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MicroRNA-Deficient NK Cells Exhibit Decreased Survival but Enhanced Function

Ryan P. Sullivan,* Jeffrey W. Leong,* Stephanie E. Schneider,* Catherine R. Keppel,* Elizabeth Germino,* Anthony R. French,* and Todd A. Fehniger*

NK cells are innate immune lymphocytes important for early host defense against infectious pathogens and malignant transformation. MicroRNAs (miRNAs) are small RNA molecules that regulate a wide variety of cellular processes, typically by specific complementary targeting of the 3′UTR of mRNAs. The Dicer1 gene encodes a conserved enzyme essential for miRNA processing, and Dicer1 deficiency leads to a global defect in miRNA biogenesis. In this study, we report a mouse model of lymphocyte-restricted Dicer1 disruption to evaluate the role of Dicer1-dependent miRNAs in the development and function of NK cells. As expected, Dicer1-deficient NK cells had decreased total miRNA content. Furthermore, miRNA-deficient NK cells exhibited reduced survival and impaired maturation defined by cell surface phenotypic markers. However, Dicer1-deficient NK cells exhibited enhanced degragulation and IFN-γ production in vitro in response to cytokines, tumor target cells, and activating NK cell receptor ligation. Moreover, a similar phenotype of increased IFN-γ was evident during acute MCMV infection in vivo. miRs-15a/15b/16 were identified as abundant miRNAs in NK cells that directly target the murine IFN-γ 3′UTR, thereby providing a potential mechanism for enhanced IFN-γ production. These data suggest that the function of miRNAs in NK cell biology is complex, with an important role in NK cell development, survival, or homeostasis, while tempering peripheral NK cell activation. Further study of individual miRNAs in an NK cell specific fashion will provide insight into these complex miRNA regulatory effects in NK cell biology. The Journal of Immunology, 2012, 188: 000–000.

N atural killer cells are an important component of the innate immune system and have a key role in early host defense against pathogens and surveillance against malignant transformation (1–4). NK cells develop from precursors arising in the bone marrow, and complete differentiation and maturation in peripheral lymphoid tissues under the direction of cytokines and transcription factors (5, 6), with IL-15 playing a central role (7, 8). During development from immature precursors, NK cells undergo an education process that results in tolerance to normal self cells and prevents NK-based autoimmunity. This tolerance is achieved through the expression of a repertoire of germline-encoded activating and inhibitory receptors that yield signals that are integrated and determine how the NK cell responds to a target cell (9–11). NK cells also constitutively express a number of cytokine receptors, and NK responsiveness is also regulated by cytokines produced by accessory immune cells sensing pathogens (12). Therefore, NK cell development and function in an immune response are distinct from adaptive T and B lymphocytes.

NK cells function to protect the host through two primary effector pathways—cytokine–chemokine production and cytotoxicity (13). Mature NK cells may be primed by cytokines produced by accessory cells sensing pathogens to optimize their cytokotic and cytokine-secretion potential upon subsequent receptor-based triggering (14–17). One aspect of NK cell priming by dendritic cell-derived IL-15 includes the rapid translation of perforin and granzyme B effector proteins (14, 15). Secretion of cytokines (e.g., IFN-γ) and chemokines (e.g., MIP-α, MIP-β, RANTES) by NK cells may directly affect virus-infected or malignant cells, promote an antiviral state in adjacent cells, recruit additional immune cells, and shape the subsequent adaptive immune response. In contrast, NK cell cytotoxicity is triggered primarily by receptor interaction with a target cell that results in granule exocytosis—the coordinated release of perforin and granzyme into a cytotoxic synapse—ultimately inducing apoptotic-like target cell death (18, 19). LAMP1 (CD107a) is retained within cytotoxic granules and is released on the NK cell surface membrane after triggering, providing a marker of recent degranulation (20). Although a number of receptors, signaling molecules, and transcription factors have been identified that regulate NK cell development and activation, our understanding of the molecular mechanisms regulating these events remains incomplete.

MicroRNAs (miRNAs) are a family of hundreds of small, non-coding RNAs that regulate numerous cellular functions by targeting sites in the 3′UTR of mRNAs, thereby suppressing translation or causing mRNA degradation (21). miRNA genes are encoded in the genome, transcribed as long primary transcripts that are subsequently cropped by the Drosha/Dgcr8 complex into precursor miRNA (premiRNA) that have a characteristic stem loop structure (22). The premiRNA is exported to the cytoplasm, where it is further processed by the Dicer complex (including Dicer1),

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Abbreviations used in this article: MCMV, murine CMV; miRNA, microRNA; miR-SEQ, microRNA sequencing; premiRNA, precursor microRNA; QRT-PCR, quantitative real-time PCR; rm, recombinant murine; WT, wild type.

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yielding mature 19–26 nucleotide miRNAs. The mature miRNA is loaded into the RNA-induced silencing complex and thereby directs downregulation of protein levels (23). Genetic disruption of Dicer1 therefore results in decreased or absent levels of a large number of miRNAs because of an interruption in miRNA biogenesis. Previous studies have shown the requirement of Dicer1 for the processing of miRNAs involved in the maturation and function of various cell types, including lymphocytes (24–29). In addition, specific miRNAs or miRNA clusters have been implicated in B and T cell development and modulate functional responses (30). However, only limited information is available on the expression and function of miRNAs in NK cells, their subsets, and developmental intermediates.

Mature murine NK cells have been shown to express more than 300 mature miRNAs using a combination of miRNA sequencing (miR-SEQ) with validation by quantitative real-time PCR (QRT-PCR) and microarrays (31). Moreover, a subset of these miRNAs are modulated with short-term IL-15 activation, including mir-223 that was shown to target the murine granzyme B 3’UTR. A number of miRNAs were expressed in common with other lymphocytes (e.g., mir-181a, mir-21, mir-142-3p/5p, mir-16, mir-15b, mir-150, and let-7i), suggesting that these regulate lymphocyte development or functional programs. A recent study has also identified mir-29 as a contributor to IFN-γ protein regulation in both innate NK cells and adaptive T cells (32). However, the functional role of the majority of expressed miRNAs in NK cells remains to be elucidated. What are the ramifications of global miRNA deficiency on developing and mature NK cells? Answering this question may provide evidence to support the role of miRNAs as regulators of specific aspects of NK cell development, survival, priming, triggering, or function. A prior study by Bezman et al. (33) evaluated the phenotype of miRNA deficiency using a global, inducible estrogen receptor (ER)-Cre model that results in Dicer1 deletion in all cell types in the mouse after tamoxifen treatment. The authors concluded that miRNA-deficient NK cells have impaired survival and proliferation, as well as reduced functional capacity as measured by IFN-γ production and degranulation. These alterations resulted in compromised function during murine CMV (MCMV) infection (33). However, the relative contribution of NK cell intrinsic miRNA deficiency to these phenotypes was challenging to assess using a global, nonselective miRNA deficiency model system.

In this study, we used a lymphocyte-restricted Cre transgenic mouse (hCD2-Cre) expressed in 30–50% of NK cells (34), coupled with Dicer1 loxP-flanked alleles (24), to generate loss of Dicer1 in the early stages of NK cell development. We used this model to analyze the role of Dicer1-dependent miRNAs in the development, maturation, and function of NK cells. We observed an in vivo developmental and phenotypic maturation defect in miRNA-deficient NK precursors and NK cells. Despite this defect, Dicer1-deficient NK cells exhibited enhanced IFN-γ production and degranulation capacity to a number of stimuli ex vivo. Moreover, miRNA-deficient NK cells responding to MCMV in vivo exhibited similar enhancement of IFN-γ production. The miR-15/16 family was identified as a potential novel contributor to IFN-γ protein suppression in NK cells. These findings are consistent with a complex role of miRNAs in NK cell biology that includes a critical role during NK development and homeostasis, and a separate role for miRNAs to dampen NK cell functional responses in mature NK cells.

Materials and Methods
Mice, cell lines, and viral infections
All mice were bred and maintained in specific pathogen-free housing, and all experiments were conducted with the guidelines of and with the approval of the Washington University Animal Studies Committee. Mice were used between 8 and 12 wk of age for all experiments. C57BL/6J, B6.RAG1<−/−> B6.Cg-Tg(CD2-cre)4Kio/J [hCD2-Cre] (34), B6.129 X 1-Gt(Rosa)26Sortm14(EYFP)Cbr/J [Rosa-LSL-YFP] (35), and B6.dicerbox/box [Dicerfl/fl] (24) were obtained from The Jackson Laboratory (Bar Harbor, ME) or investigators at Washington University. YAC-1 cells line (a gift from W. Yokoyama) were maintained in K10 media (RPMI-1640, 10% FCS, 10 mM HEPES, 1% NEAA, 1% sodium pyruvate, 1% L-glutamine, 1% penicillin–streptomycin). 293T cells (a gift from M. Sands, Washington University) were grown in D10 (DME-M, 10% FCS, 10 mM HEPES, 1% NEAA, 1% sodium pyruvate, 1% L-glutamine, 1% penicillin–streptomycin). MCMV Smith strain was injected IP as described at a dose of 5 × 10^6 PFU (14).

Reagents and mAbs
Endotoxin-free purified recombinant murine (rm) IL-15 and mIL-12 were obtained from Peprotech (Rocky Hill, NJ) and reconstituted in sterile PBS with 0.1% BSA. The following anti-mouse mAbs were obtained from BD Biosciences (San Jose, CA): IFN-γ (XMG1.2), NK1.1 (PK136), NKp46 (29A14), CD3 (145-2C11), CD45 (50-F11), CD27 (LG.3A10), CD19 (1D3), Gr-1 (RB6-8C5), CD132 (4G3), and pSTAT5 (clone 47). Additional mAbs were obtained from eBioscience (San Diego, CA): CD107a (4F4), and CD11b (M1/70). Anti-NK1.1 (PK136) was also purified by the Washington University Ab production core from hybridoma supernatant. 2.4G2 (anti-CD16/32) hybridoma supernatant was used to block nonspecific staining. CellTrace Violet was used to monitor cell division and was used according to the manufacturer’s instructions (Invitrogen, Molecular Probes, Eugene, OR). AccuCount beads were used for absolute cell number determinations via flow cytometry following the manufacturer’s instructions (Spherotech, Lake Forest, IL).

Cell isolation and sorting
Mouse tissues (spleen, bone marrow, blood, liver) were isolated from 8–12-wk old mice as described (14, 17). Single-cell suspensions were generated from spleen (mechanical disruption through a 70-μm filter or glass tissue homogenizer), bone marrow (flushing two femurs with K10), liver (mechanical disruption followed by Percoll gradient isolation), or blood (cardiac puncture). RBC lysis was performed using ACK buffer (150 mM NH4Cl, 10 mM K2CO3, 0.1 mM EDTA), and viable cell numbers were determined by trypan blue exclusion. Blood is calculated as cells per milliliter of blood obtained. Isolation of highly purified NK cells (>97% pure) was performed by flow cytometric sorting on an iCyte Reflection (iCyt, Champaign, IL) by gating on lymphocytes based on forward or side scatter and then CD45+CD3− NK1.1+YFP+ subsets.

RNA isolation and QRT-PCR
Sorted YFP+ or YFP− NK cells were immediately lysed in Trizol LS (Invitrogen, Carlsbad, CA) according to manufacturers’ instructions. Total RNA was extracted as described previously (36), cDNA was generated from RNA using either the TaqMan Reverse Transcription Kit with random hexamers or TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) according to manufacturers’ instructions. Dicer1 excision in cdna was detected by two methods: 1) internal to the loxp sites or 2) primers external to the loxp sites. Primer and probe sequences are available upon request. RNA quantification was detected by TaqMan miRNA Kit with specific primers and probes. QRT-PCR was performed on an ABI 7300 using standard settings in a 20-μl reaction. All primers and probes were manufactured by IDT (Corvalville, IA). All dicer1 samples were normalized to 18S rRNA, and all miRNA samples were normalized to housekeeping small RNAs, sn135 (mouse) or RNU48 (human), using the ∆∆CT method. Samples were further normalized to the levels in either wild type (WT) NK cells or Dicer1<−/−> YFP+ NK cells as appropriate.

Single-cell PCR
Bulk populations of YFP+ or YFP− NK cells were sorted as described above. These cells were processed essentially as described previously (37). Individual cells were isolated and amplified using the GenomiPhi v2 Whole Genome Amplification Kit (GE Healthcare, Piscataway, NJ). The whole genome amplification product (1 μl) was then used in a multiplex PCR reaction to amplify the WT, boxed, or excised Dicer1 alleles, and the resulting products were analyzed by agarose gel electrophoresis. Primer sequences are available upon request.
NanoString miRNA expression analysis

Total RNA was processed on a NanoString (Seattle, WA) nCounter instrument using the mouse miRNA Expression Assay Kit according to the manufacturer’s instructions. Data obtained were then sequentially normalized to both housekeeping miRNAs and positive miRNA-ligation reaction controls of the assay to adjust for total RNA content and ligation efficiency, respectively. This method provides a normalized absolute count of 578 mature miRNAs present in the total RNA pool. Non-specific mature miRNA probes (e.g., mir-720) defined by lack of expression based on miRNA-SEQ were excluded from this analysis (31). miRNAs from Dicer1fl/fl YFP+ NK cells that were detected at 2 SD above background were divided into categories and further analyzed for global miRNA fold change analysis by taking the geometric mean of each group of miRNAs.

Assessment of NK cell function and survival

Splenocytes (1 x 10⁶ cells) or sorted YFP+ NK cells (1 x 10⁵ cells) were cultured in 24-well plates (Corning, Corning, NY) containing either K10 plus YAC-1 at a 10:1 E:T ratio, K10 plus 10 ng/ml IL-12 and 100 ng/ml IL-15, or K10 plus plate-bound anti-NK1.1 (PK136) for 8 h as described (17, 38), or in K10 plus 5 ng/ml IL-15 or 100 ng/ml IL-15. Flow cytometry data were collected for cell surface markers including CD107a and intracellular IFN-γ on a Beckman Coulter Gallios flow cytometer. Data were analyzed using FlowJo (Tree Star, Ashland, OR) or Kaluza (Beckman Coulter, Miami, FL). In some experiments, data are presented as percent maximal CD107α or IFN-γ expression by normalizing to the condition with the highest intrassay expression in YFP+ NK cells to account for inter-experiment variability. For proliferation assays, splenocytes were stimulated as indicated after labeling in K10 with CellTrace Violet according to the manufacturer’s instructions, and data were analyzed using FlowJo (Tree Star Software, Ashland, OR). For phospho-STAT5 assays, 1 x 10⁶ splenocytes were cultured for 15 min in 5 ng/ml IL-15 before fixation and staining as described (39).

Assessment of miRNA targeting of 3’UTRs using luciferase sensor plasmid assays

Overexpression of mature miRNAs and luciferase sensor-plasmids were performed as described previously (31). Briefly, psiCheck2 (Promega, Madison, WI) with the 3’UTR of IFN-γ was cloned by amplification of the IFN-γ 3’ UTR from IFN-γ cDNA (a gift from M. Cooper). Two miR-15/16 binding sites were disrupted in two sequential rounds of mutagenesis using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) following the manufacturer’s instructions. miRNA overexpression vectors were generated by subcloning the premiRNA gene plus the 200-bp flanking genomic sequence into the PMD overexpression vector (a gift from M. Sands) (31). All primer sequences used for cloning are available upon request. 293T cells were cotransfected with 800 ng of each vector using Dharmafect Duo (Dharmacon, Lafayette, CO) and grown for 48 h in D10. Dual-Glo Luciferase Assay (Promega, Madison, WI) was then performed according to the manufacturer’s instructions on an LD400 luminescence detector (Beckman Coulter, Brea, CA). Renilla luciferase (experimental) was normalized to Firefly luciferase (control) followed by comparison of Renilla/Firefly ratios of the same psiCheck2 sensor plasmid cotransfected with an empty GFP-only control vector. Overexpression was confirmed by GFP expression and QRT-PCR of miRNA samples.

Statistical analysis

Graphical analysis and statistics were performed with GraphPad Prism 5.0. Student t test, one-way ANOVA, and two-way ANOVA were used as appropriate, with p < 0.05 being considered significant.

Results

hCD2-Cre transgenic/Rosa26-LSL-YFP mice are an NK cell Cre expression model

To investigate the role of miRNAs in NK cells, we used the lymphoid-restricted hCD2-Cre transgenic mouse (34) combined with the Cre reporter model Rosa26-LSL-YFP (35) (CD2-Cre/ RosaYFP). In this model, all cells that experience Cre-mediated excision at the Rosa26 locus, and all their daughter cells, express YFP protein (Fig. 1A). Using this model, YFP was found to be expressed in 25–50% of splenic NK cells, and expression was confirmed to be lymphocyte restricted with >95% of CD3+ T cells and CD19+ B cells, but <1% of GR-1+ myeloid cells being YFP+ (Fig. 1B, 1C). Furthermore, YFP expression was detected in early NK cell precursors in the bone marrow as defined previously (5, 40) and gradually increased in proportion throughout development in the bone marrow, with the percentage of YFP+ NK maximal at the latest stage defined as CD51 (integrin αI) CD117+ (Fig. 1D, 1E). In the spleen, YFP+ versus YFP− NK cells exhibited no difference in the expression of Ly49, NKGD2, or NKp46 receptors (Supplemental Fig. 1A, 1B). We observed a modest enrichment in stage II/II NK cells (CD27+CD11b+) or NKp46 receptors (Supplemental Fig. 1C) in YFP+ versus YFP− NK cells. Because these maturation differences could have potentially biased the results of further analysis, we performed all experiments comparing YFP+ NK cells between Dicer1 genotypes, rather than YFP− and YFP+ NK cells in the same mouse. Collectively, these analyses establish the CD2-Cre/RosaYFP model, whereby YFP+ NK cells in this mouse provide a mechanism to evaluate the role of Dicer1 alterations in NK cells.

Constitutive Dicer1-deficient NK cells have reduced miRNA content

The CD2-Cre/RosaYFP mice were further crossed to mice with various genotypes of loxP-flanked Dicer1, thereby generating Dicer1fl/fl, Dicer1fl/wt, or Dicer1wt/wt mice. Because Cre excision at the Rosa26 locus occurred in only a proportion of mature NK cells, we first evaluated loxP-flanked Dicer1 excision status in YFP+ and YFP− NK cells. Using QRT-PCR to identify excised versus WT Dicer1 miRNA (Fig. 2A) in sorted bulk NK cell populations, we compared the levels of Dicer1 excision in the Dicer1fl/wt mice to the Dicer1wt/wt mice. Surprisingly, we found that as a whole the YFP+ NK cells in both genotypes had comparable degrees of excision detected by this assay (Fig. 2B). Complete excision in the Dicer1fl/wt NK cells is expected to result in no detection with the internal primers and maximal detection with the external primers. To better investigate Dicer1 excision at the level of an individual NK cell rather than a bulk population, we performed single-cell PCR on sorted YFP+ and YFP− NK cells (Fig. 2C, 2D). This assay identifies WT, floxed, and excised Dicer1 alleles using a multiplexed PCR approach. We found that 79% of Dicer1fl/wt and 74% of Dicer1fl/fl YFP+ NK cells had excised at least one Dicer1 allele. In Dicer1fl/fl YFP+ NK cells, the single floxed allele was eliminated. In Dicer1fl/fl mice we detected Dicer1ΔΔ (63%), Dicer1Δl (11%), and Dicer1Δfl (26%) alleles in YFP+ NK cells. Thus, 86% of NK cells with evidence of Dicer1 excision had lost both Dicer1 alleles, confirming the efficiency of the hCD2-Cre model in YFP+ NK cells at the single-cell level. A fraction of YFP+ NK cells in Dicer1fl/fl mice had not excised either allele of Dicer1, which is consistent with the QRT-PCR results. Furthermore, by both assays we found that Dicer1-floxed alleles were also excised in a small fraction of YFP− NK cells (Fig. 2B, 2C), possibly because of a loss of YFP in Dicer1fl/fl cells undergoing apoptosis or imperfect Rosa26-YFP excision; this provided a second rationale for comparing YFP+ NK cells between Dicer1 genotypes to eliminate this additional potential confounder.

To confirm the functional effects of excision of Dicer1 in the Dicer1fl/wt and Dicer1wt/wt mice, we next determined the expression of mature miRNAs within various Dicer1 genotypes. We examined YFP+ NK cells for mature miRNA expression by NanoString miRNA assays (Fig. 2E, 2F) and found that there was a consistent loss of miRNA content in the Dicer1fl/fl and Dicer1wt/wt NK cells. We further confirmed this loss for three miRNAs representing a range of expression in NK cells (miR-16, miR-21, and miR-30b) (31) via QRT-PCR (Fig. 2G). Consistent with a minor proportion
of YFP\(^+\) Dicer\(^{1^{fl/wt}}\) NK cells lacking Dicer excision, miRNA expression in the Dicer\(^{1^{fl/wt}}\) NK cell population was reduced but not absent. These data suggest that examining both Dicer\(^{1^{fl/wt}}\) and Dicer\(^{1^{fl/fl}}\) YFP\(^+\) NK cells will be useful to elucidate the phenotype of miRNA-deficient NK cells.

**MiRNA-deficient NK cells exhibit defects in survival and maturation**

To evaluate effects of Dicer deficiency on NK cells in vivo, we first analyzed the percentage of YFP\(^+\) NK cells in the spleen, liver, blood, and bone marrow of CD2-Cre/RosaYFP mice with various Dicer genotypes. We observed a reduced proportion of YFP\(^+\) NK cells in Dicer\(^{1^{fl/fl}}\) mice (spleen: 14.42 ± 3.66%) compared with Dicer\(^{1^{fl/wt}}\) (spleen: 35.44 ± 7.18%) and Dicer\(^{1^{wt/wt}}\) mice (spleen: 35.74 ± 7.68%; Fig. 3A), further indicating a loss of YFP\(^+\) NK cells in the Dicer\(^{1^{fl/fl}}\) mice. In addition, although the absolute number of total NK cells was equivalent in all three genotypes (Fig. 3B), there was a significant reduction in the splenic YFP\(^+\) NK cell compartment in Dicer\(^{1^{fl/fl}}\) mice. The bone marrow showed a similar pattern, but the liver and blood showed no significant difference in the numbers of total NK and YFP\(^+\) NK cells for all three genotypes (Supplemental Fig. 1D).
Given the remarkable loss of YFP⁺ NK cells in the spleen of Dicer⁺⁻ mice, we examined whether YFP⁺ NK loss was associated with peripheral maturation stage. NK cell maturation has been defined in the periphery of mice using coexpression of CD27 and CD11b surface markers (40, 41), and we evaluated YFP expression in these NK cell maturation subsets (Fig. 3C, Supplemental Fig. 1E). Analysis was supported by at least 12 cells for all cell types except Dicer⁺⁻ YFP⁺ NK cells, for which six cells were used. Representative gel of single-cell PCR. Each lane represents a PCR reaction of a single isolated NK cell. Informative product sizes are listed to the right. Dicer⁺⁻ = 259 bp; Dicer⁺⁺ = 309 bp; Dicer⁺⁺⁺ = 390 bp. Ladder is in increments of 100 bp from 100 to 500 bp. Both negative and positive controls provided the expected results (not shown). *Failed PCR reaction. (E) MiRNA expression in YFP⁺ Dicer⁺⁻, Dicer⁺⁺⁺, and Dicer⁺⁺⁺ NK cells measured by NanoString. Absolute expression profiles of the top 15 miRNAs expressed in Dicer⁺⁺⁺ as analyzed by NanoString showing decreased miRNA expression in Dicer⁺⁻ deficient NK cells. (F) Summary of miRNA expression changes in Dicer⁺⁻, Dicer⁺⁺⁺, and Dicer⁺⁺⁺ YFP⁺ NK Cells. The geometric mean of NanoString count groups (>5000, 1000–5000, 100–1000) was compared between Dicer⁺⁺⁺ genotypes. (G) Confirmation of selected miRNA expression using QRT-PCR of miR-16, miR-21, and miR-30b. Data shown are the mean ± SEM of three independent experiments. Significance was calculated using Student t test. *p < 0.05, **p < 0.01, ***p < 0.001.
FIGURE 3. miRNA-deficient NK cells exhibit an in vivo survival defect. Mononuclear cells were isolated from spleen, liver, blood, and bone marrow. NK cells (CD45\(^{+}\)NK1.1\(^{+}\)NKp46\(^{+}\)CD3\(^{-}\)) were analyzed for YFP expression. (A) Percent YFP\(^{+}\) NK cells in indicated tissues for each genotype. (B) Total viable cells were enumerated, the percentage of YFP\(^{+}\) NK cells was analyzed by flow cytometry, and absolute total NK and YFP\(^{+}\) NK cell numbers were calculated. (C) NK cells were further analyzed for CD27 and CD11b expression, and the absolute number of cells was calculated as above for maturation stages I (CD27\(^{-}\)CD11b\(^{-}\)), II (CD27\(^{-}\)CD11b\(^{+}\)), III (CD27\(^{+}\)CD11b\(^{+}\)), and IV (CD27\(^{+}\)CD11b\(^{-}\)) as described (40–41). Significance was calculated using two-way ANOVA and is presented as Dicer\(^{fl/fl}\) (gray) or Dicer\(^{wt/wt}\) (black) versus Dicer\(^{wt/wt}\). Data shown are the mean ± SEM of five experiments (A, B) or three experiments (C). Significance in (A) and (B) was defined using one-way ANOVA with a Neuman–Keuls posttest. *p < 0.05, **p < 0.01, ***p < 0.001.
notype was cell intrinsic, sorted YFP+ NK cells were cultured in media alone or with plate-bound anti-NK1.1 (Supplemental Fig. 2C). Sorted YFP+ Dicer1fl/fl cells stimulated with anti-NK1.1 produced significantly more IFN-γ than their YFP+ Dicer1wt/wt counterparts (93 ± 7.3 versus 39 ± 18; p < 0.05). Collectively, these data show that miRNA-deficient NK cells have a cell-intrinsic enhanced capacity to produce IFN-γ and degranulate in response to multiple stimuli in vitro.

We also investigated the effect of maturation stage defined by CD27/CD11b on degranulation (CD107a), and we observed that stage II–III NK cells in the Dicer1fl/fl mice were primarily responsible for the enhanced degranulation (Supplemental Fig. 3A). Prior studies have shown that stage III NK cells exhibit a higher degranulation capacity compared with the more mature stage IV subset, which may allow increased degranulation caused by miRNA defects in these cells to be more readily apparent (40, 41). In addition, this effect was not due to altered Ly49/NKG2D receptor expression (Supplemental Fig. 3B). Therefore, neither maturation stage skewing nor Ly49/NKG2D expression changes seem responsible for the observed phenotype. Granzyme B protein levels were found to be variable in NK cells from these mice, with a trend toward increased expression in Dicer1fl/wt mice, which may control the activation threshold of NK cells.

Dicer1-deficient NK cells exhibit enhanced IFN-γ production in vivo during MCMV infection

To evaluate the effects of miRNA deficiency in NK cells during a physiologic in vivo response, we infected Dicer1fl/fl, Dicer1fl/wt, and Dicer1wt/wt with 5 × 10⁴ PFU of MCMV and evaluated IFN-γ production 36 h after infection—the expected peak IFN-γ time point. Consistent with the in vitro results, splenic YFP+ NK cells from Dicer1fl/fl mice had significantly higher IFN-γ production compared with Dicer1wt/wt mice (Fig. 6A, 6B). Furthermore, Dicer1fl/wt YFP+ NK cells demonstrated an intermediate phenotype, with IFN-γ production between Dicer1fl/fl and Dicer1wt/wt. These data suggest that miRNA-deficient NK cells have an enhanced ability to produce IFN-γ in vivo during an ongoing antiviral response.

miR-15/16 family specifically targets the IFN-γ 3′ UTR

As IFN-γ production was significantly increased in miRNA-deficient NK cells both in vitro and in vivo, we evaluated the role of individual miRNAs for their potential to directly target IFN-γ protein production via binding to the murine IFN-γ 3′UTR.
A number of miRNAs are predicted (42) by algorithms to bind to the murine IFN-γ 3'UTR, including miRs-15a/15b/16, a closely related family of miRNAs (Fig. 7A). Culture of sorted NK cells for 7 h with stimuli that induce IFN-γ protein expression: 100 ng/ml IL-15 plus 10 ng/ml IL-12, or plate-bound anti-NK1.1, resulted in a significant reduction of miR-15b in both conditions, and a significant reduction in miR-15a and miR-16 in the 12+15 condition (Fig. 7B). These miRNAs are among the most highly expressed miRNAs in IFN-γ–producing lymphocytes (Fig. 7C), and have decreased expression in Dicer1fl/fl and Dicer1fl/wt mice.

FIGURE 5. miRNA-deficient NK cells have increased functional capacity defined by in vitro degranulation and IFN-γ production. Splenocytes were cultured for 7 h in K10 medium only or stimulated with YAC-1 tumor cells, IL-12 plus IL-15, or anti-NK1.1 (plate bound PK136). (A) Example flow cytometry gating scheme that identifies NK1.1+NKp46+CD3+YFP+ NK cells expressing cell surface CD107a or intracellular IFN-γ protein in Dicer1fl/fl splenocytes. Summary data of the percent positive NK cells for CD107a (B) or IFN-γ (C) expression after 7 h with the indicated stimulus. The mean ± SEM of five independent experiments is shown expressed as the percent maximal expression within each experiment to account for expected interassay variability. Significance was calculated using one-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001.
We therefore evaluated the miR-15/16 family, along with a number of candidates using in vitro overexpression of individual miRNAs with concurrent 3' UTR luciferase sensor plasmid as a readout of targeting (Supplemental Fig. 4A). Some miRNAs, such as miR-27a and miR-29a, were found to repress the empty vector and were discarded because of a lack of specificity. The miR15/16 family was not found to target the empty psi-Check2 vector (Supplemental Fig. 4A), but was strongly predicted to target the IFN-γ 3'UTR in two sites (Fig. 7D). In cotransfection assays in which Renilla luciferase was fused to the 3' UTR of IFN-γ, overexpression of miR-15a/16-1 or miR-15b/16-2 in 293T cells (Supplemental Fig. 4B) dramatically decreased the Renilla signal compared with negative controls (GFP-only empty vector or overexpression of an irrelevant miRNA; Fig. 7E). This finding indicates that miRs-15a/15b/16 regulates the IFN-γ 3'UTR. To interrogate the specificity of the IFN-γ 3'UTR repression, we specifically mutated the top predicted site (IFNγmut-1) or the top two (IFNγmut-1+2) predicted sites (Fig. 7D). This resulted in a sequential and substantial loss and abrogation of IFN-γ 3'UTR repression by miR-15a/15b/16, indicating that the targeting was direct. These biochemical results, plus the expression pattern in resting/stimulated WT NK cells and Dicer1<sup>−/−</sup> versus Dicer1<sup>+/+</sup> mice, are consistent with miR-15/16 targeting of the 3'UTR as a possible mechanism for increased IFN-γ production in our model.

**Discussion**

In this study, we used a combination of genetic mouse models to achieve selective miRNA deficiency in NK cells, which are identifiable because of their expression of YFP. Based on the Rosa26-LSL-YFP reporter allele, Cre expression and Dicer1 excision consistently occurred early in NK cell development and persisted in mature peripheral NK cells. This model demonstrated that Dicer1-dependent miRNAs have a role in NK cell development, survival, and proliferation—when miRNAs are globally decreased NK cell development and maturation are impaireed. These results suggest that specific miRNAs or groups of miRNAs are important intrinsic regulators of NK cell development and homeostasis. However, our results also revealed that miRNA-deficient NK cells had enhanced functionality, which included increased IFN-γ production and degranulation (as a surrogate for cytotoxicity) in vitro and enhanced IFN-γ responses in vivo during MCMV infection. This phenotype indicates that miRNAs also have a role in dampening NK cell responses in mature peripheral NK cells. Indeed, we identified a family of related miRNAs—miR-15a/15b/16—that are decreased in Dicer1-deficient mice and during NK cell activation, and they directly target the IFN-γ 3'UTR. Therefore, miRNA regulation of the NK cell molecular programs is complex, with effects in development and homeostasis, as well as regulating NK cell functional responses.

Limited studies have been performed evaluating the expression and significance of miRNAs in NK cell biology. We have previously defined the expression of miRNAs in resting mature murine NK cells and following IL-15 activation using multiple profiling platforms, including miRNA-SEQ, and identified novel miRNAs in NK cells (31). Consistent with our current hypothesis regarding IFN-γ regulation, miRs-15a/15b/16 were highly expressed in resting NK cells. In the prior study, miR-223 was also identified as a potential direct regulator of another NK cell effector molecule, granzyme B, in resting NK cells. In our current study, granzyme B protein levels had a high degree of variability, with a trend toward higher granzyme B protein in Dicer1<sup>−/−</sup> NK cells, compared with...
Dicer1<sup>fl/fl</sup> or Dicer1<sup>wt/wt</sup>. Because our Dicer1-deficiency model results in the decrease of a large number of miRNAs simultaneously, there may be indirect effects on other pathways important for granzyme B expression in NK cells, or it may disrupt miRNAs that both positively and negatively regulate granzyme B expression. Current experiments using miR-223<sup>223/223</sup> mice (43) are underway to further evaluate the regulation of NK cell function by miR-223.

Bezman et al. (33) recently reported the phenotype of NK cells in the context of drug-induced Dicer1 or Dgcr8 (a critical component of the Drosha complex) loss, utilizing an estrogen receptor (ER)-Cre model to excise Dicer1<sup>fl/fl</sup> or Dgcr8<sup>fl/fl</sup> alleles. Such an approach reduces miRNAs in all cells of the mouse (including NK cells) after 5 d of tamoxifen treatment. This study identified a phenotype similar to our model for miRNA-deficiency in regard to NK cell survival; however, there were a number of phenotypic differences that warrant discussion in the context of the disparate models. In contrast, our model describes a “hypofunctional” NK cell phenotype with impaired IFN-γ production and degranulation in response to activating NK cell receptors in vitro—the opposite of the phenotype we observed using the hCD2-Cre model. In addition, the authors report increased NKG2D receptor expression in miRNA-deficient NK cells from a uniform point during NK cell development, with consistent loss of miRNAs in mature NK cells. In contrast, the ER-Cre model results in a “window” of Cre-mediated excision in both mature NK cells, and presumably their precursors, following tamoxifen treatment, thus providing a potentially heterogeneous NK cell population in regard to the timing of Dicer1 or Dgcr8 loss during NK cell development and maturation. In addition, ER-Cre eliminates Dicer1 and Dgcr8 in all mouse cells, providing no selectivity for NK cells; therefore, when using this approach it may be challenging to differentiate the effects of NK cell intrinsic versus extrinsic miRNA deficiency. These differences between the model systems may be responsible for the contrasting phenotypes we are reporting for mature NK cell function. The ER-Cre model described a “hypofunctional” NK cell phenotype with impaired IFN-γ production and degranulation in response to activating NK cell receptors in vitro—the opposite of the phenotype we observed using the hCD2-Cre model. In addition, the authors report increased NKG2D receptor expression in miRNA-deficient NK cells.
cells, which we did not observe in the hCD2-Cre model. In the setting of MCMV infection, the ER-Cre approach identified no change in IFN-γ production by splenic NK cells during MCMV infection. However, in the hCD2-Cre model there was a clear and statistically significant increase in IFN-γ protein in splenic NK cells 36 h postinfection. Similar to our phenotype with hCD2-Cre driving Dicer1-loss in NK cells, CD8⁺ T cell responses in Dicer1−/− mice achieved by a distal Lck-Cre identified enhanced activation with faster kinetics in miRNA-deficient CD8⁺ T cells (44). Recently, Eckelhart et al. and Narni-Mancinelli et al. (45, 46) have independently reported NK cell-specific NKP46-Cre mice. Future studies using these models that direct Cre expression only in NK cells or NK cell precursors will provide additional information on the functional ramifications of miRNA biogenesis or specific miRNAs in vivo.

The data presented support a role for miRNAs in the appropriate survival, maturation, and proliferation of NK cells. As the turnover of Dicer1 protein and miRNAs is rapid (26–29), and NK cell maturation takes weeks (47), Dicer1 protein and therefore Dicer1-dependent small RNAs are likely reduced at an early stage of NK development. By using a model in which Dicer1 excision occurs contemporaneously and at an early stage of NK cell development, we could analyze the role of Dicer1 in the survival and maturation of NK cells. By analyzing various organs, we observed that Dicer1fl/fl NK cells failed to reach a defined stage of peripheral NK cell development: the CD27⁺CD11blow stage, which is associated with cellular proliferation (40) and is consistent with the crucial function of miRNAs in cell division (48). In contrast, Dicer1fl/fl NK cells were underrepresented in the final stages of NK maturation, possibly indicating a failure in proper senescence (49, 50) and consistent with the intermediate level of miRNAs in these cells as analyzed by NanoString. As the numbers of YFP⁺ NK cells were only significantly decreased in the spleen and bone marrow, we cannot formally rule out that these alterations may be due to a homing defect of the Dicer1fl/fl NK cells; however, this seems less likely given the defect in the NK YFP⁺ percentage of all peripheral organs. Furthermore, there was a significant decrease in the ability of miRNA-deficient NK cells to survive ex vivo in IL-15, which was not explained by reduced IL-15R protein expression or signals via phospho-STAT5. miRNAs previously reported to have a role in regulating development and proliferation—miR-150 (51), miR-16 (52), and miR-21 (53)—are highly expressed in NK cells and are likely candidates to be involved in the repression of genes required for proper development and proliferation in maturing NK cells.

NK cell activation involves an intricate relationship between activating and inhibitory signaling, with an activation threshold determined by a variety of interactions, many of which have yet to be fully understood (54). Previous reports have shown a specific role for miR-181α in tuning the sensitivity of T cells (55, 56) by targeting the phosphatases PTPN22 and SHP-2. The increased NK cell functionality in our hCD2-Cre model implies that the net effect of reduced Dicer1-dependent miRNAs in NK cells is loss of activating signal repression, possibly via a loss of targeting of DAP12, other ITAM-containing receptors, or their downstream signaling molecules, as well as cytokine receptor signaling pathways. Because NK cell activation can be extremely sensitive to minute changes in activating and inhibitory receptor levels (57), it is a prime candidate for miRNA-based fine tuning. It is likely that, because both degragation and IFN-γ production are increased in the Dicer1fl/fl NK cells, molecules upstream of both degragation and cytokine production may be targets of miRNAs. The effect of miRNAs on the signals that ultimately are integrated from activating and inhibitory cell surface molecules are a potential mechanism contributing to whether an NK cell responds once triggered.

IFN-γ regulation is multifaceted and includes control of gene transcription as well as posttranscriptional events (58). We identified loss of miR-15/16 family miRNAs as potential factors responsible for the increased IFN-γ production in the hCD2-Cre/RosaYFP Dicer1fl/fl mice. These related miRNAs are highly expressed in resting NK cells, target the 3’ UTR of murine IFN-γ, and are decreased in Dicer1fl/fl and Dicer1fl/wt NK cells. It has been suggested previously that IFN-γ is regulated in humans by a pseudoknot element in its 5’ UTR (59) and by miR-29 (32, 60). We initially identified miR-29 as a potential IFN-γ regulator using in silico algorithms, but it did not target the mouse 3’U TR in our luciferase-based assays. Consistent with our targeting data, miR-15/16 has a much higher relative expression than miR-29 in NK cells and is highly expressed in NK and T cells, providing further biologic rationale for its regulation of IFN-γ. Moreover, the 3’UTR of IFN-γ is conserved between humans and mice, and miR-15/16 is predicted to target human IFN-γ, indicating a potential conserved mechanism between species. The miR-15/16 family is known to be highly expressed in lymphocytes as defined by miRNA-SEQ (31), suggesting that it has an integral role in lymphocyte development or function, or both. In addition, the miR-15/16 loci have been implicated in a variety of cellular roles in lymphocytes (61–63). This study’s finding that miR-15/16 targets IFN-γ, a crucial immune response molecule, provides further evidence for the vital role of this family in the immune response. Future studies will involve specifically targeting miR-15/16 family members in NK cells.

miRNAs have been found to have a role in nearly all cell types and to exert their regulation on a wide variety of cellular functions. In this study, we identify the role of miRNAs in modulating the proliferation, survival, and the essential functions of NK cells. miRNA-deficient NK cells have decreased survival and proliferation, but increased degradation and IFN-γ production, implying a central role for miRNAs in the control of physiologic NK cell responses. The role of miRNAs in dampening NK cell function may be found to have a broad impact on the fields of autoimmunity, viral defense, tumor immunotherapy, and hematopoietic stem cell transplantation, in which roles for NK cells are well established (33). Future studies, which will require more subtle manipulations of individual miRNAs or miRNA clusters, will further define the mechanism by which miRNAs control the development and activation threshold of NK cells.

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Disclosures

The authors have no financial conflicts of interest.

References

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