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Characterization of Chicken Mda5 Activity: Regulation of IFN-β in the Absence of RIG-I Functionality

Adam J. Karpala,* Cameron Stewart,* Jim McKay,† John W. Lowenthal,* and Andrew G. D. Bean*

In mammals, Mda5 and RIG-I are members of the evolutionary conserved RIG-like helicase family that play critical roles in the outcome of RNA virus infections. Resolving influenza infection in mammals has been shown to require RIG-I; however, the apparent absence of a RIG-I homolog in chickens raises intriguing questions regarding how this species deals with influenza virus infection. Although chickens are able to resolve certain strains of influenza, they are highly susceptible to others, such as highly pathogenic avian influenza H5N1. Understanding RIG-like helicases in the chicken is of critical importance, especially for developing new therapeutics that may use these systems. With this in mind, we investigated the RIG-like helicase Mda5 in the chicken. We have identified a chicken Mda5 homolog (ChMda5) and assessed its functional activities that relate to antiviral responses. Like mammalian Mda5, ChMda5 expression is upregulated in response to dsRNA stimulation and following IFN activation of cells. Furthermore, RNA interference-mediated knockdown of ChMda5 showed that ChMda5 plays an important role in the IFN response of chicken cells to dsRNA. Intriguingly, although ChMda5 levels are highly upregulated during influenza infection, knockdown of ChMda5 expression does not appear to impact influenza proliferation. Collectively, although Mda5 is functionally active in the chicken, the absence of an apparent RIG-I-like function may contribute to the chicken’s susceptibility to highly pathogenic influenza.

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Influenza is a negative (−) sense ssRNA virus that impacts on a diverse range of vertebrates. Like mammals, avians are generally able to resolve most influenza infections; however, chickens are highly susceptible to infection by high-path (HP) influenza such as H5N1 (1). Several features of HP influenza infection in chickens include rapid viral replication (2) and an acute clinical onset that is often fatal in as little as 24 h (1, 2). Although vertebrates use multiple mechanisms to thwart virus invasion, the overall clearance and outcome of HP influenza infection in chickens is critically dependent on the early protection provided by the innate immune system. Thus, it is of importance to understand the key mechanisms that may be involved in the initiation of the chicken’s immune response to influenza.

Several innate immune pathways critical for frontline protection against RNA viruses in mammals involve the recognition of the viral signature molecule, dsRNA (3). In particular, the presence of intracellular viral dsRNA leads to the upregulation of cytokines, such as type 1 IFNs (3). Associated with this, key pattern recognition receptors, including TLR3 and the RIG-like helicases RIG-I and Mda5, engage this dsRNA (4, 5). Importantly, the engagement of a particular receptor appears to depend on several features of the dsRNA, including spatial location (6, 7), 5′-triphosphate recognition (8), and the length of the dsRNA molecule (7). Although TLR3 interacts with extracellular (4, 9) or endosomal (6) dsRNA, the role of the RIG-like helicases appears to be the engagement of dsRNA within the cytoplasm (7, 8). Furthermore, RIG-I recognizes the 5′-triphosphorylated moiety of the cytoplasmic dsRNA in mammalian cells (8), whereas long cytoplasmic dsRNA molecules appear to be important for Mda5 activation (7). Thus, the specific engagement of a RIG-like helicase, RIG-I or Mda5, is probably viral dependent, as these dsRNA features derive from a particular virus (7, 10).

A number of investigations identified specific RIG-I or Mda5 activity as being associated with particular RNA viral infections in mammals (10–12). RIG-I is implicated in the antiviral response to paramyxovirus (11) and vesicular stomatitis virus (7), and, to date, RIG-I is thought to be the chief instigator of the immune response during influenza infection in mammals (12–14). These studies suggested that RIG-I recognizes (−) sense RNA viruses (12, 15). Recently, Barber et al. (16) reported the absence of RIG-I in the chicken and have suggested that the observed severity of HP influenza in chickens may be explained by the absence of RIG-I activity. However, Siren et al. (17) have suggested the idea that Mda5 might play a role in the host response to influenza in mammals because influenza-induced IFN impacts both RIG-I and Mda5 levels. Studies in mammals suggested that Mda5 recognizes (+) sense RNA viruses (12). For example, during rhinovirus infection, Mda5 plays a critical role in the activation of the host-protective responses (18), and the response to picornavirus involves Mda5 sp. act. (19). However, recent mammalian studies report the involvement of both RIG-I and Mda5 during certain viral infections, including paramyxoviridae and measles infection (20, 21). Collectively, these reports in mammalian systems indicate that RIG-I and Mda5 may both play a role in a diverse range of (−) or (+) sense RNA virus infections (20, 21). With this in mind and...
combined with the absence of RIG-I in the chicken, it may be suggested that chicken Mda5 homolog (ChMda5) could be involved in the host response to influenza infection. However, to date, there is little understanding of the functional activities of RIG-like helicases in the chicken or their role during influenza infection.

To explore the mechanisms that control the chicken immune response to virus with regard to the RIG-I–like helicases, we have identified and cloned ChMda5. We show that ChMda5 has structural differences compared with mammalian Mda5. Further to this, we observed IFN regulation of ChMda5 expression and validated that ChMda5 mediates dsRNA-induced (polyinosinic-polycytidylic acid [pIC] and viral RNA [vRNA]) responses in the chicken. In addition, we show that chicken cells do not appear to generate a response to the 5′-triphosphorylated moiety of dsRNA or differing lengths of dsRNA, as has been demonstrated in mammals (7, 8, 10). Finally, ChMda5 knockdown did not appear to affect influenza proliferation. Our data support that the RIG-I–like function reported in mammals may be absent in the chicken and is not compensated for by ChMda5, which may help to explain why chickens appear highly susceptible to HP influenza infection.

Materials and Methods

Virus

H5N1 virus A/Vietnam/1203/04 (V/1203) influenza virus was used in this study. The virus was passaged in the allantoic fluid of 10- to 14-d embryonated specific pathogen-free (SPF) chicken eggs. Allantoic fluid was harvested and aliquoted, then stored at −80°C for inoculations. The H3N2 virus A/Wyoming/3/03 was passaged in DF1 chicken fibroblasts and titrated by infectivity and hemagglutinin (HA) assay as previously described (22).

Chickens

SPF white leghorn chickens (4 wk old) were purchased from a commercial supplier (SPAFAS) for use in this study. Chickens were housed in purpose-built isolators, fed commercial rations, and had continuous access to water. All research involving animals was approved by the Australian Animal Health Laboratory Animal Ethics Committee.

Cell culture

For each experiment, four spleens were freshly harvested from 4-wk-old chickens, and single-cell suspensions of leukocytes were prepared. Splenocytes were isolated by sieving through a 70-μm strainer into DMEM (Thermo Scientific) supplemented with 1% FCS and trypsin (5 U/mL) at room temperature. For each experiment, four spleens were freshly harvested from 4-wk-old chickens, and single-cell suspensions of leukocytes were prepared. Splenocytes were isolated by sieving through a 70-μm strainer into DMEM (Thermo Scientific) supplemented with 1% FCS and trypsin (5 U/mL) at room temperature.

Virus challenge

Chickens (four per group) were inoculated intraocularly with 50 egg infectious doses of V/1203. Virus work was carried out following biocontainment practices appropriate for work with H5N1 in a BL3 biocontainment facility (Australian Animal Health Laboratory). At 12 and 24 h postinfection (p.i.), chickens were euthanized, and samples of tissue were immediately harvested for RNA extraction. The propagation of H3N2 in ChMda5 knockdown DF1 cells was carried out by incubating 500 μL diluted H3N2 (DF1-media supplemented with trypsin, 5 μg/mL) at 37°C for 1 h. Diluted virus was subsequently replaced with DF1 media supplemented with 1% FCS and trypsin (5 μg/mL). HA assay was used to determine virus titers as previously described (22).

Reagents

The synthetic dsRNA analog pIC (Invivogen) and the ssRNA analog polycytidylic acid (Invivogen) were prepared and stored as per the manufacturer’s instructions. vRNA was prepared from supernatant (SN) harvested from 3-d H3N2-infected DF1 cells. The SN was initially centrifuged at 1000 × g for 10 min to remove cell debris. Viral pellet was obtained by centrifuging SN at 48,000 × g for 90 min at 4°C, similar to the method described by Kato et al. (7). The viral pellet was resuspended in TriReagent (Sigma-Aldrich) and RNA extracted as per the manufacturer’s instructions. Dephosphorylated RNA was prepared by shrimp alkaline phosphatase (SAP, Promega) treatment of RNA as per the manufacturer’s instructions. The different lengths of dsRNA were obtained by size fractionating 10 μg pIC on a 1% agarose gel. RNA sizes of interest were excised (Qiagen) and observed on 1% agarose gel to confirm dsRNA integrity. The pIC 21-mers were generated by digesting pIC with RNaseIII as per the manufacturer’s instructions (New England Biosciences). Chicken IFN-α, IFN-β, and IL-6 were expressed in Escherichia coli and purified by nickel chromatography and for subsequent use as previously described (22). Activity of recombinant products was confirmed by virus inhibition studies (22). Nuclease acid was stored at −80°C, and IFN and IL-6 were stored at 4°C.

ChMda5 cloning

ChMda5 mRNA was PCR amplified from chicken fibroblast cDNA using Mda5-specific oligonucleotides listed in Table I. The f2–r5, f8–r4, and f6–r1-generated PCR fragments were subcloned into pGEM-T Easy vector (Promega). The f2–r5 and f8–r4-derived clones were digested with the restriction enzymes SexA1 and SalI, and the ChMda5 f8–r4 fragment was ligated to the ChMda5 f2–r5 backbone vector. The newly derived f2–r4 construct and the f6–r1 clone were digested with BstBI and SalI, and the ChMda5-specific f6–r1 band was inserted into the f2–r4 backbone to generate full-length ChMda5. All sequences were analyzed on an ABI 377 sequencer (Applied Biosystems), and the full-length ChMda5 sequence was deposited in GenBank (accession number GU570144). ChMda5 has a coding region of 3006 bp.

Small interfering RNA design

Small interfering RNA (siRNA) molecules were produced using a construction kit (Ambion) according to the manufacturer’s instructions. Three ChMda5 (GenBank number GU570144) target sites were selected: bp 2070–2088 (siMda5.1), 1788–1796 (siMda5.2), and 1029–1047 (siMda5.3). The corresponding siRNA sequences were: 5′-TGAAGAACCTAGAGGGATT-3′ (siMda5.1), 5′-CAGAACACCCTGAGAAATA-3′ (siMda5.2), and 5′-GAGAAAAAGCATCAGACGCA-3′ (siMda5.3). To confirm specific target knockdown, a GFP reporter system was produced by cloning ChMda5 region 429–2099 into the EcoR5V andXba1 restriction sites of pEGFP-C, similar to Karpala et al. (5). Transfection of each siRNA with the reporter resulted in reduced enhanced-GFP intensity compared with an irrelevant siRNA, indicating ChMda5-specific knockdown (5).

Transfections

Transfections of DF1 cells were carried out using the transfection reagent (TA) Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. This method resulted in a transfection rate of at least 70%. Briefly, dsRNA or siRNAs were diluted to 10 times the intended final concentration in DMEM, and TA was diluted into DMEM at 1 μL/cm² well area. For cotransfections, the reporter vector (0.25 μg/cm² well area) was combined with each siRNA. After 5 min, the TA and siRNA were combined 1:1, incubated for 15 min, and then added to cell culture as per the manufacturer’s instructions (Invitrogen). After 6 h, the TA/siRNA mix was removed and replaced with DMEM media, then incubated for 48 h before further treatments.

RNA isolation and reverse transcription

RNA was harvested using TriReagent (Sigma-Aldrich) according to the manufacturer’s instructions. One microgram extracted RNA was subjected to DNase treatment using a DNase 1 kit (Sigma-Aldrich) according to the manufacturer’s instructions. The DNase-treated RNA was then reverse-transcribed to cDNA using a Reverse Transcription kit (Promega) according to the manufacturer’s instructions, then diluted and stored at −20°C.

Assessment of gene expression by quantitative real-time PCR

The relative quantitation of gene expression following treatment was carried out on an ABI Prism 7700 sequence detection system (Applied Biosystems) as previously described (4), using the comparative threshold cycle method, to determine fold change per gene, as per the manufacturer’s recommendations, then reusability of the manufacturer’s instructions. Primers and probes (Table I) were designed using Primer Express software (Applied Biosystems). Where possible, the probe sets were designed across intron/exon boundaries.
Statistical analyses

To determine the significant differences between experimental groups, ANOVA or unpaired t tests were performed using the fold-change scores. The p values were set at 0.05 (p ≤ 0.05). Error bars represent the SEM (SE).

Results

Molecular cloning and bioinformatic analyses of ChMda5

Interrogation of the chicken genome using available mammalian and aquatic RIG-I and Mda5 genes identified a ChMda5 ortholog (GenBank accession number XM_422031), whereas a RIG-I ortholog could not be identified. A ChMda5-specific PCR product was obtained using cDNA derived from chicken fibroblasts (DF1) stimulated with IFN-α for 2 h (see Materials and Methods and Table I). Sequence comparisons of the ChMda5-specific PCR product to Mda5 sequence located in GenBank (accession number XM_422031) revealed sequence gaps in the actual cloned product as compared with the predicted molecule. However, the XM_422031 sequence contains two nucleotide segments, 42–168 and 233–389, that are repeated at 591–716 and 782–938, respectively. This sequence repetition is unusual in that it is not present in any Mda5 sequences available to date. Combining the coincident sequence repeats in XM_422031 with the obtained shorter than expected PCR product, the full-length ChMda5 cds was predicted to be 3006 bps, which corresponds to a start site at nt 853 in XM_422031. From this, the obtained ChMda5 sequence of 3006 bp was deposited in GenBank (accession number GU570144).

Table II. Similarity of ChMda5 (3006 nt/1001 aa) to Mda5 and other dsRNA receptors

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<th>Percent Identical aa (Total)</th>
<th>Percent Identical aa 1–300 N-terminal</th>
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ChMda5 expression is regulated by type 1 IFN

Because IFN mediates the upregulation of Mda5 in human cells (17), we investigated whether ChMda5 might also be an IFN-inducible gene. ChMda5 was upregulated in chicken splenocytes following a 2-h treatment with IFN-α (8–13-fold), IFN-β (6–13-fold), or IFN-λ (7-fold) (Fig. 2). In contrast, chicken-IL6 did not affect ChMda5 expression. When the continuous chicken cell lines HD11 and DF1 were treated with type 1 IFNs for up to 8 h, the ChMda5 levels increased at all time points (Fig. 3). Moreover, IFN treatment of HD11 cells increased ChMda5 levels up to 20-fold at 4 h (Fig. 3A), whereas treatment of DF1 cells resulted in 100-fold induction at 8 h (Fig. 3B).

pIC induces IFN-β in a time- and dose-dependent manner

To establish the conditions for the chicken cell response to dsRNA, the dsRNA-mimetic pIC was transfected into DF1 cells, and IFN-β levels were measured. When pIC was transfected into DF1 cells at a concentration of 0.05 μg/ml, IFN-β levels increased 6- and 65-fold at 4 and 8 h, respectively (Fig. 4). When the pIC concentration was increased to 0.5 or 5.0 μg/ml, IFN-β levels peaked at 4 h (∼400-fold). However, a higher concentration of pIC (50 μg/ml) resulted in reduced cell viability, impacting on the level of IFN-β. No observable IFN-β upregulation occurred following transfection with ssRNA (50 μg/ml) or when dsRNA was applied untransfected (data not shown).

pIC-induced IFN-β is mediated by Mda5 in DF1 cells

The involvement of ChMda5 in pIC-induced IFN-β expression was investigated using RNA interference (RNAi) to produce ChMda5-knockdown cells. The siRNA molecules directed against ChMda5 (siMda5.1, siMda5.2, and siMda5.3) were screened individually or in combination by transfecting them into DF1 cells. When endogenous ChMda5 was thus targeted, the levels of ChMda5 were reduced.
were reduced in DF1 cells (Fig. 5A). The level of ChMda5 knockdown achieved varied among the molecules tested, with siMda5.1 achieving the best result (87% reduction, Fig. 5A). ChMda5-knockdown DF1 cells were then transfected with pIC, and the corresponding IFN-β levels were assessed. The siMda5.1 knockdown was the most effective, leading to a 72% decrease in IFN-β mRNA levels (Fig. 5B). Similarly, ChMda5 knockdown using siMda5.2, siMda5.3, and the combination of all three siRNAs also resulted in decreased levels of IFN-β mRNA, supporting a role for ChMda5 in pCI-induced IFN-β expression.

ChMda5 responds to different sizes of RNA molecules

It has been previously reported in mammals that short RNA molecules trigger RIG-I receptor-mediated IFN-β, whereas long dsRNA molecules trigger an Mda5 receptor-mediated response (7). We therefore tested whether ChMda5 could respond to different lengths of RNA molecules. DF1 cells were transfected with unfractionated pIC, long (∼6 kb), medium (∼3 kb), or short (∼1 kb) pIC molecules to determine the response to size-specific dsRNA. IFN-β levels were found to be similarly upregulated for all pIC lengths whether they were long, medium, or short (Fig. 6A). To determine if ChMda5 was associated with the response to these various length dsRNA molecules, ChMda5-knockdown DF1 cells were stimulated with the various length dsRNA molecules. We observed that the IFN-β upregulation was reduced to approximately half the levels for all RNA lengths compared with control DF1 cells (Fig. 6B).

Chicken cells respond similarly to triphosphorylated and dephosphorylated RNA

In mammals, RIG-I is activated by 5’-triphosphorylated dsRNA (7). Given the absence of a RIG-I homolog in the chicken, we
tested whether ChMda5 could compensate for this function. pIC and vRNA were SAP-treated or left untreated and transfected into human (HeLa) cells and chicken (DF1) cells. qRT-PCR analyses showed that the untreated RNA (both pIC and vRNA) induced IFN-β to high levels in HeLa cells (∼500-fold induction, Fig. 7A). However, when these RNA were SAP-treated, IFN-β levels were induced at lower levels (pIC-SAP, 250-fold; vRNA-SAP, 15-fold; Fig. 7A). In DF1 cells, the untreated RNA also induced increased IFN-β levels (pIC, 400-fold; vRNA, 20-fold; Fig. 7B). However, in contrast to HeLa cells, the SAP-treatment RNA did not appear to affect the ability of DF1 cells to induce IFN-β mRNA expression (Fig. 7B).

Both phosphorylated and dephosphorylated dsRNA use the Mda5 pathway in chicken cells

The finding that 5′-triphosphorylated and dephosphorylated dsRNA induced similar levels of IFN-β (Fig. 7B) suggests the absence of mammalian RIG-I–like function in the chicken. However, it may be possible that 5′-triphosphorylated and dephosphorylated dsRNA interact with an alternative pathway in addition to ChMda5. To test this possibility, ChMda5-knockdown DF1 cells were stimulated with untreated or SAP-treated pIC and vRNA, and then IFN-β levels were measured. In ChMda5-knockdown...
cells, the RNA-induced IFN-β levels were reduced to approximately half the levels of the control cells (Fig. 8).

**H5N1 infection induces Mda5 in chicken cells**

To establish whether ChMda5 might also be associated with the immune response to virus, we investigated the expression of ChMda5 following H5N1 infection in the chicken. Chicken brain, lung, and spleen have similar basal levels of ChMda5 (data not shown). Chickens inoculated with H5N1 or uninfected controls were analyzed 24 h p.i. by quantitative real-time PCR (qRT-PCR) for ChMda5 expression in the brain, lung, and spleen. All three tissues displayed increased ChMda5 levels (∼30–40-fold relative to the uninfected controls, Fig. 9).

**Mda5 knockdown has minimal impact on influenza virus proliferation**

To further understand the role of the ChMda5 pathway during influenza infection, ChMda5 was knocked down in DF1 cells. Because DF1 cells did not support controlled growth of H5N1 (A.J. Karpala, unpublished observations), these cells were then infected with influenza virus (H3N2) at a multiplicity of infection (moi) of 1.0 (Fig. 10A) or moi of 0.1 (Fig. 10B). HA titers were measured at 24, 48, and 72 h to determine virus levels. No influenza virus was detected at 24 h in any of the treatments. The level of virus replication at 48 and 72 h in ChMda5-knockdown cells was the same as that observed in the control (Fig. 10).

**Discussion**

The RNA helicases RIG-I and Mda5 are critical regulators of host antiviral responses in mammals (23–25). In this study, we provide the first report, to our knowledge, on Mda5 function in the chicken. Initial observations identified an Mda5 gene in the

**FIGURE 6.** IFN-β is upregulated to similar levels regardless of the length of pIC. A. Data show the fold increase in IFN-β levels in DF1 cells following transfection with 250 ng/ml unfractionated, long (6 kb), medium (3 kb), or small (1 kb) pIC molecules for 4 h. B. ChMda5-knockdown DF1 cells were transfected with long, medium, or small pIC molecules. Data show the IFN-β levels measured at 4 h after stimulation relative to the control knockdown (silrr) cells transfected with long pIC. Experiments were performed in triplicate, and the data represent two independent experiments (A, *p ≤ 0.05 between treatment and control, unpaired t test; B, *p ≤ 0.05 between silrr and siMda5.1 knockdown, unpaired t test).

**FIGURE 7.** Untreated or SAP-treated RNA differentially upregulates IFN-β expression in human and chicken cells. pIC or vRNA were untreated or SAP-treated, then 250 ng/ml was transfected into HeLa (A) or DF1 (B) cells for 4 h. Data shown as the fold change of IFN-β (relative to untreated cells) as measured by qRT-PCR. Experiments were performed in duplicate, and data are representative of two independent experiments. *p ≤ 0.05 between treatment and control, unpaired t test.

**FIGURE 8.** ChMda5 is associated with the IFN-β response to untreated or SAP-treated dsRNA. ChMda5-knockdown DF1 cells were transfected with 250 ng/ml indicated dsRNA. Data show the relative expression of IFN-β after 4 h compared with silrr-knockdown control cells as analyzed by qRT-PCR. Experiments were performed in triplicate, and data are representative of two independent experiments. *p ≤ 0.05 between silrr and siChMda5.1 knockdown, unpaired t test.
two experiments.

ChMda5 expression in tissues of infected birds against control uninfected birds. Data are displayed as the mean of n = 4 birds and representative of two experiments.

FIGURE 9. ChMda5 is upregulated in chicken cells following infection with H5N1 influenza. SPF chickens were inoculated with H5N1 influenza (V/1203), specific tissues were harvested at 24 h, and ChMda5 expression levels were measured by qRT-PCR. Data show the fold increase in ChMda5 expression in tissues of infected birds against control uninfected birds. Data are displayed as the mean of n = 4 birds and representative of two experiments.

FIGURE 10. Knockdown of ChMda5 expression does not appear to impact on in vitro influenza virus proliferation. ChMda5-knockdown DF1 cells were infected with H3N2 influenza virus. HA titers were measured at 24, 48, and 96 h following infection. Data show HA titers following infection (A, moi 1.0; B, moi 0.1) at 48 and 96 h p.i. Experiments were performed in quadruplicate, and data represent three independent experiments.

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The sensitivity of Mda5 recognition of its ligands has also been shown to depend on dsRNA length (7). Kato et al. (7) demonstrated that 1-kb dsRNA molecules signaled through RIG-I, whereas dsRNA molecules above 2 kb in length predominantly activated Mda5 in mammalian cells. The transfection of size-fractionated pIC (1, 3, or 6 kb) appeared to have little differential impact on the induction of IFN in chicken cells. It can be suggested that the shorter 1-kb pIC signaled via a receptor other than ChMda5. However, knockdown of ChMda5 prior to transfection with the different size-fractionated pIC resulted in a similar level of the reduction in IFN-β expression. This supports that the different-length pIC molecules all interact similarly with ChMda5 and, therefore, further indicates the absence of RIG-I-like function in the chicken.

This study, in addition to mammalian investigations, suggests that ChMda5 may not play a decisive role during the immune response to influenza virus (12, 14, 26). Additionally, we have shown that RIG-I-like function, in contrast to mammals, does not appear to be present in chickens. However, it should be considered that some influenza strains may have differing RNA complements that could interact differentially with ChMda5. It is known that some influenza strains may have differing RNA complements such as H1N1, can induce different cellular responses compared with highly pathogenic strains such as H5N1 (31, 32). With this in mind, it may be interesting to test whether ChMda5 has a role in diverse influenza virus infections, especially NI2 compromised virus, thereby increasing our understanding of this receptor and its role in the immune response to viruses.

Disclosures
The authors have no financial conflicts of interest.