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MicroRNAs Activate Natural Killer Cells through Toll-like Receptor Signaling

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Running title: NK Cell Activation by MicroRNAs

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Key Points

- MicroRNAs activate natural killer cells through a TLR-NF-κB signaling pathway and may have therapeutic applications in cancer.

ABSTRACT

MicroRNAs (miRNAs) bind to complementary sequences of target mRNAs, resulting in translational repression or target degradation and thus gene silencing. MiRNAs are abundantly found in circulating blood, yet whether, as a class of regulatory molecules, they may interact with human natural killer (NK) cells has not been explored. Here we found that the treatment of human NK cells with several mature miRNAs, in the presence of a low concentration of IL-12, induced CD69 expression, IFN-γ production, and degranulation marker CD107a expression. In vivo, infusion of several miRNAs alone in murine peripheral blood also resulted in comparable NK but not T cell activation. Furthermore, miRNA administration significantly protected mice from tumor development in an NK cell-dependent manner. Mechanistically, we found that miRNA stimulation led to downstream activation of NF-κB, an effect that was blunted by a block in Toll-like receptor (TLR) 1 signaling and was attenuated in lymphoma patients. Knockdown of TLR1 resulted in less activation by miRNAs. Collectively, we show that miRNAs have a capacity to selectively activate innate immune effector cells that is at least in part via the TLR1-NF-κB signaling pathway. This may be important in the normal host defense against infection and/or malignant transformation.
Introduction

MicroRNAs (miRNAs) are small, noncoding RNAs first described in *C. elegans* in 1993 \(^1\). Later, it was found that their deletion or deregulation was associated with cancer development \(^2\). Starting with about 70-100 nucleotides (nt), the pre-miRNA are processed into an 18-25 nt mature, single-stranded RNA by the ribonuclease Dicer and the RNA-induced silencing complex (RISC) \(^3\). The mature miRNAs bind to 3’ untranslated regions (UTRs) of target mRNAs, either inducing degradation of mRNAs in the presence of the RISC complex, or blocking protein translation processes \(^4\). Computational analysis has predicted that over one-third of human protein-coding genes (including some tumor suppressor genes and oncogenes) are regulated by miRNAs \(^5\). MiRNAs play essential and pleiotropic roles in both physiologic and disease processes, including normal cell development, as well as malignant transformation and metastasis \(^6,7\).

More recent evidence has shown that miRNAs reside and circulate in the blood and other body fluids of both healthy donors and patients with diseases including cancer \(^8\). These miRNAs, which are detectable in serum or plasma, can serve as potential markers for cancer diagnosis, prognosis or even targets for treatment \(^9\). For example, levels of miR-155 increase in patients with breast cancer or lung cancer in comparison to levels found in healthy donors, and changes in miR-155 expression levels also correlate with the metastasis of breast cancer \(^10,11\). Similarly, significantly higher levels of circulating miR-21 are found in patients with hepatocellular carcinoma (HCC) and breast cancer in
comparison to healthy donors\textsuperscript{12, 13}. Interestingly, expression of miR-15b in the cerebrospinal fluid is strikingly increased in patients with glioma compared to healthy donors\textsuperscript{14}. MiR-122 is the most dominant miRNA found in the liver, and constitutes 70\% of the cloned hepatic miRNA in adult mice\textsuperscript{15}. In patients with chronic hepatitis or HCC, levels of circulating miR-122 were demonstrated to be higher in comparison to levels in healthy donors\textsuperscript{16, 17}. Some miRNAs are also found to be down-regulated in cancer patients\textsuperscript{18}. However, the biological functions of the circulating miRNAs remain largely unknown in both normal and disease states.

Natural killer (NK) cells are a critical component of innate immunity in that they often provide the first line of defense against malignant transformation and viral infection. We and others found that miRNAs are expressed by NK cells and intrinsically regulate their function and development\textsuperscript{19-22}. However, to the best of our knowledge, whether extrinsic or circulating miRNAs might be able to activate NK cells has not been explored. In this study, using both in vitro and in vivo approaches, we found that synthetic circulating miRNAs and miRNA-containing exosomes freshly isolated from healthy donors have a capacity to activate NK cells. This appears to occur via a Toll-like receptor (TLR) signaling pathway. Our study suggests that we have identified a novel role for miRNAs in the innate immune response.
Methods

Cell culture

Primary human NK cells, human PBMCs, mouse spleen cells were cultured in complete RPMI 1640 media (Invitrogen) containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were cultured at 37°C supplemented with 5% CO2. The human IL-2-dependent NK cell line NK-92, a generous gift of Dr. Hans G. Klingemann (Rush University Medical Center, Chicago, IL) was cultured similarly except that 20% FBS was used.

Mice

Eight-week old C57BL/6 and athymic nude mice were obtained from The Jackson Laboratory. All animal work was approved by The Ohio State University Animal Care and Use Committee.

Human NK cell isolation

Human NK cells were first enriched from the peripheral blood of healthy donors (American Red Cross) with the RosetteSep NK cell enrichment mixture (StemCell Technologies) and Ficoll-Paque Plus (Amersham) centrifugation. The enriched NK cells were further purified by positive selection using anti-CD56 MACS beads (Miltenyi Biotec). NK cells with purity over 99%, which was confirmed by flow cytometry, were used (Fig. S1). PBMCs from lymphoma patients were first stained with CD3-PE and CD19-PE, and subjected to negative selection for NK cells using MACS LS Columns.
(Miltenyi Biotec). The enriched NK cells were then further purified by FACS cell sorting after being stained with CD3-FITC and CD56-APC antibodies. For healthy donors, PBMCs were directly stained with CD3-FITC and CD56-APC antibodies and subjected to sorting for NK cells. The purity of sorted NK cells immediately lysed for Real-time RT-PCR analysis is over 97%. All human work was approved by The Ohio State University Institutional Review Board.

**Cell stimulation by miRNAs**

Purified human primary NK cells, PBMCs, or freshly isolated mouse splenocytes were resuspended at $1 \times 10^6$ cells per $100 \mu$l complete RPMI 1640 media, then plated on a 96-well plate in the presence of recombinant human (rh) IL-12 (Genetics Institute Inc). MiRNAs were placed in complex with DOTAP, a cationic liposomal formulation (Roche), according to manufacturer’s instruction. Briefly, 2 μg miRNAs were dissolved in 25 μl hepes-buffered saline (HBS), combined with 10 μg DOTAP solution in 25 μl HBS, and incubated for 15 minutes. Next, 50 μl of complete RPMI 1640 media were added to the miRNAs-DOTAP mixture and mixed well before adding to each well pre-seeded with cells. The final concentration of DOTAP is 50 μg/ml, and the final concentration of miRNA is 10 μg/ml. Vehicle control for all experiments consisted of 50 μg/ml of DOTAP, and RNA-control for all experiments consisted of 50 μg/ml of DOTAP complexed with 10 μg/ml of a non-specific, single-stranded control RNA called RNA41, which is similar in size to miRNA$^{24}$. The cells were stimulated for 36 h (unless specified) with miRNAs and 2.5 ng/mL rhIL-12. The dose of 2.5 ng/ml rhIL-12 is less than that typically used for stimulation of NK cells (10 ng/ml). For the TLR blocking assay, the
aforementioned cells were pre-incubated with TLR1 (InvivoGen), TLR3 (Hycult Biotech) or TLR6 (InvivoGen) blocking antibodies or control IgG (Equitech Bio) at a concentration of 10 μg/ml for 1.5 h prior to stimulation with miRNAs. The blocking Abs were also kept in the culture during the stimulation with miRNAs.

**Flow cytometric analysis**

The stimulated cells were stained with mAbs at 4°C for 20 minutes, washed with PBS, and fixed with 1% formalin, followed by FACS analysis using an LSRII (BD Bioscience) to detect surface expression of each antigen. Human NK cells were gated as CD56+CD3−, and mouse NK cells were gated as NK1.1+CD3− cells within the lymphocyte gate. Anti-human mAbs used were: CD3-FITC, CD56-APC, CD107a-PE, and CD69-PE. Anti-mouse mAbs used were: CD3-FITC, NK1.1-APC, CD69-PE, and CD107a-PE. All mAbs were purchased from BD Bioscience.

**Real-time reverse-transcriptase(RT)-PCR and enzyme-linked immunosorbent assay (ELISA)**

Total RNA was extracted and reverse-transcribed into cDNA. IFN-γ mRNA expression level was determined by Real-time RT-PCR using Taqman PCR Master Mix (Applied Biosystems), while TLRs, p65 and IRAK1 mRNA expression levels were assessed using SYBR Green Master Mix (Applied Biosystems). Expression levels were normalized to an 18S or β-actin internal control and then analyzed by the ΔΔCt method. The sequences of primers and/or probes were available upon request. To detect secreted IFN-γ protein,
cells were stimulated with miRNAs and IL-12 as described above, and cell-free supernatants were analyzed by ELISA as previously described 25.

**CD107a degranulation assay**

To detect the capacity of NK cells for cytotoxic activity, human NK cells and murine splenocytes were stimulated in vitro and treated in vivo with miRNAs, respectively. A total of 0.5 million miRNA-stimulated human and murine NK cells were then incubated at the ratio of 1:1 with K562 and YAC-1 target cells, respectively, however only human NK cells were co-cultured with a low (2.5 ng/ml) concentration of IL-12. Subsequently, 2.5 μl anti-human or 1 μl anti-mouse CD107a antibody was added to this co-culture for 1 h. Then 1μl/ml of the secretion inhibitor, monensin (eBioScience), was added for an additional incubation of 3 h. The cells were washed with PBS and stained with CD3 (human and mouse) and CD56 (human) or NK1.1 mAbs (mouse), and analyzed via flow cytometry using an LSRII.

**In vivo miRNA stimulation**

The complexes of miRNAs and vehicle, Lipofectamine 2000 (Invitrogen), were prepared according to manufacturer’s instruction. Briefly, 30 μl of Lipofectamine 2000 was mixed with 20 μg RNA-Ctl, miR-122 or miR-15b dissolved in 170 μl PBS. The liposome complexes were administered intravenously (200 μl/mouse) into mice through tail veins. Four days later, the injected mice were sacrificed, and total splenocytes were isolated for degranulation assay or stained with aforementioned mAbs for flow cytometric analysis.
Immunoblotting

Total protein lysates were prepared with T-Per (Tissue protein extraction reagent, ThermoScientific) supplemented with proteinase and phosphatase inhibitors. Proteins were resolved on a 4-20% SDS-PAGE gel and transferred onto PVDF membranes (Amersham). The antibodies used for blotting are: TLR1 (Cell Signaling), phospho (p)-p65 (Cell Signaling), p65 (Cell Signaling) and β-actin (Santa Cruz).

TLR1 shRNA knockdown

A TLR1 shRNA plasmid was constructed by inserting RNA interference (RNAi) sequences into pSUPER-retrovirus vector expressing GFP. Viruses were prepared by transfecting the shRNA plasmid and packaging plasmids into phoenix cells. Infection was performed as previously described. Briefly, NK-92 cells were co-cultured with virus-containing media and centrifuged at 1800 rpm at 32°C for 45 minutes, then incubated for 2-4 h at 32°C. This infection cycle was repeated twice. Upon completion of this infection, GFP positive cells were sorted on a FACSAria II cell sorter (BD Bioscience). Knockdown of TLR1 in the sorted NK-92 cells was confirmed by immunoblotting.

Bioluminescent imaging

Balb/C mice-derived A20 B-cell lymphoma cells were retrovirally transduced with a Pinco-pGL3-luc/GFP plasmid and the GFP positive cells were sorted by a FACSAria II cell sorter (BD Biosciences). Then $1 \times 10^5$ luciferase(luc)-expressing A20 cells were injected into each athymic nude mice via tail veins. MiRNAs were administered via a
tail-vein injection at the following three different time points: 3 days prior to, 4 days and 18 days after A20 implantation. For NK depletion, each mouse was administered 200 µg TM-β1 (IL-2/15Rβ) mAb intraperitoneally twice on day 6 prior to and day 3 after A20 cells implantation. Three weeks after A20 implantation, mice were injected with luciferin (150 mg/kg body weight, Gold Biotechnology), anesthetized with isoflurane and imaged using an IVIS-100 Imaging system (Xenogen).

Statistics

Data were compared by Student’s two tailed t test. $p < 0.05$ was considered statistically significant.
Results

MiRNAs enhance surface expression of the activation marker, CD69, on human NK cells

We synthesized several miRNAs previously demonstrated to be present in the circulation, including miR-122, miR-15b, miR-21, and miR-155 and placed them in culture with highly purified human NK cells (Fig. S1). Alone, each of these miRNAs was unable to activate NK cells (data not shown). However, when placed in culture for 36 h in the presence of a low concentration of IL-12 (2.5 ng/ml), we observed a two-to-three-fold increase in the surface expression of CD69 for each miRNA (Fig. 1A, B). In contrast, a non-specific, single-stranded control RNA termed RNA41 that is of similar size to miRNAs and was prepared in an identical fashion and incubated for an equal amount of time at an identical concentration with human NK cells did not significantly induce CD69 surface expression (Fig. 1A, B). Similar results were also obtained with either a longer (72 h) or shorter (24 h) exposure (not shown). To further verify whether this effect was sequence-specific, we mutated miR-122 and miR-15b sequences by substituting uridine (U) with guanosine (G) in the miR sequence. We found that these two mutated miRNAs completely lost their capability to activate NK cells (Fig. S2).

Consistent with these data obtained with synthetic miRNAs, we also found that CD-9 expressing-exosomes isolated from healthy donor serum and containing relatively high
concentrations of miRNAs such as miR-122 and miR-21, were able to significantly activate NK cells purified from the corresponding (autologous) donors (Fig. S3).

**Treatment with miRNAs augments human NK cell IFN-γ production and degranulation**

High expression of activation marker CD69 on NK cells is usually coupled with functional activation. We therefore assessed primary human NK cells for secretion of IFN-γ following miRNA stimulation, and we observed variable but consistently significant increases of IFN-γ protein secretion in each instance (Fig. 2A, upper panel). The data were confirmed by Real-time RT-PCR (Fig. 2A, lower panel), and were also found to be dependent of the concentration of miRNAs (Fig. 2B). Similar results were also observed in the NK cell line NK-92 (Fig. S4). Although the extent of NK-92 activation was less, most likely due to IL-2 pre-stimulation of this IL-2 dependent cell line, these data exclude the possibility that the activation of NK cells by miRNAs was caused by contamination of other immune cell subsets. Consistent with the results of CD69 surface expression, the control RNA41 did not induce IFN-γ expression in NK cells (Fig. 2A). Hereafter, we included only miR-122 and miR-15b for our experimental conditions in assessing NK cell activation because of their stronger stimulation of IFN-γ expression, compared to the other two miRNAs (miR-21 and miR-155) (Fig. 2A).
Upon activation with cytokines, human CD56\textsuperscript{bright} NK cells secrete abundant IFN-γ; while, in contrast, CD56\textsuperscript{dim} NK cells produce negligible amounts of IFN-γ in response to cytokine stimulation\textsuperscript{28, 29}. Interestingly, upon miRNA stimulation, both CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells become activated, resulting in higher expression of CD69 and IFN-γ secretion when compared to parallel cultures treated with vehicle alone (Figs. S5A and S5B).

To determine whether miRNAs also stimulate NK cell cytotoxic effector functions, we measured CD107a expression by a flow cytometric assay to quantify degranulation of primary human NK cells upon contact with K562 tumor cell targets in the presence of IL-12. We observed that, compared to the controls, CD107a expression underwent a moderate, but statistically significant, increase following stimulation with miR-122 and miR-15b (Figs. 2C and 2D). A \textsuperscript{51}Cr-release cytotoxicity assay with K562 targets was also performed, but did not reach statistical significance because of inter-donor variation in cytotoxic activity (not shown).

**MiRNAs activate NK cells in vivo**

To support the physiological relevance for our findings, we treated normal wild-type mice with miR-122 and miR-15b by injection of one dose of miRNAs, without administration of exogenous IL-12, and sacrificed the mice four days later. Flow cytometric analysis indicated that NK cells from miRNA-treated mice have 2- to 3-fold higher surface expression of the activation marker CD69 in comparison to that from
either vehicle- or RNA-Ctl sequence-treated mice (Fig. 3A). In vivo NK cell activation by miRNAs was also evidenced by ex vivo co-culture with YAC tumor cells in the absence of any exogenous IL-12, which resulted in a statistically significant increase in CD107a expression (Fig. 3B).

**MiRNAs do not activate T cells in vivo or in vitro**

In contrast to NK cells, human T cells were not activated by miRNAs when assessed in culture of whole peripheral blood mononuclear cells (PBMCs) (Fig. 4A). Moreover, mouse T cells were not found to be activated following the in vivo infusion of miRNA (Fig. 4B). These data suggest that miRNAs may selectively activate the innate immune response without activating adaptive (T-cell) immune response.

**Activation of NK cells by miRNAs occurs via the Toll-like receptor signaling pathway**

TLR9 has been shown to recognize and respond to bacterial and viral or synthetic deoxyoligonucleotides containing unmethylated CpG dinucleotide motifs $^{30}$. Murine TLR7 and human TLR8 have also been shown to recognize viral RNA $^{24,31}$. We therefore speculated that miRNAs might be activating NK cells through the TLR8 or TLR9 pathway. We first determined mRNA expression levels of TLR8 and TLR9, as well as other TLRs, in resting human NK cells as well as those co-stimulated with IL-12 and miRNAs. Surprisingly, we found that TLR8 and TLR9 are negligibly expressed in both
resting and activated human NK cells, suggesting that activation of NK cells by miRNAs is unlikely to occur through these receptors. In contrast, we found that resting human NK cells express relatively higher transcript levels of TLR1, TLR3 and TLR6 (Fig. 5A). Accordingly, we disrupted TLR signaling by blocking each of these three TLRs with their respective blocking antibodies. For TLR1, we used an antibody with a capability to block signaling activated by its ligand, Pam3CSK4 (Fig. S6). We found that blockade of TLR1 in primary NK cells significantly reduced miRNA-mediated induction of IFN-γ production by ~50%, while blockade of TLR3 and TLR6 had no significant effect on NK cell activation (Fig. 5B). To further demonstrate that TLR1 may participate in miRNA-induced NK cell activation, a short hairpin RNA (shRNA) approach was undertaken. We first confirmed that TLR1, but not TLR3, was successfully knocked down in NK-92 cells (Figs. 5C and S7). We then found that TLR1 knockdown caused NK-92 cells to lose their capability for miRNA-mediated activation as no increase of IFN-γ production was observed, while the empty vector (pSUPER)-transduced control cells remained responsive to miRNAs (Fig. 5C). A confocal microscopy study of HEK293T cells co-transfected with miRNAs and TLR1-YFP fusion plasmid indicated that miRNAs and TLR1 protein colocalize with each other within these cells (Fig. S8). Collectively, these data suggest that NK cell activation by miRNAs occurs at least in part via the TLR1 signaling pathway, while this might be different in other types of immune cells, as most recently reported 32.

NF-κB represents a signaling pathway down-stream of several TLRs, including TLR1 33. To further test our hypothesis that miRNAs activate NK cells via TLR signaling, we
assessed NF-κB activation in NK cells after treatment with miRNAs. We found that, while the total level of p65 protein, a transactivation component of NF-κB signaling, was unchanged, treatment with miRNAs induced an increase in the phosphorylation of p65 in primary NK cells primed with IL-12 (Fig. 5D). To further confirm that miRNA-induced activation of NK cells requires TLR1-mediated NF-κB signaling, we blocked TLR1 with its blocking antibody, and found that the signaling blockade inhibited phosphorylation of p65 (Fig. 5E). Importantly, when we treated purified primary human NK cells with miR-122 and miR-15b in the same way as we did to determine p65 activation, we did not observe any phosphorylation of IRF3, an activation event specifically mediated via TLR3 (data not shown).

MiRNAs enhance anti-tumor activity of NK cells in vivo to control tumor development

A hallmark of NK cell function is to kill tumor cells or virally-infected cells. To further validate the physiological relevance of NK activation by miRNAs, we treated mice with miR-122 prior to and post implantation of A20 lymphoma cells, in the presence or absence of NK cell depletion. To exclude the possible T-cell effect, athymic nude mice were utilized. As shown in Fig. 6, after 3 doses of miR-122 treatment, the growth of A20 tumor cells in mice was significantly suppressed when compared with vehicle-administered mice. However, this effect was largely abrogated when the NK cells were depleted by TM-β1 mAb treatment, suggesting that miRNA suppressed tumor growth at least in part through NK cell activation. More robust inhibition of tumor growth was
observed when we repeated our experiment with more frequent miRNA injections and a longer duration of the treatment (Fig. S9).

**Components of the TLR signaling pathway are downregulated in NK cells from cancer patients**

The data presented above provided strong evidence for miRNAs having a role in combating tumors by directly activating NK cells. Yet still, some cancer patients with disease progression have high levels of circulating miRNAs, which suggests that there may be a mechanism(s) by which tumors escape surveillance by miRNA-activated NK cells. To investigate this, we purified NK cells from PBMCs of lymphoma patients, who are reported to have elevated expression of miRNAs such as miR-155. We found that although there was no significant difference of TLR1 expression between healthy donors and cancer patients (Fig. S10), p65 and IRAK1, the two main components in the TLR1-NF-κB signaling pathway, were consistently and significantly downregulated in NK cells from lymphoma patients (Figs. 7A and 7B). We speculate that higher miRNAs in cancer patients may therefore be unable to activate NK cells in order to control tumor progression, and we will look to validate this in future studies.
Discussion

MiRNAs exist in the peripheral blood of healthy donors, as well as in patients with cancer. As supported by our in vivo anti-tumor data, it is conceivable that miRNAs may boost NK cells’ ability to participate in surveillance against malignant transformation or infectious insult in otherwise healthy individuals. On the other hand, the prevalence of circulating miRNAs in some cancer patients also imply that other factors secreted by malignant tumors, such as TGF-β, might somehow serve to disarm this form of innate immune activation. In addition, in lymphoma patients we found that both p65 and IRAK1 have consistently lower mRNA expression in NK cells when compared to those in healthy donors, suggesting that downregulation of NF-κB signaling in NK cells might be a potential underlying mechanism by which tumors evade immune surveillance by miRNAs. However, this should not imply that modulation of miRNAs in cancer patients is irrelevant. In fact, high levels of miR-21 expression are associated with a more favorable clinical outcome for diffuse large B-cell lymphoma, and during this manuscript preparation, a published report showed that systemic mi-RNA-34a delivery abrogates in vivo tumor growth of this disease.

We found the activation induced by miRNAs to be relatively specific for innate immune effector cells (i.e., NK cells), and absent in T cells, both in vitro and in vivo. This finding may have application in circumstances when selective activation of these relatively distinct arms of the immune response is desired. For example, in the setting of blood and marrow transplantation, the NK cell innate immune response can function to kill
activated T cells thereby contributing to the suppression of donor T cell-mediated graft versus host disease \textsuperscript{36, 37}

We worked to ensure that the NK cell activation noted in the presence of miRNAs was not due to non-specific binding and activation by contaminants. Our HPLC-purified RNA negative control was prepared and handled in an identical fashion to our miRNAs, making it unlikely that NK cell activation in response to the miRNAs was the result of endotoxin or other contaminants. With regard to the RNA control, we used a non-specific, single-stranded RNA called RNA41, which is similar in size to miRNAs \textsuperscript{24}, in the majority of in vitro experiments, and did not see induction of IFN-γ or CD107a degranulation. Further, to rule out the potential bias due to the use of a single RNA (RNA41) as a control, we also mutated several nucleotides of miR-122 and miR-15b and found that all NK cell activation was lost.

In addition to the potential of miRNAs for cancer diagnosis and prognosis, miRNAs are also promising for therapeutic applications and they are on their way to clinical trials. Our findings suggest that those miRNAs which function as tumor suppressors, e.g. miR-122 \textsuperscript{38} included in the current study may be promising agents for treatment of cancer because, on one hand, they may directly and specifically target oncogene expression, yet on the other hand, they are also able to activate innate immune effector cells against tumor cells. Our discovery may also shed new light on the manner in which the host mounts an immune response against infectious pathogens and/or malignant
transformation, as well the manner by which tumors or pathogens edit the immune response to escape immune activation by circulating miRNAs.

In summary, here, we provide what we believe to be the first evidence that miRNAs, as a class of regulatory molecules, directly activate both human and mouse NK cells, and this NK cell activation occurs at least in part via the TLR1 signaling pathway. This identifies a new function of miRNAs with physiological relevance, and their potential for applications in preventing or treating cancer and infections either alone or as an adjuvant.
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Authorship

S.H. designed research work, performed experiments and wrote the paper. J.C., L-C.W., H.M., Y.P., T.H., M.W., S.Y., and S.S. performed some experiments. C.A.A-B, S.V., D.M.B., C.H., X.H., K.G., S.D. designed some of the research work. J.Z. analyzed data. M.A.C. designed some of the research work and proofread the manuscript. J.Y. had a conceptual idea, designed research, performed some experiments, and wrote the paper.

Disclosure Conflict of Interest

The authors declare no competing financial interests.
References


Figure Legends

Figure 1: MiRNAs increase CD69 surface expression on NK cells. (A) Highly purified (≥ 99%) human NK cells were treated with miRNAs, DOTAP vehicle control or non-specific, single-stranded RNA, RNA41 (RNA-Ctl)\textsuperscript{24}, for 36 h in the presence of a low concentration of IL-12 (2.5 ng/ml). The treated cells were then subjected to flow cytometric analysis to determine the percentage of CD69\textsuperscript{+} cells. Representative data from 1 out of 6 donors with similar results are shown. (B) Summary of data from 4 donors obtained in 1 experiment. * indicates \( p < 0.05 \) and error bars represent S.D.

Figure 2. MiRNAs increase IFN-\( \gamma \) production by NK cells. (A) Highly purified (≥ 99%) human NK cells were treated with miRNAs, DOTAP vehicle control or non-specific, single-stranded RNA, RNA41 (RNA-Ctl)\textsuperscript{24}, for 36 h in the presence of a low concentration of IL-12. Supernatants were harvested for ELISA and cells were harvested for Real-time RT-PCR analysis to determine the levels of IFN-\( \gamma \) secretion (upper panel) and gene expression (lower panel), respectively. Gene expression of the vehicle was normalized to 1 and experimental values are each presented as fold change compared to that of the vehicle. Data shown represent 1 of 3 donors with similar results. (B) Cells were treated, and data were collected as described in (A), with the exception that concentrations of miR-122 were varied, as indicated on the X axis. Data suggest that miR-122 activates NK cells in a dose-dependent fashion. (C, D) Highly purified human NK cells were treated with miRNAs or DOTAP vehicle control for 36 h in the presence of a low concentration of IL-12. The cells were then incubated with K562 tumor cells at a
ratio of 1:1 (effector: target). After 4 h, CD107a expression was assessed by flow cytometric analysis. Shown in C are representative data from 1 out of 5 donors with similar results, and D demonstrates summary data of 5 donors. In all panels, error bars represent S.D. * indicates $p < 0.05$, and ** indicates $p < 0.01$.

**Figure 3. MiRNAs activate NK cells in vivo.** (A) Mice were treated in vivo with vehicles or 20 \( \mu \)g RNA-Ctl, miR-122 or miR-15b for 4 days. The treated mice were then sacrificed, and total splenocytes were isolated for flow cytometric analysis to measure CD69 surface expression after gating on CD3^+NK1.1^+ NK cells. Representative data from 1 out of 6 mice with similar results (left) as well as summary data from 3 mice (right) in one experiment are shown. (B) The prepared splenocytes from A were co-cultured with YAC-1 tumor cells for 3 h without any exogenous IL-12, and were subjected to flow cytometric analysis of CD107a expression after gating on CD3^+NK1.1^+ NK cells. For (A) and (B), * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, and error bars represent S.D.

**Figure 4. MiRNAs do not activate T cells in vivo.** (A) Human PBMCs were stimulated with miRNAs as described in Fig. 1A and were subjected to flow cytometric analysis of CD69 surface expression within CD3^+ T cells. Depicted are representative data from 1 of 6 donors (top), as well as summary data from 3 donors in one experiment (bottom). Data suggest that miRNAs do not significantly change human T cell surface expression of CD69. (B) Mice were treated in vivo and cells were prepared for flow cytometric analysis as described in Fig. 5A. Depicted are representative data from 1 of 6 mice with similar
data (left), as well as summary data from 3 mice in one experiment (right), suggesting that miRNAs do not significantly change murine T cell surface expression of CD69 in vivo. For (A) and (B), error bars represent S.D.

**Figure 5. Interaction of TLRs and miRNAs.** (A) Real-time RT-PCR was utilized to determine the level of TLR mRNA expressed in highly purified human NK cells. The mRNA level of TLR5 was found lowest and was normalized to 1. The mRNA level of other TLRs was presented as relative to that of TLR5. Data are shown as the average of 3 donors. (B) Purified human NK cells were pre-incubated for 1.5 h with a non-specific IgG or anti-TLR1, anti-TLR3, or anti-TLR6-blocking antibody (α). Cells were then stimulated with miR-122 as described in Fig. 1A in the presence of the blocking antibody, and supernatants were harvested to measure IFN-γ protein via ELISA. The concentration of IFN-γ in the purified NK cells incubated with IgG and miR-122 was arbitrarily set at 1. Data were averaged from 3 donors. * indicates $p < 0.05$ and error bars represent S.D. (C) NK-92 cells were infected with pSUPER-TLR1-GFP retroviruses, and stably transfected cells were sorted based on GFP expression. Both the vector-transduced cells (pSUPER) and the TLR1 knockdown cells (pSUPER-TLR1, confirmed by immunoblotting, upper panel) were stimulated with miR-122 or miR-15b as described in Fig. 1A, and cell-free supernatants were collected to assess IFN-γ secretion via ELISA (lower panel). ** indicates $p < 0.01$ and error bars represent S.D. (D) Purified human NK cells were stimulated with miR-122 or miR-15b as described in Fig. 1A, and subsequently subjected to immunoblotting using p65 and phospho-p65 (p-p65) antibodies. β-actin immunoblotting was included to demonstrate equal loading of total protein. Data shown
represent 2 of 4 donors with similar results. Numbers beneath each lane represent quantification of p-p65 by densitometry, normalized by p65. (E) The experiment was performed as in C except that anti-TLR1 blocking mAb or its control IgG was included in the culture in the presence of miR-122. Data shown represent 1 of 3 donors with similar results. Numbers beneath each lane represent quantification of p-p65 by densitometry, normalized by p65.

Figure 6. MiRNA stimulation enhances anti-tumor activity of NK cells in vivo. (A) Ventral bioluminescence imaging of mice bearing A20 lymphoma. Athymic nude mice were injected with $1 \times 10^5$ luciferase-expressing A20 cells via tail veins and subjected to miRNA stimulation alone or combined with TM-β1 treatment according to the schedule described in the Materials and Methods. The pseudo color indicates the relative signal strength for tumor growth, with strongest in red and weakest in purple. (B) Shown are quantification summary of units of photons per second per mouse from (A). Data are shown as mean ± SD from each group of mice. *, $p < 0.05$; **, $p < 0.01$.

Figure 7. Downregulation of the NF-κB signaling pathway components in NK cells from lymphoma patients. (A, B) NK cells were isolated from PBMCs of both healthy donors and lymphoma patients as described in the Materials and Methods. The purified NK cells were then immediately subjected to RNA extraction and cDNA synthesis. The expression levels of p65 (A) and IRAK1 (B) were determined by SYBR Green Real-time RT-PCR assay.
Figure 4

A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CD69 Expression</th>
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<tbody>
<tr>
<td>Vehicle</td>
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</tr>
<tr>
<td>miR-122</td>
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<td>20.3%</td>
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<tr>
<td>miR-155</td>
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B

<table>
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<tbody>
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<tr>
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<td>10.4%</td>
</tr>
<tr>
<td>miR-15b</td>
<td>11.4%</td>
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</table>
**Figure 6**

**A**

- **Vehicle**
- **miR-122**
- **miR-122 + TM-β1**

**B**

<table>
<thead>
<tr>
<th></th>
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<th>miR-122</th>
<th>miR-122 + TM-β1</th>
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</thead>
<tbody>
<tr>
<td>photons/mouse/sec</td>
<td>![Graph]</td>
<td>![Graph]</td>
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</table>

- **p = 0.21**
- **p = 0.05**
- **p = 0.01**

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Figure 7

A

$p65$

Relative mRNA level

Donors

Patients

B

$IRAK1$

Relative mRNA level

Donors

Patients