microRNAs Regulate Host Immune Response and Pathogenesis During Influenza Infection in Rhesus Macaques

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Abstract

microRNAs (miRNAs) are small noncoding RNAs that are key regulators of biological processes, including the immune response to viral infections. Differential expression levels of cellular miRNAs and their predicted targets have been described in the lungs of H1N1-infected BALB/c mice, the lungs of H5N1 influenza-infected cynomolgus macaques, and in peripheral blood mononuclear cells (PBMCs) of critically ill patients infected with 2009 pandemic H1N1. However, a longitudinal analysis of changes in the expression of miRNAs and their targets during influenza infection and how they relate to viral replication and host response has yet to be carried out. In the present study, we conducted a comprehensive analysis of innate and adaptive immune responses as well as the expression of several miRNAs and their validated targets in both peripheral blood and bronchoalveolar lavage (BAL) collected from rhesus macaques over the course of infection with the 2009 H1N1 virus A/Mexico/4108/2009 (MEX4108). We describe a distinct set of differentially expressed miRNAs in BAL and PBMCs, which regulate the expression of genes involved in inflammation, immune response, and regulation of cell cycle and apoptosis.

Introduction

MICRORNAs (miRNAs) are small (~22 nucleotides long), single-stranded noncoding RNAs that mediate post-transcriptional silencing of genes by binding to target mRNAs in a sequence-dependent manner. microRNAs have emerged as key regulators of the immune system, including regulation of cell cycle (miR-34c, miR-138, miR-193b, miR-129, and let-7f), induction of innate immunity (let-7f, miR-146b, miR-155, miR-192, miR-223, miR-451), and the development and differentiation of B and T cells (miR-34c, miR-181a, miR17–92 cluster) (4,10,17,20,26,45,50,52,53).

Influenza A viruses continue to cause respiratory tract infections resulting in significant morbidity and mortality (40), as illustrated by the emergence of the 2009 H1N1 influenza virus, which caused the first global pandemic in over 40 years (33). The host immune response to influenza infection is characterized by the induction of both innate and adaptive immunity (24). Several microRNAs have been shown to play important roles in the host response during influenza virus infection. Notably, miR-323, miR-491, miR-654, and let-7c can downregulate viral gene expression and inhibit H1N1 influenza A virus replication *in vitro* (30,39).

Differential expression levels of other miRNAs (miR-10a, miR-21, miR-29a–c, miR-30a–c, miR-31, miR-148, miR-155, miR-210, miR-223, miR-233, and miR-342) and their

predicted targets have also been observed in peripheral blood mononuclear cells (PBMCs) of critically ill patients, the lungs of H5N1 influenza-infected cynomolgus macaques, and the lungs of H1N1-infected BALB/c mice (22,38,51). In addition, temporal- and strain-specific miRNA expression was profiled in A549 cells infected with either pandemic H1N1 (2009) or H7N7 (2003), but these studies lacked the pressure normally exerted by the host immune system (28). Consequently, longitudinal changes in miRNA expression and their targets during influenza infection and how they relate to viral replication and host response *in vivo* remain poorly understood.

In this study, we defined the changes in microRNA expression and their validated targets in PBMCs and bronchoalveolar lavage (BAL) cells following infection with the 2009 H1N1 virus A/Mexico/4108/2009 (MEX4108) in rhesus macaques. Previous studies showed that cynomolgus macaques recapitulated the clinical manifestations of human infection with H5N1 and the 1918 strain (2,6,21,46). We recently showed that aged rhesus macaques infected with pandemic 2009 H1N1 California strain showed higher viral loads and a more robust inflammatory response in the BAL compared with young adults (19).

Data presented herein show that infection with MEX4108 induced a robust immune response as well as differential expression of select miRNAs and their validated mRNA targets in BAL. These targets play a role in inflammation

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and development of host immunity, as well as the regulation of cell cycle and apoptosis. Taken together, these data suggest that coordinated changes in miRNAs and their respective targets play an important role regulating host immune response to influenza infection.

Materials and Methods

Virus

A/Mexico/4108/2009 (H1N1) virus was a gift from Dr. Yoshi Kawaoka. Briefly, the virus was grown in Madin-Darby canine kidney (MDCK) epithelial cells and harvested when >70% of the cells exhibited a cytopathic effect for virus stock generation. The virus was titrated on MDCK cells using a 50% tissue culture infective dose (TCID₅₀) assay as previously described (36).

Animal studies and sample collection

The study was carried out in strict accordance with the recommendations described in the *Guide for the Care and Use of Laboratory Animals* and approved by the Oregon National Primate Research Center (ONPRC), Institutional Animal Care and Use Committee. Eight young adult (10–12 years of age) female rhesus macaques (*Macaca mulatta*) and eight aged (20–24 years of age) rhesus macaques were used in these studies ($n=8/\text{group}$). Animals were housed in adjoining individual primate cages allowing social interactions. Food and water were available *ad libitum*.

Animals were infected using a combination of intratracheal (4 mL), intranasal (0.5 mL/nostril), and conjunctival (0.5 mL/eyelid) routes for a total dose of 7×10^6 TCID₅₀. Blood samples were collected on days 0, 1, 2, 4, 7, 10, 14, 21, 28, 35, and 42 and BAL samples were collected on days 0, 4, 7, 10, 14, 21, 28, and 35 postinfection. All procedures were carried out under ketamine anesthesia by trained personnel.

Viral loads

Viral RNA was extracted from BAL supernatant and nasal, ocular, and throat swabs using a ZR viral RNA kit, as per the manufacturer's instructions (Zymo Research, Irvine, CA). Briefly, 100 μL of supernatant was transferred to a tube containing 300 μL of ZR viral RNA buffer. This mixture was bound to a Zymo-Spin IC column by centrifugation at 16,000 g for 2 min. The flowthrough was discarded, and the column was washed twice with 300 μL of RNA wash buffer. Residual wash buffer was removed by centrifugation, and the purified RNA was eluted with 12 μL of RNase-free water. Purified RNA was reverse transcribed using a high-capacity cDNA reverse transcription (RT) kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions for 20 μL reaction mixtures.

Viral loads were determined using absolute quantitative RT-polymerase chain reaction (PCR) using primers and probes specific for MEX4108 HA viral RNA and an amplicon standard and a StepOnePlus real-time PCR system from Applied Biosciences (Waltham, MA). The forward primer sequence is 5' GAT GGT AGA TGG ATG GTA CGG TTA T 3', the reverse primer sequence is 5' TTG TTA GTA ATC TCG TCA ATG GCA TT 3', and the probe sequence is 5' 6-FAM ATA TGC AGC CGA CCT GAA GAG CAC ACA

3' BHQ (FAM is 6-carboxyfluorescein and BHQ is black hole quencher dye). cDNA was subjected to 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C.

Analysis of immune cell subsets

PBMCs and BAL cells were stained with antibodies against CD3 (BD Pharmingen, San Diego, CA), CD20 (Beckman Coulter, Brea, CA), CD14 (BioLegend, San Diego, CA), HLA-DR (BioLegend), CD11c (BioLegend), and CD123 (BioLegend) to delineate monocytes (CD3⁺CD20⁺CD14⁺HLA-DR⁺) and dendritic cells (DCs, CD3⁺CD20⁺CD14⁺HLA-DR⁺). DCs were further defined into myeloid (mDCs, CD123⁺CD11c⁺) and plasmacytoid (pDCs, CD123⁺CD11c⁺) DCs. Cells were fixed according to the manufacturer's recommendations (BioLegend) and analyzed using the LSRII instrument (Beckton Dickinson, San Jose, CA) and FlowJo software (TreeStar, Ashland, OR).

To analyze T and B cell subsets, PBMCs and BAL fluid cells were stained with antibodies against CD8 β (Beckman Coulter), CD4 (eBioscience, San Diego, CA), CD28 (BioLegend), CD95 (BioLegend), CD20 (Beckman Coulter), IgD (Southern Biotech), and CD27 (BioLegend). This panel allows us to delineate naïve (CD28⁺CD95⁺), central memory (CM; CD28⁺CD95⁺), and effector memory (EM; CD28⁺CD95⁺) CD4 and CD8 T cell subsets, as well as naïve (IgD⁺CD27⁺), marginal zone-like (MZ-like; IgD⁺CD27⁺), and memory (IgD-negative CD27⁺) CD20⁺ B cell subsets (31). Cells were then fixed and permeabilized according to the manufacturer's recommendations (BioLegend) before the addition of Ki67-specific antibody (BD Pharmingen). The samples were acquired using an LSRII instrument (Beckton Dickinson, Franklin Lakes, NJ) and data were analyzed using FlowJo software (TreeStar).

Intracellular cytokine staining

Peptide libraries covering the entire genome of MEX4108 were designed as 15-mers overlapping by 9 amino acids using software available on the Sigma website (St. Louis, MO). The GenBank accession numbers of the MEX4108 genes are as follows: NS1-NEP, JF915191; PB1(F2), JF915189; M1–M2, JF915185; PB2, JF915190; NP, JF915187; PA, JF915188; NA, JF915186; and HA, JF915184. Peptides were reconstituted in DMSO and then pooled into libraries. BAL cells and PBMCs were stimulated with the overlapping peptide libraries for 16 h. At the end of the incubation, cells were first stained with anti-CD4 and anti-CD8 β antibodies. The cells were then permeabilized, followed by the addition of anti-interferon (IFN)- γ and antitumor necrosis factor (TNF)- α (eBioscience). Samples were acquired using an LSRII instrument, and the data were analyzed using FlowJo software.

Assessment of humoral immune response

IgG and IgA binding antibody titers were measured in plasma and BAL supernatant by enzyme-linked immunosorbent assay (ELISA) using plates coated with 1 $\mu\text{g}/\text{mL}$ recombinant MEX4108 HA protein (plasma) overnight at 4°C (Sino Biological, Inc., Beijing, China). Plates were then incubated with heat-inactivated (56°C, 30 min) plasma or BAL supernatant samples in threefold dilutions in triplicates. Plates were developed using horseradish peroxidase (HRP)-conjugated antirhesus IgG (Open Biosystems, Rockford, IL) or antirhesus

IgA (Fitzgerald, Acton, MA) and o-phenylenediamine dihydrochloride (OPD) substrate (Sigma). The reaction was stopped with the addition of 2 M HCl. IgG endpoint titers were calculated using the log-log transformation of the linear portion of the curve and 0.1 optical density (OD) units as the cutoff. IgG and IgA titers were standardized using a positive control sample that was included in every ELISA plate.

Hemagglutination inhibition (HI) titers were performed using chicken red blood cells as previously described (5). Results are expressed as the reciprocal serum titer at which inhibition of hemagglutination by the MEX4108 virus was no longer observed.

Assessment of cytokine, chemokine, and growth factor levels

BAL supernatant and plasma samples (stored at -80°C) were thawed and diluted 1:2 in a serum matrix for analysis with Milliplex nonhuman primate magnetic bead panel, as per the manufacturer's instructions (Millipore Corporation, Billerica, MA). Concentrations for $\text{IFN}\gamma$, interleukin (IL)-1 β , IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-12, IL-15, IL-17, TNF α , migration inhibitory factor (MIF), Monocyte chemoattractant protein-1 (MCP-1), MIP-1 α , MIP-1 β , Regulated Upon Activation, Normally T-Expressed, and Presumably Secreted (RANTES), eotaxin, Macrophage derived cytokine (MDC), interferon-inducible T cell alpha chemoattractant

(I-TAC), monokine induced by gamma interferon (MIG), IL-8, basic fibroblast growth factor (FGF-basic), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF) were determined for all samples. Values below the limit of detection of the assay were assigned a value half that of the lowest standard.

RNA extraction

RNA was extracted from 10^6 BAL fluid cells and PBMCs using TRIzol (Applied Biosciences) and the miRNeasy micro kit (Qiagen, Valencia, CA). RNA was resuspended in RNase-free water, and the RNA concentration was determined using a Thermo Scientific Nanodrop 2000 spectrophotometer (Fischer, Houston, TX).

miRNA microarray analysis

RNAs from PBMCs and BAL from three young adults and three aged animals at 0 and 7 days postinfection (dpi) were used for miRNA microarray analysis. The integrity of each RNA sample was determined using an Agilent 2100 bioanalyzer with Expert software (Agilent Technologies, Santa Clara, CA). Probe labeling and microarray slide hybridization were performed for each biological replicate

FIG. 1. Pandemic H1N1 virus replicates to similar levels in young and aged macaques. Viral loads were measured using qRT-PCR using primers and probes specific for MEX4108 hemagglutinin (HA) in throat swabs (A), nasal swabs (B), ocular swabs (C), and BAL fluid (D). Viral genome copy number data were log transformed with base 10 and longitudinal changes of viral genome copy number between aged and young adults were compared using a two-way ANOVA, followed by Bonferroni's multiple comparison post-test to determine differences in viral load. Longitudinal changes were compared using repeated-measures ANOVA, followed by Dunnett's multiple comparison post-test to explore differences between days postinfection and baseline (day 0) values, mean $\pm$ SEM are shown. (A–D) * for aged animals; † for young adult animals; **** p < 0.01, **** p < 0.001. BAL, bronchoalveolar lavage; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

using a rhesus macaque miRNA microarray kit (Agilent Technologies), according to the manufacturer's instructions. Five hundred nanograms of total RNA was used to make microRNA probes according to the manufacturer's protocol. Probes were hybridized at 56°C for 16 h. The slides were then washed according to the manufacturer's protocol.

After being washed, the slides were scanned using the Agilent Microarray scanner. Extracted raw data were background corrected using the *normexp* method with an offset of 50 and quantile normalized using the *limma* package in the R environment. Replicated probes were mean summarized, and control probes were filtered out. Microarray expression data for each animal at each time point were calculated as the ratio of its expression at day 0 postinfection. These ratios were logarithm base 2 transformed and are referred to as $\log_2$ ratios. A Student *t*-test on $\log_2$ ratios was performed to determine the probes that were differentially changed upon infection between day 0 and day 7 postinfection. Criteria for differential expression were an absolute difference between $\log_2$ ratios of day 0 and day 7 postinfection of >1.5 and a Benjamini–Hochberg-adjusted *q* value of <0.05 .

cDNA synthesis and qRT-PCR

cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems). microRNA expression was determined using TaqMan microRNA assays (Applied Biosystems). The following kits were used: U6 snRNA, assay ID 001973; hsa-let-7f, assay ID 000382; hsa-miR-18b, assay-ID 002217; hsa-miR-20a, assay-ID 245304; hsa-miR-34c, assay ID 000428; hsa-miR-129, assay ID 000590; hsa-miR-132, assay ID 000457; hsa-miR-138, assay ID 002284; hsa-miR-146b, assay ID 001097; hsa-miR-192, assay ID 000491; hsa-miR-193b, assay ID 002367; hsa-miR-451, assay ID 001141; and hsa-miR-595, assay ID 242609. Fold changes were determined using the $2^{(-\Delta\Delta CT)}$ method (27). Expression of candidate miRNAs was normalized to expression levels of U6 snRNA.

Target mRNAs were selected using miRWalk version 2.0 and their expression was determined using TaqMan gene expression assays (Applied Biosystems). The following TaqMan gene assays were used: *KRAS*, *BLIMP1*, *CDK6*, *SIRT1*, *ZAP70*, *MYC*, *BIM*, *PTEN*, *CDKN1A*, *RBI*, *CCND1*, *MIF*, and *NFKB*. Expression of mRNAs was normalized to expression levels of *RPL32*.

Statistical analysis

Graphing was performed with GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Overall, viral loads

in BAL and PBMCs were determined using area under curve (AUC), which was calculated by trapezoidal integration. A Mann–Whitney–Wilcoxon test was used for assessing differences in viral load AUCs between aged and young adult animals. ELISA titers and viral genome copy number data were log transformed with base 10 to hold the normal distribution assumption, and longitudinal changes of viral genome copy number between aged and young adults were compared using a two-way ANOVA, followed by Bonferroni's multiple comparison post-test to determine differences in viral load between groups and between dpi and baseline (day 0) values.

Longitudinal analysis of the frequency of responding and proliferating immune cells within BAL and within PBMCs was carried out using a one-way repeated-measures ANOVA model with a compound symmetric variance–covariance structure, followed by Dunnett's multiple comparison post-test to explore differences between days postinfection and baseline (day 0) values. Statistical significance for all comparisons was determined at the alpha level of 0.05.

Results

Pandemic H1N1 virus replicates to similar levels in young and aged macaques

Eight young adult and eight aged female rhesus macaques with no pre-existing immunity were infected with a total dose of 7×10^6 TCID₅₀ A/Mexico/4108/2009 (H1N1) (MEX4108) virus through the tracheal, nasal, and ocular routes as previously described (19). Animals were monitored for signs of clinical disease by visual inspection (rhinorrhea and respiratory rate) and by collecting temperatures on a daily basis for the first 4 days and then on 7, 10, 14, and 21 dpi. Overall, MEX4108 infection was asymptomatic in animals, with no significant increase in temperature, respiratory rate, or rhinorrhea (data not shown).

To determine the impact of age on MEX4108 replication, viral loads were measured in throat, nasal, and ocular swabs, as well as BAL fluid samples using quantitative RT-PCR and primers and probes specific for MEX4108 HA viral RNA (Fig. 1). Viral genome copy numbers in throat swabs were highest at 1 dpi before declining uniformly in both aged and young adults at 10 dpi (Fig. 1A). Similarly, viral RNA was detectable in nasal swabs at 1 dpi, peaking at 4 dpi, before declining at 10 dpi (Fig. 1B). In contrast, viral genomes in ocular swabs were not detected until 4 dpi, although at a lower level than in nasal or throat, and decreased to preinfection levels at 10 dpi (Fig. 1C). Last, viral loads in BAL fluid were significantly increased at 4 dpi before

FIG. 2. MEX4108 infection results in increased frequency of pDCs and robust production of cytokine, chemokine, and growth factor levels in the lungs. **(A)** The frequencies of DCs (lin⁺CD14⁺HLA-DR⁺) and macrophages/monocytes (lin⁺CD14⁺HLA-DR⁺) in BAL cells were measured by flow cytometry. **(B)** The frequencies of mDC (CD123⁺CD11c⁺) and pDC (CD123⁺CD11c⁺) in BAL cells were measured by flow cytometry. Longitudinal analyses of the frequency of immune cells within BAL were carried out using a one-way repeated-measures ANOVA model, followed by Dunnett's multiple comparison post-test to explore differences between days postinfection and baseline (day 0) values. Mean $\pm$ SEM are shown. * monocytes and mDCs; † for DCs and pDCs; *† $p < 0.05$ ***†† $p < 0.01$, ****††† $p < 0.001$ **(C–E)** Cytokine **(C)**, chemokine **(D)**, and growth factor **(E)** levels in BAL supernatant were determined using Luminex technology. The concentration of IFN α in BAL supernatant was determined by IFN α ELISA **(C)**. Left axis (white bar) and right axis (gray bar). Longitudinal changes in cytokine concentration changes were compared using repeated-measures ANOVA, followed by Dunnett's multiple comparison post-test. Mean $\pm$ SEM are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. DCs, dendritic cells; IFN, interferon; mDC, myeloid DC; pDC, plasmacytoid DC.

decreasing in both young adult and aged animals at 10 dpi (Fig. 1D). Two-way ANOVA and AUC analyses showed no differences in viral loads between young and aged animals at any given time point; therefore, for the remaining result sections, we combined data from both age groups.

MEX4108 infection results in increased frequency of pDCs in the lungs

Since innate immune cells are the first line of defense to viral infection, we characterized changes in the frequency of monocytes and DCs in BAL cells and PBMCs using flow

FIG. 3. MEX4108 infection induces a robust T cell and B cell response in BAL. The frequencies of CD20 B cells, and CD4 and CD8 T cells in BAL cells (A), as well as CM and EM subsets within CD4 (B) and CD8 T cells (E) in BAL, were measured by flow cytometry. Proliferation of CD4 (C) and CD8 (F) T cell subsets was assessed by expression of Ki67 using flow cytometry. MEX4108-specific CD4 (D) and CD8 (G) T cells in BAL samples producing IFN γ or TNF α were measured by intracellular cytokine staining following stimulation with HA-NA, PA-NP, PB1-PB1F2-NP-NSI, and PB2-M1-M2 proteins. (H) The frequencies of naïve, memory, and MZ-like B cells were measured in BAL samples by flow cytometry. (I) Proliferation of B cell subsets was assessed by measuring expression of Ki67 in naïve, memory, and MZ-like B cells in the BAL using flow cytometry. (J) H1N1-specific IgG and IgA antibody endpoint titers in BAL supernatant and in plasma and HI antibody endpoint titers in plasma. Longitudinal analyses of immune responses within BAL were carried out using a one-way repeated-measures ANOVA, followed by Dunnett's multiple comparison post-test to explore differences between days postinfection and baseline (day 0) values. Mean $\pm$ SEM are shown. (A–I) *CD4 T cells, CD4 CM, CD8 CM, naïve CD20 B cells, and HA-NA; $\dagger$ CD8 T cells, CD4 EM, CD8 EM, memory B cells, and PA-NP; $\ddagger$ CD20, MZ-like B cells, and PB2-M1-M2; $\#$ PB1-PB1F2-NP-NSI. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. CM, central memory; EM, effector memory; HI, hemagglutination inhibition; MZ, marginal zone; TNF, tumor necrosis factor.

cytometry. In the BAL, we observed a significant decrease in the percentage of monocytes at all time points postinfection with the exception of 7 dpi (Fig. 2A). In contrast, the percentage of DCs increased 7 dpi and peaked 10 dpi, returning to baseline 17 dpi (Fig. 2A). Additionally, we detected a significant increase in the relative frequency of pDCs 4 dpi that was accompanied by a concurrent decrease in mDC frequency (Fig. 2B).

In the peripheral blood, the percentage of monocytes initially decreased 4 dpi, followed by a steady increase until the end of the study at 42 dpi (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/vim). The percentage of DCs in PBMCs increased 10 dpi, followed by a return to baseline (Supplementary Fig. S1A). Within the DC population, we detected a decrease in the frequency of pDCs at 4 dpi, which persisted until 35 dpi (Supplementary Fig. S1B).

MEX4108 infection results in increased cytokine, chemokine, and growth factor levels in the lungs

Given the changes in monocyte and DC populations, we next assessed changes in the concentration of several cytokines, chemokines, and growth factors in BAL supernatant (Fig. 2C, E) and plasma (Supplementary Fig. S1C) using a multiplex assay. In BAL fluid, concentrations of IL-2, IL-4, IL-5, IL-8, IL-10, IL-17, and GM-CSF remained below the level of detection, while levels of RANTES remained unchanged (data not shown).

In contrast, we saw a significant and sharp increase in IFN α levels at 4 dpi in BAL supernatant (Fig. 2C) consistent with the observed increase in pDC frequency. Inflammatory cytokines, IL-1 β , IL-6, TNF α , and IL-15, as well as regulatory cytokines, IL-1RA and IL-10, were significantly increased at 4 and 7 dpi, returning to basal levels at 10 dpi (Fig. 2C). Levels of IL-12 also increased at 4 dpi, but remained elevated until 14 dpi, while levels of the T cell cytokine, IFN γ , increased only at 7 dpi in BAL fluid (Fig. 2C).

In addition, MEX4108 infection resulted in the upregulation of several chemokines (Fig. 2D). Levels of eosinophil attractant, eotaxin; T cell attractants, I-TAC (CXCL11) and MIG (CXCL9); monocyte attractant, MCP-1; and leukocyte attractants, MIP-1 α and MIP-1 β , were significantly increased at 4 and 7 dpi, returning to baseline by 10 dpi. Levels of MIF increased only at 7 dpi in BAL fluid. In the plasma, we only saw a transient increase in the T cell chemoattractant, I-TAC, at 7 dpi (Supplementary Fig. S1C).

MEX4108 infection also led to the production of growth factors in the BAL fluid (Fig. 3E). We detected a significant increase in the levels of FGF-basic, G-CSF, EGF, and HGF at 4 and 7 dpi with concentrations returning to basal levels by 10 dpi, with the exception of HGF, which returned to baseline 14 dpi. Levels of VEGF increased significantly at 7 and 10 dpi, returning to baseline 14 dpi.

MEX4108 infection induces a robust T cell response in BAL

To characterize the adaptive immune response to MEX4108, we first assessed changes in the frequency of T cells using flow cytometry. Although initially comparable, the relative frequency of CD4 T cells decreased starting at 10 dpi, while that of CD8 T cells increased, making them the dom-

inant lymphocyte population in the BAL (Fig. 3A). Moreover, while the frequency of CM and EM CD4 T cells remained stable (Fig. 3B), CD8 EM T cells became the dominant population at 10 dpi (Fig. 3E). In contrast to the BAL, the frequency of CD8 T cells in the peripheral blood decreased starting at 4 dpi, while the relative frequency of CD4 T cells significantly increased on days 4, 7, and 21 dpi (Supplementary Fig. S2A). Moreover, CD4 CM T cells became the dominant population, whereas the frequency of CD8 CM and EM T cells remained stable (Supplementary Fig. S2B, C).

To characterize the MEX4108-specific T cell response, we first measured T cell proliferation following H1N1 infection by assessing changes in the expression of Ki67, a nuclear protein associated with DNA replication. In BAL, proliferation of all T cell subsets significantly increased at 7 dpi and peaked 10 dpi, returning to baseline 21 dpi (Fig. 3C, F). Proliferation of all T cell subsets in peripheral blood also significantly increased 7 dpi and peaked 10 dpi, although at a lower magnitude (Supplementary Fig. S2D, E). Since proliferation can be due to bystander activation, we also determined the frequency of MEX4108-specific T cells in BAL and peripheral blood by measuring IFN γ /TNF α production by intracellular cytokine staining following stimulation with peptide pools covering the entire MEX4108 genome.

CD4 T cell responses to MEX4108 antigens were detected at 7 dpi and peaked 14 dpi before establishing a memory set point 28–35 dpi, with the exception of responses to PB2-M1-M2, which increased again 35 and 42 dpi (Fig. 3D). The frequency of MEX4108-specific CD8 T cells peaked 14–21 dpi, establishing a memory set point at 28 dpi with the exception of PB2-M1-M2-specific CD8 T cells, which as described for CD4 T cells increased at 35 and 42 dpi (Fig. 3G). No antigen-specific T cells were detected in peripheral blood (data not shown).

MEX4108 infection engenders a B cell response in BAL

Next, we characterized humoral responses. Although the frequency of CD20 B cells remained very low in the BAL

TABLE 1. MICRORNA MICROARRAY OF DIFFERENTIALLY EXPRESSED MICRORNAs IN BAL AT 7 DPI

<i>microRNAs in BAL</i>	<i>log₂ fold change</i>	<i>p</i>
Let-7f	−7.77	0.036
miR-18b	−6.1069	0.0016
miR-34c	−4.2673	0.0460
miR-129	−4.4560	0.0237
miR-146b	−3.8791	0.0491
<i>microRNAs in PBMCs</i>	<i>Fold change</i>	<i>p</i>
miR-132	−2.217	0.010
miR-138	−2.837	0.050
miR-193b	−3.017	0.003
miR-595	−1.778	0.034
miR-18b	2.097	0.050
miR-20a	1.640	0.044
miR-192	1.636	0.050
miR-451	14.527	0.001

BAL, bronchoalveolar lavage; PBMCs, peripheral blood mononuclear cells.

throughout the course of infection (Fig. 3A), we detected an increase in the frequency of naïve B cells at 4–10 dpi, followed by an increase in the frequency of MZ-like and memory B cells between 10 and 42 dpi (Fig. 3H). Proliferation of all B cell subsets was delayed compared with T cells (detected 10 dpi), but lasted longer, returning to baseline at 35 dpi (Fig. 3I). H1N1-specific IgG and IgA were detected in BAL supernatant at 7 and 14 dpi, respectively, and remained significantly increased until 28 dpi. hemagglutinin (HA)-specific IgG and HI titers were detected in plasma at 7 and 14 dpi, respectively, and remained elevated until 42 dpi (Fig. 3J). In peripheral blood, the frequency of B cells remained stable throughout MEX4108 infection (Supplemen-

tary Fig. S2A). B cell proliferation in peripheral blood was delayed and reduced compared with BAL, peaking 14 dpi and returning to baseline 17 dpi (Supplementary Fig. S2F, G).

MEX4108 infection results in different expression patterns of microRNAs in BAL and PBMCs

Considerable evidence has shown that microRNAs regulate immune function. To determine the contribution of microRNAs in coordinating the immune response to influenza infection, we performed an miRNA microarray analysis on BAL and PBMCs from three aged and three young adult animals on 0 and 7 dpi. Differentially expressed miRNAs were defined as

FIG. 4. Differentially expressed miRNAs in BAL cells following MEX4108. Expression levels of let-7f (A), miR-34c (B), miR-129 (C), miR-18b (D), miR-146b (E), miR-132 (F), miR-192 (G), and miR-138 (H) in BAL cells from rhesus macaques ($n=8$; five aged and three young adults) following MEX4108 infection were determined using qRT-PCR. Expression of miRNAs was normalized to expression of U6 snRNA. Changes in microRNA expression postinfection were determined using one-way repeated-measures ANOVA, followed by Dunnett's multiple comparison post-test. Mean $\pm$ SEM are shown. * $p < 0.05$, *** $p < 0.001$.

those with a log fold change higher than 1.5 and $p \leq 0.05$. In the BAL, five microRNAs were significantly downregulated at 7 dpi (let-7f, miR-18b, miR-34c, miR-129, and miR-146b) (Table 1). In PBMCs, four microRNAs were downregulated (miR-132, miR-138, miR-193b, and miR-595) and four were upregulated (miR-18b, miR-20a, miR-192, and miR-451) at day 7 postinfection (Table 1).

To confirm the observed differential miRNA expression, we performed longitudinal analysis using qRT-PCR on BAL samples from 8 additional animals (5 aged and 3 young adults) and PBMCs from 11 animals (5 aged and 6 young adults). In the BAL, we confirmed the downregulation of let-7f expression at 4 dpi (Fig. 4A) and miR-34c, which remained downregulated throughout the study (Fig. 4B). Expression of miR-129 was significantly decreased at 17 and 21 dpi (Fig. 4C). In contrast to the microarray data, expression levels of miR-18b showed a slight increase at 7 dpi, followed by a significant increase at 14 dpi (Fig. 4D). Similarly, instead of a decrease, expression levels of miR-146b increased in several animals 14–21 dpi, although it does not reach statistical significance (Fig. 4E).

In PBMCs, we confirmed increased expression of miRNA-18b, miR-20a, miR-192, and miR-451, although at 28 dpi (Fig. 5A–D). In contrast, expression of 138 and 193b was not decreased as suggested by the microarray data, but was rather increased at 28 dpi (Fig. 5E, F). We also confirmed decreased expression of miR-132 7 dpi (Fig. 5G), but expression of miR-595 did not change with infection (data not shown).

To determine compartment specificity of miRNA expression changes, levels of miRNAs that were differentially expressed in BAL (and confirmed via qRT-PCR) were measured in PBMCs and *vice versa*. Expression of miR-132 (downregulated in PBMCs at 4 dpi), miR-192 (upregulated in PBMCs at 28 dpi), and miR-138 (upregulated in PBMCs at 28 dpi) significantly increased at 35 and 17 dpi in BAL, respectively (Fig. 4F–H), whereas expression of miR-20a, miR-193b, and miR-451 remained unchanged (data not shown). In PBMCs, expression of let-7f (downregulated at 4 dpi in BAL) and miR-146 (upregulated in some animals at 14 dpi in BAL) significantly increased at 28 dpi (Fig. 5H–I), while miR-34c and miR-129 were not differentially expressed (data not shown).

FIG. 5. Differentially expressed miRNAs in PBMCs following MEX4108. Expression levels of miR-18b (A), miR-20a (B), miR-192 (C), miR-451 (D), miR-138 (E), miR-193b (F), miR-132 (G), let-7f (H), and miR-146b (I) in PBMCs from rhesus macaques ($n = 11$; five aged and six young adults) following MEX4108 infection were determined using qRT-PCR. Expression of miRNAs was normalized to expression of U6 snRNA. Changes in microRNA expression postinfection were determined using one-way repeated-measures ANOVA, followed by Dunnett's multiple comparison post-test. Mean $\pm$ SEM are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. PBMC, peripheral blood mononuclear cell.

Validated miRNA targets are involved in immune response and cell proliferation

To better understand the roles these microRNAs play during influenza infection, we investigated the expression of their validated targets identified using miRWalk. We selected some of the mRNA targets for validation based on several criteria: (a) targeted by multiple miRNAs within our dataset or (b) involved in inflammation, T and B cell activation, or cell proliferation (Table 2). We then analyzed the expression of these mRNAs in both BAL and PBMCs using TaqMan qRT-PCR at day 0, the same time points the miRNAs were differentially expressed, as well as the subsequent time point, or last time point (Figs. 6 and 7).

In the BAL, targets of Let-7f (downregulated at 4 dpi), *KRAS* (promotes cell growth and differentiation), *BLIMP1* (important for plasma cell differentiation), and *CDK6* (regulates cell cycle progression) were significantly upregulated at 7 and 35 dpi (Fig. 6A). Protein levels of IL-6 and IL-10, which are additional targets of Let-7f, were also increased at 4 and 7 dpi (Fig. 2C). Expression of *ZAP70* (important for TCR signaling), *MYC* (promotes cell growth and differentiation), and sirtuin 1 (*SIRT1*, a deacetylase shown to play a role in apoptosis), which are targets of miR-34c, increased at 7 and 35 dpi, respectively (Fig. 6B).

Levels of *CAMTA1* (regulates cell cycle), a target of miR-129 (downregulated at 17 and 21 dpi), were significantly increased at 17 dpi (Fig. 6C). Expression of *PTEN* (involved in cell cycle regulation), a target of miR-18b, was increased at 17 dpi right after miR-18b expression decreased (Fig. 6D). Expression of miR-132 target, *CDKN1A* (inhibits cell proliferation), miR-138 target, cyclin D1 (promotes cell cycle), and miR-192 target, *RB1* (inhibits cell proliferation), however, remained unchanged (data not shown).

This high level of concordance between miRNA and mRNA target expression was not observed in PBMCs.

Specifically, expression of *SIRT1*, which is targeted by miR-132 (downregulated 7 dpi), was upregulated at 14 and 42 dpi (Fig. 7A). Although not statistically significant, we also detected a trending increase in miR-132 target, *CDKN1A*, at 42 dpi (Fig. 7A). In contrast, expression of cell cycle regulator cyclin D1, which is targeted by miR-20a, miR-138, and miR-193b, was elevated at 28 dpi, although all three miRNAs were also upregulated (Fig. 7B).

Similarly, expression of macrophage migration inhibitory factor, *MIF* (a target of miR-451), was upregulated at the same time as miR-451 (Fig. 7C). In addition, expression of *BIM* and *PTEN*, targets of miR-20a (*PTEN* is also a target of miR-18b), increased at the same time point as miR20a (Fig. 7D, E). We measured levels of let-7f targets, but only found a significant increase in *BLIMP1* at 28 dpi, the same time point let-7f was increased (Fig. 7F). Last, we did not identify any changes in miR-192 target, *RB1*, or miR-146b target, *NFKB* (data not shown).

Discussion

Cellular miRNAs can control the molecular pathways of innate and adaptive immune responses and have been reported to participate in regulating host–pathogen interactions during viral infection. Although differential expression of cellular miRNAs following influenza infection has been characterized, no study to date has delineated these changes and their relationship to validated target expression in a temporal manner and in different compartments. In this study, we analyzed viral replication, immune response, and expression of select microRNAs and their validated targets over a longitudinal study in BAL and PBMCs of rhesus macaques infected with 2009 H1N1 virus A/Mexico/4108/2009 (MEX4108).

Our data revealed MEX4108 replicates in both the lower and upper respiratory tracts, consistent with viral replication

TABLE 2. MICRORNAS AND THEIR VALIDATED TARGETS

<i>microRNA</i>	<i>Validated targets</i>	<i>Function</i>	<i>Reference</i>
Let-7f	<i>KRAS</i>	Regulates cell proliferation and differentiation	(17)
	IL-6 and IL-10	Downregulates IL-6 and IL-10 expression	(37)
	Blimp-1	Regulates differentiation of antibody-secreting plasma cells	(34)
	<i>CDK6</i>	Regulates cell proliferation	(17)
miR-34c	<i>SIRT1</i>	Represses deacetylation of p53, increasing apoptosis.	(54)
	<i>ZAP70</i>	Regulates T cell receptor signaling	(10)
	<i>MYC</i>	Regulates cell proliferation and differentiation	(4)
miR-18b	<i>PTEN</i>	Regulates cell proliferation; regulates pro- to pretransition during B and T cell development	(52)
miR-129	<i>SOX4</i>	Regulates cell proliferation	(4,15,23)
	<i>CAMTA1</i>	Regulates cell proliferation; tumor suppressor	(13)
miR-146b	TRAF6 and IRAK	Negatively regulates Toll-like receptor signaling	(42)
miR-132	<i>SIRT1</i>	Represses deacetylation of p65, resulting in NFκB activation	(41)
miR-20a	Bim and <i>PTEN</i>	Regulates cell proliferation; regulates pro- to pretransition during B and T cell development	(52)
miR-138	Cyclin D1	Regulates cell proliferation	(55)
	Cyclin D1	Regulates cell proliferation	(26)
miR-192	MIP-2α	Downregulates MIP-2α expression	(50)
	<i>RB1</i>	Regulates cell proliferation; tumor suppressor	(11)
miR-193b	Cyclin D1	Regulates cell proliferation	(53)
miR-451	<i>MIF</i>	Downregulates MIF expression	(25)

FIG. 6. Validated miRNA targets are involved in immune response and cell proliferation in the lungs. Expression levels of (A) *KRAS*, *BLIMP1*, and *CDK6* (targets of let-7f); (B) *SIRT1*, *ZAP70*, and *MYC* (targets of miR-34c); (C) *CAMTA1* (target of miR-129); and (D) *PTEN* (target of miR-18b) in BAL from rhesus macaques (five aged and three young adults) following MEX4108 infection are shown. Expression of mRNA was normalized to expression of *RPL32*. Changes in gene expression postinfection were determined using one-way repeated-measures ANOVA, followed by Dunnett's multiple comparison post-test. Mean $\pm$ SEM are shown. (A–D) *let-7f, miR-34c, miR-129, miR-18-b, *KRAS*, *SIRT1*, *CAMTA1*, and *PTEN*; † *KRAS* and *ZAP70*; ‡ *CDK6* and *MYC*. * $p < 0.05$, *** $p < 0.01$, **** $p < 0.001$.

of other 2009 H1N1 strains (14,16,36). Although more virulent in cynomolgus macaques, duration and magnitude of MEX4108 replication were reduced in rhesus macaques compared with CA04 (19). Moreover, in contrast to our earlier observations that CA04 viral loads were higher in aged animals compared with young adults, we detected no differences in MEX4108 replication in aged versus young animals. We also detected no age-related differences in innate, T, or B cell responses. However, it is unclear at this point why MEX4108 is less pathogenic in macaques compared with CA04.

Despite limited viral replication, MEX4108 infection mobilized pDCs and a corresponding increase in IFN α production at 4 dpi in the BAL. As reported for CA04 infection (19), the increase in pDCs and IFN α occurs at the peak of viral loads in the BAL and precedes the cessation of viral replication. Interestingly, frequency of pDCs decreased in the peripheral blood at 4 dpi, suggesting potential recruitment to the site of infection, the lungs. MEX4108 infection also induced robust production of cytokines, chemokines, and growth factors in the BAL. Inflammatory cytokines, IL-1 β , IL-6, IL-12, and TNF α , were increased at 4 and 7 dpi. The initial burst of cytokine production at 4 dpi is most likely mediated by infected lung epithelial cells as well as by DCs and monocytes and correlates with a significant reduction in viral loads in the BAL.

We also show that regulatory cytokines, IL-10 and IL-1RA, were also increased at 4 and 7 dpi, which could be playing a role in mitigating the deleterious effects of proinflammatory cytokines. As we previously described for CA04 (19), levels of IL-15, which is important for NK cell

homeostasis and CD8 EM differentiation, increased at 4 and 7 dpi just before the sharp increase in CD8 EM frequency in the BAL, and IFN γ levels increased 7 dpi and correlated with T cell proliferation. Interestingly, no antigen-specific responses were detected at 7 dpi, which suggests that the IFN γ is most likely a result of bystander T cell activation. High levels of IL-12, which plays a critical role in differentiation of Th1 CD4 and CD8 EM T cells, persisted until 14 dpi, which correlated with T cell proliferation and the predominance of CD8 EM T cells.

Chemokines, MIP-1 α , MIP-1 β and MCP-1, which are involved in recruiting monocytes, memory T cells, and DCs to sites of inflammation (9,29), were significantly increased at 4 dpi and correlated with increased frequencies of DCs in the BAL. Their levels remained elevated at 7 dpi, which correlated with increased memory T cell proliferation in the BAL. Elevated levels of MCP-1 have been detected following CA04 and MEX4487 infection (19,36). Similarly, levels of T-cell chemoattractants, MIG and I-TAC, were elevated at 4 and 7 dpi and could have contributed to the recruitment of T cells into the BAL. Interestingly, the percentage of CD8 T cells in PBMCs decreased, while CD8 T cell frequency in the BAL increased, suggestive of recruitment of CD8 T cells from blood to the lungs.

In contrast to previous studies that reported increased CXCL8 expression in the lungs of H1N1 (A/CastillaLa-Mancha/RR5661/2009 and A/CastillaLaMancha/RR5911/2009)-infected ferrets, H1N1 (A/PR/8/34)-infected lung epithelial cells (1,47), and BAL of macaques infected with CA04 (19), the levels of this chemokine in the BAL did not change in our current study. This may be due to differences

FIG. 7. Validated miRNA targets are involved in immune response and cell proliferation in the peripheral blood. (A) Expression levels of *SIRT1* and *CDKN1A* (targets of miR-132); (B) *CCND1* (target of miR-20a, miR-138, and miR-193b), (C) *MIF* (target of miR-451), (D–E) *BIM* (target of miR-20a) and *PTEN* (target of miR-18b and miR-20a); and (F) *BLIMP1* (target of let-7f) in PBMCs from rhesus macaques (five aged and six young adults) following MEX4108 infection are shown. Expression of mRNA was normalized to expression of *RPL32*. Changes in gene expression postinfection were determined using one-way repeated-measures ANOVA, followed by Dunnett's multiple comparison post-test. Mean $\pm$ SEM are shown. (A–F) *miR-132, miR-20a, miR-451, miR-18b, let-7f, *SIRT1*, *CCND1*, *MIF*, *PTEN*, *BLIMP1*; †miR-138 and *BIM*; ‡miR-193b. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ††† $p < 0.001$, †††† $p < 0.0001$. *MIF*, migration inhibitory factor.

in tissues (lung vs. BAL) or severity of disease (the macaques in our study controlled viral replication more quickly than previously reported for CA04). Last, growth factors were increased in the BAL and may play a role in repairing tissue damage caused by viral replication. EGF and HGF, which are significantly increased at 4 dpi, can stimulate cell growth, proliferation, and differentiation. FGF-basic and VEGF, which are involved in angiogenesis and wound healing, were also significantly increased at 4 and 7 dpi, respectively.

MEX4108 infection also induced robust T and B cell responses as evidenced by proliferation and development of antigen-specific responses in the BAL. Interestingly, T and B cell proliferation peaked after the cessation of viral replication. Similarly, MEX4108-specific CD4 and CD8 T cells, as well as antibodies, were not detected in the BAL until 7 and 14 dpi, respectively. Although T cell proliferation was measured in peripheral blood, no antigen-specific T cell responses were detected in PBMCs. It is possible that antigen-specific T cells are quickly recruited into the lungs, where virus replication takes place. Together, these obser-

vations suggest that innate immunity might be sufficient to control MEX4108 replication and that adaptive immunity plays a more important role in protection against reinfection. This hypothesis would also explain why we did not observe any differences between aged and young animals.

Controlling influenza virus infection requires a tightly regulated antiviral immune response. The precise regulation of the immune response may be fine-tuned through transcriptional regulation by microRNAs. Thus, we profiled expression of miRNAs (selected based on their involvement in immune response) in BAL and PBMCs through qRT-PCR. MEX4108 infection changed the expression of distinct microRNAs in BAL and PBMCs. In the BAL, expression of let-7f and miR-34c was downregulated at 4 dpi. This is consistent with previous studies that have shown that both let-7f and miR-34c downregulated in the lungs of cynomolgus macaques infected with either H5N1 or 1918 influenza virus at days 2, 4, and 7 postinfection (22).

We also report an upregulation of miR-18b at 14 dpi in the BAL and at 21, 28, and 42 dpi in PBMCs. This observation is in line with previous studies of H5N3 avian

influenza infection in broiler chickens, in which miR-18b was upregulated at 4 dpi in lungs of infected chickens compared with noninfected chickens (48). We also observed a trend of increased miR-146b expression, which was previously reported to be increased at 24 h postinfection in human lung epithelial cells (A549) following *in vitro* infection with H1N1 and H3N2 influenza virus (38,44).

Additionally, our studies revealed that miR-20a and miR-451 were upregulated at 28 dpi in PBMCs as previously documented in serum (collected within 14 days from onset of infection) of patients infected with H7N9 influenza and at 8 h following *in vitro* H1N1 influenza infection of murine DCs, respectively (35,48,56). Our studies also showed elevated levels of miR-146b, which has previously been observed in whole blood (collected within 2 weeks from onset of infection) of patients infected with H1N1 (43). Finally, we identified changes in miRNAs during H1N1 infection not previously reported, including the downregulation of miR-34c and upregulation of miR-129, miR-132, miR-138, and miR-192 in the BAL. Additionally, upregulation of let-7f, miR-192, miR-193b, and miR-138 in PBMCs has not previously been documented.

To understand the biological implications of changes in miRNA expression after MEX4108 infection, we used miRWalk to identify experimentally validated targets of the differentially expressed miRNAs and investigated changes in their expression levels longitudinally through qRT-PCR. This analysis provides additional insight into how changes in microRNA expression can regulate host defense. For instance, expression of let-7f target, *KRAS*, which is involved in RAS/MAPK signaling and regulation of cell growth and differentiation (17,18), was upregulated shortly after downregulation of let-7f at 7 dpi in the BAL and correlates with increased Ki67 expression in multiple immune

cell subsets, as well as increased differentiation toward CD8 EM T cells and memory and MZ-like B cells.

Let-7f may also be involved in fine-tuning the innate immune response since inflammatory cytokine, IL-6, and anti-inflammatory cytokine, IL-10, are both targets of let-7f (37) and their levels were elevated at the same time point at which let-7f is downregulated. *BLIMP1* is another target of let-7f (34) and plays a role in B cell differentiation into Ig-secreting plasma cells. *BLIMP1* expression is upregulated at 7 dpi, shortly after downregulation of let-7f expression, and correlates with the increased frequency of memory and MZ-like B cells and antibody titers. Expression of *ZAP70*, a protein tyrosine kinase involved in T cell receptor signaling and a target of miR-34c (54), was significantly increased at 7 dpi shortly after downregulation of miR-34c in the BAL, which correlates with increased T cell proliferation at 7 dpi.

Expression of *SIRT1*, a target of miR-132 (41), which was downregulated at 7 dpi in the peripheral blood, was significantly increased at 14 and 42 dpi, which correlates with the proliferation of B cell subsets and the sustained proliferation of monocytes and DC subsets, respectively. Similarly, expression of *CAMTA1*, a transcription factor involved in inhibiting cell division (13) and a target of miR-129, was upregulated at 17 dpi in the BAL just before cessation of B and T cell proliferation. Another tumor suppressor, *PTEN* (targeted by miR-18b), was also significantly increased at both 17 and 35 dpi and may contribute to regulating cell division. Since miR-18b was upregulated, it is likely that *PTEN* is regulated by other miRNA-mediated mechanisms. Furthermore, expression of miR-146b increased 14–21 dpi, which may have contributed to regulating inflammatory responses as previously reported (8).

Levels of several cell cycle regulators were significantly increased at 35 dpi. Specifically, expression of *CDK6*, a

FIG. 8. Model of differentially expressed microRNAs, gene targets, and immune response in the lungs following MEX4108 infection.

catalytic subunit of a protein kinase complex that is important for cell cycle G1 phase progression and G1/S transition, and a target of let-7f (32) increased at 35 dpi. Similarly, *SIRT1* and *MYC*, both targets of miR-34c, were significantly increased at 35 dpi. Previous studies have shown that repression of *SIRT1* by miR-34a increases apoptosis by reducing *SIRT1*-catalyzed deacetylation of p53 (54). *MYC* functions as a transcription factor that regulates cell proliferation, cell growth, and apoptosis (7). The increased levels of *CDK6*, *SIRT1*, and *MYC* may contribute to the establishment of immunological memory, which is further supported by increased MEX4108-specific CD4 and CD8 T cells at 35 and 42 dpi.

Interestingly, the inverse correlation between expression of let-7f and miR-34c and their targets was delayed by 3 days. This might be due to the long half-life of mRNAs of those targets. Expression of some validated targets was not inversely correlated with the levels of microRNAs. For instance, expression of miR-20a, miR-138, and miR-193b and their target, cyclin D1 (*CCND1*), was increased at 28 dpi in PBMCs. Similarly, in PBMCs, expression of miR-20a, miR-18b, miR-451, and let-7f and their targets, *BIM*, *PTEN*, *MIF*, and *BLIMP1*, was upregulated at 28 dpi. These observations suggest that other validated targets might be downregulated and that *CCND1*, *BIM*, *PTEN*, *MIF*, and *BLIMP1* expression in the peripheral blood might be post-transcriptionally controlled by other regulatory mechanisms *in vivo*.

Several published studies indicate that miRNA expression is modulated in response to cytokine stimulation (3,12). A previous study reported that IL-6 stimulation of rat primary hepatocytes for 24 h resulted in an increased miR-18a expression (3). Interestingly, our study showed an increase in IL-6 and miR-18b at 4–7 and 14 dpi, respectively. Additionally, type I IFNs can modulate microRNA processing machinery. Protein levels of DICER were shown to be downregulated in JAR trophoblast cells following IFN α and polyI:C stimulation after 72 h (49). This is in line with our findings, in which let-7f and miR-34c were downregulated early in infection.

In summary, our study reveals that 2009 H1N1 influenza infection induces changes in miRNA expression that can regulate the expression of cellular targets involved in host defense and other critical cellular functions, thereby modulating the efficacy of the antiviral immune response, especially in the lungs, as outlined in Figure 8. These results provide additional insights into the regulation of host defense during respiratory viral infection and provide reference points for further investigation in modulating host defense at different stages of immune response to infection with microRNA mimics or inhibitors.

Future studies should also focus on determining the mechanisms that regulate changes in miRNA expression in addition to delineating the differential expression of miRNAs in specific immune cell subsets following influenza and other viral infections. Targeted intervention of miRNA-regulated processes could provide host-directed therapies for management of influenza infection.

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Author Disclosure Statement

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