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Identification of Resting and Type I IFN-Activated Human NK Cell miRNomes Reveals MicroRNA-378 and MicroRNA-30e as Negative Regulators of NK Cell Cytotoxicity

Pin Wang,*1 Yan Gu,*1 Qian Zhang,*1 Yanmei Han,* Jin Hou,* Li Lin,† Cong Wu,‡ Yan Bao,* Xiaoping Su,* Minghong Jiang,‡ Qingqing Wang,† Nan Li,* and Xuetao Cao*,‡

NK cells are important innate immune cells with potent cytotoxicity that can be activated by type I IFN from the host once infected. How NK cell cytotoxicity is activated by type I IFN and then tightly regulated remain to be fully elucidated. MicroRNAs (miRNAs, or miRs) are important regulators of innate immune response, but the full scale of miRNome in human NK cells remains to be determined. In this study, we reported an in-depth analysis of miRNomes in resting and IFN-α-activated human NK cells, found two abundant miRNAs, miR-378 and miR-30e, markedly decreased in activated NK cells by IFN-α, and further proved that miR-378 and miR-30e directly targeted granzyme B and perforin, respectively. Thus, IFN-α activation suppresses miR-378 and miR-30e expression to release cytolytic molecule mRNAs for their protein translation and then augments NK cell cytotoxicity. Importantly, the phenomena are also confirmed in human NK cells activated by other cytokines and even in the sorted CD16+CD56dimCD69+ human NK cell subset. Finally, miR-378 and miR-30e were proved to be suppressors of human NK cell cytotoxicity. Taken together, our results reveal that downregulated miR-378 and miR-30e during NK cell activation are negative regulators of human NK cell cytotoxicity, providing a mechanistic explanation for regulation of NK cell function by miRNAs. The Journal of Immunology, 2012, 189: 000–000.

MicroRNAs (miRNAs, or miRs) exert functions mainly through targeting the 3′-untranslated region (3′-UTR) of mRNA to induce translation suppression or mRNA degradation. It is well accepted that miRNAs participate in many physiological and pathological processes (1), and the emerging roles of miRNAs in immune development and response have been extensively explored in recent years (2). Innate immunity, mainly mediated by innate cells, including monocytes/macrophages, dendritic cells (DCs), granulocytes, and NK cells, provides the first line of host defense against invading pathogens. It has been proven that miRNAs play an indispensable role in innate immune responses via regulating the development of monocytes/macrophages (3), DCs (4), and granulocytes (5, 6), and also in regulating their responses to pathogens (7), TLR ligands (8), and viral infection (9). Whereas NK cells are of vital importance in innate immune responses against viral infection, contributing to the first line of host antiviral defense, the function of intrinsic miRNAs in NK cells remains elusive.

NK cells are a unique subset of innate immune cells that exhibit cytotoxicity function such as adoptive CTL through a ligand-recognition manner. NK cells mediate cytolsis through a variety of mechanisms, including perforin/granzyme granule-mediated exocytosis and activation of the TNF death receptor family members. Studies of gene-disrupted mice and individuals with inherited defects indicate that perforin and granzyme B are vital to the cytotoxicity function of NK cells, and they play indispensable roles in granzyme-mediated cellular apoptosis. The cytotoxicity of NK cells can be enhanced by many activating cytokines, and type I IFN is one of the most potent activators of NK cells both in humans and in mice (10), although the underlying mechanisms remain to be fully understood. Type I IFN was shown to stimulate accessory DCs to produce IL-15 and then to activate NK cells (11), whereas type I IFN signaling-induced granzyme B and perforin expression in murine NK cells was found to be required for NK cell activation in response to vaccinia virus infection (12). It seems that type I IFN activates NK cells in both direct and indirect manners. It has been reported that type I IFN can modulate miRNA expression in macrophages (13), and thus whether type I IFN could regulate miRNA expression in NK cells and subsequently regulate NK cell activity needs to be explored.

In-depth analysis of genome-wide miRNA expression profile is a powerful approach to explore the function of miRNAs in a given biological context. At present, miRNome is yielded mainly through four kinds of high-throughput technology: the short RNA cloning technique (14), the microarray technique (15), a new high-throughput microfluidic real-time quantitative PCR approach (16), and the deep sequencing platform (17). Compared to the first three high-throughput techniques, the second generation sequencing has unparalleled advantages in fully exploring the small RNA (smRNA) transcriptome including miRNome in a given tissue or

*National Key Laboratory of Medical Immunology and Institute of Immunology, Second Military Medical University, Shanghai 200433, China; Institute of Immunology, Zhejiang University School of Medicine, Hangzhou 310058, China; and National Key Laboratory of Molecular Biology, Chinese Academy of Medical Sciences, Beijing 100730, China
†P.W., Y.G., and Q.Z. contributed equally to this work.

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Address correspondence and reprint requests to Dr. Xuetao Cao, National Key Laboratory of Medical Immunology and Institute of Immunology, Second Military Medical University, 800 Xiangyin Road, Shanghai 200433, China. E-mail address: caoxt@immunol.org

The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; EGFP, enhanced GFP; GO, gene ontology; miR, microRNA; miRNA, microRNA; RISC, RNA-induced silencing complex; smRNA, small RNA; TPM, transcripts per million; 3′-UTR, 3′-untranslated region.

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cell type under a certain physiological or pathological context. Besides revealing the expression profile of known miRNAs, vast amounts of sequencing data enable exploration of novel miRNA candidates in a specific tissue or cell type and investigation of the variation of mature miRNA sequences, which is called isomiRNA. Also, it presents clues to investigate other kinds of smRNAs, for instance, repeat-associated smRNAs and PIWI-interacting RNAs.

In order to investigate the roles of miRNAs in human NK cell activation and function, we profiled smRNA components and established miRNomes of resting or IFN-α-activated human peripheral blood NK cells by deep sequencing. On the basis of comprehensive understanding of miRNAs profiling in human NK cells, we found that miR-378 and miR-30e, two of the markedly downregulated miRNAs in the activated human NK cells, directly suppressed the expression of granzyme B and perforin and their decreases during IFN-α activation granted human NK cells to be more potent cytolytic, thus providing a new mechanistic explanation for the regulation of NK cell activation by miRNAs.

Materials and Methods

Cell culture and reagents

PBMCs were isolated from periphery blood of healthy donors using Ficoll (Sigma-Aldrich, St. Louis, MO), and then the cells were labeled with fluorescent Abs according to different experimental purpose. Finally, target cellular subsets were sorted by MoFlo (Dako, Glostrup, Denmark). All human blood samples were collected with the informed consent of healthy donors and the experiments were approved by the Ethics Committee of the Second Military Medical University, Shanghai, China. Fluorescent Abs were all purchased from R&D Systems (Minneapolis, MN). Sorted human NK cells (CD56+CD3−) were cultured in IMDM medium (Life Technologies/BRL, Carlsbad, CA) supplemented with 10% FBS (PAA Laboratories, Pasching, Austria) and 10% human AB serum (TBDScience, Tianjin, China). Human NK92 cell line, K562 cell line, and HeLa cell line were obtained from the American Type Culture Collection (Manassas, VA) and were cultured as described previously (18). Recombinant human IFN-α was from R&D Systems and cycloheximide was from Sigma-Aldrich.

smRNA library generation and data production

RNA fractions enriched for smRNAs were isolated from cell pellets treated with RNalater (Life Technologies) using the mirVana miRNA isolation kit (Life Technologies). smRNAs between ~18 and 30 nt were isolated and subjected to sequencing library production (Illumina, San Diego, CA) after quality control. smRNA-Seq clean reads were mapped and classified according to annotations based on Rfam (19) miRBase (v.14.0) and normalized by RNAlater (Life Technologies) using the mirVana miRNA isolation kit. Only reads with at least 10% mapped to each transcript, and with no more than one mismatch allowed, were used to classify smRNAs. The smRNAs that were not matched to any kind of categories were subjected to sequencing library production (Illumina, San Diego, CA) after quality control. smRNA-Seq clean reads were mapped and classified according to annotations based on Rfam (19) miRBase (v.14.0) and normalized by RNAlater (Life Technologies) using the mirVana miRNA isolation kit. The smRNAs that were not matched to any kind of categories were used to predict novel miRNAs.

Gene ontology term enrichment analysis of miRNA targets

Conserved miRNAs across the five species described in miRNA conservation analysis are equally divided into five ranks according to their expression levels and analyzed total miRNAs in target GO term (GO:0002376, immune system process). The prediction of novel miRNAs was implemented in the Mireap program developed by the Beijing Genomics Institute. To identify typical and novel sequences of miRNAs in human, we adopted the following strategy. First, candidate miRNA sites were screened out from breakpoints defined by mapping of the smRNAs. Next, a minimal stringent criterion was used to select miRNA candidates, which ensured that most sequences recovered were known miRNAs. Finally, the RNA secondary structure was checked using Mfold.

Western blotting

Cells were lysed with M-PER protein extraction reagent (Pierce, Rockford, IL) supplemented with protease inhibitor mixture, and protein concentrations of the extracts were measured by bicinchoninic acid assay (Pierce). Forty micrograms of the protein was used for immunoprecipitation or was loaded per lane, subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and then blotted (20).

Abs specific to human perforin and HRP-coupled secondary Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Human granzyme B Ab was obtained from Cell Signaling Technology (Beverly, MA), and β-actin Ab was purchased from Bioworld Technology (St. Louis, MO).

RNA quantification

Total RNA containing miRNA was extracted, reverse-transcribed, and quantitative real-time PCR was amplified as described previously (9, 21). For miRNA analysis, the reverse transcription primers for has-miR-30e was 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TCC GCA CTG GAT ACG AGG CTT CCA-3' (forward) and 5'-GGC TGT AAA CAT CCT TTA C-3' (reverse) and novel sequences of miRNAs, we adopted the following strategy. First, candidate miRNA sites were screened out from breakpoints defined by mapping of the smRNAs. Next, a minimal stringent criterion was used to select miRNA candidates, which ensured that most sequences recovered were known miRNAs. Finally, the RNA secondary structure was checked using Mfold.

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Table I. Summary of data production by smRNA sequencing

<table>
<thead>
<tr>
<th>Type</th>
<th>NK-0 h</th>
<th>NK-12 h</th>
<th>NK-24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Count</td>
<td>%</td>
<td>Count</td>
</tr>
<tr>
<td>Total reads</td>
<td>16,862,552</td>
<td>17,982,973</td>
<td>17,826,283</td>
</tr>
<tr>
<td>High-quality reads&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12,470,391 (100)</td>
<td>14,582,582 (100)</td>
<td>13,777,178 (100)</td>
</tr>
<tr>
<td>Adaptor 3′ null</td>
<td>99,590 (0.80)</td>
<td>297,216 (2.04)</td>
<td>95,287 (0.69)</td>
</tr>
<tr>
<td>Insert null</td>
<td>246,533 (1.98)</td>
<td>436,647 (2.99)</td>
<td>260,607 (1.89)</td>
</tr>
<tr>
<td>Adaptor 5′ contaminants</td>
<td>177,270 (1.42)</td>
<td>276,972 (1.90)</td>
<td>291,897 (2.12)</td>
</tr>
<tr>
<td>Smaller than 18 nt</td>
<td>483,023 (3.87)</td>
<td>631,276 (4.33)</td>
<td>780,246 (5.66)</td>
</tr>
<tr>
<td>Poly(A)</td>
<td>60 (0.00)</td>
<td>56 (0.00)</td>
<td>92 (0.00)</td>
</tr>
<tr>
<td>Clean reads</td>
<td>11,463,915 (91.93)</td>
<td>12,940,415 (88.74)</td>
<td>12,349,049 (89.63)</td>
</tr>
</tbody>
</table>

<sup>a</sup>In these reads, those containing ambiguous bases were removed from total reads.
FIGURE 1. Analysis and functional annotation of miRNome of human NK cells. (A) Pie chart for percentages of total reads for categorized smRNAs in resting NK cells (NK-0 h). More information about NK-12 h and NK-24 h is directed to Table II. (B) Pairwise comparison of known miRNA expression profiles in the three libraries (NK-0 h, NK-12 h, and NK-24 h). Scatter plots show the log 10 value of TPM for each miRNA in the three libraries as indicated; each plot represents one miRNA. (C) Conserved miRNAs expressed in resting NK cells are averagely divided into five ranks according to their expression levels (the higher the rank number, the higher miRNA expression level). GO term enrichment analysis of predicted conserved targets is performed for each rank. The \( p \) value for immune system response gene set (GO: 0002376) is shown (see details in Materials and Methods). (D and E) Clustering of dynamic expression patterns of markedly changed (>2-fold or <0.5-fold, TPM ≥ 100 at least in one library) known miRNAs (D) and novel miRNA candidates (E) in human NK cells after IFN-α stimulation are shown in radar figures. Blank lines from the center separate six categories of dynamic patterns as indicated. One red dot stands for one miRNA, and the distance to the center represents the significance of changes. One gray circle represents 1-fold for miRNA variance. (F) Conserved targets of each miRNA category described in (D) are predicted by TargetScan (release 5.0) (http://www.targetscan.org). Percentage of genes in NK cell-mediated cytotoxicity pathway (Kyoto Encyclopedia of Genes and Genomes database: hsa04650) covered by these predicted targets for each category is shown. (G) Human primary NK cells were stimulated with or without 40 U/ml recombinant human IFN-α in the presence or absence of cycloheximide (CHX; 10 μg/ml) for 8 h. Cytotoxicity of these cells is measured using flow cytometry with K562 cells as target cells. The data are shown as means ± SD (\( n = 4 \)) of four independent experiments using NK cells from 4 donors. *\( p < 0.05 \).
miRNA mimic or inhibitor

miR-378 and miR-30e mimics and inhibitors from Ribobio were used for the overexpression and inhibition, respectively, of miR-378 and miR-30e in HeLa cells. Negative control mimic or inhibitor (Ribobio) was transfected as the matched controls. miRNA mimic with Cy3 labeled at the end of its antisense strand (Ribobio) was used for the overexpression of miR-378 and miR-30e in human NK cells.

Luciferase reporter plasmid constructs

Full-length 3′-UTR fragments of human GZMB and PFR1 were amplified from cDNA of human peripheral blood NK cells and cloned into a pMIR-Report construct (Ambion, Austin, TX). The mutant 3′-UTRs carrying a mutated sequence in the complementary site for the seed region of miR-378 or miR-30e, generated using the fusion PCR, were also cloned into the pMIR-Report construct.

Sponge plasmid construction

miRNA sponge plasmids were constructed on the basic structure of plasmid pEFGP-C1. Briefly, we synthesized two DNA fragments with lengths of ~250 bp containing seven sponge sites (for miR-30e sponge plasmid) or six sponge sites (for miR-378 sponge plasmid) following upstream translation stop code TAA, which was flanked with two restriction sites, BglII and EcoRI. After double digestion and ligation, these two DNA fragments were inserted at the MCS site following the enhanced GFP (EGFP) encoding sequence. All constructs were confirmed by DNA sequencing.

Assay of luciferase reporter gene expression

HeLa cells were cotransfected with 80 ng indicated luciferase reporter plasmid, 40 ng pRL-TK-Renilla-luciferase plasmid (Promega, Madison, WI), and indicated RNAs (final concentration, 20 nM) using JETSI-ENDO transfection reagents (PolyPlus Transfection, Illkirch, France). After 24 h, normalized luciferase activities were obtained as previously described (22). Alternatively, HeLa cells, which express certain level of endogenous miR-378 and miR-30e, were cotransfected with 80 ng indicated luciferase reporter plasmid, 40 ng pRL-TK-Renilla-luciferase plasmid (Promega), and 80 ng indicated sponge plasmid using JetPEI transfection reagents (PolyPlus Transfection). After 36 h, luciferase activities were measured as above.

Transfection of NK cells

Freshly isolated human NK cells were electroporated using an Amaxa human NK cell Nucleasefector kit (Lonza, Basel, Switzerland) and an NK92 cell line was electroporated using Amaxa cell line Nucleasefector kit R (Lonza, Basel), according to the manufacturer’s instructions.

Establishment of stably transfected NK92 cells

To establish stably transfected NK92 cells, G418 was added (1000 μg/ml) 48 h after transfection and maintained at 800 μg/ml in the following 3 wk for positive selection. For stably transfected cells, the expressions of EGFP were confirmed by FACS and the expressions of sponge RNA were confirmed by quantitative real-time PCR (Fig. 6A). Stably transfected NK92 cells were subsequently cultured in medium containing 500 μg/ml G418.

RNA-binding protein immunoprecipitation

NK92 cells were lysed using a complete RNA lysis buffer from a Magna RNA-binding protein immunoprecipitation kit (Millipore, catalog no. 17-701). RNA-binding protein immunoprecipitation experiments were performed according to the protocol provided with the kit. Human AGO2 Ab was purchased from Cell Signaling Technology (Beverly, MA).

Intracellular staining and flow cytometry

Cells were stained using indicated Abs with the Cytotox/Cytoperm kit (eBioscience) according to the manufacturer’s instructions, as described previously (23). Flow cytometric analysis was performed on a FACS LSRII with FACS Diva software (BD Biosciences).

Assay for NK cell cytotoxicity

NK cell cytotoxicity was analyzed using flow cytometry as previously described (23).

Statistical analysis

Statistical significance was determined by a paired two-tailed Student t test, with a p value < 0.05 considered to be statistically significant. Additionally, two-tailed Pearson correlation coefficient analysis was performed using SPSS software (version 17.0; SPSS, Chicago, IL).

Results

Analysis of miRNomes in resting and IFN-α-activated human NK cells

CD56+CD3+ NK cells freshly isolated from peripheral blood of healthy donors were used as resting human NK cells (NK-0 h), or activated by recombinant human IFN-α (40 U/ml) for 12 h (NK-12 h) or 24 h (NK-24 h). RNA components of these human NK cells were purified and subjected to smRNA Solexa sequencing. More than 107 clean reads for each sample were obtained (Table I). The mapping and categorizing of clean reads revealed that most smRNA reads were miRNAs in NK cells (Fig. 1A, Table II). Unannotated smRNAs that were not classified to any of these cate-
The abundance value of each known miRNA was normalized using transcripts per million (TPM) in each smRNA library. Known miRNAs expressed in these three libraries exhibited a similar expression profile (Fig. 1B). Only ∼1.05% of registered miRNAs were expressed abundantly (>10,000 TPM) but they accounted for ∼76.8% of all miRNA reads. The 12 most abundant miRNAs in the NK-0 h library are listed in Table III. miRNAs with more abundance may be more related to the biological characteristics of NK cells. Through GO enrichment analysis of predicted targets, we found that predicted conserved targets of miRNAs with higher expression levels were more enriched in the gene set of the immune system response term classified in the Gene Ontology database, indicated by the rising broken line in Fig. 1C. This implied, at least partially, that immune cells with specific functions might highly express functionally related miRNAs, which could be implicated in precise regulation of cell-specific gene expression pattern and thus contribute to the identity of these cells with specific functions.

**FIGURE 2.** IFN-α regulates the expression of miR-378, miR-30e, and cytolytic molecules in human NK cells. Human peripheral blood NK cells (CD56+ CD3−) were sorted from 15 healthy donors and then stimulated with recombinant human IFN-α (40 U/ml) for the indicated times. miR-378 and miR-30e levels were detected using quantitative real-time PCR (A), and intracellular granzyme B and perforin expression was detected using flow cytometry (D). The filled bar indicates the mean value of each treatment, and p < 0.01 for 0 versus 12 h and 0 versus 24 h. In those samples that exhibited a significant decrease in (A) (<=0.5-fold), NK cells were stimulated with recombinant human IFN-α at different concentrations as indicated for 24 h, and then the expression of miR-378 and miR-30e was measured (B), and pri-miR-378 and pri-miR-30e levels were also detected by quantitative real-time PCR (C). In samples exhibiting significant increase in (D) (>=2-fold), granzyme B and perforin levels were detected by Western blot, with β-actin as an input control (E), and their mRNA levels were examined by quantitative real-time PCR (F). Data are representative of five independent donors (B, C, E, F). We applied a decrement value of miR-378 relative expression (subtract miR-378 relative expression value at 24 h from its value at 0 h) versus granzyme B mean fluorescence intensity (MFI) increment value (subtract granzyme B MFI value at 0 h from the value at 24 h) (left) and decrement value of miR-30e relative expression versus perforin MFI increment value (right) for correlation statistical analysis using the Pearson correlation coefficient (G). Data are from three independent experiments (mean ± SEM). *p < 0.05, **p < 0.01, ***p < 0.001.
FIGURE 3. miR-378 and miR-30e directly target granzyme B and perforin, respectively. (A) Human GZMB and PRF1 are potential targets of miR-378 and miR-30e, respectively. Shown is a sequence alignment of miRNAs and their target sites in 3'-UTRs of their respective targets and the mutant sites in the following gene-reporting experiments. (B and C) HeLa cells (1 × 10^5) were cotransfected with 80 ng wild-type (WT) or mutant 3'-UTR firefly luciferase reporter plasmids, 40 ng pTK-Renilla-luciferase plasmids, together with miR-378 mimic or its control mimic (B, left), miR-378 inhibitor or its control inhibitor (B, right), miR-30e mimic or its control mimic (C, left), or miR-30e inhibitor or its control inhibitor (C, right) at the final concentration of 20 nM. After 24 h, firefly luciferase activity was measured and normalized by Renilla luciferase activity. (D) HeLa cells (1 × 10^5) were cotransfected with 80 ng WT or mutant 3'-UTR firefly luciferase reporter plasmids, 40 ng pTK-Renilla-luciferase plasmids, together with 80 ng miR-378 sponge plasmids or control plasmids (left) or miR-30e sponge plasmids or control plasmids (right). After 36 h, firefly luciferase activity was measured and normalized by Renilla luciferase activity. (E and F) HeLa cells (1 × 10^5) were cotransfected with 80 ng WT human GZMB 3'-UTR firefly luciferase reporter plasmids (E) or 80 ng WT human PRF1 3'-UTR firefly luciferase reporter plasmids (F), 40 ng pTK-Renilla-luciferase plasmids, together with miRNA mimic as indicated at the final concentration of 20 nM. After 24 h, firefly luciferase activity was measured and normalized by Renilla luciferase activity. (G and H) Freshly sorted primary human NK cells (3 × 10^6) were electroporated with miR-378 mimic, miR-30e mimic, or control mimic, all labeled with Cy3 (200 nM) (G) or with 2 μg miR-378 sponge plasmid, miR-30e sponge plasmid, or control plasmid (H). Ten hours after transfection, cells were stimulated with or without recombinant human IFN-α (40 U/ml) for 24 h. Then, Cy3-positive cells (PE-positive) and EGFP-positive cells (FITC-positive) were sorted for the detection of cytolytic molecules and miRNA. The mean fluorescence intensity (MFI) value was quantified in line charts as well. Flow cytometry data are representative of three independent experiments, with each experiment using NK cells from one donor. (I) NK92 cells were cultured without IL-2 for 24 h and then stimulated with recombinant human IFN-α (40 U/ml) for indicated time. RISC was immunoprecipitated with anti-human Ago2 Ab. Granzyme B and perforin mRNA levels in this complex were normalized to that in 1% input control of their respective sample and then compared with that of the 0 h sample. Data are shown as means ± SD (n = 4) of one representative experiment. Similar results were obtained in at least three independent experiments. *p < 0.05. **p < 0.01.
Next, we analyzed the dynamic expression patterns of markedly changed miRNAs (1-fold increment or decrement, and TPM > 100) in NK cell miRNome upon IFN-α stimulation. Interestingly, we found most of them were significantly downregulated in IFN-α–activated NK cells, as indicated by intensive red dots in the lower left area of the radar (Fig. 1D), although some of them increased again when IFN-α stimulation extended to 24 h. However, few miRNAs of NK cells increased during the activation. Additionally, novel miRNA candidates (TPM > 100) showed little change during IFN-α activation (Fig. 1E).

We further explored the biological significance of the changed miRNAs during NK cell activation, especially in NK cell cytotoxicity. We subjected conserved targets of six groups of significantly changed miRNAs with different expression patterns (Fig. 1D) to the NK cell cytotoxicity pathway from the Kyoto Encyclopedia of Genes and Genomes database. We found that targets of continuously downregulated miRNAs (category no. 6) were most enriched in this pathway compared with other differentially regulated miRNAs (Fig. 1F). This result implied that in IFN-α–activated human NK cells, some miRNAs were downregulated continuously to release miRNAs for protein translation. We also found that blockade of protein translation by cycloheximide attenuated cytolytic activity induced by IFN-α (Fig. 1G) whereas blockade of transcription by actinomycin D had little effect (Supplemental Fig. 2), indicating that synthesis of new proteins rather than transcriptional activation was necessary for the enhancement of human NK cell cytotoxicity after IFN-α activation.

Therefore, we wondered whether these continuously downregulated miRNAs could regulate NK cell cytotoxicity. Among them, we found that miR-378 and miR-30e, which were also among the 12 most abundant miRNAs in NK cells (Table III), were involved in the regulation of NK cell cytotoxicity.

miR-378 and miR-30e expression are negatively correlated with granzyme B and perforin protein levels, respectively, during IFN-α activation in human NK cells

Detection of the kinetic expression of miR-378 and miR-30e in IFN-α–activated human NK cells from as many as 15 donors showed varying degrees of decline (Fig. 2A). Using samples that exhibited a significant decrease (<0.5-fold), we confirmed that their decreases were IFN-α dose-dependent (Fig. 2B), and this study also revealed that the expression of the primary transcripts of miR-378 and miR-30e also decreased in IFN-α–activated NK cells (Fig. 2C).

We found that the protein levels of granzyme B and perforin, which are the predicted targets of miR-378 and miR-30e, respectively, increased in varying degrees after IFN-α activation by flow cytometry assay (Fig. 2D). We chose the samples with significant increases of granzyme B and perforin (>2-fold), which were confirmed by immunoblots (Fig. 2E), examined their mRNA levels, and found no significant differences (Fig. 2F), indicating that there is a posttranscriptional regulatory mechanism for IFN-α–upregulated granzyme B and perforin expression in human NK cells.

To further explore whether these two decreased miRNAs have certain relevance to the increased cytolytic molecules, we utilized IFN-α reactivity variation of samples from 15 donors (Fig. 2A, 2D) and performed correlation analysis for the value of miRNA decreased levels versus the value of cytolytic molecule increased levels of all human donors during IFN-α activation, and we found a strong correlation (p < 0.001) (Fig. 2G). This result strongly suggests the relevance between miRNA decrease and cytolytic molecule increase.

Additionally, the decreasing expression patterns of miR-378 and miR-30e versus increasing expression patterns of perforin and granzyme B were also observed in human NK cell line NK92 after IFN-α activation (Supplemental Fig. 1A–C).

miR-378 and miR-30e directly target the 3′-UTR of human GZMB and PRF1, respectively

As predicted by computational prediction via TargetScan (http://www.targetscan.org), human GZMB and PRF1 miRNAs are potential targets of miR-378 and miR-30e, respectively (Fig. 3A). To verify these predictions, we constructed luciferase reporter plasmid with a 3′-UTR sequence of human GZMB or PRF1, or with their mutant 3′-UTR sequence. Overexpression and inhibition experiments using synthetic miRNA mimics or inhibitors revealed that miR-378 and miR-30e targeted the 3′-UTR of GZMB and PRF1, respectively, precisely at the indicated sites (Fig. 3B, 3C).

We applied another strategy of miRNA knockdown, that is, “miRNA sponge” plasmids that express EGFP mRNA with many bulged binding sites in its 3′-UTR to absorb intrinsic miR378 or miR30e (24). The miR-378 and miR-30e sponges enhanced GZMB and PRF1 3′-UTR luciferase activity, respectively, and the enhancement was lost after site mutations (Fig. 3D).

FIGURE 4. The expression of miR-378 and miR-30e negatively correlates with expression of cytolytic molecule granzyme B and perforin in human NK cells in vivo. (A) Freshly sorted human NK cells (CD56+CD3−) of 10 healthy donors were separately subjected to quantitative PCR assay of miR-378 and miR-30e expression and flow cytometry assay of intracellular granzyme B and perforin expression. We used the ΔCt value of miR-378 (subtract miRNA Ct value from U6 Ct value) and mean fluorescence intensity (MFI) value of granzyme B (left) and the ΔCt value of miR-30e and the MFI value of perforin (right) for correlation statistical analysis using the Pearson correlation coefficient. (B and C) CD8+CD3+ cells, CD56+CD16+CD56dim cells, and CD16+CD56− cells were sorted from PBMCs of health donors. (B) The expression level of miR-378 was detected as in Fig. 2A, and the level of granzyme B was examined by flow cytometry for each of these cell subsets. (C) miR-30e level and intracellular perforin were also detected as in (B). Data are representative of five independent donors.
Besides miR-378 and miR-30e, we also investigated other miRNAs predicted to target human GZMB or PRF1. Most of them were seldomly expressed in human NK cells (TPM < 10) or showed no apparent expression changes during IFN-α stimulation. We chose 11 miRNAs that have certain abundance in human NK cells (TPM > 100) to perform the luciferase reporter gene assay, which showed that miR-378 and miR-30e were the most effective regulators (Fig. 3E, 3F). The above data show that miR-378 and miR-30e are the key regulatory miRNAs targeting GZMB and PRF1, respectively, in human NK cells.

**miR-378 and miR-30e suppress IFN-α–upregulated granzyme B and perforin expression in human NK cells**

Next, we investigated whether miR-378 and miR-30e could suppress intrinsic expression of cytolytic molecules in human NK cells. To overcome the low transfection efficiency problem in human primary NK cells, we electrotransfected human primary NK cells with miRNA mimics labeled with Cy3, a small fluorescent molecule, at the end of the antisense strand, and selected the PE-cells with miRNA mimics labeled with Cy3, a small fluorescent human primary NK cells, we electrotransfected human primary NK cells. We wondered whether the enhancement of cytolytic molecule expression by decreasing miR-378 and miR-30e was specific for type I IFN-mediated NK cell activation or a universal phenomenon.

**miR-378 and miR-30e participate in the regulation of granzyme B and perforin expression in human NK cells in vivo**

Given the variation among humans, we found that the expression level of cytolytic molecules in freshly isolated human NK cells varied a lot among individuals in our experiments. Is this variation due to the different expression levels of endogenous miR-378 and miR-30e in NK cells from different individuals? Correlation analysis revealed that there was a negative relationship between the miR-378 level and the granzyme B protein expression level in peripheral blood NK cells among healthy donors (Fig. 4A, left). The same negative relationship exists between miR-30e expression and the perforin protein expression level (Fig. 4A, right).

Moreover, among different subsets of cytotoxic cells in one individual, negative relationships were also found between cytolytic molecules and these miRNAs. As shown in Fig. 4B and 4C, cytolytic NK cells (CD16+CD56<sup>dim</sup>) with higher effector molecule expression had higher miR-378 and miR-30e expression, whereas CD16<sup>+</sup>CD56<sup>hi</sup> NK cells and NKT cells (CD56<sup>+</sup>CD3<sup>+</sup>) with a lower effector molecule expression had higher miR-378 and miR-30e expression.

These results imply that miR-378 and miR-30e control the expression level of cytolytic molecules both among different individuals and in different cytotoxic NK/NKT/CD8<sup>+</sup> T cell subsets of one human body, indicating that this is a universal regulation mechanism of cytolytic molecule (granzyme B, perforin) expression in vivo.

**Universal downregulation of miR-378 and miR-30e in the activated human NK cells**

We wondered whether the enhancement of cytolytic molecule expression by decreasing miR-378 and miR-30e was specific for type I IFN-mediated NK cell activation or a universal phenomenon.
enon for NK cell activation. We used cytokines IL-15, IL-12, and IL-2 to activate human NK cells and then analyzed the expression of miRNAs and cytolytic molecules. As shown in Fig. 5A, the expression of miR-378 and miR-30e decreased to various degrees; additionally, flow cytometry assays (Fig. 5B) and immunoblots (Fig. 5C) both confirmed that the expression of cytolytic molecules was enhanced by all of the cytokines. Interestingly, IL-15 stimulation enhanced granzyme B and perforin expression most markedly in NK cells, most significantly decreasing miR-378 and miR-30e.

To confirm that the decrease of miR-378 and miR-30e also exists in the physical process of human NK cell activation in vivo, we freshly isolated the activated human NK cell subset (CD16+ CD56dimCD3-CD69+) and resting NK cells (CD16+CD56dim CD3-CD69-) from the same donor sample to detect the expression level of miR-378 and miR-30e. Indeed, the expression of these two miRNAs was lower in the activated NK cell subset compared with that in resting NK cells from the same donor (Fig. 5D). Flow cytometry assays showed that CD69+ NK cells had a higher level of perforin and a little higher leveler of granzyme B as compared with CD69− NK cells (Fig. 5D). The data confirm that the negative relationship between miR378/miR-30e and cytolytic molecules also exists in the physical process of human NK cell activation in vivo, together with other data further suggesting that miR-378 and miR-30e negatively regulate granzyme B and perforin expression in human NK cells in vivo.

The finding that human NK cells downregulate miR-378 and miR-30e expression to increase cytolytic molecule expression shows a fundamental mechanism for the activation of human NK cells, which is not confined to IFN-α-activation.

**miR-378 and miR-30e inhibit cytotoxicity of human NK cells**

Finally, to confirm the roles of miR-378 and miR-30e in the negative regulation of NK cell cytotoxicity, we stably transfected human NK cell line NK92 with miR-378 or miR-30e sponge plasmid and confirmed the expressions of EGFP protein and sponge mRNA (Fig. 6A). Increments of granzyme B in the miR378 sponge cell line and increments of perforin in the miR-30e sponge cell line were observed (Fig. 6B). Accordingly, the NK92 cells stably transfected with miR-378 sponge plasmid or miR-30e sponge plasmid exhibited more potent cytolytic activity compared with NK92 cells stably transfected with control plasmid (Fig. 6C). Therefore, we demonstrated that miR-378 and miR-30e significantly suppress cytotoxicity of human NK cells by targeting cytolytic molecules granzyme B and perforin.

**Discussion**

In-depth miRNA profiling by deep sequencing with high-resolution views of expressed miRNAs over a wide dynamic range of expression levels has already been realized in several kinds of tissues and cell types, including human embryonic stem cells (25), human B cells (26), cell types during mouse lymphopoiesis (17), human renal cell carcinoma (27), and mouse leukemia (28). The study that revealed dynamic miRNomes during lymphopoiesis selected mouse adherent lymphokine-activated NK cells (ALAK, NK cells) as a mouse NK cell subset to get the mouse NK cell miRNome (17), which may affect the biological and physiological significance of this mouse NK cell miRNome. In our study, by deep sequencing, we performed the smRNA expression profiling of the freshly sorted human peripheral blood NK cells during the process of type I IFNs activation, paving the way to explore the relationship between smRNAs, including miRNAs, and NK cell biological function, which is of physiological relevance to deepen our understanding of the mechanism of human NK cell activity.

**FIGURE 6.** Suppression of miR-378 and miR-30e enhances cytolytic activity of human NK cells. Sponge-linked EGFP expression (A, left) and sponge mRNA expression (A, right) was confirmed in the stably transfected NK92 cell lines by flow cytometry assay and real-time PCR, respectively. Shown is the flow cytometry plot of miR-378 sponge stable cell line as representative. Intracellular granzyme B and perforin protein levels in the miRNA sponge stable NK92 cell lines were detected by flow cytometry (B). The cytolytic assay by flow cytometry with K562 cells as target cells was done using cells transfected with single sponges or two sponges together (C). Flow cytometry plots are representative of results of three independent experiments. Data are shown as means ± SD (n = 3) of one representative experiment. Similar results were obtained in at least three independent experiments. *p < 0.05.

Based on analysis of miRNomes, we went further to find that miR-378 and miR-30e, abundantly expressed in human NK cells, suppress NK cell cytotoxic activity through targeting cytolytic molecules, granzyme B and perforin, and the decreases of miR-378 and miR-30e, a common phenomenon during human NK cell activation, contribute to full activation of NK cells.

As we showed above, after IFN-α activation many miRNAs decreased whereas few miRNAs increased, and the amount of total miRNAs also decreased (Table II). It is reasonable that type I IFN signaling downregulated miRNA expression in NK cells to assist NK cells in the enhancement of some protein expressions to augment NK cell cytotoxic activity. It is probable that, apart from miR-378 and miR-30e, we are far from discovering the last miRNA taking part in the regulation of NK cell function, and we cannot exclude the possibility that changed miRNAs in IFN-α-activated NK cell feedback regulate the type I IFN signaling pathway, which is vital to many immune responses, including NK cell activation. To comprehensively assess the roles of miRNAs in type I IFN signaling and NK cells activation, integration of miRNome data with proteomics data should be helpful.

Compared with their counterparts in mice, we found that human NK cells are less sensitive to activating cytokine stimulation and have a higher basic expression level of cytolytic molecules. This is
reasonable, because human NK cells encounter so many challenges and stimulation from outside pathogens and the induced inflammatory responses, whereas specific pathogen-free laboratory mouse NK cells seldom suffer such challenge. Thus, NK cells from specific pathogen-free mice are globally naive, whereas activation levels of NK cells from different humans are of high variation. Different expression levels of miR-378 and miR-30e in NK cells from different donors probably imply their different activating level, which is supported by the accordingly different expression levels of granzyme B and perforin (Fig. 4A). NK cells from some donors with high cytolytic molecule expression and low miR-378 and miR-30e expression did not respond obviously to IFN-α stimulation (Fig. 2A, 2D), probably because they have already been activated by some challenge without apparent symptom. Previous research showed that cytotoxic activity of the CD16+/CD56hi human NK cell subset increases significantly after type I IFN activation (29). This large potential increased space of cytotoxicity in CD16+/CD56hi NK cells may be due to their high levels of miR-378 and miR-30e, which provide large decreasing space to release many cytolytic effector mRNAs for translation. It has been reported that mouse NK cells acquiring cytotoxicity ability after stimulation by activating cytokines requires the translation of a pre-existing pool of granzyme B and perforin mRNA (30), indicating that there is a posttranscriptional regulatory mechanism controlling the expression of granzyme B and perforin expression in mouse NK cells. However, we found that there is also a posttranscriptional mechanism regulating cytolytic molecule expression in human NK cells and we demonstrated that it is through miR-378 and miR-30e action. It is probable that miRNAs also participate in the posttranscriptional regulation of cytolytic molecule expression in mouse NK cells, which needs to be verified in further work. Therefore, possibly in mammals, NK cells apply this “prearmed” mechanism to ensure rapid responses to infections or other challenges from both internal and external sources.

As negative regulators of NK cytotoxicity, miR-378 and miR-30e may also participate in the dysfunction of NK cells in NK cell-associated diseases, such as hepatitis, carcinoma, and various autoimmunity diseases, which need to be validated in further researches. Our results (Fig. 4B, 4C) indicated that the posttranscriptional regulation of cytolytic molecules by miR-378 and miR-30e is not confined to the process of NK cell activation, but is universal for activation and function of all cytotoxic cellular subsets. A study revealed that type I IFN signaling-mediated up-regulation of granzyme B expression is required for the enhanced cytolytic activity of viral-specific CD8+ T cells (31), in which miR-378 downregulation is probably involved. Malfunction of these miRNAs may contribute to the irregular expression of cytolytic effectors in CD8+ T lymphocytes during chronic HIV infection (32). Furthermore, the role of these miRNAs in other cytotoxicity-related clinical disorders is worthy of exploration in further studies.

In our experiments, we developed a fluorescent labeling tactic (C3y3-labeled miRNA mimic) to overcome the obstacle of low transfection efficiency to investigate the functional roles of miRNAs in primary NK cells, which may be of use in other research for transfection of miRNA or siRNA to primary NK cells. However, the small amount of successfully transfected cells still limits the application of this method in functional assays of NK cells. Based on analysis of miRNome in human NK cells, we found that type I IFN stimulation enhances cytotoxicity of human NK cells by downregulating two abundantly expressed miRNAs, miR-378 and miR-30e, to enhance cytolytic molecule expression. During preparation of our manuscript, Fehniger et al. (33) reported the identification of miRNA transcriptome in mouse splenic NK cells (NK1.1+CD3- ) using two different kinds of second generation techniques. After thorough analysis of miRNome in resting and IL-15-activated mouse NK cells, they found that miR-223 decreased following IL-15 activation and specifically targeted the 3′-UTR of murine GZMB in vitro. With regard to human NK cells, we found that miR-223 was expressed at a very low level (TPM < 30 in our human NK cell miRNome; data not shown) and had no predicted targeting site in human GZMB 3′-UTR, implying that granzyme B expression was definitely regulated by miRNAs in mammalian NK cells whereas human and mouse NK cells applied different miRNAs in this regulation. Taken together, these two works provide a comprehensive understanding of miRNome in NK cells across mammalian species.

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Disclosures
The authors have no financial conflicts of interest.

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