RECEPTOR DETERMINANTS AND ENTRY PATHWAYS OF CORONAVIRUS AND INFLUENZA VIRUS

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ENTRY PATHWAYS AND RECEPTOR DETERMINANTS OF CORONAVIRUS AND INFLUENZA VIRUS

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Avian coronavirus infectious bronchitis virus (IBV) and influenza virus are two respiratory viruses that have great impact on veterinary and human health globally. The two respiratory viruses described in this thesis share many commonalities on the molecular mechanisms of entry in host cells. Following cell surface attachment via receptor binding, these enveloped viruses need to be internalized into subcellular compartments to initiate fusion and uncoating. Although often studied in certain prototypical cell types, many details of attachment and entry pathways are overlooked in more *in vivo*-relevant cell types.

Despite many speculations on its receptor usage, the authentic IBV receptor has not been discovered although the attachment factor sialic acid is documented. When I expressed DC-SIGN and L-SIGN, the C-type lectins, in mammalian cells, I discovered that IBV are able to infect these non-permissive cells that are usually refractory to IBV infection. In addition, the infection in DC-SIGN-expressing cells is independent on the level of sialic acid on the cell surface. When I examed whether sialic acid also plays a role in IBV infection of chicken peripheral blood derived monocytes (chPBMCs), cells that potentially harbor the authentic proteinaceous receptor for IBV, I unexpectedly found that the established attachment factor sialic acid does not play a critical role in the IBV infection in chPBMC. To further evaluate

the entry pathways utilized by IBV in chPBMCs, I examined the IBV infection in the presence chemical inhibitors used to disrupt endocytic components. Using immunofluorescence microscopy, I identified that caveolae-dependent endocytosis and macropinocytosis pathways were used by IBV entry in chPBMCs.

In a similar chemical and molecular approach, in the presence of endocytic inhibitors and dominant- negative proteins implicated in the endocytic pathways, I evaluated the influenza virus entry in polarized epithelial cells-- Madin-Darby canine kidney (MDCK) cells, a model for the cells at primary sites of influenza infection *in vivo*. My study showed that in polarized MDCK II cells, influenza virus has a differential utilization for CME pathway and requires Eps15 protein for entry

In all, these studies of coronavirus and influenza virus entry provide a clearer understanding of the molecular mechanisms involved in enveloped virus entry into host cells. The results of this study will enrich our knowledge of enveloped virus pathogenesis.

BIOGRAPHICAL SKETCH

Yueting Zhang was born in Guangzhou, China. She immigrated to the U.S. at the age of 17. She attended Sheepshead Bay High School in Brooklyn, NY and graduated second in her class in 2002. Yueting attended Binghamton University, NY for undergraduate study majoring in Biological Sciences and Mathematical Sciences. She also studied Streptococcus biofilm in Dr. Karin Sauer's laboratory as an undergraduate research assistant. She graduated in 2006 with a bachelor's degree in science and was accepted into Cornell University for Ph.D. studies in Microbiology. She studied coronavirus and influenza virus under the guidance of Dr. Gary R. Whittaker in the College of Veterinary Medicine, Cornell University.

After graduation she will work as a post-doctoral research associate in the laboratory of Dr. Julius Lucks in Department of Chemical Engineering, Cornell University. There she will study the RNA structure of influenza virus genome. With her post-doctoral training, she is looking forward to becoming an independent research scientist.

This work is dedicated to my parents, Mr. Bin Zhang and Ms. Ruzhen Wu and to my husband Mr. Roman Akhmechet.

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INTRODUCTION

Avian coronavirus infectious bronchitis virus

and

influenza virus

1.1.1 Avian Coronavirus infectious bronchitis virus: overview

As the first member of the Coronaviridae to be discovered, avian coronavirus infectious bronchitis virus has almost 80 years of history in virological research and poultry science. In the 1930's, Schalk and Hawn first described a contagious respiratory disease, infectious bronchitis (IB), in a flock of baby chicks in the United States (Schalk, 1931). The disease spread among chickens rapidly and caused high mortality. In 1936, Beach and Schalm demonstrated that IB was caused by a novel virus, which was named Infectious bronchitis virus (IBV) (Beach, 1936). In the following year, Beaudette successfully propagated the virus in embryonated eggs and discovered a lab-adapted strain of IBV (Beaudette, 1937). IBV research has provided insights into other subsequently discovered coronaviruses that are important for both veterinary and human health (Figure 1.1.).

Although other members of the coronavirus family have wide tropism and infect many tissue types, IBV mainly infects epithelia of the respiratory tract and causes respiratory disease in the chicken host. However, many studies have discovered that IBV can also replicate at comparatively lower levels and cause pathology in other tissues such as testes, oviducts, and alimentary tract (Raj and Jones, 1997). Clinical signs include, but are not limited to, coughing, sneezing, nasal discharge, loss of appetite, and reduction of egg laying (Saif, 2003b). Some strains such as Massachusetts 41 (M41) and B1648 can cause major or minor nephritis in both naturally infected or experimentally infected chickens (Lambrechts et al., 1993).

IBV has caused great economic loss in the poultry industry around the globe by reducing productivity and increasing mortality in chickens. Chickens of all ages are

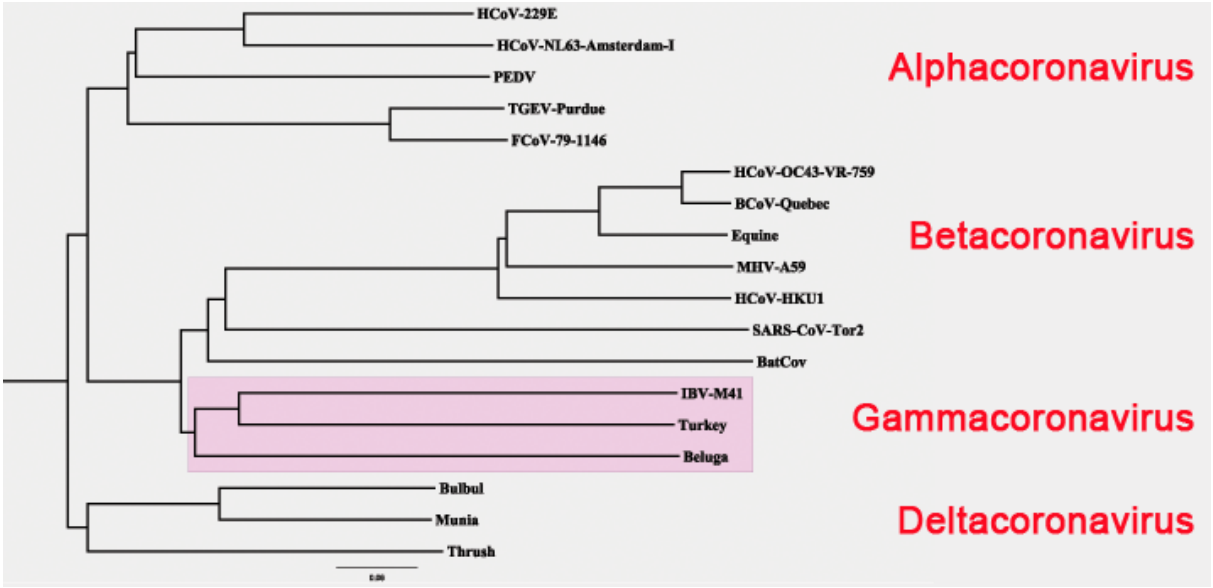

Figure 1.1. Phylogenetic tree of coronaviruses. The phylogenetic analysis was performed by using coronavirus spike protein sequences from GenBank. The accession numbers for the sequences used are PEDV, porcine epidemic diarrhea virus (ABI30278.1); TGEV-Purdue, porcine transmissible gastroenteritis virus (ABG89335.1); FCoV-79-1146, feline coronavirus (YP_004070194.1); HCoV-229E, human coronavirus (AAG48592.1); HCoV-NL63-Amsterdam, human coronavirus (AAS58177.1); BatCov-HKU2(ABQ57216.1); HCoV-OC43-VR-759, human coronavirus (AAT84354.1); BCoV-Quebec, bovine coronavirus (AAL40400.1); Equine Cov(AAQ67205.1); MHV-A59, murine hepatitis virus (AAU06356.1); HCoV-HKU1, human coronavirus (AAT98580.1); SARS-CoV-Tor2, severe acute respiratory syndrome coronavirus (NP_828851.1); IBV-M41(AAW33786.1); Turkey-Cov(ACV87265.1); Beluga Whale coronavirus SW1(ABW87820.1) ; Bulbul Cov HKU11-934 (ACJ12035.1); Thrush Covs HKU12-600 (YP_002308497.1); Munia Cov HKU13-3514(YP_002308506.1). The sequence alignment and the tree were generated by ClustalX 2.1 with the Neighbor-Joining algorithm. Scale bars represent the estimated number of substitutions per site.

susceptible to the disease; however, the severity of the disease can vary among different breeds of chicken and among different strains of IBV contracted (Cavanagh et al., 2007). IBV infections are currently under-controlled with live virus vaccination of major circulating strains. However, cross-protection for new recombined strains is fairly poor (Hofstad, 1981). Outbreaks of IBV frequently occur in vaccinated chicken flocks (Liu and Kong, 2004). Study and surveillance of IBV remains an active area in poultry science.

1.1.2 Molecular organization and life cycle of IBV

The family Coronaviridae is composed of viruses with positive single-strand RNA genomes and they are enveloped. Virion size ranges from 100 to 120 nm in diameter. The characteristic projections cover the surface of the virus, giving its name "corona" (meaning "crown" in Greek). The virion contains the nucleocapsid protein (N), which associates with the RNA genome, the surface glycoprotein spike (S), numerous copies of the integral membrane protein (M), and a smaller number of envelope proteins (E). In contrast to the Betacoronavirus, IBV does not have hemagglutinin esterase (HE) protein on its surface (Figure. 1.2.).

Like all other animal viruses, IBV starts its life cycle by attachment to the receptor on the host cell membrane via spike protein. It enters the cell through an endocytic mechanism and the fusion with host cell membrane depends on low-pH activation (Chu et al., 2006a). The viral nucleocapsid is then released into the cytosol. The RNA genome is subsequently uncoated and ready for translation and transcription. However, the detailed processes of replication are unknown (Howley, 2007). The

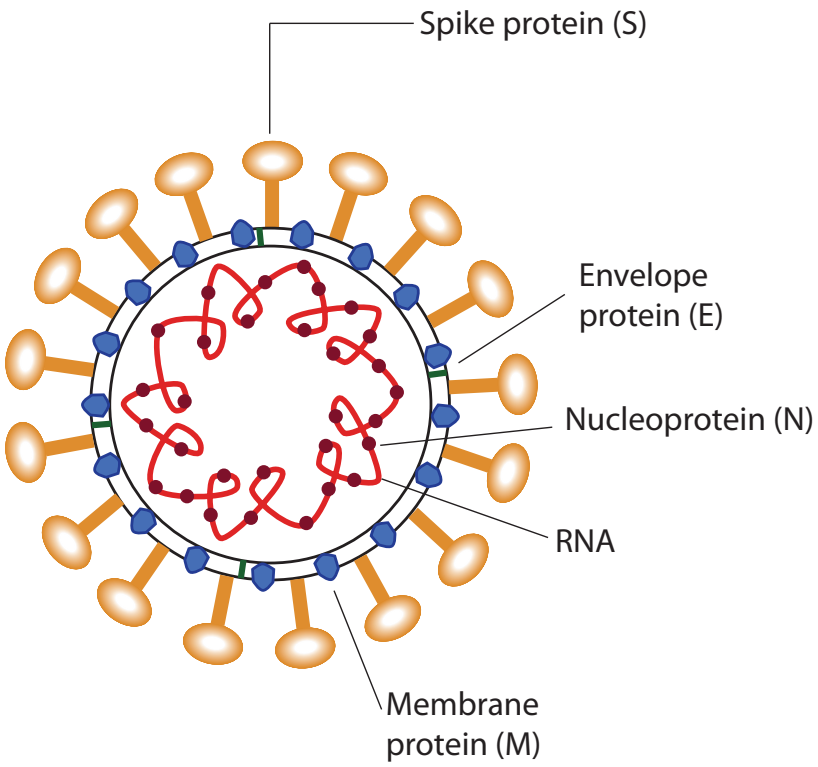

Figure 1.2. Molecular organization of IBV. The virion contains the nucleocapsid protein (N), which associates with the RNA genome, the surface glycoprotein spike (S), numerous copies of the integral membrane protein (M), and a smaller number of envelope proteins (E).

replicase-transcriptase proteins are first directly translated into two polypeptides pp1a and pp1ab. These polypeptides are then cleaved by virus-encoded proteinases into 16 viral proteins (Seybert et al., 2000). With other viral proteins and cellular proteins, the replicase-transcriptase proteins form replication-transcription complexes (RTC), which synthesize genomic and sub-genomic RNA (Prentice et al., 2004). The viral complex first synthesizes the negative strand of the RNA genome. Then by using the negative strand RNA as a template, subgenomic mRNAs are transcribed for downstream production of viral proteins including N, S, M, and E protein (Sawicki et al., 2007). The N protein and the newly synthesized RNA form nucleocapsids. Other structural proteins are inserted into and processed in the endoplasmic reticulum. The virion undergoes "internal budding" from the endoplasmic reticulum-Golgi intermediate compartments (ERGIC) and is released by an exocytic mechanism (Klumperman et al., 1994; Tooze et al., 1987). Some S proteins may travel to the host cell surface and mediate syncytium formation with neighboring cells to spread infection (Figure 1.3.)

1.1.3 IBV strains

Due to continuous global effort in IBV surveillance, over 1400 IBV strains have sequence information in the Virus Pathogen Resources Database (VIPRBRC). Currently, there are three major serotypes circulating in the U.S. represented by: M41, Connecticut 46 (Conn46), and Arkansas 99 (Ark99). Although vaccination against the major serotypes is common for poultry, it generally gives poor cross-protection to other serotypes as well as reduced protection to new variants that arise from mutation or recombination.

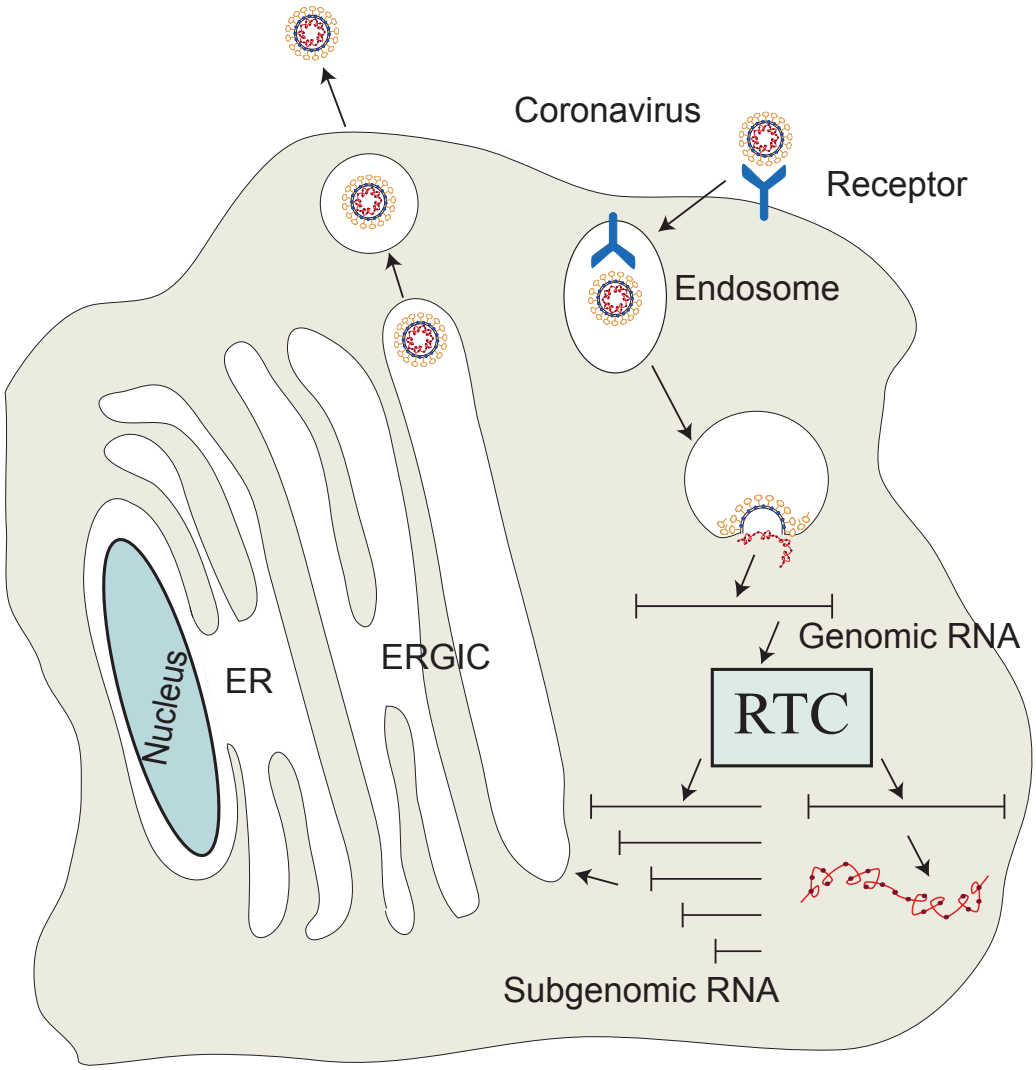

Figure 1.3. Life cycle of IBV. The IBV particle first attaches to the host cell via an unknown receptor protein. After internalization, the virus fuses with endosome membrane via its spike protein. The viral RNA is then released to the cytoplasm for translation to polyproteins. After proteolytic cleavage, the viral replicase-transcriptase proteins form replication-transcription complexes (RTC), where genomic and subgenomic RNA are synthesized. Structural and non-structural proteins are processed at the endoplasmic reticulum (ER). The progeny virions are assembled at the ER-Golgi intermediate compartment (ERGIC) and released via an exocytosis pathway.

The S1 subunit of IBV spike protein harbors specific epitotes that can induce neutralizing antibodies (Cavanagh et al., 1984; Cavanagh et al., 1986). Serotypes of IBV are distinguished by virus neutralizing assays (Cowen and Hitchner, 1975; Hopkins, 1974). Currently there are more than 20 serotypes of IBV (Gelb et al., 1991). Different serotypes of IBV can vary from 20% to 50% at the amino acid sequence level in the S1 subunit (Cavanagh, 2007). The genotype derived from the IBV S1 hypervariable region sequence correlates with serotyping in cross-neutralization assays (Wang and Huang, 2000).

Like many RNA viruses, the unique RNA replication machinery (such as the error-prone RNA-dependent RNA polymerase) and mechanisms (such as "template-switching") contribute to the genetic diversity of coronaviruses. In addition to the high mutation rate, there have been many pieces of evidence of recombination among different strains of IBV, both experimentally and in the field (Cavanagh et al., 1992; Chen et al., 2010b; Estevez et al., 2003; Jackwood et al., 2010; Jia et al., 1995; Kottier et al., 1995a, b; Kuo et al., 2010; Kusters et al., 1990; Lee and Jackwood, 2000; Mardani et al., 2010; Thor et al., 2011; Wang et al., 1993; Wang et al., 1997). Recombination near the S1 subunit could lead new serotypes or new strains of IBV in the field. (Wang et al., 1993)

The lab-adapted strain Beaudette was generated by serial passage in embryonated eggs. It has 95% sequence similarity at the amino acid level with its parental strain M41. The Beaudette strain is able to induce severe lesions and stunted growth of embryos when inoculated into 11-day-old embryonated eggs. It can also replicate to high titers in tracheal organ culture. Interestingly, the Beaudette strain does not cause symptoms when inoculated into chickens while the parental M41 strain causes severe respiratory symptoms and is able to replicate in multiple organs, including kidneys.

1.1.4 Laboratory host models for IBV

Chicken embryonated eggs have been used to cultivate IBV since the 1930's and have remained the only system to propagate the virus after isolation. Typically, IBV is inoculated into the allantoic chamber of a 10-11 day old chicken embryonated egg. Approximately 2 days post inoculation, virus can be recovered from allantoic fluid of the infected egg. IBV preparations from embryonated eggs produce large amounts of defective interfering particles. The infected chicken embryos display stunting characteristics compared to healthy embryos at the same development stage (Figure 1.2.). However, the abnormality of the IBV-infected embryo does not reflect the pathogenicity of the virus strain inoculated since the Beaudette strain, an apathogenic IBV strain in chicken, can also induce such abnormality. IBV can become attenuated, for example, in the case of Beaudette, in chickens by serial passage in embryonated eggs. The virus becomes highly lethal to chicken embryos yet is non-infectious to adult chickens (Beaudette, 1937).

The egg infectious dose 50 assay, which is the "gold standard" for titering IBV, usually does not correlate with the tissue infectious dose 50 assay using primary chicken kidney cells, which are the only cell culture system commonly used by IBV researchers. However, the reason for the discrepancy between the two tittering methods remains unclear. Another commonly used cell type for IBV field strains or clinical strains is chicken embryonated fibroblasts (Table 1.1). Other primary chicken derived cell lines are relatively inconvenient for general laboratory use. Beaudette remains the only strain that can infect a variety of mammalian cell lines thus allowing it to be used as an *in vitro* infection model. Mammalian cell lines expressing C-type lectin DC/L-SIGN are

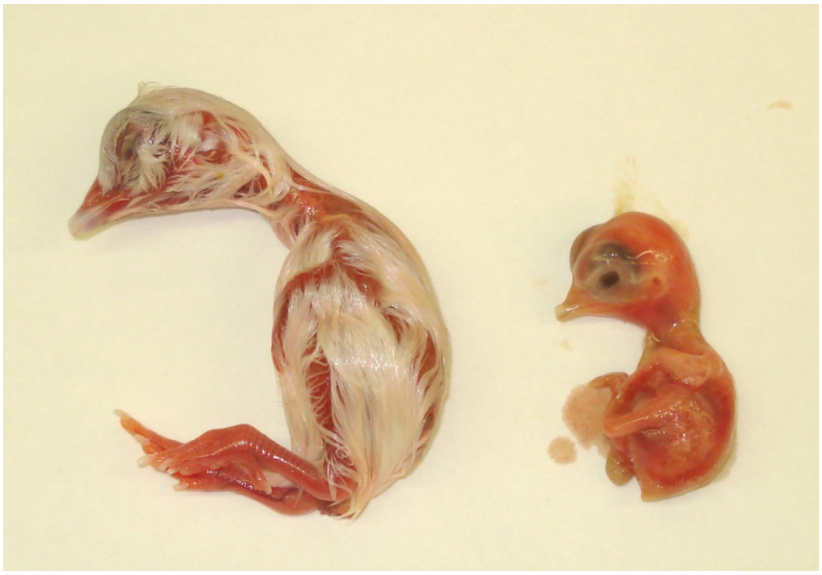

Figure 1.4. Comparison of healthy chicken embryos and IBV-M41 infected chicken embryos. 10-day-old chicken embryonated eggs were either mock infected (left) or infected with IBV-M41 virus (right). Embryos were euthanized by incubating the eggs at 4°C over night and were examined post-mortem.

Table 1.1. Cell lines susceptible to IBV

	Cell line or cell type	Susceptibility to IBV strain	Reference
Avian origin	Chicken kidney cells	All strains	(Otsuki et al., 1979a)
	Chicken embryonated fibroblasts	All strains	(Otsuki et al., 1979b)
	Chicken embryo related cell line	M41	(Ferreira et al., 2003)
	Primary chicken tracheal epithelial cells	2575/98, 2296/95	(Shen et al., 2010)
Non-avian origin	3T3-DCSIGN cell line and other DC/L-SIGN expressing cell lines	M41, Cal99, Conn46, Iowa609, Gray, Iowa97, JMK	(Zhang et al., 2012)
	Vero (African green monkey kidney) cells	Beaudette	(Cunningham et al., 1972)
	BHK-21 (Baby hamster kidney) cells	Beaudette	(Otsuki et al., 1979b)

also susceptible to IBV field strains infections. These cells will be discussed in Chapter 2 of this thesis.

1.1.5 IBV spike protein as a pathogenesis determinant

The spike glycoprotein S is a 180 kDa glycoprotein (Figure 1.3.). It has two subunits: the N-terminal S1 subunit and the C-terminal S2 subunit. The spike protein S1 subunit allows the virus to bind to the receptor or attachment factor on the host cell surface (Koch et al., 1990; Koch and Kant, 1990). The S2 subunit mediates the fusion of the viral membrane with the endosomal membrane to release the viral RNA into the cytoplasm. The S1 subunit is the major neutralizing antibody inducer in the host. This is evidenced by the lack of protective immunity or virus neutralizing antibodies in chickens immunized with IBV virus without an S1 subunit while the S2 subunit was retained (Cavanagh and Davis, 1986). The S protein is considered the major determinant of pathogenicity and cellular tropism of coronaviruses. Using reverse genetics, Cassis and coworkers demonstrated that the IBV Beaudette strain, which is pan-tropic, loses the ability to infect Vero cells and BHK cells, after having its spike protein replaced with the spike protein from the IBV-M41 strain, the tropism of which is strictly limited to primary chicken kidney cells (Casais et al., 2003).

The coronavirus S glycoprotein is synthesized as a single polypeptide chain, which oligomerises into trimers in the endoplasmic reticulum of the host cell and are processed in the Golgi apparatus (Delmas and Laude, 1990). The S protein is highly glycosylated with N-linked high mannose (Cavanagh, 1983). Sequence analysis with NetNGlyc 1.0 server shows that S protein harbors over 30 potential N-linked

glycosylation sites. These glycosylation sites may be involved in interactions with cellular components, as is the case for SARS-CoV, in which 7 N-linked glycosylation sites of the S protein are critical for DC/L-SIGN mediated entry of SARS-CoV (Han et al., 2007).

Proteolytic cleavage of S protein is an important feature of coronaviruses and other enveloped viruses such as influenza virus and respiratory syncytial virus. The S protein of IBV is cleaved into S1 and S2 subunits and is further proteolytically processed at the S2' cleavage site (Belouzard et al., 2009). The S1/S2 cleavage site does not correlate with serotype or pathogenicity (Jackwood et al., 2001). The second cleavage site within the S2 subunit is associated with furin-dependent viral entry and syncytia formation *in vitro* and is critical for cell culture infectivity (Yamada and Liu, 2009). However, the involvement of the second cleavage site of IBV S protein *in vivo* is not evident.

1.1.6 Receptor and attachment factors for IBV

The ability of IBV to infect various tissues in chickens may be linked to the receptor distribution in these tissues. The unique features of IBV tropism are often neglected at the molecular level as many studies use the pan-tropic Beaudette as a model virus for IBV. The apathogenic Beaudette strain clearly has a modified and distinct tropism compared to its pathogenic field strain counterpart M41.

The receptor for IBV has not been identified. There was speculation that feline aminopeptidase N (fAPN), which can serve as a universal receptor for Alphacoronaviruses, is the receptor for IBV, based in part on the ability of IBV to infect

Figure 1.5. Model of SARS-CoV spike. Spike protein structural model is generated in Pymol with 1T7G (Protein Data Bank). Magenta portion represents the S1 subunit and green portion represents the S2 subunit.

feline kidney cells (Miguel et al., 2002). However, expression of fAPN fails to rescue infection by a panel of field isolates of IBV in a non-permissive cell line indicates that fAPN is not a functional receptor for IBV (Chu et al., 2007).

There have been several studies showing that the tissue tropism of IBV may be linked to the distribution of its attachment factor, sialic acid, which is present on the cell surface of various enteric tissues. Winter and coworkers demonstrated that by treating Vero cells with neuraminidase, the pan-tropic IBV Beaudette strain is no longer able to infect Vero cells (Winter et al., 2006). Wickramasinghe et al further demonstrated that the S1 of IBV M41 strain exhibits sialic acid-dependent binding to chicken respiratory tract tissue sections by histochemistry. In addition, S1 of M41 is able to bind to a particular sialic acid-containing glycan: [Neu5Acα2, 3Galβ1, 3(Neu5Acα2,3Galβ1,4)-GlcNAc] on a glycan array (Wickramasinghe et al., 2011). A heparan sulfate (HS) binding site (SRRKRS or SRRRRS) within the S2 subunit of the Beaudette S protein may be used as a selective receptor for Beaudette virus entry to host cells (Madu et al., 2007). However, this sequence is only found in the S protein of Beaudette strain, which may explain for the extended host range of Beaudette in comparison to M41.

1.1.7 DC-SIGN: a novel viral receptor?

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 and L-SIGN are type-II transmembrane proteins. The structure consists of a small cytoplasmic tail, a transmembrane domain, and an ectodomain. The extracellular-oriented ectodomain has a

carbohydrate recognition domain and neck repeats, in which DC-SIGN and L-SIGN differ the most (Feinberg et al., 2001). DC-SIGN is mainly expressed on myeloid-lineage dendritic cells (DC) in the lymphoid tissues (but not on Langerhan cells) and macrophages (Soilleux, 2003). L-SIGN, which shares approximately 77% sequence homology with DC-SIGN, mainly express on liver sinusoidal endothelial cells (Bashirova et al., 2001) and endothelial cells in the lymph node and placenta (Braet and Wisse, 2002). The DC-SIGN was first discovered to be important in human immunodeficiency virus type 1 (HIV-1) pathogenesis (Geijtenbeek et al., 2000). This lectin is believed to interact with the HIV-1 surface glycoprotein gp120 and enables capturing and transporting of the virus particle from the 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 (Pei 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 (Marzi et al., 2006; Marzi et al., 2004; Regan and Whittaker, 2008; Yang et al., 2004).

1.1.8 Endocytosis of IBV

Many enveloped viruses exploit the cellular mechanism of endocytosis to gain entry into host cells. A virus can use multiple entry pathways to bring its genetic material into the cytoplasm. However, the choice of a particular pathway can be cell-type dependent. Coronaviruses use a variety of endocytic pathways to enter host cells (Table1.2.). The IBV Beaudette strain enters BHK-21 cells via a low pH dependent endocytic pathway (Chu et al., 2006a; Chu et al., 2006b). However, the exact pathway used by IBV, especially in chicken cell lines, remains unknown.

1.1.9 Apoptosis of IBV infected cells

In vitro, IBV causes extensive cytopathic effect in permissive cells. Vero cells infected by the Beaudette strain form multi-nucleared syncytia and eventually round up and detach from the tissue culture. Apoptosis of Vero cells induced by infection with the Beaudette strain is caspase-dependent and is inhibited by the pan-caspase inhibitor z-VAD-FMK (Liu et al., 2001). In addition, IBV induces cell cycle arrest in Vero cells and H1299 cells that are devoid of p53, a mediator of apoptosis (Li et al., 2007a). IBV infection in chickens is usually associated with lesions in the trachea, kidney, and other organs. However, the cytopathic effects induced by IBV *in vivo* and in chicken cells are yet to be characterized.

1.1.10 Perspectives

As the first described coronavirus, IBV still remains mysterious at the molecular level and at the clinical level; numerous questions are still puzzling researchers: what is

Table 1.2. Coronavirus endocytic pathways

	Virus	Cell type	Endocytic pathway	Reference
Alpha coronavirus	HCoV 229E	Human fibroblasts	Caveolae-mediated pathway	(Nomura et al., 2004)
	FIPV	Feline monocytes	Clathrin-independent pathway, Caveolae-independent pathway	(Van Hamme et al., 2008)
Beta coronavirus	SARS-CoV	HepG2, Cos7	Clathrin-mediated pathway	(Yang et al., 2004) (Inoue et al., 2007)
	SARS-CoV	Vero E6	Clathrin-independent pathway, Caveolae-independent pathway	(Wang et al., 2008)
	MHV A59	murine cells	Clathrin-mediated pathway, cholesterol-dependent pathway	(Choi et al., 2005; Eifart et al., 2007; Thorp and Gallagher, 2004)
	MHV-2	DBT cells	Clathrin-mediated pathway independent of Eps15	(Pu and Zhang, 2008)

its putative receptor; how does the virus interact with its receptor; what type of antigen presenting cells are the major inducers of the adaptive immune response; and so on. IBV and other coronaviruses received increased attention after the human outbreak of SARS coronavirus in 2003, which became a new model for emerging viruses. Studies on IBV not only benefit the poultry industry but also allow researchers to gain insight in the evolution of coronaviruses. Development of vaccines for IBV may also give new perspectives to future vaccine design for other human coronaviruses.

1.2.1 Influenza virus characteristics and life cycle of influenza virus

Influenza virus is a common pathogen of the upper respiratory tract of humans and a broad range of animals including birds and other mammals. Influenza virus can cause mild to severe and occasionally lethal disease. Pandemic influenza occurs irregularly; notable pandemics include the 1918 Spanish flu and the 2009 H1N1 flu. Over 200,000 influenza-associated hospitalizations occur annually in the United States (Thompson et al., 2004).

Influenza virus, a member of the *Orthomyxoviridae,* is enveloped, and has a negative-sense, segmented RNA genome. The virus includes three types: A, B and C. Among the three types, influenza A viruses are the most widespread and infect a variety of animal species; influenza B causes only mild disease in humans, while influenza C can infect humans and pigs causing mild disease signs. The eight RNA segments of influenza virus A are associated with many copies of the nucleoprotein (NP) and heterotrimeric polymerase complexes (PB1, PB2, and PA), which form the viral nucleoproteins

(vRNPs). The vRNPs are surrounded by a layer of matrix protein (M1), which connects vRNPs to the virus envelope. Two types of glycoproteins are inserted into the lipid bilayer viral membrane: hemagglutinin (HA) and neuraminidase (NA). The receptor for influenza A virus has been well characterized. The specific conformation of the sialic acid linkage (SAα-2, 3 vs. SAα-2, 6) controls species tropism of the virus. Avian influenza viruses preferentially bind to sialic acid with 2, 3 linkages to galactose. On the other hand, human influenza viruses preferentially bind to sialic acid with 2, 6 linkages to galactose (Medina and Garcia-Sastre, 2011). During virus infection, the virion binds to the host cell surface receptor via HA, followed by endocytosis into endosomal compartments. The acidic environment in the endosomes induces conformational change in the HA, and as a result, fusion peptides residing in the HA2 are exposed, triggering viral fusion with the endosomal membrane. Subsequently, due to the ion channel activity of M2 proteins anchored in the viral envelope and the matrix shell, the viral components inside the particle are exposed to the low pH of the endosome, disrupting the interaction of M1 and vRNPs. Uncoated nucleoproteins are released into the cytosol and then are transported into the nucleus to initiate viral protein synthesis. The newly synthesized viral proteins and genome are targeted to the host plasma membrane, where viral components are assembled into virions, which bud into the extracellular environment to produce progeny viruses (Pinto et al., 1992; Whittaker, 2001; Yoshimura and Ohnishi, 1984)

1.2.2 Endocytosis pathways

Endocytosis is a cellular process that involves the internalization of proteins, lipids, and soluble molecules from the cell surface or the extracellular space. Most

viruses take advantage of the cellular endocytic events to gain entry into host cells (Sieczkarski and Whittaker, 2002a). After being endocytosed, viruses can initiate replication once they penetrate or fuse with the endosomal membrane (Marsh and Pelchen-Matthews, 2000). Due to the diversity of ligands and membrane components, along with their different ultimate destinations, cells possess more than one endocytic pathway to accomplish trafficking. As obligate intracellular parasites, viruses have evolved with host cells and have gained the ability to hijack multiple cellular endocytic pathways.

Clathrin-dependent endocytosis is one of the best-characterized endocytic pathways. Cellular receptors and membrane proteins with cytoplasmic tyrosine motif (YXXΦ) or dileucine internalization motifs are recognized by the adaptor protein 2 (AP-2) (Kozik et al., 2010). With other accessory proteins such as Eps15, Epsin1, Synaptojanin1, AP-2 recruits clathrin from the cytosol reservoir to form clathrin-coated pits. The clathrin-coated pits are then pinched off at the neck by the small GTPase dynamin and other proteins to form clathrin-coated vesicles (Marsh and McMahon, 1999). After being endocytosed, the clathrin coat is disassembled and is recycled. The uncoated vesicles fuse with early endosomes, from which the internalized receptors or membrane proteins are further delivered to different destinations (Conner and Schmid, 2003; Marsh and McMahon, 1999). To date, the transferrin receptor and low-density lipoprotein (LDL) receptor are the best examples for studying endocytosis dependent on functional clathrin. Specific non-functional forms of cellular proteins have been developed to selectively block clathrin-dependent endocytosis. For example the dominant negative forms of regulatory protein Eps15 and clathrin hub fragments have been widely

used to exclusively inhibit clathrin-mediated endocytosis (Benmerah et al., 2000).

Macropinocytosis is an actin-dependent, dynamin-independent endocytic process that is involved in fluid uptake and large particles. Unlike phagocytosis, which is restricted to immune cells such as macrophages and dendritic cells, macropinocytosis can take place in all cell types upon stimulations (Swanson and Watts, 1995). As a unique endocytic process that does not form invaginations on the cell surface, macropinocytosis relies on the ruffling and blebbing of the plasma membrane. Upon growth factor stimulation and trigger of underlying receptor tyrosine kinase signaling pathways such as Pak1, Protein kinase C, Src kinase, actin filament dynamics of polymerization and turnover are changed and membrane rufflings are formed (Kasahara et al., 2007; Liberali et al., 2008; Mercer and Helenius, 2009). Small GTPases such as Ras family GTPases, Rac1, Rab5, Arf6 are involved in modulation of ruffle formations (Lanzetti et al., 2004; Ridley et al., 1992). Viruses and large particles are internalized with other fluid and solutes by macropinocytosis upon stimulation. In this case, receptor for viruses may not be required. Interestingly, adenovirus, which is endocytosed via a clathrin-dependent pathway, was found to stimulate macropinocytosis of resting cells after being internalized. However, the upregulated macropinocytosis was not employed for facilitating viral entry, but instead for virus escaping from the endosomes by an unknown mechanism (Meier and Greber, 2004).

1.2.3 Endocytic pathways of influenza virus in non-polarized cells

In early studies of influenza entry in MDCK cells, electron micrographs showed virus particles in coated pits and coated vesicles. These data suggest that influenza virus

can enter host cells via clathrin-dependent endocytosis (Matlin et al., 1981). Later studies in the influenza field demonstrated a role of dynamin-dependent endocytosis of influenza virus entry by examining the effect of dominant-negative dynamin on influenza virus entry in Mv-1 mink lung cells (Roy et al., 2000). Electron micrographs also revealed that influenza virus particles reside in non-coated invaginations in the membrane as well as smooth vesicles inside the cell (Morgan et al., 1954). These smooth vesicles were not seen in Semliki Forest virus (SFV) entry or vesicular stomatitis virus (VSV) entry. This indicates that influenza virus may exploit endocytic entry routes other than clathrin-dependent endocytosis, which is utilized by SFV and VSV. Recent studies from the Whittaker lab showed that in control to SFV, the infectivity of influenza viruses in cells expressing a dominant-negative mutant of Eps15, which inhibits clathrin-dependent endocytosis specifically, was not impaired in contrast to SFV (Sieczkarski and Whittaker, 2002a). Influenza infection is not inhibited by chemical inhibitors of caveolar-mediated endocytosis or an inhibitory mutant of caveolin-1 (Sieczkarski and Whittaker, 2002a). These observations led to the discovery of a non-clathrin-dependent, non-caveolae-dependent endocytic pathways for influenza virus entry in addition to classical clathrin-dependent pathway.

Thanks to advances in super-resolution fluorescent microscopy, individual influenza particles can be tracked in real time without disruption of endocytic pathways. The population of influenza particles entering BSC-1 cells via clathrin-dependent pathway is approximately 65%; while 35% of virus particles are endocytosed via clathrin-independent pathways (Rust et al., 2004). Examining the dynamics of the endocytic uptake also leads to the conclusions that influenza viruses exploit different

pathways with the same efficiency and that non-classical pathways are not alternative pathways for influenza virus entry (Chen and Zhuang, 2008).

Recently, by performing a detailed dissection of influenza entry pathways independent of dynamin using pharmacological inhibitors, de Vries et al. discovered that influenza virus utilizes a macropinocytosis-like route of entry that is independent of dynamin in many cell types (de Vries et al., 2011). One speculation for the reason why influenza virus uses macropinocytosis as entry pathway is the size of the influenza virus. Influenza virus has two morphotypes: spherical virions about 100nm in diameter and filamentous virions up to 20um in length. It is not surprising that instead of utilizing clathrin-mediated endocytosis route of entry, macropinocytosis is a dominant entry route by filamentous influenza virus (Rossman et al., 2012).

1.2.4 Polarized epithelial cells as sites of infection for influenza virus *in vivo*

In humans, influenza is transmitted from infected individuals through the air within aerosols generated by coughing or sneezing. The main targets of influenza virus are the ciliated epithelial cells of the upper respiratory tract, which consist of several cell types with distinct functions (Hers, 1966; Jeffery and Li, 1997). Influenza viruses can also infect many different cell types of airway epithelial cells in humans and monkeys (Rimmelzwaan et al., 2001; Tateno et al., 1966). Intracellular trafficking of influenza virus is extensively studied in non-polarized cell systems such as HeLa (cervical cancer cells), CHO (Chinese hamster ovary cells), and BSC-1 (African green monkey kidney cells) (Chen and Zhuang, 2008; Sieczkarski and Whittaker, 2002b). These molecular studies have been carried out using influenza virus infection in non-polarized cell lines

and such cells are not the *in vivo* target of viral infection.

Polarized, simple epithelial cells have a plasma membrane that is separated by tight junctions into two clearly distinct domains. The apical domain faces the tract lumen and may contain microvilli, while the basolateral surface faces the extracellular matrix. Actin filaments, microtubules, as well as an array of cellular proteins participate in the organization and maintenance of cell polarity. Simple epithelial cells have apical and basolateral endocytic pathways, each of which uses a complex series of compartments. These pathways can lead to recycling or degradation, but also to transcytosis, the selective transcellular vesicular transport from one pole of the epithelium to the opposite one (Cao et al., 2012). Previous studies have shown that influenza enters and buds from the apical domain of MDCK cells (Rodriguez Boulan and Sabatini, 1978). In either virus-infected cells or cells expressing viral proteins, each viral envelope protein (HA, NA, M2) possesses an apical sorting signal and preferentially localizes at the apical domain (Hughey et al., 1992; Jones et al., 1985; Roth et al., 1983). Since there are significant differences between polarized and non-polarized cells with regards to receptor distribution, cytoskeletal structure and the mechanism of endocytosis, it is possible that our current knowledge of influenza virus entry does not completely apply to viral infection *in vivo*.

Although we currently have much information on the entry process of influenza virus, there is still limited information on the specific route of entry into polarized epithelial cells, which are the target cells of influenza infection *in vivo*. In culture, MDCK cells have proven to be a robust and much studied model of polarized epithelia. MDCK cells are often used for studies of influenza virus, but very rarely in detailed studies of

virus entry under conditions where polarity is monitored. In general, polarized cells are a more difficult experimental system. Care must be taken to maintain a true polarized epithelium, and polarized cells are refractory to conventional transfection approaches, making the transient transfection dominant-negative methodology problematic.

Summary

The goal of this thesis work is to better understand the attachment and endocytosis of coronavirus and influenza virus entry process. In Chapter 2, the role of DC-SIGN/L-SIGN as potential attachment factor for IBV was examined. In Chapter 3, different virological aspects of IBV infection in chicken peripheral blood derived monocytes were studied. Finally, in Chapter 5, entry pathways of influenza virus in polarized epithelial cells were investigated. In all, this work substantially added to our understanding of mechanisms of virus-host interaction.

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CHAPTER 2

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

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2.1 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 Ctype 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 antilectin 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.

2.2 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, 2003b). 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, 2003). 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, 2003b).

The ability of IBV to infect different tissues in chickens may be linked to the receptor distribution within these 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 DCSIGN 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.

2.3 Materials and Methods

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 x g for 15 minutes at 4°C. For concentrated IBV-M41 virus preparation, the allantoic fluid was further centrifugated at 34500 x g for 60 minutes 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.

Cell culture and plasmids

3T3-DCSIGN 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-DCSIGN 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 0.75µl 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.

RT-PCR for viral infection detection

10^6 3T3 or 3T3-DCSIGN 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-DCSIGN 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 minutes, 30 cycles at 94°C for 30 seconds, 48°C for 30 seconds, 72°C for 1 minute, and 1 cycle of 72°C for 5 minutes. 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 were visualized on a 1% agarose gel.

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.).

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

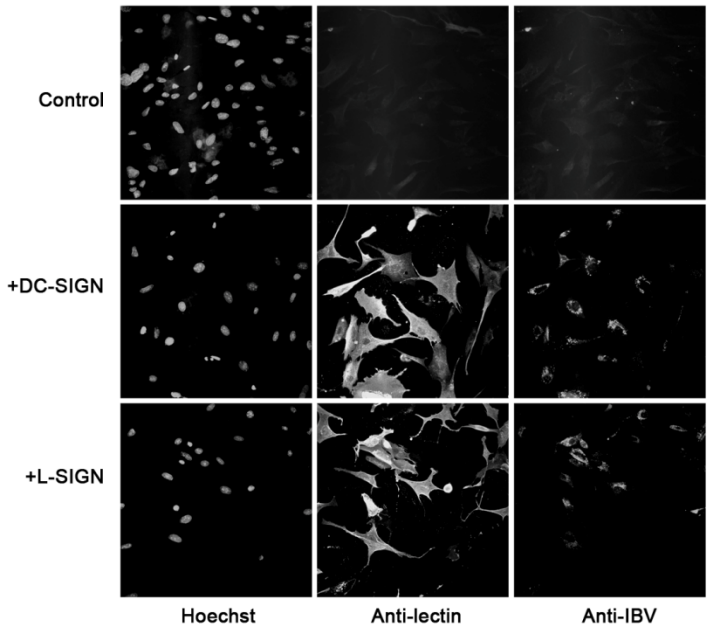
Cells were incubated with mannan at a concentration of 50µg/ml (Sigma), monoclonal antibody 9E9A8 specific for hDC-SIGN at 20mg/ml (NIAID AIDS Research and Reference Reagent Program), neuraminidase, or sugars in DMEM media for 1 h at 37°C before virus infection. Both mannose and galactose were purchased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

2.4 Results

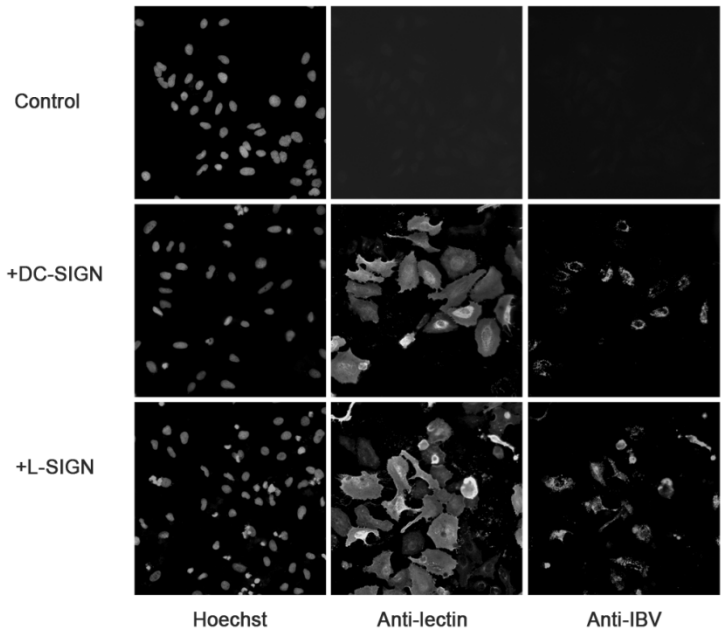
To determine a role for C-type lectins in the entry of IBV into host cells, we transiently expressed either DC-SIGN or L-SIGN in cell lines that are known to be refractory to infection by clinical strains of IBV, such as M41, and examined these cells for virus infection. Figure. 2.1. shows 3T3 (A) or 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.

Figure 2.1. 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 10^3TCID50/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.

A


Control
+DC-SIGN
+L-SIGN
Hoechst
Anti-lectin
Anti-IBV

B


Control
+DC-SIGN
+L-SIGN
Hoechst
Anti-lectin
Anti-IBV

However, in the presence of DC-SIGN or L-SIGN expression, there was a strong correlation of IBV M41 infection in lectin-expressing cells. Figure. 2.2. 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 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 (Figure 2.3.), 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

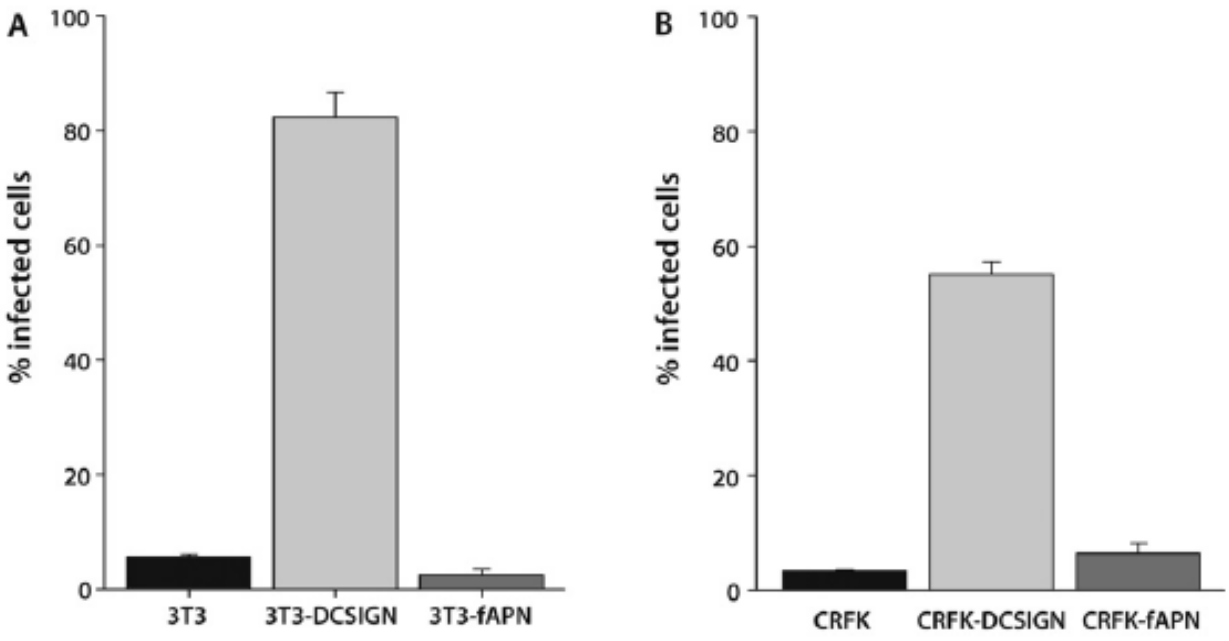
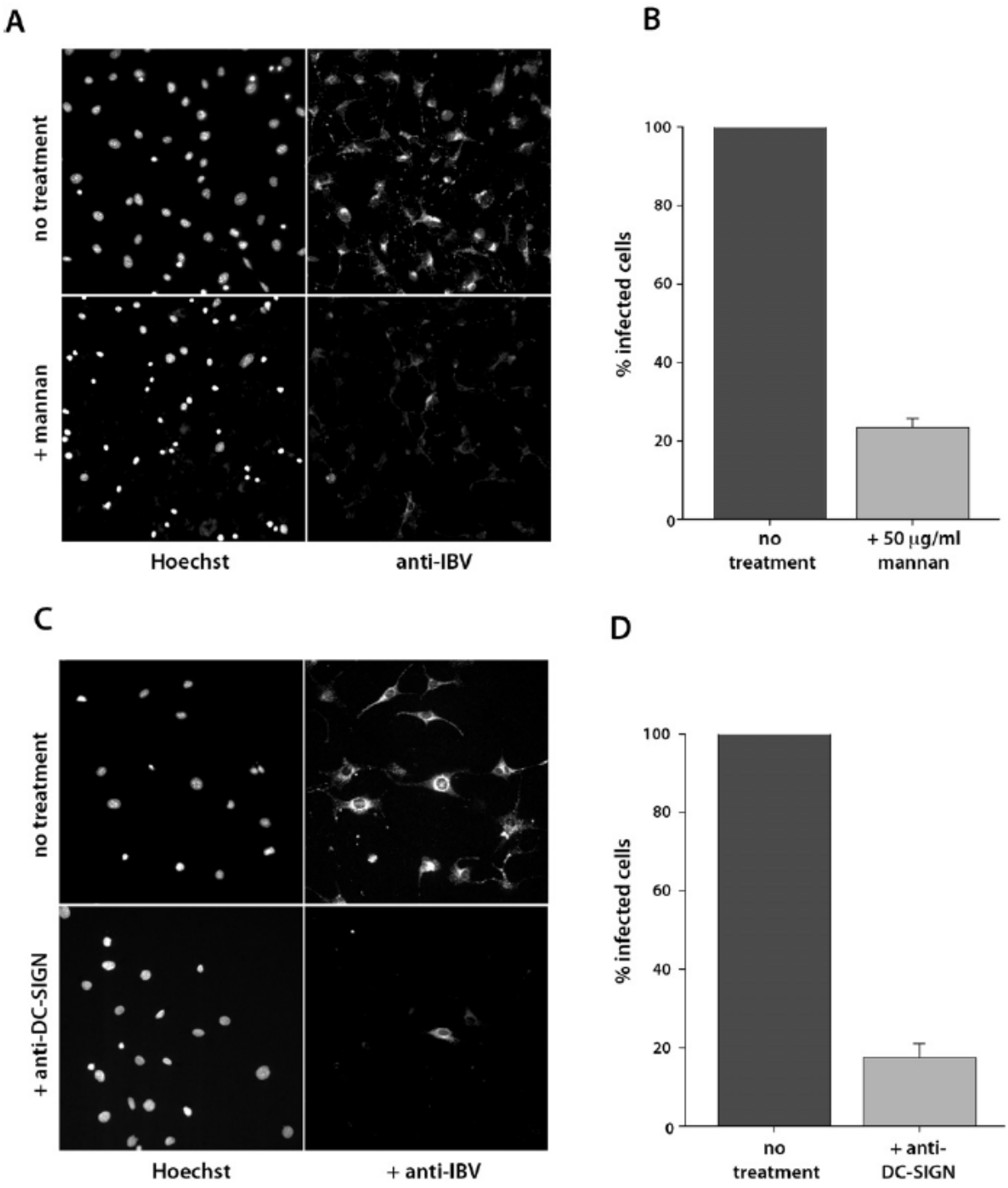


Figure 2.2. IBV-M41 infection is enhanced on 3T3 or CRFK cells stably expressing DCSIGN. 3T3 or CRFK cells stably expressing DCSIGN cells were infected with 10^3TCID50/ml of IBV-M41 for 1 h at 37°C. Cells were washed 3 times with sterile PBS and further incubated for 12 h before fixing with methanol. 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.

Figure 2.3. Specificity of enhancement of infection by IBV-M41. 3T3-DCSIGN cells were treated with 50μg/ml mannan (A) or 20mg/ml anti-DC-SIGN antibody 9E9A8 (C) prior to infection with 10^3TCID50/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.


A
no treatment
+ mannan
Hoechst
anti-IBV
B
% infected cells
100
80
60
40
20
0
no treatment
+ 50 μg/ml mannan
C
no treatment
+ anti-DC-SIGN
Hoechst
+ anti-IBV
D
% infected cells
100
80
60
40
20
0
no treatment
+ anti-DC-SIGN

complete genome replication, we extracted total RNA from either 3T3 or 3T3-DC-SIGN cells infected with IBV M41 (Figure 2.4.). 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 sialic acid and C-type lectins for entry of IBV M41 (Figure 2.5.). 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 (Figure

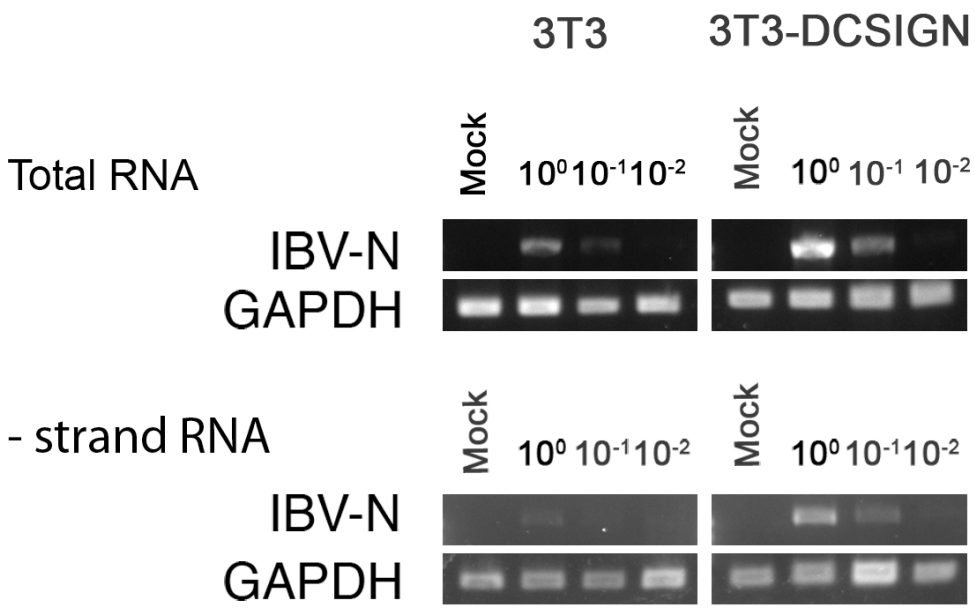


Figure. 2.4. Replication of IBV-M41 in 3T3-DC-SIGN cells. 3T3 or 3T3-DCSIGN cells were infected with 200µl virus inocula of allantoic fluid dilutions of IBV-M41 with titer of $10^{8.3}$EID50/ml for 1 h at 37°C. 8 h post infection, total RNA was extracted from cells and was subjected to RT-PCR with primers targeting to IBV nucleocapsid N gene or host GAPDH gene. Anti-sense RNA was used to derive negative strand specific cDNA.

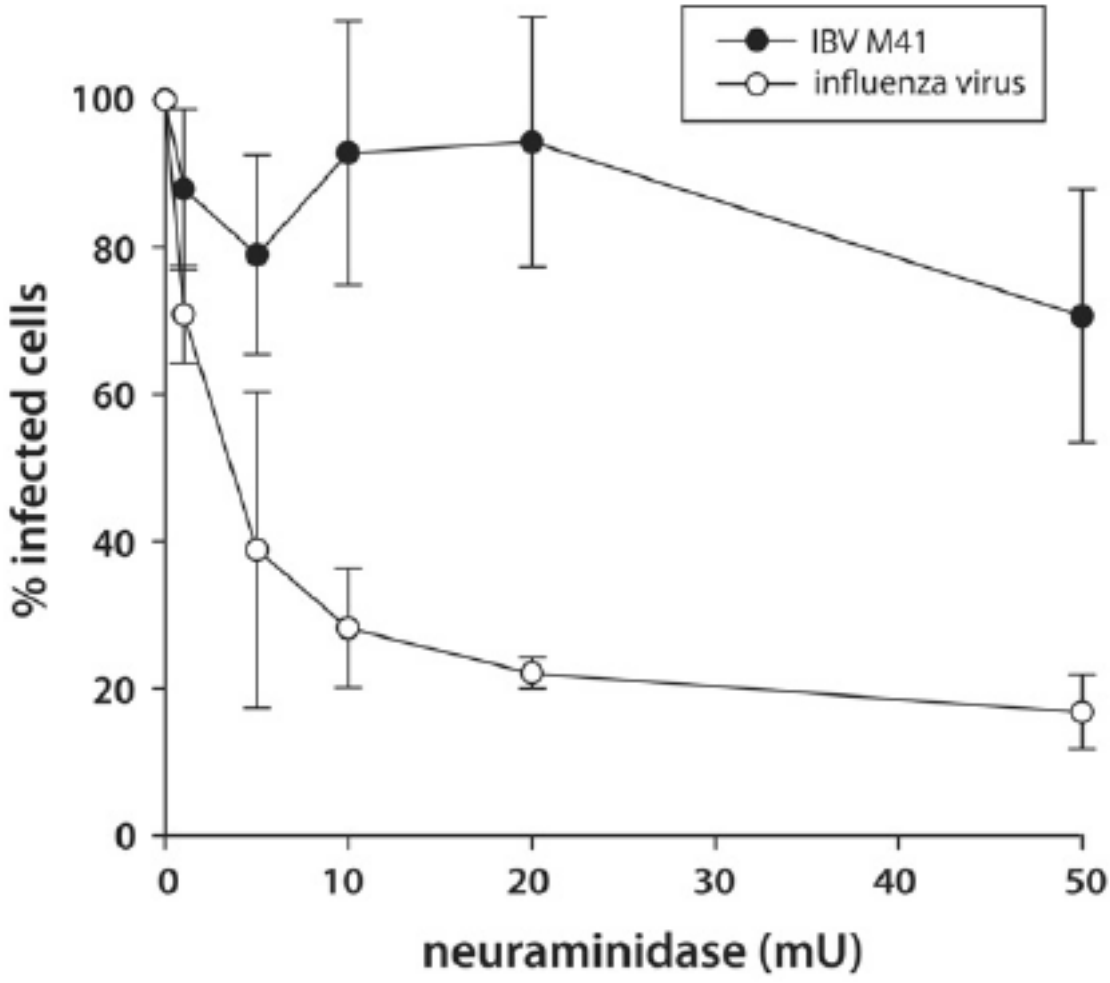
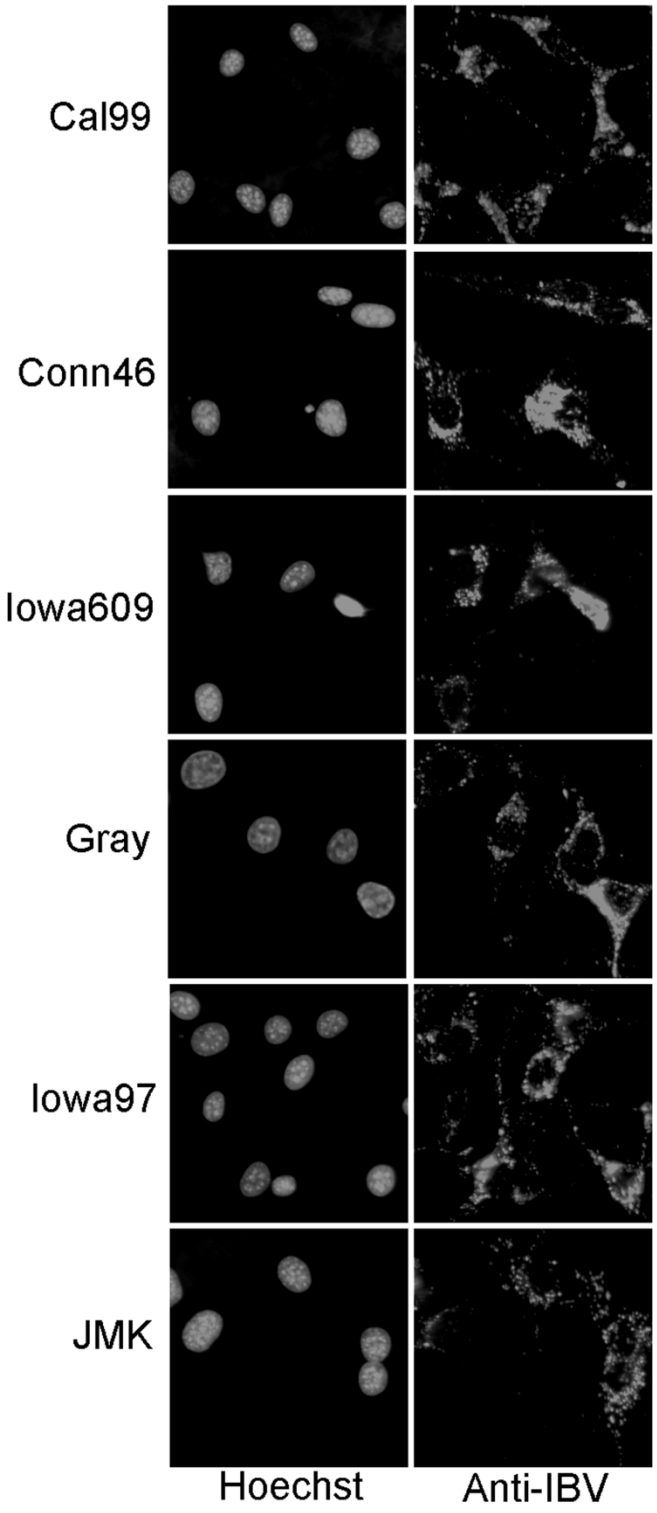


Figure 2.5. Effect of neuraminidase treatment on 3T3-DCSIGN cells infected by IBV-M41 3T3-DCSIGN cells were pre-treated with neuraminidase and then were infected by M41 (10^3TCID50/ml), or a control virus, influenza A/WSN/33 (MOI of 5). At 12 h post infection (M41) or 5 h post infection (WSN), cells 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.

Figure 2.6. The 3T3-DCSIGN cell line is susceptible to various IBV strains representing the major viral serotypes. 3T3-DCSIGN cells were infected with the allantoic fluid of embryonated eggs infected with various IBV isolates for 1h 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.


Cal99
Conn46
Iowa609
Gray
Iowa97
JMK
Hoechst
Anti-IBV

2.6.) 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 0.01M mannose, the sugar monomer enriched in mannan (Figure 2.7.). As the control, 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.

2.5 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 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; Jeffers et al., 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

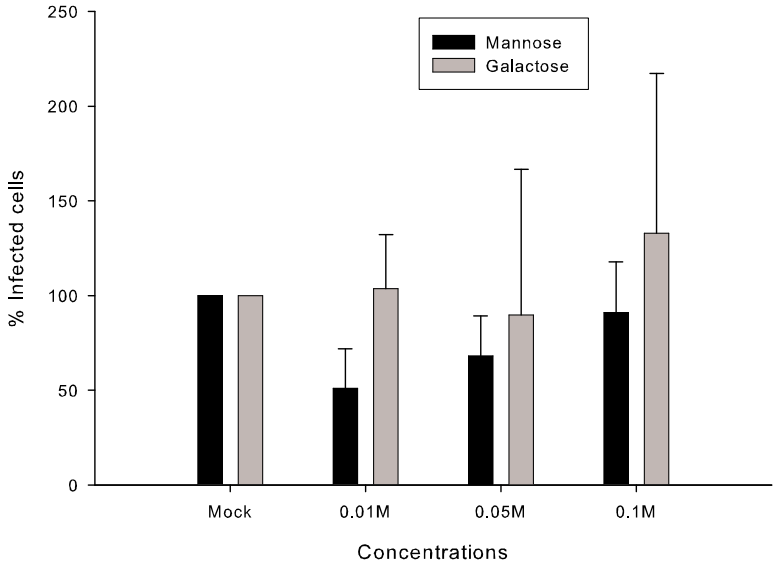


Figure 2.7. Effect of carbohydrates on IBV-M41 infectivity in chicken embryonic chicken cells. Chicken embryonic kidney cells were treated with either 0.01M mannose or 0.01M galactose before infection with IBV-M41. Cells were fixed at 8 h post infection and analyzed by immunofluoresence microscopy and quantified as previous experiments.

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 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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CHAPTER 3

Productive infection of avian coronavirus infectious bronchitis virus in chicken peripheral blood-derived monocytes

Yueting Zhang and Gary R. Whittaker

3.1 Abstract

The Coronavirus family comprises a variety of single-stranded RNA, enveloped viruses that infect a wide range of animal species, and the prototype avian coronavirus infectious bronchitis virus (IBV) is a continuing economic concern for the poultry industry. IBV typically infects the respiratory tract, but can also spread to and cause pathology in numerous other organs, including the kidney, gastrointestinal tract and the oviduct/testis. The current method of control for IBV is by vaccination, and the humoral response of IBV infection in chickens has been extensively studied. Although studies have shown that host innate immune responses to IBV contribute to the development of infectious bronchitis, little is known about the role of antigen-presenting cells for IBV immunopathogenesis. In our study, we utilized peripheral blood-derived monocytes/macrophages from chickens (chPBMC) and show that these cells are productively infected by clinical strains of IBV, such as M41. We show that IBV M41 induces apoptosis in chPBMCs within 6h post-infection, which does not require viral replication, and that chPBMCS are infected by a low pH-dependent, dynamin-independent pathway. Our data also show that sialic acid is dispensable for IBV M41 infection of chPBMCs, and that C-type lectins may function as co-receptors/attachment factors in chPBMCs. In conclusion, our data suggest that infection of chPBMCs may play a role in the IBV-mediated innate immune response, and play an important role for viral pathogenesis in chickens.

3.2 Introduction

Infectious bronchitis virus is an avian coronavirus that infects chickens, and is the prototype member of the Gammacoronavirus genus (Hudson and Beaudette, 1932; Woo

et al., 2009). Despite extensive vaccination, IBV poses continuous economic threat to the poultry industry globally. The primary site of infection for IBV is the epithelial cells of the respiratory tract; however IBV can spread to other organs of the host, with known susceptible organs that include the gastrointestinal tract, the kidneys, the reproductive tract/organs and the Haderian glands (Bezuidenhout et al., 2011; Raj and Jones, 1997). Viremia has generally been suggested as the way for internal spread of IBV, as evidenced by detection of virus in the blood of infected chickens (Chen et al., 2010a; Hofstad and Yoder, 1966). However, the exact mechanism of internal spread of IBV remains unknown. Other coronaviruses, such as SARS-coronavirus (SARS-CoV) (Ding et al., 2003), and feline coronavirus (FCoV) (Rottier, 1999), exhibit well recognized systemic infection. In these cases, monocytes, macrophages, and dendritic cells (DCs), originating from a common myeloid progenitor, act to disseminate the virus and also act as an important immunopathogenic component in disease development (Perlman and Dandekar, 2005). For instance, SARS-CoV has an abortive infection in human macrophages or DCs, but is capable of inducing pro-inflammatory cytokines in these cells (Frieman and Baric, 2008). Likewise, feline macrophages can be productively infected by FCoV and can be utilized by FCoV to disseminate to various organs, with macrophage activation leading to the production of pro-inflammatory cytokines critical to pathogenesis (Kipar et al., 2010).

In the case of IBV, previous studies have shown that chicken bone marrow-derived macrophages were resistant to infection *in vitro* (von Bulow and Klasen, 1983). However, macrophages from different anatomical sites of a host can elicit distinct functions or responses to foreign pathogens (Murray and Wynn, 2011; Perlman and

Dandekar, 2005). To explain the known presence of IBV in the blood of infected chickens (Chen et al., 2010a; Hofstad and Yoder, 1966), we reasoned that peripheral blood-derived monocytes/macrophages from chickens (chPBMCs) could be susceptible to IBV infection. Here we show that chPBMCs are highly susceptible to IBV infection *in vitro*, with rapid induction of apoptosis in these cells, suggesting that infection of chPBMCs plays a role in the IBV-mediated innate immune response, and has an important role for viral pathogenesis in chickens.

3.3 Materials and methods

Virus preparation

IBV strains used in this study were M41, Cal99, Conn46, and Iowa97. For preparation of virus stocks for infection, approximately 10^2 EID50 of IBV was inoculated into 10-day-old specific-pathogen-free chicken embryos. At 48 h post inoculation, allantoic fluid from infected embryos was collected and clarified by centrifugation at 1800 x g for 15 minutes at 4°C. To further concentrate IBV-M41, the allantoic fluid was ultracentrifugated at 34500 x g for 60 minutes at 4°C using a Ti45 rotor (Beckman Coulter). The virus pellet was resuspended in phosphate buffered saline (PBS) (Cellgro). The virus stocks were stored at -80°C. IBV preparations were titered by egg infectious dose 50 (EID50) assay. Concentrated IBV-M41 preparations were titered by tissue culture infectious dose 50 (TCID50) assay

Cell culture

Chicken peripheral blood monocytes were prepared as described (20). Briefly, fresh blood was obtained by cardiac puncture from 6-8 week-old specific-pathogen-free White

Leghorn chickens maintained by the Department of Microbiology and Immunology, Cornell University. All work with chickens was carried out according to the Cornell University Animal Care and Use program and complied with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Monocytes were isolated by Ficoll gradient (GE) and were cultured in RPMI 1640 supplemented with 10% heat inactivated chicken serum and 1% Penicillin/Streptomycin (Cellgro). 24 h after cell seeding, non-adherent or dead cells were washed away with Dulbecco's PBS (Cellgro) and PBS was replaced with fresh culture media. Monocytes for each independent experiment were obtained from an individual blood donor.

Virus infection

Virus stocks were diluted in RPMI 1640 medium containing 0.2% bovine serum albumin (Sigma), 1mM HEPES, pH 6.8. 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 culture medium.

Immunofluorescence assay

Cells were seeded on glass cover slips. After infection and incubation, cells were fixed with 100% methanol for detection of IBV viral antigens, otherwise, cells were fixed with 3% paraformaldehyde (PFA). 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 IBV-Cal99, IBV-Conn46, and IBV-Iowa97. Monoclonal antibody KUL01 was purchased from Abcam. The secondary antibodies AlexaFluor goat anti-mouse 488 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). Cells were quantified by scoring the percentage of cells positive for viral antigen compared to the total number of cells. >200 cells were quantified from three independent experiments.

RT-PCR for viral detection

10^6 PBMCs were seeded onto 6 cm dishes and infected with 7x 10^2 $TCID_{50}$ of IBV-M41. At indicated time points, culture supernatant was collected and was filtered through a 0.22 μm filter (Corning). Viral RNA was extracted from 140 μl of the supernatant with Qiagen Viral RNA extraction kit. RT-PCR reaction was conducted with SuperScript One-Step RT-PCR kit (Invitrogen) in a Bio-Rad DNA Engine Peltier Thermal Cycler using the following PCR cycles: 1 cycle of 94°C for 2 minutes, 30 cycles at 94°C for 30 seconds, 48°C for 30 seconds, 72°C for 1 minute, and 1 cycle of 72°C for 5 minutes. IBV N gene primers were previously described by (17).

Neuraminidase and lectin treatment

PBMCs were fixed with 3 % PFA before incubating with 5 μg/mL biotinylated *Maackia Amurensis* (MAA) I, 5 μg/mL *Maackia Amurensis* (MAA) II, or 1 μg/mL *Sambucus Nigra Lectin* (SNA). Lectins were purchased from Vector Labs. Streptavidin conjugated with AlexaFluor 488 was used to visualize the bound lectin on the cells by fluorescence microscopy. The lectins described above were incubated with chPBMCs were present before infection with IBV, during viral adsorption, and during incubation. Neuraminidase (Sigma-Aldrich) was pre-incubated at the indicated concentrations in RPMI media for 1 h at 37°C, and was removed by washing the cells three times with PBS before infection with IBV.

Treatment with carbohydrates and endocytic inhibitors

Cells were incubated with carbohydrates (Sigma-Aldrich) or endocytic inhibitors (Sigma-Aldrich) in RPMI 1640 media for 1 h at 37°C before infection, for 1 h during viral adsorption, as well as for the incubation periods. Treatment with EGTA (Calbiochem) was the same except that EGTA was removed 2 h after infection due to its toxicity. Post treatments were following the same viral infection protocol and drugs were added after viral adsorption.

Transferrin (Tfn) uptake assay

PBMCs were seeded on glass clover slips. Prior to performing the uptake assay, cells were serum starved for 30 min. 50 μg/ml Tfn conjugated with AlexaFluor 568 (Molecular Probes) was added to cells in RPMI 1640 for 20 minutes on ice for binding. Cells were washed with RPMI and then warmed to 37°C for 15 minutes. Tfn on cell surface was washed off with acidic buffer (0.1M NaCl and 0.1M glycine, pH 3). Cells were then fixed with 3% PFA for microscopy.

Apoptosis assays

PBMCs were infected with $2x10^3$ $TCID_{50}$ IBV-M41 and at 6 h post infection, reagents from a Green FLICA caspase 3/7 assay kit (ImmunoChemistry Technologies) were added using the manufacturer's instructions. Apoptotic cells were visualized by fluorescence microscopy. For the DNA laddering assay, total cellular DNA was extracted from infected or uninfected PBMCs plated in 6-cm culture dishes using a Qiagen DNeasy Blood & Tissue kit at 24 h post infection, and laddering was visualized on 2% agarose gel. To inactivate IBV-M41, viruses were placed on ice for 30 minutes under a UV-lamp with a wavelength of 254nm. Inactivation was confirmed by loss of infectivity in

chPBMCs. Ammonium chloride (NH_4Cl) was added to PBMCs before viral infection and was present during viral adsorption and incubation.

3.4 Results

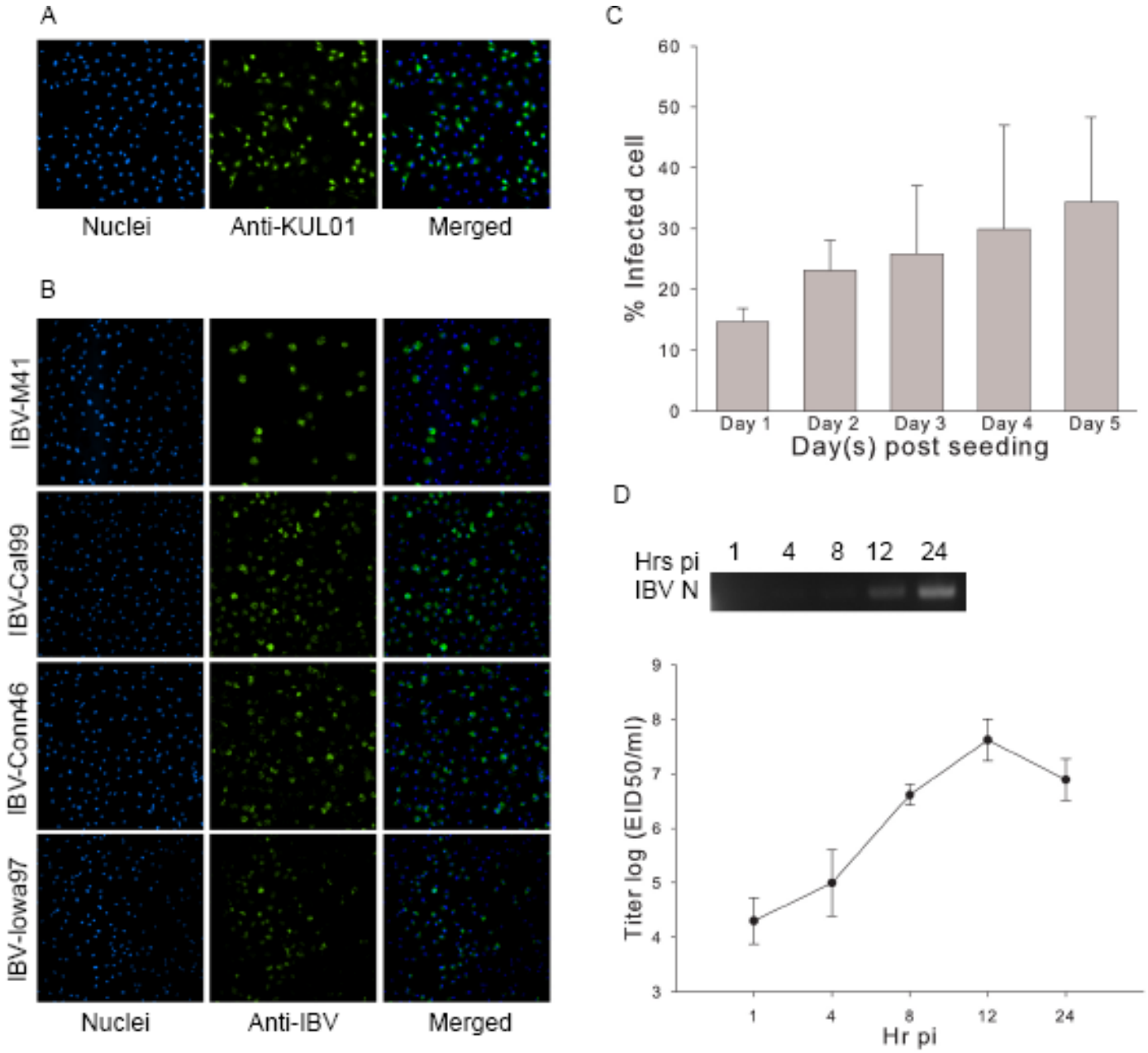
Peripheral blood-derived monocytes from chickens can be productively infected by IBV

To investigate a role for myeloid cells in IBV dissemination and viral pathogenesis, we isolated peripheral blood-derived monocytes from chickens (chPBMCs) and confirmed that they were positive for the monocyte/macrophage marker KUL01 (Figure 3.1A). Due to the lack of available reagents for avian species, we were unable to further distinguish whether these chPBMCs were matured or differentiated into macrophages or dendritic cells, or to determine the composition of unique cell populations with specific cell surface antigens. In contrast to previous studies that showed bone-marrow-derived macrophages were resistant to IBV infection *in vitro* (von Bulow and Klasen, 1983), we found that chPBMCs are highly susceptible to the IBV field strains M41, Cal99, Conn46, and Iowa 97 (Figure 3.1B).

To examine whether the stage of maturation or differentiation of the adherent chPBMCs had an effect on the ability of IBV to infect these cells, we tested the infection of chPBMCs at different days post-seeding. Infectivity with IBV- M41 was determined using an immunofluorescence assay (Figure 3.1C). The percentage of infected cells increased with time, reaching a maximum infectivity of 35% at day 5. However, beyond day 3 to 4

Figure 3.1. Chicken PBMCs can be productively infected by field strains of IBV

(A) ChPBMCs (3-day-old culture) were stained with mouse anti-KUL01 avian macrophage/monocyte marker antibody; followed by incubation with secondary antibody goat anti-mouse AlexaFluor-488. (B) ChPBMCs were infected with IBV-M41, IBV-Cal99, IBVConn46, or IBV-Iowa97 for 1 h at 37°C. Cells were washed with PBS and further incubated for 9 h for IBV-M41 and 23 h for other IBVs. Viral spike protein antigen was detected by immunofluorescence microscopy using anti-M41 –specific S1 mouse monoclonal antibody 15:88 followed by goat anti-mouse AlexaFluor-488. Infection of other virus strains was detected with the mouse anti-M protein monoclonal antibody 9:19 followed by goat anti-mouse AlexaFluor-488. (C) Infectivity of IBV-M41 in day 0 to 5 cultures of chPBMCs were determined by immunofluorescence microscopy. For each day of culture, monocytes were infected with IBV-M41. Cells were quantified by scoring the percentage of cells positive for viral antigen S or M. >200 cells were quantified from each of three independent experiments. (D) Filtered supernatants were harvested from IBV-M41 infected chPBMC at indicated time points. RT-PCR against IBV N gene was performed and amplified DNA products were visualized on an agarose gel. The infectious titer of the supernatants containing IBV particles was also determined in 10-day-old embryonated eggs by EID50 assay.


A
Nuclei
Anti-KUL01
Merged
B
IBV-M41
IBV-Cal99
IBV-Conn46
IBV-Iowa97
Nuclei
Anti-IBV
Merged
C
% Infected cell
60
50
40
30
20
10
0
Day 1
Day 2
Day 3
Day 4
Day 5
Day(s) post seeding
D
Hrs pi
1
4
8
12
24
IBV N
Titer log (EID50/ml)
9
8
7
6
5
4
3
1
4
8
12
24
Hr pi

post-seeding, we observed a large proportion of giant multinucleated cells, as observed by previous investigators (Weiss and Fawcett, 1953). In all further experiments, we utilized day 3 or day 4 cultures due to difficulty in imaging and quantifying the giant cells.

We next determined whether IBV infection of the chPBMCs produces progeny viral particles. RT-PCR was performed to detect the IBV N gene of the released particles in the supernatant of infected PBMCs. Starting at 8 h post-infection, we could detect the viral RNA in the infected culture, reaching a maximum RNA level at 24 h post-infection. To determine whether these progeny virions were infectious, we performed an EID50 assay on the supernatant samples. The peak viral titer was 10^8 EID_{50}/mL at 12 h post-infection. At 24 h post-infection, the viral titer decreased slightly even though RT-PCR indicated more virus particles present at this time point (Figure 3.1D). The reduced infectivity may be due to aggregation of viral particle between 12 and 24 h post infection.

IBV induces rapid apoptosis in chPMBCs

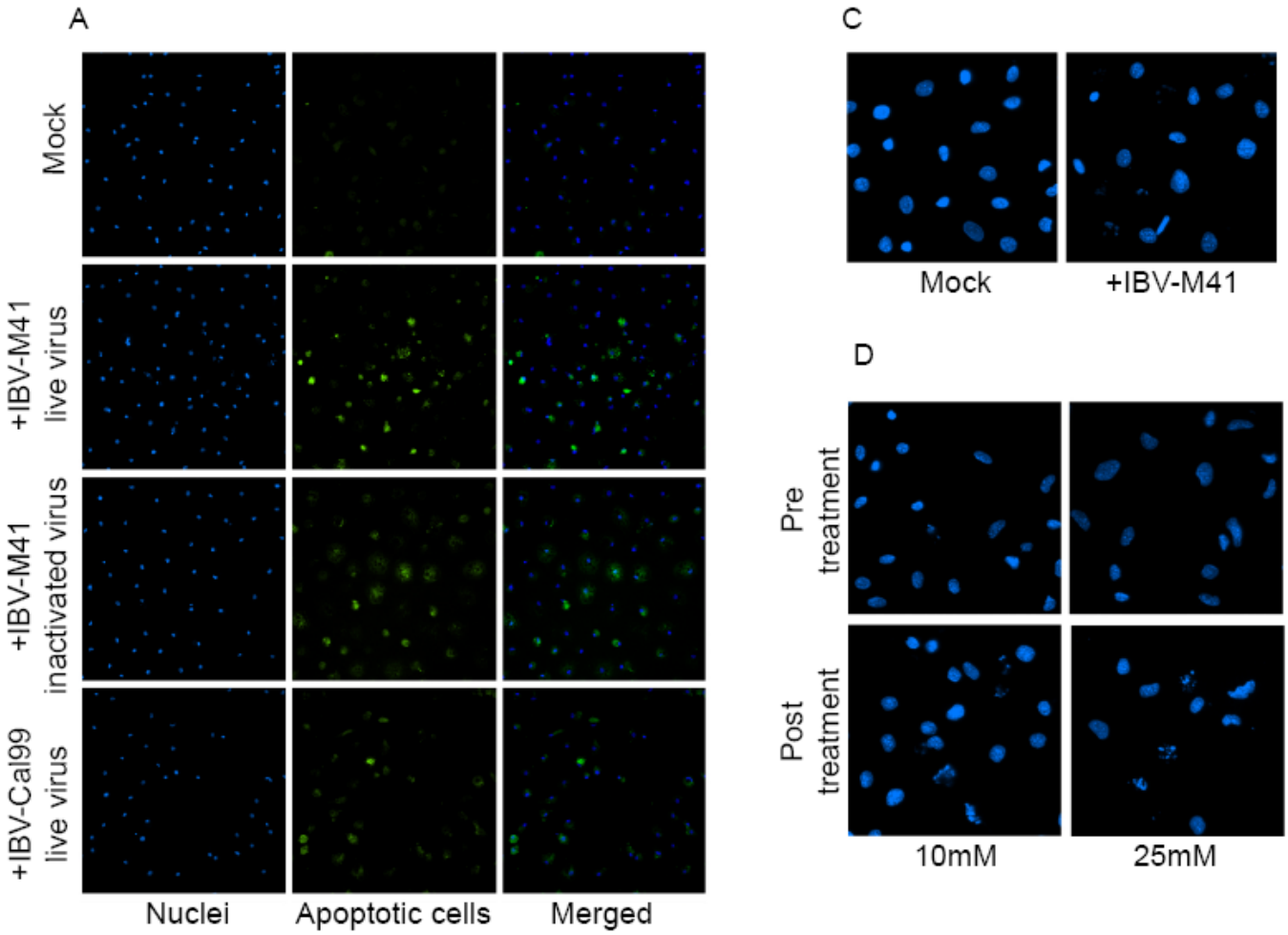
It is known that the IBV Beaudette strain, which is a highly laboratory-adapted virus, can induce caspase-dependent apoptosis in Vero cells at 16 h post-infection (Liu et al., 2001). To determine whether IBV can induce apoptosis in chPBMCs, we examined the activation of caspase 3/7 after infection by IBV-M41 and IBV-Cal99 virus, using a FLICA assay, which employs the caspase 3/7 inhibitor FAM-DEVD-FMK that binds to activated caspase 3/7 irreversibly. At 6 h post-infection, there was an increase in the fluorescence signal in the IBV-infected cells, compared to mock-infected cells,

suggesting activation of caspase 3/7 in these cells. Interestingly, upon addition of UV-inactivated IBV-M41 ($2x10^3$ $TCID_{50}$), chPBMCs also displayed an increase in fluorescence signal, indicating that replication-defective IBV is also able to trigger apoptosis (Figure 3.2A). To further confirm the IBV-induced apoptosis, cellular DNA from mock infected or infected chPBMCs were collected after 24 h post-infection and analyzed by agarose gel electrophoresis. DNA from infected cells was fragmented and displayed an apoptosis-characteristic laddering, while no obvious laddering was observed for mock-infected cells.

When we added a small amount ($5x10^2$ $TCID_{50}$) of UV-inactivated IBV-M41 was added to chPBMCs, such that the same quantity of inactivated particles were used compared to infectious IBV M41 particles, DNA fragmentation was no longer observed. Since the quantity of defective particles per cell was approximately 200-fold less than the quantity used in the experiment in Figure 3.2A, the DNA fragmentation was not able to be visualized from such a small population of apoptotic cells. While some viruses such as HIV are able to induce bystander cell death in uninfected CD4+ T cells(Holm et al., 2004), our data implied that the IBV-induced apoptosis observed in chPBMCs was not due to the bystander effect (Figure 3.2B). Our data imply that even though chPBMCs could undergo apoptosis by encountering a defective IBV virus, neighboring uninfected chPBMCs would not be affected.

IBV entry occurs via endosomes, in a low-pH dependent manner (Chu et al., 2006a). To examine whether IBV-induced cell death requires endosomal fusion, we treated the chPBMCs with NH_4Cl, a cell permeable weak base, to raise the pH in the endosomes before infecting with IBV-M41. Presumably IBV particles were arrested in

Figure 3.2. IBV-M41 induces rapid apoptosis in chPBMCs (A) Mock infected, live IBV-M41, or inactivated IBV-M41 infected chPBMCs were assayed at 6 h post infection. Apoptosis was revealed by green fluorescence FLICA caspase 3/7 detection assay. (B) Mock infected, IBV-M41(5x102 TCID50) infected, or UV- inactivated IBV-M41(5x102 TCID50) infected chPBMCs seeded on 6 cm tissue culture dishes were harvested and total cellular DNA were extracted at 24 h post infection and analyzed on agarose gel. (C) chPBMCs were mock infected, IBV-M41 infected before nuclei were stained with Hoechst at 10 h post infection. Apoptotic nuclei were visualized by fluorescence microscopy. (D) chPBMCs were treated with different concentrations of NH4Cl before adding IBV-M41. At 10 h post infection, apoptotic nuclei were stained with Hoechst and visualized by fluorescence microscopy.

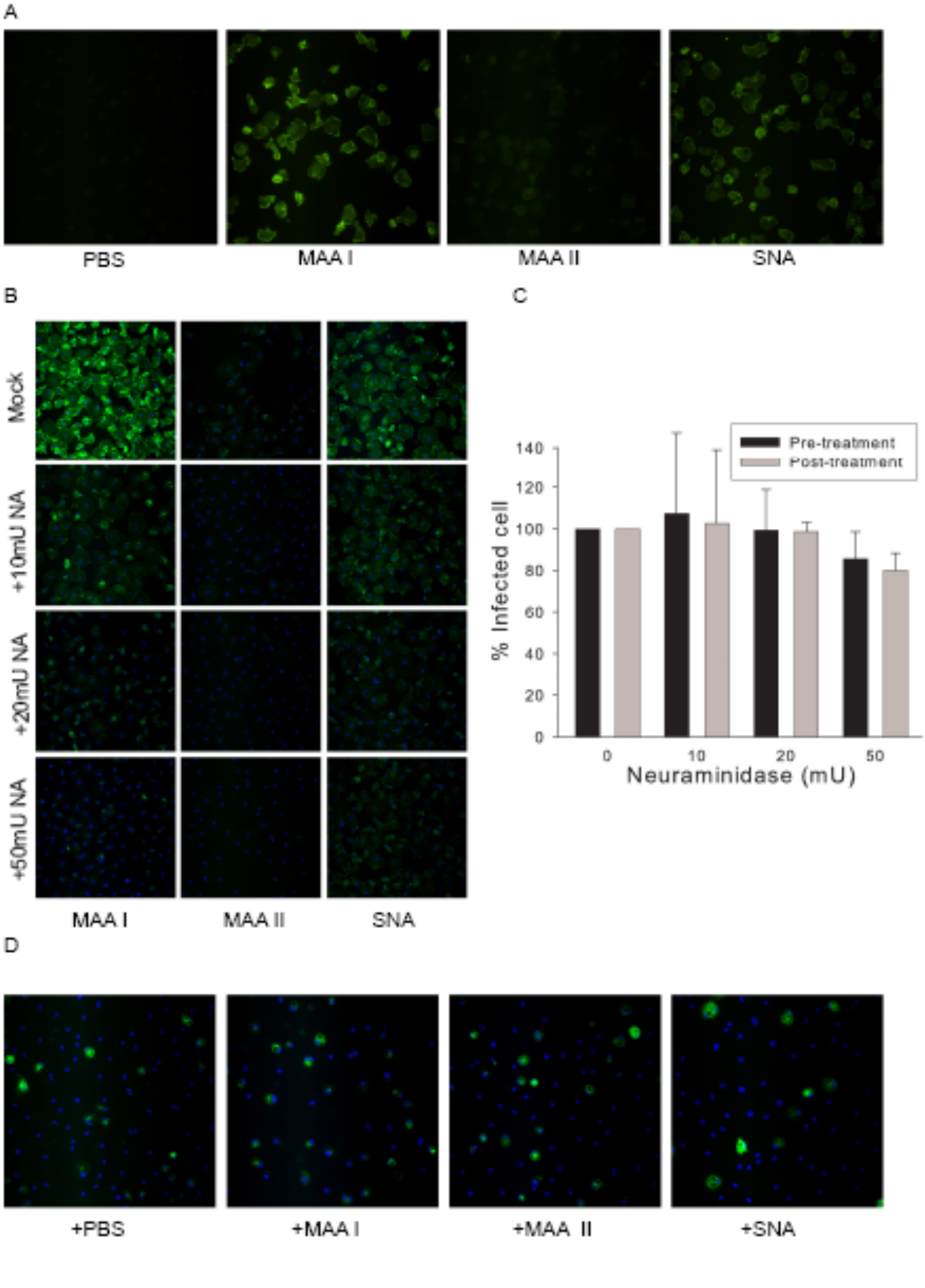

A
Mock
+IBV-M41 live virus
+IBV-M41 inactivated virus
+IBV-Cal99 live virus
Nuclei
Apoptotic cells
Merged
C
Mock
+IBV-M41
D
Pre treatment
Post treatment
10mM
25mM
B
Ladder
Mock infection
Infectious IBV-M41
Inactivated IBV-M41

the endosomes after NH_4Cl treatment. Nuclear staining with DNA binding dye Hoechst showed that pre-treatment with NH_4Cl prevents the condensation of the chromosomes (Figure 3.2D). In contrast, non-treated cells and cells treated with NH_4Cl after infection display fragmentation of the nuclei (Figure 3.2C). These data indicate that IBV-triggered apoptosis may require endosomal fusion.

IBV infection in chPBMCs does not depend on the level of cell surface sialic acid

It has been previously shown that IBV infection of epithelial cells is dependent on cell surface sialic acid. However, avian species are known to have different levels of sialic acid at the surface of various tissues such as trachea, kidney, intestine (Ito et al., 2000; Wickramasinghe et al., 2011). Therefore, we first investigated the sialic acid linkages present on our isolated chPBMCs. Biotinylated sialic-acid specific lectins from plants: *Maackia amurensis* lectin I, II and *Sambucus nigra* lectin have been used to distinguish between α2,3 sialic acid linkages (MAA I, II) and α2,6 sialic acid linkages (SNA). We found a clear, strong binding of MAA I to chPBMCs, while MAA II binding was minimal (Figure 3. 3A). The different binding patterns from these two isoforms of MAAs may be due to the specificity of the two lectins: MAA I recognizes the SAα2,3 Gal β1, 4 GlcNAc sugar moiety while MAA II recognizes SAα2,3 Gal β1, 3GalNAc (Geisler and Jarvis, 2011; Nicholls et al., 2007). Surprisingly, we also observed some binding of SNA to the chPBMCs, indicating the possibility that chPBMCs surface glycoproteins or glycolipids contain α2, 6-linked sialic acid. The signal of MAAI, MAA II, and SNA binding was reduced in a dose-dependent manner upon treatment with neuraminidase, which cleaves terminal sialic acids from cell surface glycoproteins

Figure 3.3. IBV-M41 infection in chPBMCs does not depend on cell surface the sialic acid levels (A) chPBMCs were stained with biotinylated MAA I, MAA II, or SNA lectins and sialic acid level was visualized by fluorescence microscopy. (B) Staining of sialic acid specific lectins (MAAI, MAAII, and SNA) on chPBMCs treated with different concentrations of neuraminidase. (C) chPBMCs were pre-treated with neuraminidase and then were infected by IBV-M41. Post-treatment with neuraminidase occurred 1 h post infection by IBV-M41. At 10 h post infection (M41), cells were fixed and stained for immunofluorescence microscopy with anti-S1 mouse monoclonal antibody. >200 cells from each of three independent experiments were scored by positive viral antigens. Error bars represent the standard deviation from the mean. (D) chPBMCs were pre-treated with sialic acid specific lectins (MAA I, MAA II, SNA) before infection with IBV-M41. Viral spike protein antigen was visualized by immunofluorescence microscopy


A
PBS
MAA I
MAA II
SNA
B
Mock
+10mU NA
+20mU NA
+50mU NA
MAA I
MAA II
SNA
C
Pre-treatment
Post-treatment
% Infected cell
140
120
100
80
60
40
20
0
0
10
20
50
Neuraminidase (mU)
D
+PBS
+MAA I
+MAA II
+SNA

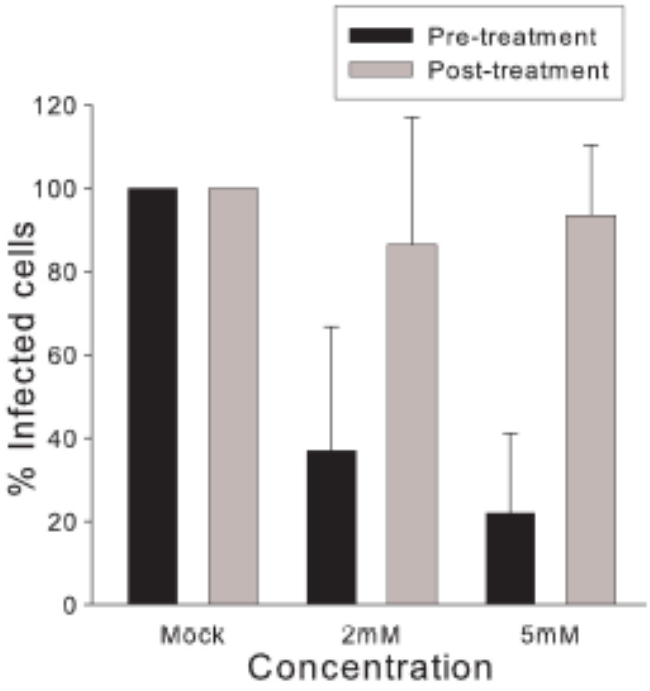
(Figure 3.3B). To investigate the role of cell surface sialic acid for IBV infection of chPMBCs, we treated cells with various concentrations of neuraminidase before infecting with IBV. Surprisingly, we did not find a significant reduction in the infection level of IBV-M41 in the neuraminidase-treated cells (Figure 3.3C). The slight decrease on the infectivity of IBV at 50mU neuraminidase post-infection may be due to the toxicity of sialidiase as the neuraminidase post-infection treatment displayed a similar reduction of infectivity. When we used the same concentrations of neuraminidase on chicken embryonic kidney cells prior to IBV-M41 infection, we confirmed a decrease in IBV infectivity, as previously described in the literature (Winter et al., 2006). To further confirm our findings, we also used biotinylated lectins as sialic acid-binding competitors for IBV-M41 infection of chPBMCs. Upon pre-treatment with these lectins, we did not observe a decrease in IBV-M41 infectivity (Figure 3.3D). Overall, our data indicate that sialic acids on the surface of chPBMCs do not play a role in IBV infection.

Inhibition of IBV infection in chPBMCs by EGTA and purified monosacchrides

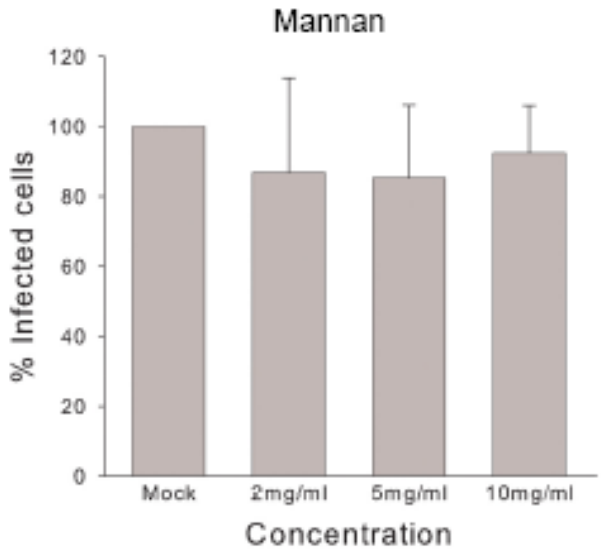
Myeloid-origin cells such as monocytes, macrophages, and dendritic cells are critical for pathogenesis of numerous coronaviruses, which have been shown to be able to utilize C-type lectins such as DC-SIGN or L-SIGN as receptors or attachment factors (Hofmann et al., 2006; Marzi et al., 2004; Regan and Whittaker, 2008; Yang et al., 2004). Our previous studies determined that human DC-SIGN, when expressed in non-permissive mammalian cell lines, allows for IBV infectivity (Zhang et al., 2012). To investigate whether C-type lectins in chPBMCs can play a role in IBV infection, we utilized the calcium-chelating agent EGTA. As C-type lectins require calcium for

Figure 3.4. IBV-M41 infection in chPBMCs is sensitive to calcium chelating agent EGTA but is insensitive to treatment with a panel of carbohydrates. (A) chPBMCs were pre-treated with EGTA at 2 mM or 5 mM concentrations before (pre-treatment) or after (post-treatment) infection by IBV-M41. The bar graph represents quantification of immunofluoresence microscopy from three independent experiments. Cells were scored for positive viral spike antigen. Error bars represent the standard deviation from the mean. (B) Mannan at indicated concentrations was added to chPBMCs prior to IBV-M41 infection. (C) Carbohydrates at indicated concentrations were added to chPBMC culture before infection by IBV-M41.

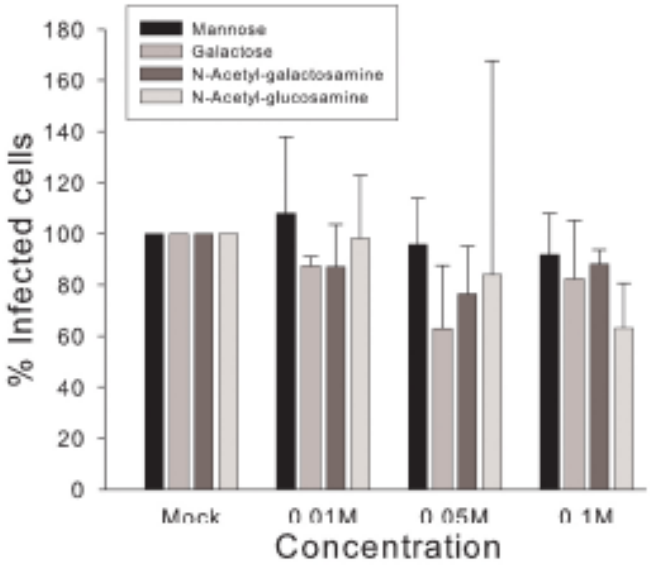
A


Pre-treatment
Post-treatment
% Infected cells
120
100
80
60
40
20
0
Mock
2mM
5mM
Concentration

B


Mannan
% Infected cells
120
100
80
60
40
20
0
Mock
2mg/ml
5mg/ml
10mg/ml
Concentration

C


Mannose
Galactose
N-Acetyl-galactosamine
N-Acetyl-glucosamine
% Infected cells
180
160
140
120
100
80
60
40
20
0
Mock
0.01M
0.05M
0.1M
Concentration

binding, EGTA acts as a general inhibitor of C-type lectins. We observed that IBV-M41 infection of chPBMC decreased by 80% at 5 mM concentration of EGTA (Figure 3.4A). EGTA treatment after IBV infection on the cells did not yield a significant decrease in infectivity, suggesting that the reduction of IBV infection occurs during IBV entry and is mediated via interactions with C-type lectins. However, we cannot rule out the possibility that other cellular components that are critical for IBV infection also require calcium ions.

Next, we treated the chPBMCs with a panel of purified monosaccharides or oligosaccharides to investigate whether IBV infection requires a particular sugar moiety. Our experiments show that we were unable to inhibit the IBV-M41 infection with mannan, mannose, galactose, N-acetyl-galactosamine, or N-acetyl-glucosamine (Figure 3. 4B and 3.4C). While not providing support for IBV interaction with C-type lectin, it is very possible that there is a specific avian species of carbohydrates that interacts with IBV, which remains unidentified and so could not been tested.

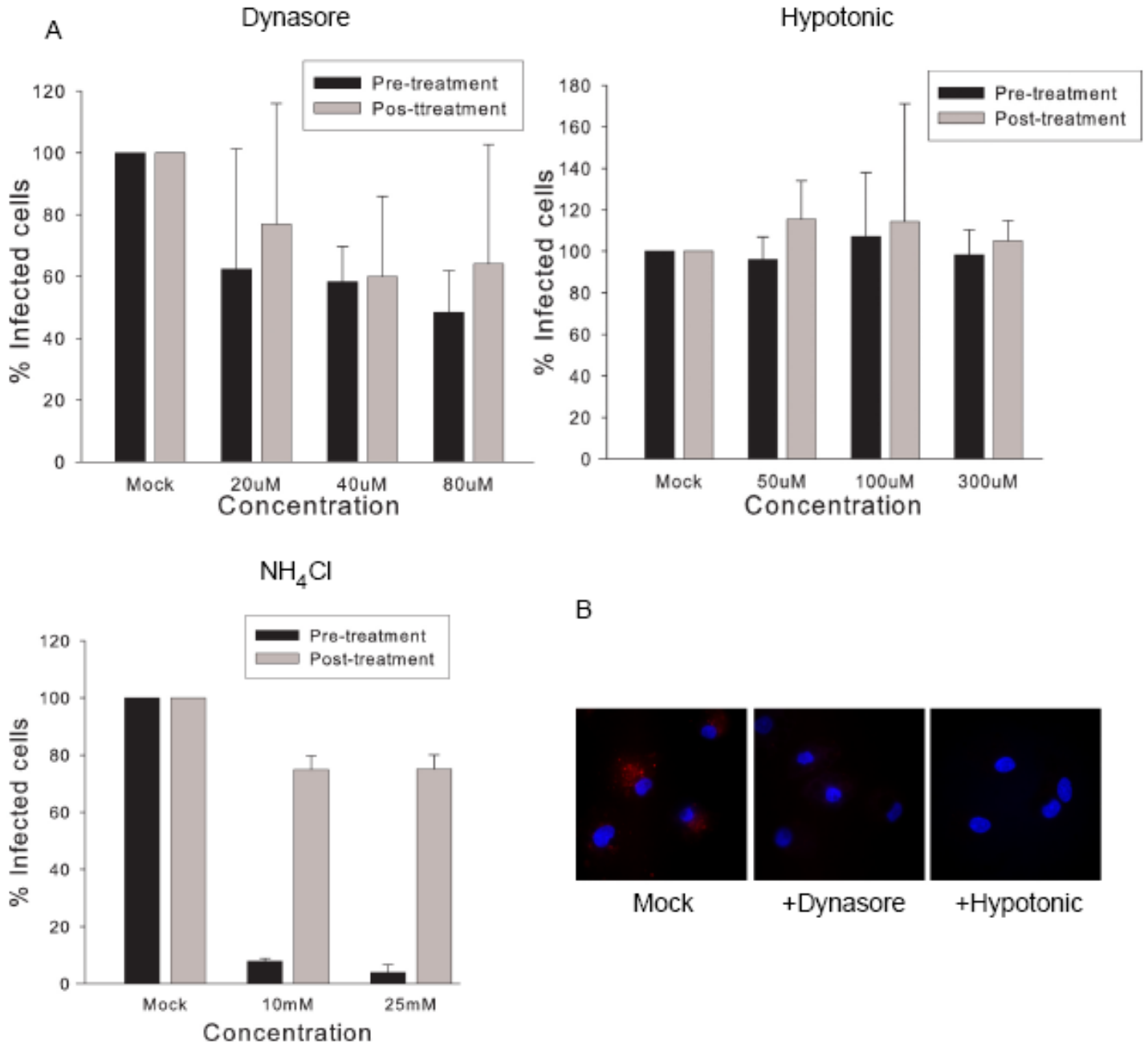
Entry mechanism by IBV-M41 into chPBMC

Coronaviruses usually enter host cells via receptor-mediated endocytosis and require low pH to fuse the viral membrane with host endosomal membrane. For instance, SARS-coronavirus is believed to enter cells via a clathrin- and caveolae- independent endocytic route (Wang et al., 2008). Our laboratory has previously reported that IBV enters cells via endocytosis and requires low pH-dependent fusion activation of the spike protein (Chu et al., 2006a; Chu et al., 2006b). To examine the entry route that IBV-M41 uses to enter the chPBMCs, we used dynasore, a specific inhibitor of the GTPase activity of dynamin. Dynamin is a small GTPase that plays an important role in pinching off both

clathrin- and caveolae-mediated endocytic vesicles. We pre-incubated chPBMCs with various concentrations of dynasore prior to infection with IBV-M41. Infection of the cells was scored by the positive fluorescence signal of viral spike protein. Our data revealed that there was no significant difference of the percentage of infected cells between the various concentrations of dynasore treatment. When we pre-treated chPBMCs with a hypotonic solution of sucrose, which inhibits clathrin-coated pit formation in the cell membrane, there was also no significant reduction on the percentage of infected cells. As a control for the experiments, NH_4Cl, a cell permeable weak base commonly used to raise the endosomal pH, was added to the chPBMCs before or after virus adsorption. In agreement with previous literature, our result confirmed that IBV-M41 is sensitive to pre-treatment of NH_4Cl and IBV enters chPBMCs via a low pH-dependent route (Chu et al., 2006a) (Figure 3.5A). As a control, both the dynasore and the hypotonic sucrose treatment effect were confirmed by the blocking uptake of fluorescent transferrin (Tfn), a well-characterized endocytosis marker (Figure 3.5B). We also used another pharmacological inhibitor, chlorpromazine (CPZ), which prevents proper clathrin lattice assembly, to examine the role of clathrin-mediated endocytosis. However, CPZ was found to be toxic to the chPBMC, and therefore, could not be used to elicit the desired inhibition of the endocytosis (data not shown). Our data demonstrate that IBV-M41 utilizes a non-clathrin mediated, dynamin-independent, low pH-dependent entry route into chPBMCs.

Many viruses hijacked caveolaes as internalization pathway. We next examined the role of caveolae-dependent endocytosis by treating the chPBMCs with caveolar-inhibiting drugs during IBV infection. Both MBCD and nystatin bind and

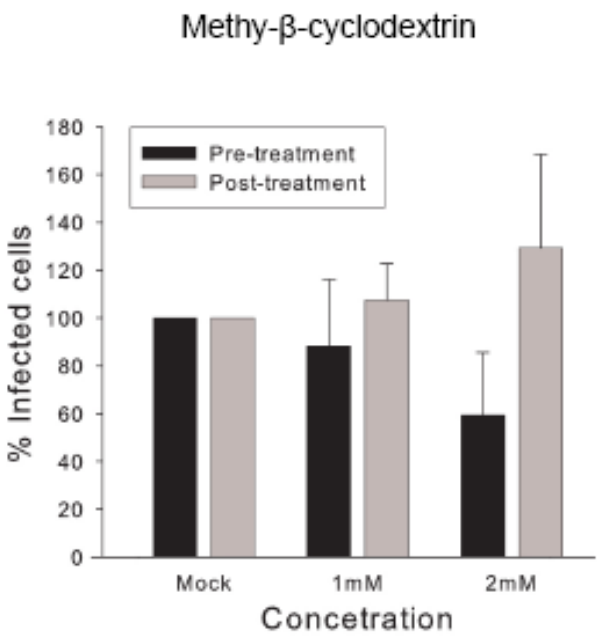
Figure 3.5. IBV-M41 entry in chPBMCs is independent of clathrin-mediated endocytosis but requires low-pH. (A) ChPBMCs were treated with indicated concentrations of dynasore, hypotonic solution or NH4Cl before infection by IBVM41. Cells were further incubated for 9 h before fixing for immunofluoresence microscopy. Post-treatment with drugs was performed 1 h post infection. (B) chPBMCs were pre-treated with dynasore or hypotonic solution. AlexaFluor 568-conjugated transferrin was bound to cells on ice for 30 minutes. Cells were allowed to warm up to 37C for transferrin uptake for 10 minutes. Cells were washed with acidic buffer 3 times and then fixed with 3% PFA. Nuclei were stained with Hoechst. Transferrin uptake was evaluated by fluorescence microscopy.


A
Dynasore
Pre-treatment
Pos-ttreatment
% Infected cells
120
100
80
60
40
20
0
Mock
20uM
40uM
80uM
Concentration
Hypotonic
Pre-treatment
Post-treatment
% Infected cells
180
160
140
120
100
80
60
40
20
0
Mock
50uM
100uM
300uM
Concentration
NH4Cl
Pre-treatment
Post-treatment
% Infected cells
120
100
80
60
40
20
0
Mock
10mM
25mM
Concentration
B
Mock
+Dynasore
+Hypotonic

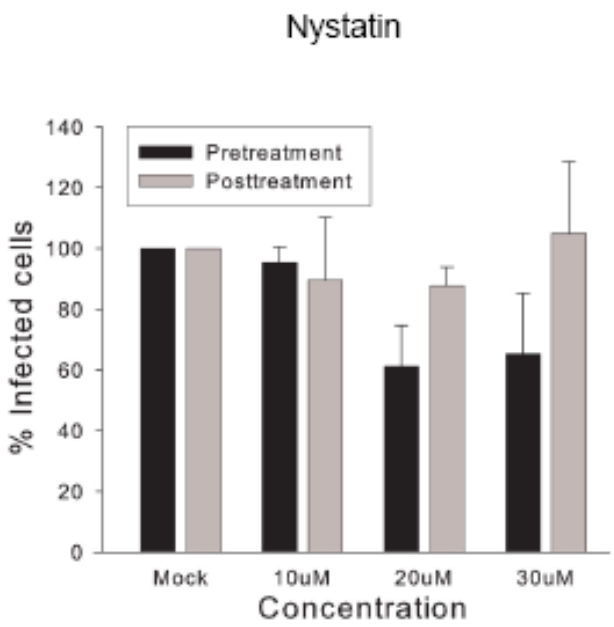
sequester cholesterol and prevents formation of caveolae. Immunofluorescence microscopy analysis revealed that the percentage of IBV infected cells reduced by increasing concentrations of MBCD treatment or nystatin treatment while the percentage of IBV infected cell in post-treatment with drug was comparable to mock treatment (Figure 3.6A, 3.6B). This reduction of infection rate indicates that cholesterol plays an important role in the IBV infection and IBV utilizes a caveolae-dependent endocytosis route in chPBMCs. Monocytes are important antigen-presenting cells in the immune surveillance. They constantly internalize foreign antigens by macropinocytosis, an actin-dependent cellular process. To examine whether macropinocytosis can be utilized by IBV to gain entry in chPBMCs, we treated the cells with EIPA, a macropinocytosis inhibitor that Inhibits Na^+/H^+ exchanger activity, during IBV infection. At 50uM concentration of EIPA treatment, the percentage of cells was reduced to 50% of percentage of infected cells of mock treatment (Figure 3.6C). To confirm the usage of caveolae-dependent pathway and macropinocytosis, which are actin-dependent pathways, cytochalasin D, an actin polymerization inhibitor, was used to treat the cells during IBV infection. The percentage of IBV-infected cells reduced to approximately 50% at the highest concentration of cytochalasin D used in our study (Figure 3.6D). In addition, we observed that cells treated with MBCD, nystatin, EIPA and cytochalasin D after infection of IBV had a higher percentage of infection comparing to mock treatment. The apparent increased infectivity may be due to induction of paralleled endocytic pathways. Like many other viruses, IBV does not enter host cells in multiple endocytici pathways. Taken together, our data suggest that IBV does not enter chPBMCs through classical clathrin-mediated endocytosis but utilizes caveolae-mediated

Figure 3.6. IBV-M41 entry in chPBMC requires cholesterol and by macropinocytosis. ChPBMCs were treated with indicated concentrations of methy-beta-cyclodextrin (A), nystatin (B), EIPA (C), or Cytochalasin D (D) before infection by IBVM41. Cells were further incubated for 9 h before fixing for immunofluoresence microscopy. Post-treatment with drugs was performed 1 h post infection. The bar graphs represent quantification of immunofluorescence microscopy images from three independent experiments.

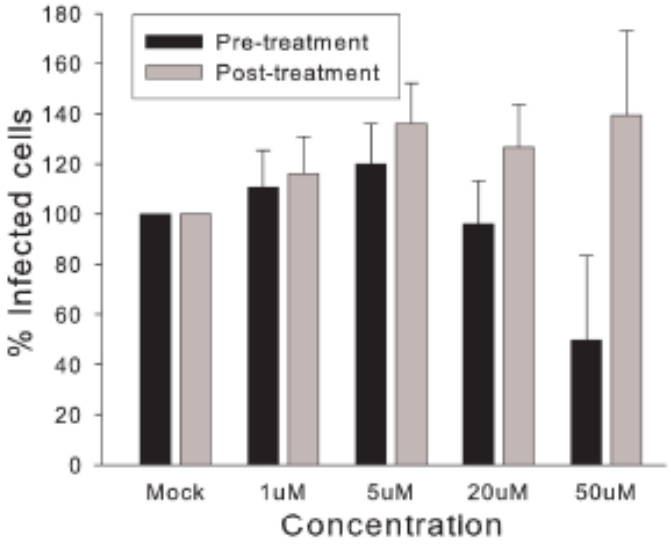
A


Methy-β-cyclodextrin
Pre-treatment
Post-treatment
% Infected cells
180
160
140
120
100
80
60
40
20
0
Mock
1mM
2mM
Concetration

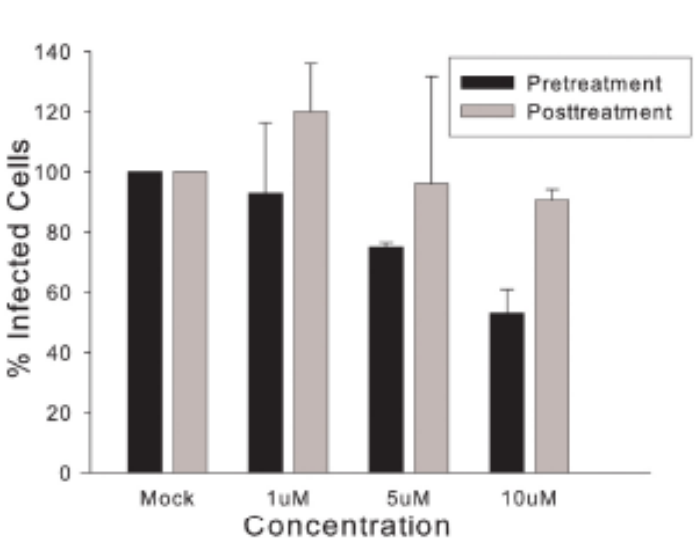
B


Nystatin
Pretreatment
Posttreatment
% Infected cells
140
120
100
80
60
40
20
0
Mock
10uM
20uM
30uM
Concentration

C


EIPA
Pre-treatment
Post-treatment
% Infected cells
180
160
140
120
100
80
60
40
20
0
Mock
1uM
5uM
20uM
50uM
Concentration

D


Cytochalasin D
Pretreatment
Posttreatment
% Infected Cells
140
120
100
80
60
40
20
0
Mock
1uM
5uM
10uM
Concentration

endocytosis as well as macropinocytosis as a way to enter chPBMCs.

3.5 Discussion

IBV mainly replicates in the epithelial cells of the respiratory tract, but also in the epithelial cells of other organs such as kidneys, oviducts and alimentary tract, as evidenced by immunohistochemistry of tissue sections from infected chickens (Dolz et al., 2011). Yet little information on IBV replication in different tissues and cell types is available. Our study demonstrates that IBV is able to replicate to high titer (10^8 EID_{50}/mL) in chicken peripheral blood-derived monocytes/macrophages (chPMBCs), cells that are imperative for immune surveillance and play a key role in the immune response to viral infection. It is known that monocytes originate from the bone-marrow, and then circulate in the blood before differentiating into macrophages, dendritic cells, or osteoclasts (Gordon and Taylor, 2005). The chPBMCs population that we used in this study represents the precursors for macrophages and dendritic cells, and may have heterogeneous immunological functions and various levels of susceptibility to IBV. Our data show that infection of IBV in chPBMCs follows typical viral growth kinetics, with fast growth of the virus, peaks at approximately 12 hr post infection, eventually causing cytopathic effect as observed for IBV in other cell types such as chicken kidney cells (Saif, 2003a).

IBV field strains such as Massachusetts 41 (M41), California 99 (Cal99), Connecticut 46 (Conn 46) are usually cultivated in chicken embryos, while primary chicken kidney cells remain the only option for virus infection and propagation in cell culture. No established cell lines allow propagation of clinical strains of IBV (Saif,

2003a). The lack of knowledge in IBV receptor leaves a big gap in our understanding of the relatively wide chicken tissue tropism and strict species tropism of IBV. However the broad tissue tropism of IBV *in vivo* has been suggested to be related to the distribution of the attachment factor sialic acid (Wickramasinghe et al., 2011), especially in the case of the infection of the epithelium of various organs. However, we show here that infection of chPMBCs is sialic acid-independent. The interaction of IBV and cellular sialic acid remains puzzling: first, IBV does not possess a viral component that destroys sialic acid/receptor as for some Betacoronaviruses and all Toroviruses (de Groot, 2006), and second, from the IBV glycan array data published by Wickramsinghe et al, the avidity of IBV spike protein binding to sialic acid is relatively low compared to influenza virus (Wickramasinghe et al., 2011). We speculate that the contribution of sialic acid-IBV spike interaction may be secondary for IBV entry, under the assumption that an authentic receptor as-yet unidentified, is the primary component in the IBV spike protein-receptor complex. The discrepancy between the roles of sialic acid for IBV infection in epithelial cells and in chPBMCs can be one feature of the intricate pathogenesis of IBV in the host. Our findings on the dispensable role of sialic acid in the IBV infection on chPBMCs reveal a more complex virus-receptor/host factor interaction than previously thought.

DC-SIGN, a widely-expressed C-type lectin receptor on immune cells, has been proposed to be an attachment factor for many enveloped viruses, by interacting with the viral glycoproteins (Svajger et al., 2011) and recent data has further established that DC-SIGN has a critical role for both the pathogenesis and interspecies transmission of enveloped virus such as bunyaviruses (Lozach et al., 2011). In the coronavirus family, human DC-SIGN and/or L-SIGN (a closely related C-type lectin) have been shown to be

able to enhance infection of SARS coronavirus, human coronavirus-229E, human coronavirus-NL63, as well as feline coronavirus viral entry (Hofmann et al., 2006; Marzi et al., 2004; Regan and Whittaker, 2008; Yang et al., 2004). It is reasonable to speculate that coronaviruses have evolved to resort to a lectin molecule as a common mechanism for systemic spread. Our previous data showed that IBV infectivity in non-permissive cell lines can be rescued by introducing human DC-SIGN or L-SIGN(Zhang et al., 2012). However, the chicken homolog of DC-SIGN remains unidentified. Interaction with receptors or attachment factors plays an important role in determining the species and tissue tropism. In our study, we show that infection of PBMCs was inhibited by EGTA, a Ca^{2+} chelating agent, indicating a role for a calcium-dependent C-type lectin. Formal proof for the involvement of a C-type lectin could not be obtained, likely due to the lack of information on specific avian species of carbohydrates that interact with IBV.

Coronavirus-induced apoptosis has been documented for a number of viruses in the family. For instance, SARS-CoV-infected patients displayed severe organ damage that has been suggested to be due to virus-induced apoptosis (Tan et al., 2007). Similar gross and microscopic lesions, as well as tissue damage of the respiratory tract and kidneys, have also been observed in IBV-infected chickens (Dolz et al., 2011). For IBV, apoptosis has been observed in Vero cell cultures infected by laboratory-adapted strain Beaudette (Liu et al., 2001). However, there is little information on IBV-induced cell death in chicken-specific cell types. In our study, chPBMCs are able to undergo apoptosis without IBV replication. However, by blocking acidification of the endosomal compartments and arresting IBV in these vesicles, apoptosis was no longer observed. We can rationalize that a fusion event in the endosomes after acidification is required for

apoptosis and that the IBV-induced apoptosis take place following virus fusion but prior to replication.

Productive infection of monocytes can potentially lead to dissemination of virus to distal organs, causing a systemic infection. Programmed cell death can halt the productive infection of the monocytes, and can be viewed as a host defense mechanism by lowering the viral load in the blood in order to reduce the potential systemic spread of viral infection. However, IBV may also utilize apoptosis of monocytes as a strategy to evade surveillance that could initiate an immune response against IBV infection. Even with a sub-optimal productivity due to premature cell death, it is nevertheless beneficial for IBV to spread from the primary site of infection to other organs. Our data suggest that IBV infection of peripheral blood derived monocytes/macrophages might be an important component in the pathogenesis of IBV in the chicken host and might be critical for viral dissemination through the blood to distal organs *in vivo*.

3.6 Acknowledgements

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CHAPTER 4

Influenza entry pathways in polarized MDCK II cells

Yueting Zhang and Gary R. Whittaker

4.1 Abstract

In non-polarized cell culture model, influenza virus enters host cells via multiple endocytic pathways: classical clathri-mediated endocytotic (CME) route, clathrin-, caveolae-, independent routes, and macropinocytosis. However, little is known about the entry route of influenza virus in differentiated epithelia, *in vivo* site of infection for influenza virus. Here we show that in polarized Madin Darby canine kidney type II (MDCK II) cells, influenza virus has a differential utilization for CME pathway and requires Eps15 protein for entry.

4.2 Introduction, results and discussion

Most animal viruses take advantage of the diverse cellular endocytic pathways to gain access to the host cells (Sieczkarski and Whittaker, 2002a). After being endocytosed, viruses can initiate replication once they penetrate or fuse with the endosomal membrane (Marsh and Pelchen-Matthews, 2000). A role of dynamin-dependent endocytosis of influenza virus entry was demonstrated by examining the effect of dominant-negative (DN) dynamin, a small GTPase mediating the scission of clathrin-coated vesicles from plasma membrane, on influenza virus entry in Mv-1 lung cells (Roy et al., 2000). Studies from our group showed that the infectivity of influenza viruses in cells expressing a DN mutant of Eps15 (Epidermal growth factor receptor pathway substrate), which inhibits clathrin-dependent endocytosis specifically, was not impaired (Sieczkarski and Whittaker, 2002b). When chemical inhibitors as well as DN mutant of caveolin-1 were used to disrupt caveolae-dependent endocytosis in host cells, influenza infectivity was retained as that in untreated cells (Sieczkarski and Whittaker, 2002b). These observations lead to the discovery of non-clathrin-dependent, non-caveolae-

dependent endocytic pathways for influenza virus entry in addition to classical clathrin-dependent pathway. Recently, with detailed dissection of influenza entry pathways independent of dynamin using pharmacological inhibitors, de Vries et al. discovered that influenza virus can utilize a macropinocytosis-like route in many cell types (de Vries et al., 2011). In the recent advancements of super-resolution fluorescent microscopy, individual influenza particle can be tracked in real time without disruption of endocytic pathways, a novel technique in discovering redundant or parallel endocytic pathways. With the new technique, Epsin1 (Epn1), an adaptor protein that interacts with clathrin, adaptors AP2, and Eps15 in clathrin-coated pits, is demonstrated to be an influenza cargo specific adaptor for entry via CME pathway in BSC-1 cells (Chen and Zhuang, 2008). Examining the dynamics of the endocytic uptake also leads to the conclusions that influenza viruses exploit different pathways with the same efficiency and those non-classical, less-characterized pathways do not act as alternative pathways for influenza virus entry.

Polarized, simple epithelial cells have a plasma membrane that is separated by tight junctions into two clearly distinct domains: the apical domain facing the tract lumen and the basolateral surface facing the extracellular matrix. Actins, microtubules, as well as an array of cellular proteins participate in the organization and maintenance of cell polarity. It is well recognized that influenza enters and buds from apical domain of MDCK cells (Rodriguez Boulan and Sabatini, 1978). Previous study from our laboratory demonstrated that actin microfilaments play different roles in influenza virus infection in polarized epithelial cells and non-polarized cells (Sun and Whittaker, 2007). In contrast to their dispensable role in viral infection of non-polarized cells, intact actin filaments are

obligatory for influenza virus infection in polarized epithelial cells. Since there are significant differences between polarized and non-polarized cells with regard to receptor distribution, cytoskeletal structure, trafficking events, and mechanism of endocytosis, it is possible that our current knowledge of influenza virus entry in non-polarized cells such as HeLa, MDCK, BSC-1 cells, does not completely apply to *in vivo* viral infection, which is initiated at the differentiated airway epithelial cells. This is the first study that examined influenza virus entry pathways by pharmacological inhibitors and DN mutant proteins in fully polarized MDCK II cells, a robust model for differentiated epithelia (Mostov et al., 2003).

In order to obtain polarized MDCK II epithelial cell culture, MDCK II cells (provided by Dr. Colin Parrish, Cornell University) were grown in DMEM media supplemented with 10% fetal bovine serum and 1% Penicillin and Streptomycin(Cellgro) on 0.4μm semi-permeable Transwell filters (Corning). The semi-permeable filter supports allowed nutrients to reach both apical domain and basal domain of the cells. Since care must be taken to maintain a true polarized epithelium, polarity was monitored by measuring the transepithelial electrical resistance (TEER) of the monolayer cultivated on the semi-permeable filter. We used an EVOMX meter along with the electrodes for cell culture inserts (World Precise Instruments). Before measurement, culture media was changed to fresh warm media for all filter inserts. After being confluent for 3 to 4 days on Transwell filter, MDCK II reached an average TEER of 230 ohms.cm^2 (Figure 4.1.), which was consistent with observations in literature (Shaw, 2002). MDCK cells (ATCC) that displayed both fibroblast-like and epithelia-like morphology were used as control due to their inability to form tight monolayer. Indeed, the measured TEER of MDCK

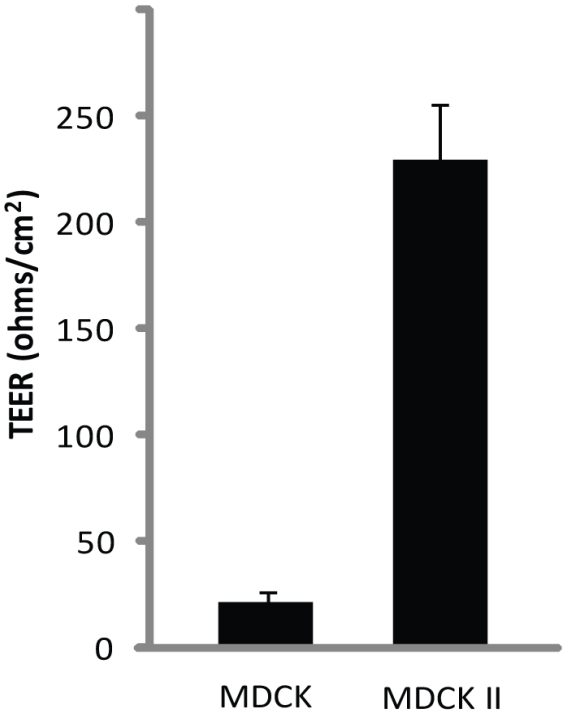
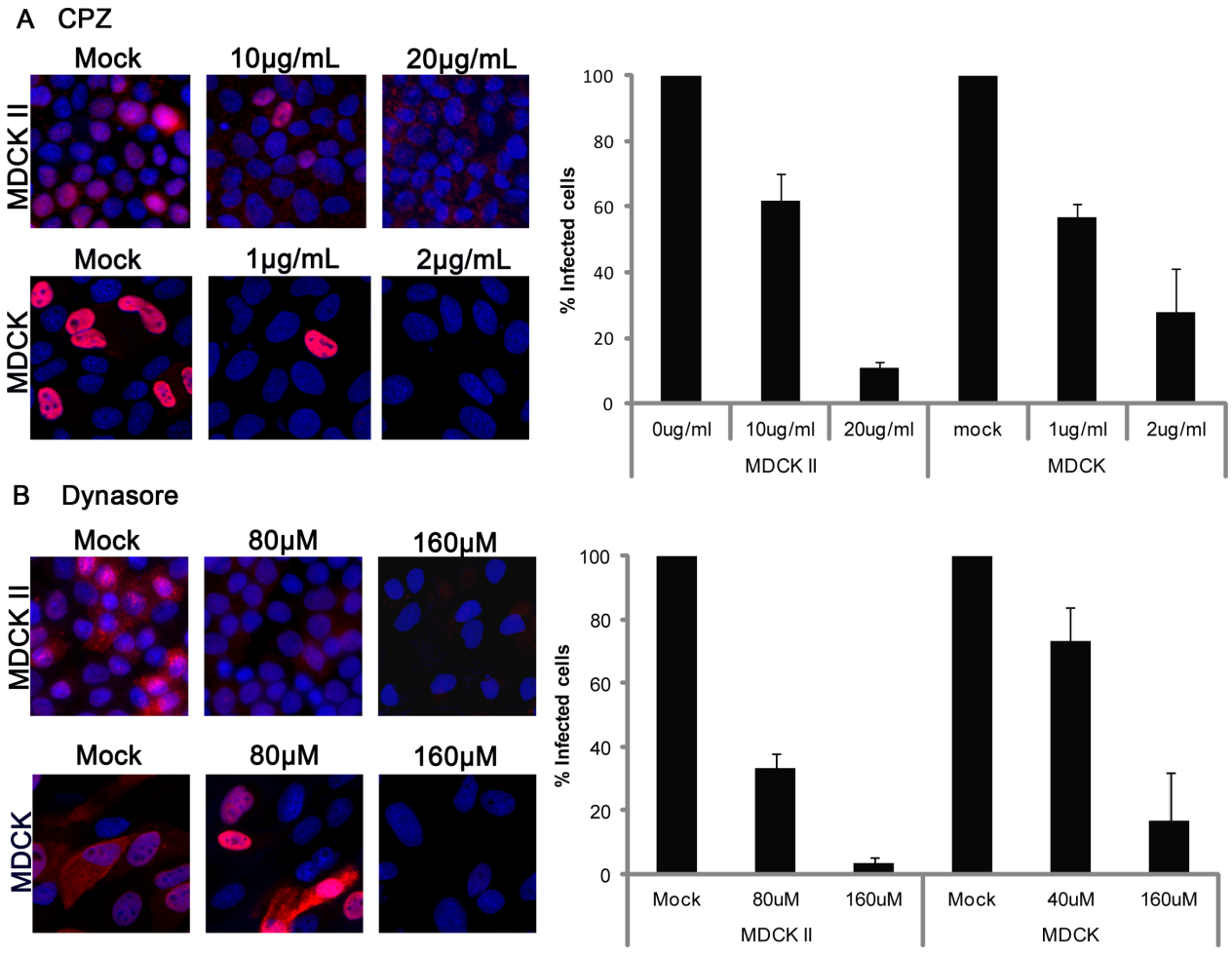


Figure.4.1. TEER measurements for non-polarized MDCK cells or polarized MDCK II cells in this study. MDCK cells or MDCK II cells were grown on 0.4μm semi-permeable Transwell filters. TEER of both cell types was measured 3-4 days after reaching 100% confluency.

cells grown under the same culture conditions of MDCK II cells on Transwell filters was 10-fold lower than the TEER of those MDCK II cells. (Figure 4.1.)

To examine the CME pathway, the dominant route used by influenza virus in non-polarized cell system, we first utilized pharmacological inhibitors that target the CME pathways in host cell. Chlorpromazine (CPZ), which prevents clathrin assembly, has been widely used to block CME including the study on the entry mechanism of influenza viruses in non-polarized cells (Sieczkarski and Whittaker, 2002a). Dynasore has inhibitory effect on GTPase activity of dynamin with low cell toxicity (Macia et al., 2006). Since the discovery of Dynasore, it has been widely used as specific dynamin inhibitor for various virus entry studies such as those of VSV, densovirus, HPV type 16 (Johannsdottir et al., 2009; Schelhaas et al., 2008; Vendeville et al., 2009). CPZ or dynasore were used to treat fully polarized MDCK II cells and non-polarized MDCK cells for 30 min before infection with influenza virus A/WSN/33 (H1N1), which was propagated and titered by plaque assay in MDCK cells. The inhibitor-treated cells were then incubated with influenza virus at an MOI of 1 (MDCK) or an MOI of 5 (MDCK II) for 1 hour at 37˚C in the presence of inhibitors. After virus adsorption, the inoculum was replaced with fresh media (DMEM supplemented with 2% fetal bovine serum) and inhibitor for the incubation duration. At 5 h post infection, cells were fixed with 3% paraformaldehyde (PFA) and stained with mouse anti-nucleoprotein (NP) antibody (H16, L10-4R5, ATCC) and AlexaFluor 568 secondary antibody against mouse (Molecular Probes) for immunofluorescence microscopy. MDCK cells were more sensitive to the toxicity of CPZ than MDCK II cells; thus lower concentrations of CPZ were used to treat MDCK cells. The quantity of inhibitors used in the treatment did not affect cell viability

Figure 4.2. Differential utilization of clathrin-mediated endocytosis pathway by influenza virus in non-polarized MDCK cells and polarized MDCK II cells. MDCK cells or MDCK II cells were pretreated with different concentrations of CPZ(A) or Dynasore (B) for 30 minutes at 37°C prior to infection with influenza virus at an MOI of 1 (MDCK) or an MOI of 5 (MDCK II). At 5h post infection, cells were stained with mouse anti-NP antibody and AlexaFluor 568 secondary antibody against mouse for immunofluorescence microscopy. NP-positive cells were quantified from images from three independent experiments. Error bar represents standard deviation of the mean. Viral infectivity in MDCK cells or MDCK II cells with mock treatments was normalized to 100%. (C) MDCK cells or MDCK II cells were infected with influenza virus after pretreatment with CPZ or Dynasore (DNS). Viral protein M from whole cell lystate was analyzed by western blot with mouse anti-M1. Tubulin (Tub) was used as loading control.


A CPZ
Mock
10μg/mL
20μg/mL
MDCK II
Mock
1μg/mL
2μg/mL
MDCK
% Infected cells
100
80
60
40
20
0
0ug/ml
10ug/ml
20ug/ml
mock
1ug/ml
2ug/ml
MDCK II
MDCK
B Dynasore
Mock
80μM
160μM
MDCK II
Mock
80μM
160μM
MDCK
% Infected cells
100
80
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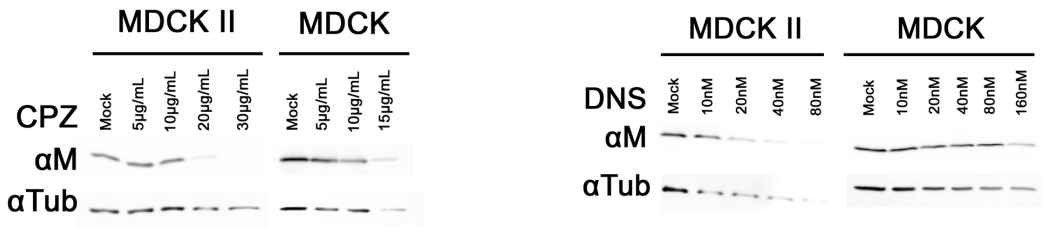
MDCK II
MDCK
CPZ
Mock
5μg/mL
10μg/mL
20μg/mL
30μg/mL
Mock
5μg/mL
10μg/mL
15μg/mL
αM
αTub
MDCK II
MDCK
DNS
Mock
10nM
20nM
40nM
80nM
Mock
10nM
20nM
40nM
80nM
160nM
αM
αTub

(data not shown). Treatment with CPZ on both MDCK and MDCK II caused a substantial reduction on the number of NP expressing cells after influenza infection (Figure 4.21A.) However, the Dynasore treatment had a more obvious impact on polarized MDCK II cells than their non-polarized counterpart (Figure 4.2B.). This evidence indicates a potential role of dynamin in the entry pathway of polarized MDCK II cells, while non-polarized MDCK does not depend on the dynamin usage for entry. The effect of CPZ and dynamin on the canine kidney cells were further confirmed by western blot with infected whole cell lysate with an antibody against viral Matrix protein (M2-1C6-4R3, ATCC) (Figure 4.2C).

The pharmacological approaches mentioned above indicate an important role of CME in influenza virus infection in both polarized MDCK II cells and non-polarized MDCK cells. However, we wished to examine specific components of the CME pathway. Conventional transfection methods with cationic lipid or adenovirus transduction are either of low transfection efficiency or not ideal for our study. To overcome the difficulty of recombinant protein expression, we used electroporation on monolayer cells as described in (Deora et al., 2007). 30 μg/ml of plasmid DNA was mixed with electroporation buffer (Eppendorf) and was delivered using an electrode (CUY512-5, Nepagene) connected to ECM 830 electroporation system (BTX Instrument). We electroporated wild type (WT) or dominant negative (DN) constructs of Dynamin 2 (Figure 4.3A), Eps15 (Figure 4.3B), or Epn1 (Figure 4.3C) into fully polarized MDCK II cells and infected MDCK II cells with influenza virus at a MOI of 5 after 18 h post electroporation (Table 4.1). Cells were then fixed and stained for confocal microscopy after 5 h of infection. Expression of DN form of Dynamin 2 or Epn1 had reduced

Table 4.1. List of plasmid constructs used

Protein	Construct	Source
Dynamin 2	Dynamin2-GFP (WT) Dynamin2-GFP-K44A (DN)	Dr. Mark McNiven, Mayo Clinic Cancer Center
Eps15	Eps15-DIIIΔ2-GFP (WT) Eps15-Δ95/295-GFP (DN)	Dr. Jennifer Lippincott-Schwartz, NIH
Epsin1 (Epn1)	Epsin1-ECFP Epsin1ΔUIM-ECFP	Dr. Xiaowei Zhuang, Harvard University
Caveolin 1	Caveolin1-GFP (WT) Caveolin1-GFP (DN)	Dr. Ari Helenius, ETH Zürich

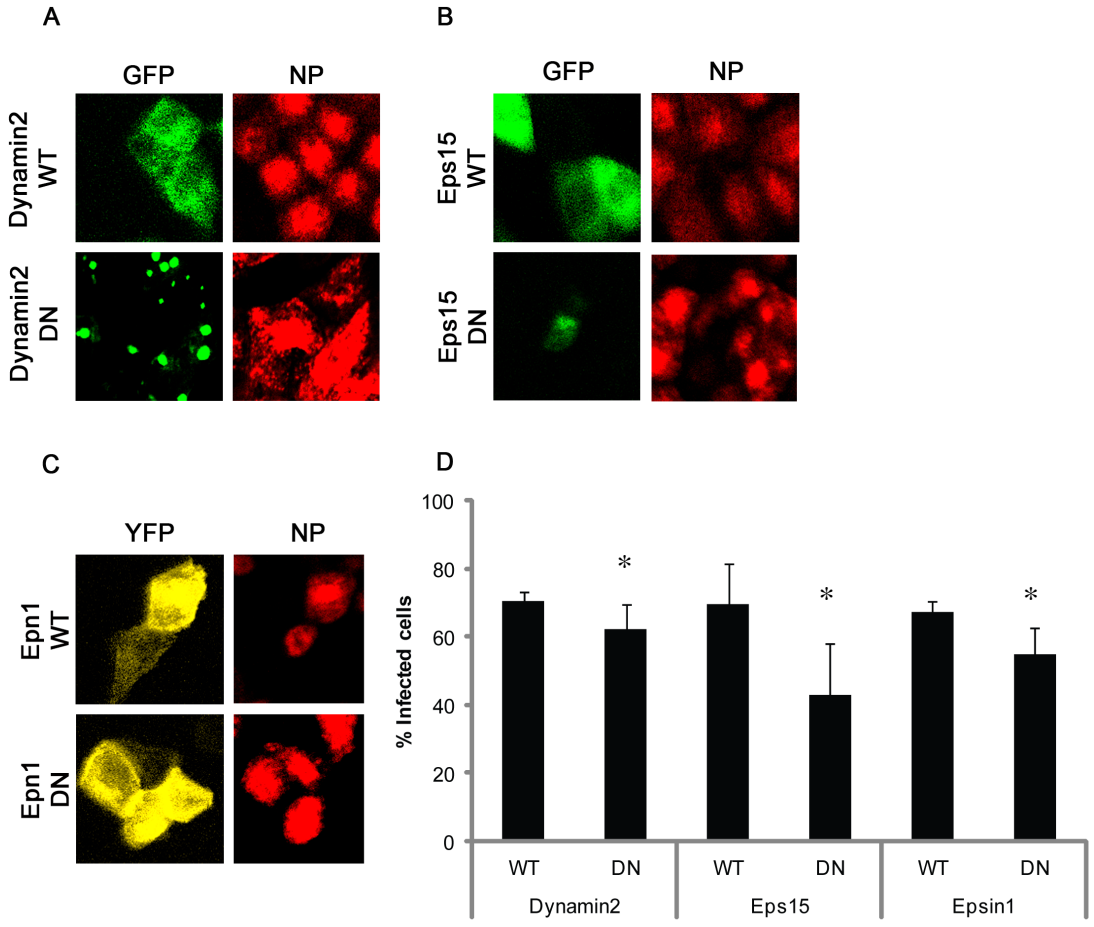

Figure 4.3. Eps15 is required for influenza virus infection on polarized MDCK II cells. MDCK II cells were polarized on Transwell filters for 3-4 days and then were electroporated with either wild type (WT) or dominant negative (DN) constructs of Dynamin2 (A), Eps15 (B), or Epsin1 (C). 18 h post electroporation, cells were infected with influenza virus at MOI of 10. At 5 h post infection, cells were fixed and stained with mouse anti-NP antibody for confocal microscopy. (D) Both transfected and infected cells were scored and quantified from images from three independent experiments. Student's t test was used to calculate the statistical significance between differences of % infected cells in cells expressing WT and DN proteins. * indicates $p<0.05$.

percentage of GFP or EYFP expressing cells that were positive for viral NP expression, comparing to the control WT version of the respective construct (Figure 4.3D). However, unlike in non-polarized cell such as HeLa cells described in previous study (Sieczkarski and Whittaker, 2002b), Eps15-Δ95/295-GFP DN construct expression in MDCK II cells greatly reduced the percentage of cells infected with influenza virus and expressing viral NP, comparing to its WT counterpart Eps15-DIIIΔ2-GFP expressing cells.

Recently influenza virus has been shown to be able to enter host cell via macropincytosis pathway (de Vries et al., 2011; Rossman et al., 2012). To examine whether influenza virus can also utilize macropinocytosis in polarized MDCK II cells, we used 5-ethylisopropyl amiloride (EIPA; Sigma), a macropinocytosis inhibitor that Inhibits Na^+/H^+ exchanger activity to treat the canine kidney cells before infecting with influenza virus (Mercer and Helenius, 2009). By examination of viral protein M1 in whole cell lysate using western blot analysis, we found that neither MDCK cells nor MDCK II cells were significantly inhibited by EIPA, regardless of drug treatment concentrations (Figure 4.4). Our data suggest that similar to non-polarized MDCK cells, there exist redundant entry pathways (presumably CME) in polarized MDCK cells and macropinocytosis is not a preferential major pathway utilized by influenza virus.

In non-polarized cell system, influenza has been shown to enter host cells via caveolae-independent entry routes (Sieczkarski and Whittaker, 2002b). To examine whether influenza virus enters polarized MDCK II cells via caveolae-independent entry pathway as in previous studies on non-polarized cells, we first treated the MDCK or MDCK II cells with different concentrations of caveolae-inhibiting drugs such as Methyl-β-cyclodextrin (MBCD) and nystatin. As revealed by expression of viral protein M1 on

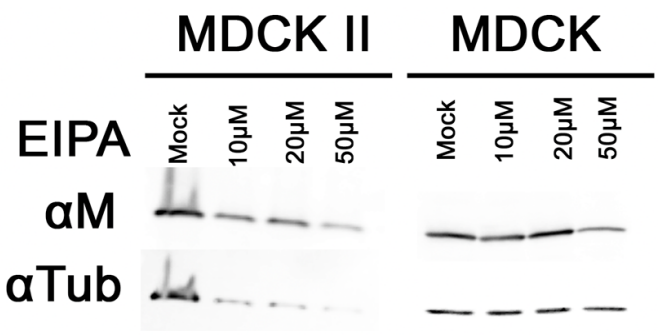

Figure 4.4. Macropinocytosis inhibitor does not affect influenza virus infection in MDCK II cells. Polarized MDCK II cells were treated with EIPA before infection with influenza virus.Viral protein M from whole cell lystate was analyzed by western blot with mouse anti-M1. Tubulin (Tub) was used as loading control.

western blot, treatment with high concentrations of MBCD was able to inhibit both polarized MDCK II cells and non-polarized MDCK cells (Figure 4.5A). In contrast, nystatin did not have an inhibitory effect on either cell type. To examine the caveolar-mediated pathway more specifically in polarized MDCK II cells, we electroporated GFP-tagged Caveolin-1 WT or DN plasmids into polarized MDCK II cells before infection with influenza virus. Caveolin-1 is a major structural protein in the caveolae. Using confocal microscopy, both Cav1 WT and Cav1 DN expressing cells were able to be infected by influenza virus as shown by the positive signal of viral NP in the transfected cells (Figure 4.5C, 4.5D). The discrepancy in influenza virus infectivity in MDCK II cell after treatment with MBCD and with nystatin may be due to the discrete modes of action by the two drugs: MBCD inhibition on influenza virus entry in both MDCK and MDCK II cells was not by inhibiting caveolae-dependent endocytosis , by altering the structure of cholesterol-rich domain in plasma membrane. A similar observation was also described in SARS-coronavirus entry studies (Li et al., 2007b; Wang et al., 2008). Taken together, our data demonstrated that, similar to non-polarized MDCK cells, influenza virus does not enter polarized MDCK II through caveolae-dependent endocytosis.

To our knowledge, this is the first time that endocytic pathways employed by influenza virus in a fully polarized epithelial cell system grown on Transwell filter were examined. Polarized epithelial cells are usually refractory to transfections and more difficult to work with compared to non-polarized cells. Components in the CME pathway have shown to be regulators of cell polarity (Deborde et al., 2008; Shivas et al., 2010). To avoid DN cellular proteins disrupting formation of proper cellular polarity, we chose to introduce the DN constructs to the MDCK II cells after the cells formed a tight

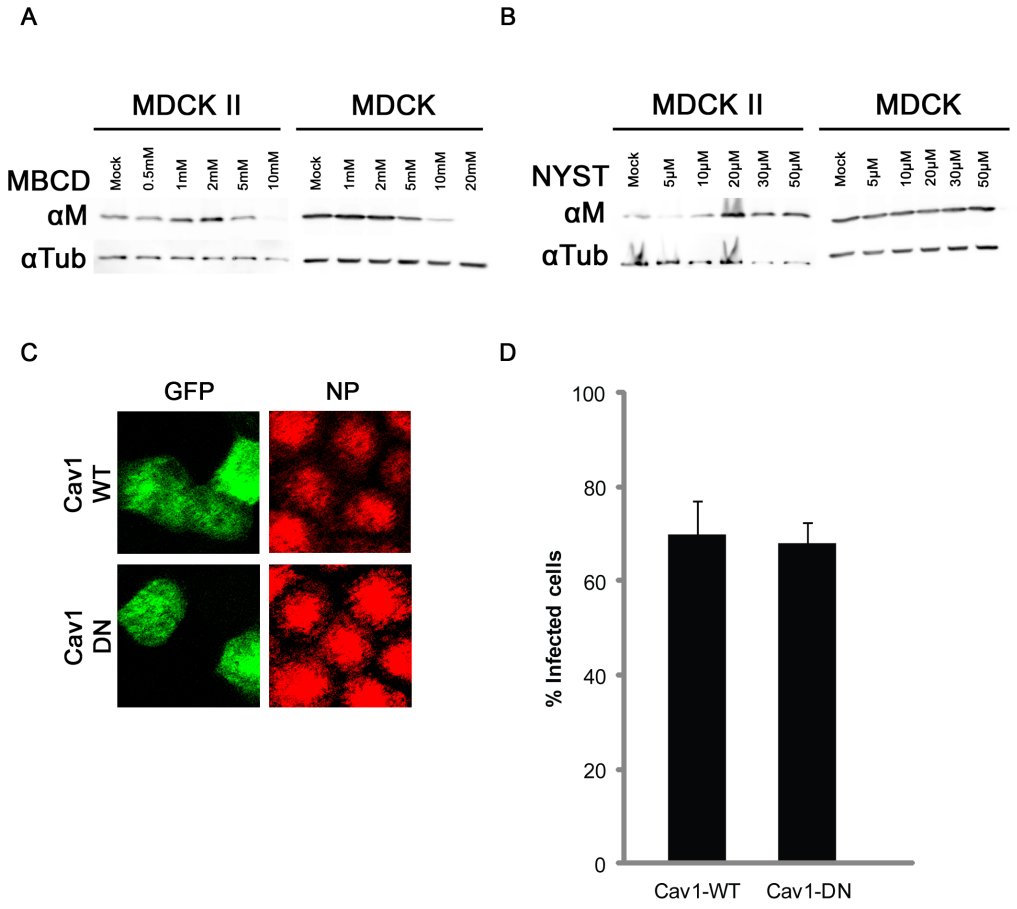

Figure 4.5. Influenza virus does not enter polarized MDCK II cells via caveolae-mediated endocytosis. MDCK cells or MDCK II cells were either pretreated with MBCD (A) or nystatin (B) for 30 min before infected with influenza virus. Viral protein M was analyzed with mouse anti-M1 antibody on western blot from infected whole cell lysates. (C) Polarized MDCK II cells were infected with influenza virus after electroporation with Caveolin-1 (Cav1) WT plasmid or Cav1-DN plasmid and were analyzed with confocal microscopy with mouse anti-NP antibody. (D) Both transfected and infected cells were quantified from confocal images from three independent experiments.

monolayer and were fully polarized. Conventional transfection method utilizing cationic lipid often yields low transfection efficiency in polarized epithelial cells (data not shown). Adenovirus transduction to introduce recombinant protein into polarized epithelia is another frequently used method. However, adenovirus can induce macropinocytosis and other undesirable signaling events after gaining access into host cell (Amstutz et al., 2008; Hayashi and Hogg, 2007; Sirena et al., 2004; Wickham et al., 1993).

Dynamin2 and Epn1 DN proteins had a small reduction on the percentage of MDCK II cells infected with influenza virus. This may be due to a masking effect by other endocytic pathways that the virus particles enter through while CME pathway was inhibited (at least partially) by Dynamin 2 or Epn1 DN proteins. Unexpectedly, a large portion of polarized MDCK II cells expressing Eps15 DN were not able to be infected by influenza virus (Figure 4.3D) Eps15 is an adaptor protein that interacts with Epsin1, AP180, and synaptojanin via the Eps homology (EH) domains and recruits cargo adaptor protein AP-2 to plasma membrane (Salcini et al., 1997). The inhibitory effect of Eps15-Δ95/295 DN protein on influenza virus entry in polarized MDCK II cells suggests that Eps15 plays an important role for influenza virus entry at CME pathway. The requirement of functional Eps15 for influenza virus at clathrin-coated pit could involve a different adaptor protein that is associated with Eps15.

In non-polarized cell system, it is well documented that influenza virus enters host cells through CME (Chen and Zhuang, 2008; Sieczkarski and Whittaker, 2002b). Other entry routes such as clathrin, caveolae-independent routes, and macropinocytosis have also been described (de Vries et al., 2011; Sieczkarski and Whittaker, 2002b). In addition,

influenza infectious entry utilized different endocytic routes in different non-polarized epithelial cell types(De Conto et al., 2010). By using pharmacological and molecular approaches, our study demonstrated that influenza virus enters polarized MDCK II cells preferentially via clathrin-dependent, caveolae-independent route. The utilization of endocytic pathway was similar to the route observed in non-polarized MDCK cells. Under the treatment of CME inhibitors such as CPZ and dynasore, there was a portion of MDCK II cells that allowed influenza virus gains entry to the cells (Figure 4.2A, 4.2B). In addition, we observed that a significant percentage of these cells expressed both Eps15 DN and viral NP (Figure 4.2D), indicating that the influenza virus was able to overcome the effect of dysfunctional Eps15 protein in polarized MDCK II cells. These data implied that there exist other endocytic pathways that are utilized by influenza virus to gain entry into the polarized cells.

4.3 Acknowledgement

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CHAPTER 5

Summary and conclusions

A typical enveloped virus life cycle is initiated with binding to a receptor or attachment factor on the host cell surface. Then the virus particle is internalized by a cell-driven process-endocytosis into cellular compartments such as endosomes. Upon the acidification of the endosome, this enveloped virus can fuse its viral membrane with host vesicle membranes. The genetic material, e.g. viral RNA complexes, can be deposited into the cytosol for subsequent viral replication. My thesis research, which studied IBV and influenza virus, has focused on the first two steps of the viral entry processes: attachment and endocytosis.

Attachment: receptor determinants of IBV

Attachment of virus to host cells and receptor determinants of the prototype avian coronavirus IBV in non-permissive and permissive cells were investigated in this thesis. Even though the putative receptor for IBV is not identified, sialic acid, a terminal sugar on glycoproteins or glycolipids, is proposed to be an attachment factor for IBV (Winter et al., 2006). Intriguingly, IBV is able to infect many different tissues or organs in the host chicken; yet IBV is unable to infect any cells from other animal species than chicken. The sialic acid binding pattern in multiple chicken organs is implicated in pathogenesis in the host, but the correlation of binding cannot explain the limited susceptibility of IBV to non-chicken cells since sialic acid is ubiquitously expressed in animal tissues (Wickramasinghe et al., 2011). The lack of knowledge of IBV receptor leaves a big gap in our understanding of the relatively wide chicken tissue tropism and strict species tropism of IBV. When DC-SIGN, a human C-type lectin that may resemble a chicken

homolog lectin, was expressed by either stable transfection or transient transfection in mammalian cell lines, I found that IBV infection was rescued in these cell lines, suggesting a potential role for C-type lectin as receptor or attachment factor for IBV. After neuraminidase treatment, which removed most of the sialic acid from the cell surface, I also demonstrated that the C-type lectin rescue of IBV infection was not dependent on the level of sialic acid on the cell surface. In addition, I examined whether sialic acid also plays a role in IBV infection in chicken PBMCs, host cells that potentially harbor the authentic proteinaceous receptor for IBV. Unexpectedly, I found that the established attachment factor sialic acid did not play a critical role in the IBV infection in PBMC. Overall, the data suggest that, sialic acid may be dispensable or may play a lesser role in virus-receptor complex formation in the presence of a potential high-affinity receptor molecule e.g. DC-SIGN.

The IBV virus-receptor complex is more intricate than previously thought. Based on my data and previous published data from the literature, there can be several possible scenarios for the interaction of IBV virus-receptor complex, assuming there exists an unknown proteinaceous receptor for IBV (Chu et al., 2007; Wickramasinghe et al., 2011). In the respiratory, alimentary, and reproductive tract, IBV may rely heavily on the attachment factor sialic acid to enter host epithelial cells. This hypothesis is supported by the evidence in a report published by Winter and coworkers as well as my data that IBV is not able to enter epithelial cells after sialic acid was removed by neuraminidase (Winter et al., 2006). However, in cells (non-epithelial, possibly immune cells) expressing C-type lectins that are similar to human DC-SIGN or L-SIGN, IBV may be able to enter without the help of sialic acid and C-type lectins can be the sole receptor

allowing IBV entry. The third scenario is the one that was observed in chicken monocytes: an unknown proteinaceous receptor is highly expressed in chicken monocytes and sialic acid plays a minor role in the virus-receptor complex.

The discovery of the capacity of a C-type lectin to allow IBV infection in non-avian cells may shed light on future research on IBV receptor studies. However, the authentic host receptor for IBV is yet to be determined. My studies on the C-type lectin rescue of IBV infection in non-permissive mammalian cells suggested that C-type lectin could be a promising candidate for IBV receptor. C-type lectin serves as a receptor or an attachment factor for numerous animal viruses, including viruses in the coronavirus family (Han et al., 2007; Regan and Whittaker, 2008). However, my attempt to identify a chicken homolog of human DC-SIGN or L-SIGN has not been successful and it is unclear what chicken molecule that resembles the said C-type lectin can allow IBV entry in chicken cells. Initially, a BLAST analysis with human, monkey, dog DC-SIGN amino acid sequence in the chicken genome database yielded several potential candidates. The most promising candidate -chicken hepatic lectin (CHL) was cloned into the same expression vector as human DC-SIGN (pCDNA 3.1) and was expressed in non-permissive cells (3T3 cells). However, IBV infection could not be rescued in these cells. Although CHL was considered structurally as a chicken equivalent of DC-SIGN of human and other species (Lin et al., 2009), the carbohydrate binding specificity of CHL and DC-SIGN are different: DC-SIGN binds to high mannose and/or fucose residues on glycoproteins (Guo et al., 2004) while CHL is specific for terminal N-acetyl-glucosamine on glycoproteins (Kawasaki and Ashwell, 1977). Since the C-type lectin interaction with IBV may be due to the binding of a carbohydrate recognition domain of the lectin to the

sugar moiety on the spike protein, the discrepancy between the binding specificities of two C-type lectins could be the reason why CHL fails to rescue IBV infection.

Aminopeptidase N (APN) was considered as a universal receptor for Alphacoronaviruses (Tusell et al., 2007). There are speculations that APN is a possible receptor for IBV (Miguel et al., 2002). To test whether APN can act as a receptor for IBV, chicken APN was expressed in 3T3 cells. However, over-expression of the chicken homolog of APN did not rescue infection by IBV. This suggests that IBV cannot use chicken APN either as a sole receptor or as a receptor within the receptor-virus complex.

Future directions

There were several observations during my research on IBV projects that are very interesting and may be worthwhile for follow-up. First, infection in DC-SIGN stable cell lines (such as 3T3-DCSIGN cells) was not a productive infection. In chicken kidney cells, the typical cells used for IBV infection in laboratories, IBV usually caused cytopathic effect in the infected cells (Liu et al., 2001). The kidney cells then produced infectious virions approximately 24-48 h post infection. However, no infectious virions were produced in IBV-M41 infected 3T3-DCSIGN cells 24 h post infections, although I observed cytopathic effect about 12 h post infection. I could not see any IBV infection (by immunofluorescence assay) in new cells with supernatant from infected 3T3-DCSIGN cells. Moreover, I could detect little of the viral protein N in the infected cell supernatant using western blot. The level of viral protein in the infected 3T3-DCSIGN cell supernatant was similar to that of the basal level of supernant from the infected 3T3 cells. There are a couple of possible explanations for non-productive infection in the 3T3-DCSIGN cells. Although I observed high expression of the viral protein spike in the

infected cells, it is possible that the progeny virions were associated with the infected cells and were not released into the cell culture media. To address the possibility, I used a syringe with a 26g5/8 needle to lyse the cells post infection to allow virions to dissociate from infected cells. However, the lysis with mechanical tearing did not yield any infectious virions. It is also possible that progeny virus associated with DC-SIGN molecules with high affinity thus the progeny virions remained tightly attached to the infected cell surface or cell debris. This possibility had not yet been addressed. Scanning electron microscopy of the infected 3T3-DCSIGN cell surface can be used to visualize potential virions attached to the cell surface. Other possibilities for an unknown block in assembly and budding of IBV in these murine cells cannot be ruled out. It will be very interesting to find out the requirement for IBV to replicate in mammalian cells such as 3T3 cells. IBV Beaudette, a strain that was derived from M41 and has high sequence homology to M41, is able to replicate and to produce progeny virions in Vero cells, a cell type that is deficient in interferon. It is interesting to see whether interferon can play a hindering factor for IBV M41 to produce progeny virus in the murine 3T3 cells.

The second interesting observation was that IBV Beaudette could not infect chicken monocytes. To ensure the virus prep was viable, the same Beaudette that I used to infect chicken monocytes was able to infect BHK-21 (hamster cells) shown by immunofluorescence for viral protein spike. Then I performed a binding assay to further examine the infection block in the chicken monocytes. M41 or Beaudette was allowed to bind to monocyte surface on ice and then was detected by immunofluorescence and flow cytometry of viral spike protein. M41 bound to monocytes in a virus concentration dependent manner as expected. However, Beaudette was not able to bind to the

monocytes. This observation is intriguing and leads to my hypothesis that Beaudette uses a different receptor than M41. The difference in the ability of M41 and Beaudette to infect chicken monocytes may be an important factor for the drastic difference of the IBV pathogenesis between these two viruses. M41 is pathogenic in chickens while Beaudette does not cause any diseases (Saif, 2003b). The spike protein of M41 and Beaudette has 96% sequence identity. S1 subunit is responsible for receptor-binding (Cavanagh and Davis, 1986; Koch et al., 1990). Using sequence alignment of Beaudette and M41 spike protein amino acid sequences, I found that there are 27 amino acid differences between the two viruses in the S1 subunit. It is possible that by mutagenesis of unique M41 amino acid and examining the abolished binding effect on chicken monocytes we will be able to find key residues for true receptor binding.

IBV entry in chicken kidney cells may reply heavily on sialic acid attachment. The true receptor—IBV interaction, which is masked by the sialic acid-IBV interaction, may be difficult to identify. Thus, using chicken kidney cells as a source for receptor identification or isolation can be problematic since IBV uses sialic acid as attachment in chicken kidney cells (Winter et al., 2006). At the start of my research on IBV, I attempted to identify the IBV receptor by a pull-down method or a virus-overlay blot method using chicken kidney cells as a source of receptor. However, the attempts were not successful and only inconclusive results were obtained. In the future, to look for the putative proteinaceous receptor for IBV, chicken monocytes will be an excellent source for the receptor. Virus overlay blot method is an excellent way to identity virus receptor. It will be useful to remove sialic acid beforehand to reveal the interactions between virus spike and cellular receptor.

Endocytosis

The second focus of my thesis research was endocytosis of virus in host cells. The endocytosis of IBV in chicken peripheral-blood monocytes and endocytosis of influenza virus in polarized MDCK epithelial cells were investigated in this thesis. Enveloped viruses usually employ sophisticated, receptor-mediated endocytosis pathways to gain entry into host cells, although endocytosis is a normal cellular activity for uptake of nutrients. Many studies on virus entry pathways rely on continuous cell lines that are easy to cultivate and to manipulate. However, viruses can utilize distinct endocytic pathways in different cell types (Choi et al., 2005; De Conto et al., 2010; Li et al., 2007b; Pu and Zhang, 2008; Wang et al., 2008). In my work described here, endocytosis pathways were investigated in primary cells (chicken monocytes) and differentiated cells (polarized MDCK cells). This work provides an increased understanding of the uptake of coronavirus or influenza virus in host cells.

IBV endocytosis

Previous members of the Whittaker laboratory demonstrated that IBV Beaudette enters BHK-21 cells by low-pH dependent endocytic pathways (Chu et al., 2006a; Chu et al., 2006b). In my study here, using chemical inhibitors treatment, a clear description can be made of the internalization of IBV field strain M41 in host cells: primary chicken monocytes. I found that IBV did not use a clathrin-mediated endocytic pathway in monocytes since viral infection was not inhibited by treatment with dynasore or hypotonic solution. As a control, Tfn (transferrin) uptake was abolished by treatment with the clathrin-mediated endocytosis inhibitors. To further investigate the pathway that IBV

employs to enter chicken monocytes, I also used cholesterol-depleting inhibitors to treat the cells before infection with IBV. A reduction of infection of IBV in monocytes after treatment of MBCD and nystatin indicated a potential role of cholesterol in the uptake of IBV in these cells. Macropinocytosis inhibitor EIPA inhibited IBV infection in chicken monocytes. Treatment with the actin-disrupting agent cytochalasin D treatment also decreased IBV infection in monocytes, providing supporting evidence for the important role of macropinocytosis in IBV entry. It is not surprising that macropinocytosis is used as a dominant route of entry by IBV in monocyte since peripheral-blood derived monocytes constitutively internalize foreign antigens by macropinocytosis as their innate immune function (Mercer and Helenius, 2009).

Influenza endocytosis

Influenza virus entry pathways are well characterized among the enveloped viruses. However, it is unclear whether the entry pathways in cells other than non-polarized cells is similar to what was known. By using chemical inhibitor and expression of dominant negative acting proteins, I found that influenza virus enters polarized MDCK II cells predominantly by clathrin-mediated endocytosis pathway. Similar to non-polarized cells, influenza virus does not use caveolae-mediated endocytosis as an entry route in polarized MDCK II cells.

One experiment that would be interesting to append to this particular study is to examine the effect of inhibitors or dominant negative proteins with another known cellular protein or a virus that utilizes a certain endocytic pathway. Traditionally, Tfn

uptake is used as a control to assess the function of the clathrin-mediated pathway, and cholera toxin (CTX) uptake is used as a control for caveolae-mediated endocytosis. In the 3-dimensional polarized cell system, the apical domain and basolateral domain have distinct receptor distributions and endocytosis machineries, which are not taken into consideration in the flat, 2-dimensional non-polarized cell system. The uptake of Tfn and CTX takes place in the basolateral domain of polarized epithelial cells (Gottlieb et al., 1993; Torgersen et al., 2001). In contrast, influenza virus is known to infect and bud from the apical domain of polarized epithelial cells (Rodriguez Boulan and Sabatini, 1978). Evaluation of basolatoeral domain endocytosis after manipulation of the cells may not provide precise information for apical domain endocytosis. Thus, conventional uptake assays with Tfn and CTX to assess functions of endocytic pathways are not suitable for influenza virus. Well-characterized viruses such as SFV (Semliki forest virus) or VSV (vesicular stomatitis virus) are usually used as control virus for the clathrin-dependent pathway (Helenius et al., 1980; Johannsdottir et al., 2009; Sun et al., 2005). However, examination of virus entry pathways in polarized cell system is a novel study field, no information is available on endocytic pathways of "reference" viruses. Instead, I chose to compare the virus infectivity in polarized MDCK II cell to its non-polarized counterpart with same manipulations. Both polarized MDCK II cells and non-polarized MDCK cells were derived from the same origin (ATCC). The two types of MDCK cells differ in their morphology due to clonal selection (ManWarren et al., 1997; Nichols et al., 1986). In addition, cultivation of MDCK II cells in semi-permeable Transwell filter ensures proper formation of polarity (Handler, 1983).

In the experiment where dominant-negative proteins (Dyn2, Eps15, Epsin1)

implicated in the clathrin-dependent pathways were introduced into polarized MDCK II cells, I found that influenza virus infectivity was reduced. The data suggested that influenza virus used clathrin-dependent endocytosis in polarized MDCK II cells. It is clear that there is a difference in the level of reduction of influenza virus infectivity in cells expressing these DN proteins. The influenza virus entry was slightly reduced in Dyn2 DN expressing MDCK II cells. It is possible that influenza also uses a dynamin-2 independent pathway to enter the cells; thus a large portion of influenza virus can still enter cells overexpressing DN Dyn2 protein. Eps15 DN expressing MDCK II cells revealed a great reduction on influenza virus entry. This is unexpected since in non-polarized cells e.g. HeLa cells, influenza virus entry is not inhibited by Eps15 DN expression (Sieczkarski and Whittaker, 2002b). The data presented here suggest that influenza virus has a different of utilization of components in the clathrin-mediated endocytosis pathway. Influenza virus entry in polarized MDCK II cells, instead of using Epsin1 as a cargo-specific adaptor as in non-polarized cells, may depend on a different adaptor (e.g. AP-2) that is associated with Eps15, since Eps15 does not directly bind to influenza virus (Chen and Zhuang, 2008). Nevertheless, an alternative explanation of the different levels of reduction of influenza infectivity in cells expressing the DN protein of Dyn2, Eps15, Epsin1 can be the different levels of knock-down effect by the DN proteins.

MDCK cells are a robust and widely used system to study cellular polarity (Mostov et al., 2003). Polarized MDCK cells are used here as a model for the differentiated epithelial cells in the airway, where *in vivo* influenza infection occurs. My study here is an initial step to investigate the entry mechanisms of influenza virus in

polarized epithelial cells. Future studies will need to use more indicative models of *in vivo* infection. Many literature studies have focused on influenza infection on human airway epithelial cells or human bronchial/tracheal cells (Ilyushina et al., 2012a; Ilyushina et al., 2012b; Oshansky et al., 2011). The primary cells such as human bronchial cells have limited doublings in laboratory culture and are more difficult to work with than continuous cell lines such as MDCK cells. Nevertheless, these human polarized cells are usually cultured in similar conditions to MDCK II cells. The chemical and molecular approaches described in the study here can be applied to the human polarized epithelial cells to investigate the influenza entry pathways. In the past, manipulations of polarized cells especially introduction of dominant negative plasmids into fully polarized cells were problematic (Sun and Whittaker, 2007). My study here demonstrated that the protocol difficulties were overcome by using electroporation on monolayer cells. This particular method not only allows efficient expression of recombinant proteins from plasmid DNA but also is superior over other transfection methods such as nucleofection and viral transduction.

Conclusion

Although there are still many unanswered questions remaining on the topic of my thesis research, overall, my work has provided a better understanding of reeptor determinants and the entry pathways of two respiratory viruses: IBV and influenza virus.

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