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miR-451 Regulates Dendritic Cell Cytokine Responses to Influenza Infection

Carrie M. Rosenberger,* Rebecca L. Podyminogin,* Garnet Navarro,* Guo-Wei Zhao,† Peter S. Askovich,* Mitchell J. Weiss,† and Alan Aderem*‡

MicroRNAs (miRNAs) are important posttranscriptional regulators in immune cells, but how viral infection regulates miRNA expression to shape dendritic cell (DC) responses has not been well characterized. We identified 20 miRNAs that were differentially expressed in primary murine DCs in response to the dsRNA agonist polyinosinic-polycytidylic acid, a subset of which were modestly regulated by influenza infection. miR-451 was unique because it was induced more strongly in primary splenic and lung DCs by live viral infection than by purified agonists of pattern recognition receptors. We determined that miR-451 regulates a subset of proinflammatory cytokine responses. Three types of primary DCs treated with antisense RNA antagomirs directed against miR-451 secreted elevated levels of IL-6, TNF, CCL5/RANTES, and CCL3/MIP1α, and these results were confirmed using miR-451−/− cells. miR-451 negatively regulates YWHAZ/14-3-3ζ protein levels in various cell types, and we measured a similar inhibition of YWHAZ levels in DCs. It is known that YWHAZ can control the activity of two negative regulators of cytokine production: FOXO3, which is an inhibitory transcription factor, and ZFP36/Tristetraprolin, which binds to AU-rich elements within 3′-untranslated regions to destabilize cytokine mRNAs. Inhibition of miR-451 expression correlated with increased YWHAZ protein expression and decreased ZFP36 expression, providing a possible mechanism for the elevated secretion of IL-6, TNF, CCL5/RANTES, and CCL3/MIP1α. miR-451 levels are themselves increased by IL-6 and type I IFN, potentially forming a regulatory loop. These data suggest that viral infection specifically induces a miRNA that directs a negative regulatory cascade to tune DC cytokine production. The Journal of Immunology, 2012, 189: 5965–5975.

Dendritic cell (DC) recognition of microbes is instrumental for instructing innate and adaptive immunity to clear pathogens and establish immunological memory. The use of purified agonists has revealed the critical role of pattern recognition receptors in orchestrating DC responses, which has important implications for vaccine adjuvant design (1, 2). Influenza is an enveloped negative-sense ssRNA virus that is thought to be detected primarily by RNA-specific pattern recognition receptors. This concept is supported by impaired cytokine responses by influenza-infected cells lacking the RNA sensors TLR3, TLR7, and MAVS and no clear demonstration of host recognition of the 11 influenza-encoded proteins (3–7). Viral entry, assembly, and budding perturb normal cell biology, but it is unclear how sentinel cells integrate these potential signals from live viral infection with foreign RNA detection.

This study identifies a microRNA (miRNA) that is induced more strongly by influenza infection than stimulation with the purified dsRNA agonist polyinosinic-polycytidylic acid [poly(I:C)], a viral mimic that is known to be detected by endosomal TLR3 and cytosolic RIG-I (8–10). miRNAs are important posttranscriptional regulators in immune cells, but their roles within DCs have not been well characterized (11). These small noncoding RNAs negatively regulate protein levels by interacting with target mRNAs by partial base pair complementarity, which blocks translation or triggers mRNA degradation (12). miRNAs can act as fine-tuners to titrate the levels of translatable mRNA as well as switches to repress protein production by maintaining mRNA levels below a threshold (13). Fine-tuning of protein levels by miRNAs has been shown to regulate developmental programs and cellular responses to infection and provide a restraint on inflammation (14–17). Expression of miRNAs and target mRNAs can be cell type–restricted, resulting in a cell lineage–specific role for this class of negative regulators in many systems (18).

The identification of negative regulatory networks is particularly relevant to understanding and manipulating DC biology. These cells rapidly secrete high levels of cytokines that orchestrate inflammatory responses aimed at controlling replicating pathogens. Cytokines drive a number of positive-feedback loops, and their production must therefore be tightly controlled to limit chronic inflammatory sequelae. Proinflammatory cytokine production can be inhibited at multiple regulatory points: signaling, transcription, RNA stability, translation, and secretion (19). The transcription factor FOXO3 negatively regulates proinflammatory cytokine gene expression, and FOXO3−/− DCs secrete increased IL-6, TNF, and CCL2/MCP-1 following viral infection (20). The protein ZFP36/Tristetraprolin postranscriptionally represses the expression of numerous proinflammatory cytokines, including TNF, IL-6, CCL2/MCP-1, CCL3/MIP1α, CCL4/MIP-1β, and CXCL2/MIP-2.
by binding to AU-rich elements in their mRNA 3′-untranslated regions (UTRs) and promoting mRNA decay (21–24). Both FOXO3 and ZFP36 are inhibited by YWHAZ/14-3-3, an adaptor protein that modulates the activity of binding partners by controlling subcellular localization or kinase activity (25). YWHAZ binds FOXO3 and ZFP36 via phosphoserine-dependent interactions to inhibit the activity of these negative regulators by sequestering them from nuclear acid interactions (26–30). Regulation of YWHAZ itself is less well characterized. YWHAZ function is altered by its phosphorylation state, and its levels are stoichiometrically limiting in cells, rendering YWHAZ activity sensitive to the regulation of its expression level (25). In this study, we demonstrate that YWHAZ levels are inhibited by an miRNA specifically induced by viral infection. Reduced YWHAZ levels can relieve repression of ZFP36, resulting in negative regulation of proinflammatory cytokine expression by DCs.

Materials and Methods

Cell culture and mice

C57BL/6 mice (Charles River Laboratories) or MyD88-/- mice (Institute for Systems Biology, Seattle, WA) were injected s.c. between the shoulder blades with 6 × 10^6 B6-melanoma cells expressing FLRT3 to expand the DC compartment. After 2 wk, spleens and livers were isolated, enzymatically dissociated using Liberase Blendzyme III (Roche), and DCs isolated by AutoMACS magnetic bead purification using pan-DC beads (specific for CD11c and plasmacytoid DC Ag-1 [PDCA1]; Miltenyi Biotec). Cell purity was measured by flow cytometry of CD11c expression and was >95% in each experiment. This purification scheme yielded a mixture of conventional CD11c DCs as well as PDCA1+ plasmacytoid cells, and the ratio of these two populations was consistent between experiments. These splenic DCs were used for all experiments, unless otherwise noted. To isolate specific DC populations, FLRT3-expanded spleens were enzymatically dissociated, RBCs removed using ACK lysis solution (Sigma-Aldrich), B cells depleted using CD19+ AutoMACS microbeads, stained using CD11c-FITC (BD Biosciences) and PDCA1-allophycocyanin (Miltenyi Biotec), and sorted for CD11c+PDCA1+ (myeloid DC) and PDCA1+ (plasmacytoid DC expression using an FACSaria (BD Biosciences). Bone marrow was isolated from the femurs and tibia of C57BL/6 mice. RBCs removed by ACK lysis, and cells cultured at a density of 5 × 10^5 cells/ml in RPMI 1640 containing 10% heat-inactivated FBS, 2 mM l-glutamine, penicillin, and streptomycin, 100 μg/ml gentamicin, and 100 ng/ml FLRT3 for 8 d without medium change. JAWS II DC line was obtained from American Type Culture Collection and cultured as recommended. The J4C RBC line was used to compare miRNA expression between erythroid cells and DCs. CD8+ T cells were isolated from the spleens of OT-1 mice (The Jackson Laboratory) by mechanical dissociation and positive selection using CD8-specific microbeads and AutoMACS purification. Cells were stained using 1 μM CFSE in PBS containing 5% FBS for 10 min at room temperature, followed by three washes with PBS, miR-144/miR-451miDr mice were generated by the deletion of a 388-bp segment of genomic DNA containing both the pre-miR-144 and pre-miR-451 sequences by homologous recombination in 12 embryonic stem cells (26). Mice were backcrossed five times onto the C57BL/6 background (verified to be >95% C57BL/6) and wild-type littersmates used for infection experiments. These mice are deficient for both miR-451 and miR-144, but miR-144 expression is undetectable in DCs. Experiments using mice were approved by an International Animal Care and Use Committee and performed in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals.

Nucleofection

Antigens were introduced by nucleofection using the Amaxa mouse DC nucleofection kit and program Y-01 according to the manufacturer’s protocol (Lonza). A total of 2 × 10^6 cells was combined with 24 μl 5 nM antigen in 100 μl. Media was gently added to cells without centrifugation, and cells centrifugation were added to the induced stimuli.

Retroviral transduction of DCs

A total of 1 × 10^6 Phoenix cells plated in a 10-cm petri dish in 5 ml complete media without antibiotics was transfected with 12 μg DNA and 36 μl Lipofectamine 2000 (Invitrogen) in 3 ml OptiMEM (Life Technologies). After 18 h, the transfection mix was replaced with 10 ml complete media and the virus allowed to grow for 3 d. A total of 7 to 8 × 10^6 bone marrow cells from C57BL/6 mice femurs was cultured in 10 ml complete media with 10 ng/ml IL-3, 20 ng/ml IL-6, and 25 ng/ml stem cell factor for 3 d. After 3 d of culture, 1 × 10^6 bone marrow cells was transduced by centrifuging at 1800 relative centrifugal force at 32 C for 2 h with 5 ml retroviral supernatant and 4 μg/ml polybrevine and then incubated for 3 more h at 37 C followed by a media change containing IL-3, IL-6, and stem cell factor. After 3 d, media was replaced with fresh media containing 100 ng/ml FLRT3 and then incubated for 5 d before selection with 5 μg/ml puromycin for 3 d. An expression construct expressing murine miR-451 along with flashing sequences 100 b upstream and 198 bp downstream was cloned into a retroviral stem cell virus GFP vector. Packaged virus was generated using Phoenix ecotropic cells, transduced onto JAWS II cells, and stably selected using 15 μg/ml puromycin. Retroviral constructs expressing short hairpin RNAs specific for Ywzhuc (control) or overexpressing Ywzh were described in previous studies (26).

Cell stimulation

Cells were infected with H1N1 influenza A/Puerto Rico/8/34 (PR8; Charles River Laboratories) or H1N2 influenza X31 where indicated; Paul Thomas, St. Jude’s, Memphis, TN) at a multiplicity of infection (MOI) of 10 virions/DC and encyphalomyocarditis virus at the MOI of 2. Cells were treated with 30 μg/ml poly(I:C), 30 nM miR-451 antagomir in 100 μl OptiMEM (Life Technologies), B cells depleted using CD19+ AutoMACS microbeads, stained with 1 μM CFSE in PBS containing 5% FBS, and 2 μM EdU (Invitrogen) and incubated for 30 min in the dark. Cells were then fixed with 4% PFA, permeabilized with 0.1% Triton X-100, stained with 1 μg/ml anti-Ki67 Ab (BD Biosciences), and analyzed by flow cytometry using a high-speed FACSAria (BD Biosciences). CFSE dilution in live (7-aminoactinomycin D (7-AAD)-negative) cells was shown for culturing conditions DCs treated with miR-451 antagomir or control antigen. DC−T cell cocultures were cultured at a ratio of 1:2, treated as described, and supernatants collected after 24 or 72 h.

Flow cytometry

Cells were blocked using 1 μg/ml anti-FcR Ab (2.4G2) in PBS containing 2% FBS and 2 mM EDTA, stained using fluorescently labeled CD11c, CD80, MHC II (MHC II), CD80-specific Abs (BD Biosciences), or PDCA1-specific Ab (Miltenyi Biotec), and fixed in 10% formalin. Fluorescence of Ab-stained cells or cells nucleofected with FITC-conjugated antigens was measured using an FACSCalibur (BD Biosciences).

miRNA array profiling

miRNA profiling was performed using a miRNA array (Exiqon) on primary DCs that were infected with poly(I:C) or left uninfected (control). The microarrays were designed, ordered, and produced by Exiqon. The Hy3-labeled samples and an Hy5-labeled reference sample were mixed pairwise and hybridized to the miRCURY LNA array version 10.0 (Exiqon), which contains capture probes targeting all miRNAs for all species registered in the miRBASE version 11.0 at the Sanger Institute. The hybridization was performed according to the miRCURY LNA array manual using a Tecan HS4800 hybridization station (Tecan, Grödig, Austria). After hybridization, the microarray slides were scanned and stored in an ozone-free environment (ozone level <2 ppb) to prevent potential bleaching of the fluorescent dyes. The miRCURY LNA array microarray slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies), and the image analysis was carried out using the ImaGene 8.0 software (BioDiscovery). The quantified signals were normalized using the global Locally Weighted Scatterplot Smoothing regression algorithm. 30 RNAs were 1 μg/ml/10^5 cells to be expressed if measured above the detection limit in all three independent biological replicates of at least one experimental condition. miRNAs were considered to be differentially expressed if the ratio relative to mock was ≥1.5 in either influenza-infected or poly(I:C)-treated samples, and the p value was <0.01. The Student t test was performed in stimulated cells relative to mock. The full data set has been deposited in Gene Expression Omnibus (accession GSE36316; http://www.ncbi.nlm.nih.gov/geo/).

Molecular biology

For quantitative RT-PCR (qRT-PCR) measurements, RNA was isolated using TRIzol (Invitrogen), CDNA was synthesized from DNase-treated RNA.
using random primers or mir-specific primers, and qRT-PCR was performed using gene- or miRNA-specific primers and probes (Applied Biosystems). Expression was normalized to Ef-1 (Efi1a) (miRNA) or sno202 (miRNA). Viral RNA was quantified by RT-PCR using primers specific for influenza M gene (forward, 5'-CAT GGA ATG GCT AAA GAC AAG ACC-3'; reverse, 5'-CCA TTA AGG GCA TTT TGG ACA-3'; probe FAM-5'TTT GTG TTC ACG CTC ACC GTG CCC A-TAMRA-3') and normalized to the level of mouse EF-1. Western blot analysis of total protein was performed on cellular lysates prepared using RIPA buffer containing protease and phosphatase inhibitors and nuclear extracts prepared by hypotonic lysis. Equal quantities were run on 4–12% gradient SDS-PAGE gels, transferred to polyvinylidene difluoride, and serial incubations of membranes were performed with the indicated Abs (143-3-3/YWHAZ [sc-1019; Santa Cruz Biotechnology], FOXO3a [#2497; Cell Signaling Technology], phospho-FOXO3a [Ser294; #5538; Cell Signaling Technology], ZFP36/Tetradecapotin [sc458; Santa Cruz Biotechnology], LAMINB1 [Invitrogen], or β-ACTIN-HRP [ab20271; Abcam]). Species-specific secondary Abs conjugated to HRP were visualized by enhanced chemiluminescent detection, and the signal was quantified by densitometry.

Cytokine levels in cell supernatants were measured using a 16-32-plex Luminex panel (Millipore).

**Statistical analysis**

Means ± SEM for independent biological replicates are shown unless stated otherwise in the text. The p values were determined using an unpaired two-tailed Student t test, assuming equal variances on all experimental datasets.

**Results**

**Viral infection regulates miRNA expression**

To explore how viral infection modulates DC gene expression post transcriptionally, we profiled 578 miRNAs in primary murine splenic DCs infected in vitro with influenza A virus or stimulated with poly(I:C) for 8 h. Influenza is a negative-sense ssRNA virus that activates DCs through RIG-I, TLR3, and TLR7 (3, 7, 31), whereas poly(I:C) is a dsRNA agonist that stimulates cells via TLR3, MDA5, and RIG-I–dependent signaling. The expression of 246 miRNAs was detectable in at least one experimental condition, and the concentrations of the majority of these miRNAs were not altered by these stimuli. However, 21 miRNAs exhibited expression changes of ≥1.5-fold (p < 0.001) following poly(I:C) treatment or viral infection relative to mock-treated cells. Eighteen of these differentially expressed miRNAs were upregulated, and three were downregulated (Fig. 1A, 1B). Regulated expression of these miRNAs within DCs has not been described previously, except for miR-155, which is known to be induced in DCs and macrophages by inflammatory stimuli (32, 33). Poly(I:C) stimulation affected miRNA expression more potently than influenza infection for nearly all miRNAs, which is consistent with the overall weaker effect of influenza infection on global gene expression in DCs (data not shown). dsRNA, either from viruses or poly(I:C), is a well-characterized agonist of DC responses. However, it is unclear how additional aspects of viral infection, including expression of viral proteins or disruption of cellular processes by the viral life cycle, regulate host transcription. The induction of miR-451 expression by influenza infection and not poly(I:C) stimulation was therefore notable (Fig. 1A). miR-451 regulates erythroid lineage differentiation (26, 34–36) and has not been characterized within DCs.

We used qRT-PCR to confirm that miR-451 is increased following influenza infection for 8 h (Fig. 1C). We also validated the increased expression of miR-155, which is more strongly induced by poly(I:C) when compared with influenza, the decreased expression of miR-685 by poly(I:C) more than by influenza infection, and the stable expression of Let-7a, which was not altered by stimulation in the miRNA array profiling dataset (Fig. 1C). We explored the range of stimuli that increase miR-451 expression in primary DCs using doses that trigger robust transcriptional responses in DCs. There was not a requirement for live virus, as both live and UV-inactivated influenza induced miR-451 (Fig. 2A). miR-451 expression was increased most strongly by the H1N1 influenza strain PR8, the cytokines type I IFN (IFN-β) or IL-6, but not by a variety of TLR agonists (poly(I:C), LPS, R848) relative to mock-treated cells (Fig. 2A). Induction of miR-451 was partially dependent on type I IFN signaling: IFNAR null DCs, which cannot respond to the type I IFNs that are produced following influenza infection, exhibited lower miR-451 induction than wild-type cells (Fig. 2B). To test whether elevated IFN production by influenza-infected DCs could provide a possible mechanism for the high expression level of miR-451 in virally infected cells compared with cells treated with poly(I:C) or R848, we measured type I and type III IFN expression. Influenza infection did not induce significantly more IFN-α, IFN-β, or IFN-λ compared with poly(I:C) or R848 stimulation (Fig. 2C and data not shown).

**FIGURE 1.** Viral stimulation regulates miRNA expression in DCs. **(A)** The expression of 578 miRNAs was measured in splenic DCs infected with influenza or stimulated with poly(I:C) for 8 h in vitro and compared with unstimulated cells cultured in parallel (Mock). The means ± SEM for three independent biological replicates were calculated, and the fold change relative to unstimulated cells is shown. The dotted line indicates where expression is equivalent to unstimulated cells. miRNAs with a ≥1.5-fold increase in expression in stimulated relative to unstimulated cells and p < 0.001 are shown; n = 3. **(B)** The miRNAs with a ≥1.5 mean fold decrease in expression and p < 0.001 following stimulation are shown, as described in (A); n = 3. **(C)** qRT-PCR was performed on miRNAs isolated from splenic DCs infected with influenza, stimulated with poly(I:C), or cultured in parallel without stimulation for 8 h. Expression of the indicated miRNAs was normalized to sno202 expression and the data are displayed as fold change in stimulated cells relative to mock; means ± SEM for n = 3–8 independent biological experiments are shown. *p < 0.05, **p < 0.01.

### Materials and Methods

- **miRNA extraction and analysis:**
  - Total RNA was extracted from DCs using the TRIzol reagent (Invitrogen).
  - Reverse transcription was performed using the miScript II RT Kit (QIAGEN) according to the manufacturer’s instructions.
  - qPCR was performed using the miScript SYBR Green II Kit (QIAGEN) on a 7500 Fast Real-Time PCR System (Applied Biosystems).
  - Amplification was performed with 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.
  - Data was analyzed using the ΔCt method.

- **Statistical analysis:**
  - Data is presented as mean ± SEM.
  - Statistical significance was determined using Student’s t-test or one-way ANOVA followed by Tukey’s multiple comparison test.

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**Figure 1:**

(A) The expression of 578 miRNAs was measured in splenic DCs infected with influenza or stimulated with poly(I:C) for 8 h in vitro and compared with unstimulated cells cultured in parallel (Mock). The means ± SEM for three independent biological replicates were calculated, and the fold change relative to unstimulated cells is shown. The dotted line indicates where expression is equivalent to unstimulated cells. miRNAs with a ≥1.5-fold increase in expression in stimulated relative to unstimulated cells and p < 0.001 are shown; n = 3. (B) The miRNAs with a ≥1.5 mean fold decrease in expression and p < 0.001 following stimulation are shown, as described in (A); n = 3. (C) qRT-PCR was performed on miRNAs isolated from splenic DCs infected with influenza, stimulated with poly(I:C), or cultured in parallel without stimulation for 8 h. Expression of the indicated miRNAs was normalized to sno202 expression and the data are displayed as fold change in stimulated cells relative to mock; means ± SEM for n = 3–8 independent biological experiments are shown. *p < 0.05, **p < 0.01.
miR-451 expression is more strongly induced by virus, IL-6, and type I IFN compared with purified pattern recognition receptor agonists. (A) qRT-PCR was performed on splenic DCs stimulated with the indicated agonists for 6 h, and miR-451 expression was normalized to sno202 expression. *p = 0.01. (B) qRT-PCR of miR-451 expression was performed as described in (A) on wild-type (WT) or IFNAR−/− splenic DCs stimulated with the indicated agonists for 6 h; n = 2 and is representative of ≥2 independent experiments. (C) qRT-PCR was performed on splenic DCs stimulated with the indicated agonists for 4 h. Type I IFN (IFN-β1 and IFN-α4) and type III IFN (IL-28b/IFN-λ) gene expression was normalized to Ef-1, and fold induction relative to mock-cultured cells is shown; n = 3 independent experiments. (D) MyD88−/− splenic DCs were cultured for 4 or 8 h with or without influenza PR8 infection and miR-451 expression was quantified by qRT-PCR as described in (A); n = 2. (E) qRT-PCR was performed as described in (A) on primary lung DCs treated with equivalent numbers of live or UV-inactivated influenza PR8 virions (MOI 10) for 8 h; means ± SEM for n = 3–6 are shown. (F) Conventional myeloid DCs (mDC; CD11c+PDCA1−) and plasmacytoid DCs (pDC; PDCA1+) were isolated by FACS and infected with influenza, stimulated with poly(I:C), or cultured in media alone for 18 h. miR-451 expression was measured by qRT-PCR and normalized to sno202 expression; means ± SEM for n = 3 are shown. p = 0.01. (G) miR-451 expression was measured by qRT-PCR in splenic DCs infected with influenza PR8 for 6 h and compared with Jc3 RBCs and expression plotted relative to 5S rRNA expression. *p < 0.05, **p < 0.01.

not shown). Although R848 is a well-established inducer of type I IFN expression via TLR7-MyD88-dependent signaling cascades, miR-451 is upregulated in the absence of MyD88 (Fig. 2D). miR-451 expression also increased in influenza-infected primary murine lung DCs (Fig. 2E). The DCs isolated from spleens and lungs are comprised of both myeloid and plasmacytoid DCs. To determine which type of DC upregulates miR-451 following influenza infection, we isolated myeloid (CD11c+PDCA1−) and plasmacytoid (PDCA1+) cells by FACS and observed that miR-451 was upregulated in infected myeloid DCs (Fig. 2F). DCs express ~10-fold less miR-451 than the high levels measured in erythroid cells (Fig. 2G) (36). In erythroid cells, miR-451 and miR-144, encoded in the same bicistronic locus, are both expressed. In contrast, miR-144 expression was not detectable by microarray (Gene Expression Omnibus accession number GSE36316) or qRT-PCR (data not shown) in DCs.

Antagomir knockdown of miR-451 expression does not alter DC stimulatory activity

To determine the functional consequence of miR-451 expression by DCs infected with influenza, we introduced a locked nucleic acid–stabilized RNA oligonucleotide that is antisense to miR-451 (miR-451 antagonist) into primary splenic DCs by nucleofection and compared responses to cells identically treated with a scrambled oligonucleotide control (control antagonist). We observed uniform uptake of the FITC-conjugated antagomirs (Fig. 3A) and functional inhibition of miR-451 expression following treatment with the miR-451 antagonist by qRT-PCR after 21 h (Fig. 3B). miR-451 levels were decreased by >99% based on the detection limit of the assay. miR-451 antagonist treatment did not decrease expression levels of other miRNAs compared with control antagonist treatment (data not shown) and did not decrease miR-451 levels when RNA was isolated immediately after nucleofection and before the antagonist could lead to degradation of its target (Fig. 3B). These controls confirm the specificity of the RT-PCR assay and show that the presence of the antagonist does not simply inhibit the qRT-PCR reaction. Cells responded to the process of nucleofection of RNA antagonirs, in which RNA is physically delivered to the cytosol, by inducing miR-451 expression (Fig. 3C). This induction of miR-451 expression is less than that produced by influenza infection and more than that produced by poly(I:C) treatment (Fig. 1C).

Ag presentation is a principal function of DCs. We observed that wild-type DCs treated with miR-451 antagonist or control antagonist or cells derived from wild-type and miR-451−/− mice were similar in their ability to activate T cells. The surface expression of two molecules necessary for Ag presentation to CD4+ T cells, MHC II and the costimulatory molecule CD80, was independent of miR-451 expression (Fig. 3D). Loss of miR-451, by either antagonist treatment or genetic deletion, did not alter DC survival (data not shown). Inhibition of miR-451 expression did not alter the T cell stimulatory capacity of influenza-infected DCs, as assessed by IFN-γ production (Fig. 3E), or CFSE dilution by Ag-specific CD8+ T lymphocytes (data not shown), and similar results were obtained following poly(I:C) stimulation (data not shown).

miR-451 regulates the production of a distinct set of cytokines by influenza-infected DCs

Inhibition of miR-451 expression had a striking effect on cytokine production by primary splenic DCs following influenza infection in vitro. Levels of secreted IL-6, TNF, CCL5/RANTES, and CCL3/MIP-1α were all consistently increased in cells treated with miR-451 antagonist relative to cells treated with control antagonist (Fig. 4A). Additionally, in some experiments, secretion of CXCL2/MIP-2, CCL4/MIP-1β, CXCL1/KC, and CCL2/MCP-1 by cells treated with miR-451 antagonist was also elevated relative to control-treated cells. In contrast, there were no significant differences in the secretion of CXCL10/IP-10, IL-1β, CXCL9/MIG, GM-CSF, IL-10, CCL11/eotaxin, IL-9, IL-13, IL-7, IL-15, or G-CSF (Supplemental Fig. 1). Cytokine secretion by DCs treated with miR-451 antagonist and control antagonist was equivalent following stimulation with poly(I:C), an agonist that does not induce miR-451 expression (Fig. 4A).

miRNAs can negatively regulate protein levels by blocking translation or by targeting miRNAs for degradation. To explore the mechanism of action of miR-451, qRT-PCR was performed and increased steady-state mRNA levels of IL-6, Tnf, and Cc55/Rantes mRNA were measured in cells treated with antagonist specific for miR-451 (Fig. 4B). To establish whether miR-451 negatively
regulates expression of this group of cytokines in multiple types of DCs, we performed ELISAs on supernatants collected from murine splenic, lung, and FLT3L-derived bone marrow DCs 24 h after influenza infection and measured increased levels of IL-6, TNF, and CCL5/RANTES secreted by all three types of DCs (Fig. 4C). We also detected increased levels of IFN-β mRNA and protein secretion by miR-451 antagomir-treated DCs in response to influenza infection (Fig. 5A, 5B). We measured the expression of a panel of type I IFN–regulated genes and identified an increased type I IFN signature in miR-451 antagomir-treated cells (Fig. 5A). This did not correlate with improved antiviral capacity to limit viral transcription in miR-451 antagomir-treated cells, as viral RNA loads were equivalent to those in control cells (Fig. 5C).

The cytokine phenotype observed after miR-451 suppression by antagomir was confirmed in primary DCs by comparing cytokine production in wild-type and miR-451null DCs, although the differences observed were more subtle. When we measured a panel of 26 cytokines and chemokines (measured in Fig. 1 and Supplemental Fig. 1), we observed increased secretion of IL-6, TNF, CXCL2/MIP-2, and CXCL1/KC by miR-451null DCs following influenza infection (Fig. 6A). We measured analogous increases in Il6, Tnf, and Ifnβ at the mRNA level (Fig. 6B). To test whether antagomir treatment modulated DC cytokine production independently of the decrease in miR-451 expression, miR-451null splenic DCs were treated with miR-451 antagomir or control antagomir and compared with wild-type cells. IL-6 and CCL3/MIP-1α production in response to influenza infection was equivalent in miR-451null and wild-type DCs treated with miR-451 antagomir. Furthermore, the increase in secretion of these two cytokines was similar in miR-451null DCs and wild-type DCs treated with miR-451 antagomir (Supplemental Fig. 2A).

Overexpression of miR-451 led to a reciprocal decrease in cytokine secretion following infection with two different influenza A viral strains. Stable lines of the JAWS II DCs overexpressing miR-451 or vector alone were generated by retroviral transduction and drug selection. miR-451 overexpression impaired the secreted levels of IL-6, CCL3/MIP-1α, and CCL5/RANTES (Fig. 6C), whereas TNF production by these cells was below the assay de-
Decreased levels of CXCL2/MIP2 and CCL4/MIP1β were also measured along with normal levels of all other assayed cytokines (data not shown). These data were corroborated using bone marrow–derived DCs transduced to overexpress miR-451, which resulted in decreased levels of IL-6, CCL3/MIP-1α, CCL5/RANTES, and TNF (Fig. 6D). Although poly(I:C) does not induce miR-451 expression or result in differential cytokine production in miR-451 antagomir-treated cells (Fig. 4A), poly(I:C) stimulation of cells engineered to overexpress miR-451 results in increased cytokine secretion compared with control cells (Fig. 6D).

miR-451 regulates cytokine expression by targeting Ywhaz

We and others (26, 35, 37, 38) have demonstrated that miR-451 targets the 3′-UTR of Ywhaz/14-3-3zm mRNA in multiple cell types. In agreement with these studies, YWHAZ protein levels were significantly increased in miR-451null DCs compared with wild-type cells (Fig. 7A). Moreover, Ywhaz mRNA was increased by miR-451 antagomir treatment in DCs (Fig. 5A). Thus, expression of miR-451 correlates with suppression of Ywhaz mRNA and protein expression in DCs, similar to what we reported for erythroid cells (26). YWHAZ belongs to the 14-3-3 family of phosphoserine binding proteins that interact with functionally diverse signaling proteins to modulate their activities. YWHAZ can bind and sequester ZFP36 and FOXO3 from interacting with nucleic acids, thereby inhibiting the actions of these two negative regulators of IL-6 and TNF expression (20, 28). YWHAZ sequesters FOXO3 in the cytoplasm, preventing it from repressing transcription in the nucleus (28, 29). ZFP36 promotes mRNA destabilization by binding to AU-rich elements in the 3′-UTRs in some inflammatory cytokine mRNAs, including TNF, IL-6, and CCL3, and is negatively regulated by interactions with YWHAZ (27, 30). ZFP36 protein levels were reduced in miR-451null cells, which have increased YWHAZ expression (Fig. 7A). We measured increased phosphorylation of FOXO3 on Ser294, which targets this protein for degradation, and increased nuclear translocation of FOXO3, both of which reflect decreased FOXO3 activity in cells with increased YWHAZ levels (Fig. 7A).

We have shown that decreased expression of miR-451 correlates with increased YWHAZ expression, decreased nuclear FOXO3, reduced ZFP36 expression, and increased levels of IL-6, TNF, CCL3/MIP1α, and CCL5/RANTES. Although causal relationships between these observations have been established in other systems, YWHAZ has not been directly connected to FOXO3 or ZFP36 activities or cytokine expression in DCs. We were unable to investigate these mechanisms through YWHAZ knockdown experiments due to observed nonspecific effects of short hairpin RNAs on IL-6 secretion in DCs (Supplemental Fig. 2C). However, overexpression of Ywhaz by retroviral transduction in FLT3L-expanded bone marrow–derived DCs decreased ZFP36 protein, similar to what we observed in miR-451null cells in which Ywhaz is derepressed. In contrast, FOXO3 nuclear localization was increased in cells overexpressing YWHAZ (Fig. 7B), which is not concordant with our observation that reduced miR-451 levels correlate with increased YWHAZ expression and decreased nuclear localization of FOXO3 (Fig. 7A). Fig. 7C shows comparable regulation of Ywhaz, Zfp36, IL-6, Ccl3/Mip1a, and Tnf in cells with decreased miR-451 levels using antagonirs, genetic ablation, or cells exogenously overexpressing Ywhaz.

These data can be synthesized into a working model shown in Fig. 8. Influenza infection increases miR-451 expression, which...
levels of secreted IFN-

was also noteworthy that the expression of the vast majority of

gain-of-function, loss-of-function, and mechanistic studies, as used

tated expression of these miRNAs remains to be assessed using
downregulated miRNAs and confirmed the known induction of
pathways resulted in the novel description of 16 upregulated and 3
expression in primary DCs responding to live viral infection or

responses to infection. Global profiling of differential miRNA

Discussion

analogous decrease in ZFP36 and increase in IL-6 and TNF ex-

pression, increased YWHAZ protein and mRNA levels correlate

with reduced miR-451 levels following antagomir treatment

protein expression (26, 35, 37, 38). Increased levels of YWHAZ in

cells with reduced miR-451 levels following antagomir treatment

or genetic ablation can be linked to increased levels of inflammatory
cytokine expression by two possible mechanisms. YWHAZ binds

is a potent chemoattractant for T cells along with eosinophils

activate expression of a module of antiviral effectors during acute

viral infections as well as during underlying chronic inflammatory
diseases such as rheumatoid arthritis, and TNF-blocking Abs are

a standard of care for treating rheumatoid arthritis. Type I IFNs

activate expression of a module of antiviral effectors during acute

viral infections as well as during underlying chronic inflammatory
diseases such as lupus and type 1 diabetes (41, 42). CCL5/RANTES
is a potent chemoattractant for T cells along with eosinophils and basophils, and CCL3/MIP1o chemoattracts neutrophils and monocytes. miR-451 knockdown had inconsistent effects on increasing secretion of other chemokines (CCL4/MIP-1b, CXCL2/ MIP2, CXCL9/MIG, and CXCL1/keratinocyte chemoattractant), with differences reaching statistical significance in some experiments but not all. We observed specificity in the cytokines affected by miR-451 expression, which did not alter secretion of IL-10, etoxin, and IL-1b. Together, the proinflammatory cytokines and chemokines that are more highly expressed in the absence of miR-451 activate a broad range of inflammatory cells, highlighting the importance of stringent regulatory mechanisms.

miR-451 has been shown in multiple systems to inhibit YWHAZ
protein expression (26, 35, 37, 38). Increased levels of YWHAZ in
cells with reduced miR-451 levels following antagomir treatment

or genetic ablation can be linked to increased levels of inflammatory
cytokine expression by two possible mechanisms. YWHAZ binds
two inducible attenuators of DC activation: ZFP36 and FOXO3.
Zfp36 expression is highly induced by stimulation with TLR agonists or cytokines such as TNF or type I IFN (21). ZFP36 is an RNA binding protein that targets AU-rich mRNAs such as TNF, IL-6, and CCL3/MIP1α for degradation. YWHAZ has been shown in multiple cell types to sequester ZFP36 from binding target mRNAs. Increasing YWHAZ levels by genetic overexpression or miR-451 antagonist treatment correlated with decreased ZFP36 levels (Fig. 7). YWHAZ is also a known negative regulator of FOXO3, a transcription factor that interacts with the transcriptional machinery to negatively regulate transcription of cytokines such as IL-6 and TNF (20, 43). FOXO3 is controlled by four mechanisms: transcription, posttranslational modifications, cellu-
miR-451 negatively regulates Ywhaz expression in DCs. (A) Whole-cell lysates were prepared from splenic DCs isolated from wild-type (WT) or miR-451null (KO) mice and infected in vitro with influenza PR8 for 6 h. Western blots were performed using Abs specific for YWHAZ, ZFP36, or phosphorylated FOXO3, signals quantified using densitometry, normalized to the expression level of actin, and displayed relative to the levels in wild-type cells. Nuclear extracts were prepared from cells treated under identical conditions, probed for FOXO3 expression, normalized to the level of LaminB1, and displayed relative to the levels in WT cells. Means ± SEM are shown for two to seven samples. (B) Whole-cell lysates or nuclear extracts were prepared from splenic DCs stably expressing YWHAZ or vector alone, infected in vitro with influenza PR8 for 6 h, and Western blotting performed as described in (A). Means ± SEM; n = 2. (C) Splenic DCs were prepared from WT or miR-451null (KO) mice, and WT cells were treated with control antagonist or miR-451 antagonist or transduced to overexpress YWHAZ or vector alone. Following infection with influenza PR8 for 24 h, RNA was isolated, and qRT-PCR was performed. Gene expression was calculated relative to Eif-1, and means ± SEM are shown for two to three samples. *p < 0.05.
and IFN-$\beta$ lower overall expression of the locus. miR-451 targeting of Ywhaz expressed and processed similarly in both erythroid and DCs but miR-144 in erythroid cells (26, 36). It could be that the locus is alternatively, miR-451 is expressed at much higher levels than that can be processed by Ago2 in the absence of Dicer (45–47), detectable in DCs (data not shown). miR-451 is the only miRNA both be measured in DCs, mature miR-144 expression was un-
lar localization, and degradation (44). FOXO3 expression is induced following TLR stimulation, and phosphorylation of FOXO3 can lead to degradation or binding to YWHAZ, which excludes FOXO3 from the nucleus to prevent transcriptional repression (28). We have previously demonstrated that miR-451 expression increased FOXO3 activity (26). In the current study, we detected a similar positive correlation between miR-451 and FOXO3 nuclear localization (and thereby activity). However, overexpression of YWHAZ did not lend mechanistic support to this observed correlation. This could result from a technical limitation, due to the sensitivity of DCs to perturbation by the experimental manipulation of gene expression, or result from a unique pattern of regulation of FOXO3 by YWHAZ in DCs. Our data propose one miR-451 target that could explain the observed cytokine phenotype, but does not exclude a role for other YWHAZ binding partners or other miR-451 target mRNAs that could contribute to our observed miR-451–dependent cytokine phenotype.

miR-451 plays an important role in erythroid lineage differentiation, and miR-451 expression had not previously been described in DCs. miR-144 is encoded on the same pri-miRNA transcript as miR-451, and the two mature miRNAs are coexpressed in erythroid cells; in contrast, whereas pri–miR-451 and pri–miR-144 could both be measured in DCs, mature miR-144 expression was undetectable in DCs (data not shown), miR-451 is the only miRNA that can be processed by Ago2 in the absence of Dicer (45–47), and differences in miRNA processing between myeloid and erythroid cells provide a possible mechanism for the unique expression pattern of processed mature miR-451 and miR-144. Alternatively, miR-451 is expressed at much higher levels than miR-144 in erythroid cells (26, 36). It could be that the locus is expressed and processed similarly in both erythroid and DCs but that mir-144 is found at subthreshold levels in the latter because of lower overall expression of the locus. miR-451 targeting of Ywhaz is conserved between erythroid and DCs, yet the consequences, erythroid differentiation and resistance to oxidant stress and DC proinflammatory cytokite production, are distinct. The cell lineage–specific expression of YWHAZ binding partners (ZFP36 is highly expressed in activated DCs) and distinct FOXO3–dependent transcriptional programs (erythroid cells lack IL-6 and TNF expression) could explain the different phenotypic effects of miR-451 expression between cell types.

miR-451, ZFP36, and FOXO3 are all transcriptionally induced by viral stimuli, and the phenotypes of Zfp36$^{null}$ and Foxo3$^{null}$ mice show that their contribution to negative feedback is more important than providing a homeostatic restraint on inflammation (20, 22), miRNAs have modest effects on the protein levels of targets, decreasing concentrations by an average of 2-fold (48, 49). The phenotypic effects of miRNAs can be larger than their effects on steady-state protein levels when target expression is at a threshold, when they target multiple members of the same pathway, or when multiple miRNAs bind to a given mRNA target (13). Signaling molecules and transcription factors are both particularly sensitive to changes in concentration (11). miRNAs can therefore more potently inhibit cytokine transcripts by targeting the regulators of cytokine gene expression. In this study, we characterize an miRNA that targets a multifunctional protein, YWHAZ, that is present in limiting quantity (25), providing another mechanism for amplifying the effect of small modulations in protein levels.

Cells most efficiently process information using biochemical networks with high sensitivity to changes in input signals and low sensitivity to stochastic fluctuations. Regulatory circuits enable cells to buffer propagated noise while maintaining sensitivity to signals (50). Although positive regulatory circuits are instrumental in cellular differentiation programs, negative regulatory circuits generate homeostasis or oscillatory behavior. Negative feedback can serve as a buffer by attenuating noise as well as providing stability by limiting the range over which the concentrations of network components can fluctuate (51, 52). Our data add a third negative regulator in a negative-feedback cascade that buffers proinflammatory cytokine secretion by influenza-infected DCs. It will be interesting to determine whether induction of miR-451 by influenza infection and the correlating restraint on cytokine secretion offer a net benefit to the host or pathogen.

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References


