template, IAV-specific dsRNAs were produced by an *in vitro* transcription and replication system, and the dsRNA molecules were further digested into 25- to 27-nt-long IAV-specific DsiRNAs by the Dicer enzyme (Fig. 1). With this approach we could combine the benefits of DsiRNAs and siRNA swarms. Thus, the generated cIAV DsiRNA swarm was expected to have higher specificity and better genome coverage and potentially lower off-target effects than the traditionally used canonical single-site siRNAs. Moreover, since single-stranded RNA (ssRNA) and dsRNA templates were produced by using T7 polymerase and φ6 P2 replicase, respectively, the produced dsRNA molecules contain 5′ triphosphate groups. Thus, DsiRNA swarm contains some siRNA molecules which bear 5′ triphosphate groups after being digested from dsRNA template, and these molecules may induce some IFN gene expression. Therefore, pretransfection with DsiRNA swarm may inhibit IAV replication by both inducing weak IFN responses and silencing viral gene expression.

We first tested the efficacy of cIAV siRNAs at inhibiting the replication of avian IAVs in human primary macrophages and moDCs. The inhibition was more significant in human macrophages since the expression of all studied viral proteins of H5N1 and H7N9 viruses was almost completely inhibited by pretransfection of the cells with cIAV siRNAs (Fig. 2). Plaque-forming assay and qRT-PCR analysis with supernatant samples from H5N1-infected cells also showed that pretreatment with cIAV-specific DsiRNA could efficiently inhibit the productive replication of H5N1 virus in human primary macrophages and moDCs (Fig. 3). The experiments carried out in moDCs also demonstrated the efficacy of cIAV DsiRNAs in inhibiting the replication of a broad range of IAV strains (Fig. 4). For instance, the expression of the PB1 protein of all IAV strains, including the two avian IAVs, was significantly blocked by the cIAV DsiRNAs. PB1 protein is the catalytic subunit of the IAV polymerase, and it is essential for IAV replication (51). Targeting the viral RNAs that encode the components of the polymerase, especially PB1, the minimal subunit of viral RNA polymerase, is a good strategy since the polymerase proteins are likely the rate-limiting factors for IAV replication.

In order to validate the general applicability, efficacy, and specificity of this approach, cIAV siRNAs were pretransfected into human lung epithelial Calu-3 cells, followed by challenging the cells with several influenza virus strains including one IBV

strain. The qRT-PCR and Western blotting results showed that transfection with cIAV siRNAs can largely reduce viral RNA and protein expression of IAVs but failed to reduce IBV RNA and protein expression (Fig. 5). Even though A549 cells are more resistant to RNA transfection than other cell types, they showed similar inhibition levels of IAV replication by cIAV DsRNA as quantitated by M gene-specific qRT-PCR (Fig. 6). This indicates that our technology is functional in many types of cells and shows high specificity for the target virus, i.e., IAV in this case.

One of the major challenges in the clinical use of siRNAs is to find a safe and effective delivery system for humans. Primary cells are resistant to siRNA transfection compared to other cell types. Presently, mainly two ways to introduce siRNA into primary cells have been used: electroporation and liposome transfection (lipofection). Although electroporation may show higher efficiency of transfection, it can easily reduce the viability of the primary cells due to their sensitivity to manipulation (52). In contrast, liposome transfection appears to be less toxic to primary cells. In the present study, we screened several liposome reagents and found that TransIT-X2 showed the highest efficiency for delivering siRNAs into the cells, and it was also the least harmful to cell viability (data not shown). We also carried out experiments to explore whether siRNA was effective in cells that were already infected by IAV. The data indicated that in order to efficiently inhibit IAV replication, the cIAV siRNAs must be present in cells before the infection (Fig. 5). However, in previous studies siRNA-induced IAV inhibition has been documented when siRNAs have been transfected into previously infected MDCK cells and embryonated chicken egg cells (25). This discrepancy in findings may be due to substantial differences between the experimental designs, the nature of the siRNAs, and the cells used. Moreover, our data do not necessarily imply that preinfection delivery is an essential prerequisite for clinical efficacy. Rather, it is plausible that during early phases or even during later phases of IAV infection, most of the epithelial and immune cells in the respiratory tissues of a patient are still uninfected, and, thus, there may be a sufficient time window for siRNAs to prevent further spread of the infection.

dsRNAs can be recognized by different types of cellular RNA receptors, and siRNAs are small dsRNA molecules that can also stimulate innate immune antiviral responses (53). Previous studies have shown that chemically synthesized single-site siRNAs can activate the innate immune response in mammalian cells (54–57). It has also been shown that long chemically synthesized siRNAs are more potent activators of IFN responses than canonical siRNAs (58, 59). In the present study, we also tested the potential of siRNAs and DsiRNAs to elicit IFN responses since we noticed that negative-control siRNA or eGFP DsiRNA swarm weakly inhibited viral M1 mRNA expression of some IAV strains (Fig. 2A and 4A). As mentioned above, since our DsiRNA swarm contains some siRNA molecules which bear 5′ triphosphate groups after being digested from dsRNA templates and since commercial negative siRNAs also bear 5′ monophosphate group, they may induce weak IFN antiviral responses. Our data indeed showed that the transfection with all tested siRNAs, 21- to 23-nt siRNA (negative siRNA) and the longer 25- to 27-nt DsiRNAs generated by *Giardia intestinalis* Dicer (eGFP DsiRNA and cIAV DsiRNA), weakly induced IFN-β and IFN-λ1 mRNA expression and MxA expression in human macrophages and moDCs (Fig. 8A and B). The level of IFN gene expression induced by siRNA or DsiRNA was very low compared to that induced by an 88-bp dsRNA, which has been shown to efficiently induce IFN responses (42). The induced IFN antiviral response by siRNA or DsiRNA may further inhibit viral replication during IAV infection. However, further analysis in mouse KO cells, in which IFN antiviral signaling pathways are partially or completely impaired, demonstrated that inhibition of IAV viral mRNA or protein expression by IAV DsiRNA is mainly not due to induced IFN antiviral responses (Fig. 9). Our study suggests that the treatment with a cIAV-specific DsiRNA swarm may inhibit IAV replication by two mechanisms: it can inhibit IAV replication through gene silencing and through induction of some IFN antiviral responses although the first pathway apparently plays a dominant role.

There is a controversy about whether inducing IFN responses by siRNA is beneficial

or harmful for siRNA-based antiviral activity. Some studies have shown that siRNAs can result in global inhibition of protein translation and cell death, whereas other researchers have reported that the nonspecific immunostimulatory effects induced by siRNAs could contribute to more effective antiviral activity (53, 60). Specifically, it has been demonstrated that airway macrophages play a critical role in limiting lung injury by restricting the accumulation of virus products in macrophages, which tightly control the subsequent activation of inflammation and may lead to impaired host responses (61). In our experimental setting, pretreatment with the cIAV-specific DsiRNA swarm was able to induce very limited IFN antiviral responses compared to those induced by treatment with an 88-bp dsRNA (Fig. 8A and B), but it could still inhibit viral M1 mRNA and viral protein expression during IAV infection (Fig. 2 and 9). Even though 5′-triphosphate-positive DsiRNA molecules may induce some IFN responses, they likely play a minor role in the cIAV DsiRNA-mediated antiviral response since in mouse KO cells defective in the IFN system, influenza virus-specific DsiRNA effectively inhibited IAV (Fig. 9). Moreover, we also noticed that pretransfection with an siRNA swarm could induce only limited inflammatory responses (data not shown), and these responses together with limited IFN antiviral responses are beneficial to the host for preventing IAV replication or further infection. Previously, several groups have demonstrated that H5N1 avian IAV can induce a "cytokine storm" resulting in an excessive inflammatory reaction (62). And this reaction would lead to severe lung injury and unfavorable prognosis of IAV infection. So the treatment with a cIAV DsiRNA swarm which induced limited antiviral/inflammatory responses is likely not leading to excessive inflammatory reactions (cytokine storm/acute respiratory distress syndrome [ARDS]) in the host.

In the present study, we have generated a broad-spectrum IAV-specific DsiRNA swarm that efficiently inhibits both avian and seasonal influenza virus replication in various types of human cell systems. The findings have significant implications for the use of DsiRNA swarms for the prophylaxis and therapy of IAV infections, and our observations will open up the way for further studies on the efficacy of siRNAs and DsiRNAs in *in vivo* IAV infection models.

MATERIALS AND METHODS

Cell cultures. Human primary monocytes were purified from the freshly collected, leukocyte-rich buffy coat layer in centrifuged blood samples obtained from four healthy blood donors as described previously (63). Monocytes were differentiated into either macrophages or immature DCs.

Mononuclear cells were allowed to adhere to plates (Falcon; Becton, Dickinson) for 1 h at +37°C in RPMI 1640 medium (Sigma-Aldrich) to obtain monocytes for macrophage differentiation. The cells were washed using cold phosphate-buffered saline (PBS; pH 7.35), and the remaining monocytes were cultured in macrophage/serum-free medium (Life Technologies) supplemented with human recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) (10 ng/ml; Nordic Biosite). Cells were differentiated into macrophages for 6 days, with a change to fresh culture medium every 2 days.

The differentiation of monocyte-derived dendritic cells (moDCs) was achieved by cultivating monocytes in the presence of 10 ng/ml of recombinant human GM-CSF (BioSource International) and 20 ng/ml of recombinant human interleukin-4 (IL-4) (R&D Systems) in RPMI 1640 medium. The cells were cultivated for 6 days, and fresh medium was added every 2 days.

Cultured human airway epithelial cell lines Calu-3 (ATCC HTB-55) and A549 (ATCC CCL185) and Madin-Darby canine kidney (MDCK) cells (ATCC CCL-34) were maintained by continuous growth in Eagle minimal essential medium (Eagle-MEM) (Sigma-Aldrich). Wild-type mouse embryonal fibroblasts (MEFs) and IRF3/IRF7 double and RelA/c-Rel/Nfkb1 triple KO cells were kindly provided by Alexander Hoffmann, Signaling Systems Lab, Los Angeles, CA, and IFN-α/β receptor 1 (IFNAR1) KO cells were kindly provided by Peter Staeheli, University of Freburg, Germany. Wild-type and KO cell lines were obtained after rigorous passaging of MEFs until a stable immortalized cell line was obtained. The MEF cell lines were cultured in Dulbecco's MEM.

All cell culture media were supplemented with 0.6 μg/ml penicillin, 60 μg/ml streptomycin, 2 mM L-glutamine, 20 mM HEPES, and 10% (vol/vol) fetal bovine serum (Integro). All cells were maintained at 37°C in a humidified atmosphere in the presence of 5% CO_2.

Analysis and selection of conserved sequences within the IAV genome. Highly conserved regions of the IAV genome were identified by aligning the genomic sequences of several H5N1 strains using the Clustal W program (64). The IAV strains included in this analysis were avian/swine A/chicken/Jiangsu/cz1/2002, A/goose/Jilin/hb/2003, A/swine/Henan/wy/2004, A/wild duck/Hunan/211/2005, and A/avian/Hong Kong/0828/2007 and human A/Hong Kong/482/97, A/Viet Nam/1194/2004, and A/Anhui/1/2005 viruses. Conserved sequences identified within six genome segments of H5N1 that encoded PB2, PB1, PA, NP, M, and NS proteins were further analyzed and scored using siVirus (65). Sites with potential off-target specificity were identified by siVirus, and subsequently the most conserved sequences that had minimal

off-target potential were reverse transcribed into cDNA by Moloney murine leukemia virus reverse transcriptase (Fermentas) using viral genomic RNAs of A/wild duck/Hunan/211/2005 (GenBank accession numbers EU329189.1, EU329188.1, EU329187.1, EU329185.1, EU329183.1, and EU329182.1) as the template. The produced cDNAs were amplified by PCR, and the individual DNA fragments were ligated to produce a 2,756-bp-long chimeric DNA molecule that comprised the conserved sequences. This chimera was cloned into the multiple cloning site of plasmid pLD18 (42), and the resulting plasmid, pLD19, was subsequently used as a template for dsRNA production (66).

RNA preparations. Full-length chimeric IAV or eGFP gene-specific dsRNAs and an 88-bp-long phage φ6-specific control dsRNA were produced by *in vitro* transcription and replication. The transcription and replication of IAV or eGFP gene-specific dsRNAs and the 88-bp-long phage φ6-specific control dsRNA were accomplished using bacteriophage T7 DNA-dependent RNA polymerase and bacteriophage φ6 RNA-dependent RNA polymerases with plasmids pLD19 (42), pCR3.1-eGFP, and pLM659 (67) as the templates, respectively. The eGFP- and IAV-specific DsiRNA swarms were subsequently generated from the produced dsRNAs using recombinant *Giardia intestinalis* Dicer (40). The 88-bp dsRNA and all the produced siRNAs were desalted in NAP5 columns (GE Healthcare) and purified by high-performance liquid chromatography (HPLC) purification (GenFax PAC, Waters, or CIM Monolithic columns [BIA Separations]) as described previously (68). A commercial 21-nt siRNA with no sequence similarity with IAV was used as a negative control (Ambion/Thermo Fisher Scientific).

Influenza viruses. The human isolates of avian A/Vietnam/1194/04 (H5N1) and A/Anhui/1/13 (H7N9) viruses and seasonal human IAVs A/Udorn/307/1972 (H3N2), A/Beijing/353/1989 (H3N2), A/Wisconsin/67/2005 (H3N2), A/New Caledonia/20/1999 (H1N1), A/Brisbane/59/2007 (H1N1), A/Finland/643/2009 (H1N1), and IBV B/Shandong/7/97 were grown in allantoic cavities of 11-day-old embryonated chicken eggs at +36°C for 3 days. Virus titers from supernatant samples were determined by a plaque assay in MDCK cells, as previously described (41, 69, 70). The MOI is given according to the titers determined in the MDCK cells (41). The propagation of the avian IAV stocks and infection experiments with avian IAVs were carried out under biosafety level 3 (BSL-3) conditions.

RNA transfection and virus infection in monocyte-derived macrophages and DCs. All experiments were performed with macrophages or moDCs obtained from four blood donors. The cell cultures that originated from the individual donors were transfected with RNAs separately. DsiRNA swarms and an 88-bp dsRNA were transfected using TransIT-X2 transfection reagent (TransIT-X2 Dynamic Delivery System; Mirus Bio) into primary cells according to the manufacturer's instructions. UV irradiations of the stock viruses were performed at 600 mJ of UV light before the viruses were added to the cells. The infectivity of IAVs was completely destroyed by the dose of UV light used (69). At 21 h after transfection, the cells were infected with either UV-irradiated (control) or live IAVs for different periods of time, as described in the figure legends.

IAV infection in the Calu-3 and A549 cells. Cells were plated onto 12-well culture plates (5×10^4 cells/well) 1 day before transfection, at which time different siRNAs were transfected into the cells. At 21 h after the transfection, the cells were infected with different strains of IAV for 24 h. The cells were collected and then used for isolation of total cellular RNA or lysed in passive lysis buffer (Dual Glo kit; Promega) for the subsequent analyses of viral protein expression.

Quantitative reverse transcriptase PCR. Total cellular RNA was isolated from macrophages, moDCs, Calu-3, or A549 cells using an RNeasy Mini RNA Isolation kit (Qiagen). As the amounts of cells were limited and as there was a considerable individual variation in the responses of the cells of different blood donors, we used pooled cellular RNA specimens to obtain a more global view of siRNA protective efficacy against IAV infection in macrophages or moDCs.

DNase-treated total cellular RNA (1 μg) was reverse transcribed into cDNA by using the TaqMan Reverse Transcriptase kit (Applied Biosystems). cDNA samples were then amplified using TaqMan Universal PCR Mastermix buffer (Applied Biosystems) and a commercial gene expression system assay (Applied Biosystems) with primers and probes for human IFN genes IFN-β (Hs00277188_s1), IFN-λ1 (Hs00601677_g1), and β-actin (Hs99999903_ml). The IAV M1 gene-specific primer-probe pair that detects a highly conserved sequence in the M genes of all IAVs was designed by Ward and colleagues and was used with minor modifications (71). The IBV NP gene-specific primer-probe pair has been described previously (70). Each cDNA sample was amplified in duplicate with an Mx3005P quantitative PCR (QPCR) system (Stratagene). The relative amount of cytokine or viral RNAs was calculated with the delta-delta comparative threshold cycle ($\Delta\Delta C_T$) method using human β-actin mRNA in the standardization.

To quantify viral RNA from the supernatant samples, siRNA or DsiRNA nontransfected/pretransfected macrophages, moDCs, and A549 cells were infected as described above, and the supernatant samples were collected at 1 and 24 h after the infection. RNA isolation and cDNA synthesis have been described previously (41). qRT-PCR was performed using a Qiagen QuantiTect Multiplex PCR NoRox kit (Qiagen) with the same influenza A virus M1 primer-probe pair as described above.

Western blot analysis. Whole-cell lysates were prepared from pooled macrophages and moDCs derived from the four blood donors or from the Calu-3 and A549 cells. Passive lysis buffer of a Dual Luciferase Assay kit (Promega) containing 1 mM Na_3VO_4 was used as a protease inhibitor for the cell lysates. Protein aliquots of whole-cell lysates (30 μg) were separated on 10% SDS-polyacrylamide gels using a Laemmli buffer system (72). Proteins were transferred onto Immobilon-P membranes, followed by blocking with 5% milk in PBS (or in Tris-buffered saline [TBS] for Cell Signaling antibodies). Previously produced antibodies against IAV PB1, NS1, NP, and M proteins and IBV anti-NP and anti-NS1 antibodies were used (69, 70). Antibody against β-actin (actin H-300; SC-10731) was obtained from Santa Cruz Biotechnology, and antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Cell Signaling Technology. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit

antibody (DakoCytomation) was used as the secondary antibody. Antibody binding was visualized by an enhanced chemiluminescence (ECL) system (Pierce ECL Western Blotting substrate; Thermo Scientific) on HyperMax films (GE Healthcare).

Densitometry analyses. The software Image J was used for densitometric analyses of Western blot bands.

Statistical analyses. Results of all experiments are presented as means ± the standard deviations (SD) of the means. Statistical analyses were performed by the use of an unpaired two-tailed Student's *t* test; the statistical analysis results were adjusted by Bonferroni correction.

Ethics statement. Adult human blood was obtained from anonymous healthy blood donors through the Finnish Red Cross Blood Transfusion Service (permission no. 33/2015, renewed annually). The permission to import the avian virus strains for research purposes was obtained from the Finnish Food Safety Authority (permission no. 8634/0527/2012). All experiments using infective H5N1 and H7N9 viruses were performed within a BSL-3 laboratory of the National Institute for Health and Welfare, Helsinki, Finland. Animal immunization was approved by the Ethics Committee of National Institute for Health and Welfare (permission KTL 2008-02).

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M.J. conceived the study, designed and coperformed laboratory experiments, analyzed the results, and cowrote the paper; P.Ö. codesigned the study, performed experiments, and cowrote the paper; V.W. coperformed laboratory experiments; M.M.P., D.H.B., D.G., and I.J. codesigned the study and cowrote the paper.

We have no financial conflicts of interest.

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