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Major Histocompatibility Complex Class II Expression and Hemagglutinin Subtype Influence the Infectivity of Type A Influenza Virus for Respiratory Dendritic Cells[▽]

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Dendritic cells (DC) play a key role in antiviral immunity, functioning both as innate effector cells in early phases of the immune response and subsequently as antigen-presenting cells that activate the adaptive immune response. In the murine respiratory tract, there are several respiratory dendritic cell (RDC) subsets, including $CD103^+$ DC, $CD11b^{hi}$ DC, monocyte/macrophage DC, and plasmacytoid DC. However, little is known about the interaction between these tissue-resident RDC and viruses that are encountered during natural infection in the respiratory tract. Here, we show both *in vitro* and *in vivo* that the susceptibility of murine RDC to infection with type A influenza virus varies with the level of MHC class II expression by RDC and with the virus strain. Both $CD103^+$ and $CD11b^{hi}$ RDC, which express the highest basal level of major histocompatibility complex (MHC) class II, are highly susceptible to infection by type A influenza virus. However, efficient infection is restricted to type A influenza virus strains of the H2N2 subtype. Furthermore, enhanced infectivity by viruses of the H2N2 subtype is linked to expression of the I-E MHC class II locus product. These results suggest a potential novel role for MHC class II molecules in influenza virus infection and pathogenesis in the respiratory tract.

Influenza virus, a member of the *Orthomyxoviridae* family of viruses, is a human pathogen of great interest that causes respiratory tract infections that result in yearly epidemics and occasional severe pandemics (7). Influenza virus primarily infects epithelial cells of the upper respiratory tract, but certain highly pathogenic strains can infect pneumocytes of the lower respiratory tract as well (26, 34, 38, 41). Additionally, we have recently shown that murine respiratory dendritic cells (RDC) are susceptible to infection with at least one influenza virus strain (A/Japan/305/57) (13). It is has not been established whether different influenza virus strains can infect RDC with comparable efficiencies. Previous studies evaluating DC susceptibility to infection by influenza virus have utilized splenic and bone marrow-derived DC, both of which differ in properties from DC resident in the respiratory tract (4, 27, 28). Therefore, very little is currently known about the interaction of influenza virus with tissue-resident RDC that encounter infectious influenza virus during natural infection in the respiratory tract.

Dendritic cells (DC) are a distinct lineage of hematopoietic cells derived from myeloid and lymphoid progenitors in the bone marrow that reside in both lymphoid organs and peripheral tissues, where they play key roles in the immune response to viral infection (3, 11). As innate defenders in the periphery, DC recognize virus-associated molecular patterns via endosomal Toll-like receptors and cytoplasmic RIG-I-like receptors and subsequently produce a variety of inflammatory cytokines and chemokines involved in immune cell activation and recruitment to the site of infection. Additionally, specific subsets of DC have been shown to produce molecules, i.e., type I interferons, with direct antiviral activity (14, 16, 22, 25). Furthermore, maturation of DC that acquire viral antigens (Ag) in the periphery, either through phagocytosis of infected cells or direct infection by the virus, is followed by migration to draining lymphoid organs, where the DC serve as antigen-presenting cells (APC) that stimulate the adaptive immune response (12, 15).

The plethora of functions exhibited by DC is highlighted by the recent identification of a number of unique tissue-specific subsets of these cells. Within the respiratory tract, there are at least four distinct RDC subsets that have been identified thus far (18, 35, 39, 40). Airway $CD103^+$ RDC are localized to intraepithelial and subepithelial spaces (and perivascular sites), and recent work in our laboratory demonstrated that, following influenza virus infection, these cells acquire influenza virus Ag and migrate to lung-draining lymph nodes, where they present Ag to both $CD4^+$ and $CD8^+$ T cells and induce their proliferation and differentiation into effector cells (15). A subset of $CD103^-$ $CD11b^{hi}$ RDC localized to the lung interstitium and perivascular regions also present influenza Ag to $CD4^+$ T cells and induce effector differentiation in lung-draining lymph nodes (15). Plasmacytoid DC (pDC) in the lung parenchyma, although less abundant than $CD103^+$ and $CD11b^{hi}$ RDC, function as potent producers of type I interferons (9, 17) and are required for influenza virus-specific antibody production (12).

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Finally, a substantial population of monocytic RDC (MoRDC) also resides in the lung interstitium, but the function of these cells has not been well characterized. Because many, if not all, of these RDC subsets play a major role in the anti-influenza virus immune response, it is vital that we gain a better understanding of their interaction with influenza virus.

The influenza virus hemagglutinin (HA) serves as the receptor for virus attachment to cells, a necessary first step for virus infection. The HA recognizes and binds terminal sialic acids in certain preferred carbohydrate linkages potentially displayed by a diverse array of glycolipids and glycoproteins on the cell surface (10, 33, 36), although there is evidence that influenza virus may preferentially interact with one or more host cell N-linked glycoproteins for efficient entry and infection (6). In this study, we isolated murine RDC from healthy (uninfected) lungs (13, 24) as a source of cells for *in vitro* analysis of susceptibility to infection by type A influenza virus strains of different subtypes. We found that, among the major RDC subsets, only the $CD103^+$ and $CD11b^{hi}$ RDC subsets were susceptible to infection by type A influenza virus either *in vitro* or *in vivo*. Of note, these two RDC subsets also express the highest basal level of major histocompatibility complex (MHC) class II molecules. Furthermore, only type A influenza virus strains of the H2N2 subtype could efficiently infect these RDC subsets. A link between influenza virus infectivity and MHC class II expression was suggested by the finding that binding of monoclonal antibody to the I-E MHC class II locus product substantially inhibited infection of these two RDC subsets by the H2N2 subtype viruses. The potential significance of these findings with respect to influenza virus infection in the respiratory tract is discussed here.

MATERIALS AND METHODS

Mice. Eight- to 10-week-old female BALB/c ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$) mice were purchased from Taconic Farms (Germantown, NY) and maintained in a pathogen-free environment. All experiments were performed in accordance with regulatory standards and guidelines approved by the University of Virginia Animal Care and Use Committee.

Antibodies. The following monoclonal antibodies were used in the analysis of RDC populations: anti-MHC class II-fluorescein isothiocyanate (FITC) (2G9), $I\text{-}A^d$-FITC (AMS-32.1), I-E-phycoerythrin (PE) (14-4-4S), CD11c-PE-Cy7 (HL3), CD45R/B220-PerCP (RA3-6B2), and CD11b-PerCP-Cy5.5 (M1/70) (all purchased from BD Pharmingen, San Diego, CA). CD103-PE (2E7) was purchased from eBioscience (San Diego, CA); monoclonal antinucleoprotein (anti-NP) (H16) was a gift from Walter Gerhard (Wistar Institute, Philadelphia, PA). The anti-NP antibody was conjugated to Alexa Fluor 647 dye in accordance with the instructions provided with a conjugation kit purchased from Molecular Probes (Eugene, OR).

For antibody blockade experiments, RDC were resuspended in fluorescence-activated cell sorter (FACS) buffer without NaN_3 and Fc blocked for 5 min at 4°C before being incubated for 30 min in the presence of 5 μg of one of the following unconjugated antibodies: anti-$I\text{-}E^d$ (14-4-4s), anti-$I\text{-}A^d$ (AMS-32.1), anti-$H2\text{-}D^d$ (34.2.12), or mouse IgG2a isotype control (MOPC-173).

Cell lines. Madin-Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection (Manassas, VA) and were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA), 2 mM L-glutamine, 100 U/ml penicillin (Gibco BRL), 100 μg/ml streptomycin (Gibco BRL), and 50 mM 2-ME (2-mercaptoethanol). Cells were passaged at 80% confluence.

Preparation and administration of Flt3L plasmid. The pUMVC3-hFlex plasmid encoding Flt3 ligand (Flt3L) was obtained from Hardy Kornfeld (University of Massachusetts Medical School, Worcester, MA). *Escherichia coli* One Shot Top10 competent cells (Invitrogen Life Technologies, Carlsbad, CA) were transformed with the plasmid and grown to a large quantity. The Flt3L plasmid was purified from the transformed bacteria by the use of an EndoFree plasmid Giga kit (Qiagen, Valencia, CA). No residual endotoxin was detected in the plasmid preparation, as measured by the *Limulus* amebocyte lysate assay (Charles River Laboratories, Wilmington, MA). Flt3L-encoding plasmid was resuspended in sterile 0.9% saline solution at a final concentration of 5 μg/ml, and 2 ml of the preparation was rapidly injected intravenously (i.v.) via the lateral tail vein of each mouse over 5 to 10 s in order to achieve hypotonic *in vivo* transfection. This procedure was repeated 6 days later, and the lungs of plasmid-treated mice were isolated for RDC preparation 6 days after the second plasmid administration.

Preparation of lung single-cell suspension. Mice were sacrificed by cervical dislocation. The lungs were perfused via the right ventricle of the heart with 5 ml of sterile phosphate-buffered saline (PBS) to remove the intravascular pool of cells from the lung vasculature. Lungs were minced and digested in 183 U/ml type II collagenase (Worthington, Lakewood, NJ) in Iscove's modified Dulbecco's medium (IMDM) (Gibco BRL, Gaithersburg, MD) at 37°C in 7% CO_2 for 40 min. Afterward, the minced lung tissue was homogenized, passed through cell strainers (BD), and washed twice with IMDM. Lung cell pellets were resuspended either in magnetism-activated cell sorter (MACS) buffer (PBS supplemented with 0.5% bovine serum albumin [BSA] and 2 mM EDTA [Promega Corporation, Madison, WI]) for RDC isolation or in FACS lysing solution (BD, San Jose, CA) for flow cytometry staining.

Purification of RDC. Lung single-cell suspensions were magnetically labeled with anti-CD11c microbeads (N418) purchased from Miltenyi Biotec GmbH and isolated using a MACS according to the protocols of the manufacturer. Preparations were consistently >98% $CD11c^+$ (data not shown).

Viruses and infection. Mouse-adapted influenza A/PR/8 virus (H1N1) and A/Japan/305/57 (H2N2) virus were grown in hen eggs that had been embryonated for 10 days and stored at −80°C. Other wild-type influenza A virus strains used included A/Bel (H1N1), A/Taiwan/1/64 (H2N2), A/HK/68 (H3N2), and A/Mem/1/71 (H3N2). Reassortant influenza virus strains used included A/Jap/Bel (H2N1), A/Nip/Bel (H2N1), A/Ned/Bel (H2N1), and A/Mem/Bel (H3N1). The recombinant influenza A virus PR/8-Japan HA strain was generated using a reverse-genetics approach and pPOLI plasmids carrying the hemagglutinin of influenza A/Japan/305/57 virus and the remaining seven gene segments of influenza A/PR/8 virus.

For infection of RDC *in vitro*, purified RDC from either untreated or Flt3L plasmid-treated mice were seeded at a concentration of 1.0e6 cells/ml in a 6-well flat-bottom plate (Corning Incorporated, Corning, NY) and incubated for 1 h in complete medium (IMDM supplemented with 2 mM L-glutamine, 10% fetal bovine serum [Atlanta Biologicals, Norcross, GA], 100 U/ml penicillin [Gibco BRL], 100 μg/ml streptomycin [Gibco BRL], and 50 mM 2-ME). Following the 1-h incubation, RDC were washed once with prewarmed IMDM to remove residual serum proteins and nonadherent cells in the cultures. Serum-free IMDM (1 ml) was added to the wells, and virus was added at a multiplicity of infection (MOI) of 10. RDC were then incubated on ice for 10 min, followed by 1 h of incubation at 37°C in 7% CO_2. Following this incubation, RDC were washed twice with serum-free IMDM and then cultured in 1 ml of complete medium for the indicated time period. In some experiments, virus was subjected to heat inactivation at 55°C for 30 min prior to infection; in those cases, heat inactivation reduced the hemagglutinating activity of the virus 2-fold (data not shown), and we compensated for this reduction by doubling the inoculum size (i.e., equivalent to an MOI of 20) used to treat the RDC.

For *in vivo* infections, mice were lightly anesthetized with halothane prior to intranasal (i.n.) infection with a 50-μl volume of influenza virus diluted in serum-free IMDM. Lungs of infected mice were harvested as described at 24 h postinfection (p.i.).

MDCK cells were infected in serum-free IMDM suspensions as indicated for 10 min on ice followed by 1 h at 37°C in 7% CO_2 with light agitation every 5 to 10 min. Following infection, cells were washed twice with serum-free IMDM, resuspended in MDCK culture media, and plated in 6-well flat-bottom plates at a concentration of 1.0e6 cells/ml for the indicated time period.

Intracellular and surface staining for flow cytometry analysis. Purified RDC or MDCK cells from *in vitro* cultures or total lung cell suspensions from infected mice were fixed in FACS lysing solution for 10 min at room temperature, followed by resuspension in FACS buffer (PBS supplemented with 0.5% BSA and 0.02% NaN_3). Cells were Fc blocked for 5 min at 4°C and then surface stained by incubation with the indicated monoclonal antibodies for 30 min at 4°C in the dark. Cells were then washed with FACS buffer and permeabilized with Perm/Wash (BD). To block nonspecific intracellular antibody binding, cells were incubated with Perm/Wash containing 5% healthy goat serum for 20 min on ice in the dark. Following this incubation, Alexa Fluor 647-conjugated anti-NP antibody was added directly to the cells for 30 min at 4°C in the dark. Labeled cells were detected by flow cytometry using either a FACSCalibur system (BD,

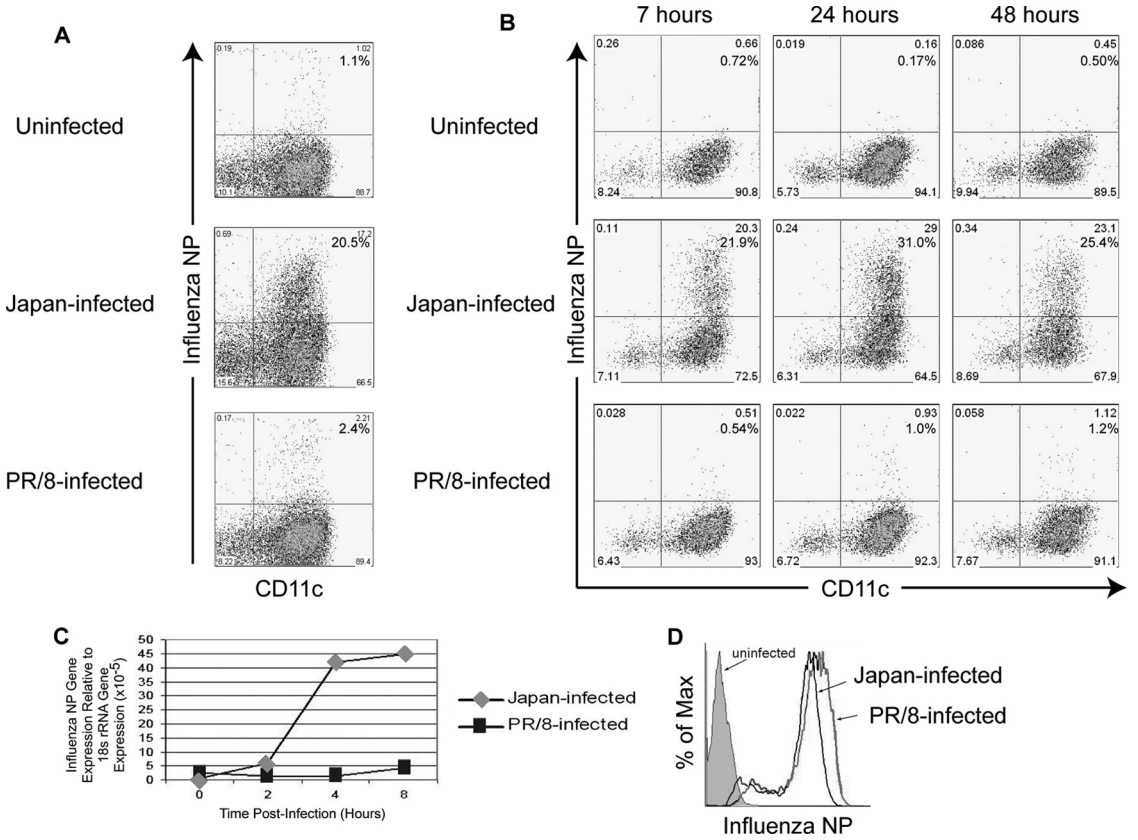

FIG. 1. Differential susceptibilities of RDC to infection by influenza A/PR/8 virus (H1N1) and A/Japan/305/57 (H2N2) *in vitro*. (A) RDC isolated from the lungs of Flt3L-encoding plasmid-treated BALB/c mice were infected *in vitro* with the indicated virus at an MOI of 10. At 18 h postinfection, RDC were collected, surface stained with monoclonal antibody against CD11c, and then stained for intracellular NP, followed by flow cytometry analysis. Percent values indicate the percentages of $CD11c^+$ cells that were also NP^+. (B) Kinetic analysis of the accumulation of influenza NP in RDC infected with the indicated virus. (C) Real-time RT-PCR analysis of influenza virus NP gene expression over time in RDC infected with the indicated virus. (D) MDCK cells were infected with the indicated virus at an MOI of 10 and assessed by intracellular staining for the accumulation of NP at 18 h postinfection. Data in each panel are representative of the results of >3 independent experiments.

Mountain View, CA) or a FACS Canto system (BD, Mountain View, CA) and were analyzed using Flowjo software (Tree Star).

Real-time RT-PCR. For analysis of influenza virus NP gene expression in infected RDC, *in vitro*-cultured RDC were harvested and lysed with buffer RLT for RNA extraction using an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Extracts were digested with amplification grade DNase I (Invitrogen Life Technologies, Carlsbad, CA), and total RNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). Total RNA (0.5 μg) was used for reverse transcription-PCR (RT-PCR) with random hexamer primers (Invitrogen Life Technologies, Carlsbad, CA) to generate cDNA. Real-time PCR was performed using an ABI 7000 instrument with cDNA samples, specific influenza virus NP primers, and SYBR green technology (Applied Biosystems, Foster City, CA). The A/Japan/305/57 NP primers used were as follows: forward, 5′-TCTTGTCTGCCTGCCTGTGT-3′; reverse, 5′-GTGTGCTGGATTCTCGTTCG-3′. The influenza A/PR/8 virus NP primers used were as follows: forward, 5′-GAACAACCGTTATGGCAGCA-3′; reverse, 5′-CATGTCAAAGGAAGGCACGA-3′. The 18s rRNA primers used were as follows: forward, 5′-TCGAACGTCTGCCCTATCAA-3′; reverse, 5′-GGGTCGGGAGTGGGTAATTT-3′. Relative NP gene expression levels were calculated by normalizing influenza virus NP threshold cycle (C_T) values against 18s rRNA C_T values for each sample.

RESULTS

RDC differ in susceptibility to infection with different type A influenza virus strains. Previous work in our laboratory has shown that murine RDC are susceptible to infection with the influenza virus strain A/Japan/305/57 (13). We wished to determine whether RDC are equally susceptible to infection by other influenza virus strains which, like A/Japan/305/57, can induce a potent innate and adaptive immune response in the infected mice. We compared RDC infection by influenza A/Japan/305/57 virus, an H2N2 subtype influenza virus strain, with that by the prototype H1N1 influenza virus strain A/PR/8. RDC isolated from the lungs of mice subjected to RDC enrichment by hypotonic *in vivo* transfection with a plasmid encoding Flt3L as previously described (see reference 13 and Materials and Methods) were infected *in vitro* with either strain of influenza virus. While this procedure significantly increased the number of RDC that could be isolated from the murine lungs, we have previously shown that RDC generated in this way do not differ from RDC in untreated mice either in their phenotypes or in their relative subset distributions (13). To ensure adequate exposure of RDC to virus, the cells were infected under single-cycle conditions at a multiplicity of infection of 10. At 18 h postinfection, cells were harvested and the extent of infection assessed using flow cytometry to detect *de novo* viral protein synthesis by intracellular staining with an antibody specific for influenza virus nucleoprotein (NP). As shown in Fig. 1A, the A/Japan/305/57 influenza virus strain

efficiently infected a significant fraction of the total RDC. Detection of NP in RDC exposed to influenza A/Japan/305/57 virus did not reflect phagocytosis of virus particles, as we did not detect this protein by intracellular staining when RDC were exposed to either UV- or heat-inactivated virus or when the RDC were infected in the presence of a protein synthesis inhibitor (13). It should also be noted that influenza A/Japan/305/57 virus-infected RDC could not be formally examined for the release of infectious particles, because the RDC were cultured in the presence of fetal bovine serum (FBS) to retain viability. Also, to our knowledge these RDC do not express the trypsin-like serine endoprotease necessary to cleave the hemagglutinin of assembling particles in order to render released virions infectious. As such, there was only a 2-fold decrease in RDC viability in influenza A/Japan/305/57 virus-infected cells compared to uninfected cell results (data not shown). In contrast to results obtained with influenza A/Japan/305/57 virus, only background levels of NP staining were observed when RDC were exposed to influenza A/PR/8 virus (Fig. 1A). Additionally, the differences between RDC exposed to influenza A/Japan/305/57 virus and those exposed to influenza A/PR/8 virus with respect to NP detection were not due to differences in the kinetics of influenza A/PR/8 virus replication, as NP did not accumulate in influenza A/PR/8 virus-infected RDC up to 48 h postinfection, while NP gene expression in influenza A/Japan/305/57 virus-infected RDC approached the maximal level by 7 h postinfection (Fig. 1B). Identical results were obtained for RDC exposed to influenza A/PR/8 virus at MOI ranging from 1 to 100 (not shown). These findings suggested that the highly mouse-adapted influenza A/PR/8 virus is unable to efficiently infect (replicate in) the differentiated RDC *in vitro*. In keeping with these results, NP gene expression measured by real-time RT-PCR confirmed that influenza A/PR/8 virus gene expression did not occur in the RDC whereas viral gene expression was rapidly detected in influenza A/Japan/305/57 virus-infected RDC (Fig. 1C). Importantly, influenza A/PR/8 virus efficiently infected MDCK cells; also, the anti-NP monoclonal antibody employed in the flow-based analysis detected influenza A/Japan/305/57 virus NP and influenza A/PR/8 virus NP equally (Fig. 1D). Together, these data demonstrate that RDC exhibit differential levels of susceptibility to infection by these two prototype mouse-adapted influenza virus strains.

The efficiency of influenza virus infection of RDC correlates with expression of H2N2-subtype hemagglutinin. It is noteworthy that both influenza A/Japan/305/57 virus and influenza A/PR/8 virus replicate to high titers in the murine lungs, can produce lethal infection, and induce potent immune responses (19, 20, 21). Therefore, the observed differences in the susceptibilities of RDC to infection by these 2 influenza virus strains are not readily explained by significant differences in the degree of adaptation to replication in the murine respiratory tract. Since influenza virus hemagglutinin (HA) influences the cellular tropism of influenza viruses (10, 33, 36), we explored the possibility that the differences in RDC susceptibility to infection may be linked to the HA subtype. Therefore, we screened a number of different type A influenza virus strains representative of the H1N1, H2N2, and H3N2 subtypes for the ability to infect RDC in order to determine whether infectivity correlated with the HA subtype of the virus strain. Interestingly, the H2N2 strains evaluated efficiently infected RDC,

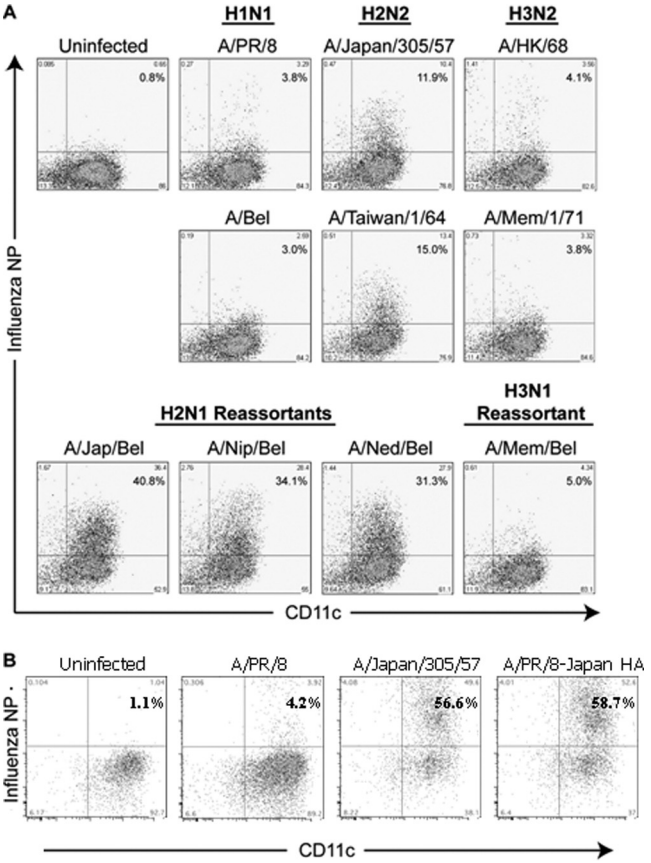

FIG. 2. RDC susceptibility to infection by influenza virus correlates with expression of H2 HA by the virus. RDC isolated from the lungs of BALB/c mice were infected *in vitro* with influenza virus as indicated. At 18 h postinfection, RDC were collected, surface stained with monoclonal antibody against CD11c, and then stained for intracellular NP, followed by flow cytometry analysis. Percent values indicate the percentages of $CD11c^+$ cells that were also NP^+. Data are representative of the results of 3 independent experiments.

whereas the infectivity of H1N1 strains was poor (Fig. 2A). Importantly, the H3N2 strains evaluated also infected RDC poorly, suggesting that the HA of the H2N2 subtype, and not the N2 neuraminidase, is the viral gene product likely to be responsible for the efficient infection of RDC by H2N2 influenza virus strains. In support of the idea of this role for H2 HA in the infection of RDC, robust infection was observed for a number of H2N1 reassortant influenza virus strains that express antigenically distinct but related HAs of the H2N2 subtype but not for an H3N1 reassortant influenza virus strain (A/Mem/Bel) (Fig. 2A). While expression of the H2N2-subtype HA was a common feature of the virus strains capable of infecting RDC, other viral genes, differentially expressed among the parental (e.g., A/Japan and A/Bel) and reassortant (A/Jap/Bel) influenza virus strains, could also influence influenza virus infectivity and tropism for RDC. To formally address the role of the H2 HA in virus tropism, we used influenza virus reverse genetics to construct a recombinant influenza virus containing the influenza A/Japan virus HA gene in the backbone of the 7 remaining genes from the influenza A/PR/8 virus (designated influenza virus A/PR/8-Japan HA). As shown

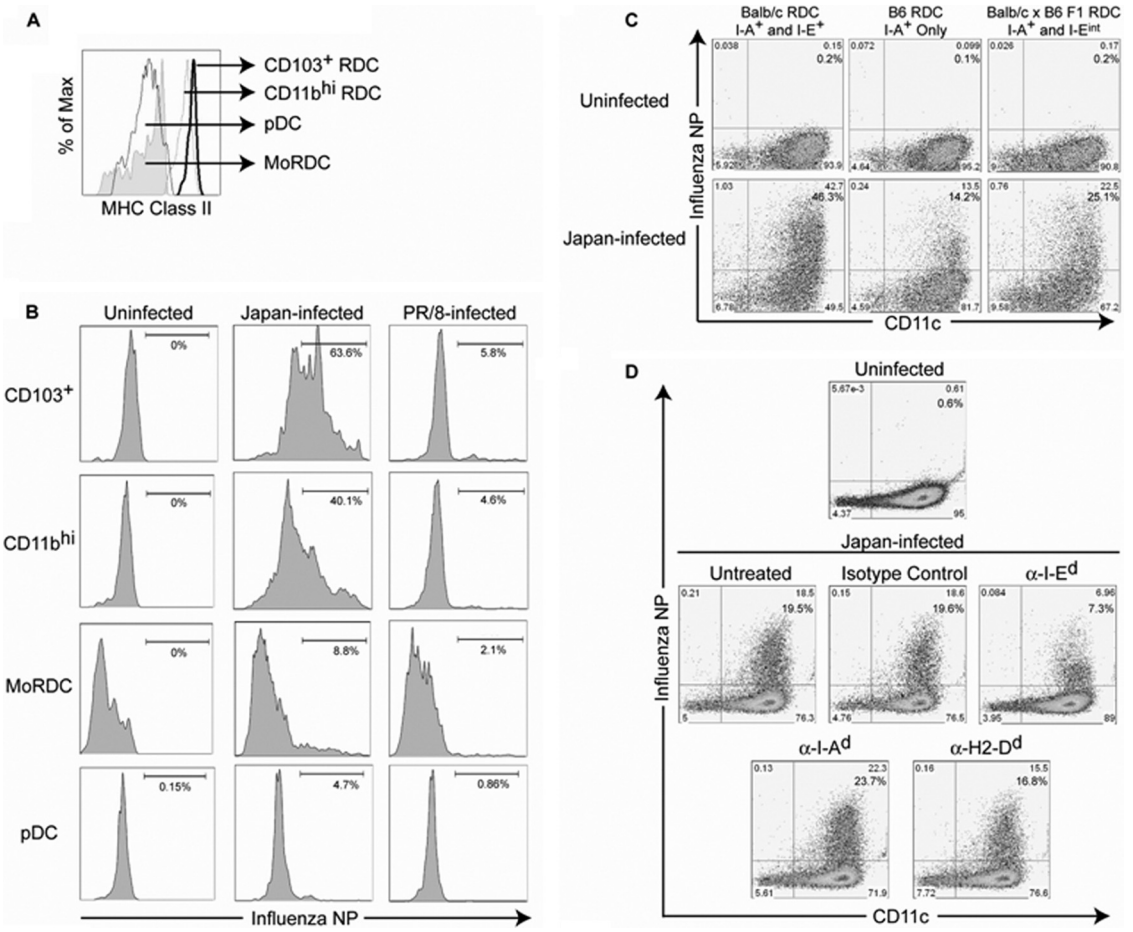

FIG. 3. Efficient infection of murine RDC by influenza virus of the H2N2 subtype requires an interaction of the virus with the MHC class II I-E locus on RDC. (A) MHC class II expression by the major subsets of RDC. (B) RDC isolated from the lungs of Flt3L-encoding plasmid-treated BALB/c mice were infected *in vitro* with influenza virus as indicated. At 18 h postinfection, individual RDC subsets were gated as described previously (13) and assessed for infection by intracellular staining for influenza NP. (C) RDC were isolated from the lungs of Flt3L-encoding plasmid-treated BALB/c, C57BL/6, or BALB/c × C57BL/6 F1 mice and infected with influenza virus as indicated. Accumulation of influenza NP was determined 18 h postinfection by intracellular staining. (D) Prior to infection, RDC were incubated with blocking antibody against the indicated MHC class I or class II molecules. Cells infected as indicated were harvested 6 h postinfection and assessed for accumulation of intracellular influenza NP. Data are representative of the results of 3 independent experiments.

in Fig. 2B, the reverse-genetics-engineered recombinant virus infected RDC isolated from untreated BALB/c mice as efficiently as the wild-type influenza A/Japan/305/57 virus strain, thus demonstrating that efficient infection of RDC by influenza virus depends solely on expression of the H2 HA subtype.

Infection of RDC by influenza virus requires an interaction with the I-E^d MHC class II molecule. The findings described above demonstrated an association between the susceptibility of murine RDC to influenza virus infection and expression by the virus of the HA of the H2N2 subtype. These results demonstrate a striking parallel with those of earlier studies that demonstrated a preferential interaction between murine B lymphocytes and influenza virus strains of the H2N2 subtype. This virus-cell interaction (which resulted in B-cell activation) required MHC class II molecule expression by the B cell (1, 2, 30, 31, 32). We therefore evaluated expression of MHC class II in the major subsets of RDC (12, 13, 15, 18, 35). As shown in Fig. 3A, the CD103$^+$ and CD11b^{hi} RDC subsets expressed very high levels of MHC class II molecules whereas the MoRDC and pDC expressed low levels of these molecules. Since, as demonstrated above, only a fraction (i.e., 20% to 40%) of the total RDC were susceptible to virus infection, it was of interest to determine whether the susceptibility of RDC to influenza virus infection correlated with the level of MHC class II displayed by these RDC subsets. When we analyzed the susceptibility of these major RDC subsets to infection by two prototype influenza virus strains, we observed that infection of RDC by the influenza A/Japan/305/57 virus strain was largely restricted to the MHC class II^{hi} RDC subsets (Fig. 3B). Thus, the CD103$^+$ and CD11b^{hi} RDC subsets, while representing only a fraction of the total RDC isolated (13), likely represent the majority of the infected (NP$^+$) RDC under *in vitro* conditions of infection. We considered the possibility that H2N2 viruses might interact (through their HA) with MHC class II^{hi} RDC and, as a result of the interaction, deliver a signal that renders these RDC subsets susceptible to influenza virus infection. To test this possibility, we exposed RDC to mixtures of heat-inactivated influenza A/Japan/305/57 virus (which lacks

infectivity but retains hemagglutinating activity) and infectious influenza A/PR/8 virus over a range of ratios of noninfectious virus to infectious virus. Using this strategy, we were unable to demonstrate the ability of RDC to support influenza A/PR/8 virus infection and gene expression (data not shown).

Earlier studies analyzing the interaction of H2N2 influenza virus strains with splenic B cells implicated the I-E locus of MHC class II molecules as critical in the H2N2 virus–B cell interaction that results in B cell activation (1, 2, 30, 31, 32). For the studies described to this point, we employed RDC from BALB/c mice that express both the I-A and I-E locus products of the H-2^d haplotype. To evaluate the role that I-E^d displayed on RDC plays in the infection process, we compared the efficiency of infection of RDC isolated from BALB/c mice, which express both I-E^d and I-A^d MHC class II molecules, with that of RDC isolated from C57BL/6 mice, which express only I-A^b (29). In contrast to the efficient infection of BALB/c-derived RDC by influenza A/Japan/305/57 virus, the infection of C57BL/6-derived RDC by this strain was dramatically reduced (Fig. 3C). We also found that BALB/c × C57BL/6 F1-derived RDC, which express I-E^d but at reduced levels, displayed infection by influenza A/Japan/305/57 virus that was intermediate to that seen with RDC from the mouse strains exhibiting high (BALB/c) and low (C57BL/6) susceptibility (Fig. 3C). Additionally, RDC from another I-E-expressing mouse line (MA/My) demonstrated susceptibility to infection comparable to that of BALB/c-derived RDC (data not shown).

The results described above indicated a link between the level of expression of the MHC class II I-E locus product by RDC and the susceptibility of the DC to infection by H2N2-subtype HA-expressing influenza virus strains. It was therefore of interest to determine whether blocking the accessibility of the virus to the I-E locus product on the RDC would affect the susceptibility of the RDC to influenza virus infection. To this end, we treated RDC with a monoclonal antibody directed to the I-E^d molecule prior to virus infection. When I-E^d on RDC was blocked prior to infection by influenza A/Japan/305/57 virus, NP levels were dramatically reduced compared to those seen with the untreated or isotype control antibody-treated RDC (Fig. 3D). Importantly, this phenomenon was specific to I-E^d, as antibody blockade of I-A^d or the H2-D^d MHC class I molecule had no effect on the infectivity of influenza A/Japan/305/57 virus (Fig. 3D). Importantly, although the I-E^d blockade did not completely abrogate infection of RDC by influenza A/Japan/305/57 virus, it should be noted that pretreatment with blocking antibody did not completely saturate the cell surface I-E^d (data not shown) and that this could account for the inability to completely inhibit virus infectivity. Nevertheless, the results suggest that a novel interaction between certain type A influenza virus strains and MHC class II^{hi} RDC expressing specific MHC class II locus products affects the susceptibility of RDC to influenza virus infection.

Influenza A/Japan/305/57 virus, but not influenza A/PR/8 virus, efficiently infects $CD103^+$ and $CD11b^{hi}$ RDC *in vivo*. The observations described above suggested that the susceptibility of RDC to infection by influenza virus was dependent on the influenza virus strain as well as the mouse strain donating the RDC and that infection of RDC was restricted to distinct RDC subsets. Since these findings were obtained using induced RDC from the healthy murine lungs infected *in vitro*, it was of interest to determine whether a similar pattern of susceptibility to infection occurred *in vivo* during natural influenza virus infection of the respiratory tract. To evaluate this, we infected naïve BALB/c mice by the intranasal route with an inoculum consisting of a 10× 50% lethal dose (LD_{50}) of influenza A/Japan/305/57 virus or influenza A/PR/8 virus to ensure both high-level virus replication in the respiratory tract at an early postinoculation time point and accessibility of RDC to infectious virus at that time. At 24 h postinfection, lungs were harvested and RDC infection was measured by intracellular staining for influenza NP *ex vivo*. The same hierarchy of RDC subset susceptibilities to infection observed *in vitro* was observed for influenza A/Japan/305/57 virus *in vivo*, and, importantly, influenza A/PR/8 virus failed to efficiently infect any RDC subsets *in vivo* (Fig. 4). These data demonstrate that influenza A/Japan/305/57 virus infects RDC more efficiently than influenza A/PR/8 virus *in vivo* as well as *in vitro* and that the RDC subsets most susceptible to infection are the $CD103^+$ RDC and the $CD11b^{hi}$ RDC.

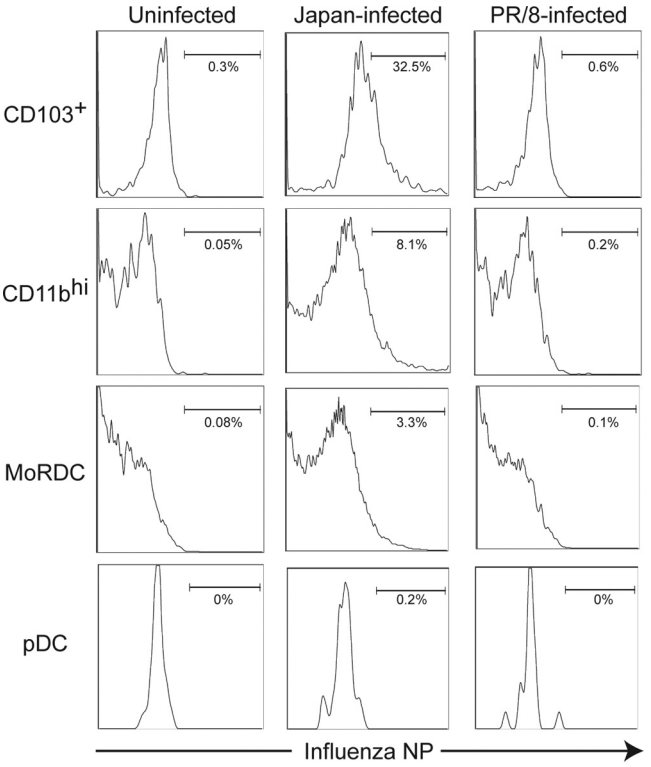

FIG. 4. Differential susceptibilities of RDC to infection by influenza A/PR/8 virus (H1N1) and influenza A/Japan/305/57 (H2N2) virus *in vivo*. BALB/c mice were infected intranasally with 10× LD_{50} of either influenza A/PR/8 virus or influenza A/Japan/305/57 virus. At 24 h postinfection, lungs were harvested, and single-cell suspensions were stained *ex vivo* for detection of intracellular influenza NP within RDC subsets. Numbers indicate the percentage of NP^+ cells within each RDC subset. Data are representative of the results of 3 independent experiments.

DISCUSSION

RDC represent a heterogeneous set of peripheral tissue-resident DC that both function as innate effector cells and orchestrate the induction of adaptive immune responses to microorganisms and inert antigens present in the respiratory

tract. In this report, we characterize the interaction between RDC and influenza virus *in vitro* and *in vivo* in the murine model. We demonstrate that specific RDC subsets are differentially susceptible to infection by influenza virus and that infection of these RDC is dependent on the virus strain. The MHC class II^{hi} intrapepithelial and subepithelial $CD103^+$ RDC and the interstitial $CD103^-$ $CD11b^{hi}$ RDC are efficiently infected by the influenza A/Japan/305/57 virus strain both *in vitro* and *in vivo*, whereas MHC class II^{lo} MoRDC and pDC are not susceptible to infection following exposure to this virus. In contrast, influenza A/PR/8 virus does not efficiently infect any of these RDC subsets. Interestingly, we found efficient infection of RDC by a number of H2N2 influenza virus strains and poor infection by several H1N1 and H3N2 strains. Evidence from the infection of RDC by reassortant influenza virus strains and a reverse genetics-constructed influenza A/PR/8-Japan HA recombinant virus demonstrates that expression of HA of the H2N2 subtype facilitates efficient infection of murine RDC by type A influenza viruses. The susceptibility of RDC to infection by H2N2 virus was dependent upon their expression of MHC class II molecules, with the MHC class II^{hi} $CD103^+$ and $CD11b^{hi}$ RDC subsets displaying the greatest susceptibility to infection. Importantly, susceptibility was further shown to be associated with expression by RDC of the murine MHC class II locus I-E; also of interest, binding of antibody to I-E on RDC prior to virus exposure inhibited virus infectivity.

The restriction of infection of RDC to influenza virus strains expressing HA of the H2N2 subtype was unexpected, as was the requirement for expression of the murine MHC class II I-E locus product by the RDC. Influenza viruses bind to cell surfaces through HA-mediated recognition of terminal sialic acids displayed by cell surface molecules. Although the HA molecules of different type A influenza virus subtypes differ in specificity of sialic acid recognition according to sialic acid linkage to subterminal sugars (34), it is unlikely that the HA of an H1N1 influenza virus strain such as influenza A/PR/8 virus would differ sufficiently from influenza A/Japan/305/57 virus HA in sialic acid binding specificity to render the virus incapable of binding murine RDC. In this regard, it is noteworthy that both of these mouse-adapted type A influenza virus strains are capable of high-level replication in the murine respiratory tract (19, 20, 21), again suggesting that the influenza A/PR/8 virus is fully capable of efficiently binding to murine cells. We did not formally evaluate the impact of binding of antibody to MHC class II on the extent of virus binding to the RDC. Since RDC display abundant sialic acids on their surface glycoproteins and glycolipids, we considered it unlikely that blocking the interaction of virus with MHC class II molecules would lead to a diminution of virus binding to the RDC cell surface that was sufficient to be detected by standard quantitative means. Instead, the link between virus infectivity and MHC class II expression by RDC could argue for a role of MHC molecules in facilitating infection of cells by influenza virus. However, most cell types susceptible to influenza virus infection, most notably ciliated and nonciliated respiratory (airway) epithelial cells, do not express MHC class II molecules. Consequently, the MHC class II dependence of susceptibility to influenza virus infection observed in this study would most likely be restricted to infection of DC and related cell types.

At present, the mechanism accounting for the dependence of RDC infection by influenza virus on MHC class II expression and on hemagglutinin of the H2N2 subtype is not as yet known with certainty. As discussed above, it is unlikely that any subtle differences in the sialic acid specificity of the influenza A/PR/8 virus or influenza A/Japan/305/57 virus HA would affect the initial virus attachment to cells. However, although it seems unlikely, we cannot formally exclude the possibility that the MHC class II I-E molecule displays a specific carbohydrate array recognized and found only by HAs of the H2N2 subtype. Nevertheless, the impact of MHC class II and the subtype of the type A influenza virus HA would most likely be at the level of virus uptake by RDC or postentry events associated with virus replication. In this regard, following exposure of RDC to virus strains (such as influenza A/PR/8 virus) whose HA is nonpermissive, we were unable to detect any *de novo* viral gene expression and amplification in RDC exposed to such viruses. Thus, a block to nonpermissive influenza A/PR/8 virus replication postentry into RDC would be at an extremely early step, presumably following virus uncoating and viral genome entry into the cell cytoplasm and certainly prior to any *de novo* viral gene expression in the cell nucleus. Also the finding that the susceptibility of RDC to infection maps to the HA gene (as demonstrated by the efficient infection of RDC by the influenza A/PR/8-Japan HA recombinant virus) further argues against a direct effect in the postfusion function of the RNP and polymerase complexes in the cytosol or nucleus, as these genes and their products are identical in the wild-type and the recombinant virus.

An alternative explanation is suggested by the requirement for I-E expression by RDC to support infection by H2N2-subtype influenza virus as well as by the ability of antibody to the I-E gene products to inhibit infection. These findings raise the possibility that the I-E molecule may serve as a novel receptor or coreceptor required to initiate and/or support infection of murine RDC by H2N2-subtype influenza viruses. MHC class II has been reported to serve as a receptor for other viruses (8, 23). Furthermore, recent evidence has emerged to suggest that, in addition to the requirement for binding to sialic acid ligands, influenza virus must also interact with one or more cell surface N-linked glycoproteins as a requirement for infection. These N-linked glycosylated glycoproteins are not required for influenza virus binding but are required for virus internalization (6). MHC class II I-A and I-E locus-encoded molecules are known to differ with respect to the sites and diversity of N-linked glycosylation (37). Since, as noted above, MHC class II expression is not required for the infection of most cell types by type A influenza viruses, the role of MHC class II as a coreceptor and the link to the H2N2-subtype HA may reflect a feature of virus uptake and endocytosis unique to dendritic cells.

The idea of an interaction between influenza viruses (specifically influenza virus HA) and MHC class II locus molecules is not without precedent. An earlier study (5) demonstrated that type A influenza viruses, and influenza virus strains of the H2N2 subtype in particular, serve as lymphocyte mitogens. Subsequent studies refined this phenomenon and demonstrated that influenza virus HA (and specifically the HA of the H2 and H6 subtypes) provided a strong mitogenic stimulus to murine B cells (1, 2, 30, 31, 32). This mitogenic activity was

dependent on a direct interaction between the influenza HA and the MHC class II I-E gene product. The earlier observations raise the possibility that in RDC, the H2N2-subtype HA may, through an interaction with the I-E molecule, provide the activation stimulus necessary to support virus replication in RDC. To address this issue, we infected RDC simultaneously with heat-inactivated influenza A/Japan/305/57 virus, which does not replicate in RDC but retains a functional HA molecule, and wild-type influenza A/PR/8 virus to determine whether the interaction between H2 HA from influenza A/Japan/305/57 virus and I-E^d allowed the RDC to support replication of A/PR/8. However, influenza A/PR/8 virus failed to replicate efficiently in the RDC exposed to the influenza A/Japan/305/57 H2 virus HA (data not shown). In companion experiments, we were likewise unable to prevent infection of RDC by influenza A/Japan/305/57 virus following pretreatment of the cells with influenza A/PR/8 virus, implying that the encounter of RDC with this virus strain does not result in active suppression of influenza replication. Thus, we are unable to identify a virus strain-dependent activating or suppressive signal triggered by a virus interaction with RDC which could account for this restricted virus tropism.

We also observed that *in situ* (*in vivo*) infection of RDC by influenza A/Japan/305/57 virus and influenza A/PR/8 influenza virus strains directly paralleled the pattern of infection observed *in vitro*, i.e., RDC were readily infected by the prototype H2N2 strain but only minimally infected by the prototype H1N1 strain, as determined by NP expression in a flow cytometry-based analysis performed 24 h following intranasal influenza virus infection. Since, as we and others have demonstrated (12, 15), RDC which have migrated during influenza virus infection from the respiratory tract to the draining lymph nodes play an essential role in initiating the adaptive immune T cell response, these results might imply that infection of RDC by influenza A/PR/8 virus is not essential for efficient presentation of viral Ag to naïve T cells in the draining nodes. In this connection, we had previously reported in studies that employed infections by both influenza A/Japan/305/57 virus (21) and influenza A/PR/8 virus (15) that NP^+ migrant RDC are demonstrable in the lung draining lymph nodes at day 3 following infection. While influenza A/PR/8 virus only minimally infects lung-resident RDC *in vivo* during the first 24 h following intranasal virus inoculation, we have observed in preliminary studies that, by day 3 postinfection, NP^+ lung-resident DC are readily demonstrable in the influenza A/PR/8 virus-infected respiratory tract (K. M. Hargadon and T. J. Braciale, unpublished data). The reason for this transition in susceptibility status within the RDC population from NP^- (uninfected) to NP^+ (and presumably infected) is currently unknown. It has, however, been well documented that, following infection of the respiratory tract, there is an influx of DC into the lungs in response to this inflammatory stimulus (12, 15), and the properties of these infiltrating DC are likely to be different from those resident at the onset of an infection.

This report provides the first demonstration that bona fide RDC can differ with respect to susceptibility to infection by different influenza virus strains. Moreover, the link between RDC expression of the I-E MHC class II locus and infection by influenza virus strains of the H2N2 subtype suggests a potential role for MHC polymorphisms in regulating the susceptibility of RDC and other cells of the respiratory tract to infection by different influenza virus strains. Such a role would have significant implications for the relevance of this finding to human infection by influenza virus. Therefore, the impact of RDC infection by influenza virus on the immune response to the virus is of great interest, and we are currently exploring whether differences in the susceptibilities of RDC to infection by different influenza virus strains have an impact on the efficiency of virus clearance or, alternatively, viral virulence. The differences in susceptibility of RDC to infection with the influenza A/Japan/305/57 virus and the influenza A/PR/8 virus strains do not appear to have a significant effect on the capacity of RDC to serve as APC for the induction of the adaptive immune T cell response to these two viruses. Infection of RDC may, on the other hand, significantly affect the early innate immune response in the respiratory tract. Indeed, we have observed that type 1 interferon gene expression is dramatically upregulated *in vivo* following infection by influenza A/Japan/305/57 virus but is absent following infection by influenza A/PR/8 virus; this finding correlates with the type I interferon gene and protein expression profiles of RDC exposed to these virus strains *in vitro* (Hargadon and Braciale, unpublished). It should be interesting to evaluate additional cytokine and chemokine profiles for RDC following exposure to different influenza virus strains. The mechanistic basis for the virus strain-dependent differences in immune responses made to infection, the role of these responses in immune-mediated virus clearance versus pathogenesis, and the role of RDC in regulating the activities of other cells within the respiratory tract during influenza virus infection are topics for future analysis.

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