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Evaluation of the innate immune responses to influenza and live-attenuated influenza vaccine infection in primary differentiated human nasal epithelial cells

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ABSTRACT

The host innate immune response to influenza virus is a key determinant of pathogenic outcomes and long-term protective immune responses against subsequent exposures. Here, we present a direct contrast of the host responses in primary differentiated human nasal epithelial cell (hNEC) cultures following infection with either a seasonal H3N2 influenza virus (WT) or the antigenically-matched live-attenuated vaccine (LAIV) strain. Comparison of the transcriptional profiles obtained 24 and 36 h post-infection showed that the magnitude of gene expression was greater in LAIV infected relative to that observed in WT infected hNEC cultures. Functional enrichment analysis revealed that the antiviral and inflammatory responses were largely driven by type III IFN induction in both WT and LAIV infected cells. However, the enrichment of biological pathways involved in the recruitment of mononuclear leukocytes, antigen-presenting cells, and T lymphocytes was uniquely observed in LAIV infected cells. These observations were reflective of the host innate immune responses observed in individuals acutely infected with influenza viruses. These findings indicate that cell-intrinsic type III IFN-mediated innate immune responses in the nasal epithelium are not only crucial for viral clearance and attenuation, but may also play an important role in the induction of protective immune responses with live-attenuated vaccines.

1. Introduction

Influenza A viruses (IAV) remain one of the most relevant human pathogens with annual epidemics resulting in 250,000–500,000 deaths worldwide [16]. Annual vaccination against influenza viruses is required due to frequent antigenic drift and inefficient cross-protective immunity from prior infections or vaccination. Although vaccination remains the best approach against infection and disease, the identification of new circulating strains and the time for production and distribution present constraints on vaccine development. Additionally, other host-driven factors can contribute to the efficacy of influenza vaccines [9]. Defining host molecular responses—and the viral and host factors interactions that trigger and regulate them—is therefore essential to developing effective vaccines.

Live-attenuated influenza vaccine (LAIV) strains are based on the cold-adapted master donor virus A/Ann Arbor/6/1960 (H2N2). The 6:2 reassortant vaccine viruses contain the RNA segments encoding the hemagglutinin (HA) and neuraminidase (NA) surface antigens derived from circulating wild type influenza virus (WT) and the six internal RNA segments derived from the master donor virus. LAIV has been described to have three phenotypic changes that relate to its reduced ability to cause disease: (i) it

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causes reduced disease in animal models compared to seasonal strains of influenza (attenuation, or *at*) (ii) has restricted replication at temperatures at or above 39 °C (temperature sensitivity or *ts*) and (iii) is able to efficiently replicate at temperatures as low as 25 °C (cold-adaptation or *ca*). Although several pre-clinical [5,6,27,28] and clinical studies [17,19,30] have demonstrated that LAIVs are safe, efficient, and an effective means of vaccination, the mediators and correlates of protection still remain poorly understood.

In this study, we use a whole-genome transcriptional approach to elucidate the early response to wild-type (WT) A/Victoria/361/2011 (H3N2) influenza A virus (A/Victoria/361/2011) and an antigenically matched LAIV strain in primary differentiated human nasal epithelial cell (hNEC) cultures. We demonstrate that LAIV elicits robust antiviral responses, which may also restrict viral replication. LAIV also induces enhanced chemokine secretion and that downstream transcriptional profiles following infection with vaccine strains are predictive of increased leukocyte and lymphocyte recruitment to sites of virus replication which may contribute to the generation of a strong adaptive immune responses. These responses are reflective of those observed in the nasal epithelium during acute infection of humans with IAV. Thus, primary differentiated hNEC cultures can model the innate immune responses present in influenza-infected individuals and provide insight into the molecular mechanisms of LAIV efficacy.

2. Materials and methods

2.1. Viruses

Seasonal influenza A virus A/Victoria/361/2011 (H3N2) and an antigenically-matched live attenuated influenza vaccine virus A/Victoria/361/2011 LAIV (LAIV; HA and NA RNA segments from A/Victoria/361/2011, and other RNA segments from A/Ann Arbor/6/1960) were utilized in this study. Virus seed stocks were obtained from Medimmune and virus sequences verified using MiSeq to obtain sequences for all virus segments. The viruses are referred to as H3N2 WT or LAIV in the manuscript. Working stocks were generated by infecting confluent MDCK cells at an MOI of 0.01 for 48–72 h in DMEM supplemented with 0.3% bovine serum albumin, 2 mM GlutaMax, 100 U/ml Penicillin, 100 μg/ml Streptomycin, and 4 μg/ml N-acetyl Trypsin (NAT). The supernatants were then harvested, clarified by low speed centrifugation, and aliquoted for storage at −70 °C.

2.2. Cell culture conditions

Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 2 mM GlutaMax, 100 U/ml Penicillin and 100 μg/ml Streptomycin and maintained in a humidified environment at 37 °C with 5% CO_2.

Human nasal epithelial cell (hNECs) cultures (male and female donors) were obtained from non-diseased tissue during endoscopic sinus surgery for non-infection related conditions and grown in culture at the air-liquid interface (ALI) as previously described [25,32,33]. Female and male donors were used and the ages were between 18 and 49 years old. Tissue processing, differentiation medium, and culture conditions have been previously described in detail [12].

2.3. Infection of primary differentiated hNECs

Differentiated hNEC cultures were infected at a multiplicity of infection (MOI) of one (high MOI) or 0.03 (low MOI) 50% tissue culture infectious dose ($TCID_{50}$) per cell. All incubations were performed at 32 °C in a humidified environment with 5% CO_2. Prior to infection, the apical surface of the cultures was washed with 200 μl DMEM (supplemented with 0.3% BSA, 2 mM Glutamax, 100 U/ml Penicillin, and 100 μg/ml Streptomycin) and the basolateral media was replaced with 500–1000 μl of fresh LHC Basal Medium:DMEM-H. The virus inoculum was added to the apical compartment in a volume of 100 μl and incubated for 1 h, after which the inoculum was aspirated and the apical surface was washed three times with 200 μl phosphate buffered saline containing calcium and magnesium (PBS+). The plates were then returned to the incubator. Both apical washes and basolateral medium were collected and stored at −70 °C. Virus production in apical washes was quantified by determining the 50% tissue culture infectious dose ($TCID_{50}$) using MDCK cells [24] and the Reed-Muench algorithm [34].

2.4. Microarray experiments and data processing

RNA was isolated from Trizol homogenates of mock, WT or LAIV infected cells harvested at 24hpi and 36hpi Fluorescent-labeled probes were generated from each sample using Agilent one-color LowInput Quick Amp Labeling Kit (Agilent Technologies). All infected samples were confirmed to express viral M2 mRNA by quantitative RT-PCR [13,29]. Individual cRNA samples were then hybridized to oligonucleotide microarrays for gene expression profiling using SurePrint G3 Human Gene Expression v2 Microarray Kit (G4851A; Agilent Technologies.

The primary transcriptomic data was extracted and quantile normalized using the 'normalizeBetweenArrays' method available in the 'limma' package of the R statistical computing software suite and adjusted for batch effects using the ComBat software [22]. Differential expression (DE) of H3N2 WT and LAIV was determined by comparing the average ratio of virus-infected replicates to time-matched and donor-matched mock-infected samples based on a linear model fit using the 'limma' package. Criteria for differential expression were an absolute fold-change of 1.5 and an adjusted *p*-value of <0.05, calculated by Benjamini-Hochberg correction [3].

2.5. Functional enrichment analysis

Functional analysis of DE genes was done using Ingenuity Pathway Analysis (IPA, Ingenuity Systems). IPA canonical pathway enrichment was calculated using a right-tailed Fisher's exact test with a threshold of significance set at *p*-values 0.05. Enrichment of Diseases and Biological Functions and upstream regulators analysis were based on activation |Z-scores| > 2 and *p*-values < 0.05. Virus-infected cells were compared to time- and donor-matched mock-infected samples.

2.6. Gene expression profile correlations

Correlation analysis of influenza signatures to cytokine response gene expression profiles derived from hNECs was performed using data available from the NCBI-GEO database under the following accession number: GSE19182. **Spearman** correlation coefficients (r) and confidence intervals were calculated using the 'psych' package in R statistical computing software suite. Virus-infected cells were compared to time- and donor-matched mock-infected samples.

2.7. Chemokine and cytokine measurements

Secreted chemokines and proinflammatory cytokines were quantified from the basolateral samples of both low and high MOI infections using multiplex ELISAs (MesoScale Diagnostics):

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