JVI Journals.ASM.org

The Macrophage Galactose-Type Lectin Can Function as an Attachment and Entry Receptor for Influenza Virus

Wy Ching Ng,[a] Stella Liong,[a]* Michelle D. Tate,[a]* Tatsuro Irimura,[b] Kaori Denda-Nagai,[c] Andrew G. Brooks,[a] Sarah L. Londrigan,[a] Patrick C. Reading[a,d]

Department of Microbiology and Immunology, University of Melbourne, Victoria, Australia[a]; Institute for Medical Innovation, St. Luke's International Medical Center, Tokyo, Japan[b]; Laboratory of Cancer Biology and Molecular Immunology, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan[c]; WHO Collaborating Centre for Reference and Research on Influenza, Victorian Infectious Diseases Reference Laboratory, North Melbourne, Victoria, Australia[d]

Specific protein receptors that mediate internalization and entry of influenza A virus (IAV) have not been identified for any cell type. Sialic acid (SIA), the primary attachment factor for IAV hemagglutinin, is expressed by numerous cell surface glycoproteins and glycolipids, confounding efforts to identify specific receptors involved in virus infection. Lec1 Chinese hamster ovary (CHO) epithelial cells express cell surface SIA and bind IAV yet are largely resistant to infection. Here, we demonstrate that expression of the murine macrophage galactose-type lectin 1 (MGL1) by Lec1 cells enhanced Ca^{2+}-dependent IAV binding and restored permissivity to infection. Lec1 cells expressing MGL1 were infected in the presence or absence of cell surface SIA, indicating that MGL1 can act as a primary receptor or as a coreceptor with SIA. Lec1 cells expressing endocytosis-deficient MGL1 mediated Ca^{2+}-dependent IAV binding but were less sensitive to IAV infection, indicating that direct internalization via MGL1 can result in cellular infection. Together, these studies identify MGL1 as a cell surface glycoprotein that can act as an authentic receptor for both attachment and infectious entry of IAV.

The first step in influenza A virus (IAV) infection of host cells is the attachment of virions to cell surface *N*-acetylneuraminic acid (sialic acid; SIA). SIA is the terminal component of oligosaccharide chains expressed by cell surface glycoproteins and glycolipids, and numerous studies have implicated SIA as an essential component of cellular receptors for influenza virus (reviewed in reference 1). Although interactions between SIA and the viral hemagglutinin (HA) are of low affinity (2), the abundance of SIA on the surface of mammalian cells nevertheless results in virions binding with high avidity to most cells.

Once bound to SIA, virus can be internalized by receptor-mediated endocytosis using clathrin- or caveolin-dependent pathways or pathways independent of both clathrin and caveolin (reviewed in reference 3). In addition, recent studies identified macropinocytosis as an alternative entry route for IAV (4–6). Following internalization, virus is trafficked through the endosomal maturation pathway until endosomal acidification triggers a conformational change in the viral HA, exposing its fusion peptide and facilitating fusion of viral and endosomal membranes (7). However, binding of IAV to sialylated cell surface receptors does not always result in receptor-mediated internalization (8, 9), and engagement of specific cellular signals may be required to initiate endocytosis of IAV. Consistent with this, IAV attachment to epithelial cells induces a signaling platform, activating receptor tyrosine kinases to facilitate virus uptake (10). Epsin 1 has been identified as a cargo-specific adaptor for clathrin-mediated internalization of IAV (11); however, the specific cellular receptors linking virus recognition and entry to epsin 1 (or to other adaptors) are not known.

The identification of specific IAV entry receptors is complicated by the ability of the virus to enter cells via multiple pathways. Studies utilizing a ganglioside-deficient fibroblast cell line (GM-95) indicated that sialoglycoproteins were critical for IAV infection, whereas gangliosides were not (12). Chu and Whittaker presented evidence that infection of Chinese hamster ovary (CHO) cells by IAV required host cell *N*-linked glycoprotein(s). In these studies, a mutant CHO line lacking a functional *N*-acetylglucosaminyltransferase I (GnT1) gene (Lec1 cells) was shown to be resistant to IAV infection despite efficient SIA-dependent binding of virus to the cell surface (13). Recent studies confirmed Lec1 cells to be resistant to IAV infection but reported efficient entry of IAV into other GnT1-deficient cell lines (5), leading the authors to propose that Lec1 cells harbor additional defects that affect IAV entry in the absence of sialylated *N*-linked glycans.

Detection of respiratory pathogens, including IAV, by airway macrophages (MΦ) is a critical component of innate immunity to infection. Infection of mouse MΦ by seasonal IAV is abortive (14–17), but it does result in release of antiviral and proinflammatory cytokines (18, 19), which may control early virus replication and regulate inflammatory responses to infection. Depletion of airway MΦ using clodronate-loaded liposomes results in enhanced IAV replication and exacerbated disease severity in mice (20, 21) and in pigs (22). Previous studies from our group reported that seasonal IAV strains, such as BJx109 (which induces very mild disease in mice), infected murine MΦ to high levels *in vitro*, whereas the mouse-adapted PR8 strain (which induces se-

Received 19 July 2013 **Accepted** 12 November 2013

Published ahead of print 20 November 2013

Address correspondence to Patrick C. Reading, preading@unimelb.edu.au.

* Present address: Stella Liong, Department of Obstetrics & Gynaecology, University of Melbourne, Parkville, VIC, Australia, and Mercy Perinatal Research Centre, Mercy Hospital for Women, Heidelberg, VIC, Australia; Michelle D. Tate, Centre for Innate Immunity and Infectious Diseases, Monash Institute of Medical Research, Monash University, Clayton, Victoria, Australia.

Sarah L. Londrigan and Patrick C. Reading are the senior authors of this work.

doi:10.1128/JVI.02014-13

vere disease in mice) did not (14, 15, 23). Moreover, depletion of airway MΦ from the lungs of mice markedly altered the course of BJx109 infection, resulting in severe disease (20), arguing that efficient infection of MΦ is an important factor limiting disease severity.

C-type lectin receptors (CLR) recognize carbohydrate structures and are involved in pathogen uptake and antigen presentation by immune cells, including MΦ and dendritic cells (DC) (reviewed in references 24–26). The macrophage galactose-type lectin (MGL) is a type II transmembrane CLR containing a single carbohydrate recognition domain (CRD) that is specific for the monosaccharides galactose and *N*-acetylgalactosamine (GalNAc). A single gene encodes human MGL, whereas 2 orthologs, murine MGL1 and MGL2, are expressed in mice (27). Recent studies from our group used direct binding methods to demonstrate that MGL bound to IAV in a Ca^{2+}-dependent manner, and MGL was implicated in mediating IAV infection of murine MΦ (23). Highly glycosylated IAV strains that infected MΦ to high levels were recognized efficiently by MGL, whereas the PR8 strain was not, arguing that (i) MGL acts as an attachment and/or entry receptor for IAV into MΦ, (ii) differences in MGL-mediated recognition determine the tropism of virus strains for murine MΦ, and (iii) that this, in turn, modulates the virulence of different IAV strains in mice. However, direct studies to confirm the role of MGL in IAV attachment, entry, and infection are lacking.

As Lec1 cells are largely resistant to IAV infection (5, 13), they provide a system that can be exploited for transfection and expression of putative receptors for IAV. Here, we expressed MGL1 in Lec1 cells to confirm its ability to function as an IAV attachment receptor and to investigate its ability to promote IAV infection in the presence or absence of cell surface SIA. Lec1 cells expressing endocytosis-defective MGL1 also bound IAV effectively but showed a reduced susceptibility to infection. To our knowledge, this is the first description of a specific glycoprotein that recognizes IAV to facilitate receptor-mediated internalization of virus, resulting in cellular infection.

MATERIALS AND METHODS

Cell lines and primary macrophages. CHO Pro-5 (CHO; ATCC CRL-1781) and Lec1 CHO (Lec1; ATCC CRL-1735) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in alpha minimal essential media (αMEM; Gibco BRL, New York, NY) supplemented with 10% fetal calf serum (JRH Biosciences, KS), 4 mM L-glutamine, 100 IU penicillin, 10 μg/ml streptomycin, nonessential amino acids (Gibco), and 50 μM beta-mercaptoethanol. The RAW 264.7 MΦ cell line (ATCC TIB-71) was maintained in Dulbecco's minimal essential medium (DMEM; Gibco) supplemented as described above. In some experiments, RAW 264.7 MΦ cells were cultured in the presence of 400 ng/ml of recombinant murine interleukin-4 (IL-4; Jomar Biosciences, Adelaide, Australia) for 48 h. RAW 264.7 MΦ cells were also transfected with MGL1-specific short interfering RNA (siRNA; target sequence, 5′ CTGAGAGCCACTTTAGACA 3′) (Sigma-Aldrich) using Lipofectamine 2000 transfection reagent (Life Technologies, Australia) by following manufacturer's guidelines. They were washed and cultured for 48 h before use in experiments. Scrambled control siRNA to an irrelevant protein was also provided by Sigma-Aldrich.

C57BL/6 and mannose receptor-deficient ($MMR^{-/-}$) mice (28) were bred and housed in specific-pathogen-free conditions at the Department of Microbiology and Immunology, The University of Melbourne. Mice aged 6 to 10 weeks were used in experiments conducted according to the guidelines of The University of Melbourne Animal Ethics Committee. Peritoneal exudate cells (PECs) from C57BL/6 and $MMR^{-/-}$ mice were obtained as described previously (15), and MΦ (2.5×10^5 cells) were seeded into 8-well glass chamber slides (Lab-Tek) and incubated for 4 h at 37°C. Cell monolayers were washed to remove nonadherent cells. The next day, slides were washed in serum-free media to remove any remaining nonadherent cells, and the adherent MΦ population was used in virus infection assays as described below.

Viruses. The IAV strains used in this study were BJx109 (a high-yielding reassortant of A/PR/8/34 [PR8, H1N1] with A/Beijing/353/89 [H3N2], expressing the H3N2 surface glycoproteins) and PR8. Viruses were grown in 10-day-old embryonated eggs and titrated on Madin-Darby canine kidney (MDCK) cells by standard procedures. Viruses were purified from allantoic fluid by rate zonal sedimentation on 25 to 75% (wt/vol) sucrose gradients as described previously (29). Purified virus was biotin labeled via exposed lysine groups using EZ-link-Sulfo-NHS-LC-LC biotin reagent (Thermo Scientific, IL) according to the manufacturer's instructions, dialyzed against Tris-buffered saline (TBS; 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.2), and stored at 4°C.

Reassortant IAV were generated by 8-plasmid reverse genetics as described previously (30). The viruses used were (i) 7:1 reassortants consisting of the PR8 backbone with the HA from BJx109 (RG-BJx109) or A/Brazil/11/78 (RG-Braz) or (ii) eight genes from PR8 (RG-PR8). The rescued viruses were recovered after 3 days and amplified in the allantoic cavity of 10-day-old embryonated eggs.

Parainfluenza virus type-3 (PIV-3) from the Victorian Infectious Diseases Reference Laboratory, Victoria, Australia, was propagated in HEP-G2 cells. Titers of infectious virus were determined following immunofluorescence staining of HEP-G2 monolayers and expressed as fluorescent focus-forming units (FFU)/ml.

Generation of Lec1 cells expressing MGL1. RNA isolated from the spleen of C57BL/6 mice was used as a template to amplify full-length MGL1 by PCR using specific primers (forward primer, 5′ CACCATGAT ATACGAAAACCTCCAGAACTC 3′; reverse primer, 5′ CTAGCTCTCC TTGGCCAGC 3′) that were then inserted into the pcDNA3.1/V5-His-TOPO expression vector (Invitrogen, CA). MGL1 mutant lacking 45 nucleotides from its cytoplasmic domain (ΔMGL1) was generated by PCR using specific primers (forward primer, 5′ GGGAAGCTTACCATGCTC CTGTTCTCCCTGGGC 3′; reverse primer, 5′ CCCCTCGAGCTTCTAG CTCTCCTTGGCCAGCTT 3′), and amplified products were purified following agarose gel electrophoresis and ligated into pcDNA3.1/V5-His-TOPO. Competent *Escherichia coli* (DH5α strain) cells were transfected, and vectors were purified using a Miniprep kit (Qiagen) according to the manufacturer's instructions. MGL1 inserts were confirmed by sequencing, and the full-length sequence was identical to NCBI reference sequence NM_010796.

Lec1 cells were transfected with pcDNA3.1/V5-His-TOPO expression vectors containing either full-length MGL1 or ΔMGL1 using FuGene 6 transfection reagent (Roche Diagnostic, Switzerland) according to the manufacturer's instructions. As controls, CHO and Lec1 cells were transfected with pcDNA3.1/V5-His-TOPO expressing cytoplasmic hen egg ovalbumin (OVA) lacking the sequence for cell surface trafficking, as previously described (31). Stable transfectants expressing full-length MGL1 (Lec1-MGL1), the MGL1 mutant (Lec1-ΔMGL1), or cytoplasmic OVA (CHO-ctrl, Lec1-ctrl) were selected in the presence of 1 mg/ml Geneticin (G418; Invitrogen). Transfected cells were screened for cell surface expression of MGL1 using a biotin-labeled monoclonal antibody (MAb) specific for murine MGL (clone ER-MP23; AbD Serotec, Oxford, United Kingdom) followed by streptavidin conjugated to allophycocyanin (APC; BD Biosciences, USA), and single cells with high cell surface MGL1 expression were isolated using a FACSAria cell sorter (BD Biosciences) and expanded in culture for use in experiments.

Western blot and virus overlay protein blot assays (VOPBA). Whole-cell lysates were prepared by adding 1 ml lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% [vol/vol] Triton X-100, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and broad-spectrum protease inhibitor cocktail; Roche, Manheim, Germany) to a confluent TC75 flask for 1 h on ice. Cells

were collected and clarified by centrifugation (10,000 × *g*, 3 min), and the protein concentration was determined by Bradford assay (Bio-Rad protein dye; Bio-Rad, CA). Lysates (~10 μg protein) were boiled for 5 min and analyzed by SDS-PAGE under nonreducing conditions using 10 to 12.5% gels, followed by transfer to polyvinylidene difluoride (PVDF) membrane (Millipore, MA) in Tris-glycine transfer buffer (25 mM Tris containing 192 mM glycine and 10% [vol/vol] methanol; pH 8.3).

To detect MGL1 by Western blotting, membranes were blocked in phosphate-buffered saline (PBS) containing 0.5% (wt/vol) bovine serum albumin (BSA) (or 2% skim milk) and 0.1% (vol/vol) Tween 20, and all subsequent wash and antibody binding steps were performed in PBS containing 0.05% (vol/vol) Tween 20. Membranes were incubated for 1 h at room temperature (RT) with MAb LOM-8.7 (specific for MGL1), and bound MAb was detected using horseradish peroxidase (HRP)-conjugated rabbit anti-rat immunoglobulins (Dako, Denmark) in conjunction with enhanced chemiluminescence (ECL; Western Lightning plus ECL; Perkin-Elmer, CT). Blots were developed using a Kodak Image Station 4000Mm, and images were managed using Adobe Photoshop software.

To detect virus binding, PVDF membranes were blocked with VOPBA buffer (TBS containing 1% [wt/vol] BSA, 0.05% [vol/vol] Tween 20, and 5 mM $CaCl_2$) for 4 h and incubated overnight with purified BJx109 (2 μg/ml). Membranes were washed and incubated with 2 μg/ml biotinylated MAb C1/1, which binds to the HA of BJx109 (a kind gift from Lorena Brown and Georgia Deliyannis, Department of Microbiology and Immunology, The University of Melbourne). After washing, bound virus was detected using streptavidin-conjugated HRP and ECL imaging as described above. All steps were performed using VOPBA buffer, and incubation steps were performed at 4°C. To determine if interactions between MGL1 and BJx109 were Ca^{2+} dependent, $CaCl_2$ was omitted from VOPBA buffer and replaced with 10 mM EDTA.

Binding of plant lectins and IAV to cells. Adherent cell lines were detached from TC75 flasks using 1 mM EDTA in PBS and washed in binding buffer (TBS containing 0.1% BSA [wt/vol] and 10 mM $CaCl_2$). For lectin and virus binding, cells were preincubated for 60 min at 37°C with serum-free media (mock) or 100 mU/ml of bacterial sialidase derived from *Vibrio cholerae* (type III; sialidase; Sigma-Aldrich, MO). Following incubation, cells were labeled on ice with 10 μg/ml of biotinylated *Maackia amurensis* lectin II (MAA; binds α-2,3gal-linked SIA; EY Laboratories, CA), 10 μg/ml of biotinylated BJx109, or 5 μg/ml of biotinylated *Phaseolus vulgaris*-L (L-PHA; binds *N*-linked glycans; EY Laboratories), and then they were washed, stained with streptavidin-APC, and analyzed using a FACSCalibur (BD Biosciences). Note that for L-PHA staining, BSA was omitted from the binding buffer. To test the lectin activity of MGL1, cells were incubated with 10 μg/ml α-D-galactose-PAA-fluorescein isothiocyanate (FITC) (galactose-PAA; Glycotech, Maryland) on ice, washed in binding buffer, and analyzed by flow cytometry. To determine if ligand binding to MGL1 was Ca^{2+} dependent, $CaCl_2$ was omitted from binding buffer and replaced with 0.5 to 5 mM EDTA as indicated. Values indicated by mean fluorescence intensity (MFI) indicate the geometrical mean value.

Antibody-mediated MGL1 internalization assay. The MGL1 internalization assay was adapted from the protocol used by Van Vliet et al. to examine internalization of MAb by human MGL (32). Briefly, cells were detached and incubated with 0.1 mg/ml biotin-labeled anti-mouse MGL MAb (clone ER-MP23; AbD Serotec) in TBS containing 10 mM $CaCl_2$ (TBS-Ca) for 30 min on ice. Unbound MAb was removed by washing in TBS-Ca, and cells were then placed on ice or at 37°C for 1 min or 30 min, washed again, and incubated with streptavidin-APC before analysis by flow cytometry. The MFI of samples incubated at 37°C were compared to those of control samples incubated on ice to determine the percentage of MAb remaining on the cell surface.

Virus infection assays. CHO, Lec1, CHO-ctrl, Lec1-ctrl, Lec1-MGL1, or Lec1-ΔMGL1 cells (5×10^4 cells/250 μl) were seeded into 8-well chamber slides (Lab-Tek) and infected with IAV, and the percentage of IAV-infected cells was determined as described previously (15, 23, 33). Briefly, after overnight culture, slides with confluent cell monolayers were washed and incubated with IAV in serum-free media for 1 h at 4°C (to allow virus binding but to inhibit virus entry) or at 37°C (to allow virus binding and entry). After removal of virus inoculum, cells were washed and incubated for a further 1 to 8 h at 37°C in serum-free media. Slides were washed with phosphate-buffered saline (PBS) and then fixed with 80% (vol/vol) acetone. IAV-infected cells were stained using (i) MAb MP3.10g2.1C7 (WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia), which is specific for the nucleoprotein (NP) of type A influenza viruses, or (ii) MAb C1/1, which detects the HA of BJx109, followed by FITC-conjugated goat anti-mouse Ig (Millipore, MA). The percentage of virus-infected cells was determined by costaining with 4′,6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) and counting the total number of cells versus FITC-positive cells under ×100 magnification. A minimum of 4 random fields were selected for counting, assessing >200 cells for each sample. Images were acquired with a Leica DMLB microscope (Leica Microsystems, Germany) and a Leica DFC 490 camera in conjunction with Leica IM50 Image Manager software. In some experiments, cell monolayers were pretreated with (i) 100 mU/ml of bacterial sialidase for 60 min at 37°C to remove cell surface SIA or (ii) 5 mg/ml of asialofetuin (ASF; Sigma-Aldrich) or 10 mg/ml of mannan (Sigma-Aldrich) in serum-free media for 30 min at 37°C to block C-type lectin receptors prior to addition of virus inoculum. In other experiments, 5 mM ammonium chloride (NH_4Cl) or 50 μM dynasore (Dy; Sigma-Aldrich) was added to cell monolayers at various times as indicated.

To detect IAV-infected cells by flow cytometry, cells in 12-well plates were washed and incubated with IAV as described above. At various times, cells were detached, fixed with 2% paraformaldehyde (PFA), and permeabilized for 20 min in 1% (vol/vol) Triton-X (Sigma-Aldrich). Cells were then stained in PBS containing 0.5% Triton-X and 1% fetal bovine serum using MAb MP3.10g2.1C7 or MAb C1/1, followed by FITC-conjugated goat anti-mouse Ig.

The number of adherent cells remaining after exposure to IAV was determined as described previously (20). Briefly, 24 h after exposure, cells were incubated in 80% (vol/vol) acetone in water for 2 min and then stained with 10 μg/ml propidium iodide (PI). Nuclear morphology was assessed, intact nuclei were counted in 4 or more independent fields, and these data were used to determine the percentage of viable cells. Levels of MCP-1, tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6) in clarified supernatants from mock- or IAV-infected cells were determined using a mouse cytokine bead array (CBA) flex set (Becton, Dickinson, USA), and MIP-2 was detected by enzyme-linked immunosorbent assay (ELISA) (Roche Diagnostics Corp.) according to the manufacturer's instructions.

Binding of recombinant MGL1 or plant lectin to IAV (ELISA). Wells of a polyvinyl microtiter plate were coated overnight at 4°C with a series of concentrations of purified IAV in PBS, blocked for 1 h with BSA (10 mg/ml), and washed with TBS containing 0.05% Tween 20 (TBST). To detect binding of MGL1 to IAV, wells were incubated for 2 h at room temperature with 1 to 10 μg/ml of recombinant MGL1 diluted in TBST containing 5 mg/ml of BSA and either 10 mM $CaCl_2$ (BSA_5-TBST-Ca^{2+}) or 5 mM EDTA (BSA_5-TBST-EDTA) and then washed. Binding of MGL1 was detected by the addition of biotinylated anti-MGL-specific MAb LOM-14 followed by streptavidin-HRP (GE Healthcare, Buckinghamshire, United Kingdom).

To determine binding of plant lectin *Ricinus communis* agglutinin I (RCA), wells coated with purified IAV were incubated for 2 h with 2 μg/ml of biotin-labeled RCA (Vector Laboratories, CA) in BSA_5-TBST-Ca^{2+} and washed, and bound lectin was detected using streptavidin-HRP followed by substrate. In some experiments, biotinylated RCA was incubated in BSA_5-TBST-Ca^{2+} supplemented with 5 mg/ml ASF to inhibit binding to IAV. To confirm equivalent coating levels of different IAV, duplicate wells were probed with a carbohydrate-specific MAb (MAb 165) which

recognizes the cross-reactive host antigen common to all egg-grown IAV (34).

Statistical analysis. Graphing and statistical analysis of data were performed using GraphPad Prism (GraphPad Software, San Diego, CA). An unpaired Student's *t* test was used to compare two sets of data. When comparing three or more sets of values, the data were analyzed by one-way analysis of variance (ANOVA; nonparametric) followed by *post hoc* analysis using Tukey's multiple comparison test. $P \leq 0.05$ was considered significant.

RESULTS

MGL1 plays a role in the infectious entry of IAV into mouse MΦ lacking MMR expression. In previous studies, we used biochemical approaches to demonstrate interactions between IAV and the C-type lectin receptors MMR (specific for Man-type glycans) and MGL1 (specific for Gal-type glycans), and both receptors were implicated in IAV infection of mouse MΦ (23). RAW264.7 MΦ express low levels of cell surface MGL1 (23) and do not express MMR (23, 27). Therefore, we modulated MGL1 expression on RAW264.7 MΦ to determine if this altered sensitivity to IAV infection. Consistent with previous reports (35), culture of RAW264.7 MΦ in the presence of recombinant murine IL-4 increased cell surface expression of MGL. In 3 independent experiments, IL-4-cultured RAW 264.7 MΦ showed a 3.4- ± 2.8-fold increase in MGL expression compared to mock-treated MΦ as determined by flow cytometry (data not shown). Moreover, enhanced MGL expression correlated with a significant increase in susceptibility of RAW264.7 MΦ to infection by IAV strain BJx109 (H3N2) (Fig. 1Ai, white bars), and infection was blocked by pretreatment of the cells with ASF, a multivalent ligand of MGL (Fig. 1Ai, black bars). Treatment of RAW264.7 MΦ with MGL1-specific siRNA resulted in a 2.1- ± 0.6-fold reduction (in triplicate samples in 2 independent experiments) in cell surface MGL1 expression compared to MΦ treated with scrambled control siRNA (data not shown), and knockdown of MGL1 correlated with reduced susceptibility to IAV infection (Fig. 1Aii). Note that no significant differences were observed in the percentage of IAV-infected cells between untransfected RAW264.7 MΦ and cells transfected with control siRNA (data not shown).

MMR is expressed by primary MΦ from C57BL/6 mice but not by MΦ from $MMR^{-/-}$ mice (Fig. 1Bi) (28), whereas both expressed cell surface MGL (Fig. 1Bii). MΦ isolated from the peritoneal cavity of C57BL/6 and $MMR^{-/-}$ mice were purified by adherence to 8-well glass chamber slides incubated with BJx109, and the percentage of IAV-infected cells was determined 8 h postinfection as previously described (14, 15, 23). MΦ from C57BL/6 and $MMR^{-/-}$ mice were equally susceptible to infection by BJx109. Consistent with previous studies (15, 23), pretreatment of C57BL/6 MΦ with either mannan (mannose-rich ligand for MMR) or ASF (galactose-rich ligand for MGL1) significantly reduced susceptibility to infection by BJx109 (Fig. 1Biii), consistent with a role for both MMR and MGL in infectious entry of IAV. In contrast, mannan did not inhibit IAV infection of $MMR^{-/-}$ MΦ but ASF did ($P \leq 0.001$) (Fig. 1Biii), indicating that in the absence of MMR, MGL1 is a likely candidate receptor involved in mediating efficient IAV infection of primary mouse MΦ.

MGL1 is expressed by MΦ and not by epithelial cells, and the latter represent the primary cellular targets that support productive IAV replication. To understand the consequences of MGL1-mediated infection, we examined MΦ responses following exposure of $MMR^{-/-}$ MΦ to BJx109 (which infects MΦ to high levels) and the mouse-adapted PR8 strain (which infects MΦ poorly) (14, 15, 23). First, we confirmed that 8 h after exposure, BJx109 infected $MMR^{-/-}$ MΦ to high levels whereas PR8 did not (Fig. 1Ci). By 24 h, only ~10% of BJx109-infected MΦ remained adherent, whereas the numbers of adherent MΦ in PR8-infected cultures were similar to those in mock controls (Fig. 1Cii). Titers of infectious virus were similar in cell-free supernatants taken from IAV-infected $MMR^{-/-}$ MΦ at 2 and 24 h after exposure to IAV (data not shown), consistent with previous reports demonstrating abortive infection in murine MΦ (15–17). Finally, $MMR^{-/-}$ MΦ produced IL-6, TNF-α, MCP-1, and MIP-2 following exposure to BJx109 but not PR8 (Fig. 1Ciii). Thus, IAV infection of murine $MMR^{-/-}$ MΦ results in cell death without release of infectious virions as well as induction of soluble mediators likely to modulate inflammation and immunity to infection.

Lec1 cells are less sensitive to IAV infection than CHO cells. As a first step toward utilizing an expression-based approach to specifically address the role of MGL1 as a receptor for IAV, we screened a range of epithelial and fibroblast cell lines for resistance to IAV infection. CHO, Caco-2, L929, HeLa, HEK293, BHK-21, and Vero cells were all susceptible to IAV infection, as assessed by immunofluorescence at 8 to 24 h postinfection (data not shown). Lec1 cells are derived from CHO Pro5 cells and cannot synthesize mature *N*-linked glycans due to a defect in the *N*-acetylglucosaminyltransferase I (GnT1) gene (36, 37). CHO cells express high levels of α(2,3)-linked, but not α(2,6)-linked SIA, due to a deficiency in galactosidase α-2,6-sialytransferase (38). Consistent with this, plant lectin MAA [specific for α(2,3)-linked SIA] bound efficiently to CHO Pro 5 cells and Lec1 cells (Fig. 2Ai, black histograms), and binding was markedly reduced if cells were pretreated with bacterial sialidase (Fig. 2Ai, gray histograms). As predicted, we did not detect sialidase-sensitive binding of plant lectin SNA [specific for α(2,6)-linked SIA] to either cell line (data not shown). L-PHA, a plant lectin that recognizes complex oligosaccharides, bound to CHO cells but not Lec1 cells (Fig. 2Aii), confirming that Lec1 cells do not express cell surface *N*-linked glycans.

The ability of IAV strain BJx109 (H3N2) to bind to CHO and Lec1 cells next was determined by flow cytometry. BJx109 bound to both CHO cells and Lec1 cells (Fig. 2B), and binding was abrogated if cells were pretreated with bacterial sialidase to remove cell surface SIA. Overall, binding of IAV to CHO cells was markedly higher than that to Lec1 cells. Together, these studies confirm the phenotype of Lec1 cells relative to parental CHO Pro5 cells and demonstrate SIA-dependent binding of IAV to both cell types, albeit less efficiently to Lec1 cells. Of note, BJx109 is a human IAV with HA preference for α(2,6)-linked SIA (39). While interactions between the IAV HA and SIA are of low affinity (40), simultaneous binding to multiple α(2,3)-linked SIA on the surface of CHO and Lec1 cells is likely to increase binding avidity.

Previous studies demonstrated Lec1 cells were resistant to infection by IAV (strain A/WSN/33 [H1N1]) and influenza B viruses (5, 13). To confirm these findings, monolayers of CHO cells and Lec1 cells were incubated with BJx109 (H3N2) for 1 h at 37°C, washed, and fixed 1 h later (i.e., 2 h after first exposure) or cultured an additional 7 h prior to fixing (i.e., 8 h postexposure). Monolayers were then stained for expression of viral NP or HA and examined by indirect immunofluorescence to count fluorescent and total cells. Representative images shown in Fig. 2Ci demonstrate negligible staining for viral NP or HA at 2 h compared to high levels at 8 h. Similarly, NP and HA are not detected in Lec1 cells at

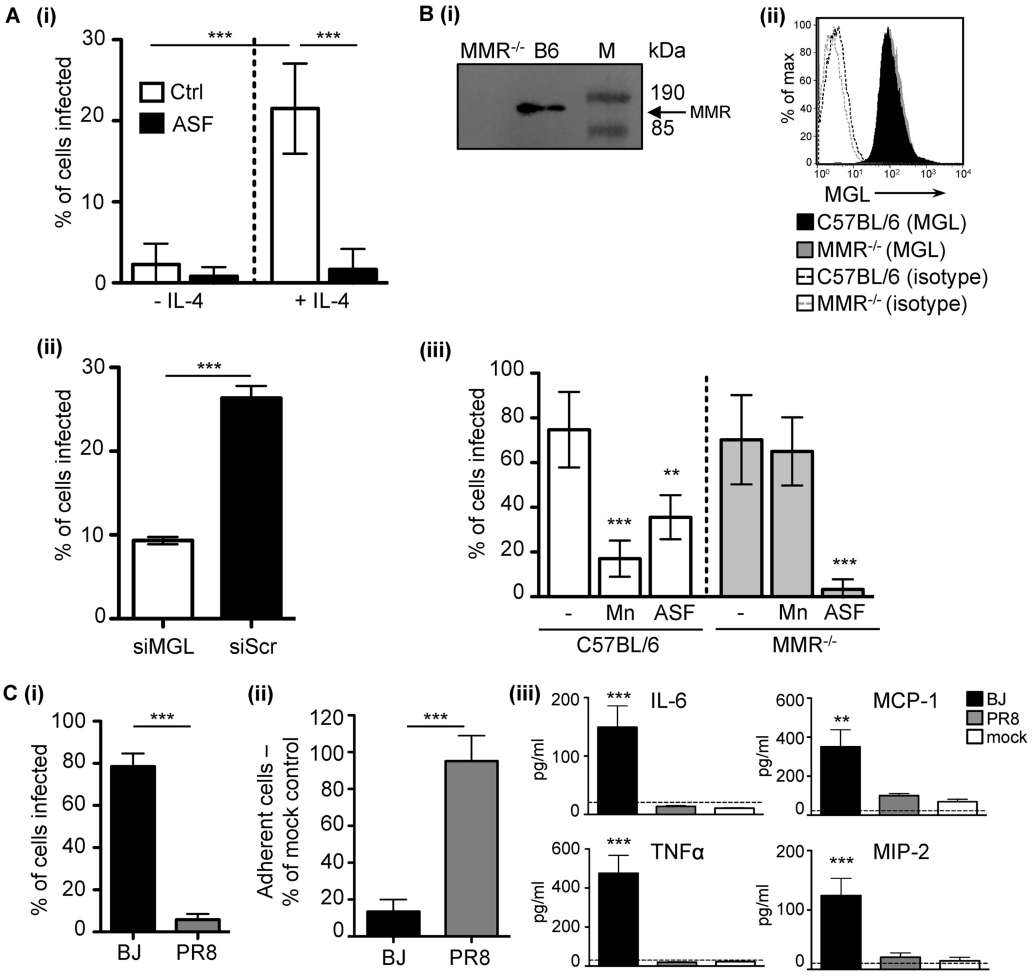

FIG 1 Expression of MGL1 on murine MΦ correlates with susceptibility to infection by IAV. (Ai) RAW 264.7 MΦ were cultured for 48 h with or without 400 ng/ml of recombinant murine IL-4, incubated with or without 5 mg/ml ASF for 30 min at 37°C, and infected with 10^4 PFU of BJx109 for 1 h at 37°C. After washing, cells were incubated a further 7 h in the presence of ASF, fixed, stained for expression of newly synthesized viral NP, and examined by immunofluorescence. Data show the mean percent infection ± 1 standard deviation (SD) from 3 independent experiments. (Aii) RAW 264.7 MΦ were transfected with control (siScr) or MGL1-specific (siMGL) siRNA, cultured an additional 48 h, and infected with 10^5 PFU of BJx109 in serum-free medium and cultured, fixed, and stained as described for panel Ai. Data from 1 of 2 independent experiments are shown. For panels i and ii, Student's *t* test was used for statistical analysis (***, $P \leq 0.001$). (Bi) Western blot analysis confirming expression of MMR by PEC MΦ from C57BL/6 (B6) but not MMR$^{-/-}$ mice. Proteins were resolved by SDS-PAGE under nonreducing conditions, transferred to PVDF membrane, and detected using MMR-specific MAb. Molecular weight markers (M) are shown. (Bii) Cell surface expression of MGL by PEC MΦ of C57BL/6 mice or MMR$^{-/-}$ mice. Cells were stained with biotin-labeled MGL-specific MAb (solid histograms) or isotype control (dashed histograms). (Biii) Susceptibility of MΦ to infection by BJx109. Monolayers of PEC MΦ were pretreated with 10 mg/ml mannan (Mn) or 5 mg/ml ASF or were mock treated (−) and infected with 10^6 PFU BJx109. Data are representative of 2 independent experiments. One-way ANOVA was used for statistical analysis ($P \leq 0.01$ [**] and $P \leq 0.001$ [***] compared to percent infection of the respective mock-treated samples). (C) Responses of MMR$^{-/-}$ to IAV strains BJx109 (BJ) and PR8. PEC MΦ from MMR$^{-/-}$ mice were infected with 10^6 PFU of each virus. (Ci) At 8 h, cells were fixed, stained for expression of viral NP, and assessed by immunofluorescence. (Cii) At 24 h, cells were fixed and stained for nucleic acids using PI, and the number of adherent cells remaining was determined by immunofluorescence. Data from panels Ci and Cii are representative of 3 independent experiments and were analyzed using Student's *t* test (***, $P \leq 0.001$). (Ciii) At 24 h, cell supernatants were clarified and assayed for IL-6, MCP-1, TNF-α, and MIP-2 as described in Materials and Methods. Detection limits of each assay are indicated by the dotted lines. One-way ANOVA was used for statistical analysis ($P \leq 0.01$ [**] and $P \leq 0.001$ compared to mock-treated samples).

2 h, and the number of infected Lec1 cells expressing either viral protein at 8 h is markedly reduced compared to that of CHO cells (Fig. 2Cii).

To determine the percentage of IAV-infected cells at 8 h postinfection, DAPI$^+$ cells and fluorescent NP$^+$ or HA$^+$ cells were counted. Staining for viral NP or HA confirmed that CHO cells were more sensitive than Lec1 cells to infection by BJx109 (Fig. 2D). These findings were confirmed using flow cytometry, as expression of viral NP by CHO or Lec1 cells was low at 2 h (Fig. 2Ei), but by 8 h CHO cells expressed significantly higher levels of NP than Lec1 cells (Fig. 2Eii). Virus titers were not significantly different in supernatants removed 2 and 24 h after infection of CHO or Lec1 cells with BJx109 (data not shown), indicating that IAV replication was not productive in these cell lines. Thus, these data are consistent with previous reports (5, 13) demonstrating that Lec1 cells were markedly less sensitive to IAV infection than parental CHO cells.

Generation of Lec1 cell lines expressing the C-type lectin receptor MGL1. We generated Lec1 cell lines expressing MGL1

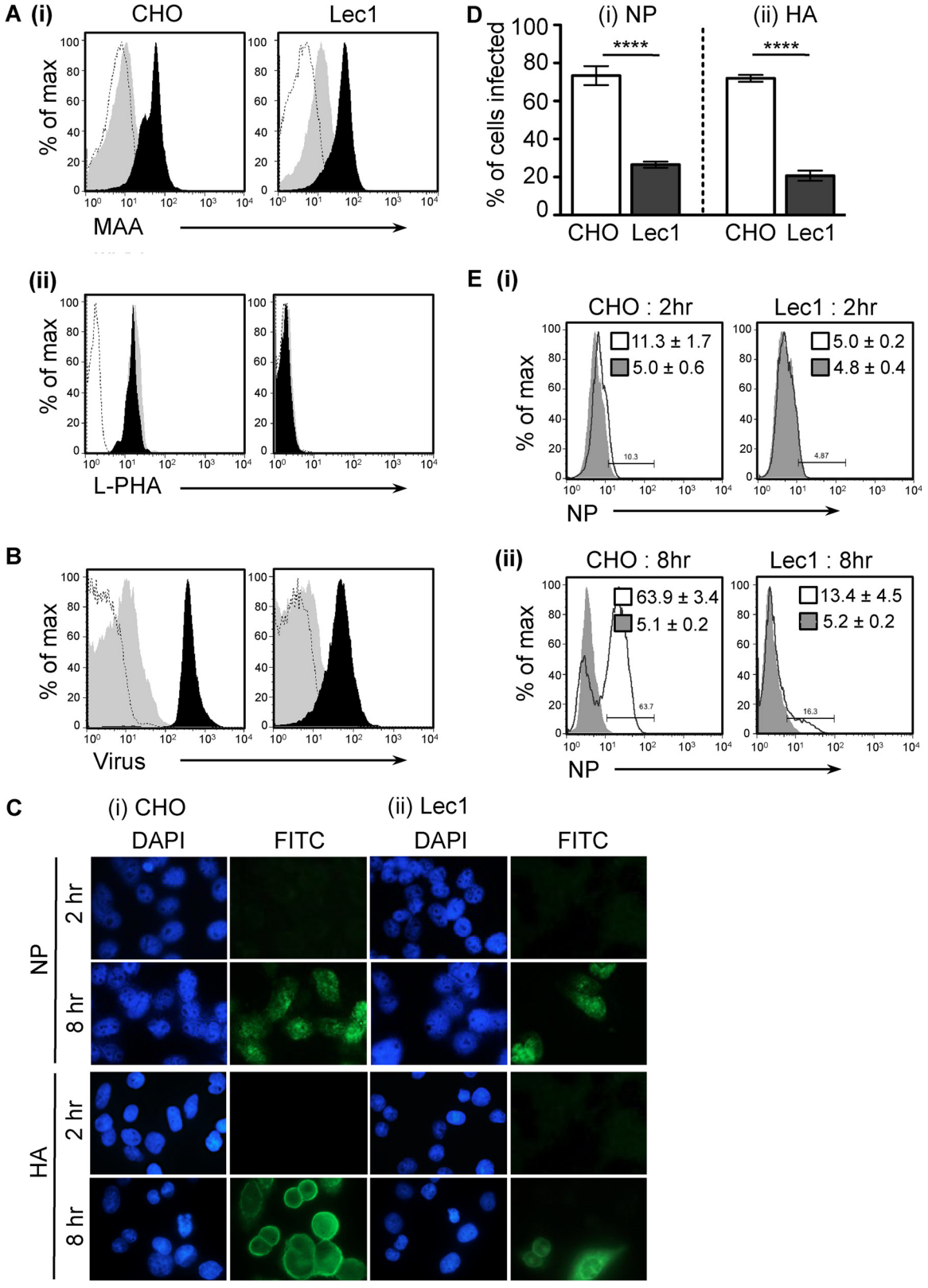

FIG 2 Lec1 cells are less susceptible to IAV infection than parental CHO cells. (A and B) CHO and Lec1 cells were incubated in medium alone (untreated; black histograms) or medium supplemented with 100 mU/ml of bacterial sialidase (sialidase; gray histograms), washed, and used in binding assays. (A) Binding of biotin-labeled MAA (i) or L-PHA (ii) was determined by flow cytometry. Representative histograms of triplicate samples are shown, and dashed lines represent cells stained with streptavidin-APC alone. MFI (± 1 SD) of binding to mock- or sialidase-treated cells for MAA (i) were the following: CHO, 31.2 ± 17.5 and 5.2 ± 3.6, respectively; Lec1, 35.6 ± 1.7 and 7.9 ± 1.4, respectively. MFI (± 1 SD) for L-PHA (ii) were the following: CHO, 13.6 ± 2.5 and 14.1 ± 1.4, respectively;

(Lec1-MGL1 cells) to determine if expression of MGL1 could enhance the susceptibility of Lec1 cells to IAV infection. Cell surface expression of MGL on Lec1-transfected cells was confirmed by flow cytometry (Fig. 3A), and Western blot analyses of cell lysates showed a single band of ~35 kDa in Lec1-MGL1 transfectants corresponding to MGL protein (Fig. 3B). As MGL1 expresses two potential sites of *N*-linked glycosylation in its extracellular domain (27) and Lec1 cells exhibit defects in expression of *N*-linked glycans (37), it was important to confirm that MGL1 expressed by Lec1 cells retained C-type lectin activity. Therefore, we examined binding of galactose-PAA, a multivalent ligand of MGL1, to the surface of Lec1-MGL1 cells. As seen in Fig. 3C, Lec1-MGL1 cells bound PAA-galactose in a Ca^{2+}-dependent manner, whereas Lec1-ctrl cells did not.

Lec1 cells expressing MGL1 show enhanced susceptibility to IAV infection. To determine if MGL1 on Lec1 cells acts as an attachment factor for IAV, Lec1-MGL1 cells and Lec1-ctrl cells were incubated on ice with purified BJx109 in the presence of $CaCl_2$ or EDTA and washed, and the amount of virus attached to the cell surface was determined. Overall, CHO-ctrl cells (Fig. 4Ai) bound higher levels of BJx109 than Lec1-ctrl cells (Fig. 4Aii), and binding to either cell type was Ca^{2+} independent, consistent with recognition of cell surface SIA by IAV HA. In contrast, binding to Lec1-MGL1 cells was significantly enhanced in the presence of Ca^{2+} (Fig. 4Aiii). In addition, VOPBA confirmed Ca^{2+}-dependent interactions between BJx109 and an ~35-kDa species in lysates from Lec1-MGL1 cells (Fig. 4B).

The susceptibility of Lec1-ctrl cells and Lec1-MGL1 cells to IAV infection was examined next. By immunofluorescence, BJx109 infected Lec1-MGL1 cells at significantly higher levels than Lec1-ctrl cells (Fig. 4C), and these data were confirmed using flow cytometry to detect NP (Fig. 4Di) or HA (Fig. 4Dii) at 8 h postinfection. IAV infection of Lec1-ctrl and Lec1-MGL1 resulted in nonproductive virus replication with no increase in infectious virus detected in supernatants 2 or 24 h after exposure to virus inoculum (data not shown). Thus, expression of MGL1 by Lec1 cells was associated with Ca^{2+}-dependent binding of IAV to the cell surface and enhanced susceptibility to IAV infection.

The lectin activity of Lec1-MGL1 contributes to dynamin-dependent infection of Lec1-MGL1 cells. Expression of MGL1 by Lec1 cells increases permissivity of IAV infection such that it is equivalent to parental CHO-ctrl cells (Fig. 5Ai). However, preincubation of cells with ASF blocked infection of Lec1-MGL1 cells but not CHO-ctrl cells (Fig. 5Aii), consistent with lectin-mediated infection of Lec1-MGL1 cells but not CHO-ctrl cells.

We next investigated the role of cell surface SIA in MGL1-mediated enhancement of IAV infection of Lec1 cells. Pretreatment of CHO-ctrl cells with sialidase almost completely abolished

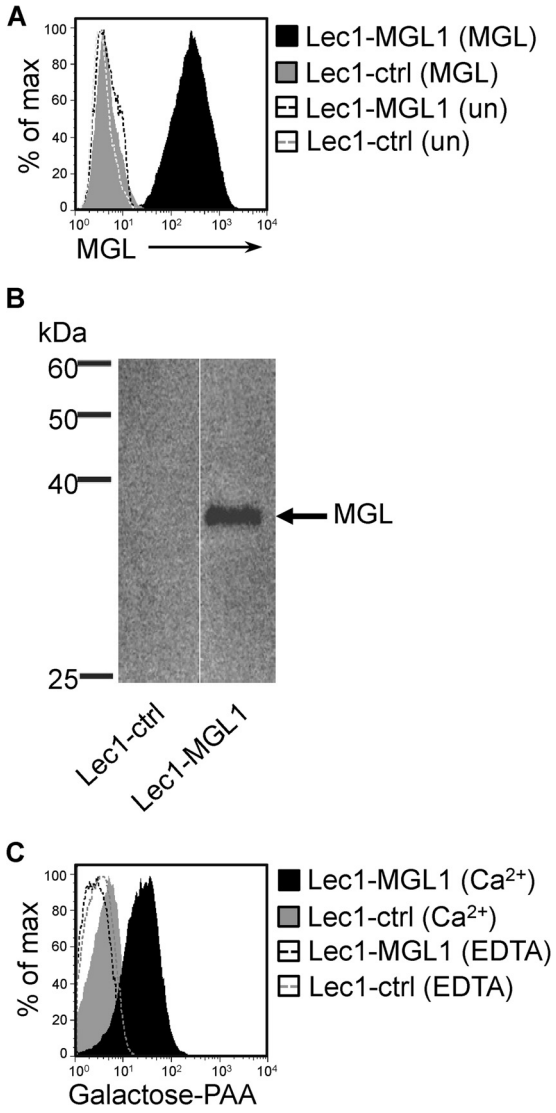

FIG 3 MGL1 expressed by Lec1 cells retains calcium-dependent C-type lectin activity. (A) Cell surface expression of MGL1 (solid histograms) on Lec1-MGL1 (black histogram) and Lec1-ctrl cells (gray histogram) was determined by flow cytometry using an MGL-specific MAb. Unstained cells (un; dashed lines) are included for comparison. (B) Western blot analysis confirming expression of MGL in cell lysates from Lec1-MGL1 cells but not Lec-1-ctrl cells. Proteins were resolved by SDS-PAGE under nonreducing conditions, transferred to PVDF membrane, and detected using MGL-specific MAb. Data are representative of 3 independent experiments. (C) Binding of galactose-PAA to Lec1-MGL1 (black histogram) but not Lec1-ctrl cells (gray histogram) in buffer containing either 10 mM $CaCl_2$ (solid histograms) or 5 mM EDTA (dashed lines). Data are representative of 2 independent experiments.

Lec1, 1.4 ± 0.2 and 1.4 ± 0.2, respectively. (B) Binding of 5 μg/ml biotin-labeled BJx109 was determined by flow cytometry. Representative histograms of triplicate samples are shown, and dashed lines represent cells stained with streptavidin-APC alone. MFI (± 1 SD) for binding to mock- or sialidase-treated cells are shown (CHO cells, 395.3 ± 2.3 and 5.5 ± 0.1, respectively; Lec1 cells, 41.4 ± 4.2 and 3.7 ± 0.1, respectively.) (C and D) Monolayers of CHO or Lec1 cells were infected with 10^7 PFU of BJx109 at 37°C for 1 h as described in Materials and Methods. At 2 or 8 h, cells were fixed and stained for expression of viral NP or HA. (C) Representative images of CHO cells (i) and Lec1 cells (ii) using a Leica DMLB microscope (used to determine the percentage of IAV-infected cells) are shown. Images show NP or HA (FITC) and double-stranded nucleic acid (DAPI). (D) Fluorescent and total cells were counted in at least 4 random fields (>200 cells) of CHO and Lec1 cells at 8 h postinfection. Data show the mean percent infection (± 1 SD) and are representative of 5 independent experiments. Student's *t* test was used to determine significance (***, $P \leq 0.001$). (E) Flow cytometry to determine expression of IAV NP at 2 h (i) or 8 h (ii) after infection with BJx109. Representative histograms from IAV-infected (white histograms) or mock-infected (gray histograms) triplicate samples and mean percentages of infected cells (± 1 SD) are shown. A gate was set to include 5% of cells in uninfected controls, and the percentage of infected cells was determined relative to this. Data are representative of 3 independent experiments.

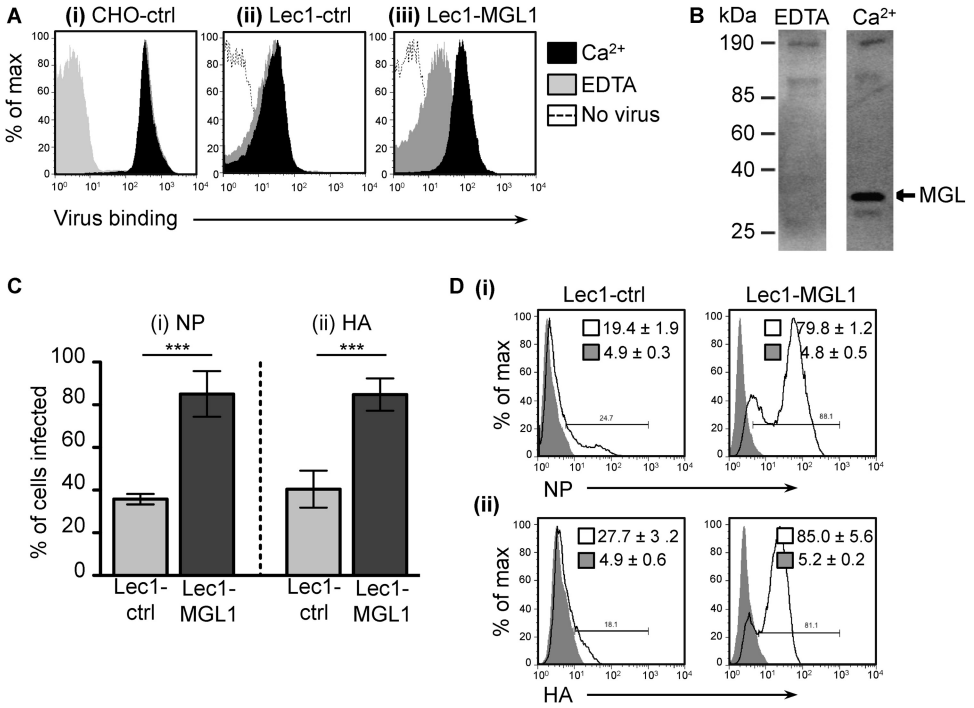

FIG 4 Expression of MGL1 by Lec1 cells results in enhanced IAV binding and increased susceptibility to IAV infection. (A) Binding of 5 μg/ml biotin-labeled BJx109 to CHO-ctrl (i), Lec1-ctrl (ii), and Lec1-MGL1 (iii) cells was determined by flow cytometry in the presence of 10 mM $CaCl_2$ (black histograms) or 0.5 mM EDTA (gray histograms). Representative histograms of triplicate samples are shown, and data were used to calculate mean MFI (± 1 SD) for each cell type in Ca^{2+} or in EDTA: CHO-ctrl, 389.7 ± 19.0 and 400 ± 28.6, respectively; Lec1-ctrl, 16.5 ± 3.2 and 13.5 ± 1.7, respectively; Lec1-MGL1, 77.2 ± 1.4 and 18.8 ± 6.4, respectively ($P \leq 0.001$ [***] compared to Lec1-MGL1 in the presence of Ca^{2+}; Student's *t* test). (B) Proteins in cell lysates of Lec1-MGL1 cells were resolved by SDS-PAGE under nonreducing conditions, transferred to PVDF membrane, and probed with purified BJx109 in VOPBA as described in Materials and Methods. Arrow indicates Ca^{2+}-dependent binding to a species of ~35 kDa, corresponding to the molecular mass of MGL1. (C) Lec1-ctrl or Lec1-MGL1 cells were infected with 10^7 PFU of BJx109, and expression of newly synthesized viral NP (i) or HA (ii) was determined 8 h later. Data represent mean percent infection (± 1 SD) and are representative of 2 independent experiments (***, $P \leq 0.001$; Student's *t* test). (D) Flow cytometry to determine expression of IAV NP (i) or HA (ii) at 8 h after infection with BJx109. Representative histograms from IAV-infected (white histograms) or mock-infected (gray histograms) cells are shown along with the mean percentage of infected cells (± 1 SD) from triplicate samples. A gate was set to include 5% of cells in uninfected controls, and the percentage of infected cells was determined relative to this. Data are representative of 2 independent experiments.

IAV infection (Fig. 5Aiii), whereas this treatment reduced, but did not abrogate, susceptibility of Lec1-MGL1 cells. Moreover, ASF completely blocked infection of sialidase-treated Lec1-MGL1 cells (Fig. 5Aiv). Note that MAA staining of CHO-ctrl and Lec1-MGL1 cells confirmed that sialidase effectively removed cell surface SIA from both cell lines (data not shown). Together, these data indicate that MGL1 mediates lectin-mediated attachment and infection of Lec1 cells by IAV in the absence of cell surface SIA. However, MGL1-mediated infection is enhanced when SIA is expressed on the surface of Lec1 cells.

To elucidate the entry pathway of IAV into Lec1-MGL1 cells we included dynasore, a small molecule inhibitor of dynamin, which blocks both clathrin- and caveolin-mediated endocytosis (41), in media during both virus inoculation and subsequent culture. As seen in Fig. 5B, 50 μM dynasore reduced BJx109 infection of CHO-ctrl and Lec1-MGL1 cells to <5%, confirming that dynamin-dependent endocytosis was required for IAV infection of each cell type. PIV-3 is a paramyxovirus that enters cells via direct fusion rather than by endocytosis (42). Consistent with this, dynasore did not affect infection of CHO-ctrl cells by PIV-3 (Fig. 5B).

IAV strains differ in the expression of galactose-rich glycans and in their ability to infect cells expressing MGL1. IAV strains differ markedly in the number of potential *N*-linked glycosylation sites expressed on the head of the viral HA (1, 43) and in their ability to infect murine MΦ (15, 23). BJx109 expresses 4 potential sites of *N*-linked glycosylation on the head of its HA and infects MΦ to high levels compared to PR8, which lacks glycosylation on the head of its HA and is particularly poor in its ability to infect murine MΦ (15, 23). Therefore, we investigated interactions between different IAV and MGL1. The galactose-specific plant lectin RCA-1 bound to BJx109 more efficiently than to PR8 (Fig. 6A), and binding was inhibited in the presence of ASF. Moreover, recombinant MGL1 also bound to BJx109 more efficiently than to PR8, and binding was blocked by chelation of Ca^{2+} with EDTA (Fig. 6B) or in the presence of ASF (data not shown). Thus, BJx109 expressed increased levels of Gal-type glycans and was recognized more efficiently by MGL1 than PR8.

Lec1-MGL1 cells were incubated with an equivalent infectious dose of either BJx109 or PR8 and washed, and the percentage of IAV-infected cells was determined at 8 h postinfection. Again, mock-treated or sialidase-treated Lec1-MGL1 cells were markedly more sensitive than Lec1-ctrl cells to BJx109 infection (Fig. 6Ci). In contrast, mock-treated Lec1-MGL1 cells showed only a modest increase in sensitivity to PR8 infection, and pretreatment with sialidase reduced infection levels to <1% (Fig. 6Ci). To confirm the importance of the viral HA, we used genetically defined viruses

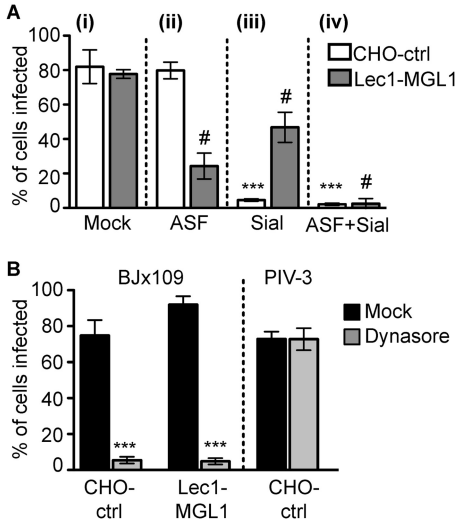

FIG 5 Role of cell surface sialic acid and dynamin-dependent endocytosis in MGL1-mediated infection of Lec1 cells by IAV. (A) Monolayers of parental CHO-ctrl and Lec1-MGL1 cells were mock treated or treated with 100 mU/ml bacterial sialidase (Sial) for 60 min at 37°C, followed by incubation with 5 mg/ml ASF or medium alone at 4°C for 30 min. Cells were then incubated with 10^7 PFU of BJx109 for 1 h at 4°C to allow virus binding, washed, and incubated for a further 7 h at 37°C. (B) Monolayers of parental CHO-ctrl and Lec1-MGL1 cells were incubated with 10^7 PFU of BJx109 alone (mock) or in the presence of 50 μM dynasore for 1 h at 37°C, washed, and incubated for a further 7 h in the presence or absence of dynasore. Cells were fixed and stained for expression of newly synthesized viral NP as described in Materials and Methods. Cells were fixed, stained, and examined by immunofluorescence as described in the text. CHO-ctrl cells incubated with 10^6 FFU of PIV-3 in the presence or absence of dynasore were fixed and stained at 16 h postinfection. Data represent the mean percent infection (± 1 SD) and are representative of 2 independent experiments. ***, significantly different from mock-treated control cells ($P \leq 0.001$; one-way ANOVA); #, significantly different from mock-treated Lec1-MGL1 cells ($P \leq 0.001$; one-way ANOVA).

which expressed 7 genes from PR8 in conjunction with the HA gene of either BJx109 (RG-BJx109) or PR8 (RG-PR8). As such, these viruses express an identical NA glycoprotein and differ only in respect to the origin of the viral HA. Only expression of the BJx109 HA was associated with increased infection of mock-treated or sialidase-treated Lec1-MGL1 cells (Fig. 6Cii). A genetically defined virus expressing 7 genes from PR8 with the HA from Braz/78 (an H1 subtype HA, like PR8, but with 4 potential glycosylation sites on the head of its HA [44]) also infected mock-treated or sialidase-treated Lec1-MGL1 cells to higher levels than Lec1-ctrl cells (Fig. 6Cii).

Generation of Lec1 cells expressing an endocytosis-defective mutant of MGL1. Expression of MGL1 by Lec1 cells could enhance IAV infection by a number of distinct mechanisms. MGL1 may act as an attachment factor, concentrating virions at the cell surface to promote interaction with other (unknown) receptors, which then mediate dynamin-dependent internalization of virus. Alternatively, MGL1 itself could act as both an attachment and entry receptor for IAV. Therefore, we generated an MGL1 construct with 45 nucleotides (corresponding to 15 amino acids, including the putative internalization motif YENL [27, 32, 45]) deleted from its cytoplasmic domain (Fig. 7A). Lec1 cells were transfected with this construct, and stable cell lines expressing the MGL1 deletion mutant were generated (Lec1-ΔMGL1) and matched with Lec1-MGL1 for cell surface expression of the receptor (Fig. 7B). As expected, the MGL1 species expressed by Lec1-ΔMGL1 showed a reduced molecular mass by Western blotting (Fig. 7C) and retained lectin activity equivalent to that of Lec1-MGL1 cells (Fig. 7D).

A biotin-labeled MAb specific for MGL1 was used to compare the endocytic activity of MGL1 expressed by Lec1-MGL1 and Lec1-ΔMGL1 cells. Cells were incubated at 4°C with anti-MGL1 MAb, washed, and moved to 37°C to facilitate internalization of cell surface MAb. After either 1 or 30 min, cells were washed in ice-cold buffer, fixed, and stained with streptavidin-APC, and analyzed by flow cytometry to determine the amount of MAb remaining at the cell surface. By 30 min, levels of MAb bound to MGL1 on the surface of Lec1-MGL1 cells were significantly reduced (Fig. 7E), indicative of antibody-mediated internalization. In contrast, levels of MAb on the surface of Lec1-ΔMGL1 cells at 1 or 30 min were not significantly different, confirming the importance of the intracellular domain in efficient MGL1-mediated endocytosis. To control for the off-rate of MAb, cells were fixed with 2% paraformaldehyde (PFA) prior to incubation with MAb. The MFI associated with anti-MGL MAb detected at the cell surface of fixed Lec1-MGL1 and Lec1-ΔMGL1 was not significantly different following incubation at 37°C for 1 or 30 min (data not shown).

MGL1 can act as an endocytic receptor for infectious entry of IAV into cells. We next compared Lec1 cells expressing MGL1 or ΔMGL1 for sensitivity to IAV. First, equivalent levels of BJx109 bound to the surface of Lec1-MGL1 and Lec1-ΔMGL1 cells (Fig. 8A), and levels were significantly enhanced compared to those of Lec1-ctrl cells. We next compared Lec1-ctrl, Lec1-MGL1, and Lec1-ΔMGL1 cells for susceptibility to infection by BJx109. BJx109 infected Lec1-ΔMGL1 cells less efficiently than Lec1-MGL1 cells ($P \leq 0.001$ by one-way ANOVA) (Fig. 8B). While there was a trend for Lec1-ΔMGL1 cells to be more susceptible to IAV infection than Lec1-ctrl, this was not significant across multiple independent experiments.

We next examined the rate of virus entry (leading to infection) in CHO-ctrl, Lec1-MGL1, and Lec1-ΔMGL1 cells. In these experiments, cell monolayers were inoculated with BJx109 at 4°C for 30 min (to allow virus binding but not endocytosis), washed, and cultured at 37°C for various times before the addition of NH_4Cl to retain virus in endosomes and prevent further infection. All wells were then fixed at 8 h postinoculation and stained for expression of IAV NP (Fig. 8C). Note that gray bars indicate wells that did not receive NH_4Cl and were fixed at 8 h. Inoculation of CHO-ctrl and Lec1-MGL1 with BJx109 (with no NH_4Cl added was the mock condition) resulted in ~90 and ~70% infection, respectively, whereas addition of NH_4Cl immediately after incubation with virus ($t = 0$ h) reduced infection levels to <1%. Infectious entry of BJx109 into CHO-ctrl cells (Fig. 8Ci) and Lec1-MGL1 cells (Fig. 8Cii) occurred during the first 30 min at 37°C, whereas infectious entry into Lec1-ΔMGL1 cells was not detected at this time (Fig. 8Ciii), consistent with defects in MGL-mediated endocytosis. By 8 h after virus exposure, the percentage of IAV-infected Lec1-ΔMGL1 cells was approximately half that of Lec1-MGL1 cells (Fig. 8, compare gray bars in panels ii and iii). Under similar infection conditions using an inoculum of 10^7 PFU of BJx109, the percentage of IAV-infected Lec1-ΔMGL1 cells had increased further by 24 h (Lec1-ΔMGL1 cells, 72.8% ± 7.8% infected; Lec1-MGL1 cells, 85.2% ± 3.2% infected), consistent with delayed kinetics of MGL1-mediated virus entry into Lec1-ΔMGL1 cells (data not shown).

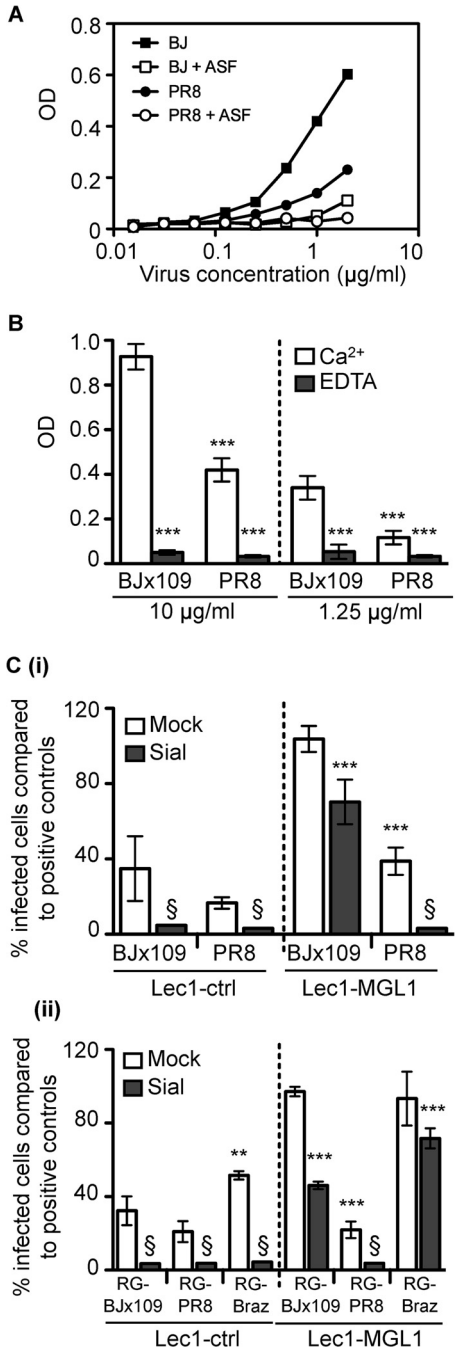

FIG 6 IAV strains differ in expression of galactose-rich glycans and in the ability to infect cells expressing MGL1. (A) Binding of plant lectin RCA to Gal-type glycans expressed by purified BJx109 and PR8. Wells coated with increasing concentrations of purified virus were probed with biotin-labeled RCA in the presence or absence of 5 mg/ml ASF. MAb 165 was used to confirm equivalent coating levels of each of the purified viruses (data not shown). (B) Binding of recombinant MGL1 to purified IAV. Wells coated with 10 μg/ml or 1.25 μg/ml of BJx109 or PR8 were incubated with 2 μg/ml recombinant MGL in buffer containing 25 mM $CaCl_2$ (white bars) or 5 mM EDTA (black bars). Data shown are the means from triplicate samples (± 1 SD) and are representative of 3 independent experiments. $P < 0.001$ (***) compared to binding to an equivalent coating concentration of BJx109 in the presence of $CaCl_2$. OD, optical density. (C) Monolayers of Lec1-ctrl and Lec1-MGL1 cells were mock treated or treated with 100 mU/ml of bacterial sialidase (sial) for 1 h at 37°C before infection with 10^7 PFU of BJx109 or PR8 (i) or 7:1 viruses (ii) generated by reverse genetics. Cells were fixed at 8 h and stained for newly synthesized NP as described in the text. Data represent the mean percent infection (± 1 SD) and are expressed relative to the positive control (CHO-ctrl cells) for each virus infection. Experiments were performed 3 times with similar results. $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) compared to infection of mock-treated cells by BJx109 (i) or RG-BJx109 (ii). §, percent infection was <1% of total cells.

DISCUSSION

Although specific entry receptors required for infectious entry of influenza virus into host cells have not been identified, these molecules are likely to differ depending on cell type. MMR and MGL are expressed on MΦ but not epithelial cells, and our studies have implicated both CLRs in infectious entry of IAV into murine MΦ (23) (Fig. 1 and 5A). In addition, BJx109 and PR8 infect epithelial cells to equivalent levels, whereas PR8 is particularly poor in its ability to infect primary MΦ and MΦ cell lines (Fig. 1C) (15, 23). Here, we demonstrate that BJx109, but not PR8, infected MΦ from $MMR^{-/-}$ mice to high levels, consistent with the notion that MGL1 alone can facilitate IAV entry and infection. Moreover, expression of MGL1 in an epithelial cell line deficient in endogenous entry/internalization receptors (Lec1 cells) resulted in enhanced IAV attachment and infection. MGL1-mediated infection occurred in the absence of cell surface SIA but was enhanced when SIA was expressed on the surface of Lec1 cells. IAV also bound to Lec1 cells expressing endocytosis-defective MGL1, but these cells were markedly less sensitive to virus infection. Together, these studies demonstrate that MGL1 can act as an attachment and entry receptor for influenza virus infection.

It is well established that IAV can enter host cells via clathrin-mediated and clathrin-independent endocytic pathways (46–48), with the majority of virions entering via clathrin-mediated endocytosis (CME) (48). The particular receptors engaged by IAV are likely to determine selection of particular endocytic pathways. Experimental cell culture conditions also modulate endocytic pathways (49–53), and addition of serum was shown to induce dynamin-independent macropinocytosis as an alternative IAV entry pathway in addition to CME (5, 54). Compared to spherical virions, filamentous IAV use macropinocytosis as the primary mechanism of virus entry, likely avoiding the size restriction of clathrin-coated vesicles (6). Identification of MGL1 as a specific receptor for virus attachment and entry is an important step toward the dissection and study of IAV entry pathways. Surprisingly little is known regarding how IAV enters immune cells, such as MΦ and DC, which naturally express MGL1. Future studies will investigate the specific pathways used by IAV to enter immune cells, the role of C-type lectin receptors in this process, and how IAV entry might be modulated in the presence of serum or by incubation in the presence of protein-rich airway fluids.

GnT1-deficient cell lines do not express sialylated *N*-linked glycans, although SIAs are present on *O*-linked glycoproteins and glycolipids (55–57). Complementation of GnT1 restored IAV entry in GnT1-deficient CHO 15B cells but was much less efficient in Lec1 cells, consistent with the proposal that GnT1-deficient Lec1 cells harbor an additional defect(s) that affects IAV entry in the absence of sialylated sugars (5). Our approach to demonstrate the receptor function of MGL1 for IAV depended on identification of a cell type that showed some resistance to IAV irrespective of the cellular defect associated with reduced virus entry. Despite virus

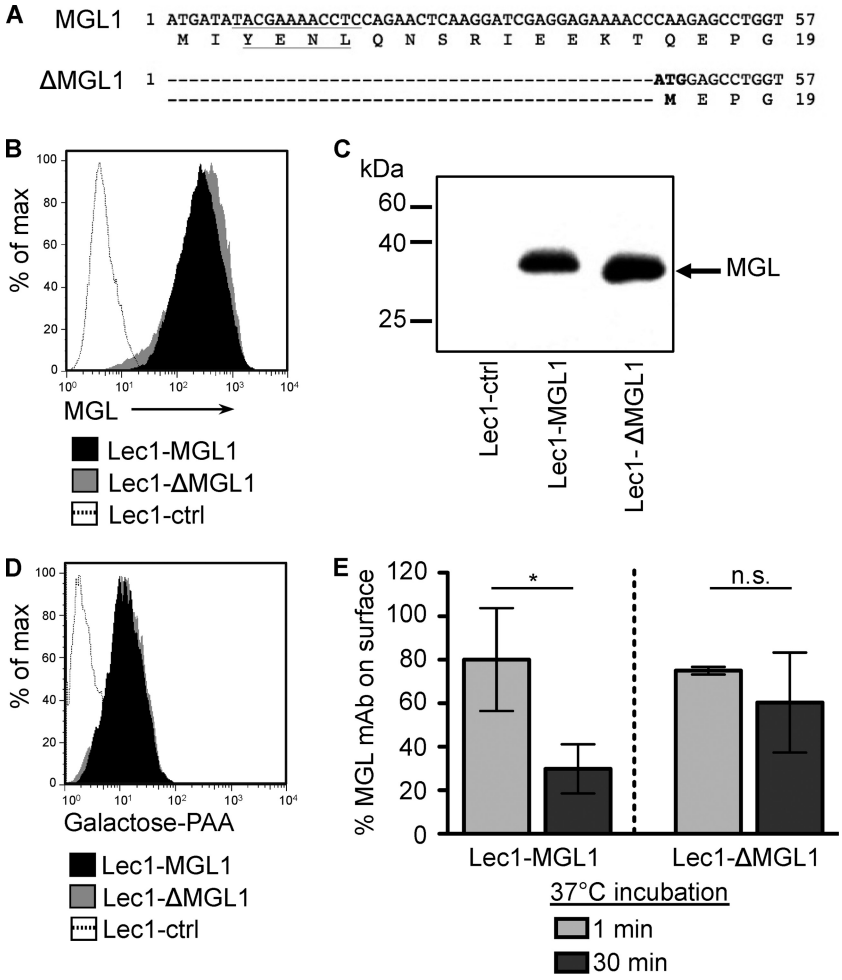

FIG 7 Mutant of MGL1 lacking 15 amino acids from its cytoplasmic domain retains C-type lectin activity but shows reduced endocytic activity when expressed by Lec1 cells. (A) Nucleotide and deduced amino acid sequences of the cytoplasmic domain of wild-type MGL1 and a deletion mutant lacking 15 amino acids from the cytoplasmic domain (ΔMGL1). The putative internalization domain YENL is underlined. (B) Cell surface expression of MGL1 on Lec1-MGL1 (black histogram), Lec1-ΔMGL1 (gray histogram), or Lec1-ctrl cells (dashed line). (C) Western blot detecting MGL in lysates from Lec1-ΔMGL1 cells. (D) MGL1 on Lec1-ΔMGL1 cells retains lectin function equivalent to that of MGL1 on Lec1-MGL1 cells. Data are representative of 2 independent experiments. (E) Reduced endocytic capacity of MGL1 expressed on Lec1-ΔMGL1 cells. MGL1-mediated endocytosis was determined as described in Materials and Methods. The asterisk indicates cell surface MAb levels that were significantly different between samples after 1 and 30 min ($P \leq 0.05$; Student's *t* test). n.s., not significant.

binding to cell surface SIA (presumably to *O*-linked glycans and gangliosides), the limited sensitivity of parental Lec1 cells to IAV infection suggests that specific signaling required to initiate virus entry was inefficient. Expression of MGL1 was sufficient to overcome such defects, and Lec1-MGL1 cells could be infected by IAV to levels comparable to those of CHO-ctrl cells (Fig. 5Ai). In this context, binding of IAV to MGL1 induced appropriate signaling, and Lec1 cells expressing MGL1 were fully susceptible to virus entry and infection.

Chu and Whittaker reported a dramatic reduction in susceptibility of Lec1 cells to IAV infection (~95% compared to <1% for CHO and Lec1 cells following incubation with virus strain A/WSN/33) (13), and recent studies confirmed these findings (5). In our hands, BJx109 infected CHO cells and Lec1 cells to ~80 and ~25%, respectively, suggesting that Lec1 cells differ in their susceptibility to different virus strains. Reassortant viruses expressing the HA of Beij/89, Braz/78, and PR8 also mediated SIA-dependent infection of Lec1-ctrl cells to levels higher than those previously reported using A/WSN/33 (Fig. 6), suggesting that the HA/NA balance of particular IAV strains is critical for effective binding to sialylated receptors on Lec1 cells which lack sialylated *N*-glycans. Consistent with this, IAV infection of Lec1-MGL1 cells generally was more efficient when cells expressed cell surface SIA. Together, these data indicate that both levels of galactose-rich glycans expressed by different IAV as well as interactions between the viral HA/NA and cell surface SIA can modulate MGL1-mediated entry and infection by IAV.

Recombinant human MGL retained C-type lectin activity comparable to that of native MGL (58), consistent with our finding that recombinant MGL1 mediated Ca^{2+}-dependent binding to IAV (Fig. 6B). As recombinant MGL1 expressed in *E. coli* lacks appropriate glycosylation, these data confirm that the two sites of *N*-linked glycosylation on MGL1 are not required for lectin function. When expressed by Lec1 cells, MGL1 mediated Ca^{2+}-dependent binding to galactose-PAA (Fig. 3) and to IAV (Fig. 4Aiii), and the enhanced susceptibility of Lec1-MGL1 cells to IAV infection

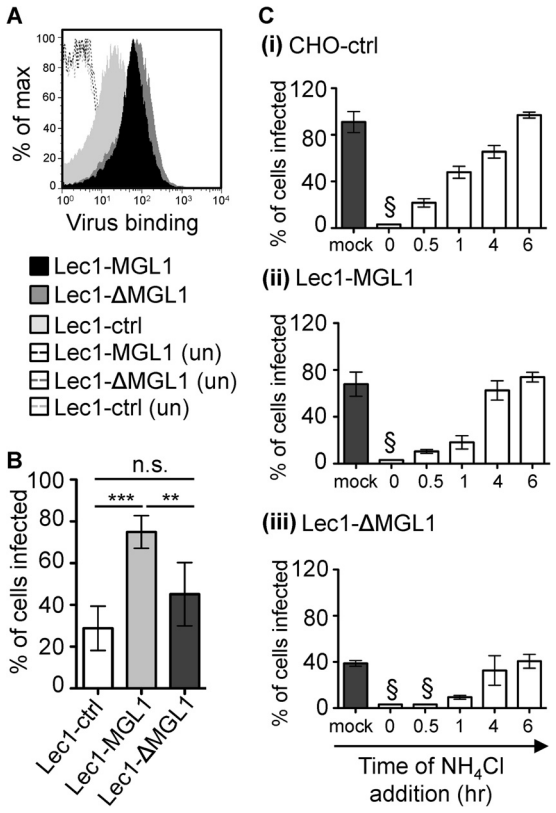

FIG 8 Lec1 cells expressing endocytosis-defective MGL1 show reduced sensitivity to IAV infection. (B) Binding of 5 μg/ml biotin-labeled BJx109 to cells in the presence of 10 mM $CaCl_2$ was determined by flow cytometry. Representative histograms of triplicate samples are shown, and dashed lines represent cells stained with streptavidin-APC alone (un). MFI (± 1 SD) of BJx109 binding to Lec1-ctrl, Lec1-MGL1, and Lec1-ΔMGL1 cells (14.4 ± 2.8, 48.4 ± 7.1, and 65.9 ± 22.5, respectively). **, $P < 0.01$ by one-way ANOVA compared to Lec1-ctrl. (C) Monolayers of Lec1-ctrl, Lec1-MGL1, and Lec1-ΔMGL1 were infected with 10^7 PFU of BJx109 as described in Materials and Methods. Cells were fixed at 8 h, stained, and then examined by immunofluorescence. Data show the mean percent infection (± 1 SD) from 6 independent experiments. (**, $P \leq 0.01$; ***, $P < 0.001$; n.s., not significant; one-way ANOVA). (D) Monolayers of CHO-ctrl (i), Lec1-MGL1 (ii), and Lec1-ΔMGL1 (iii) were incubated with 10^7 PFU of BJx109 at 4°C for 30 min to allow virus binding and then moved to 37°C. At various times, supernatants were removed and replaced with media containing 5 mM NH_4Cl to prevent further infection. NH_4Cl was not added to mock-treated cells. All cells were fixed at 8 h and stained for expression of viral NP. Data represent the mean percent infection (± 1 SD) and are representative of 3 independent experiments. §, percent infection was <1% of total cells.

was blocked by ASF (Fig. 5Aii). MMR is a complex C-type lectin that expresses multiple sites for *N*- and *O*-linked glycosylation. Inhibitors of oligosaccharide processing did not affect C-type lectin activity of MMR (59); however, expression of MMR by Lec1 cells was associated with severe defects in MMR-mediated recognition and internalization of mannose-specific ligands (60). It is well established that *N*-linked glycosylation can be an important regulator of molecular and cellular interactions, and as such, the transfection/expression-based approach described here will not be appropriate to examine all putative virus receptors.

MMR and DC-SIGN are Man-specific CLRs that bind glycans expressed on IAV HA/NA, and they have been implicated in promoting infection of different MΦ and dendritic cell populations (15, 23, 33, 61). These and additional CLRs have been reported to act as attachment factors and promote infection by a range of other viruses; however, in most studies the mechanism(s) underlying CLR-mediated infection enhancement has not been elucidated (reviewed in reference 31 and 62). That said, removal of the putative internalization motif from DC-SIGN prevented antibody-mediated internalization of DC-SIGN but still allowed efficient replication of dengue virus (DV) (63), indicating that DC-SIGN acts as an attachment factor but not as an essential entry receptor for DV. However, cells expressing an endocytosis-defective mutant of DC-SIGN were resistant to phlebovirus uptake and infection (64), confirming that DC-SIGN can act as a true viral entry receptor in other instances. Currently, less is known regarding the role of MGL as an attachment factor and/or virus entry receptor. Human MGL binds to the highly glycosylated mucin-like domain within the envelope glycoprotein of filoviruses to enhance virus attachment and infection (65, 66); however, the mechanisms by which MGL promotes infection are not clear. Our findings that MGL1 and endocytosis-defective MGL1 are able to bind equivalent amounts of IAV but differed dramatically in their ability to facilitate infectious virus entry demonstrate that MGL1 can function as a true entry receptor for IAV.

ACKNOWLEDGMENTS

We thank Peter Cowan, Immunology Research Centre, St. Vincent's Hospital, Melbourne, Australia, and Michel C. Nussenzweig, The Rockefeller University, New York, NY, for provision of the mannose receptor-deficient ($MMR^{-/-}$) mice. We thank St. Jude Children's Research Hospital (Memphis, TN, USA) for providing the pHW2000 plasmid for reverse genetics. We thank Karen Laurie, WHO Collaborating Centre for Reference and Research on Influenza, Victorian Infectious Diseases Reference Laboratory, North Melbourne, Victoria, Australia, for helpful advice in the design and use of siRNA.

This study was supported by project grant 1027545 from the National Health and Medical Research Council (NHMRC) of Australia. P.C.R., S.L.L., and A.G.B. are all recipients of funding from the NHMRC. W.N. is a recipient of an NHMRC Dora Lush Biomedical Research Scholarship. S.L.L. is a recipient of a University of Melbourne Early Career Research Grant and a University of Melbourne, Melbourne Research Fellowship.

The Melbourne WHO Collaborating Centre for Reference and Research on Influenza is supported by the Australian Government Department of Health.

REFERENCES

1. **Skehel JJ, Wiley DC.** 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu. Rev. Biochem. **69:**531–569. http://dx.doi.org/10.1146/annurev.biochem.69.1.531.
2. **Lees WJ, Spaltenstein A, Kingery-Wood JE, Whitesides GM.** 1994. Polyacrylamides bearing pendant alpha-sialoside groups strongly inhibit agglutination of erythrocytes by influenza A virus: multivalency and steric stabilization of particulate biological systems. J. Med. Chem. **37:**3419–3433. http://dx.doi.org/10.1021/jm00046a027.
3. **Lakadamyali M, Rust MJ, Zhuang X.** 2004. Endocytosis of influenza viruses. Microbes Infect. **6:**929–936. http://dx.doi.org/10.1016/j.micinf.2004.05.002.
4. **de Vries E, Tscherne DM, Wienholts MJ, Cobos-Jiménez V, Scholte F, García-Sastre A, Rottier PJM, De Haan CAM.** 2011. Dissection of the influenza A virus endocytic routes reveals macropinocytosis as an alternative entry pathway. PLoS Pathog. **7:**e1001329. http://dx.doi.org/10.1371/journal.ppat.1001329.
5. **de Vries E, De Vries RP, Wienholts MJ, Floris CE, Jacobs M-S, van den Heuvel A, Rottier PJM, De Haan CAM.** 2012. Influenza A virus entry into cells lacking sialylated N-glycans. Proc. Natl. Acad. Sci. USA **109:**7457–7462. http://dx.doi.org/10.1073/pnas.1200987109.
6. **Rossman JS, Leser GP, Lamb RA.** 2012. Filamentous influenza virus

enters cells via macropinocytosis. J. Virol. **86:**10950–10960. http://dx.doi.org/10.1128/JVI.05992-11.
7. **Hamilton BS, Whittaker GR, Daniel S.** 2012. Influenza virus-mediated membrane fusion: determinants of hemagglutinin fusogenic activity and experimental approaches for assessing virus fusion. Viruses **4:**1144–1168. http://dx.doi.org/10.3390/v4071144.
8. **Carroll SM, Paulson JC.** 1985. Differential infection of receptor-modified host cells by receptor-specific influenza viruses. Virus Res. **3:**165–179. http://dx.doi.org/10.1016/0168-1702(85)90006-1.
9. **Williams SP, Robertson JS.** 1993. Analysis of the restriction to the growth of nonegg-adapted human influenza virus in eggs. Virology **196:**660–665. http://dx.doi.org/10.1006/viro.1993.1522.
10. **Eierhoff T, Hrincius ER, Rescher U, Ludwig S, Ehrhardt C.** 2010. The epidermal growth factor receptor (EGFR) promotes uptake of influenza a viruses (IAV) into host cells. PLoS Pathog. **6:**e1001099. http://dx.doi.org/10.1371/journal.ppat.1001099.
11. **Chen C, Zhuang X.** 2008. Epsin 1 is a cargo-specific adaptor for the clathrin-mediated endocytosis of the influenza virus. Proc. Natl. Acad. Sci. U. S. A. **105:**11790–11795. http://dx.doi.org/10.1073/pnas.0803711105.
12. **Ablan S, Rawat SS, Blumenthal R, Puri A.** 2001. Entry of influenza virus into a glycosphingolipid-deficient mouse skin fibroblast cell line. Arch. Virol. **146:**2227–2238. http://dx.doi.org/10.1007/s007050170033.
13. **Chu VC, Whittaker GR.** 2004. Influenza virus entry and infection require host cell N-linked glycoprotein. Proc. Natl. Acad. Sci. U. S. A. **101:**18153–18158. http://dx.doi.org/10.1073/pnas.0405172102.
14. **Reading PC, Whitney PG, Pickett DL, Tate MD, Brooks AG.** 2010. Influenza viruses differ in ability to infect macrophages and to induce a local inflammatory response following intraperitoneal injection of mice. Immunol. Cell Biol. **88:**641–650. http://dx.doi.org/10.1038/icb.2010.11.
15. **Reading PC, Miller JL, Anders EM.** 2000. Involvement of the mannose receptor in infection of macrophages by influenza virus. J. Virol. **74:**5190–5197. http://dx.doi.org/10.1128/JVI.74.11.5190-5197.2000.
16. **Rodgers B, Mims CA.** 1981. Interaction of influenza virus with mouse macrophages. Infect. Immun. **31:**751–757.
17. **Cline TD, Karlsson EA, Seufzer BJ, Schultz-Cherry S.** 2013. The hemagglutinin protein of highly pathogenic H5N1 influenza viruses overcomes an early block in the replication cycle to promote productive replication in macrophages. J. Virol. **87:**1411–1419. http://dx.doi.org/10.1128/JVI.02682-12.
18. **Tate MD, Schilter HC, Brooks AG, Reading PC.** 2011. Responses of mouse airway epithelial cells and alveolar macrophages to virulent and avirulent strains of influenza A virus. Viral Immunol. **24:**77–88. http://dx.doi.org/10.1089/vim.2010.0118.
19. **Herold S, Wulffen von, Steinmueller WM, Pleschka S, Kuziel WA, Mack M, Srivastava M, Seeger W, Maus UA, Lohmeyer J.** 2006. Alveolar epithelial cells direct monocyte transepithelial migration upon influenza virus infection: impact of chemokines and adhesion molecules. J. Immunol. **177:**1817–1824.
20. **Tate MD, Pickett DL, van Rooijen N, Brooks AG, Reading PC.** 2010. Critical role of airway macrophages in modulating disease severity during influenza virus infection of mice. J. Virol. **84:**7569–7580. http://dx.doi.org/10.1128/JVI.00291-10.
21. **Wijburg OL, DiNatale S, Vadolas J, Van Rooijen N, Strugnell RA.** 1997. Alveolar macrophages regulate the induction of primary cytotoxic T-lymphocyte responses during influenza virus infection. J. Virol. **71:**9450–9457.
22. **Kim HM, Lee YW, Lee KJ, Kim HS, Cho SW, Van Rooijen N, Guan Y, Seo SH.** 2008. Alveolar macrophages are indispensable for controlling influenza viruses in lungs of pigs. J. Virol. **82:**4265–4274. http://dx.doi.org/10.1128/JVI.02602-07.
23. **Upham JP, Pickett D, Irimura T, Anders EM, Reading PC.** 2010. Macrophage receptors for influenza A virus: role of the macrophage galactose-type lectin and mannose receptor in viral entry. J. Virol. **84:**3730–3737. http://dx.doi.org/10.1128/JVI.02148-09.
24. **Geijtenbeek TBH, Gringhuis SI.** 2009. Signalling through C-type lectin receptors: shaping immune responses. Nat. Rev. Immunol. **9:**465–479. http://dx.doi.org/10.1038/nri2569.
25. **Van Kooyk Y.** 2008. C-type lectins on dendritic cells: key modulators for the induction of immune responses. Biochem. Soc. Trans. **36:**1478. http://dx.doi.org/10.1042/BST0361478.
26. **Robinson MJ, Sancho D, Slack EC, Leibundgut-Landmann S, Reis e Sousa C.** 2006. Myeloid C-type lectins in innate immunity. Nat. Immunol. **7:**1258–1265. http://dx.doi.org/10.1038/ni1417.
27. **Tsuiji M, Fujimori M, Ohashi Y, Higashi N, Onami TM, Hedrick SM, Irimura T.** 2002. Molecular cloning and characterization of a novel mouse macrophage C-type lectin, mMGL2, which has a distinct carbohydrate specificity from mMGL1. J. Biol. Chem. **277:**28892–28901. http://dx.doi.org/10.1074/jbc.M203774200.
28. **Lee SJ.** 2002. Mannose receptor-mediated regulation of serum glycoprotein homeostasis. Science **295:**1898–1901. http://dx.doi.org/10.1126/science.1069540.
29. **Anders EM, Hartley CA, Jackson DC.** 1990. Bovine and mouse serum beta inhibitors of influenza A viruses are mannose-binding lectins. Proc. Natl. Acad. Sci. U. S. A. **87:**4485–4489. http://dx.doi.org/10.1073/pnas.87.12.4485.
30. **Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, Hughes M, Perez DR, Donis R, Hoffmann E, Hobom G, Kawaoka Y.** 1999. Generation of influenza A viruses entirely from cloned cDNAs. Proc. Natl. Acad. Sci. U. S. A. **96:**9345–9350. http://dx.doi.org/10.1073/pnas.96.16.9345.
31. **Londrigan SL, Tate MD, Brooks AG, Reading PC.** 2011. Cell-surface receptors on macrophages and dendritic cells for attachment and entry of influenza virus. J. Leukoc. Biol. **92:**97–106. http://dx.doi.org/10.1189/jlb.1011492.
32. **Van Vliet SJ, Aarnoudse CA, Broks-Van Den Berg VCM, Boks M, Geijtenbeek TBH, Van Kooyk Y.** 2007. MGL-mediated internalization and antigen presentation by dendritic cells: a role for tyrosine-5. Eur. J. Immunol. **37:**2075–2081. http://dx.doi.org/10.1002/eji.200636838.
33. **Londrigan SL, Turville SG, Tate MD, Deng Y-M, Brooks AG, Reading PC.** 2011. N-linked glycosylation facilitates sialic acid-independent attachment and entry of influenza A viruses into cells expressing DC-SIGN or L-SIGN. J. Virol. **85:**2990–3000. http://dx.doi.org/10.1128/JVI.01705-10.
34. **Reading P, Morey L, Crouch E, Anders E.** 1997. Collectin-mediated antiviral host defense of the lung: evidence from influenza virus infection of mice. J. Virol. **71:**8204–8212.
35. **Raes G, Brys L, Dahal BK, Brandt J, Grooten J, Brombacher F, Vanham G, Noël W, Bogaert P, Boonefaes T, Kindt A, Van den Bergh R, Leenen PJM, De Baetselier P, Ghassabeh GH.** 2005. Macrophage galactose-type C-type lectins as novel markers for alternatively activated macrophages elicited by parasitic infections and allergic airway inflammation. J. Leukoc. Biol. **77:**321–327. http://dx.doi.org/10.1189/jlb.0304212.
36. **Stanley P, Caillibot V, Siminovitch L.** 1975. Selection and characterization of eight phenotypically distinct lines of lectin-resistant Chinese hamster ovary cell. Cell **6:**121–128. http://dx.doi.org/10.1016/0092-8674(75)90002-1.
37. **Puthalakath H, Burke J, Gleeson PA.** 1996. Glycosylation defect in Lec1 Chinese hamster ovary mutant is due to a point mutation in N-acetylglucosaminyltransferase I gene. J. Biol. Chem. **271:**27818–27822. http://dx.doi.org/10.1074/jbc.271.44.27818.
38. **Lee EU, Roth J, Paulson JC.** 1989. Alteration of terminal glycosylation sequences on N-linked oligosaccharides of Chinese hamster ovary cells by expression of beta-galactoside alpha 2,6-sialyltransferase. J. Biol. Chem. **264:**13848–13855.
39. **Tate MD, Brooks AG, Reading PC.** 2011. Correlation between sialic acid expression and infection of murine macrophages by different strains of influenza virus. Microbes Infect. **13:**202–207. http://dx.doi.org/10.1016/j.micinf.2010.10.004.
40. **Sauter NK, Bednarski MD, Wurzburg BA, Hanson JE, Whitesides GM, Skehel JJ, Wiley DC.** 1989. Hemagglutinins from two influenza virus variants bind to sialic acid derivatives with millimolar dissociation constants: a 500-MHz proton nuclear magnetic resonance study. Biochemistry **28:**8388–8396. http://dx.doi.org/10.1021/bi00447a018.
41. **Doherty GJ, McMahon HT.** 2009. Mechanisms of endocytosis. Annu. Rev. Biochem. **78:**857–902. http://dx.doi.org/10.1146/annurev.biochem.78.081307.110540.
42. **Moscona A, Peluso RW.** 1993. Relative affinity of the human parainfluenza virus type 3 hemagglutinin-neuraminidase for sialic acid correlates with virus-induced fusion activity. J. Virol. **67:**6463–6468.
43. **Kim JI, Park M-S.** 2012. N-linked glycosylation in the hemagglutinin of influenza A viruses. Yonsei Med. J. **53:**886. http://dx.doi.org/10.3349/ymj.2012.53.5.886.
44. **Tate MD, Brooks AG, Reading PC.** 2011. Specific sites of N-linked glycosylation on the hemagglutinin of H1N1 subtype influenza A virus determine sensitivity to inhibitors of the innate immune system and virulence in mice. J. Immunol. **187:**1884–1894. http://dx.doi.org/10.4049/jimmunol.1100295.

45. **Suzuki N, Yamamoto K, Toyoshima S, Osawa T, Irimura T.** 1996. Molecular cloning and expression of cDNA encoding human macrophage C-type lectin. Its unique carbohydrate binding specificity for Tn antigen. J. Immunol. **156:**128–135.
46. **Matlin KS, Reggio H, Helenius A, Simons K.** 1981. Infectious entry pathway of influenza virus in a canine kidney cell line. J. Cell Biol. **91:**601–613. http://dx.doi.org/10.1083/jcb.91.3.601.
47. **Sieczkarski SB, Whittaker GR.** 2002. Dissecting virus entry via endocytosis. J. Gen. Virol. **83:**1535–1545.
48. **Rust MJ, Lakadamyali M, Zhang F, Zhuang X.** 2004. Assembly of endocytic machinery around individual influenza viruses during viral entry. Nat. Struct. Mol. Biol. **11:**567–573. http://dx.doi.org/10.1038/nsmb769.
49. **Francis CL, Ryan TA, Jones BD, Smith SJ, Falkow S.** 1993. Ruffles induced by Salmonella and other stimuli direct macropinocytosis of bacteria. Nature **364:**639–642. http://dx.doi.org/10.1038/364639a0.
50. **Hoffmann PR.** 2001. Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells. J. Cell Biol. **155:**649–660. http://dx.doi.org/10.1083/jcb.200108080.
51. **Amyere M, Mettlen M, Van Der Smissen P, Platek A, Payrastre B, Veithen A, Courtoy PJ.** 2002. Origin, originality, functions, subversions and molecular signalling of macropinocytosis. Int. J. Med. Microbiol. **291:**487–494.
52. **Donaldson JG, Porat-Shliom N, Cohen LA.** 2009. Clathrin-independent endocytosis: a unique platform for cell signaling and PM remodeling. Cell. Signal. **21:**1–6. http://dx.doi.org/10.1016/j.cellsig.2008.06.020.
53. **Mayor S, Pagano RE.** 2007. Pathways of clathrin-independent endocytosis. Nat. Rev. Mol. Cell Biol. **8:**603–612. http://dx.doi.org/10.1038/nrm2216.
54. **van der Vlist M, De Witte L, de Vries RD, Litjens M, De Jong MAWP, Fluitsma D, de Swart RL, Geijtenbeek TBH.** 2011. Human Langerhans cells capture measles virus through Langerin and present viral antigens to CD4+ T cells but are incapable of cross-presentation. Eur. J. Immunol. **41:**2619–2631. http://dx.doi.org/10.1002/eji.201041305.
55. **Gottlieb C, Skinner AM, Kornfeld S.** 1974. Isolation of a clone of Chinese hamster ovary cells deficient in plant lectin-binding sites. Proc. Natl. Acad. Sci. U. S. A. **71:**1078–1082. http://dx.doi.org/10.1073/pnas.71.4.1078.
56. **Stanley P.** 1984. Glycosylation mutants of animal cells. Annu. Rev. Genet. **18:**525–552. http://dx.doi.org/10.1146/annurev.ge.18.120184.002521.
57. **Reeves PJ, Callewaert N, Contreras R, Khorana HG.** 2002. Structure and function in rhodopsin: high-level expression of rhodopsin with restricted and homogeneous N-glycosylation by a tetracycline-inducible N-acetylglucosaminyltransferase I-negative HEK293S stable mammalian cell line. Proc. Natl. Acad. Sci. U. S. A. **99:**13419–13424. http://dx.doi.org/10.1073/pnas.212519299.
58. **Sato M, Kawakami K, Osawa T, Toyoshima S.** 1992. Molecular cloning and expression of cDNA encoding a galactose/N-acetylgalactosamine-specific lectin on mouse tumoricidal macrophages. J. Biochem. **111:**331–336.
59. **Pontow SE, Blum JS, Stahl PD.** 1996. Delayed activation of the mannose receptor following synthesis. Requirement for exit from the endoplasmic reticulum. J. Biol. Chem. **271:**30736–30740.
60. **Su Y, Bakker T, Harris J, Tsang C, Brown GD, Wormald MR, Gordon S, Dwek RA, Rudd PM, Martinez-Pomares L.** 2005. Glycosylation influences the lectin activities of the macrophage mannose receptor. J. Biol. Chem. **280:**32811–32820. http://dx.doi.org/10.1074/jbc.M503457200.
61. **Hillaire MLB, van Eijk M, van Trierum SE, van Riel D, Saelens X, Romijn RA, Hemrika W, Fouchier RAM, Kuiken T, Osterhaus ADME, Haagsman HP, Rimmelzwaan GF.** 2011. Assessment of the antiviral properties of recombinant porcine SP-D against various influenza A viruses in vitro. PLoS One **6:**e25005. http://dx.doi.org/10.1371/journal.pone.0025005.
62. **Cambi A, Koopman M, Figdor CG.** 2005. How C-type lectins detect pathogens. Cell. Microbiol. **7:**481–488. http://dx.doi.org/10.1111/j.1462-5822.2005.00506.x.
63. **Lozach P-Y, Burleigh L, Staropoli I, Navarro-Sanchez E, Harriague J, Virelizier J-L, Rey FA, Desprès P, Arenzana-Seisdedos F, Amara A.** 2005. Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN)-mediated enhancement of dengue virus infection is independent of DC-SIGN internalization signals. J. Biol. Chem. **280:**23698–23708. http://dx.doi.org/10.1074/jbc.M504337200.
64. **Lozach P-Y, Kühbacher A, Meier R, Mancini R, Bitto D, Bouloy M, Helenius A.** 2011. DC-SIGN as a receptor for phleboviruses. Cell Host Microbe **10:**75–88. http://dx.doi.org/10.1016/j.chom.2011.06.007.
65. **Takada A, Fujioka K, Tsuiji M, Morikawa A, Higashi N, Ebihara H, Kobasa D, Feldmann H, Irimura T, Kawaoka Y.** 2004. Human macrophage C-type lectin specific for galactose and N-acetylgalactosamine promotes filovirus entry. J. Virol. **78:**2943–2947. http://dx.doi.org/10.1128/JVI.78.6.2943-2947.2004.
66. **Usami K, Matsuno K, Igarashi M, Denda-Nagai K, Takada A, Irimura T.** 2011. Involvement of viral envelope GP2 in Ebola virus entry into cells expressing the macrophage galactose-type C-type lectin. Biochem. Biophys. Res. Commun. **407:**74–78. http://dx.doi.org/10.1016/j.bbrc.2011.02.110.