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

Ryan P. Sullivan,* Leslie A. Fogel,† Jeffrey W. Leong,* Stephanie E. Schneider,* Rachel Wong,* Rizwan Rome,* To-Ha Thai,‡ Veronika Sexl,§ Scot J. Matkovich,¶ Gerald W. Dorn, II,¶ Anthony R. French,† and Todd A. Fehniger*

NK cells are innate 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–deficient 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 are 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 (155F0E), 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 155F0E mouse models. Unexpectedly, both 155F0E and 155−/− decreased MCMV titers, suggesting that the increased function of miR-155, 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-Mir155tm1(Hki)J. 155F0E mice were generated by crossing Tg(Ncr1-iCre)265Stx mice previously described (31), with mice containing an miR-155 LoxP-STOP-LoxP expression cassette knocked in to the Rosa locus (26). Rosa26-stop-eYFP mice were obtained from The Jackson Laboratory as B6.129S1-Gt(ROSA)26Sortm1(EYFP)Cos/J and have been backcrossed at least six generations onto a C57BL/6 background. All other mice were originally generated on a C57BL/6 background. Mouse NK cells were sorted to 99% purity from RAG−/− mice, weiced and maintained in specific pathogen-free housing, and all experiments were conducted in accordance with the guidelines of and with the approval of the Washington University Animal Studies Committee.

Abs

Anti-mouse mAbs were obtained from BD Biosciences (San Jose, CA): IFN-γ (XMG1.2), NK1.1 (PK136), NK46 (29A1.4), CD3 (145-2C11), CD45 (30-F11), CD27 (LG.3A10), CD11b (M1/70), CD19 (1D3), CD212 (144), CD132 (4G3), Ly49A (J90-318), Ly49C/I (5E6), Ly49G2 (4D11), Ly49D (4E5), and Ly49H (3D10); eBioscience (San Diego, CA): CD107a (114), CD132 (4G3), Ly49A (JR9-318), CD107a (114), CD132 (4G3), Ly49H (3D10); BioLegend: IFN-γ (B27).

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 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 ΔΔthreshold 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).
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-18 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. 1). 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-18 (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-18 (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.

**Mice with NK cell–specific miR-155 overexpression (155FOE) have a normal NK cell compartment and produce more IFN-γ following activation**

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 exhibited 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–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+ (Rosa26FPV) 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 an increased percentage of IFN-γ+ NK cells following activation with NK1.1 ligation, IL-12 plus IL-15, or IL-12 plus IL-18 (Fig. 5A, 5C–E), with either no change or a modest reduction of IFN-γ protein per NK cell, determined by MFI (Fig. 5A, 5F–H). In contrast, NK cells from 155FOE mice had increased per-NK cell expression of IFN-γ (MFI) following activation with NK1.1 ligation or IL-12 plus IL-15 (Fig. 5B, 5F–H), without alterations in the percentage of NK cells responding (Fig. 5B, 5C–E). In the IL-12 plus IL-18 experiments, the percentage positive and MFI for IFN-γ were maximal in control NK cells, thereby providing a minimal dynamic range to detect IFN-γ increases after miR-155 alteration. Because our genetic mouse models of miR-155 loss and overexpression in NK cells revealed different cellular mechanisms of enhanced IFN-γ production, we analyzed per-NK cell IFN-γ production in mature human and mouse NK cells following LV overexpression of miR-155. Consistent with 155FOE mice, LV-GFP/155–transduced human (Supplemental Fig. 2A–C) and mouse
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. 5I). 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...
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 (cyclosporin A), and NF-kB (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-\(\gamma\) production, whereas inhibition of NF-kB led to a differential response only at concentrations above its IC\(_{50}\) (Fig. 8C), and an inhibitor of p38 kinase, SB203580, had no effect on IFN-\(\gamma\) production (Fig. 8D). These data indicate that the PI3K and calcineurin pathways contribute to the increased production of IFN-\(\gamma\) 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-\(\gamma\) 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](http://www.jimmunol.org/)

**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: RIR >1.2; group 2: 1 < RIR < 1.2; group 3: 0.85 < RIR < 1; and group 4: RIR < 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^−/− NK cells also produced more IFN-γ upon cytokine activation. How can miR-155 overexpression and deficiency lead to similar IFN-γ 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, 155 FOE NK cells also secreted increased amounts of IFN-γ upon activation. We hypothesized that miR-155 loss or overexpression had distinct mechanisms that lead to similar apparent IFN-γ phenotypes. Indeed, a higher proportion of NK cells from 155^−/− mice produced IFN-γ following activation, whereas NK cells from 155^{5OE} mice exhibited increased per NK cell expression of IFN-γ 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-γ when stimulated. In contrast, forced overexpression of miR-155 in NK cells, either in vitro (LV) in mature NK cells or in vivo (155^{5OE}) in developing NK cells, allows an NK cell, once it crosses the activation threshold, to have an amplified IFN-γ 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-γ production. Although the Ly49C^+/Ly49C^− ratio of IFN-γ production (the licensing ratio) is not altered per se (Supplemental Fig. 1K), we do note that the vast majority of IFN-γ-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^−/− and the 155^{5OE} 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 155^{5OE} NK cells develop with increased
levels of miR-155, the increased percentage of activated NK cells in the LV-GFP/155 further supports a developmental/maturity 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 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 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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