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Murine MHC class II H2-E binds Influenza H19 head distinct from the sialic acid binding site but sterically conflicts with TCR binding

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Abstract

Receptor recognition by influenza A virus (IAV) hemagglutinin (HA) is a key determinant of viral entry and host range, traditionally-known to be mediated by sialylated glycans. Previous studies have suggested that major histocompatibility complex class II (MHC II), the antigen-presenting molecule recognized by $CD4^+$ T cells, can serve as an entry receptor for several IAVs. The molecular basis of this alternative receptor recognition and its potential implications for MHC II-associated immune functions remain incompletely understood. Here, we report that *in vitro* expressed H19 bound murine H2-E (I-E), whereas H17 and MHC II from other species tested showed no detectable binding, and a 2.65 Å cryo-electron microscopy structure of the H19/H2-E complex was determined. The H2-E α1 domain binds the lateral surface of the HA head outside the canonical sialic acid-binding pocket, forming three contact regions without directly contacting the peptide presented by H2-E. The key residue candidates within the interaction interface indicate the species-dependent recognition of murine and mallard compared to other species. We also observed two or three H2-E bound to a single H19 trimer, but with limited particle numbers. The structure of two H2-E with one H19 trimer was solved to 6.06 Å and an apo H19 structure was also solved here to 2.67 Å. Structural superposition suggested steric incompatibility of the H19 docking with T cell receptor or HLA-DM binding configurations. These findings define the molecular architecture of H19/MHC II complex recognition and suggest a potential link between influenza receptor usage and MHC II-associated immune functions.

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Introduction

As a typical zoonotic pathogen, the natural reservoir of classical influenza A viruses (IAVs) is recognized as waterfowls, but with a broad host range, including diverse avian and mammalian species, together with humans[1,2]. Seasonal influenza viruses impose a substantial constant burden on population health worldwide each year[3]. Furthermore, IAVs from the natural reservoir can occasionally spill over into mammals or humans lacking pre-existing immunity via genome reassortment and genetic mutations[4], especially with new IAV subtypes carrying the potential to cause devastating pandemics[5,6,7]. Based on the genetic and antigenic differences of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), IAV is categorized into different subtypes based on HA and NA as HxNy, which have diverse host tropism[2,5]. Through engagement with host cell receptors, HA of IAVs initiates host cell infection. Sialic acid (SA), as terminal sugar moieties on cellular glycoproteins and glycolipids, is the canonical receptor used by HA to promote entry and initiate the replication cycle[8,9]. Functional and structural evidence indicates that the architecture of SA linking to glycoconjugates confers a key determinant for host range of IAVs. Human and other mammalian IAVs have recognition preference for alpha-2,6-linked SA, whereas avian IAVs display preferential binding of alpha-2,3-linked SA[10,11].

In contrast to conventional HAs, the bat-origin H17 and H18 HA subtypes, as well as avian-origin H19, were shown to fail to bind of SAs for cell entry, but unexpectedly, they use the major histocompatibility complex class II (MHC II) as an entry receptor[12–17]. As a heterodimeric transmembrane protein complex, MHC II molecules consist of alpha (α)- and beta (β)-chains, each comprising two extracellular domains, i.e., juxtaposed membrane-distal domains α1 and β1 and membrane-proximal barrel-shaped Ig-like α2 and β2[18]. Mainly expressed on professional antigen-presenting cells (APCs) such as macrophages, dendritic cells and B cells, MHC II utilizes the α1 and β1-formed peptide-binding groove and presents antigenic peptides of extracellularly derived proteins to $CD4^+$ T cells[19]. The MHC II human leukocyte antigen DR isotype (HLA-DR) was firstly determined as the entry receptor for bat H17 and H18 viruses, and MHC II from different bat species, pigs, mice or chickens also confer susceptibility to infection of H18N1[14,20]. A series of studies expanded the MHC II receptor usage from bat H17, H18 to H1, H2, H3, H5, H7, H9, and H19 viruses[13,15,21]. Although with an obvious species-specific usage by different HAs, the MHC II of diverse species have been shown as the IAV entry receptor, including human, bat, pig, mouse, chicken, duck, and swan[14,15,21]. It was demonstrated that H2-E expression rendered MDCK cells susceptible to

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H19-virus-like particle (VLP) entry and recombinant H19N6 IAV could replicate in MDCK cells expressing H2-E[15].

Though without precise interaction modes between HA and MHC II, chimeric or mutated protein expression indicated the potential binding sites. Studies demonstrated that mutations near the SA receptor binding site of the H3N2 HA1 domain could alter species-specificity of MHC II-mediated entry[22]. For the HA-binding sites on MHC II, the use of chimeric H2-E transfected into HEK293T cells suggested that the MHC II α chain, but not the β chain, was sufficient to facilitate H19 entry, with amino acids in the α1 chain conferring the H2-E-specific cell entry[15]. However, a recent study identified the α2 domain of HLA-DR as key for H18-mediated entry, although some conserved amino acids in the α1 and β1 domains of HLA-DR were also shown to have viral entry-modulating function[13]. Solid knowledge of the interaction interface between HA and MHC II is pivotal for the understanding of the species-specific usage of MHC II as an entry receptor of different IAVs and the explanation of potential interspecies transmission and tropism[21].

Herein, we investigated the interaction between influenza HA and MHC II to define the molecular basis of species-specific receptor recognition. We determined the structure of the H19/H2-E complex at a resolution of 2.65 Å, revealing an α1-mediated recognition interface spatially distinct from the canonical SA-binding site. Comparative binding and sequence analyses provided insights into receptor specificity, while structural superposition suggested potential steric conflicts with TCR and HLA-DM binding configurations. Beyond defining a distinct protein-based receptor-binding mode of IAVs, these findings raise the possibility that influenza HA engagement may influence the accessibility of MHC II to its immune-interacting partners.

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Methods

Expression constructs

A knob-into-hole (KIH)-based expression was used to produce soluble H2-E as previously described[23,24]. Briefly, signal peptide along with the ectodomains of H2-E α chain (NCBI: NM_010381), or HLA-DRA*01:01 (NCBI: NM_019111.4), B-L α chain (NCBI: NM_001245061.1) and DLA α chain (NCBI: NP_001011723.1) featuring thrombin cleavage site and Fc with human IgG1Fc (knob Fc), along with 6xHis tag following alpha chain, were cloned into a pcDNA3.4 vector. Simultaneously, signal peptide is covalently linked to the positive peptide[25](HPPSYVYSQFEKSYRHKR) via a GS linker, and linked to the ectodomains of H2-E β chain (NCBI: NM_010382.2), or positive peptide for HLA-DRB1*01:01 (NCBI: NM_001243965.1), positive peptide for B-L β chain (NCBI: NM_001318995.2), CLIP peptide for DLA β chain (NCBI: NP_001014768.1) incorporating thrombin cleavage site and Fc with human IgG1Fc (hole Fc), along with avi tag, were cloned into pcDNA3.4 vector.

The extracellular domains of H19 (A/Lesser Scaup/CA/3087/2010), and H17 (A/little yellow-shouldered bat/Guatemala/060/2011) HA were separately amplified and cloned into the pFastBac1 insect baculovirus transfer vector, as previously reported[4,26]. A gp67 signal peptide was fused at the N-terminus of the sequence, while a thrombin cleavage site, a trimerization fold of the sequence, avi tag and 6xHis tag were fused at the C-terminus to facilitate protein expression and purification.

Protein expression and purification

MHC II proteins were expressed in Expi293F cells with OPM-293 serum-free medium (OPM Biosciences). The α and β chains of MHC II were co-transfected into Expi293F cells using PEI MW40000 (YEASEN) transfection reagent and expressed at 37°C for 7 days. For protein purification of MHC II with a His-tag, culture supernatant was passed through a Histrap HF column (Cytiva) and further purified by gel filtration (Superdex 200 Increase 10/300 GL, GE Healthcare). For surface plasmon resonance (SPR), MHC II was biotinylated by Biotin-Protein Ligase (GeneCopoeia) overnight at 4°C, followed by gel filtration chromatography purification on a Superdex 200 Increase 10/300 GL column with PBST as running buffer.

HA protein was expressed in Hi5 insect cells using recombinant baculovirus as described[4,26]. Culture supernatants were collected at 48 h after infection, removed by centrifugation and purified by HisTrap HP column followed by SEC (Superdex 200 Increase

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10/300 GL, GE Healthcare). For the SPR assay, the purified HA was digested with thrombin overnight at 4°C and further purified by SEC in PBST.

SPR assay

Interactions between MHC II and HA were tested using an SPR assay on a BIAcore 8K (Cytiva) platform. The assays were performed using a SA chip at 25°C in multi-cycle mode with PBST buffer. Biotinylated MHC II was immobilized on a Sensor Chip SA and different concentrations of HA protein were flowed over the chip surface. After each reaction, the chip was regenerated with 10 mM NaOH. The equilibrium dissociation constant (KD) of each interaction was determined using a 1:1 binding model in BIAcore Insight Evaluation 2.0.15.12933 software (Cytiva).

Cryo-EM sample preparation

Purified H19 HA trimer and H2-E were incubated in 20 mM Tris–HCl (pH 8.0) and 50 mM NaCl at a final total protein concentration of 1 mg/mL and incubated for 2 h at 4°C. A 4μL aliquot was applied to glow-discharged gold grids (UltraAuFoil R1.2/1.3, 300 mesh), followed by plunge-freezing in liquid ethane using a Vitrobot Mark V (Thermo Fisher Scientific) at 8°C and 100% relative humidity. The grids were blotted with a blot force of -4 for 1 s.

Cryo-EM data acquisition

Cryo-EM data were collected on a Titan Krios G4 microscope (Thermo Fisher Scientific) operated at 300 kV and equipped with a Falcon 4i direct electron detector and a Selectris X energy filter. Movies were acquired at a nominal magnification of ×165,000, corresponding to a calibrated pixel size of 0.729 Å, with 40 frames per movie and a total exposure of 50 e^{-} /$Å^{-2}$. Data were collected at a nominal defocus range of -1.0 to -2.0 μm.

Cryo-EM image processing and atomic model refinement

A total of 27,903 micrographs were motion-corrected and subjected to contrast transfer function (CTF) estimation in cryoSPARC (version 5.0.2, exact build to be confirmed)[27]. An initial subset of 3,303 micrographs was used for blob picking, binned particle extraction and 2D classification. Selected particles (n = 22,522) were used for ab initio reconstruction to generate particle-picking templates. Subsequent template-based picking and classification across the full dataset yielded 5,162,843 particles. Following homogeneous refinement and H2-E-focused 3D classification, 1,054,740 particles were selected for further classification. Final unbinned particles (n = 106,397) were refined without imposed symmetry (C1) using non-

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uniform refinement[28], yielding a 2.65 Å reconstruction at the gold-standard Fourier shell correlation (FSC) 0.143 criterion. We also determined the apo H19 HA trimer structure at 2.67 Å resolution, which closely resembles the previously reported H19 HA structure (PDB: 8VCC) in its overall architecture (Supplementary Fig. 2 and Supplementary Table 1).

Post-processing for map interpretation was performed with EMReady[29]. Initial models of H19 HA (PDB 8VCC) and an MHC II template (PDB 1I3R) were fitted into the map in UCSF ChimeraX (v1.9)[30], followed by real-space refinement in PHENIX (v2.1.6)[31] and manual model adjustment in Coot (v0.9.8.95)[32]. Refinement and model-validation statistics are summarized in Supplementary Table1.

A separate lower-resolution reconstruction consistent with two H2-E heterodimers bound to one H19 trimer was obtained at 6.06 Å (Supplementary Fig. 3). The 1:3 H19-/H2-E assembly was generated by applying C3 symmetry to the single H19/H2-E protomer complex and was not determined independently by cryo-EM.

Structural interface analysis

Molecular representations and interface inspection were performed in UCSF ChimeraX[28]. The locations of H19/H2-E complex contact regions were assigned from the refined atomic model. For reproducible quantitative reporting of interface contacts, buried solvent-accessible surface area was computed via PDBePISA, while electrostatic potential and hydrophobicity visualizations were generated in ChimeraX using APBS and the Kyte-Doolittle scale, respectively.

Sequence conservation analysis of the HA and MHC II

MHC II α chain protein sequences were retrieved from UniProt and IPD-MHC. Mouse H2-E α chain (P01904) was used as the reference. Following multiple-sequence alignment, residues corresponding to positions 18–65 were extracted. For each species, the predominant amino acid and the fraction of sequences matching the reference residue were calculated at each position. Within-species conservation was assessed using mean pairwise amino acid identity. Species were grouped by taxonomy and hierarchically clustered within each group. Position-specific conservation was visualized using a custom Python script.

HA sequences from H17 and H19 influenza viruses were subjected to multiple-sequence alignment. Fifteen selected candidate contact positions were extracted. Amino acids at

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corresponding positions were compared across subtypes and classified as conserved or divergent relative to H19[33]. Sequence differences were visualized using a custom Python script.

Structural superposition and steric clash analysis

To assess the potential steric interference between H19 HA and MHC II-interacting proteins, published structures of MHC II complexes with CD4 (PDB: 1JL4), LAG-3 (9BF9), Epstein–Barr virus gp42 (1KG0), TCR (1FYT), HLA-DM (4FQX), and bacterial superantigens SEB (1SEB), SEI (2G9H) and SPE-C (1HQR) were retrieved from the Protein Data Bank. The MHC II molecules in these structures were structurally superimposed onto H2-E in the H19/H2-E complex using UCSF ChimeraX (v1.9). The associated binding partners were retained in their aligned orientations to generate comparative structural models. Potential steric clashes between H19 HA and these proteins were assessed by visual inspection of their spatial overlap. These models were used for structural comparison and do not represent experimentally determined ternary complexes.

Results

Influenza H19 HA bound to murine MHC class II H2-E *in vitro*

To define the MHC II-binding profiles of influenza virus HA, we examined the interactions of H19 and H17 HAs with murine H2-E, human HLA-DR, chicken B-L and canine DLA-DR. Recombinant H19 and H17 HAs, and four MHC II molecules were purified by size-exclusion chromatography and assessed by SDS-PAGE (Supplementary Fig. 1).

SPR analysis revealed concentration-dependent binding of H19 HA to murine H2-E, with apparent equilibrium dissociation constants (KD) of 4.54 μM (Fig. 1A). In contrast, no detectable binding of H17 HA to H2-E was observed (Fig. 1E). Furthermore, none of the two HAs exhibited appreciable positive binding responses to human HLA-DR, chicken B-L or canine DLA-DR (Fig. 1B-D,F-H). These results indicate that H19, but not H17 HA, prefers to recognize murine H2-E among the HA/MHC II combinations examined.

Cryo-EM reveals lateral engagement of the H19 head by H2-E

To determine how H19 recognizes H2-E, we analyzed the H19/H2-E complex by cryo-electron microscopy (Cryo-EM). From 27,903 micrographs, successive particle classification and refinement yielded a final reconstruction from 106,397 particles at an overall resolution of 2.65 Å (Supplementary Fig. 2). The map resolved an H19 HA trimer associated with one H2-E heterodimer, allowing the three HA protomers, the H2-E α and β chains and the bound

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peptide to be assigned (Fig. 2A). The fitted atomic model showed that H2-E approaches the trimer laterally and contacts the membrane-distal HA head, rather than the stalk, at the surface of one protomer. The peptide-binding platform faces the HA head in this arrangement, defining the overall orientation of the recognition interface.

Notably, the 2D class averages revealed particle projections suggestive of two or potentially three H2-E molecules associated with a single H19 trimer (Fig. 2B). In addition to the single H2-E-bound complex resolved at high resolution, a separate 6.06 Å Cryo-EM reconstruction showed two H2-E molecules associated with one H19 trimer (Supplementary Fig. 3A). The corresponding two H2-E assembly is illustrated in the atomic model (Fig. 2C). A structural model accommodating three H2-E molecules around the trimer was also generated (Supplementary Fig. 3B), although this three-receptor arrangement was not resolved experimentally. The two-H2-E reconstruction indicates that H19 can accommodate more than one H2-E molecule in the observed *in vitro* assembly, while the model illustrates a possible higher-occupancy arrangement.

Reciprocal surface views delineated a discrete H19-binding footprint on H2-E and the corresponding H2-E-binding region on the lateral surface of the HA head (Fig. 2D). PISA analysis revealed an average buried interface area of approximately 514 Å^2 per binding partner. Mapping of electrostatic potential and hydrophobicity onto these surfaces further defined the physicochemical context of the interface (Fig. 2D). The opposing binding surfaces exhibited complementary electrostatic features and hydrophobic patches, suggesting favourable physicochemical interactions at the H19/H2-E complex interface. These data establish the overall architecture of H19/H2-E complex recognition and reveal that engagement of the HA head can occur at more than one site on an H19 trimer.

H19 recognizes the H2-E α1 domain in a peptide-independent binding mode

The H19/H2-E complex interface is centered on the H2-E α1 domain. In particular, the α1 helix and adjacent β-sheet floor contact the HA head, whereas the bound peptide remains within the MHC II groove and does not make direct contacts with H19 in the resolved structure (Fig. 3A). H19 therefore recognizes the H2-E protein surface rather than the exposed peptide itself. This geometry is consistent with a peptide-independent mode of direct recognition.

Inspection of the opposing interfaces identified three contact regions, termed Core A, Core B and Core C on the HA head (Fig. 3B). Core A (residues 75-80, in H3 numbering), comprising H19 key residues Glu75, Asp77, Leu78 and Leu80, directly engages H2-E residues Lys18,

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Gln57, Leu60 and Ala64. The close proximity of H19 Leu78 and Leu80 to H2-E Leu60 suggests nonpolar packing, accompanied by neighboring polar interactions. Core B (120-152) comprises three contact clusters. In Core B1 (residue 120), H19 Arg120 interacts with H2-E Gln57, whereas Core B2 (residue 132) involves H19 Tyr132 and H2-E Glu40. Core B3 (146-152) is formed by H19 Ser146, Arg149 and Arg152, which are positioned near H2-E Ile36, Glu37, Lys38 and Ser39, forming a network of potential hydrogen-bonding and electrostatic interactions. Core C (255-255a, "a" represents insertion at the H3 numbering stage) involves H19 Tyr255a positioned near H2-E Glu55, forming an additional localized contact. The cryo-EM densities further support the assignment of key interfacial residues (Supplementary Fig. 3C). Residue-level interaction analysis identified 145 van der Waals contacts and eight potential hydrogen bonds across the H19/H2-E interface (Supplementary Table 2). Superposition of H2-E in the H19/H2-E complex onto H2-E in the peptide–MHC II complex alone showed only minor differences at the H19-binding site (Fig. 3C). In contrast, H19 in the complex differed modestly from the apo H19 structure determined here, with the conformational changes confined to loops at the H2-E-binding site (Fig. 3D).

Together, these three regions define a distributed interface dominated by the H2-E α1 domain and lacking direct contacts with the presented peptide. They identify candidate residues and structural elements for the determinants of H19/H2-E binding.

Conservation of interface residues across MHC II species and HA subtypes

Conservation of the H19/H2-E interface was first assessed across the four MHC II molecules tested (Fig. 4A, C). Among the seven H2-E residues forming hydrogen bonds to H19, Glu55 and Gln57 in the α1 helix are invariant in mouse, human, dog and chicken, whereas residues in the other five positions vary. And for the sequential residues "I-E-K-S-E" motif (36-40) in the A-B turn of H2-E, especially Ile36 is conservatively replaced by Met in human and dog. Position 37 Glu of H2-E loses its charge as an Ala in humans and shortens to Asp in chicken. Position 38 Lys of H2-E becomes Ala in chicken. Position 39 Ser of H2-E acquires Lys in humans and dogs, opposing a second positive charge to the Arg152 guanidinium, or becomes Ala in chicken. Chicken B-L additionally replaces Glu40 with Gln. These substitutions remove or reposition donor and acceptor groups, and would be expected to perturb the hydrogen bonds formed by Arg152 and Tyr132. They are consistent with the absence of detectable binding to human, dog and chicken MHC II (Fig. 1).

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To place these four molecules in a broader context, the region corresponding to H2-E residues 18-65 was compared across vertebrate DRA sequences (Supplementary Fig. 4). Positions 36-40 carry lineage-specific combinations, with primates mostly retaining the "M-A-K-K-E" motif while rodents, carnivores, ungulates and cetaceans use alternative sets, and avian α chains diverging further. Among the non-murine α chains examined, mallard duck Ap-DR is the closest match to the "I/L-E-K-S-E" motif at the interface, preserving six of the seven hydrogen-bond partners and differing only by the conservative I36L change (Fig. 4A).

On the HA side, three of the six hydrogen-bond partners differ between H19 and H17. Arg120 turns into Lys, Tyr132 becomes Thr and Arg152 becomes Asn from H19 to H17, whereas Ser146, Arg149 and Tyr255 are conserved (Fig. 4B, D). Lys120 and Thr132 retain potential donors, but Asn152 cannot reproduce the bidentate contacts that the Arg152 guanidinium makes with Lys38 and Ser39 of H2-E. H17 additionally carries non-conservative replacements at Core A (Asn75, Asn78) and at positions 133, 135 and 255, but without detectable binding to H2-E (Fig. 1).

The same comparison identified H9 as the only subtype besides H19 that conserves all six hydrogen-bond partners, together with the remaining Core A and Core C residues (Supplementary Fig. 5 and 6). H9 differs from H19 at only three positions (Ser75, Ile135 and Gly145).

Steric conflicts of the H19/H2-E complex prevent H2-E binding to either TCR or HLA-DM

To investigate the structural compatibility of H19-bound H2-E with other MHC II-interacting proteins, we superimposed the H19/H2-E complex onto previously-determined MHC II complexes involved in antigen recognition and immune regulation (Fig. 5). Structural alignment with the TCR/MHC II/peptide complex (PDB 1FYT) revealed substantial steric overlap between the TCR and H19 HA (Fig. 5A). Although H19 binds laterally to the H2-E α1 domain without directly contacting of the presented peptide, its binding footprint partially overlaps the corresponding TCR-contacting region of the MHC II α1 helix, and its position overlaps with the space occupied by the TCR above the MHC II peptide-binding platform. The superimposed model therefore indicates that the H19-bound configuration is sterically incompatible with the reference TCR docking orientation, suggesting that HA engagement could restrict TCR access to peptide-loaded MHC II. Notably, this spatial conflict does not require direct occupation of the peptide presented by H19.

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A similar structural comparison with the HLA-DM–MHC II complex (PDB 4FQX) revealed pronounced spatial overlap between H19 and HLA-DM (Fig. 5B), indicating steric incompatibility between the two binding configurations. In contrast, superposition with CD4 (PDB 1JL4) and LAG-3 (PDB 9BF9) revealed no obvious steric overlap with H19 in the corresponding models (Fig. 5C, D). Both proteins occupied regions spatially separated from the H19-binding interface, suggesting that their reference binding orientations could be accommodated without direct structural interference from H19.

We further extended the comparison to pathogen-derived MHC II-binding proteins. Superposition with Epstein–Barr virus gp42 (PDB 1KG0) revealed no apparent spatial conflict with H19 (Supplementary Fig. 7D), whereas the bacterial superantigens SEB (PDB 1SEB), SEI (PDB 2G9H) and SPE-C (PDB 1HQR) exhibited steric overlap in the corresponding models (Fig. S7A, B, C).

Together, these structural comparisons reveal distinct patterns of spatial compatibility between H19-bound H2-E and other MHC II-associated proteins. In particular, the predicted steric overlap with TCR and HLA-DM contrasts with the absence of obvious overlap with CD4 and LAG-3, indicating that H19 binding may selectively constrain the accessibility of different MHC II interaction surfaces.

Discussion

MHC II molecules serve as entry receptors for influenza A viruses[13–15,21,22,34,35], substantially expanding the conventional paradigm of influenza receptor usage. Although MHC II-dependent entry has been demonstrated for bat-derived H17 and H18, avian H19, and several conventional influenza HA subtypes, the molecular determinants governing receptor recognition and host specificity remain incompletely understood. Here, we determined a 2.65 Å Cryo-EM structure of H19 HA in complex with murine H2-E, revealing an interaction interface centered on the membrane-distal α1 domain of MHC II. H2-E engages the lateral surface of the HA head through three distinct contact regions, involving a combination of hydrophobic and polar interactions, without directly affecting the presented peptide. These findings extend previous functional studies of MHC II-mediated influenza entry by defining the molecular organization of the H19/H2-E complex interface and providing a structural basis for investigating the determinants of receptor specificity.

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The H19/H2-E complex interface lies largely outside the canonical SA receptor-binding site (RBS), which is formed by the 130-loop, 190-helix and 220-loop[12,35]. The H-2E recognition of H19 involves its protein surface through a broader interface located along the side of the HA head. This arrangement is consistent with the finding that wild-type H19 lacks detectable binding to conventional sialylated receptors, whereas substitutions at positions 134, 137 and 226 within its putative RBS can restore SA-dependent entry without abolishing MHC II-mediated entry[15]. Moreover, human and avian H2N2 viruses and selected H3N2 viruses can use both SAs and MHC II as entry receptors[22,34]. These observations suggest that the two receptor-recognition functions can coexist within the same HA architecture and need not be mutually exclusive. Mutations outside a direct binding interface may alter local conformation, receptor accessibility or the conformational dynamics of HA. Indeed, mutations near the conventional SA RBS influence MHC II-dependent entry of some H3N2 viruses[22]. Thus, HA possesses a structurally adaptable receptor-binding head in which glycan and protein recognition can be differentially regulated, providing a potential evolutionary route between predominantly SA-dependent, predominantly MHC II-dependent and dual-receptor phenotypes of IAV subtypes[36].

The differential MHC II-binding profiles of H17 and H19 as shown in SPR further emphasize that receptor recognition cannot be inferred solely from overall HA structural similarity. Our SPR analysis demonstrated binding of H19 to murine H2-E, whereas H17 showed no detectable binding. Previous studies nevertheless established MHC II-dependent entry by H17 and H18 viruses[14,20]. Notably, functional studies associated H19-mediated entry predominantly with determinants in the MHC II α1 domain[15], consistent with the direct recognition interface revealed by our structure. In contrast, mutational analysis of H17/H18-mediated entry identified the MHC II α2 domain as important for infection[13]. Although residues required for viral entry do not necessarily represent direct binding contacts, these observations suggest that related HA subtypes may differ in their recognition of MHC II.

To further examine HA-side determinants, we compared 15 H19 interface-associated positions across H1-H19 subtypes. These positions exhibited varying degrees of conservation, with H9 showing a relatively similar residue pattern to H19. Recent studies have also demonstrated MHC II-dependent entry by several conventional HA subtypes, including H9, while deep mutational scanning of H5 and H7 revealed that individual HA substitutions can substantially influence MHC II-dependent entry[21]. Together, these biochemical, structural and

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functional observations suggest that MHC II recognition may involve both shared and subtype-specific HA surface features, rather than a universally-conserved set of interface residues.

Recently, two separate preprint studies reported cryo-EM structures of H5 mutant HA/tufted duck MHC II complexes at a resolution of 4.8 Å and H18 HA/HLA-DR was reported, respectively, although the limited resolution precluded detailed characterization of their receptor-binding interfaces[21,37]. However, the 2.65 Å structure of the H19 HA/H2-E complex determined here provides a high-resolution view of the molecular interactions, which reveals precise structural insights into HA/MHC II recognition and provides a molecular basis for understanding receptor specificity across divergent influenza HA subtypes.

The species specificity of H19 HA recognition may reflect the physicochemical compatibility of the MHC II binding interface rather than sequence variation alone. Previous mutational studies demonstrated that replacing human HLA-DR residues M61/A62/K64 with their murine counterparts partially restored H19-mediated entry, whereas additional substitutions in the α1 domain further enhanced receptor compatibility[15]. Our structure provides a possible molecular explanation for these observations. The corresponding H2-E region, particularly Glu37, Lys38 and Ser39, lies near the positively-charged H19 residues Arg149 and Arg152. In contrast to the “I/L-E-K-S-E” motif shared by murine and mallard MHC II, the corresponding position 39 is occupied by Lys in several non-compatible orthologues, including human HLA-DR and canine DLA-DR. Introduction of an additional positive charge at this position could create unfavorable electrostatic interactions with the HA surface and alter local side-chain packing. However, the presence of Lys38 within the compatible H2-E interface suggests that the spatial arrangement of charged residues, rather than charge alone, is critical. Moreover, the incomplete restoration of H19-mediated entry by individual residue-group substitutions indicates that receptor compatibility cannot be attributed to a single motif. Together, these observations suggest that species-dependent H19 recognition arises from the combined effects of local electrostatic complementarity and cooperative interactions across the broader MHC II interface.

The H19/H2-E complex structure concerns the relationship between receptor recognition and the antigen-presenting function of MHC II. Although the observed complex contains a peptide in the MHC II binding groove, H19 does not contact it[38]. This indicates that the principal direct recognition determinants reside within the MHC II protein itself, rather than in the exposed side chains of the presented antigen. Such a binding mode could allow H19 to

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recognize multiple peptide-loaded forms of a compatible MHC II molecule. However, MHC-bound peptides can influence the conformation and dynamic properties of the peptide-binding platform, potentially affecting interactions at neighboring protein surfaces[39]. Furthermore, the contribution of β-chain residues to H5 recognition raises the possibility that the peptide repertoire may indirectly modulate certain HA–MHC II interactions[40]. Therefore, the present structure supports a peptide-independent direct contact interface, whereas functional peptide independence remains to be established by comparing H2-E complexes loaded with different peptides.

The potential interference with TCR recognition is particularly intriguing because H19 engages the MHC II protein surface without directly contacting the presented peptide. If H19 can recognize different peptide-loaded forms of H2-E, its binding could potentially restrict multiple antigen-specific $CD4^+$ T cell responses rather than being limited to a particular peptide epitope[41]. A relevant precedent is provided by Epstein-Barr virus gp42, which uses MHC II as an entry co-receptor. Structural and functional studies demonstrated that gp42 interferes with TCR recognition of peptide-loaded HLA-DR and reduces antigen-specific $CD4^+$ T cell activation without detectably disrupting MHC II maturation or surface expression[40,42]. Although H19 and gp42 engage MHC II through distinct structural arrangements, the predicted steric conflict between H19 and the TCR docking region raises the possibility of a related mechanism of interference with antigen recognition[43-45]. Nevertheless, the extent of such interference would depend on the peptide-loaded MHC II conformations recognized by H19 and the proportion of surface MHC II molecules occupied by HA[46].

Beyond TCR recognition, H19 engagement may have different consequences for other MHC II-associated immune interactions. HLA-DM facilitates peptide exchange and selection within endosomal compartments[47], and structural studies have demonstrated that this process involves substantial rearrangements of the MHC II peptide-binding platform. The predicted spatial incompatibility between H19 and HLA-DM therefore raises the possibility that HA engagement could affect peptide editing, although this would require their association with MHC II in the same intracellular compartment and compatible conformational states. In contrast, the absence of obvious steric overlap with CD4 and LAG-3 suggests that their MHC II-binding sites may remain accessible. Moreover, the binding of MHC II/H19 is also not overlapped with the bacterial superantigens, which may indicate an additive inhibition of MHC II by virus and bacteria simultaneously[48,49]. These observations suggest that H19 engagement may influence different stages of MHC II-mediated immune recognition.

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The trimeric architecture of HA introduces an additional consideration regarding receptor engagement. Although the principal high-resolution H19/H2-E reconstruction contains one H2-E heterodimer bound to an HA trimer, a second reconstruction at 6.06 Å revealed a complex consistent with the association of two H2-E molecules. Structural modelling also suggested that three H2-E heterodimers could potentially be accommodated around the trimer. This arrangement is conceptually consistent with the multivalent nature of viral attachment and with previous models of H18-mediated MHC II engagement[16,37]. Multiple receptor contacts could increase the effective avidity of HA for MHC II-expressing membranes, promote receptor clustering or facilitate stable attachment during the early stages of entry. The experimentally observed two-receptor complex therefore supports the structural feasibility of multivalent engagement, whereas the proposed three-receptor configuration and its functional consequences require further investigation.

Finally, MHC II recognition represents an additional dimension of influenza host adaptation. Its expression on antigen-presenting cells may provide alternative routes of viral entry beyond conventional SA-dependent pathways[35]. Previous studies demonstrated MHC II-mediated entry of H2N2 viruses into human airway cultures and macrophages, while investigations of bat influenza highlighted the importance of receptor compatibility alongside other viral determinants of host adaptation[14,34,50]. Although productive infection and transmission depend on additional viral and host factors, HA–MHC II receptor compatibility emerges as a molecular component of influenza host adaptation and a potentially informative indicator for assessing cross-species infection.

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Data Availability

Cryo-EM data and structures will be released upon formal publication.

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Figure 1. Binding characteristics of H19 and H17 HAs toward MHC class II molecules from different species

(A – D) SPR sensorgrams of H19 HA binding to murine H2-E (A), human HLA-DR (B), canine DLA-DR (C), and chicken B-L (D). (E – H) SPR sensorgrams of H17 HA binding to murine H2-E (E), human HLA-DR (F), canine DLA-DR (G), and chicken B-L (H). Curves of different colors represent different HA concentrations; black dashed lines indicate kinetic fitting. The x-axis shows time (s), and the y-axis shows response units (RU). KD denotes the equilibrium dissociation constant.

Figure 2. Cryo-EM structure and binding-interface characteristics of the H19/H2-E complex

(A) Three-dimensional cryo-EM reconstruction of the H19/H2-E complex shown in front, back, and top views. (B) Representative two-dimensional class averages of the H19/H2-E complex. (C) Atomic model of the H19/H2-E complex in front and top views. (D) Electrostatic potential, surface hydrophobicity, and binding-surface distributions at the H19/H2-E interface. The three HA protomers are colored cyan, purple, and pale yellow; the H2-E α and β chains are colored pink and yellow, respectively, and the bound peptide is colored green. Red and blue indicate negative and positive electrostatic potential, respectively; yellow and blue denote hydrophobic and hydrophilic surface regions, respectively. Binding surfaces are highlighted in pink and cyan. Arrows indicate the direction of structural rotation.

Figure 3. Molecular structural basis of H19/H2-E interactions

(A) Overall structure and enlarged views of the H19 HA/H2-E binding interface, showing the H2-E structural elements involved in binding and three principal interaction regions on the HA surface. (B) Key interacting residues and intermolecular contacts within Core A, Core B, and Core C; Core B is further subdivided into B1, B2, and B3. (C) Comparison of local conformations in the H2-E binding-associated region. (D) Overall and local structural comparison of unbound H19 HA and H19 HA in the H2-E-bound state. Core A, Core B, and Core C are shown in orange-red, dark blue, and brown, respectively. The H2-E α chain is

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shown in pink. Key amino acid residues are displayed as sticks, yellow dashed lines indicate potential hydrogen bonds, and dashed boxes indicate enlarged regions.

Figure 4. Sequence conservation and variation of key residues at the H19/H2-E binding interface

(A) Amino acid sequence alignment of the MHC class II α chains from murine H2-E, duck Ap-DR, human HLA-DR, canine DLA-DR, and chicken B-L. (B) Amino acid sequence alignment of the H2-E-binding regions of H19 and H17 HAs from different isolates. (C) Comparison of key H19 HA-interacting residues in MHC class II α chains from different species. (D) Comparison of corresponding H2-E-interacting residues in H19, H18, and H17 HAs. Red backgrounds in the sequence alignments indicate conserved amino acids, and blue boxes indicate aligned regions. Yellow dots mark residues involved in hydrogen bonds, whereas green dots indicate other intermolecular contacts.

Figure 5. Potential spatial effects of H19 HA binding on interactions with MHC class II-associated proteins

(A) Structural superposition of the H19/H2-E complex with the TCR/MHC class II complex (PDB: 1FYT). (B) Structural superposition of the H19/H2-E complex with an HLA-DM-associated complex (PDB: 4FQX). (C) Structural superposition of the H19/H2-E complex with a CD4-associated complex (PDB: 1JL4). (D) Structural superposition of the H19/H2-E complex with an LAG-3-associated complex (PDB: 9BF9). TCR, HLA-DM, CD4, and LAG-3 are shown in red; the H2-E α and β chains are shown in pink and yellow, respectively, and HA is shown in cyan. Front and top views are shown for each model. “Clash” indicates a steric clash in the structural model, whereas “No clash” indicates no apparent steric clash. Red circles and red surfaces denote the ligand-binding surface; blue circles and blue surfaces denote the H19-binding surface on H2-E; purple indicates regions of spatial overlap.

Figure S1. Protein purification and biochemical characterization of MHC class II molecules from different species and H19 HA

(A-E) Size-exclusion chromatography and SDS-PAGE analyses of human HLA-DR (A), murine H2-E (B), chicken B-L (C), canine DLA-DR (D), H19 HA (E)and H17 HA (F). Blue curves show absorbance at 280 nm; the x-axis indicates elution volume (mL), and the y-axis indicates A280 (mAU). M denotes molecular weight markers, and +DTT and −DTT indicate reducing and nonreducing conditions, respectively.

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Figure S2. Cryo-EM data processing and three-dimensional reconstruction of the H19/H2-E complex

(A, F) Representative raw cryo-EM micrographs. (B, G) Representative two-dimensional class averages and three-dimensional reconstructed density maps. (C, H) Cryo-EM data processing and reconstruction workflows. (D, I) Particle orientation distributions in the final reconstructions. (E, J) Fourier shell correlation (FSC) curves. Panels A – E show the workflow for the H19/H2-E complex, whereas panels F – J show the workflow for the HA trimer. A total of 27,903 micrographs were collected, and 106,397 particles were used to obtain a 2.65 Å reconstruction. The final density map is colored by local resolution; the overall resolution was determined using the FSC = 0.143 criterion.

Figure S3. Structural models of H19 HA bound to multiple H2-E molecules and local density features

(A) Cryo-EM density map and structural model of an H19 HA trimer bound simultaneously to two H2-E molecules. (B) Structural model of one HA trimer bound to three H2-E molecules, constructed on the basis of the single-H2-E binding mode. (C-D) Local cryo-EM density fitting in the H2-E peptide-binding region and adjacent structural elements of the H19 HA/H2-E complex. HA is colored cyan; the H2-E α and β chains are colored pink and yellow, respectively; and the bound peptide is colored green. Semitransparent gray represents the cryo-EM density. The overall resolution of the two H2-E-bound complex in (A) is 6.06 Å.

Figure S4. Structural and sequence conservation of the HA-binding-associated region of vertebrate MHC class II α chains

Top: Representative structures of MHC class II molecules from mouse, duck, rat, and human. Bottom: Amino acid sequence alignment of residues 18 – 65 of vertebrate MHC class II α chains and analysis of within-species sequence identity. Red backgrounds indicate residues identical to the mouse reference sequence, whereas white backgrounds indicate amino acid substitutions. Arrowheads above the alignment mark selected positions. Numbers in parentheses indicate the numbers of sequences analyzed; within-species identity (%) is shown on the right.

Figure S5. Sequence variation at key H2-E interface residues among H1 – H19 HA subtypes

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Comparison of amino acids at 15 selected interface-associated positions across representative sequences of H1 – H19 HA subtypes. Residues are numbered according to the H3 reference. Solid red backgrounds indicate positions fully conserved across all compared sequences; red letters indicate amino acid substitutions relative to the H3 reference.

Figure S6. Amino acid sequence alignment of the HA head region across H1 – H19 subtypes

Amino acid sequence alignments of representative H1 – H19 HA sequences corresponding to H3 residues 65-114, 115-159, 159a-210, and 211-261, with key H19/H2-E interaction residues highlighted. Residues are numbered according to the H3 reference. Solid red backgrounds denote fully conserved positions, red letters denote substitutions relative to the reference sequence.

Figure S7. Spatial compatibility of H19 HA with MHC class II-associated superantigens and viral proteins

(A) Structural superposition of SEB (PDB: 1SEB) with the H19/H2-E complex. (B) Structural superposition of SEI (PDB: 2G9H) with the H19/H2-E complex. (C) Structural superposition of SPEC (PDB: 1HQR) with the H19/H2-E complex. (D) Structural superposition of EBV gp42 (PDB: 1KG0) with the H19/H2-E complex. The corresponding superantigens or viral protein are shown in red, HA is shown in cyan, and the H2-E α and β chains are shown in pink and yellow, respectively. Front and top views are shown for each model.

Table S1. Cryo-EM data collection, reconstruction, and atomic model refinement statistics for the H19/H2-E complex and related HA structure

The table summarizes cryo-EM data acquisition parameters, particle numbers, three-dimensional reconstruction resolutions, model composition, and geometric quality metrics. FSC, Fourier shell correlation; RMSD, root-mean-square deviation; CTF, contrast transfer function.

Table S2. Amino acid residue contacts at the H19/H2-E binding interface

The table summarizes key interacting residues between H19 HA and H2-E and the corresponding numbers of van der Waals contacts and potential hydrogen bonds. Regular numbers represent the numbers of van der Waals contacts, whereas underlined numbers indicate the numbers of potential hydrogen bonds. Van der Waals contacts were counted

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using a 4.5 Å distance cutoff; hydrogen bonds were analyzed using PDBePISA with a 3.5 Å distance cutoff.