Expression of the C-type lectins DC-SIGN or L-SIGN alters host cell susceptibility for the avian coronavirus, infectious bronchitis virus

Yueting Zhang [a], Elizabeth Buckles [b], Gary R. Whittaker [a,*]

[a] Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, United States
[b] Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, United States

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ABSTRACT

Infectious bronchitis virus (IBV), an avian coronavirus, is a cause of great economic loss in the poultry industry. The virus mainly infects respiratory epithelium, but can be also detected in other organs. The functional receptor for the virus has not been found and field strains of IBV do not infect conventional cell lines. Recently, it has been shown that the C-type lectins DC-SIGN/L-SIGN can promote entry of several coronaviruses. Here we examine whether DC-SIGN/L-SIGN are entry determinants for IBV. We show that by introducing human DC-SIGN/L-SIGN into non-permissive cells, infection by the IBV is dramatically increased. DC-SIGN mediated infection was inhibited by mannan and anti-lectin antibodies, and was independent of sialic acid levels on the cell. Enhancement of IBV infection also occurred for different serotypes of IBV. Our findings demonstrated that even in the absence of avian-specific receptor, DC-SIGN-like lectins are capable of mediating efficient IBV infection.

1. Introduction

Infectious bronchitis virus is the type species of the family *Coronaviridae*, and is part of the *Gammacoronavirus* genus (Woo et al., 2009). Within the *Coronaviridae*, individual species are able to infect a wide array of animals, including but not limited to humans, dogs, cats, pigs, cows, birds, bats, and whales. IBV mainly infects chickens, and globally causes great economic loss for the poultry industry every year (Saif, 2008). Although IBV mainly infects the ciliated epithelia in the respiratory tract of chickens and causes respiratory disease, many studies have discovered that IBV can also spread to other organs of the chicken and can cause pathology in other issues, such as alimentary tract, testes, oviduct, and Harderian gland (Bezuidenhout et al., 2011; Raj and Jones, 1997). Clinical

signs include coughing, sneezing, nasal discharge, loss of appetite, and reduction in egg laying (Saif, 2008). In addition to the original Massachusetts serotype, dozens of serotypes and genotypes of IBV have now been detected (Meir et al., 2010). Many strains can cause major or minor nephritis in both naturally infected or experimentally infected chickens (Lambrechts et al., 1993).

Susceptibility of virulent strains of IBV to different cell lines has been sporadically reported; *e.g.* chicken fibroblasts (Nazerian and Cunningham, 1968), HeLa cells (Chen et al., 2007), primary chicken tracheal epithelial cell (Shen et al., 2010), but currently the only way to efficiently propagate IBV is by using embryonated chicken eggs. In cell culture, while most field strains of IBV can infect primary chicken derived cells (*e.g.* chick kidney and tracheal cells), no cell lines are generally considered to be susceptible to IBV infection. The exception to this is the Beaudette strain, which is a highly embryo- and cell-culture adapted non-virulent virus and can infect cell lines derived from a variety of mammalian species (Saif, 2008). The ability of IBV to infect different tissues in chickens may be linked to the receptor distribution within these

* Corresponding author at: Department of Microbiology and Immunology, Veterinary Medical Center, C4127, Cornell University, Ithaca, NY 14853, United States. Tel.: +1 607 253 4019; fax: +1 607 253 3385.
E-mail address: grw7@cornell.edu (G.R. Whittaker).

tissues. Although the virus was first identified almost 80 years ago, the primary receptor for IBV has not been identified. There was speculation that feline aminopeptidase N (fAPN), which can serve as a common receptor for many Alphacoronaviruses, is a receptor for IBV (Miguel et al., 2002). This was based in part on the ability of the Ark99 strain of IBV to infect feline kidney cells (Miguel et al., 2002). However, these studies were not confirmed with additional isolates of IBV (Chu et al., 2007) and the current view is that APN is not a functional receptor utilized by IBV. There have also been studies showing that the tissue tropism of IBV may be linked to the use of sialic acid as an attachment factor, which is present on cell surface of various tissues (Winter et al., 2006). In addition, heparin sulfate has been reported as an attachment factor specifically for the Beaudette strain of IBV (Madu et al., 2007).

Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) and its closely related Liver/lymph node-specific ICAM-3 grabbing non-integrin (L-SIGN) are calcium-dependent lectins. DC-SIGN was first discovered to be important in human immunodeficiency virus type 1 (HIV-1) pathogenesis (Geijtenbeek et al., 2000). This lectin, expressed mainly on dendritic cells, is believed to interact with the HIV-1 surface glycoprotein gp120 and enable capturing and transporting of the virus particle from mucosal infection site to secondary lymphoid tissues (Geijtenbeek et al., 2000). DC-SIGN is also able to enhance HIV-1 infection in cis as an attachment factor (Lee et al., 2001). With a similar structure to DC-SIGN, L-SIGN is able to capture HIV-1 in the same manner. Many enveloped viruses such as Ebola virus, hepatitis C virus, and Sindbis virus also have been shown to be interacting with DC-SIGN and/or L-SIGN presumably via interaction with high-mannose glycoproteins on the viral particles (Alvarez et al., 2002; Klimstra et al., 2003; Navarro-Sanchez et al., 2003; Pohlmann et al., 2003).

For coronaviruses, there have been increasing numbers of reports demonstrating a role for human DC-SIGN and/or L-SIGN in pathogenesis of SARS coronavirus, human coronavirus-229E, human coronavirus-NL63, as well as feline coronavirus by enhancement of infection (Hofmann et al., 2006; Marzi et al., 2004; Regan and Whittaker, 2008; Yang et al., 2004). Based on the evidence that human DC-SIGN is able to promote infection of non-human viruses, i.e. feline immunodeficiency virus and feline coronavirus (de Parseval et al., 2004; Regan and Whittaker, 2008), we were interested in whether human DC-SIGN or L-SIGN might be an entry determinant for IBV and whether they may function in combination with fAPN.

In our study, we demonstrate that by introducing DC-SIGN or L-SIGN into non-permissive cells, infection of IBV strain M41 is dramatically increased. This enhancement of infection also applies to various field strains of virulent IBVs and is independent from the previous reported attachment factor sialic acid. Our findings indicate that there may be a role for DC-SIGN for IBV spread from one organ to another, but that an additional receptor is involved in the infection of epithelial cell types, which is likely to be distinct from APN.

2. Materials and methods

2.1. Virus strains

IBV strains used in this study were M41, Cal99, Conn46, Iowa609, Gray, Iowa97, and JMK. For preparation of virus stocks, approximately 10^2 EID50 of IBV was inoculated into 10-day-old specific-pathogen-free chicken embryos. Allantoic fluid from infected embryos was collected 48 h post inoculation, and subjected to clarification by centrifugation at $1800 \times g$ for 15 min at 4 °C. For concentrated IBV-M41 virus preparation, the allantoic fluid was further centrifuged at $34,500 \times g$ for 60 min at 4 °C using a Ti45 rotor (Beckman Coulter). The virus pellet was resuspended in phosphate buffered saline (Cellgro). IBV allantoic fluid was titered by egg infectious dose 50 assay. IBV concentrated preparation was titered by tissue culture infectious dose 50 assay. Influenza virus strain A/WSN/33 was propagated in MDBK cells and supernatant was collected 48 h post infection. Influenza virus preparation was titered by plaque assay.

2.2. Cell culture and plasmids

3T3–DC-SIGN cells were obtained from the NIAID AIDS Research and Reference Reagent Program. NIH3T3, CRFK, Vero E6, and BHK-21 cells were purchased from ATCC. 3T3-fAPN, CRFK-fAPN, and CRFK-DC-SIGN cells were kindly provided by Dr. Andrew D. Reagan, Cornell University. 3T3, CRFK, Vero E6, and BHK-21 cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin (Pen/Strep). The stable cell lines were grown in DMEM supplemented with 10% fetal bovine serum, 1% Penicillin/Streptomycin, and 400 μg/ml G418. Chicken embryonic kidney cells were purchased from Charles River Laboratories and were cultured in basal media (Invitrogen) supplemented with 10% calf serum and 1% Pen/Strep. Plasmids of hDC-SIGN and hL-SIGN were obtained from the NIAID AIDS Research and Reference Reagent Program. For transfection assays, 250 ng of plasmid DNA were mixed with 0.75 μl of Lipofectamine 2000 (Invitrogen) in 50 μl of Opti-MEM (Gibco) at room temperature according to manufacturer's protocol. Cells seeded on glass cover slips were transfected at 37 °C over night before viral infection.

2.3. RT-PCR for viral infection detection

10^6 3T3 or 3T3–DC-SIGN cells were seeded onto 6 well plates and infected with different dilutions of allantoic fluid of IBV-M41. Total RNA was extracted from IBV-M41 infected 3T3 or 3T3–DC-SIGN cells 8 h post infection using a Qiagen RNeasy Mini Kit following manufacturer's protocols. Total RNA was reversed transcribed into cDNA by using SuperScript One-Step RT-PCR kit (Invitrogen). Negative-strand RNA was reversed transcribed into cDNA using ThermoScript Reverse Transcriptase (Invitrogen) with anti-sense primer. PCR reaction with High Fidelity Platinum Taq polymerase (Invitrogen) was conducted in a Bio-Rad DNA Engine Peltier Thermal Cycler with conditions of 1 cycle of 94 °C for 2 min, 30 cycles at 94 °C for 30 s, 48 °C for 30 s, 72 °C for 1 min, and 1 cycle of 72 °C for 5 min.
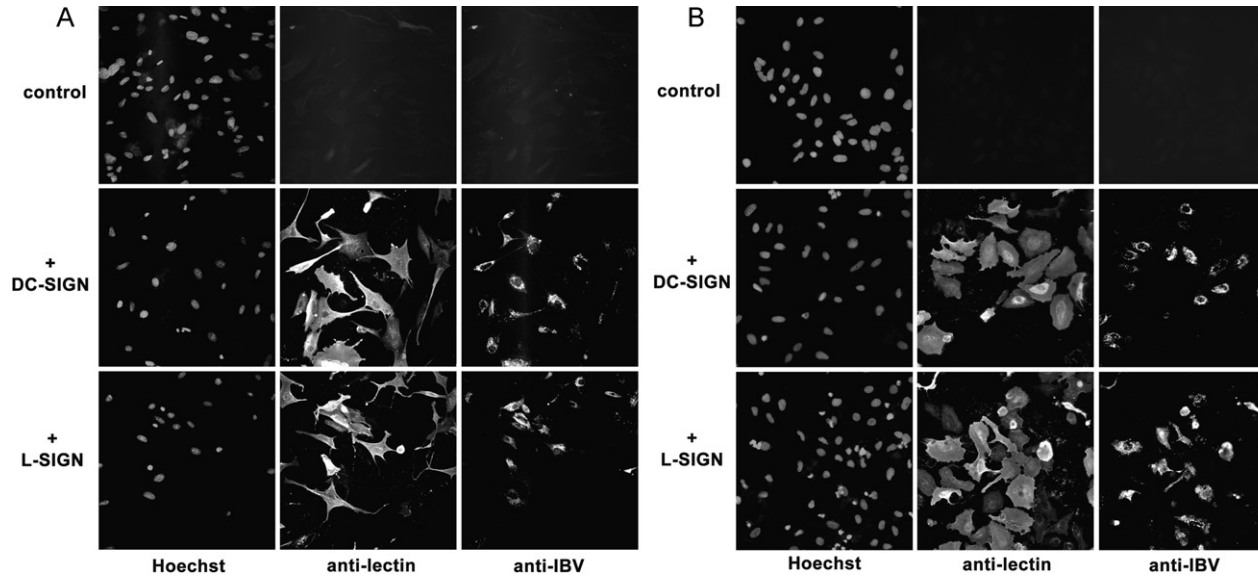
Fig. 1. IBV-M41 infection of 3T3 and CRFK cells is enhanced by introduction of hDC-SIGN or hL-SIGN. 3T3 (A) or CRFK (B) cells were transfected with expression plasmids for hDC-SIGN or hL-SIGN and infected with 10^3 TCID50/ml of IBV-M41 for 1 h at 37 °C. Cells were washed 3 times with sterile PBS and further incubated for 12 h before fixing with methanol. Viral S protein was detected by immunofluorescence microscopy using the anti-S1 mouse monoclonal antibody 15:88 following by goat anti-mouse AlexaFluor-568. Lectin expression was detected using the 14EG7 monoclonal antibody. Cell nuclei were counterstained with Hoechst 33258.

Primers for detecting nucleocapsid protein (N) of IBV and host glyceraldehyde 3-phosphate dehydrogenase protein (GAPDH) were described by (Shen et al., 2010). 10 μL of the PCR products was visualized on a 1% agarose gel.

2.4. Virus infection and immunofluorescence assay

Virus stocks were stored at −80 °C, and for infection were diluted in RPMI 1680 medium containing 0.2% bovine serum albumin (Sigma) and adjusted pH to 6.8 with HEPES. Cells were infected for 1 h at 37 °C without CO_2 on a rocking platform, and then were washed 3 times with PBS before incubating in 2% FBS/DMEM/1%Pen/Strep incubation medium. For immunofluorescence assay, cells were seeded on glass cover slips for 24 h, infected or transfected and fixed with 100% methanol for detection of IBV viral antigens or 3% paraformaldehyde for other antigens. For detection of IBV viral antigen, the anti-S1 monoclonal antibody 15:88 was used for IBV-M41. The anti-M monoclonal antibody 9:19 was used for the panel of IBV isolates. The anti-NP monoclonal antibody H10, L16-4R5 was used for influenza infection detection (ATCC). For detection of DC-SIGN, monoclonal antibody 14EG7 (NIAID AIDS Research and Reference Reagent Program) was used. The secondary antibodies AlexaFluor goat anti-mouse 488 or AlexaFluor goat anti-mouse 568 with isotype specificity against primary antibodies were purchased from Molecular Probes. Nuclei were stained with Hoechst 33258 (Molecular Probes). Cover slips with cells were mounted on glass slides using Mowiol and were examined on a Nikon Eclipse E600 fluorescence microscope equipped with a SensiCam EM (Cooke Corp.).

2.5. Treatment with mannan, DC-SIGN specific antibody, neuraminidase or sugars

Cells were incubated with mannan at a concentration of 50 μg/ml (Sigma), monoclonal antibody 9E9A8 specific for hDC-SIGN at 20 mg/ml (NIAID AIDS Research and Reference Reagent Program), neuraminidase, or sugars in DMEM media for 1 h at 37 °C before virus infection. Both mannose and galactose were purchased from Sigma and were used at a concentration of 0.01 M.

3. Results

To determine a role for C-type lectins in the entry of IBV into host cells, we transiently expressed either DC-SIGN or L-SIGN in cell lines that are known to be refractory to infection by clinical strains of IBV, such as M41, and examined these cells for virus infection. Fig. 1 shows 3T3 (A) or CRFK cells (B) transfected with plasmids expressing DC-SIGN or L-SIGN, or with a control plasmid, and then infected with IBV M41. Cells expressing DC-SIGN or L-SIGN were identified with anti-lectin antibodies. In the absence of lectin expression, we observed no infection with IBV M41. However, in the presence of DC-SIGN or L-SIGN expression, there was a strong correlation of IBV M41 infection in lectin-expressing cells. Fig. 2 shows a quantification of the rescue of IBV infection, in this case using 3T3 and CRFK cells lines stably expressing DC-SIGN, as well as 3T3 and CRFK cells expressing feline aminopeptidase N (fAPN)—which has been previously proposed to be a receptor for IBV. Both 3T3 and CRFK cells expressing DC-SIGN were efficiently infected with IBV M41, whereas there was no apparent rescue of infection in 3T3 or CRFK cells expressing fAPN. Overall, these data strongly suggest that over expression of the C-type lectins L-SIGN or DC-SIGN can act as part of an IBV receptor complex and allow infection of 3T3 or CRFK cells that are otherwise resistant to IBV infection, and further support the idea that fAPN is not a functional receptor for IBV.

To further examine the role of C-type lectins for IBV entry, we treated DC-SIGN-expressing cells with either mannan or anti-DC-SIGN antibodies, and then infected the
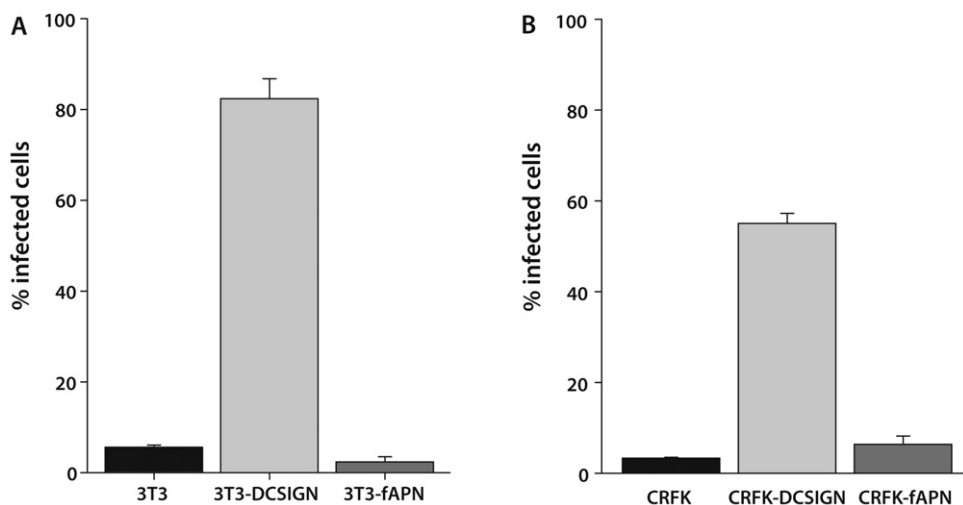

Fig. 2. IBV-M41 infection is enhanced on 3T3 or CRFK cells stably expressing DC-SIGN. 3T3 or CRFK cells stably expressing DC-SIGN cells were infected with 10^3 TCID50/ml of IBV-M41 for 1 h at 37 °C. Cells were washed 3 times with sterile PBS and further incubated for 12 h before fixing with methanol. Viral S protein was detected by immunofluorescence microscopy using the anti-S1 mouse monoclonal antibody 15:88 following by goat anti-mouse AlexaFluor-568. Cells were quantified by scoring the percentage of cells positive for viral antigen. >200 cells were quantified from three independent experiments. Error bars represent the standard deviation from the mean.
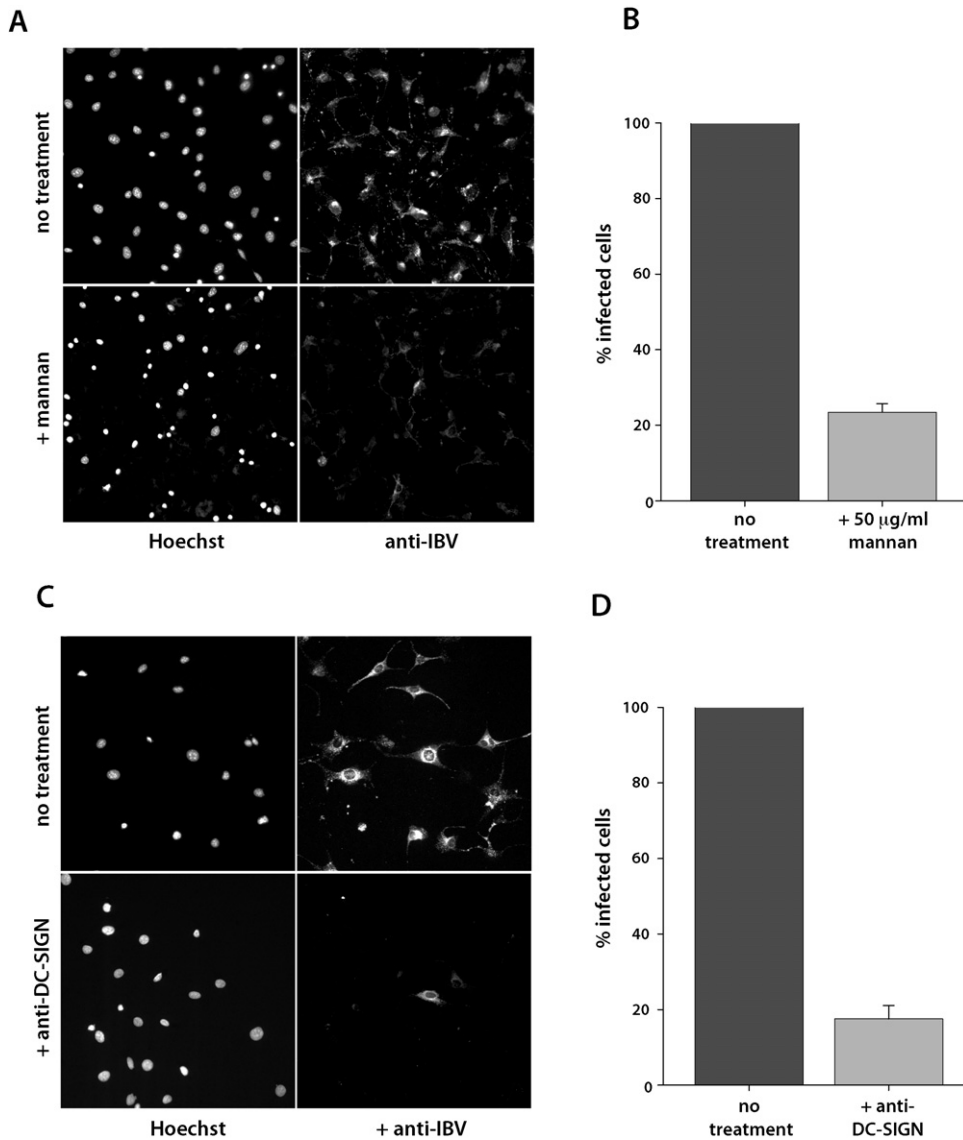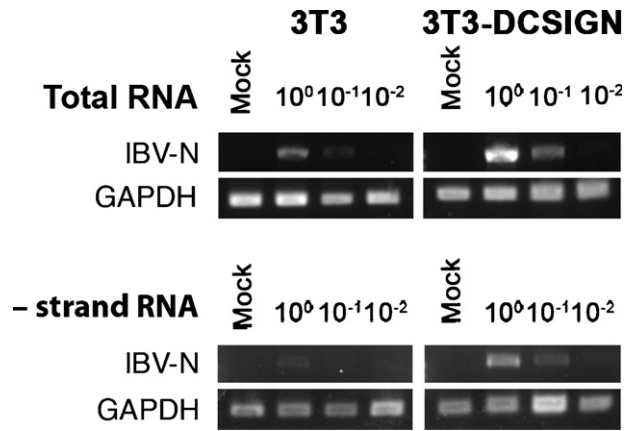
Fig. 3. Specificity of enhancement of infection by IBV-M41. 3T3–DC-SIGN cells were treated with 50 μg/ml mannan (A) or 20 mg/ml anti-DC-SIGN antibody 9E9A8 (C) prior to infection with 10^3 TCID50/ml of IBV-M41. Cells were fixed at 24 h post infection and analyzed by immunofluorescence microscopy. Cell nuclei were counterstained with Hoechst 33258. For each treatment, cells were quantified by scoring the percentage of cells positive for viral antigen (B and D). >200 cells were quantified from three independent experiments. Error bars represent the standard deviation from the mean.

cells with IBV M41. Mannan is a polymer of mannose that is well recognized to compete with mannose-containing carbohydrates on glycoproteins and block interactions of viruses with C-type lectins. Both mannan and anti-DC-SIGN antibodies inhibited infection by IBV M41 (Fig. 3), further supporting a specific role for DC-SIGN as part of the IBV receptor complex, and indicating that the interactions are mediated through mannose-containing carbohydrate residues present on the viral spike protein. In addition, infection was inhibited by the Ca^{2+}-sequestering agent EGTA (data not shown), further indicating a specific role for C-type lectins, which are known to be Ca^{2+}-dependent for their function.

To confirm that DC-SIGN-mediated entry of IBV M41 into cells allowed complete genome replication, we extracted total RNA from either 3T3 or 3T3–DC-SIGN cells infected with IBV M41 (Fig. 4). We then performed RT-PCR to detect the presence of both total and negative-sense viral RNA. In 3T3 cells, we could detect a low level of total viral RNA, but with no detectable negative-sense viral RNA. This indicates that there was some of the original virus inoculum remaining in the sample, but that viral replication had not taken place. In contrast, 3T3–DC-SIGN cells showed a strong signal for both total and negative-sense viral RNA confirming that DC-SIGN expression can rescue replication of IBV M41 in cells that are otherwise refractory to infection.

Sialic acid has been proposed to be part of the receptor complex for IBV M41 (Winter et al., 2006), and so we examined whether there may be any interplay between

3T3   3T3-DCSIGN

Total RNA | Mock | 10^0 10^{-1} 10^{-2} | Mock | 10^0 10^{-1} 10^{-2}

IBV-N

GAPDH

– strand RNA | Mock | 10^0 10^{-1} 10^{-2} | Mock | 10^0 10^{-1} 10^{-2}

IBV-N

GAPDH

Fig. 4. Replication of IBV-M41 in 3T3–DC-SIGN cells. 3T3 or 3T3–DC-SIGN cells were infected with 200 µl virus inocula of allantoic fluid dilutions of IBV-M41 with titer of $10^{8.3}$ EID50/ml for 1 h at 37 °C. 8 h post infection, total RNA was extracted from cells and was subjected to RT-PCR with primers targeting to IBV nucleocapsid N gene or host GAPDH gene. Anti-sense RNA was used to derive negative strand specific cDNA.
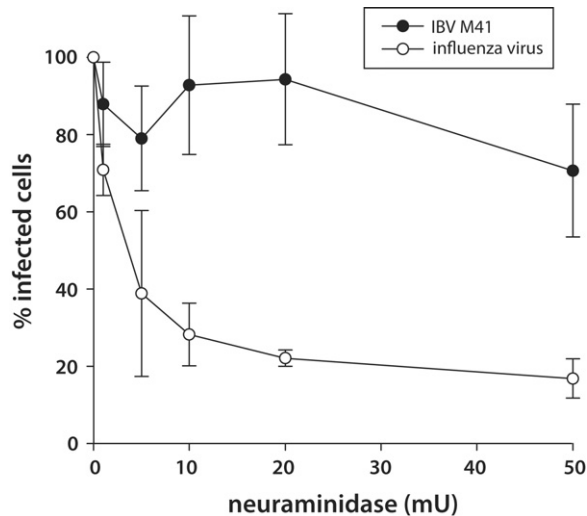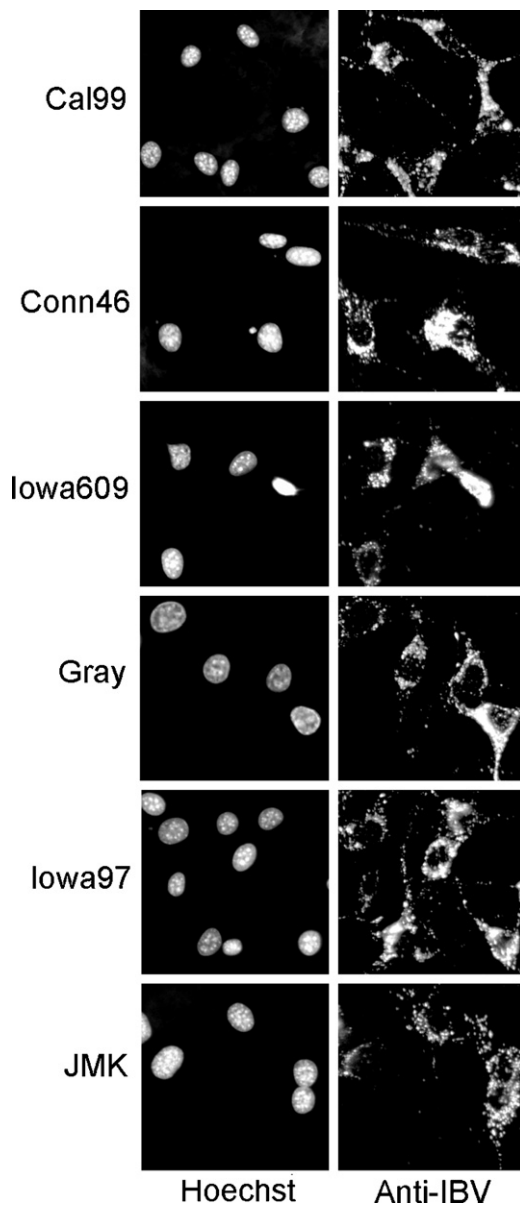

Fig. 5. Effect of neuraminidase treatment on 3T3–DC-SIGN cells infected by IBV-M41 3T3–DC-SIGN cells were pre-treated with neuraminidase and then were infected by M41 (10^3 TCID50/ml), or a control virus, influenza A/WSN/33 (MOI of 5). At 12 h post infection (M41) or 5 h post infection (WSN), cells were fixed and stained for immunofluorescence microscopy with 15:88 anti-S1 mouse monoclonal antibody (M41) or anti-NP mouse monoclonal antibody (WSN/33). Cells were quantified by scoring the percentage of cells positive for viral antigen. >200 cells were quantified from three independent experiments. Error bars represent the standard deviation from the mean.

sialic acid and C-type lectins for entry of IBV M41 (Fig. 5). 3T3–DC-SIGN cells were infected with IBV M41 in the presence of varying concentrations of neuraminidase, which would cleave sialic acids on the cell surface but not affect C-type lectin function. As a control we used influenza virus, which is well established to use sialic acid as a functional receptor. As expected, influenza infection was strongly inhibited by neuraminidase treatment in a dose-dependent manner. In contrast, there was no overall effect of neuraminidase treatment on IBV M41 infection. These data indicate that there is no functional interplay between sialic acid and C-type lectins as part of the IBV receptor complex, and that C-type lectin-mediated interactions dominate over sialic acid-mediated interactions for IBV M41 infection.

IBV exists in several serotypes, which are antigenically distinct and so may differ in their receptor requirements.

To determine whether C-type lectins can promote entry of a range of different IBVs, we infected 3T3 (not shown) or 3T3–DC-SIGN cells (Fig. 6) with the IBV strains Cal99, Conn46, Iowa609, Gray, Iowa97 and JMK, which cover the major virus serotypes. In all cases we observed efficient infection of 3T3–DC-SIGN cells with the IBV strain used, confirming that C-type lectins such as DC-SIGN can promote entry of a wide range of distinct IBV strains.

To examine the role of C-type lectin in IBV infection *in vivo*, we used primary chicken kidney cells, a chicken cell type that is naturally susceptible to IBV infection. While we were not able to inhibit the IBV-M41 strain infection in chicken kidney cells with mannan at 50 µg/ml concentration (data not shown), we observed notable reduction in infection with presence of 0.01 M mannose, the sugar monomer enriched in mannan (Fig. 7). As the control,
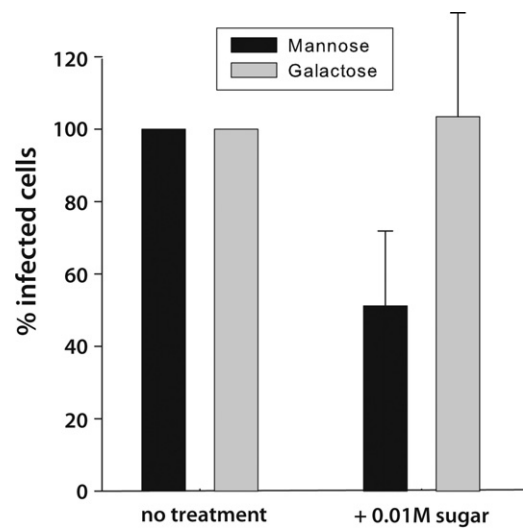
Fig. 6. The 3T3–DC-SIGN cell line is susceptible to various IBV strains representing the major viral serotypes. 3T3–DC-SIGN cells were infected with the allantoic fluid of embryonated eggs infected with various IBV isolates for 1 h at 37 °C. Cells were fixed at 24 h post infection and analyzed by immunofluorescence microscopy using the 9:19 anti-M antibody. Cell nuclei were counterstained with Hoechst 33258.

Fig. 7. Effect of carbohydrates on IBV-M41 infectivity in chicken embryonic chicken cells. Chicken embryonic kidney cells were treated with either 0.01 M mannose or 0.01 M galactose before infection with IBV-M41. Cells were fixed at 8 h post infection and analyzed by immunofluorescence microscopy and quantified as previous experiments.

cell lines, mouse 3T3 and feline CRFK cells can be efficiently infected by the prototype IBV strain M41 when these cells lines express the C-type lectins L-SIGN or DC-SIGN. Similar rescue of coronavirus infection has also been observed for a range of different coronaviruses, including SARS-CoV, HCoV-229E and feline coronaviruses (Jeffers et al., 2006, 2004; Regan and Whittaker, 2008). While it is presently unclear whether L-SIGN and DC-SIGN act as coronavirus attachment factors or *bone fide* receptors, it is clear that the use of a C-type lectin during coronavirus entry can lead to a major shift in host cell tropism. In the case of IBV, the data presented here show efficient infection of mouse or feline cells, and we expect that infection would not be limited to these cells, but would occur in many cell types. It is possible the utilization of a C-type lectin may be involved in host range changes that are known to occur with coronavirus infections. Alternatively, use of L-SIGN and DC-SIGN may be involved in the spread of the virus to new cell types or target organs within an infected animal. Although it is evident that IBV viral replication took place in the mouse cell line expressing DC-SIGN, the level of released viruses into the infected cell culture supernatant was not observed, possibly due to defects in virus assembly or release in these mouse cells (data not shown).

Our studies rely on the use of a human lectin to rescue IBV infection. A chicken holmolog of L-SIGN and DC-SIGN has not been identified and so it remains unclear what lectins might be involved in IBV infection in the chicken. We performed BLAST analysis of the chicken genome in an attempt to reveal potential chicken homologs of DC-SIGN. Three lectins, chicken hepatic lectin, the chicken C-type lectin receptor B-NK and chicken B-lec, were identified as the closest homologs to human DC-SIGN by amino acid sequence. Each of these homologs was cloned and expressed in 3T3 cells, but in each case the expressed

galactose treatment at the same concentration did not render a similar decrease in infection. Our data suggest that there may be a role for a C-type mannose-binding lectin during IBV infection of the chicken host.

4. Discussion

Infection of cells and cell lines in culture by field strains of the avian coronavirus infectious bronchitis virus (IBV) is typically restricted to primary chicken cells, such as chicken kidney (CK) cells. Here we show that two standard

lectin was unable to rescue infection of IBV (data not shown). The failure to rescue IBV infection may be due to differences in carbohydrate specificity. It is thought that DC-SIGN binds to high mannose and/or fucose residues on the glycoproteins (Guo et al., 2004) including the coronavirus spike protein (Khoo et al., 2008) and the potential chicken DC-SIGN homologs tested may have different carbohydrate-binding specificity. For example, chicken hepatic lectin, which has the highest homology to human DC-SIGN, is known to be specific for terminal N-acetylglucosamine on glycoproteins (Kawasaki and Ashwell, 1977). Thus a functional equivalent to DC-SIGN remains to be identified in the chicken.

Previous reports have indicated a low level of IBV infection in feline CRFK cells, suggesting that feline APN (fAPN) is a possible IBV receptor. While feline CRFK cells were rescued for IBV infection by DC-SIGN expression, the same situation occurred for mouse 3T3 cells. Assuming that DC-SIGN is acting in concert with an additional IBV receptor, it appears that both mouse and feline homologs of this receptor are able to act, at least in the presence of high levels of DC-SIGN expression. In addition to testing for the function of over-expressed fAPN, we also cloned and expressed the chicken aminopeptidase N (chAPN). However, like fAPN, this potential receptor failed to rescue infection by IBV (data not shown). Cell lines in culture typically do not express C-type lectins such as DC-SIGN. However we observed that there was is always a very low amount of infection observed in most avian and mammalian cell lines tested, perhaps due to a very low level of C-type lectin expression or the inefficient use of a homologous (non-chicken) receptor.

While C-type lectins can allow infection of cells by IBV, they are unlikely to be the sole component of the IBV receptor complex in chicken epithelial cells, since we were not able to find a critical role of mannose-binding molecules in the infection of chicken kidney cells by IBV. While *in vivo* infection with most IBV strains is primarily localized to the respiratory tract, it is known that certain strains can spread to other organs, *e.g.* kidney and oviduct. This *in vivo* spread is likely to be highly influenced by C-type lectin expression and distribution, especially in relation to the proposed role of hematopoetic cells, such as macrophages, which often express high levels of C-type lectins and can readily disseminate the virus to distant organs. Nevertheless, the C-type lectin molecules DC-/L-SIGN are able to mediate efficient IBV infection even in the absence of a chicken—specific receptor. Overall, our findings on lectin–virus interactions reveal important parts of the IBV receptor complex, the full intricacy of which remains to be determined.

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