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MicroRNA-155 Tunes Both the Threshold and Extent of NK Cell Activation via Targeting of Multiple Signaling Pathways

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NK cells are innate lymphocytes important for host defense against viral infections and malignancy. However, the molecular programs orchestrating NK cell activation are incompletely understood. MicroRNA-155 (miR-155) is markedly upregulated following cytokine activation of human and mouse NK cells. Surprisingly, mature human and mouse NK cells transduced to overexpress miR-155, NK cells from mice with NK cell–specific miR-155 overexpression, and miR-155+/− NK cells all secreted more IFN-γ compared with controls. Investigating further, we found that activated NK cells with miR-155 overexpression had increased per-cell IFN-γ with normal IFN-γ+ percentages, whereas greater percentages of miR-155+/− NK cells were IFN-γ+. In vivo murine CMV–induced IFN-γ expression by NK cells in these miR-155 models recapitulated the in vitro phenotypes. We performed unbiased RNA-induced silencing complex sequencing on wild-type and miR-155 per-cell IFN-γ pathways were mechanistically involved in regulating IFN-γ production by miR-155+/− NK cells. These data indicate that miR-155 regulation of NK cell activation is complex and that miR-155 functions as a dynamic tuner for NK cell activation via both setting the activation threshold as well as controlling the extent of activation in mature NK cells. In summary, miR-155+/− NK cells are more easily activated, through increased expression of proteins in the PI3K, NF-κB, and calcineurin pathways, and miR-155+/− and 155-overexpressing NK cells exhibit increased IFN-γ production through distinct cellular mechanisms. The Journal of Immunology, 2013, 191: 000–000.

Natural killer cells are innate immune lymphocytes important for host protection against infection and mediate antitumor effector responses, especially against hematologic malignancies (1, 2). NK cells develop from the common lymphoid progenitor along with T and B cells (3), but undergo a distinct developmental pathway without DNA rearrangement of a clonal Ag receptor. Mature NK cells integrate activating and inhibitory signals mediated by a wide variety of surface receptors (4). These receptors signal through intracellular or adapter-containing ITAM/ITIM motifs (5). Upon cytokine receptor activation, NK cells acquire enhanced functional competence and also produce NK cell–derived cytokines/chemokines, including IFN-γ, GM-CSF, TNF-α, and MIP1-α (2). When triggered through cell-surface receptors, NK cells release cytotoxic granules and kill target cells (6). It is through these primary functions that NK cells provide a rapid response to infected or tumor target cells, as well as participate in complex cross-talk with other immune cell types.

In the physiologic setting of a complex receptor–ligand environment, NK cells continually adapt, highlighted by studies in which transgenic overexpression of activating receptor ligands leads to NK cell functional anergy (7) or transfer of NK cells from an MHC class I–sufficient environment into an MHC class I–deficient environment, where the NK cells rapidly become hypofunctional (8). This ability of NK cells to adapt to their environment by altering their functionality is summarized by a model known as the tuning or rheostat model (9), which proposes that the relative strength of the activating and inhibitory signals that an NK cell receives tunes up (arms) or down (disarms) the NK cell responsiveness. This education process both prevents inappropriate NK cell activation, which could lead to autoimmune inflammation and disease, and is responsible for graft-versus-leukemia effects by setting a threshold for activation (10). Although some of the molecular events responsible for regulating NK cell activation and tuning have been defined (11, 12), our understanding of the molecules and intracellular changes that control these processes is incomplete.

MicroRNAs (miRNAs) are a family of small, noncoding RNAs that mediate downregulation of targeted mRNA transcripts by binding to complementary sites primarily in the 3′ untranslated regions of mRNAs (13). miRNAs have been shown to have a wide variety of roles in cancer (14), inflammation (15), and immune responses (16). In NK cells, miRNAs have been shown to regulate NK cell
proliferation and survival and alter functionality (17–19). Recently, individual miRNAs have been shown to influence NK cell development and function (20–22), including miR-155 (23, 24).

miR-155 is encoded within the BIC noncoding RNA, and its role in T cells, B cells, and macrophages has been characterized (25–28). miR-155 is expressed in resting NK cells (29) and has been shown to be overexpressed in NK cell type lymphoma/leukemia (20). A recent report has identified that miR-155 regulates IFN-γ production by human NK cells partially via repression of SHIP-1, a phosphatase involved in negative regulation of PI3K signaling (23). In addition, mice expressing a Lck-driven miR-155 transgene have NK cell IFN-γ alterations, further linking miR-155 repression of SHIP-1 to NK cell activation (24). miR-155–deficient mice were further found to have alterations in murine CMV (MCMV) responses (30). However, mice that are deficient in miR-155 (155–/−), or that overexpress miR-155 in an NK cell–restricted fashion (155FOE), have not been directly compared and evaluated in depth for alterations in NK cell responses.

In this study, we compared the effects of multiple miR-155 alterations, including lentiviral (LV) overexpression in mature human and murine NK cells, as well as NK cells from 155−/− and 155FOE mouse models. Unexpectedly, both 155FOE and 155−/− mice were found to have alterations in murine CMV (MCMV) responses (30). However, mice that are deficient in miR-155 (155−/−), or that overexpress miR-155 in an NK cell–restricted fashion (155FOE), have not been directly compared and evaluated in depth for alterations in NK cell responses.

In this study, we compared the effects of multiple miR-155 alterations, including lentiviral (LV) overexpression in mature human and murine NK cells, as well as NK cells from 155−/− and 155FOE mouse models. Unexpectedly, both 155FOE and 155−/− NK cells exhibit an augmented IFN-γ response. We found that 155−/− NK cells had increased IFN-γ secretion due to an increased percentage of IFN-γ+ NK cells without a change in per-cell expression, indicating a novel role for miR-155 in altering the NK cell activation threshold. Conversely, 155FOE NK cells produced more IFN-γ per cell. The cellular mechanism for miR-155’s dual effect on IFN-γ was recapitulated in vivo during MCMV infection of 155−/− and 155FOE mice. Notably, 155−/− mice had decreased MCMV titers, suggesting that the increased functionality of 155−/− NK cells has a biologically significant impact on the early NK-mediated anti-viral cytokine response. Using RNA-induced silencing complex (RISC) sequencing (RISC-Seq) analysis of activated NK cells from wild-type (WT) and 155−/− mice, we identified and validated novel miR-155 mRNA targets in NK cells. We further used chemical inhibitors of multiple activation pathways to eliminate IFN-γ production differences, indicating that miR-155 extensively regulates molecules involved in NK cell activation, thereby regulating the NK cell activation threshold.

**Materials and Methods**

**Mice**

155−/− mice were described previously (26) and obtained from The Jackson Laboratory as B6.Cg-Mir155 tm1.1Rsky/J. 155FOE mice were generated by the Jackson Laboratory as B6.129 (µMT)-1Gt(ROSA)26Sortm1(EYFP)Cos/J and have been bred and maintained in specific pathogen-free housing, and all other mice were originally generated on a C57BL/6 background. All mice have been bred and maintained in specific pathogen-free housing, and all other mice were originally generated on a C57BL/6 background. All mice have been bred and maintained in specific pathogen-free housing, and all other mice were originally generated on a C57BL/6 background. All mice have been bred and maintained in specific pathogen-free housing, and all other mice were originally generated on a C57BL/6 background.

**Abs**

Anti-mouse mAbs were obtained from BD Biosciences (San Jose, CA): IFN-γ (XMG1.2), NK1.1 (PK136), NKP46 (29A.14), CD3 (145-2C11), CD45 (30-F11), CD27 (LG.3A10), CD11b (M170), CD19 (1D3), CD212 (114), CD132 (4G3), Ly49A (J93-318), Ly49C/I (5E6), Ly49G2 (4D11), Ly49D (4E5), and Ly49H (3D10); eBioscience (San Diego, CA): IFN-γ (1D4B), NKG2A (16a11), NKG2ACE (20d5), CD94 (18d3), and CD122 (1D3); and BioLegend (San Diego, CA): granzyme B (GB12); Caltag Laboratories: granzyme B (GB12); BioLegend (San Diego, CA): CD107a (114), CD132 (4G3), Ly49A (JR9-318), Ly49C/I (5E6), Ly49G2 (4D11), and Ly49H (3D10). Abs were obtained from Beckman Coulter: CD3 (UCHT1); CD56 (N901), CD158a.h (EB6B), and CD158hi.h2,j (GL183); BD Biosciences: CD16 (3G8) and CD94 (HP-3D9); and BioLegend: IFN-γ (B27).

**Cell lines and reagents**

Endotoxin-free purified recombinant cytokines (recombinant murine [rm]IL-12), rmIL-18, recombinant human [rh]IL-12, rhIL-15, and rhIFN-γ were obtained from PeproTech (Rocky Hill, NJ) and reconstituted in sterile PBS with 0.1% BSA. Anti-NK1.1 (PK136) was purified by the Washington University Ab production core from hybridoma supernatant. Chemical inhibitors (Ly294002, BAY 11-7082, and cyclosporin A) were obtained from EMD Millipore.

**IFN-γ ELISA**

Sorted GFP+ (LV-GFP, LV-GFP/155), Cre+ (155FOE), or bulk (155+/−) NK cells were plated in duplicate and cultured for the indicated periods of time in complete RPMI 1640 (cRPMI) + 10% FBS + 10 ng/ml each indicated cytokine or immobilized Ab. Cell-free supernatants were collected and frozen at −80 °C, thawed only once, and analyzed by ELISA from eBioscience according to the manufacturer’s instructions.

**IFN-γ stimulation assay/inhibitor assay**

IFN-γ stimulation was performed as previously described (18). Licensing assays were performed as described previously (32). For inhibition assays, IC50 values were obtained from the manufacturer or, in cases in which the inhibitor had been tested in NK cells, were used at the previously demonstrated value [cyclosporin A = 35.7 nM (33); BAY 11-7082 = 10 μM (manufacturer); and Ly294002 = 1.2 μM (34)]. Conditions with NK cell toxicity were excluded.

**LV transduction**

High-titer MND-ΔU3 (35) lentivirus was generated using standard third-generation packaging systems pseudotyped with vesicular stomatitis virus-G in ultracentrifuged supernatants from 293T cells. Human NK cells were purified from peripheral blood to >90% purity using RossetSep ( Stem-Cell Technologies, Vancouver, Canada). Mouse NK cells were sorted to >99% purity from RAG−/− spleens. A total of 1 × 106 human NK cells were cultured in cRPMI + 10% HAB serum + 100 ng/ml rhIL-15, whereas mouse NK cells were cultured in cRPMI + 10% FBS + 100 ng/ml rmIL-15. Lentivirus was then added at a multiplicity of infection of 20, and the cells were centrifuged (spinfected) at 2000 rpm, 30°C, 45 min in a standard swinging bucket table-top centrifuge. Cells were cultured overnight at 37°C in 5% CO2, and spinfection was repeated the next day (day 1). On day 2, media was replaced by removing half of the media, taking care not to disturb the cells, and adding cRPMI + 10% FBS +10 ng/ml rmIL-15 or HAB + 10 ng/ml rhIL-15 as appropriate. Media was replaced in this manner every other day until harvest. Mouse NK cells were harvested at day 7, whereas human NK cells were harvested at days 4 or 9, and then sorted to >99% purity for GFP+ NK cells.

**Flow cytometry**

All flow cytometry was conducted on a Beckman Coulter Gallios machine, and all cells were sorted using a BD FACSAria II (BD Biosciences) at the Washington University Pathology Flow Cytometry and Fluorescence Activated Cell Sorting Core. Flow cytometry was analyzed using FlowJo (Tree Star, Ashland, OR).

**Statistical analysis**

Graphical analysis and statistics were performed with GraphPad Prism 5.0 (GraphPad). Student’s t test, one-way ANOVA, and two-way ANOVA were used as appropriate, with p < 0.05 considered significant: *p < 0.05, **p < 0.01, ***p < 0.001.

**MCMV infection and plaque assays**

MCMV infections, stock preparations, and titering were performed as described previously (36, 37). Mice were infected with 10,000 PFU MCMV (Smith Strain) and injected with BrdU 4 d postinfection (p.i.). Four hours post-BrdU injection, spleens were harvested and assessed using standard BrdU intracellular flow cytometry.

**Quantitative RT-PCR**

Quantitative PCR (qPCR) was performed on sorted NK cells as indicated by isolating total RNA from TRIZol (Invitrogen), according to the manufacturer’s instructions. TaqMan Assays (Applied Biosystems) were used to create cDNA and real-time quantitative PCR (RT-qPCR) was run on an ABI 7300,
according to the manufacturers’ instructions. Relative quantification was determined by the ΔΔ threshold cycle method, by normalizing either to sno-135 (mouse) or RNU48 (human).

**RISC-Seq**

A total of $1 \times 10^6$ to $3 \times 10^6$ WT or 155/−/− NK cells was sorted and allowed to recover for 2 d in 100 ng/ml IL-15. The NK cells were then activated with 10 ng/ml each IL-12, IL-15, and IL-18 for 24 h. RISC-Seq was then performed exactly as described (38).

**Luciferase target validation**

Performed as previously described (18) with primers available upon request.

**Results**

miR-155 expression is markedly upregulated after NK cell activation in vitro and in vivo

miR-155 is moderately expressed in resting mouse NK cells when evaluated by next-generation small RNA sequencing (Fig. 1A) (29). However, miR-155 is markedly upregulated after cytokine activation, especially after stimulation with IL-12, IL-15, and IL-18 (Fig. 1B) (23). This upregulation was consistent between human and murine NK cells (Fig. 1B). Furthermore, miR-155 is induced in murine NK cells 36 h p.i. with MCMV (Fig. 1C). This

**FIGURE 1.** miR-155 is expressed in NK cells and induced after NK cell activation in vitro and in vivo. miR-155 expression was assessed by deep sequencing (A) or qPCR (B, C) after cytokine stimulation (B) or MCMV infection (C). (A) miR-155 is expressed in resting mouse NK1.1+CD3 splenic NK cells. Data shown are the rank order of all expressed NK cell miRNAs by small RNA sequencing from mouse NK cells (29). Sequencing reads were normalized to total miR reads and plotted as log 2 (normalized read counts) versus rank order of expression in resting NK cells. The large circle indicates miR-155. (B) Human CD56+CD3− peripheral blood NK cells (left panel) and mouse NK1.1+CD3− splenic NK cells (right panel) were enriched to >95% purity and evaluated for miR-155 expression at rest or at the indicated time points after cytokine activation. Relative fold change was assessed by miR-155 RT-qPCR. Data summarize five mice and four donors from two to three independent experiments. (C) miR-155 is induced following MCMV infection in NK cells. Ly49H+ and Ly49H− NK1.1+CD3− NK cells were sorted from the spleens of naive control mice or control mice infected with MCMV (5 × 10^5 PFU/mouse) at 1.5 or 7 d p.i. miR-155 mRNA levels were compared by RT-qPCR and found to be significantly increased in both NK cell subsets at 1.5 d p.i. Data summarize 10 mice from three independent experiments. (D) Summary data showing the robust IFN-γ production at day 1.5 p.i. Data summarize 10 mice from three independent experiments. **p < 0.01, ***p < 0.001.

**FIGURE 2.** Overexpression of miR-155 in human and mouse mature NK cells results in increased IFN-γ protein secretion. (A) Relative miR-155 expression measured by qPCR from human and mouse LV-transduced NK cells (mean ± SEM fold change), including both resting and IL-12 + IL-18–stimulated NK cells. Data summarize five to six donors from three independent experiments and 4–12 mice from two to three independent experiments. (B) IFN-γ protein production by human NK cells by ELISA after 12 h of stimulation with the indicated cytokines. Data summarize 8–10 donors from two independent experiments. (C) IFN-γ protein production by mouse NK cells measured by ELISA after 12 h of stimulation with the indicated cytokines. Data show one experiment of two groups (n = 2 to 3 mice/group), representative of four groups of n = 2 to 3 mice from three independent experiments. For (B) and (C), data are shown as mean ± SEM IFN-γ concentration. *p < 0.05, ***p < 0.001.
corresponds to the peak of IFN-γ production by splenic NK cells (Fig. 1D) (37). Therefore, miR-155 expression is markedly increased in both mouse and human NK cells after in vitro stimulation and in the context of a physiologic antiviral response.

**Overexpression of miR-155 in mature mouse and human NK cells augments activation-induced IFN-γ**

Because miR-155 expression increases in activated NK cells, we evaluated the impact of forced LV overexpression of miR-155 on the IFN-γ response in both mouse and human NK cells. Both control LV-GFP and LV-GFP/155 were able to efficiently transduce human and mouse NK cells (data not shown), and LV-GFP/155 virus resulted in miR-155 overexpression in resting and cytokine-activated mouse and human NK cells (Fig. 2A). Mature NK cells transduced with LV-GFP/155 or LV-GFP control were sorted for GFP+ NK cells and stimulated to secrete IFN-γ. When miR-155 was overexpressed, we observed enhanced IFN-γ production in human NK cells after stimulation with IL-12 plus IL-18, but not IL-12 plus IL-15 alone, due to a high degree of human donor variability (Fig. 2B, Supplemental Fig. 2), miR-155 overexpression also increased the median fluorescence intensity (MFI) of staining with an anti-CD158b1/b2/j mAb (but not other killer Ig-like receptors) on the surface of human NK cells (Supplemental Fig. 2D). Similar experiments with mature splenic WT NK cells showed that mature mouse NK cells transduced with LV-GFP/155 also produced more IFN-γ protein when stimulated with IL-12 plus IL-18 or with IL-12 plus IL-15 (Fig. 2C). Therefore, forced overexpression of miR-155 results in increased IFN-γ production.

**NK cells from 155−/− mice have an intact NK cell compartment and enhanced cytokine-induced IFN-γ production**

To further investigate the role of miR-155 in regulating NK cells, we examined 155−/− mice (Fig. 3A) (26). An in-depth characterization of the NK cell compartment in 155−/− mice found no changes in NK cell numbers, percentages, development, maturation, survival, or homeostasis from all tissues examined (Supplemental Fig. 1). NK cells from 155−/− mice have normal expression of most NK cell receptors (Supplemental Fig. 1), although we observed significantly decreased expression of Ly49G2 and a slight increase in Ly49A expression (Supplemental Fig. 1D). Therefore, prior to activation, 155−/− NK cells appear similar to WT B6 NK cells. Because NK cells transduced to overexpress miR-155 produced more total IFN-γ, we expected that the 155−/− NK cells would have the opposite phenotype: decreased IFN-γ production after activation. However, we were surprised to find that the 155−/− NK cells secreted more IFN-γ after stimulation with IL-12 plus IL-15 (Fig. 3B) or IL-12 plus IL-18 (Fig. 3C) as measured by ELISA. Furthermore, 155−/− NK cells produce more granzyme B upon stimulation with IL-15 (Supplemental Fig. 1F) and have

**FIGURE 3.** NK cells from 155−/− mice have enhanced IFN-γ production. (A) NK cells sorted from 155−/− mice express no detectable miR-155 at rest or following 24 h of IL-12 + IL-15 + IL-18 combined activation. Data shown (mean ± SEM fold change) are normalized to WT resting NK cells and summarize four pools of four to five mice from three independent experiments. (B and C) 155−/− NK cells exhibit increased IFN-γ production after 6 or 12 h of cytokine stimulation. IFN-γ protein was measured by ELISA from cell-free culture supernatants after 6 or 12 h with IL-12 + IL-15 (B) or IL-12 + IL-18 (C). Data shown are the mean ± SEM from one experiment (two to three mice/genotype), representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 4.** NK cells from 155FOE mice have increased levels of miR-155 and increased IFN-γ production. (A) miR-155 expression was measured using RT-qPCR in sorted resting WT GFP+ NK cells, 155FOE GFP+ NK cells, and GFP- NK cells from the spleen, normalized to WT GFP+ NK cells with sno135 as the calibrator. Data summarize at least three mice per group from three independent experiments. IFN-γ secretion as measured by ELISA of cell-free supernatants by flow sorted GFP+ NK cells after 6 or 12 h of stimulation with IL-12 + IL-15 (B) or IL-12 + IL-18 (C). Data shown are mean ± SEM IFN-γ concentration of one experiment of two to three mice per group, representative of two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
increased levels of surface CD107a after NK1.1 ligation (Supplemental Fig. 1G), suggesting a global enhancement in responses following activation or triggering. We confirmed that Ly49G2 and Ly49A expression was not associated with this IFN-γ phenotype (Supplemental Fig. 1I), and 155−/− and control NK cells had similar Ly49C-based licensing ratios (Supplemental Fig. 1J, 1K). Despite this increase in global activation, the killing of YAC-1 tumors by 155−/− and control NK cells after 48 h of IL-15 stimulation was not significantly different (Supplemental Fig. 1H). This may reflect an alteration of threshold following high-dose IL-15 stimulation in vitro. Collectively, these data suggest that 155−/− NK cells are more responsive to activation.

Distinct cellular mechanisms are responsible for enhanced production in mature mouse NK cells.

We hypothesized that the incongruous phenotype between 155−/− and LV-GFP/155 models could be explained by in vitro culture and/or LV transduction. To further investigate this premise, we generated a conditional miR-155 overexpression knockin model (26) combined with an NK cell–specific Cre (Ncr1-iCre) (31) to allow for specific miR-155 overexpression in NK cells (155FOE). In this model, miR-155 overexpression commences at an early stage of dedicated NK cell development and persists throughout the lifespan of the mature NK cell, with Cre− NK cells marked by GFP (NK cells routinely ≥85% Cre−). Cre+ NK cells from 155FOE mice exhibit increased miR-155 expression, compared with both WT Cre− NK cells, or the small number of Cre− NK cells within 155FOE mice (Fig. 4A). Similar to 155−/− mice, resting 155FOE NK percentages, numbers, maturation, surface receptor expression, and ex vivo expansion were normal (Supplemental Fig. 1D, IL-15-P), with the exception of an increased percentage of Ly49G2+ NK cells (Supplemental Fig. 1D).

We next investigated IFN-γ production by cytokine-activated 155FOE NK cells. Sorted GFP+ 155FOE NK cells or control Cre− (Rosa26YFP) NK cells were stimulated with IL-12 plus IL-15 or IL-12 plus IL-18 and analyzed for IFN-γ production by ELISA (Fig. 4B, 4C). In this model, IFN-γ production was also increased after stimulation compared with controls. Therefore, forced miR-155 overexpression initiated early in NK development again led to increased total IFN-γ production in mature mouse NK cells.

Distinct cellular mechanisms are responsible for enhanced IFN-γ production by 155−/− versus 155FOE NK cells.

In an effort to better understand the seemingly disparate finding that both 155−/− and 155FOE NK cells produce more IFN-γ than control NK cells, we investigated per-cell IFN-γ production by intracellular flow cytometry. We found that 155−/− NK cells had a minimal dynamic range to detect IFN-γ increases after miR-155 alteration. Because our genetic mouse models of miR-155 loss and LV-overexpression of miR-155. Consistent with 155FOE mice, LVGFP/155–transduced human (Supplemental Fig. 2A–C) and mouse

**FIGURE 5.** NK cells from 155−/− and 155FOE mice produce increased IFN-γ by different cellular mechanisms. Representative intracellular IFN-γ flow cytometry histograms of 155−/− (A, light gray) and 155FOE (B, dark gray) compared with WT NK cells (A, black) or GFP+ control NK cells (B, black), after stimulation with IL-12 + IL-15 for 6 h. (C–E) Normalized summary data for percent IFN-γ positive NK cells demonstrate a significantly higher percentage of 155−/− NK cells are positive for IFN-γ, whereas 155FOE NK cells are unchanged, compared with control NK cells. (F–H) The IFN-γ expression on a per-cell basis (MFI) of IFN-γ− NK cells was unaffected or modestly decreased within 155−/− NK cells, whereas 155FOE NK cells exhibited a significant increase in the expression of IFN-γ on a per-cell basis, in all conditions except IL-12 + IL-18. (I) 155−/− NK cells are activated to produce IFN-γ at concentrations that do not activate WT NK cells, indicating an activation threshold defect. Data in (C–H) summarize 5–20 mice per group from two to seven independent experiments. Data in (I) are from three mice per group in one experiment, representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
IFN-γ+ NK cells and the amount of IFN-γ produced by each cell recapitulates and explains the increased total IFN-γ measured by ELISA in both of these models (Figs. 2–4), suggesting a role for an activation threshold alteration in the 155−/− NK cells. Indeed, anti-NK1.1 mAb dilutions revealed a concentration at which 155−/− NK cells produced IFN-γ, but WT NK cells did not (Fig. S1). Though these differences occur primarily in Ly49C+ cells, which are the primary IFN-γ producers, we observed no alterations in the licensing ratio in the 155−/− NK cells (Supplemental Fig. 1J, 1K). Therefore, distinct cellular mechanisms explain the phenomenon of increased total IFN-γ production in all three models.

MCMV infection recapitulates in vitro IFN-γ responses of 155−/− and 155FOE NK cells and leads to decreased viral titers in 155−/− mice

To determine whether the increased activation of 155−/− NK cells was relevant in vivo, we infected 155−/− and B6 WT mice, or 155FOE and RosaYFP control mice, with MCMV. At 36 h p.i., an increased percentage of 155−/− NK cells responded to MCMV infection by producing IFN-γ (Fig. 6A–C) compared with control NK cells. In contrast, 155FOE NK cells had increased IFN-γ MFI with no alteration in the IFN-γ+ NK cell percentage (Fig. 6D–F). Therefore, these in vivo results recapitulated the in vitro IFN-γ phenotype. We next evaluated whether the increased NK cell–derived IFN-γ at 1.5 d p.i. affected the course of MCMV infection by measuring MCMV viral titers of 155−/− mice at 3 d p.i. (Fig. 6G). 155−/− mice had decreased MCMV titers at 3 d p.i., consistent with the observed enhanced IFN-γ response. Thus, NK cells from 155−/− mice exhibit enhanced early IFN-γ production during the cytokine-dependent phase, and miR-155 disrupts the anti-MCMV NK cell response.

RISC-Seq identifies novel miR-155 targets in multiple NK cell activation pathways

To determine relevant targets of miR-155 involved in threshold and IFN-γ production, WT and 155−/− NK cells were activated with IL-12, IL-15, and IL-18 for 24 h to induce mRNA targets that are likely regulated by miR-155 at its peak expression level in NK cells. We then sequenced (RISC-Seq) paired total RNA and RNA from immunoprecipitated RISC, which contains miRNAs and their targets from both WT and 155−/− NK cells (Supplemental Fig. 3A) (38, 39). An mRNA transcript that is a target of miR-155 is expected to be enriched in the RISC of WT NK cells compared

**FIGURE 6.** 155−/− and 155FOE NK cells produce more IFN-γ during MCMV infection in vivo. (A–C) Age- and sex-matched 155−/− or B6 mice were infected with 5 × 10⁶ PFU MCMV, and splenic NK cells were assessed for intracellular IFN-γ production by flow cytometry after 36 h. Representative flow plots (A) and summary data (B, C) showing increased mean ± SEM IFN-γ+ percentage (B) and MFI (normalized to WT in each experiment) (C) of NK cells in 155−/− mice, compared with WT controls at 36 h p.i. Data summarize six to seven mice per group from two independent experiments. Representative flow plot (D) and summary data (E, F) showing increased mean ± SEM IFN-γ+ percentage (E) and MFI (F) of NK cells in 155FOE mice, compared with Cre− controls at 36 h p.i. Data summarize four to six mice per group from two independent experiments. (G) Groups of MCMV-infected WT and 155−/− mice were assessed for splenic viral titers 3 d p.i. (mean ± SEM viral titer). Dotted line represents the limit of detection for the assay (0.92). Data summarize seven to eight mice/group from two independent experiments. *p < 0.05, **p < 0.01.
with $155^{-/-}$ NK cells (i.e., a RISC incorporation ratio [RIR] > 1) (Fig. 7A). To improve the signal/noise ratio of this analysis, we filtered by predicted miR-155 targets in TargetScan (Fig. 7B) (40). This resulted in marked enrichment of targets with an RIR > 1.2, with an almost complete loss of targets with an RIR < 0.83. This filter approach did not produce any mRNA target enrichment when performed with an irrelevant miR target prediction (Supplemental Fig. 3B, 3C). We next biochemically validated miR-155 targets identified using miR-155 overexpression coupled with a luciferase sensor plasmid containing the 3’ untranslated region of the putative target mRNAs (18, 29) (Fig. 7C) and Western blot of $155^{-/-}$ and WT NK cells for SHIP-1, SLP-76, and IKBKE (Fig. 7D). Notably, all candidates identified by RISC-Seq that were tested by either luciferase assay or Western blot were confirmed to be true miR-155 targets by these validation methods. Together, these data indicate that miR-155 targets multiple molecules in NK cell activation pathways.

mRNA transcripts with an RIR > 1 SD from the mean were analyzed using DAVID pathway analysis, and the KEGG lymphocyte (T cell) activation pathway was found to be significantly overrepresented. We hypothesized that the decreased activation threshold of $155^{-/-}$ NK cells was due to an increase in the protein levels of the miR-155–targeted members of this pathway and that treatment with specific inhibitors would elucidate the proteins critical for miR-155 threshold effects. To directly test these confirmed targets in the acquisition of a decreased threshold of activation in the $155^{-/-}$ NK cells, we used chemical inhibitors of NK cell activation pathways; specifically, PI3K (Ly294002), calcineurin (cyclopasin A), and NF-κB (BAY 11-7082). We used these inhibitors in an NK1.1 ligation assay because this stimulus demonstrated the greatest threshold effect. We found that inhibition of either calcineurin (Fig. 8A) or PI3K (Fig. 8B) led to a dose-responsive decrease in the differential between $155^{-/-}$ and WT NK cell IFN-γ production, whereas inhibition of NF-κB led to a differential response only at concentrations above its IC₅₀ (Fig. 8C), and an inhibitor of p38 kinase, SB203580, had no effect on IFN-γ production (Fig. 8D). These data indicate that the PI3K and calcineurin pathways contribute to the increased production of IFN-γ in $155^{-/-}$ NK cells and, collectively, form the basis of the decreased threshold of activation in these cells (Fig. 8E).

**Discussion**

In this study, we assessed the impact of miR-155 alterations in human and mouse NK cells using LV overexpression, as well as genetic mouse models of global miR-155 deficiency and NK-specific miR-155 overexpression. We demonstrate that miR-155 is induced upon cytokine activation of both mouse and human NK cells in vitro, as well as in vivo during an ongoing NK cell response to MCMV. We confirmed results of a previous study (23) showing that miR-155 overexpression in mature human NK cells leads to increased IFN-γ production following NK cell activation. Furthermore, we found identical results in mature mouse NK cells, demonstrating consistent miR-155 regulation of NK cell activation in mice and humans in this context. However, we unexpectedly

**FIGURE 7.** RISC-Seq identifies miR-155 targets in NK cells. (A) Unfiltered results of RISC-Seq, displayed as WT total RNA expression versus RIR (WT/155⁻⁻⁻⁻). Graph depicted is limited to targets with a fold change between 0.5 and 2 to align with (B). A total of 116 transcripts lie outside of these depicted boundaries, but none of these were targets predicted by TargetScan. Targets validated in (C) are shown as predicted (open circle) or not predicted (open square) by TargetScan, and the open diamond represents SHIP-1, a previously validated target. Numbers above graphs in (A) and (B) represent the percentage of total mRNA transcripts within each RIR quartile: group 1: RI > 1.2; group 2: 1 < RI < 1.2; group 3: 0.83 < RI < 1; and group 4: RI < 0.83. Data represent two biological replicates for groups of WT and $155^{-/-}$ NK cells following IL-12, IL-15, and IL-18 activation. (B) Dataset in (A) filtered by cross-referencing with miR-155 predicted targets (TargetScan). (C) Luciferase validation in 293T cells of RISC-Seq targets, as indicated in (A) and (B). Data summarize two independent experiments of duplicate wells. (D) Western blot analysis of miR-155 target expression in WT and $155^{-/-}$ NK cells. Relative overexpression for $155^{-/-}$ NK cells is indicated. UTR, Untranslated region.
discovered that $155^{+/2}$ NK cells also produced more IFN-$\gamma$ upon cytokine activation. How can miR-155 overexpression and deficiency lead to similar IFN-$\gamma$ phenotypes in NK cells? To address this, we generated a novel 155 FOE model that specifically overexpresses miR-155 in developing and mature NK cells, finding that similar to lentivirus-transduced NK cells that overexpress miR-155, 155FOE NK cells also secreted increased amounts of IFN-$\gamma$ upon activation. We hypothesized that miR-155 loss or overexpression had distinct mechanisms that lead to similar apparent IFN-$\gamma$ phenotypes. Indeed, a higher proportion of NK cells from $155^{+/2}$ mice produced IFN-$\gamma$ following activation, whereas NK cells from $155^{+/2}$ mice exhibited increased per NK cell expression of IFN-$\gamma$ following activation. These findings, in multiple complementary experimental systems, suggest a dual role for miR-155 by regulating NK cell activation and cytokine production. When miR-155 is deficient during NK cell development and maturation (e.g., $155^{+/}$), the NK cell threshold for future activation is lowered, allowing more NK cells to express IFN-$\gamma$ when stimulated. In contrast, forced overexpression of miR-155 in NK cells, either in vitro (LV) in mature NK cells or in vivo (155FOE) in developing NK cells, allows an NK cell, once it crosses the activation threshold, to have an amplified IFN-$\gamma$ response (Supplemental Fig. 4). This is likely due to the previously postulated mechanism of miR-155 negatively regulating SHIP-1, a negative regulator of IFN-$\gamma$ production. Although the Ly49C+/Ly49C− ratio of IFN-$\gamma$ production (the licensing ratio) is not altered per se (Supplemental Fig. 1K), we do note that the vast majority of IFN-$\gamma$-producing cells in these mice are Ly49C+, and thus the licensed subset of NK cells is the major subset affected by miR-155 loss. It remains possible, therefore, that miR-155 may have a role in NK cell licensing. Licensing alterations in both the $155^{+/2}$ and the 155FOE NK cells are an area of ongoing study. Furthermore, because LV-GFP/155 NK cells develop in a context of normal levels of miR-155, whereas 155FOE NK cells develop with increased

![Figure 8](http://www.jimmunol.org/)

**FIGURE 8.** miR-155 targets NK cell activation pathways. NK cells were activated via NK1.1 ligation using plate-bound anti-NK1.1 in the presence of an inhibitor of calcineurin (A), PI3K (B), NF-κB (C), or p38 kinase (D) to demonstrate loss of threshold differentials after including inhibitors. Solid lines represent previously determined IC$_{50}$ values (see Materials and Methods), whereas dashed lines represent IC$_{50}$ values obtained by our experiments. The data shown are mean ± SEM and representative of three independent experiments. (E) Model/summary of miR-155’s role in targeting genes involved in the acquisition of NK cell threshold. RIR incorporation values are included with each gene. RISC-Seq–identified (RIR >1.2) miR-155 targets (dark gray boxes) and inhibitor targets (light gray boxes) are indicated.
levels of miR-155, the increased percentage of activated NK cells in the LV-GFP/155 further supports a developmental/maturational role for miR-155 in suppressing future activation via threshold modulation. Thus, the combined use of both human and murine mature NK cells in concert with genetic mouse models provides a comprehensive picture of the complex role of miR-155 in directing NK cell activation and IFN-γ production.

The findings presented in this study that both mature human and mouse NK cells that overexpress miR-155 exhibit enhanced IFN-γ production upon activation are in agreement with a recent study focused primarily on human NK cells (23). Furthermore, the enhanced NK cell IFN-γ phenotype demonstrated in 155FOE mice was also observed in a recent report in an Lck-miR-155-transgenic mouse model (24). However, the Lck-miR-155–transgenic mice were reported to have increased NK cell percentages, numbers, and proliferation, whereas the 155FOE mice had a normal complement of NK cells in all tissues examined. The level of overexpression of miR-155 was similar in both of these mouse models, suggesting that disparate miR-155 overexpression was not the explanation. The 155FOE is NK cell specific, tracked using a Cre-reporter allele, and was analyzed on a B6 background. We suspect that cell specificity of miR-155 overexpression, strain of the mice used in the different models, or integration effects may play a role in the different phenotypes observed in these models.

What are the downstream targets that are responsible for miR-155’s effects in mature NK cells? miR-155 target mRNAs have been shown to be cell-context specific (41), and thus we demonstrate in this study the first miR-155 target set in primary NK cells. In this study, we report a RISC-Seq approach that identified >20 high-probability candidate mRNA targets and validated many using luciferase sensor assays and Western blot. Notably, this included SHIP-1 (Inpp5d), which has been previously identified and validated as a target of miR-155 (23, 28). Indeed, specific knockdown of SHIP-1 (Inpp5d), which has been previously identified and validated as a target of miR-155 (23, 28), indeed, specific knockdown of SHIP-1 has been shown to phenocopy miR-155 deletion in macrophages. SHIP-1 functions as a negative regulator of IFN-γ production, and thus miR-155 inhibition of SHIP-1 in activated NK cells should result indirectly in greater IFN-γ production. Our data fit with this model of miR-155 directly targeting SHIP-1, with increased IFN-γ MFI in LV-GFP/155 and 155FOE NK cells and a modestly decreased IFN-γ MFI in some activating conditions in our 155+/− NK cells. Further, a recent study (42) has shown that SHIP-1 deficiency in developing NK cells, contrary to what would be expected for deletion of a negative regulator of activation, actually leads to a decreased percentage of mature cells becoming IFN-γ positive after stimulation. These findings are provocative and consistent with our data, because miR-155 deletion would lead to increased SHIP-1 levels in development and therefore may alter the NK cell activation threshold, leading to increased IFN-γ percentages in mature NK cells. This concept is similar to the known TCR signal amplitude tuning by miR-181a targeting of multiple phosphatases, identified in T cells (43). Thus, SHIP-1 represents one validated miR-155 target that may contribute to the NK cell phenotype. However, previous studies examining NK cell SHIP-1 gain-of-function resulted in only a partial restoration of the NK cell activation phenotype (23), suggesting that targets other than SHIP-1 play a role in this phenotype.

In this study, we identified a number of these additional miR-155 mRNA targets directly in primary NK cells in our RISC-Seq experiments. A number of miR-155 NK cell mRNA targets are important members of signaling cascades central to NK cell activation, providing an alternative explanation for how miR-155 may tune the activation threshold of NK cells (Fig. 8). Additionally, we found that novel targets identified in the PI3K, NF-κB, and calcineurin pathways are able to contribute to the increased IFN-γ production in NK cells, as chemical inhibition of these pathways results in a loss of the enhanced IFN-γ production phenotype, though to a far lesser extent for NF-κB, indicating that this pathway is less important for miR-155’s impact on IFN-γ. Thus, these additional miR-155 targets in the NK cell activation pathway contribute to NK cell activation phenotypes. The individual and collective contribution of these targets to tuning the NK cell threshold will be evaluated further in future studies.

We also demonstrate a regulatory role for miR-155 during early antiviral NK cell responses in vivo, with effects on early cytokine-dependent IFN-γ production. IFN-γ production by 155+/− NK cells was increased in vivo at day 1.5 post-MCMV infection, which correlated with a decreased viral titer at 3 d, and was consistent with our in vitro cytokine-dependent IFN-γ phenotype. MCMV studies of 155FOE mice are ongoing. These findings may have powerful implications in the human NK cell response to MCMV, and extending the study of miR-155 in this setting is of exceptional interest. A recently published study (30) identified suppressor of cytokine signaling 1 and Noxa as targets of miR-155 and demonstrated a blunted Ly49H+ expansion at later time points. Future studies in an miR-155 floxed knockout model that selectively lacks miR-155 in NK cells will be used to investigate the NK cell–intrinsic role of miR-155.

In this study, we have defined a novel dual role for miR-155 in the regulation of both mature human NK cell IFN-γ production and setting a threshold of activation during NK cell development and maturation. This regulation of activation by miR-155 is evident both in vitro and in vivo during an NK cell response to viral infection. Although SHIP-1 represents a validated target of miR-155 that is likely important in these processes, novel mRNA targets in activation signaling pathways identified in this study contribute to setting the NK cell activation threshold. miR-155 also regulates the NK cell IFN-γ response during MCMV infection, indicating that the observed miR-155–mediated changes in IFN-γ influence host defense against a viral pathogen. These findings further support the role for miR-155, and miRNAs in general, as tuners of immune cell activation. Future studies of miR-155, especially in the context of cell-specific deletion or overexpression, will undoubtedly uncover more of miR-155’s diverse functions in NK cells.

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Disclosures

The authors have no financial conflicts of interest.

References


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Fig. S1
**Supplemental Figure 1. miR-155\(^{-/-}\) and 155\(^{FOE}\) mice have an intact NK cell compartment at baseline.** (A) The lymphocyte compartment of miR-155\(^{-/-}\) mice demonstrates slight alterations in B and T cell percentages, but no significant differences in absolute number, and no alterations in NK cell percentage or number. N=8-12 mice per group, from 3 independent experiments. (B) NK cells from miR-155\(^{-/-}\) mice exhibit similar expression of NKG2A, combined NKG2C,A,E, and IL-18R1 compared to WT controls. (C) NK cells from miR-155\(^{-/-}\) mice exhibit normal maturation in the spleen, liver, bone marrow and lung, compared to littermate controls as defined by CD27 and CD11b. Stage 1: CD27^CD11b\(^{hi}\), Stage 2 CD27^CD11b\(^{lo}\), stage 3: CD27^CD11b\(^{hi}\), stage 4: CD27^CD11b\(^{lo}\). Shown are representative spleens (top) and summary data (bottom) from N=7-10 mice per group from at least 3 independent experiments. (D) The NK cell Ly49 repertoire is slightly altered in miR-155\(^{-/-}\) and 155\(^{FOE}\) mice, with miR-155\(^{-/-}\) NK cells exhibiting significantly decreased Ly49G2 and modestly increased Ly49A percentages, and 155\(^{FOE}\) NK cells exhibiting increased Ly49G2 percentages. Ly49C/I, Ly49D, and Ly49H expression are similar in NK cells from miR-155\(^{-/-}\) and control mice. Of note, the ligands for Ly49G2 are not present in C57Bl/6 mice, and are thus unlikely to affect NK cell responses in this genetic model. Neither Ly49G2 or Ly49A are direct targets of miR-155. N= 8-28 mice per group, from 2-8 independent experiments. (E) NK cells from miR-155\(^{-/-}\) mice exhibit no difference in survival, as measured by 7-AAD positivity, after IL-15 culture, compared to WT controls, after culture in cRPMI without IL-15, with 5ng/mL rmIL-15, or 100 ng/mL rmIL-15 for 24, 48, or 72 hours. N=6 mice per group from 2-3 independent experiments. (F) NK cells from WT or miR-155\(^{-/-}\) mice were cultured in rmIL-15 for 24, 48, or 72 hours and assayed for intracellular granzyme B expression by flow cytometry. A significantly higher proportion of miR-155\(^{-/-}\) NK cells were positive for GzmB protein following activation. Data shown are mean ± SEM percent GzmB positive NK cells, and summarize at least 8 mice per group from 4 independent experiments. (G) miR-155\(^{-/-}\) NK cells also had increased degranulation (surface CD107a) after activating NK receptor ligation (anti-NK1.1). NK cells were cultured for 6 hours with plate-bound α-NK1.1, and then assayed for cell surface CD107a by flow cytometry as a surrogate of degranulation. These data are mean ± SEM normalized CD107a+ NK cells from at least 12 mice per group from 4 independent experiments. (H) NK cells from miR-155\(^{-/-}\) mice do not display enhanced killing. (I) Ly49G2 and Ly49A expression does not determine IFN-γ responsiveness in 155\(^{-/-}\) NK cells. (J-K) No differences in licensing were detected between 155\(^{-/-}\) and WT NK cells. Licensing ratio is indicated in lower part of upper right quadrant. (L) NK cell percentages and numbers are equivalent in the spleens of miR-155\(^{FOE}\) and WT mice. Left: Representative flow plots. Middle: Summary data showing the percentage of NK cells is the same in miR-155\(^{FOE}\) and WT mice. Right: Summary data showing the absolute number of NK cells in the spleen is the same in miR-155\(^{FOE}\) and WT mice. (M) Representative bivariate flow plots showing similar NK cell maturation as defined by CD27 and CD11b in miR-155\(^{FOE}\) and WT mice. (N) Summary data showing similar NK cell maturation (mean + SEM percentage of each stage) in miR-155\(^{FOE}\) and WT mice. N=2-4 mice per group from 2-3 independent experiments. (O) # of NK cells after culture for 3 days in 100ng/mL IL-15, normalized to input. N = 3 groups of mice from 2 independent experiments. (P) Further surface phenotyping of resting miR-155KO and miR-155FOE NK cells. N = 4-5 mice per genotype from 2 independent experiments.
Supplemental Figure 2. LV-GFP/155 transduced human and mouse NK cells produce more IFN-γ due to increased IFN-γ+ percentages and increased IFN-γ MFI. Human (A-C) and mouse (D-F) NK cells were transduced as in (Fig. 2), and then analyzed by flow cytometry for IFN-γ production. Shown is a representative donor for human transduction, stimulated with media only, K562 / 12+15, 12+15, or 12+18 (A), and summarized MFI (B) and %IFN-γ+ (C). Similarly, a representative mouse sample is shown, in which NK cells were transduced, GFP+ NK cells were sorted, and stimulated with plate bound α-NK1.1, 12+15, or 12+18 (D), with summarized MFI (E) and %IFN-γ+ (F). Data in (A-C) are representative of 8-10 donors from 4 independent experiments. Data in (D-F) are representative of 4-6 pools of 2-4 mice each from 4-6 independent experiments. (G) Human NK cells transduced similarly to (A-C), but stimulated 4 days after culture. Data are from 5 donors in 2 independent experiments. (H) Data from Fig. 2 12+15, normalized to each donor. (I) Number of cells obtained 9 days after transduction with the indicated viruses. (J) Surface repertoire analysis of transduced NK cells from 7 donors in 3 independent experiments.
Supplemental Figure 3. Supplemental data for RISC-Seq. (A) Schematic of RISC-Seq, a technique used to identify NK-specific miR-155 targets. First, sorted NK cells are lysed, and separated into two fractions: 1) The RISC-IP fraction, which is immunoprecipitated using anti-Ago2 antibodies, and 2) the total RNA fraction. RNA from both fractions is isolated using Trizol, and then sequenced using an Illumina RNA-seq pipeline. The sequences are aligned to the transcriptome, and mRNAs enriched in the RISC are identified. (B) When filtered using TargetScan results for an irrelevant miRNA, no enrichment is observed of the Group 1 (>1.2 RISC Incorporation Ratio) transcripts. (C) Quantification of the fold change of the graph in (B). (D) The targets in the Lymphocyte (T Cell) Signaling Pathway that exist in our data set are highly enriched in the increased RISC Incorporation Ratio fraction of transcripts.
Supplemental Figure 4. Model for miR-155’s role in NK cells. (A) A summary of the effects of miR-155 alterations on a population of stimulated NK cells. Black = unactivated, blue = activated with low IFN-γ production, red = activated with high IFN-γ production.

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