Cell-surface receptors on macrophages and dendritic cells for attachment and entry of influenza virus

Sarah L. Londrigan,* Michelle D. Tate,*,† Andrew G. Brooks,* and Patrick C. Reading*,†,2

*The Department of Microbiology and Immunology, The University of Melbourne, Victoria, Australia; and †WHO Collaborating Centre for Reference and Research on Influenza, Victorian Infectious Diseases Reference Laboratory, North Melbourne, Victoria, Australia

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ABSTRACT

Airway Mφ and DCs are important components of innate host defense and can play a critical role in limiting the severity of influenza virus infection. Although it has been well established that cell-surface SA acts as a primary attachment receptor for IAV, the particular receptor(s) or coreceptor(s) that mediate IAV entry into any cell, including Mφ and DC, have not been clearly defined. Identifying which receptors are involved in attachment and entry of IAV into immune cells may have important implications in regard to understanding IAV tropism and pathogenesis. Recent evidence suggests that specialized receptors on Mφ and DCs, namely CLRs, can act as capture and/or entry receptors for many viral pathogens, including IAV. Herein, we review the early stages of infection of Mφ and DC by IAV. Specifically, we examine the potential role of CLRs expressed on Mφ and DC to act as attachment and/or entry receptors for IAV. J. Leukoc. Biol. 92: 97–106; 2012.

Introduction

Influenza viruses are important respiratory pathogens belonging to the Orthomyxoviridae family of enveloped viruses and can be classified into three distinct types: A, B, and C, based on antigenically distinct, internal proteins. IAVs are the major etiological agent causing epidemics in humans and have the potential to cause pandemics. It is well established that cell-surface SA acts as a primary attachment receptor for IAV, following recognition by the viral HA. However, the process of virus entry into cells, resulting in productive replication in airway epithelial cells or uptake by airway Mφ and DC, remains poorly defined. Specialized receptors expressed by Mφ and DC, including those of the CLR family, have emerged as important receptors for attachment and uptake of a range of viruses. We and others [1–4] have recently provided evidence that CLRs may also play a role in IAV infection of Mφ and DC.

VIRAL DETERMINANTS OF IAV INFECTION: THE ENVELOPE GLYCOPROTEINS

The IAV genome is comprised of eight segments of single-stranded, negative-sense RNA, which encode at least 11 proteins. Two viral glycoproteins—the HA and the NA—protrude from the surface of the virion [5] and are key determinants in attachment, entry, and infection of target cells. The HA exists as a trimer of three identical monomers formed by noncovalent association. Each monomer consists of a globular head and a stalk region anchored in the viral envelope by a short hydrophobic sequence. Within the globular head is the receptor-binding site, a shallow pocket of highly conserved amino acids that interact with N-acetyllneuraminic acid (commonly referred to as SA), expressed on oligosaccharide side-chains of cell-surface glycoproteins and glycolipids [6–8]. Following attachment, virus is internalized via endocytosis, and HA undergoes conformational changes in the low pH of the endosomal compartment to expose the fusion peptide at the N-terminus of the HA1 subunit [9, 10]. Fusion of viral and endosomal membranes allows delivery of the viral nucleocapsid into the cell cytoplasm before entry into the nucleus to initiate viral replication [11].

NA is a tetrameric glycoprotein formed by the association of four identical monomers. Each monomer is composed of a globular head and a stalk region embedded in the viral enve-
lope. The majority of antigenic sites is found on the globular head, as well the enzyme-active site that contains a number of charged amino acid residues [12]. The enzymatic function of NA is to cleave SA residues from the cell surface, enabling newly synthesized virions to detach from infected cells. In addition, NA cleaves SA from the IAV glycoproteins, thereby preventing HA-mediated self-aggregation of virus. Hence, HA and NA have opposing functions, such that high NA activity could result in inadequate attachment of HA to cell-surface SA, and conversely, excessive HA activity may limit release of newly formed virions from infected cells. A balance between the activities of the HA and NA is therefore critical in determining the efficiency of infection by different IAV [13, 14].

N-linked glycosylation is a common post-translational modification of mammalian glycoproteins, where oligosaccharide side-chains are attached through N-glycosidic linkages to the Asn residues of the Asn-X-Ser/Thr motif (where X may represent any amino acid except proline) [15]. HA and NA have potential sites for N-linked glycosylation, and the attached oligosaccharides are commonly a mixture of high-mannose (type I: branched structures terminating in the sugar mannose), complex (type II: branched structures terminating in sugars, such as mannose, galactose, GalNAc, and/or fucose), or hybrid-type oligosaccharides [16–19].

On HA, oligosaccharides, attached to the stalk region, are well-conserved between IAV strains and are important for HA stability and conformation [20–22], whereas glycosylation of the globular head can vary markedly in number, location, and type of oligosaccharide [23, 24]. HA serves as the major target for neutralizing antibodies, and glycans on the head of HA are likely to shield or modify antigenic sites [6]. Analysis of H1 sequences (1918–2010) indicate no glycans present on the top of the receptor-binding domain from 1918 and 2009 pandemic IAV strains [25], whereas the majority of seasonal H1 IAV strains is characterized by the presence of three to five glycosylation sites [26, 27]. Since their appearance in the human population in 1968, H3N2 viruses also acquired additional glycans on the head of HA [23, 28, 29], and recent strains carry as many as eight to 10 potential glycosylation sites [27]. These findings are consistent with a role for glycosylation in mediating evasion of antibody-mediated neutralization in the human population.

Loss or gain of glycans on HA can affect biological properties of IAV, such as changing the affinity of HA for host cell-surface receptors [30–34] or enhancing sensitivity to recognition by collectins of the innate immune system [34–38]. The mouse-adapted PR8 (H1N1) strain is notable for its lack of glycosylation on the head of HA [39]. We recently reported that addition of glycans to the PR8 HA or removal of glycosylation sites from the HA of seasonal strain A/Brazil/11/78 (H1N1) modulated sensitivity to collectins and virulence in a mouse model [40]. Therefore, the amount of glycosylation added to HA to circumvent humoral immune responses may be limited by the increased sensitivity of glycosylated IAV to components of innate defense.

**CELLULAR TARGETS OF IAV INFECTION**

In humans, IAV infection is predominantly confined to the upper respiratory tract [41]. Thus, airway epithelial cells and antigen presenting cells (APCs) represent primary targets of IAV infection. It is well-established that attachment to and entry of IAV into epithelial cells result in productive infection, characterized by genomic replication, synthesis of viral proteins, assembly of virions, and release of infectious progeny. MΦ and DC from humans or mice are susceptible to IAV, yet the outcomes of infection are less clear. IAV infection of MΦ/DC has been associated with productive infection [42–44] but also with nonproductive infection, whereby genomic replication and synthesis of at least some viral proteins occur, yet the infectious cycle is blocked prior to virus release [2, 3, 45–50]. These differences may reflect the heterogeneous nature of MΦ and DC, particularly in the lung, as well as susceptibility of MΦ/DC to infection by the particular IAV strain(s) used.

**CELLULAR DETERMINANTS OF IAV INFECTION: CELL-SURFACE RECEPTORS**

Virus attachment and entry into host cells are generally complex multistep processes involving sequential and/or simultaneous recognition of multiple cell-surface receptors. By definition, virus receptors are host-cell molecules (usually membrane-associated), which bind virus attachment proteins and are required for entry [51]. Coreceptors are cell-membrane proteins that bind specifically to viral proteins and are required for entry, in addition to the primary receptor (typically to ensure the continuation of the entry process after binding) [51].

**SA—the primary attachment receptor for IAV**

SA is the primary attachment receptor for IAV, and there is an abundance of SA on the surface of mammalian cells [6]. In nature, SA is generally attached to the underlying galactose residues of glycans by α(2,3)-Gal or α(2,6)-Gal linkages [6]. The conformation of the SA linkage on host cells is an important determinant of virus tropism, in fact, residues within or in the vicinity of the receptor-binding pocket of the viral HA modulate which SA linkages are preferentially recognized [52–54]. In general, human IAV prefer SA linked in an α(2,6)-Gal conformation, which is abundant in the human respiratory tract [52, 55–58], whereas avian IAV strains show a preference for α(2,3)-Gal, which is expressed throughout the avian gastrointestinal tract [59–61]. Differences in receptor specificity between human and avian IAV are likely to be critical factors in limiting interspecies transmission, as well as modulating virulence.

Many studies have described the importance of SA in promoting IAV infection of epithelial and immune cells. Pretreatment of cells with bacterial sialidases removes cell-surface SA and renders cells resistant to IAV [2, 7, 62–64]. In addition, enzymatically swapping the linkage of SA expressed on the cell surface can alter susceptibility to infection by IAV strains with a particular receptor specificity [60, 64, 65].
SA-independent entry of IAV

Although it is generally accepted that treatment with bacterial sialidase prevents IAV infection, desialylated mammalian MDCK cells can support infection, albeit at reduced levels [66], and desialylated human airway epithelial cells were permissive to IAV entry and at least the early stages of infection [57]. One study attempting to elucidate the nature of IAV receptors reported that a mutant CHO cell line deficient in N-linked glycans (Lec1 cells) was largely resistant to IAV infection, despite retaining full capacity for virus binding [67]. This led the authors to propose that SA expressed on glycolipids alone were insufficient for infection of CHO cells and that N-linked glycoprotein(s) were critical for infectious entry. Other studies have confirmed that binding of IAV to sialylated cell-surface receptors does not always result in receptor-mediated internalization [68, 69]. Rappoport et al. [70] examined binding of oligosaccharide probes to MDCK and Vero epithelial cell lines and obtained results consistent with the presence of cell-surface galectins and/or mannose-binding lectins, which they proposed could potentially recognize IAV and contribute to infection. Collectively, these findings imply that the presence of cell-surface SA is not always sufficient for IAV infection. Moreover, attachment and entry of IAV into cells can occur independently of SA. As proposed by Stray et al. [66], SA may enhance binding to the cell surface to increase subsequent and/or simultaneous interaction with secondary and/or coreceptors that are required for virus entry.

C-TYPE LECTINS ON MΦ AND DC AS RECEPTORS FOR IAV

MΦ and DCs are immune cells equipped with an array of specialized pattern recognition receptors (PRRs), including scavenger receptors, toll-like receptors (TLRs), and CLRs, to facilitate recognition and response to a range of microbial pathogens, including viruses [71–76]. Although the specific molecules mediating IAV entry into MΦ and DC have not been defined, we and others [1–4] have reported interactions between IAV and mannose-specific CLRs (e.g., MMR and DC-SIGN/L-SIGN) and galactose-specific CLRs (e.g., MGL).

MMR

The MMR (CD206) is a 42-kDa single-transmembrane protein containing a cysteine-rich domain and a fibronectin domain [77]. The cytoplasmic tail contains two internalization motifs consistent with a role for MMR as a recycling receptor in the endocytic compartment [78–82]. MMR has been detected on human alveolar MΦ [83], mouse peritoneal MΦ [84], and rat splenic MΦ [85], as well as monocyte-derived human DCs [79, 86] and subsets of endothelial cells [87–89]. A wide range of bacteria, fungi, and protozoa is recognized by the MMR, including but not limited to Mycobacterium tuberculosis, Candida albicans, and Leishmania spp. [72, 74].

The role of MMR as a viral receptor is summarized (see Table 1). MMR has been reported to enhance dengue virus infection of MΦ via recognition of oligosaccharides expressed on the viral envelope glycoprotein [90]. MMR also binds the gp120 envelope glycoprotein of HIV-1 [91] and promotes non-productive infection of MΦ [92]. For dengue virus and HIV-1, it is currently unclear if enhanced infection results from direct MMR-mediated endocytosis or by MMR-mediated attachment, promoting binding to additional receptor(s), which mediate virus entry. Aside from pathogen recognition, MMR also mediates antigen uptake and presentation to T lymphocytes [78, 86, 93–95] and has been implicated in lymphocyte homing and adhesion to lymphatic endothelium [96].

MGL

MGL (CD301) is a 42-kDa single-transmembrane glycoprotein with a single CRD (Fig. 1), which forms homo-oligomers on the cell surface. It is a known endocytic receptor with internalization motifs located within the cytoplasmic tail [97–101]. In mice, two distinct isoforms of MGL, namely MGL-1 and MGL-2, have been described, which share 91.5% amino acid homology and have similar expression patterns [102]. However, MGL-1 displays Ca$^{2+}$-dependent specificity for terminal galactose, Lewis-X structures, and terminal GalNAc residues, whereas MGL-2 binds exclusively to terminal GalNAc residues [103, 104]. Like murine MGL-2, rat and human or-
\textbf{CLR-MEDIATED ENHANCEMENT OF IAV INFECTION OF MΦ AND DC}

\textbf{IAV and MMR}

Current evidence suggests that lectin-mediated interactions between IAV and MMR play a critical role in the infection of MΦ. First, CRDs of the MMR have been shown to recognize N-linked glycans on the HA/NA of IAV in a Ca^{2+}-dependent manner [2]. In addition, IAV infection of primary mouse MΦ but not epithelial cells was inhibited by mannan, a complex polymer of mannose residues [2, 3], and susceptibility of the J774 MΦ cell line to IAV infection correlated with levels of MMR expression [2]. Recently, we used direct-binding techniques to further characterize interactions between MMR and IAV. SA-dependent binding of IAV HA to MMR was reported, as well as SA-independent recognition of glycans on viral HA/NA by the lectin activity of MMR [3]. However, sialidase treatment of MΦ greatly reduced susceptibility to IAV infection, demonstrating that efficient infection requires contributions from SA and the lectin activity of MMR.

\textbf{IAV and MGL}

In our efforts to characterize IAV-MMR interactions in more detail, virus-binding assays also revealed Ca^{2+}-dependent binding of murine MGL to IAV [3]. Although MGL is sialylated, binding of MGL to IAV was blocked completely in the presence of galactose, indicating that SA expressed by MGL was not recognized by HA (or at least not by the HA of IAV strains used in the study). Moreover, IAV infection of MΦ was blocked by addition of asialofetuin, a multivalent ligand of MGL. These studies were not designed to discriminate between binding to MGL-1 or MGL-2; however, the MΦ cell lines used expressed only MGL-1, pointing to a role for this receptor in IAV infection of murine MΦ. As for MMR, treatment of MGL-1 MΦ with bacterial sialidase led to a marked reduction in susceptibility to IAV infection [3].

\textbf{IAV and DC-SIGN}

Our studies examining murine MMR and MGL as IAV receptors were informative but relied on (i) correlation between receptor levels and susceptibility to infection and (ii) ability of receptor ligands to block IAV infection. For many viruses, identification of cell-surface receptors has been demonstrated following transfection of gene(s) encoding putative receptor(s) into cell lines that are resistant to infection, such that cells are rendered susceptible to virus entry. When studying IAV, such approaches are complicated by the abundant cell-surface SA on mammalian cells, and it has been difficult to find cell lines that are resistant to infection. Recently, we demonstrated that Lec2 cells, a mutant CHO cell line deficient in cell-surface SA [129, 130], bound IAV poorly and were largely resistant to IAV infection [1]. These studies defined an experimental system, in which SA-independent interactions between IAV and putative cell-surface receptors could be investigated. Expression of DC-SIGN (or its homologue L-SIGN) by SA-deficient Lec2 cells resulted in Ca^{2+}-dependent IAV attachment and enhanced susceptibility to infection [1]. As infection was blocked by mannan, but not by pretreatment with bacterial sialidases, we concluded that DC-SIGN mediated recognition of mannose-rich glycans on IAV to promote SA-independent IAV infection. Wang et al. [4] also used a transfection-based approach to report that DC-SIGN can act as a receptor for H5N1 IAV. In this model, DC-SIGN-mediated H5N1 infection...
of transfected cells was dependent on the presence of cell-surface SA (for infection in cis), and captured virus particles were also transferred to other permissive cells (for infection in trans).

**PROPOSED MODELS FOR CLR-MEDIATED ENHANCEMENT OF IAV INFECTION**

As described above, MMR, MGL, and DC-SIGN can bind IAV and enhance IAV infection, yet the specific mechanisms by which they do this are not clear. A model for CLR-mediated IAV infection of SA-deficient Lec2 cells and **MΦ/DC** is depicted in Fig. 2.

**Lectin-mediated interactions among IAV and MMR, MGL, and DC-SIGN**

Lectin-mediated binding of MMR, MGL, and DC-SIGN to glycans, expressed on the HA/NA glycoproteins of IAV, can occur independently of SA. Highly glycosylated strains of IAV bind these CLRs in a Ca^{2+}-dependent manner [1, 3], and CLR-mediated infection was blocked by multivalent ligands of each CLR in a manner that corresponds to their expression on target cells [1–3]. Poorly glycosylated IAV, such as PR8, did not bind CLRs efficiently and were poor in their ability to infect CLR^{-} cells [1, 3]. The contribution of specific glycans on the head of H1 [40] and H3 [38] IAV in determining sensitivity to soluble C-type lectins has been defined recently, and similar approaches will yield important information as to which glycans on different IAV subtypes are recognized by membrane-associated CLRs.

**SA-mediated interactions**

Endogenous MMR, MGL, and DC-SIGN expressed on mammalian cells are sialylated, but each CLR retains lectin-binding activity for IAV in the absence of SA [1, 3]. Despite the ability of CLRs to bind IAV independently of SA, our studies using murine MΦ (which expressed MMR and/or MGL) suggest a dual dependence on SA expression and lectin-binding activity, as desialylated MΦ were not susceptible to IAV infection [2]. Studies using DC-SIGN-transfected cell lines also showed that pretreatment with sialidase abrogated CLR-mediated enhancement of H5N1 IAV infection [4].

Although interactions between SA and IAV HA are of low affinity [131, 132], the abundance of SA on the surface of mammalian cells provides influenza virus with multiple receptors to increase binding avidity. Therefore, simultaneous binding of multiple HAs to SA would strengthen IAV binding to the cell surface and promote lectin-mediated binding of CLR. The ability of SA to concentrate virions at the cell surface might be particularly important on MΦ/DC, as CLRs are expressed at relatively low levels. For example, there are 10^{3}–10^{5} surface-binding sites for mannosylated ligand/cell for mouse and rabbit MΦ [133–135] and ~1 × 10^{5} MGL molecules/cell for rat peritoneal MΦ [136]. Although detailed binding characteristics between IAV HA/NA and CLRs are yet to be determined, CLRs have been reported to bind other viral glycoproteins with high affinity [137].

For SA-independent infection of epithelial cells, Stray et al. [66] proposed that the requirement for initial interaction with SA to enrich IAV at the cell surface might be circumvented at higher virus concentrations. Similarly, the high levels of CLR expressed on SA-deficient Lec2-DC-SIGN cells [1] may also bypass the need for SA-mediated attachment and enrichment at the cell surface. Hence, although transfection-based approaches allow for isolation of putative IAV receptors in the absence of the confounding complexities of SA–HA interactions, it is critical to confirm the role of CLRs on relevant cell types (i.e., MΦ/DC), where SA is expressed. Such studies will allow us to determine the relevant contributions of SA and CLR to IAV infection of appropriate target cells.

**CLRs—attachment or entry receptors for IAV?**

Our studies using MΦ/DC or CLR-transfected Lec2 cells do not discern between CLR-mediated endocytosis of IAV or whether CLRs represent an (additional) attachment receptor that passes IAV to other entry receptor(s) (Fig. 2A and B). For many viruses, it is unclear whether enhanced infection results

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**Figure 2. Models for CLR-mediated enhancement of IAV infection.** (A) IAV infection of SA-deficient Lec2 CHO cells via CLR. (i) Lectin-mediated binding of the DC-SIGN CRD (shown in blue) to mannose-rich glycans on IAV HA/NA could lead to direct DC-SIGN-mediated endocytosis. Alternatively, (ii) IAV could be passed from DC-SIGN to additional cell-surface receptor(s) (the identity of which is currently unknown; shown in green), resulting in enhanced infection of Lec2 cells. Note that CLRs could also remain associated with IAV to facilitate entry via additional coreceptors. (B) IAV infection of MΦ and DC: a multistep process involving CLRs. (i) IAV HA binds to SA on cell-surface glycoproteins or glycolipids. Abundant cell-surface SA provides multiple sites for IAV attachment, thereby concentrating IAV at the cell surface. (ii) Attachment to cell-surface SA facilitates lectin-mediated binding of CLRs to glycans on IAV HA/NA. Lectin-mediated binding may be strengthened by HA-mediated recognition of SA residues expressed on CLRs. Direct CLR-mediated entry may result, or (iii) CLRs may pass IAV to additional unidentified receptor(s) (shown in green) for virus entry. Binding of IAV to additional receptors could be SA-dependent or -independent. Interactions between IAV and unidentified receptors could also occur independently of CLRs [i.e., Step (i) followed by Step (iii)]. In addition, IAV could be endocytosed directly after binding to sialylated receptors in Step (i).
from CLR-mediated endocytosis or following subsequent interaction with other entry receptors (Table 1). WT and endocytosis-defective DC-SIGN permitted dengue virus infection of transduced cells, indicating that endocytosis via DC-SIGN itself was not essential for infection [140]. Similar approaches will be used to determine whether CLRs can act as direct entry receptors for IAV. As well as promoting infection in cis, CLRs on MΦ/DC can capture and sequester virus, which may then be passed to other permissive cells. In this way, DC-SIGN promotes in trans infection by H5N1 IAV [4], HIV-1 [142], HCV [146], CMV [149], and Ebola viruses [127]. MMR on MΦ has also been reported to facilitate in trans infection of T cells by HIV-1 [138]. These represent additional mechanisms by which CLR-virus interactions can modulate virus dissemination during infection.

CONCLUDING REMARKS, IMPLICATIONS, AND SIGNIFICANCE

Many viruses use a two-step infection process, whereby virus initially binds to an abundant receptor (e.g., SA or heparin sulfate), via a low-affinity interaction, to promote contact with additional receptor(s), which are required for virus entry. For IAV, it seems likely that multiple low-affinity interactions between the viral HA and SA concentrate virus at the cell surface, allowing it to “browse” or “roam” the cell surface until it contacts secondary receptor(s), as posited by Burnet [152]. On MΦ and DC, lectin-mediated binding by CLRs may represent one pathway by which IAV entry and infection can occur. However, at present, it is not clear whether CLRs themselves act as endocytic receptors for IAV or if additional receptors and/or coreceptors are required for virus entry. Understanding the specific mechanisms by which MΦ and DC recognize and internalize IAV may provide important information relevant to the tropism of different IAV for particular airway cells and therefore, pathogenesis. For example, mouse-adapted IAVs, such as PR8, evade detection by CLR [2, 3], infect airway MΦ poorly [1, 2, 153], and induce severe disease in mice following intranasal infection [154]. Moreover, in mice α(2,6)-Gal SA is the predominant linkage of MΦ compared with α(2,3)-Gal SA on epithelial cells [64, 155, 156], suggesting that the particular linkage of SA may be an important factor in recruiting particular IAV to different airway cells.

AUTHORSHIP

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