Distinct Requirements of MicroRNAs in NK Cell Activation, Survival, and Function

Natalie A. Bezman, Elizabeth Cedars, David F. Steiner, Robert Bleloch, David G. T. Hesslein and Lewis L. Lanier

*J Immunol* published online 30 August 2010
http://www.jimmunol.org/content/early/2010/08/30/jimmunol.1000980

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2010/08/30/jimmunol.1000980.DC1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Distinct Requirements of MicroRNAs in NK Cell Activation, Survival, and Function

Natalie A. Bezman,*† Elizabeth Cedars,*†‡ David F. Steiner,*†‡ Robert Blelloch,§∥ and Lewis L. Lanier*†‡

MicroRNAs (miRNAs) are small noncoding RNAs that have recently emerged as critical regulators of gene expression within the immune system. In this study, we used mice with conditional deletion of Dicer and DiGeorge syndrome critical region 8 (Dgcr8) to dissect the roles of miRNAs in NK cell activation, survival, and function during viral infection. We developed a system for deletion of either Dicer or Dgcr8 in peripheral NK cells via drug-induced Cre activity. We found that Dicer- and Dgcr8-deficient NK cells were significantly impaired in survival and turnover, and had impaired function of the ITAM-containing activating NK cell receptors. We further demonstrated that both Dicer- and Dgcr8-dependent pathways were indispensable for the expansion of Ly49H+ NK cells during mouse cytomegalovirus infection. Our data indicate similar phenotypes for Dicer- and Dgcr8-deficient NK cells, which strongly suggest that these processes are regulated by miRNAs. Thus, our findings indicate a critical role for miRNAs in controlling NK cell homeostasis and effector function, with implications for miRNAs regulating diverse aspects of NK cell biology. The Journal of Immunology, 2010, 185: 000–000.

N atural killer cells are a key component of the innate immune system, providing early cellular defense against viruses and intracellular pathogens, and contributing to the early detection and destruction of transformed cells (1). NK cells develop from a common lymphoid precursor in the bone marrow and undergo terminal maturation in the periphery, acquiring optimal cytolytic and effector functions (2). Mature NK cells are relatively static in terms of proliferative capacity until transferred into a lymphopenic environment (3, 4) or challenged with pathogens (5).

NK cell activation and function are determined by a balance of signals transmitted by inhibitory and activating NK cell receptors (NKR) (1). Many of the inhibitory NKR, including Ly49 receptors in mice and inhibitory killer cell Ig-like receptors in humans, recognize self-ligands, such as MHC class I molecules. Activating NKR recognize host-encoded molecules induced by transformation or viral infection (e.g., NKG2D ligands) (6), or nonself-ligands, including the mouse cytomegalovirus (MCMV) m157 gp, which is recognized by Ly49H (7, 8). Some activating receptors associate with adapter proteins that contain ITAMs, including DAP12, FcεRIγ, and CD3ζ. Ligation of ITAM-containing receptor complexes results in recruitment and activation of protein tyrosine kinases, which initiate the signaling cascade, resulting in secretion of effector cytokines, such as IFN-γ, and target cell cytotoxicity.

Lack of NK cells renders both human and mice susceptible to certain infections, particularly the herpesviruses, including human cytomegalovirus and MCMV (5). Therefore, experimental infection of mice with MCMV provides a useful model for studying factors that are important for NK cell activation in vivo. Although much has been learned about the signaling molecules and transcription factors that control activation of NK cells, less is known about the posttranscriptional mechanisms that regulate NK cell function.

MicroRNAs (miRNAs) are short (~22 nt) noncoding RNAs, expressed from endogenous genes, that act on protein-encoding mRNAs, targeting them for translational repression or degradation (9). The biogenesis of miRNAs involves two processing steps. Primary miRNA transcripts are first cleaved by the nuclear “microprocessor” complex containing the RNase III enzyme Drosha and its dsRNA-binding partner DiGeorge syndrome critical region 8 (Dgcr8), resulting in short hairpin structures (precursor miRNAs) (10, 11). These precursor miRNAs are then exported to the cytoplasm, and are further processed by another RNAse III enzyme, Dicer, to generate the mature duplex miRNAs (12). Finally, a single strand of the mature miRNA duplex, loaded into the RNA-induced silencing complex, targets specific sequences within mRNA 3’ untranslated regions resulting in mRNA degradation and/or translational repression.

Genetic studies showed a critical requirement for Dicer in vivo. Ablation of Dicer in the mouse germline produces a lethal phenotype (13), and conditional deletion of Dicer in various hematopoietic lineages has been shown to have detrimental effects, such as impaired cell differentiation, proliferation, and survival (14–21). The function of Dicer, however, is not limited to miRNA biogenesis. Dicer is also required for the processing of small inhibitory RNAs (siRNAs) derived from endogenous dsRNA transcripts or exogenous sources, including viral dsRNAs (22). Endogenous siRNAs were discovered in mammalian oocytes and embryonic stem cells (23–25) and were shown to be essential for oocyte maturation (26). Thus, although the loss of miRNA-dependent regulation has

Abbreviations used in this paper: Dgcr8, DiGeorge syndrome critical region 8; LNA, locked nucleic acid; MCMV, mouse cytomegalovirus; MFI, mean fluorescence intensity; miRNA, microRNA; NKR, NK cell receptor; PL, postinfection; siRNA, small inhibitory RNA; YFP, yellow fluorescent protein.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10/$16.00
been implicated in the phenotypes caused by Dicer deficiency, at least in some cases they are the consequence of the loss of other small RNA classes. Unlike Dicer, Dgcr8 is essential for the biogenesis of canonical miRNAs, but not in the generation of siRNAs (23, 27). In this study, we developed a system for the deletion of either Dicer or Dgcr8 in NK cells via drug-induced Cre activity to dissect the roles of miRNAs in NK cell activation, survival, and function during infection.

Materials and Methods

Mice and infections

The Cre-ER\textsuperscript{T2} C57BL/6 transgenic mice were kindly provided by Dr. M. Matloubian (University of California San Francisco, San Francisco, CA) (28), R26R-yellow fluorescent protein (YFP) knockin C57BL/6 mice were provided by Dr. N. Killeen (University of California San Francisco) (29), and Ly\textsuperscript{5/6}-deficient (K\textalpha/\textdelta\textgamma\textvepsilon\textzeta) mice were from Dr. S. Vidal (McGill University, Montreal, Quebec, Canada). Mice carrying the conditional floxed alleles of Dicer (Dicer\textsuperscript{floxed}) (14) and Dgcr8 (Dgcr8\textsuperscript{floxed}) (30) were backcrossed onto a C57BL/6 background for eight generations. To generate bone marrow chimeras, 6- to 8-wk-old CD45.1-congenic C57BL/6 mice (National Cancer Institute, Frederick, MD) were lethally irradiated with 1000 rad. Donor bone marrow was harvested from Dicer\textsuperscript{floxed}, Dicer\textsuperscript{floxed}/Dgcr8\textsuperscript{floxed}, or Dgcr8\textsuperscript{floxed} mice carrying Cre-ER\textsuperscript{T2} transgene and mixed 1:1 with bone marrow harvested from CD45.1 C57BL/6 mice. Cells were then injected into irradiated recipients by the intracardial route in the retro-orbital plexus. The chimera were treated with tamoxifen 7 wk after reconstitution and infected i.p. with 5 \times 10\textsuperscript{4} PFU of a salivary stock of MCMV (Smith strain) 2 wk later (31). All experiments were conducted in accordance with University of California San Francisco Institutional Animal Care and Use Committee guidelines.

Tamoxifen treatment

Mice were administered tamoxifen (Sigma-Aldrich, St. Louis, MO) dissolved in corn oil via oral gavage for 5 consecutive days. Unless otherwise stated, animals were sacrificed for analysis 10–20 d posttreatment.

Flow cytometry and functional assays

Single-cell suspensions were used for flow cytometry. FC receptors were blocked with anti-CD16 and CD32 mAb (clone 2.4G2) at 10 \mu g/ml prior to surface staining with the indicated Abs (all purchased from BD Biosciences, San Jose, CA, eBioscience, or BioLegend, San Diego, CA). Rae-1 expression was measured using anti–pan-Rae-1 mAb (clone 186107). Splenocytes were generated using tissue culture plates treated with N-(1-(3-dimethoxyloxy) propyl)-N,N,N-trimethylammonium methylsulfate (Sigma-Aldrich) and coated with anti–NK1.1, anti–Nkp46, anti–Ly49H, or control mAb for 4 h at 37 \degree C in the presence of GolgiStop (BD Pharmingen, San Diego, CA), followed by staining for lysosome-associated membrane protein-1 (CD107\alpha, BD Pharmingen) and intracellular IFN-\gamma (BD Pharmingen). For control experiments, splenocytes were stimulated with IL-12 (20 ng/ml) and IL-18 (10 ng/ml). For apoptosis analysis, freshly isolated splenocytes were first stained with anti–NK1.1, anti–TCR-\beta, and Live/Dead fixable near-IR stain (Invitrogen, Carlsbad, CA), washed, and then stained with annexin V-PE (BD Pharmingen), according to the manufacturer’s protocol. For expansion of NK cells in vitro, splenocytes were stained with anti-CD4, anti-CD8, anti–Ter119, and anti–GR-1 mAb and then incubated with goat anti-rat IgG-coated and goat anti-mouse IgG-coated magnetic beads (Miltenyi Biotec, Auburn, CA) to deplete T cells, B cells, and GR-1\textsuperscript{*} cells, respectively. NK cells were stained with PE-conjugated DX5 mAb and positively selected by using an anti-PE-mAb-conjugated magnetic bead system (Miltenyi Biotec, Auburn, CA). The resulting NK cells were cultured with 1333 \mu M recombinant human IL-2 (National Institutes of Health Biological Resources Branch Preclinical Repository, Frederick, MD). NK cells were counted and stained with PE-conjugated anti–Nkp46, PerCP-Cy5.5-conjugated-CD3, and annexin V-conjugated A647 after culture for 4 d. Flow cytometry was performed by using a LSRII flow cytometer and analyzed with FlowJo software (TreeStar, Ashland, OR).

Cell sorting

NK cells from spleen were enriched with mAbs against CD5, CD4, CD8, Ter119, Gr-1, and CD11b (University of California San Francisco Ab Core), and anti-rat IgG-coated magnetic beads (Miltenyi Biotec). NK cells were then stained with anti–NK1.1 and anti–TCR-\beta mAbs, and NK1.1\textsuperscript{*} TCR-\beta\textsuperscript{*} cells were sorted using a FACSAria (Becton Dickinson, San Jose, CA). For human NK cells, the low-buoyant density cells from the Percoll interface layer were isolated from PBMCs (Stanford Blood Center, Palo Alto, CA), stained with anti-CD56 and anti-CD3 mAbs, and CD56\textsuperscript{+} CD3\textsuperscript{−} lymphocytes were sorted. The purity of the recovered NK cells was typically >98%.

In vivo BrdU labeling

Mice were injected i.p. with 200 \mu g BrdU (Sigma-Aldrich) every 24 h for 3 consecutive days (Fig. 3B) or once for 2 h (Fig. 3E) and then sacrificed. For the detection of incorporated BrdU, cells were first stained for surface Ags, then permeabilized, treated with DNase I, and stained with allophycocyanin-conjugated anti-BrdU mAb (BD Pharmingen).

CFSE labeling and adoptive transfer

A 1:1 splenocyte mixture of CD45.1\textsuperscript{*} (from wild-type C57BL/6 mice) and CD45.2\textsuperscript{*} (from either Dgcr8\textsuperscript{floxed} or Dgcr8\textsuperscript{floxed}/Dgcr8\textsuperscript{floxed}) mice was treated with ACK lysis buffer to remove RBCs, labeled for 8 min with 0.5 \mu M CFSE, and washed twice in PBS. A total of 1 \times 10\textsuperscript{5} labeled cells were transferred i.v. into Ly5/6-deficient C57BL/6 mice. Twenty-four hours later, recipient mice were infected with MCMV and sacrificed 4 d later. Adoptively transferred NK cells were analyzed for Ly5/6 expression and CFSE dilution by flow cytometry.

MiRNA array profiling

Total RNA was extracted from sorted mouse splenics and human peripheral blood NK cells by using TRIzol (Invitrogen). RNA quality was verified by an Agilent 2100 Bioanalyzer profile. A mixture of equal amounts of RNA from mouse and human NK cells were made as reference. Total RNA (5 \mu g) from the samples (mouse or human NK cells) and a reference pool were labeled with Hy5 and Hy3 fluorescent dye label, respectively, using the miRCURY locked nucleotide acid (LNA) miRNA array labeling kit (Exiqon, Denmark). The Hy5-labeled samples and Hy3-labeled reference pool RNA samples were mixed pairwise and hybridized to the LNA array (Gladstone Genomics Core, San Francisco, CA), which contains capture probes targeting human, mouse, and rat miRNAs. The hybridization was performed on a GeneChip hybridization station (Genomic Solutions, Boston, MA). The slides were scanned by Axon GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA). GPR files containing fluorescent ratios (sample/control) were generated using GenePix Pro 6.0 software.

Quantitative RT-PCR

Total RNA was isolated from sorted YFP\textsuperscript{+} NK cells by using the mirVana miRNA Isolation kit (Ambion, Austin, TX). The expression levels of specific miRNAs were examined by using the TaqMan MicroRNA Assay kit (Applied Biosystems, Foster City, CA) with gene-specific stem-loop PCR primers and TaqMan probes (IDT, Coralville, IA) to detect mature miRNA transcripts. Quantitative RT-PCR reaction was performed on an Applied Biosystems 7500 Fast Real-Time PCR System. snoRNA202 was used as endogenous control. Relative quantification using the \Delta\Deltact method in tamoxifen-treated Dicer\textsuperscript{floxed} or Dgcr8\textsuperscript{floxed} versus control mice was carried out and relative changes were calculated for each miRNA.

PCR analysis of Dicer\textsuperscript{floxed} and Dgcr8\textsuperscript{floxed} mice

The floxed Dicer allele was genotyped as previously described (18). The same primers were used to quantitate the floxed Dicer alleles by real-time PCR with the SYBR green PCR Master Mix (Applied Biosystems). The PU.1 sequences 5’-CTTCACTGGCCCATCATTGCTATCA-3’ (forward) and 5’-GCTGGGACAAGTTGTTAAGGAA-3’ (reverse) were used for normalization. The floxed Dgcr8 allele was genotyped using primers 5’-CTTGAATGGCCATTGAGCTTTC-3’ (forward) and 5’-CCTGATTACCTTTTACAACAACC-3’ (reverse).

Statistical analysis

All data shown are the mean \pm SEM unless stated otherwise. Comparisons between samples were performed by using a two-tailed Student t test. Statistics were determined using Prism software (GraphPad Software, La Jolla, CA). The p values were denoted in the following manner: *p < 0.05, **p < 0.01, ***p < 0.001.

Results

Inducible deletion of either Dicer or Dgcr8 leads to a global miRNA deficiency in NK cells

We determined the expression profile of miRNAs in mouse and human NK cells. The miRNAs obtained from sorted NK1.1\textsuperscript{*} TCR-\beta\textsuperscript{*} mouse NK cells and from CD56\textsuperscript{+} CD3\textsuperscript{−} human NK cells were profiled with a LNA-based microarray (Supplemental Table I). The
preliminary analysis indicated that 80% of all miRNAs present in human NK cells were also expressed in mouse NK cells, and 59% of mouse miRNAs were present in human NK cells (Supplemental Fig. 1A). Quantitative RT-PCR confirmed expression of the top 20 common miRNAs in mouse NK cells (Supplemental Fig. 1B).

To understand the role of miRNAs in NK cell biology, we induced ablation of the conditional Dicer^f/f (14) and Dgcr8^f/f (30) alleles using a drug-inducible Cre recombinase. This inducible system is dependent upon three sets of genes. The first are the genes encoding Dicer and Dgcr8 in which both alleles are flanked by two loxP sites (Dicer^f/fP and Dgcr8^f/fP, respectively). The second gene is a Cre recombinase-human estrogen receptor (Cre-ER^T2) chimeric molecule under the control of the ubiquitin promoter. This fusion protein is sequestered within the cytoplasm in the absence of the estrogen analog, tamoxifen (28, 32). In the presence of tamoxifen, the Cre-ER^T2 protein shuttles into the nucleus and gains access to loxP sites. This approach allows for the constitutive expression of a Cre recombinase yet prevents it from acting on loxP sites until tamoxifen is administered. The third component is an enhanced YFP gene inserted into the Rosa-26 locus (29). The YFP cassette is preceded by a loxP-flanked tpA transcriptional stop signal. This construct permits expression of YFP and identification of cells that currently or previously expressed an activated Cre. When tamoxifen is administered to mice, Cre becomes active and deletes the currently or previously expressed an activated Cre. When tamoxifen is administered. Cre becomes active and deletes the floxed Dicer or Dgcr8 alleles and the R26R stop cassette, resulting in Dicer^f/fYFP and Dgcr8^f/fYFP phenotypes, respectively (Fig. 1A). To serve as a control for these mice, we generated mice in which loxP sites flank only one allele, the other allele is wild-type (Dicer^f/fYFP and Dgcr8^f/fYFP mice). Following tamoxifen treatment, these mice would be referred to as Dicer^f/fYFP and Dgcr8^f/fYFP, respectively.

We determined the kinetics of YFP expression and concomitant reduction of miRNA expression in NK cells from Dicer^f/fYFP and Dgcr8^f/fYFP mice after administration of tamoxifen. YFP expression appeared on day 5 after tamoxifen treatment was initiated (data not shown) and was maximal by day 14 posttreatment (Fig. 1B). Only ~25% of NK cells in Dicer^f/fYFP and Dgcr8^f/fYFP control mice produced the YFP protein, suggesting that this system underreports Dicer (and perhaps Dgcr8) deletion, as has been reported for other genes (33). In fact, Dicer was also excised in YFP+ NK cells in Dicer^f/fYFP mice, albeit not as efficiently as in YFP+ NK cells (Supplemental Fig. 2A). To confirm that deletion occurred in the studied cells, all experiments were performed on cells gated as YFP+.

By day 14 posttreatment, miRNA expression in YFP+ NK cells from Dicer^f/fYFP mice decreased to 7–22% of the miRNA expression level in NK cells from untreated Dgcr8^f/fP mice (Fig. 1C). Because the half-life of naive NK cells is ~17 d (4) [and potential global effects of Dicer and Dgcr8 deficiency on the mouse immune system may exist (18, 21, 34)], we limited our analysis of NK cells from these mice to days 10–20 posttamoxifen treatment. Approximately 50–75% reduction of select miRNAs was confirmed in YFP+ NK cells from several Dicer^f/fYFP and Dgcr8^f/fYFP mice (Fig. 1D, Supplemental Fig. 2B). Importantly, miRNAs expressed at high (miR21 and miR16), medium (miR29a and miR30b), and low levels (miR222) were similarly affected by Dicer and Dgcr8 deletion. These studies demonstrate that inducible Cre-ER^T2-mediated deletion of either Dicer or Dgcr8 leads to a significant depletion of miRNAs in YFP+ NK cells.

Reduction of the NK cell compartment in Dicer^f/fYFP and Dgcr8^f/fYFP mice
To investigate the role for Dicer- and Dgcr8-controlled miRNAs in the regulation of NK cell homeostasis, we first determined the frequency and number of NK cells in peripheral organs of Dicer^f/fYFP and Dgcr8^f/fYFP mice. Flow cytometric analyses of YFP+ lymphocytes in the spleen, liver, and blood showed a marked reduction of the overall percentage of NK1.1+ TCR-β+ cells (2-fold reduction on average) in Dicer^f/fYFP and Dgcr8^f/fYFP mice compared with littermate controls (Fig. 2A, 2B). Using the same R26-YFP reporter mice, it was previously demonstrated that in the absence of Dicer, there is a strong deletion of CD4+ and CD8+ T cells that have expressed Cre (16). We also found a smaller proportion of YFP+ cells in the splenic and liver NK populations in Dicer^f/fYFP and Dgcr8^f/fYFP mice compared with controls (Fig. 2C). Taken together with the reduced frequency of NK cells within the YFP+ lymphocytes, the absolute numbers of YFP+ NK cells were significantly decreased in the spleen of Dicer^f/fYFP and Dgcr8^f/fYFP mice (2.4-fold, p < 0.0001, and 3.3-fold, p = 0.0024, respectively). Similar reduction in numbers of YFP+ NK cells were seen in liver of Dicer^f/fYFP and Dgcr8^f/fYFP mice (6.3-fold, p = 0.0066, and 3.3-fold, p = 0.033, respectively) (Fig. 2D). This decrease in peripheral NK cell numbers in Dicer^f/fYFP and Dgcr8^f/fYFP mice occurs in the context of reduced, but not abolished, miRNA function. As such, it is possible that the reason that we observed only 50–75% reduction in miRNA levels (Fig. 1D) is because these are the only cells that are viable.

In contrast to the NK cell compartment, the frequencies of B cells and CD4+ T cells were unchanged in the spleens of Dicer^f/fYFP and Dgcr8^f/fYFP mice compared with controls. CD8+ T cells were consistently moderately reduced in Dicer^f/fYFP and Dgcr8^f/fYFP mice, although this difference did not reach statistical significance (Fig. 2E, 2F). Spleen cellularity in Dicer- and Dgcr8-deficient mice, however, was significantly reduced, as compared with controls (67.6 × 10^7 ± 8.5 × 10^6 [n = 9 Dicer^f/fYFP] versus 39.8 × 10^6 ± 4.9 × 10^6 [n = 14 Dicer^f/fYFP]; p = 0.006 and 59.4 × 10^6 ± 12.8 × 10^6 [n = 8 Dgcr8^f/fYFP] versus 30.0 × 10^6 ± 2.8 × 10^6 [n = 8 Dgcr8^f/fYFP]; p = 0.04). As a result, the numbers of B cells, CD4+ T cells, and CD8+ T cells, were lower (~2-fold, 2.3 fold, and 5-fold, respectively) in Dicer^f/fYFP and Dgcr8^f/fYFP mice compared with that in controls. This is consistent with prior reports showing that Dicer deletion resulted in a dramatic reduction of B cells and T cells (15;17). Although inKcT cell development was recently reported to be severely impaired in the absence of Dicer (19, 20), we detected only minor reduction in the number of NK1.1+ TCR-β+ cells in our Dicer^f/fYFP and Dgcr8^f/fYFP mice (~1.3-fold and ~1.2-fold reduction compared with controls, respectively) (data not shown). Taken together, these data show that miRNAs are required for the maintenance of normal lymphocyte numbers, particularly those of the NK cell lineage.

**MiRNAs regulate survival and turnover of NK cells**
To determine whether the reduction in NK cell numbers in Dicer^f/fYFP and Dgcr8^f/fYFP mice was the result of increased cell death, impaired expansion of immature NK cells, or both, we stained freshly isolated cells with annexin V and a viability dye to assess apoptosis (live, annexin V−) and death (dead, annexin V+). There was a consistent increase in both apoptotic and dead NK cells from Dicer^f/fYFP and Dgcr8^f/fYFP versus control mice (Fig. 3A, 3B). The rate of apoptosis and death of Dicer^f/fYFP NK cells was further increased and significantly higher than that of Dicer^f/fYFP NK cells if cells were cultured in the presence of IL-2 in vitro (Fig. 3C, 3D). In addition, we examined NK cell turnover using BrdU incorporation. Approximately 10% of wild-type NK1.1+ TCR-β+ NK cells incorporated BrdU over 3 d of labeling, consistent with previous reports (4). In contrast, Dicer^f/fYFP and Dgcr8^f/fYFP NK cells incorporated BrdU at a lower rate, 6.3 ± 0.3% and 5.8 ± 2.0% of NK cells, respectively (Fig. 3E). Consistent with the decreased turnover rate, Dicer^f/fYFP and Dgcr8^f/fYFP NK cells did not exhibit activated phenotypes, shown by the similar expression levels of CD69 and KLRG1 (Fig. 4A). These
results indicate that Dicer- and Dgcr8-deficiency affects both the turnover rate, as well as survival, of NK cells.

**Phenotype and receptor repertoire of NK cells in Dicer\(^{\Delta/-}\) and Dgcr8\(^{\Delta/-}\) mice**

Dicer-deficiency has been shown to affect the development of Treg and iNKT lineages, and the differentiation of CD4\(^+\) T cell into Th1 and Th2 cells (15, 16, 18–21). To determine whether miRNAs could affect the maturation of peripheral NK cells, we analyzed the expression of maturation markers and surface receptors on freshly isolated YFP\(^+\) NK cells. The maturation status of peripheral NK cells is classified based on their expression of CD11b and CD27, with CD27\(^+\) CD11b\(^+\) NK cells being the most mature population (35). The percentage of this mature NK cell subset in Dicer\(^{\Delta/-}\) and Dgcr8\(^{\Delta/-}\) mice was decreased compared with control mice (Fig. 4B). Expression levels of NK1.1, NKp46, Ly49H, Ly49D, CD94, Ly49G2, Ly49C/I, and Ly49A receptors were unaffected in Dicer\(^{\Delta/-}\) and Dgcr8\(^{\Delta/-}\) mice (Fig. 4C, Supplemental Table II). The only exception was NKG2D whose level was often diminished on NK cells from Dicer\(^{\Delta/-}\) and Dgcr8\(^{\Delta/-}\) mice. NKG2D recognizes...
several cellular ligands, which are expressed at low levels in healthy cells, but are often upregulated in stressed, virally infected, or tumor cells (36). We found that the reduced expression of NKG2D correlated with higher expression of Rae-1 ligands on Dicer\^{\text{\textDelta/\textDelta}} (Fig. 4D), and Dgcr8\^{\text{\textDelta/\textDelta}} (data not shown) NK cells. Together, our results indicate that Dicer- and Dgcr8-deficiency effects

**FIGURE 2.** Preferential reduction of NK cells in Dicer\^{\text{\textDelta/\textDelta}} and Dgcr8\^{\text{\textDelta/\textDelta}} mice. Flow cytometric analysis of lymphocytes from Dicer\^{\text{\textDelta/\textDelta}} (n = 16), Dgcr8\^{\text{\textDelta/\textDelta}} (n = 9), and control Dicer\textsuperscript{+/+} (n = 13) and Dgcr8\textsuperscript{+/+} (n = 11) mice. A, Contour plots depict frequency of NK (NK1.1\textsuperscript{+} TCR-\beta\textsuperscript{+}) cells in spleen (top panels), liver (middle panels), and blood (bottom panels). Gated on YFP\textsuperscript{+} lymphocytes. B, Summary (mean \pm SEM) of data shown in A. C, Selective loss of YFP\textsuperscript{+} Dicer\^{\text{\textDelta/\textDelta}} and YFP\textsuperscript{+} Dgcr8\^{\text{\textDelta/\textDelta}} NK cells. Histograms depict relative cell number versus Cre-induced YFP fluorescence within NK cells in spleen (top row) and liver (bottom row). Gated on NK1.1\textsuperscript{+} TCR-\beta\textsuperscript{+} cells and percentages of YFP-expressing cells are indicated. D, Bar graph shows the number of YFP\textsuperscript{+} NK cells from spleen (left panel) and liver (right panel) of Dicer\^{\text{\textDelta/\textDelta}}, Dgcr8\^{\text{\textDelta/\textDelta}}, and control Dicer\textsuperscript{+/+}, Dgcr8\textsuperscript{+/+} mice. E and F, Comparison of B (CD19\textsuperscript{+}), CD4\textsuperscript{+} (CD4\textsuperscript{+} TCR-\beta\textsuperscript{+}), and CD8\textsuperscript{+} T (CD8\textsuperscript{+} TCR-\beta\textsuperscript{+}) cell frequencies from spleen of Dicer\^{\text{\textDelta/\textDelta}}, Dgcr8\^{\text{\textDelta/\textDelta}} and control Dicer\textsuperscript{+/+}, Dgcr8\textsuperscript{+/+} mice. E, Flow cytometric analysis of populations in total YFP\textsuperscript{+} splenocytes (top panel) and YFP\textsuperscript{+} TCR-\beta\textsuperscript{+} splenocytes (bottom panel). Percentages are shown. F, Summary (mean values \pm SEM) of data shown in E from four to six mice per group. Data are representative of at least five experiments.
NK cell maturation and elicits upregulation of NKG2D ligand expression, but does not globally affect the NKR repertoire. Dicer and Dgcr8 are critical for Ly49H+ NK cell expansion during MCMV infection.

**FIGURE 3.** Increased apoptosis and reduced basal turnover of Dicer- and Dgcr8-deficient NK cells. A and B, Annexin V staining of freshly isolated splenic NK cells. A, Percentages of apoptotic (live annexin V−) and dead cells in control versus DicerΔΔ and Dgcr8ΔΔ mice are indicated within the quadrants. B, Summary (mean values ± SEM) of data shown in (A) from three to five mice per group. C, Enumeration of NK cells after 4 d culture in medium supplemented with IL-2. Values shown represent the mean ± SEM of NK cell numbers (triplicate wells). D, Histogram shows annexin V expression by DicerΔ/Δ (black line) and DicerΔΔ (gray line) NK cells after 4 d culture in medium supplemented with IL-2. E, Intracellular staining for the incorporation of BrdU in control, DicerΔΔ, and Dgcr8ΔΔ mice. Percentage of NK cells that incorporated BrdU is shown. A–E, Gated on YFP+ NK cells. Data are representative of two to three independent experiments.

DicerΔΔ and Dgcr8ΔΔ NK cells produce less CD107a and IFN-γ when stimulated through ITAM-containing receptors

Many of the activating NKRs, such as NK1.1, NKp46, and Ly49H, associate with the ITAM-containing FcεRIγ or DAP12 adapter proteins (1). Ligation of an ITAM-associated NKR results in degranulation, cytokine production, and killing of the target cell. Several studies have recently reported that miRNAs regulate activation of signaling pathways downstream of ITAM receptors (37, 38). We asked whether Dicer- or Dgcr8-deficient NK cells show impaired function of their ITAM-containing receptors. Freshly isolated NK cells were activated by plate-bound anti-NK1.1, anti-NKp46, and anti-Ly49H Abs, and surface CD107a (LAMP-1) and intracellular IFN-γ were measured. There were fewer CD107a+ cells among gated YFP+ NK populations from DicerΔΔ and Dgcr8ΔΔ mice compared with controls (Fig. 5A). Furthermore, fewer DicerΔΔ and Dgcr8ΔΔ NK cells produced IFN-γ when activated by Abs to NK1.1, NKp46, or Ly49H (Fig. 5B). To determine whether this is a generalized hyporesponsiveness or an exclusive defect associated with ITAM-containing receptors, we stimulated NK cells with IL-12 and IL-18. Interestingly, DicerΔΔ, Dgcr8ΔΔ, and control NK cells produced comparable amounts of IFN-γ in the presence of IL-12 and IL-18 (Fig. 5C). This demonstrates that Dicer- and Dgcr8-deficient NK cells are fully capable of responding through their cytokine receptors; however, function of their ITAM-containing receptors is impaired. Dicer and Dgcr8 are critical for Ly49H+ NK cell expansion during MCMV infection

To investigate whether miRNAs regulate NK cell function in vivo, we asked whether Dicer and Dgcr8 are necessary for NK cell cytokine production and expansion in response to viral infection. During infection with MCMV, NK cells bearing the activating
Ly49H receptor recognize the viral protein m157 (7, 8), and respond to inflammatory cytokines, such as IL-12 produced by dendritic cells (39). Signaling primarily via ITAM-containing DAP12, and augmented through DAP10, Ly49H+ NK cells mount a rapid antiviral response, secrete cytokines, including IFN-γ, and undergo expansion (40).

To measure MCMV-specific immune responses by Dicer- and Dgcr8-deficient NK cells, we generated chimeric mice by reconstituting lethally irradiated mice with 1:1 mixed bone marrow from wild-type (CD45.1+) and experimental (DicerF/F, Dgcr8F/F, and control DicerF/+, Dgcr8F/+ [CD45.2+]) mice (Fig. 6 A). Prior to tamoxifen treatment, wild-type and experimental bone marrow reconstituted the NK cell compartment equally well (Supplemental Fig. 3 A). Posttamoxifen treatment, however, the frequency of NK cells was significantly decreased in the CD45.2+ Dicer−/− and Dgcr8−/− compartments of chimeric mice, compared with that in the Dicer+/+ and Dgcr8+/+ chimeric mice, consistent with our earlier findings (Supplemental Fig. 3 A, 3B). Importantly, NK cell frequency in the CD45.1+ compartment was similar in Dicer−/−, Dgcr8−/−, and Dicer+/+ mice, thus providing an internal control as a standard for each sample.

FIGURE 4. Dicer- and Dgcr8-deficient NK cells do not possess an activated phenotype and have normal receptor expression with the exception of NKG2D. Splenocytes from Dicer−/−, Dgcr8−/−, and control Dicer+/+ and Dgcr8+/+ mice were stained with mAbs against activation markers CD69 and KLRG1 (A), maturation markers CD11b and CD27 (B), and activating NKRs NK1.1, NKp46, Ly49H, and NKG2D (C). Gated on YFP+ NK1.1+ TCRβ cells. Data are representative of at least five experiments. D, Expression levels of Rae-1 on YFP+ NK1.1+ TCR-β cells in the spleens of Dicer−/− and Dicer+/+ mice. Mean fluorescence intensity (MFI) values are shown as mean ± SD using two mice per group. Data are representative of three experiments.

Ly49H receptor recognize the viral protein m157 (7, 8), and respond to inflammatory cytokines, such as IL-12 produced by dendritic cells (39). Signaling primarily via ITAM-containing DAP12, and augmented through DAP10, Ly49H+ NK cells mount a rapid antiviral response, secrete cytokines, including IFN-γ, and undergo expansion (40).

To measure MCMV-specific immune responses by Dicer- and Dgcr8-deficient NK cells, we generated chimeric mice by reconstituting lethally irradiated mice with 1:1 mixed bone marrow from wild-type (CD45.1+) and experimental (Dicer−/−, Dgcr8−/−, and control Dicer+/+, Dgcr8+/+) mice (Fig. 6A). Prior to tamoxifen treatment, wild-type and experimental bone marrow reconstituted the NK cell compartment equally well (Supplemental Fig. 3A). Posttamoxifen treatment, however, the frequency of NK cells was significantly decreased in the CD45.2+ Dicer−/− and CD45.2+ Dgcr8−/− compartments of chimeric mice, compared with
When we infected Dicer\textsuperscript{Δ/Δ} and Dgcr8\textsuperscript{Δ/Δ} chimeric mice with MCMV, both mice showed an increase in Ly49H\textsuperscript{+} NK cell frequency (Fig. 6D), and numbers (Fig. 6E) at day 7 PI. With precursor numbers of \(\sim 0.8 \times 10^5\) (Dicer\textsuperscript{Δ/Δ}) and \(\sim 0.66 \times 10^5\) (Dgcr8\textsuperscript{Δ/Δ}) total Ly49H\textsuperscript{+} NK cells in the spleen, the absolute number of Ly49H\textsuperscript{+} NK cells at day 7 PI expanded \(\sim 10.6\)- and \(\sim 13.8\)-fold, respectively (Fig. 6E). In contrast, fewer Ly49H\textsuperscript{+} NK cells were found in both Dicer\textsuperscript{Δ/Δ} and Dgcr8\textsuperscript{Δ/Δ} chimeric mice at day 7 PI. The absolute number of Ly49H\textsuperscript{+} NK cells in Dicer\textsuperscript{Δ/Δ} and Dgcr8\textsuperscript{Δ/Δ} chimeric mice expanded only \(\sim 3\)- and \(\sim 2.1\)-fold,
respectively. Importantly, wild-type CD45.1+ Ly49H+ NK cells from every set of chimeric mice (Dicer<sup>+/+</sup>, Dicer<sup>-/-</sup>, Dgcr8<sup>+/+</sup>, or Dgcr8<sup>-/-</sup>) were treated with tamoxifen and then given PBS (uninfected) or infected with MCMV. In the experiments, intracellular staining for IFN-γ in NK cells immediately ex vivo at day 1.5 PI showed representative scatter plots gated on CD45.2<sup>+</sup> NK1.1<sup>-</sup> TCR-β<sup>-</sup> cells from Dicer<sup>+/+</sup>, Dicer<sup>-/-</sup>, Dgcr8<sup>+/+</sup>, and Dgcr8<sup>-/-</sup> chimeric mice are shown, and the numbers represent percentages of cells in the indicated quadrants. Cumulative results (n = 4–8 mice/group) are quantified. Percentages (D) and absolute numbers (E) of CD45.2<sup>+</sup> Ly49H<sup>+</sup> NK cells in Dicer<sup>+/+</sup>, Dicer<sup>-/-</sup>, Dgcr8<sup>+/+</sup>, or Dgcr8<sup>-/-</sup> chimeric mice at day 7 PI. Absolute number of wild-type CD45.1<sup>+</sup> Ly49H<sup>+</sup> NK cells in the same Dicer<sup>+/+</sup>, Dicer<sup>-/-</sup>, Dgcr8<sup>+/+</sup>, or Dgcr8<sup>-/-</sup> chimeric mice at day 7 PI. E and F. The number of Ly49H<sup>+</sup> NK cells from uninfected chimeric mice (gray bars) and from MCMV-infected mice (black bars) is shown. Data are mean ± SEM of three mice per group and are representative of three independent experiments.

**FIGURE 6.** Dicer and Dgcr8 are necessary for Ly49H<sup>+</sup> NK cell expansion during MCMV infection. A–F. Mixed bone marrow chimeric mice (1:1 mixture of wild-type [CD45.1<sup>+</sup>] and either Dicer<sup>+/+</sup>, Dicer<sup>-/-</sup>, Dgcr8<sup>+/+</sup>, or Dgcr8<sup>-/-</sup> [CD45.2<sup>+</sup>] cells) were treated with tamoxifen and then given PBS (uninfected) or infected with MCMV. B and C. Intracellular staining for IFN-γ in NK cells immediately ex vivo at day 1.5 PI. B. Representative scatter plots gated on CD45.2<sup>+</sup> NK1.1<sup>-</sup> TCR-β<sup>-</sup> cells from Dicer<sup>+/+</sup>, Dicer<sup>-/-</sup>, Dgcr8<sup>+/+</sup>, and Dgcr8<sup>-/-</sup> chimeric mice are shown, and the numbers represent percentages of cells in the indicated quadrants. C. Cumulative results (n = 4–8 mice/group) are quantified. Percentages (D) and absolute numbers (E) of CD45.2<sup>+</sup> Ly49H<sup>+</sup> NK cells in Dicer<sup>+/+</sup>, Dicer<sup>-/-</sup>, Dgcr8<sup>+/+</sup>, or Dgcr8<sup>-/-</sup> chimeric mice at day 7 PI. E and F. The number of Ly49H<sup>+</sup> NK cells from uninfected chimeric mice (gray bars) and from MCMV-infected mice (black bars) is shown. Data are mean ± SEM of three mice per group and are representative of three independent experiments. MIRNAs regulate survival of Ly49H<sup>+</sup> NK cells during MCMV infection

Defective expansion of Dicer<sup>-/-</sup> and Dgcr8<sup>-/-</sup> Ly49H<sup>+</sup> NK cells could result from decreased NK cell proliferation and/or impaired survival during MCMV infection. To discriminate between these possibilities, we monitored proliferation in vivo by analyzing BrdU incorporation. Following a 2-h pulse at day 5 PI, similar percentages of Dicer<sup>-/-</sup>, Dgcr8<sup>-/-</sup>, and Dicer<sup>+/+</sup>, Dgcr8<sup>-/-</sup> NK cells from mixed bone marrow chimeric mice incorporated BrdU (Fig. 7A). Adoptive transfer of CFSE-labeled NK cells into Ly49H-deficient recipients further confirmed that both Dgcr8<sup>-/-</sup> and Dgcr8<sup>-/-</sup> Ly49H<sup>+</sup> cells were dividing to a similar extent, fully diluting their CFSE during the 4-d following infection (Fig. 7B). We also monitored apoptosis by annexin V staining at the same time point PI. Notably, a higher percentage of apoptotic cells was detected in Dgcr8<sup>-/-</sup> mice compared with that in Dgcr8<sup>-/-</sup> control mice (Fig. 7C). Together these data indicate that miRNAs are not
required for the proliferative responsiveness of Ly49H⁺ NK cells, but regulate the survival of NK cells during MCMV infection.

**Discussion**

In this study, we have shown identical phenotypes when either *Dicer* or *Dgcr8* gene was inactivated in NK cells. These results strongly suggest that these two molecules function in the same biological pathway in miRNA biogenesis, and demonstrate that the deficiency in miRNAs is the primary cause underlying the observed phenotypes. Although it is possible that Dicer-deficient cells also exhibit other more subtle phenotypes, such as the derepression of retrotransposons (24, 25), it appears that it is miRNAs, rather than other Dgcr8-independent, Dicer-dependent small RNAs, that are critical for NK cells.

Ablation of the miRNA biogenesis pathway, through deletion of Dicer or Dgcr8, led to increased apoptosis of peripheral NK cells. Similarly, Dicer deletion in developing B cells (17), thymocytes (15, 16), or iNKT cells (19) resulted in increased cell death. These results suggest that the miRNA pathway plays an important role in controlling cell survival. Potential mechanisms include mitotic defects due to centromere dysfunctions (43), defects in heterochromatin maintenance (44), and aberrant over expression of proapoptotic protein Bim (17). In addition, our preliminary studies indicate that Bcl-2 mRNA level was consistently decreased in Dicer⁻/⁻ and Dgcr8⁻/⁻ NK cells (Supplemental Fig. 4). Yamanaka et al. (38) recently showed that inhibition of miR21 in the human NK cell line led to increased apoptosis associated with the upregulation of proapoptotic miR21 targets PTEN, PDCD4, and Bim. Although it is very likely that multiple miRNAs take part in the coordinate regulation of NK cell survival, it would be informative to test whether miR21 is a key regulator of NK cell survival.

Maturation of NK cells is characterized by a decrease in the expression of CD27, active proliferation, and a concomitant increase in the levels of CD11b and effector functions (35, 45). Analysis of Dicer⁻/⁻ and Dgcr8⁻/⁻ mice revealed a relative accumulation of more immature CD27⁺⁺ CD11b⁺⁺ NK cells. This could be due to a selective loss of more mature CD27⁻ CD11b⁻⁺ NK cells or a maturation defect. We did not detect any preferential difference in the death or turnover in the CD27⁺⁺ CD11b⁺⁺ or CD27⁻⁺ CD11b⁺⁺ NK cell subsets (N. Bezman, unpublished observations). Furthermore, we found a similar accumulation of CD27⁺⁺ CD11b⁺⁺ NK cells in CD45.2⁺ subsets of Dicer⁻/⁻ and Dgcr8⁻/⁻ mixed bone marrow chimeras (data not shown). These data indicate that impaired transition from CD27⁺⁺ CD11b⁺⁺ to CD27⁻⁺ CD11b⁺⁺ of Dicer- and Dgcr8-deficient NK cells is hematopoietic cell intrinsic and cell autonomous, suggesting that miRNAs might regulate NK cell maturation. Interestingly, miR150 has been shown to regulate B cell development and this regulation occurs through the inhibition of Myb expression (46, 47). MiR150 is dynamically regulated during NK cell maturation (N. Bezman, unpublished observations).

**FIGURE 7.** Robust proliferation, but decreased survival, of Dicer- and Dgcr8-deficient NK cells during MCMV infection. A, BrdU incorporation (day 5 PI) by NK1.1⁺ TCR-β⁻ cells from mixed bone marrow chimeric mice (Fig. 6). Percentages of BrdU incorporation by wild-type (CD45.1⁺) cells (lower panel) and either Dicer⁻/⁻, Dicer⁻/Δ, Dgcr8⁻/⁻, or Dgcr8⁻/Δ (CD45.2⁺⁺) cells (top panel) are shown. Data are representative of two experiments. B, CFSE-labeled 1:1 mix of wild-type (CD45.1⁺) and either Dgcr8⁻/⁻ or Dgcr8⁻/Δ (CD45.2⁺⁺) NK cells were transferred into Ly49H-deficient recipients. Ly49H⁺ NK cells were analyzed at day 4 PI. Data are representative of three independent experiments. C, NK cells from Dgcr8⁻/⁻ or Dgcr8⁻/Δ mice were transferred into Ly49H-deficient recipients and were stained for annexin V at day 4 PI. Bar graph indicates the percentage of NK1.1⁺ TCR-β⁻ cells (mean ± SD) that are live and are staining positive for annexin V (indicating apoptotic). Data are representative of three independent experiments using two mice per group.
showed that reducing levels of miR21 in the human NK cell line Src kinase, Lck, and Erk (37). In addition, Yamanaka et al. (38) enhanced basal activation of the TCR signaling molecules, including known whether they regulate NK cell activation. In T cells, (52); with the exception of SHP-2, however, it is currently not necessary for ITAM-based activation.

levels of Rae-1 would likely lead to down modulation of NKGD on NK cells, as has previously been demonstrated (36).

We have shown that degranulation and IFN-γ production were impaired in Dicer- and Dgcr8-deficient NK cells following stimulation via NK1.1, NKp46, or Ly49H. This impairment in IFN-γ production might be caused by the reduction in the CD27 the CD11b the NK cell subset in Dicer the and Dgcr8 mice, because this subset has been demonstrated to play a dominant role in cytokine production (45). To this end, we costained NK cells with anti-CD27 and anti-CD11b Abs and measured intracellular IFN-γ levels. Both CD27 the CD11b and CD27 the CD11b NK cell subsets from Dicer and Dgcr8 mice produced less IFN-γ compared with that in the corresponding NK cell subsets from the control mice (data not shown). Thus, a decline in CD27 the CD11b NK cells in Dicer and Dgcr8 mice does not explain the reduction in cytokine generation. Because stimulation with IL-12 and IL-18 induced comparable amounts of IFN-γ in Dicer and Dgcr8 mice, and control NK cells, we conclude that miRNAs are necessary for ITAM-based activation.

Li et al. (37) recently demonstrated that miR181a modulates signal strength of the TCR by downregulating expression of several protein tyrosine phosphatases, including SHP-2, PTPN22, DUSP5, and DUSP6. These phosphatases are expressed in mouse NK cells (52); with the exception of SHP-2, however, it is currently not known whether they regulate NK cell activation. In T cells, miR181a-mediated regulation of these phosphatases leads to enhanced basal activation of the TCR signaling molecules, including Sdc kinase, Lck, and Erk (37). In addition, Yamanaka et al. (38) showed that reducing levels of miR21 in the human NK cell line resulted in the downregulation of phosphorylated AKT. Thus, a potential explanation for the impaired function of ITAM-containing receptors in Dicer- and Dgcr8-deficient NK cells is that specific miRNA(s) might regulate signaling downstream of these ITAM-containing receptors.

NK cells have long been compared with effector and memory CD8 T cells in phenotype and function (53), and recently several groups have demonstrated that NK cells can become long-lived cells and mount secondary responses against viral Ags (31, 54). In this study, we determined the miRNA expression profile of mouse and human NK cells. Naive NK cells and CD8 T cells share a large part of their miRNA profile: miR142-3p, miR142-5p, miR150, miR16, miR23a, miR15b, miR29a, miR29b, miR30b, and miR26a are highly expressed in both naive NK cells and CD8 T cells [Supplemental Fig. 1 (55, 56)]. Similar to NK cells, the frequency and number of naive CD8 T cells were preferentially reduced in Dicer- and Dgcr8-deficient mice. Thus, miRNAs shared by naive NK cells and CD8 T cells might be implicated in the regulation of common molecular pathways, such as regulation of homeostasis.

NK cells also share a miRNA profile with effector and memory CD8 T cells. MiR21, miR221, and miR222 are expressed in both NK and effector CD8 T cells, whereas miR146a is found in both NK cells and memory CD8 T cells [Supplemental Fig. 1 (56)]. It is intriguing to speculate that the miRNAs shared by NK cells, effector CD8 T cells, and memory CD8 T cells might be implicated in the regulation of common pathways that lead to the acquisition of the effector phenotype and survival. Our identification of a requirement of Dicer and Dgcr8 for Ly49H NK cell expansion during MCMV infection predicts miRNAs that might target factors that are important for maintaining the NK cell population during viral infection. In conclusion, our results present evidence for miRNAs regulating diverse aspects of NK cell biology, including basic processes, such as turnover and survival, as well as the function of activating NKRs during MCMV infection.

Acknowledgments

We thank Drs. Michael McManus and Eric Brown for mice. We also thank Jessica Jarjoura for assistance with cell sorting, Hernan Consengco for assistance with genotyping, and members of the Lanier laboratory for critical reading of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

References

12 DICER- AND DGCR8-DEPENDENT PATHWAYS REGULATE NK CELL FUNCTION


