Title

Stage-specific regulation of natural killer cell homeostasis and response against viral infection by microRNA-155

Journal

Proceedings of the National Academy of Sciences of the United States of America, 110(17)

ISSN

0027-8424

Authors

Zawislak, Carolyn L

Beaulieu, Aimee M

Loeb, Gabriel B

et al.

Publication Date

2013-04-23

DOI

10.1073/pnas.1304410110

Peer reviewed

Stage-specific regulation of natural killer cell homeostasis and response against viral infection by microRNA-155

Carolyn L. Zawislak[a,1], Aimee M. Beaulieu[a,1], Gabriel B. Loeb[a,b], Jenny Karo[a], David Canner[a,b], Natalie A. Bezman[c], Lewis L. Lanier[c], Alexander Y. Rudensky[a,b,2], and Joseph C. Sun[a,2]

[a]Immunology Program and [b]Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, NY 10065 and [c]Department of Microbiology and Immunology, University of California, San Francisco, CA 94143

Contributed by Alexander Rudensky, March 8, 2013 (sent for review February 13, 2013)

Natural killer (NK) cells function in the recognition and destruction of host cells infected with pathogens. Many regulatory mechanisms govern the potent responses of NK cells, both at the cellular and molecular level. Ablation of microRNA (miRNA) processing enzymes demonstrated that miRNAs play critical roles in NK cell differentiation and function; however, the role of individual miRNAs requires further investigation. Using mice containing a targeted deletion of microRNA-155 (miR-155), we observed defects in NK cell maintenance and maturation at steady state, as well as in homeostatic proliferation in lymphopenic mice. In addition, we discovered that miR-155 is up-regulated in activated NK cells during mouse cytomegalovirus (MCMV) infection in response to signals from the proinflammatory cytokines IL-12 and IL-18 and through signal transducer and activator of transcription 4 (STAT4) signaling. Although miR-155 was found to be dispensable for cytotoxicity and cytokine production when triggered through activating receptors, NK cells lacking miR-155 exhibited severely impaired effector and memory cell numbers in both lymphoid and nonlymphoid tissues after MCMV infection. We demonstrate that miR-155 differentially targets Noxa and suppressor of cytokine signaling 1 (SOCS1) in NK cells at distinct stages of homeostasis and activation. NK cells constitutively expressing Noxa and SOCS1 exhibit profound defects in expansion during the response to MCMV infection, suggesting that their regulation by miR-155 promotes antiviral immunity.

The natural killer (NK) cell response against mouse cytomegalovirus (MCMV) infection has been shown to consist of several distinct phases (1, 2). Early after viral infection, NK cells respond to type I interferons and proinflammatory cytokines, and produce cytokines and lytic molecules. The subset of NK cells bearing the Ly49H receptor, which recognizes the m157 glycoprotein encoded by MCMV, is able to specifically kill virally infected cells through the secretion of perforin and granzymes (1, 2). Interestingly, Ly49H$^+$ NK cells are able to undergo a clonal-like proliferation to amass a large number of virus-specific effector NK cells (1, 2). After contraction of the majority of the effector NK cells, a small pool of long-lived memory NK cells reside in both lymphoid and nonlymphoid organs for months after systemic MCMV infection is resolved (3). In addition, NK cells undergo homeostatic proliferation in lymphopenic environments and also generate long-lived progeny able to proliferate robustly and mediate effector functions against pathogens (4). The factors that promote and regulate the distinct stages of both the virus-specific NK cell response and the homeostatic proliferation of NK cells remain to be elucidated.

Recent studies have shown that microRNAs (miRNAs) play an important role in the regulation of NK cell development and function (5–7). Conditional gene ablation of the miRNA-processing enzymes Dicer or Dgcr8, which leads to a global loss of miRNAs, resulted in an impaired survival of maturing NK cells (6, 8). Furthermore, NK cells lacking miRNAs have been shown to exhibit defects in proliferation and IFN-γ secretion after viral infection (6, 8). Although individual miRNAs that regulate the development and function of T-cell and B-cell subsets and myeloid lineage cells have been identified (9, 10), few reports have investigated a similar role for specific miRNAs in NK cell development and effector function. Recently, miR-150 was shown to regulate the development of NK cells by antagonizing the expression of transcription factor c-Myb, as mice with a targeted deletion of miR-150 are impaired in NK cell maturation and function (11).

The function and several gene targets of the highly conserved miR-155 have been well characterized in multiple immune cell populations (10, 12). The product of a non–protein-encoding transcript of the *Bic* gene (13, 14), miR-155 is abundantly expressed by many cells of the immune system, especially in response to activating stimuli (10, 12). Several groups have reported an immunodeficiency and widespread immune dysregulation in miR-155–deficient mice (15, 16). miR-155 has been demonstrated to regulate B-cell responses and the germinal center reaction (16–19), helper CD4$^+$ T-cell differentiation and function (15, 16, 20), generation and homeostasis of regulatory T cells (21), and maturation and activation of macrophages and dendritic cells (22, 23). Although miR-155 is expressed in resting NK cells and is further up-regulated on activation, its precise role in NK cell development and function has not been investigated until now. Here we show that miR-155 is critically required for NK cell maturation and maintenance at steady state, as well as for NK cell responses to viral infection in vivo.

Results

Accelerated Maturation of NK Cells from miR-155–Deficient Mice. miR-155 regulates functions in both innate (macrophages and dendritic cells) and adaptive (B and T cells) immune cells (10, 12, 23). Because NK cells develop from the same common lymphoid progenitor cell that gives rise to B and T cells, we investigated a role for miR-155 in development. We evaluated NK cell numbers, subsets, phenotype, and function in various tissues of miR-155–deficient and WT mice. The overall number of NK cells in the spleens and livers did not differ significantly between *miR-155*$^{-/-}$ and WT mice (Fig. 1*A*). Interestingly, the most mature NK cell population, characterized as CD27^{lo}CD11b^{hi} and expressing Ly49D receptors, Ly49H receptors, or both, was elevated in the *miR-155*$^{-/-}$ mice compared with WT mice (Fig. 1 *B* and *C*). A corresponding decrease in the less-mature NK cell population

Author contributions: C.L.Z., A.M.B., G.B.L., J.K., D.C., N.A.B., and J.C.S. designed research; C.L.Z., A.M.B., G.B.L., J.K., D.C., N.A.B., and J.C.S. performed research; C.L.Z., A.M.B., G.B.L., and J.C.S. contributed new reagents/analytic tools; C.L.Z., A.M.B., G.B.L., J.K., D.C., N.A.B., and J.C.S. analyzed data; and C.L.Z., A.M.B., G.B.L., L.L.L., A.Y.R., and J.C.S. wrote the paper.

The authors declare no conflict of interest.

[1]C.L.Z. and A.M.B. contributed equally to this work.

[2]To whom correspondence may be addressed. E-mail: rudenska@mskcc.org or sunj@mskcc.org.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1304410110/-/DCSupplemental.

IMMUNOLOGY

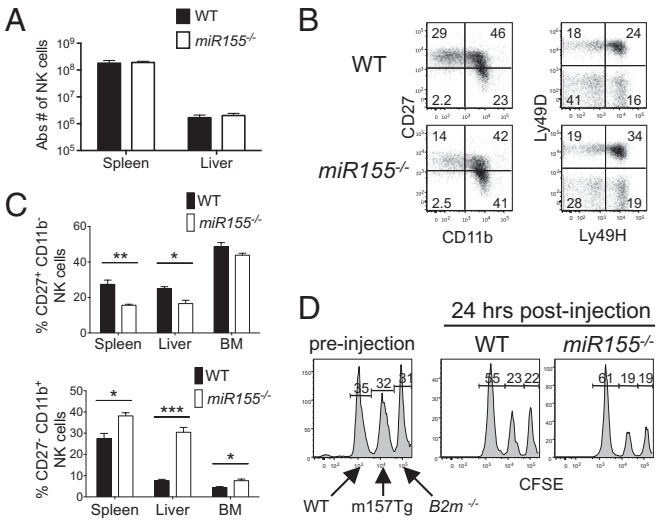

Fig. 1. NK cells from miR-155-deficient mice exhibit a more mature phenotype. (*A*) Absolute numbers of NK cells (TCR-β$^-$ NK1.1$^+$) in the spleen and liver of WT and *miR-155*$^{-/-}$ mice were determined. Error bars show SEM (n = 3–5). (*B*) Flow cytometry plots were gated on TCR-β$^-$ NK1.1$^+$ cells and analyzed for expression of CD27, CD11b, Ly49D, and Ly49H. (*C*) Percentages of less-mature (CD27^{hi}CD11b^{hi}) and more-mature (CD27^{lo}CD11b^{hi}) NK cells were determined in spleen, liver, and bone marrow (BM) from WT and *miR-155*$^{-/-}$ mice. *P < 0.05; **P < 0.01; ***P < 0.001. Error bars show SEM (n = 3–5). (*D*) WT, m157Tg, and *B2m*$^{-/-}$ splenocytes were labeled with low, medium, or high concentrations of CFSE, respectively, and coinjected into WT or *miR-155*$^{-/-}$ mice. Transferred cells were analyzed in spleens of recipient mice at 24 h after injection.

(CD27^{hi}CD11b^{lo} or Ly49D$^-$H$^-$) was evident in spleen and liver, but not in bone marrow, of *miR-155*$^{-/-}$ mice (Fig. 1 *B* and *C*). Thus, miR-155 appears to be less critical for NK cell development in the bone marrow, but has an important role in NK cell maturation and homeostasis in peripheral lymphoid and nonlymphoid tissues.

To investigate the in vivo cytotoxic potential of NK cells in *miR-155*$^{-/-}$ and WT mice, both "missing self" *B2m*$^{-/-}$ and viral m157-expressing (m157Tg) splenocytes were injected along with WT splenocytes as a control. Both groups of mice eliminated the *B2m*$^{-/-}$ and m157Tg targets equally well (Fig. 1*D*), demonstrating that *miR-155*$^{-/-}$ NK cells exhibited normal lytic function at steady state. In accordance with these in vivo killing assays, WT and *miR-155*$^{-/-}$ NK cells were equally able to kill a variety of NK-sensitive target cells (Fig. S1*A*) and produce IFN-γ when triggered through activating receptors or with proinflammatory cytokines (Fig. S1*B*). Thus, although homeostatic maturation of NK cells is dependent on miR-155, miR-155 deficiency does not impact cytotoxicity or cytokine secretion by peripheral NK cells.

miR-155 Deficiency Results in Reduced Homeostasis of NK Cells. To investigate a cell-intrinsic role for miR-155 in NK cells, we generated mixed bone marrow chimeric mice by reconstituting lethally irradiated hosts with an equal number of bone marrow cells from *miR-155*$^{-/-}$ (CD45.2) and WT (CD45.1) mice. Although the early hematopoietic compartment was present at a 1:1 WT:*miR-155*$^{-/-}$ ratio, WT NK cells dominated the NK cell compartment by 11 wk postreconstitution, with 75–80% WT NK cells compared with only 20–25% *miR-155*$^{-/-}$ NK cells (Fig. 2*A*). In WT and *miR-155*$^{-/-}$ mice given BrdU, *miR-155*$^{-/-}$ NK cells exhibited less uptake of BrdU in the spleen, liver, and bone marrow (Fig. 2*B*). Taken together, these findings suggest that NK cells lacking miR-155 either are not able to undergo homeostatic proliferation to fill the peripheral NK cell "niche" or are less able to survive in the periphery.

To directly address whether *miR-155*$^{-/-}$ NK cells can undergo homeostatic proliferation in a lymphopenic setting, we cotransferred *miR-155*$^{-/-}$ and WT NK cells into *Rag2*$^{-/-}$ × *Il2rg*$^{-/-}$ mice deficient in NK, B, and T cells. Although the percentages of the two transferred NK cell populations were similar at day 2, the WT NK cells began to dominate the NK cell pool by day 7, corresponding to a diminished percentage of *miR-155*$^{-/-}$ NK cells (Fig. 2*C*). The percentage of *miR-155*$^{-/-}$ NK cells continued to decline over time, and by day 45 represented less than 15% of the total NK cell population (Fig. 2*D*). Although the rate of division during homeostatic proliferation did not differ significantly between the two cell populations (Fig. S2*A*), the reduced *miR-155*$^{-/-}$ NK cell percentage was observed in multiple tissues (Fig. S2*B*). Taken together, these findings suggest that the differences in cell numbers observed on day 7 and beyond reflect a survival defect in *miR-155*$^{-/-}$ NK cells during homeostatic proliferation.

Proinflammatory Cytokines Drive High miR-155 Expression in NK Cells. Because miR-155 is critical for the homeostasis of NK cells, we investigated whether miR-155 also plays a role in the NK cell response against pathogens. We first investigated whether miR-155 is modulated in NK cells during viral infection. During MCMV infection in WT mice, miR-155 expression was rapidly up-regulated in NK cells at day 2 postinfection (PI), with levels returning to baseline by days 4 and 7 PI (Fig. 3*A*). To identify the specific signals that drive miR-155 expression, we cultured purified NK cells overnight with various proinflammatory cytokines and measured induction of miR-155 by quantitative RT-PCR (qRT-PCR). A combination of IL-12 and IL-18 resulted in a significant up-regulation of miR-155 in cultured NK cells, whereas individual cytokines alone induced smaller increases in miR-155 levels (Fig. 3*B*). Ligation of Ly49H did not result in appreciable

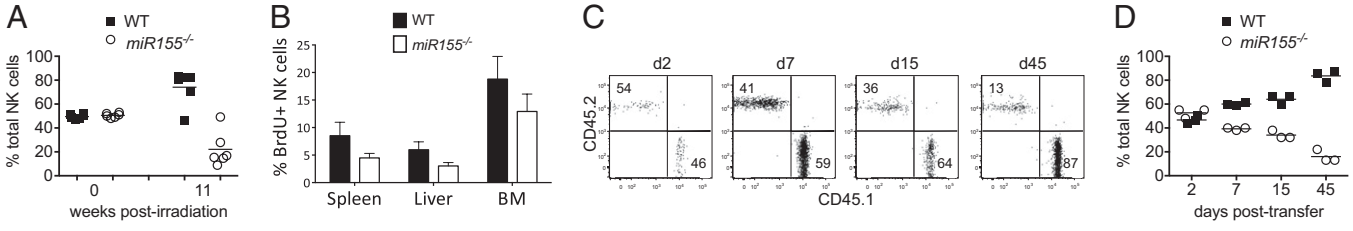

Fig. 2. Diminished homeostatic proliferation in miR-155–deficient NK cells. (*A*) Mixed 1:1 WT:*miR-155*$^{-/-}$ bone marrow chimeric mice were generated, and percentages of WT (CD45.1) and *miR-155*$^{-/-}$ (CD45.2) NK cells were determined in peripheral blood at various time points postreconstitution. The bar in the graph represents the mean value for each group. (*B*) WT and *miR-155*$^{-/-}$ mice were given BrdU over 3 d, and percentages of BrdU$^+$ NK cells in spleen, liver, BM, and lymph nodes (LN) were determined. (*C*) Enriched WT (CD45.1) and *miR-155*$^{-/-}$ (CD45.2) NK cells (5×10^5) were cotransferred into *Rag2*$^{-/-}$ × *Il2rg*$^{-/-}$ mice, and the percentage of each NK cell population was determined at various time points after adoptive transfer. (*D*) Graph shows the relative contribution of WT and *miR-155*$^{-/-}$ cells to the overall NK cell population within individual mice. The bar represents the mean value.

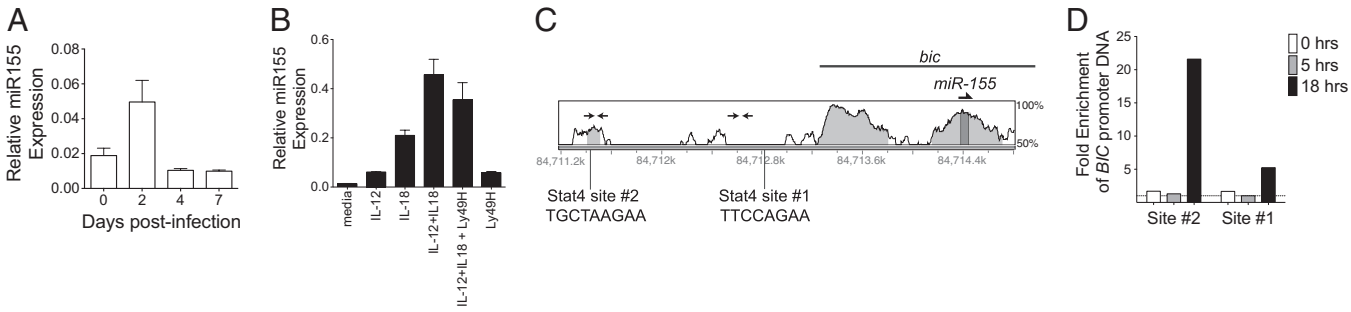

Fig. 3. Proinflammatory cytokines induce miR-155 in NK cells. (*A*) Splenic NK cells were sorted from uninfected or MCMV-infected mice on days 2, 4, and 7 PI. Relative expression of miR-155 was measured by qRT-PCR. Error bars show SEM. (*B*) Sorted NK cells were stimulated overnight with various combinations of IL-12, IL-18, and plate-bound anti-Ly49H mAbs. Relative miR-155 expression was determined by qRT-PCR. Error bars show SEM. (*C*) Evolutionarily conserved sequences within the *Bic* promoter and gene are shown in gray, and two consensus STAT4-binding sites in the promoter region are highlighted along with the primers used for qRT-PCR. miR-155 within the *Bic* gene is highlighted. (*D*) Sorted WT NK cells were stimulated with IL-12 and IL-18 for 0, 5, and 18 h, and STAT4 binding at the *Bic* promoter was assessed by ChIP followed by qRT-PCR. Target DNA levels were normalized to input. Data are expressed as the fold enrichment of target DNA after anti-STAT4 pull-down compared with control IgG pull-down.

miR-155 up-regulation, further suggesting that miR-155 is driven by proinflammatory cytokines produced immediately after infection, and not by activating receptor signaling.

We investigated what signaling pathways might mediate the effect of proinflammatory cytokines on miR-155 induction. Because IL-12 signals are known to induce phosphorylation and nuclear translocation of the transcription factor signal transducer and activator of transcription 4 (STAT4), we investigated the presence of consensus STAT4-binding motifs in the promoter of the *Bic* gene that encodes miR-155, and identified several putative STAT4-binding sites (Fig. 3*C*). ChIP of resting versus activated NK cells using an anti-STAT4 antibody followed by qRT-PCR confirmed activation-dependent binding of STAT4 to a site within the conserved noncoding sequence ~2-kb upstream from the *Bic* promoter (Fig. 3*D*). This finding suggests that STAT4 mediates enhanced expression of miR-155 in activated NK cells. Together, these experiments demonstrate that up-regulation of miR-155 in NK cells occurs during viral infection in response to proinflammatory cytokines.

Defective Expansion of *miR-155*$^{-/-}$ NK Cells During MCMV Infection. We investigated whether miR-155 plays a role during the viral-driven proliferation of NK cells. To exclude pleiotropic differences between WT and *miR-155*$^{-/-}$ mice, we took advantage of the mixed bone marrow chimeric mice described above and NK cell adoptive transfer systems (3, 4). We generated chimeric mice with equal numbers of mature WT and *miR-155*$^{-/-}$ NK cells by reconstituting irradiated recipients with twice as many bone marrow cells from *miR-155*$^{-/-}$ mice as from WT mice (Fig. 4*A*). On MCMV infection, we observed a preferential expansion of WT effector Ly49H^{+} NK cells at day 7 PI (Fig. 4*A*), with an average of 85% WT cells and only 15% *miR-155*$^{-/-}$ cells (Fig. 4*B*). Thus, miR-155 is absolutely crucial for the productive expansion of effector NK cells in response to MCMV infection.

Early after MCMV infection of the mixed bone marrow chimeras, WT and *miR-155*$^{-/-}$ NK cells demonstrated similar activation phenotypes and function, along with comparable up-regulation of activation markers, phosphorylation of STAT proteins, and production of IFN-γ and granzyme B (Fig. S3*A*). Consistent with miR-155–mediated effects on expansion at later time points in the NK cell response against MCMV infection, phenotypic differences were observed between WT and *miR-155*$^{-/-}$ NK cells at day 7 PI. A higher percentage of WT NK cells expressed killer cell lectin-like receptor subfamily G member 1 (KLRG1) and were CD27^{lo}CD11b^{hi} (Fig. S3*B*), demonstrating an enhanced activation state that presumably correlates with the increased expansion; interestingly, cytotoxicity was comparable in the WT and *miR-155*$^{-/-}$ NK cells. These findings indicate that miR-155 exerts specific influences over only certain NK cell effector mechanisms and not others during viral infection.

We also evaluated NK cells that had already undergone homeostatic proliferation for their ability to undergo antigen-driven expansion in response to MCMV infection. At 50 d after the initial transfer of WT or *miR-155*$^{-/-}$ NK cells into *Rag2*$^{-/-}$ × *Il2rg*$^{-/-}$ mice, with the ratio of WT:*miR-155*$^{-/-}$ Ly49H^{+} NK cells at ~2:1 (Fig. S4*A*), the mice were challenged with MCMV infection. Strikingly, the ratio of WT:*miR-155*$^{-/-}$ Ly49H^{+} NK cells grew to nearly 10:1 (Fig. S4*B*). To determine whether the number of *miR-155*$^{-/-}$ Ly49H^{+} NK cells was also diminished in lymphoid and nonlymphoid organs, we killed the mice at day 10 PI and

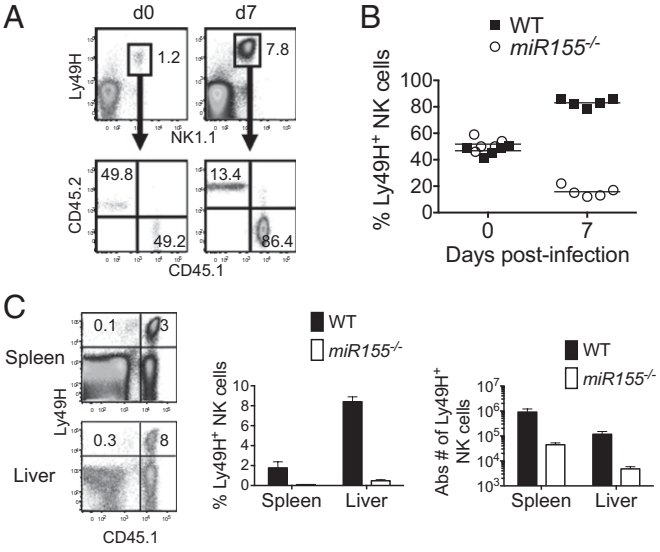

Fig. 4. *miR-155*$^{-/-}$ NK cells exhibit a profound defect in expansion during MCMV infection. (*A*) Mixed chimeric mice were infected with MCMV. Percentages of WT (CD45.1) and *miR-155*$^{-/-}$ (CD45.2) cells within the Ly49H^{+} NK cell population at day 0 and day 7 PI are shown. (*B*) Graph shows the relative percentages of WT and *miR-155*$^{-/-}$ Ly49H^{+} NK cells at day 0 and day 7 PI. The bar represents the mean value. (*C*) 10 d after MCMV infection (day 60 posttransfer), the percentages of WT and *miR-155*$^{-/-}$ Ly49H^{+} NK cells in spleen and liver were determined. WT is shown in the top right quadrant; *miR-155*$^{-/-}$ in the top left quadrant. Graphs show percentages and absolute numbers of WT and *miR-155*$^{-/-}$ Ly49H^{+} NK cells in spleen and liver. Error bars show SEM ($n = 3$). $P < 0.001$ for both organs.

IMMUNOLOGY

found a ratio of WT:*miR-155*$^{-/-}$ Ly49H^{+} NK cells of 30:1 in the spleen and 20:1 in the liver at this later time point (Fig. 4*C*). In both spleen and liver, the defective *miR-155*$^{-/-}$ Ly49H^{+} NK cell response was characterized by reduced down-regulation of CD62L and up-regulation of KLRG1, in addition to a decrease in mature or activated CD27^{lo}CD11b^{hi} NK cells compared with the less-mature CD27^{hi}CD11b^{lo} NK cell population (Fig. S4*C*). The pronounced phenotype observed in the adoptive transfer compared with the mixed chimera setting may be explained in part by the greater fold expansion of Ly49H^{+} NK cells during viral infection after adoptive transfer of NK cells.

Generation of NK Cell Memory Depends on miR-155. Because recent studies in both mice and humans have suggested that the lifespan of previously activated NK cells is on the order of months and years rather than days and weeks (1), we investigated whether miR-155 influences the longevity of "memory" NK cells after MCMV infection. Equal numbers of purified WT or *miR-155*$^{-/-}$ Ly49H^{+} NK cells were adoptively transferred into Ly49H-deficient mice, and percentages of Ly49H^{+} NK cells in each group were determined at various time points after MCMV infection. WT Ly49H^{+} NK cells proliferated robustly in the Ly49H-deficient hosts, in contrast to the *miR-155*$^{-/-}$ Ly49H^{+} NK cells (Fig. 5*A*). After the contraction of effector NK cells, larger numbers of long-lived memory NK cells were found within the WT group compared with the *miR-155*$^{-/-}$ group (Fig. 5*A*). The ratio of WT:*miR-155*$^{-/-}$ cells increased incrementally from day 7 PI (5:1) to day 14 PI (7:1) and finally to day 45 PI (13:1) (Fig. 5*B*), suggesting that miR-155 limits the contraction of effector NK cells during the memory maintenance phase.

Interestingly, although the overall numbers of Ly49H^{+} NK cells begin to favor the WT over *miR-155*$^{-/-}$ NK cells as early as day 4 PI, little difference in proliferation, as measured by dilution of carboxyfluorescein succinimidyl ester (CFSE), was observed between the two groups at day 4 or day 5 PI (Fig. 5*C*). The *miR-155*$^{-/-}$ Ly49H^{+} NK cells at day 45 PI do not preferentially home to nonlymphoid organs, as demonstrated by the similar ratios of WT: *miR-155*$^{-/-}$ NK cells detected in the blood, spleen, and liver (Fig. 5*D*). Taken together, these findings suggest that *miR-155*$^{-/-}$ NK cells likely have a normal trafficking pattern, but a reduced capacity for survival after viral infection.

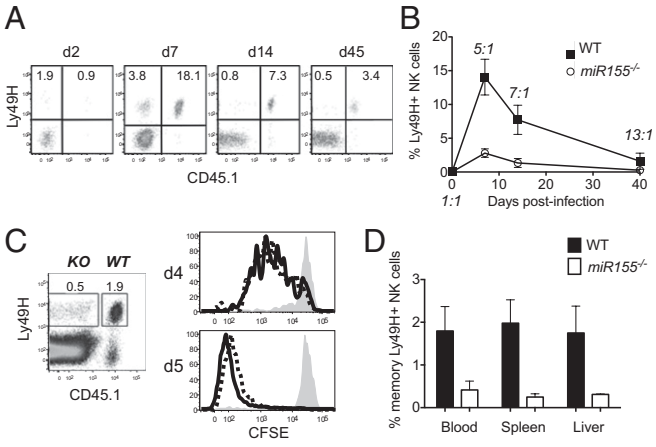

Fig. 5. miR-155 is required for robust memory NK cell generation after MCMV infection. (*A*) 10^5 WT (CD45.1) and *miR-155*$^{-/-}$ (CD45.2) Ly49H^{+} NK cells were cotransferred into Ly49H-deficient mice (CD45.2) that were then infected with MCMV. Plots are gated on total NK cells. Percentages of adoptively transferred Ly49H^{+} NK cells (WT in the top right quadrant and *miR-155*$^{-/-}$ in the top left quadrant) for each time point PI are shown. (*B*) Percentages of adoptively transferred WT and *miR-155*$^{-/-}$ Ly49H^{+} NK cells within the total NK cell population are graphed for indicated time points. Error bars show SEM ($n = 4$). Ratios of WT:*miR-155*$^{-/-}$ Ly49H^{+} NK cells are included at each time point. (*C*) CFSE-labeled WT and *miR-155*$^{-/-}$ NK cells were adoptively transferred, and after MCMV infection, the NK cells were recovered at days 4 and 5 PI. Histogram plots show data from WT Ly49H^{+} (CD45.1^{+} and solid line), *miR-155*$^{-/-}$ Ly49H^{+} (CD45.1^{-} gate and dotted line), and WT Ly49H^{-} (CD45.1^{+} and gray) NK cells. (*D*) Graph shows the percentage of memory WT and *miR-155*$^{-/-}$ Ly49H^{+} NK cells in blood, spleen, and liver of mice at day 45 PI. Error bars show SEM ($n = 4$). $P < 0.05$ for all organs.

miR-155 Regulates NK Cell Responses by Targeting Noxa and Suppressor of Cytokine Signaling 1. We investigated the genes that miR-155 may be targeting during NK cell homeostasis and activation, which might contribute to the expansion and survival defects observed in *miR-155*$^{-/-}$ NK cells after viral infection. We considered miR-155–binding sites that were identified through Argonaute cross-linking immunoprecipitation with high-throughput sequencing (CLIP-seq) (24). CLIP-seq identified two miR-155–binding sites in the 3′ UTR of the Noxa transcript (Fig. 6*A*), one of which was highly conserved across species (Fig. 6*B*). A previous report suggested that Noxa, a proapoptotic Bcl-2 homology domain (BH3)-only protein, is an important regulator of survival in NK cells (25). The CLIP-seq results also support miR-155 targeting of previously defined sites in suppressor of cytokine signaling 1(SOCS1), an important regulator of T-cell homeostasis (21).

To confirm direct regulation of Noxa and SOCS1 by miR-155, we generated luciferase reporter constructs containing the 3′ UTR of Noxa or of SOCS1 and cotransfected each of these with constructs expressing miR-155 or a control miRNA, miR-146a. Cotransfection of the Noxa 3′ UTR with miR-155 resulted in significant repression of reporter activity (Fig. 6*C*). The SOCS1 3′ UTR was similarly repressed by miR-155, although to a lesser degree (Fig. 6*C*).

To determine whether miR-155 targets Noxa and SOCS1 in resting or activated NK cells, we sorted WT and *miR-155*$^{-/-}$ Ly49H^{+} NK cells from WT:*miR-155*$^{-/-}$ chimeric mice at day 0, 2, 4, and 7 after MCMV infection and determined transcript levels of *Noxa* and *Socs1* by qRT-PCR. Compared with WT NK cells, *miR-155*$^{-/-}$ NK cells expressed higher levels of *Noxa* mRNA at day 0 PI (uninfected mice) and *Socs1* mRNA at day 7 PI (Fig. 6*D*).

To examine whether the elevated levels of Noxa or SOCS1 detected in *miR-155*$^{-/-}$ NK cells could be responsible for the expansion phenotype exhibited by these cells, we generated retrogenic mice in which Noxa or SOCS1 (or a control GFP vector) were constitutively expressed in half of the hematopoietic compartment (Fig. 6*E*). After MCMV infection, the NK cell response of the "Noxa Tg" or "SOCS1 Tg" populations was compared with that of the control retrogenic WT (GFP only) population. We observed that both Noxa- and SOCS1-expressing NK cells did not proliferate comparably to the control WT NK cells after MCMV infection, with WT NK cells expanding >10-fold and >6-fold more, respectively (Fig. 6*F*). Surprisingly, the known miR-155 target, Src homology-2 domain-containing inositol 5-phosphatase 1 (SHIP-1), was not elevated in *miR-155*$^{-/-}$ NK cells during infection (Fig. S5*A*), and overexpression experiments with SHIP-1 demonstrated no differences in NK cell responses (Fig. S5*B*). Together, these studies suggest that miR-155–mediated regulation of Noxa and SOCS1 can promote a robust NK cell response against MCMV infections.

Discussion

The activation and regulation of potent NK cell responses is known to occur at the cellular and molecular levels, and to include both cell-extrinsic and -intrinsic signals. Cross-talk between NK cells and other cells of the immune system via proinflammatory cytokines and surface receptors acts to drive NK cell activation as well as inhibition (26). For example, cytokines such as IL-12, IL-18, and type I IFNs, produced by cells such as dendritic cells, initiate NK cell responses against pathogens (1).

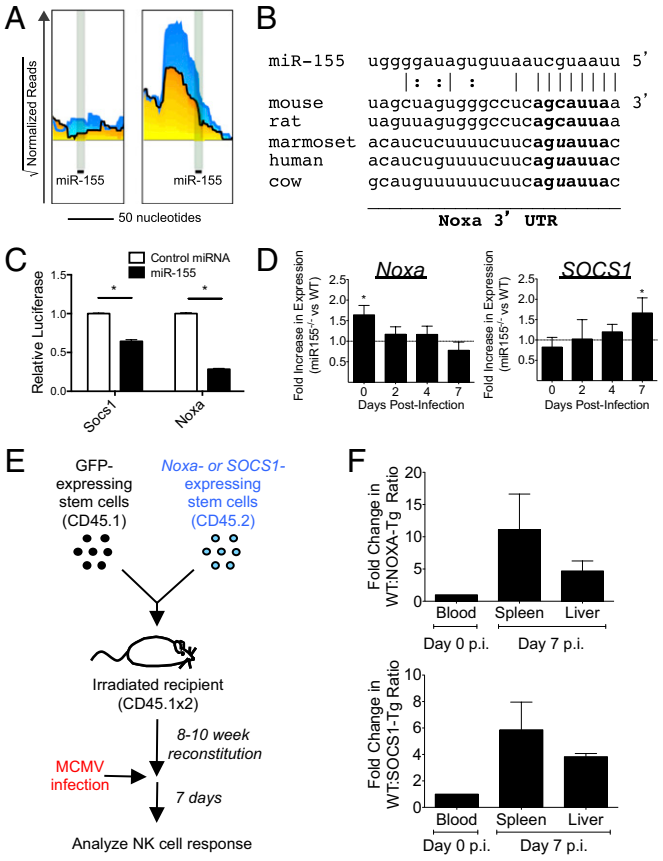

Fig. 6. miR-155 targets Noxa and SOCS1 in NK cells for enhanced survival and proliferation. (*A*) miR-155–binding sites in the 3′ UTR of Noxa demonstrated by differential CLIP-seq. Each blue shade represents Argonaute CLIP-seq reads from a single WT replicate, and each yellow shade represents Argonaute CLIP-seq reads from a *miR-155*$^{-/-}$ replicate. Twelve replicates for each genotype are stacked for visualization. Gray bars indicate the location of the miR-155 seed in the binding site. (*B*) Multiple species sequence alignment performed on a sequence from the Noxa 3′ UTR in which miR-155 is predicted to bind (shown in bold). (*C*) Repression of the Noxa and SOCS1 3′ UTRs by miR-155 assessed in luciferase reporter assays ($n = 4$). *$P < 0.001$. (*D*) WT and *miR-155*$^{-/-}$ Ly49H$^+$ NK cells from mixed chimeric mice infected with MCMV were sorted at indicated time points, and qRT-PCR was performed to determine levels of Noxa and SOCS1 mRNA. Data are shown as fold increase in expression in *miR-155*$^{-/-}$ NK cells compared with WT NK cells. Asterisks highlight time points at which the increase in mRNA expression exceeded 1.5-fold. (*E*) Experimental schematic shows generation of mixed bone marrow chimeric mice containing WT (CD45.1$^+$ and GFP$^+$) and Noxa- or SOCS1-expressing (CD45.2$^+$ and GFP$^+$) NK cells. After reconstitution of the hematopoietic compartment, mice were infected with MCMV and the NK cell response analyzed. (*F*) Graphs show fold-expansion observed in WT versus experimental (Noxa Tg or SOCS1 Tg) Ly49H$^+$ NK cell populations in spleen and liver over 7 d of MCMV infection. The baseline ratio of WT to experimental populations in each uninfected mouse was determined from peripheral blood and compared with the day 7 PI ratio in spleen and liver in the same mouse. Error bars show SEM ($n = 4$).

At the same time, potent NK cells must be restrained after pathogen clearance to limit "collateral damage" to healthy tissues; regulatory cytokines, such as IL-10 and TGF-β, have recently been suggested to constrain NK cell activity (27, 28). NK cells also express both activating and inhibitory receptors, which when ligated dictate the magnitude and duration of the effector response (26). NK cell responses may be further regulated by changes in gene expression. The role of miRNAs in regulating the transcriptome of many immune cell populations has been described (12, 29). Studies in which global miRNA ablation was achieved through the deletion of genes encoding miRNA-processing enzymes Dicer and Dgcr8 in mice have demonstrated severe defects in NK cell development and function (6, 8). In the present study, we present evidence indicating that a specific miRNA, miR-155, is critical for regulating the in vivo maturation, homeostasis, and antiviral response of NK cells.

Deletion of miR-155 resulted in enhanced maturation of NK cells, along with a reduction in immature NK cell populations that are less differentiated but have greater proliferative potential. An increase in the number of terminally differentiated resting NK cells in miR-155–deficient populations may explain why these cells cannot compete with WT NK cells in homeostatic and lymphopenic environments. Given that the proapoptotic molecule Noxa has been implicated as an antagonist of myeloid cell leukemia sequence 1 (Mcl-1), a critical factor in IL-15–mediated survival of NK cells (25), and that we have validated Noxa as a functionally important target of miR-155, a mechanism might exist in which developing and resting NK cells require low levels of miR-155 to suppress Noxa levels for enhanced NK cell survival and homeostasis (Fig. S6*A*). Consistent with this hypothesis, a recent study suggested that miR-155 protects macrophages from apoptosis through its regulation of multiple proapoptotic genes, including Noxa (30). Although *Noxa* mRNA levels did not differ greatly in WT and *miR-155*$^{-/-}$ NK cells after viral infection, miR-155–mediated suppression of Noxa, in addition to numerous other gene targets, may still be a contributing factor to the preferential expansion and memory maintenance of WT compared with *miR-155*$^{-/-}$ or Noxa-expressing NK cells observed after MCMV infection.

During MCMV infection, miR-155 was absolutely required for a robust NK cell response. A recent study in regulatory T cells demonstrated that miR-155 targets SOCS1, resulting in loss of protein, but not of SOCS1 mRNA expression (21). In contrast, activated NK cells lacking miR-155 had elevated levels of SOCS1 transcripts compared with WT NK cells after viral infection (Fig. S6*B*). Given that SOCS1 is known to suppress various STATs downstream of proinflammatory cytokine signaling (31), along with our recent demonstration that IL-12 and STAT4 signaling are required for the generation of robust effector and memory cell numbers (32), we hypothesized that miR-155–mediated SOCS1 repression might allow for enhanced NK cell proliferation. In support of our hypothesis, both miR-155–deficient NK cells and NK cells with constitutive SOCS1 expression failed to proliferate substantially after MCMV infection (Fig. S6*B*). Perhaps regulation of this potent suppressor of STAT signals by miRNAs has evolved to confer extensive proliferative and survival advantages during cellular responses against pathogens.

Previous studies have identified SHIP-1 as an important target of miR-155 in human NK cells, as well as in macrophages and B cells (33–36). However, we found no differences in SHIP-1 transcript in WT and *miR-155*$^{-/-}$ NK cells, or any defects in development or MCMV-induced expansion in NK cells from mixed bone marrow chimeric mice in which SHIP-1 was constitutively expressed by retroviral transduction. Whether regulation of SHIP-1 by miR-155 is a cell type- or species-specific phenomenon that does not occur in mouse NK cells, or whether regulation occurs but is nevertheless dispensable for mouse NK cell function, is unknown. With respect to the latter, we have previously shown that both resting and activated mouse NK cells harbor very high levels of SHIP-1 (37, 38), such that targeting by miR-155 may have an inconsequential effect on the overall abundance of SHIP-1. Whether the plethora of SHIP-1 mRNA acts as a "sink" for miR-155 in NK cells (39) and reduces the availability of miR-155 for other mRNA targets remains to be investigated. Although additional targets of miR-155 regulate NK cell development, homeostasis, and response against pathogens remain to be identified, the present study suggests that

miR-155 is able to influence the NK cell response in a multifaceted manner, targeting distinct cell-intrinsic pathways of effector cell function, cell cycle, and survival.

Materials and Methods

Mice, Adoptive Transfer, Chimeras, and Viral Infection. Detailed descriptions of the WT and KO strains of mice used in this study are provided in *SI Materials and Methods*. Adoptive transfer experiments were performed and mixed bone marrow chimeric mice were generated as described previously (3). Mice overexpressing Noxa, SOCS1, and SHIP-1 were generated as described previously (40); details are provided in *SI Materials and Methods*. Mice were infected by i.p. injection of Smith strain MCMV (10^4 pfu).

NK Cell Stimulation and Quantitative RT-PCR. Total RNA (including miRNA) was extracted from NK cells stimulated with antibodies or cytokines, or sorted from naïve and infected mice. Amounts of miR-155, Noxa, SOCS1, and controls were measured by qRT-PCR. Detailed descriptions of protocols and primers are provided in *SI Materials and Methods*.

ChIP, Argonaute CLIP-seq, and Luciferase Reporter Assays. ChIP was performed on NK cells activated with recombinant mouse IL-12 (20 ng/mL) and IL-18 (10 ng/mL) using an anti-STAT4 Ab or control IgG, and relative amounts of target DNA were determined by qRT-PCR. Argonaute CLIP-seq was performed on WT and miR-155–deficient T cells (24). Luciferase reporter assays were performed by transfection with expression vectors containing miR-155 (or control miRNA), along with vectors containing the 3′ UTR of target genes. Detailed descriptions of protocols, plasmids, and primers are provided in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank members of the J.C.S. laboratory for technical support and experimental assistance, and members of the Memorial Sloan-Kettering Cancer Center NK Club for insightful comments and helpful discussions. A.M.B. is supported by a National Institutes of Health (NIH) T32 award (CA009149). G.B.L. is supported by a predoctoral Cancer Research Institute training grant. N.A.B. is supported by an American Cancer Society Postdoctoral Fellow grant. L.L.L. is an American Cancer Society Professor and is funded by NIH Grant AI068129. A.Y.R. is an investigator with the Howard Hughes Medical Institute and is funded by NIH Grant R37 AI034206. J.C.S. is supported by the Searle Scholars Program, the Cancer Research Institute, and the NIH (Grants AI085034 and AI100874).

1. Sun JC, Lanier LL (2011) NK cell development, homeostasis and function: Parallels with CD8+ T cells. *Nat Rev Immunol* 11(10):645–657.
2. Yokoyama WM, Kim S, French AR (2004) The dynamic life of natural killer cells. *Annu Rev Immunol* 22:405–429.
3. Sun JC, Beilke JN, Lanier LL (2009) Adaptive immune features of natural killer cells. *Nature* 457(7229):557–561.
4. Sun JC, Beilke JN, Bezman NA, Lanier LL (2011) Homeostatic proliferation generates long-lived natural killer cells that respond against viral infection. *J Exp Med* 208(2): 357–368.
5. Beaulieu AM, et al. (2013) MicroRNA function in NK cell biology. *Immunol Rev* 253: 1–13.
6. Bezman NA, et al. (2010) Distinct requirements of microRNAs in NK cell activation, survival, and function. *J Immunol* 185(7):3835–3846.
7. Fehniger TA, et al. (2010) Next-generation sequencing identifies the natural killer cell microRNA transcriptome. *Genome Res* 20(11):1590–1604.
8. Sullivan RP, et al. (2012) MicroRNA-deficient NK cells exhibit decreased survival but enhanced function. *J Immunol* 188(7):3019–3030.
9. O'Connell RM, Baltimore D (2012) MicroRNAs and hematopoietic cell development. *Curr Top Dev Biol* 99:145–174.
10. O'Connell RM, Rao DS, Baltimore D (2012) microRNA regulation of inflammatory responses. *Annu Rev Immunol* 30:295–312.
11. Bezman NA, Chakraborty T, Bender T, Lanier LL (2011) miR-150 regulates the development of NK and iNKT cells. *J Exp Med* 208(13):2717–2731.
12. Xiao C, Rajewsky K (2009) MicroRNA control in the immune system: Basic principles. *Cell* 136(1):26–36.
13. Tam W (2001) Identification and characterization of human *BIC*, a gene on chromosome 21 that encodes a noncoding RNA. *Gene* 274(1-2):157–167.
14. Tam W, Ben-Yehuda D, Hayward WS (1997) *bic*, a novel gene activated by proviral insertions in avian leukosis virus-induced lymphomas, is likely to function through its noncoding RNA. *Mol Cell Biol* 17(3):1490–1502.
15. Rodriguez A, et al. (2007) Requirement of bic/microRNA-155 for normal immune function. *Science* 316(5824):608–611.
16. Thai TH, et al. (2007) Regulation of the germinal center response by microRNA-155. *Science* 316(5824):604–608.
17. Dorsett Y, et al. (2008) MicroRNA-155 suppresses activation-induced cytidine deaminase-mediated Myc-Igh translocation. *Immunity* 28(5):630–638.
18. Teng G, et al. (2008) MicroRNA-155 is a negative regulator of activation-induced cytidine deaminase. *Immunity* 28(5):621–629.
19. Vigorito E, et al. (2007) microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. *Immunity* 27(6):847–859.
20. O'Connell RM, et al. (2010) MicroRNA-155 promotes autoimmune inflammation by enhancing inflammatory T cell development. *Immunity* 33(4):607–619.
21. Lu LF, et al. (2009) Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein. *Immunity* 30(1):80–91.
22. O'Connell RM, et al. (2008) Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *J Exp Med* 205(3):585–594.
23. Turner ML, Schnorfeil FM, Brocker T (2011) MicroRNAs regulate dendritic cell differentiation and function. *J Immunol* 187(8):3911–3917.
24. Loeb GB, et al. (2012) Transcriptome-wide miR-155 binding map reveals widespread noncanonical microRNA targeting. *Mol Cell* 48(5):760–770.
25. Huntington ND, et al. (2007) Interleukin 15-mediated survival of natural killer cells is determined by interactions among Bim, Noxa and Mcl-1. *Nat Immunol* 8(8):856–863.
26. Lanier LL (2005) NK cell recognition. *Annu Rev Immunol* 23:225–274.
27. Marcoe JP, et al. (2012) TGF-β is responsible for NK cell immaturity during ontogeny and increased susceptibility to infection during mouse infancy. *Nat Immunol* 13(9): 843–850.
28. Yu J, et al. (2006) Pro- and antiinflammatory cytokine signaling: reciprocal antagonism regulates interferon-gamma production by human natural killer cells. *Immunity* 24(5):575–590.
29. O'Connell RM, Rao DS, Chaudhuri AA, Baltimore D (2010) Physiological and pathological roles for microRNAs in the immune system. *Nat Rev Immunol* 10(2):111–122.
30. Koch M, Mollenkopf HJ, Klemm U, Meyer TF (2012) Induction of microRNA-155 is TLR- and type IV secretion system-dependent in macrophages and inhibits DNA-damage induced apoptosis. *Proc Natl Acad Sci USA* 109(19):E1153–E1162.
31. Yoshimura A, Naka T, Kubo M (2007) SOCS proteins, cytokine signalling and immune regulation. *Nat Rev Immunol* 7(6):454–465.
32. Sun JC, et al. (2012) Proinflammatory cytokine signaling required for the generation of natural killer cell memory. *J Exp Med* 209(5):947–954.
33. Trotta R, et al. (2012) miR-155 regulates IFN-γ production in natural killer cells. *Blood* 119(15):3478–3485.
34. Costinean S, et al. (2009) Src homology 2 domain-containing inositol-5-phosphatase and CCAAT enhancer-binding protein beta are targeted by miR-155 in B cells of Emicro-MiR-155 transgenic mice. *Blood* 114(7):1374–1382.
35. Cremer TJ, et al. (2009) MiR-155 induction by *F. novicida* but not the virulent *F. tularensis* results in SHIP down-regulation and enhanced pro-inflammatory cytokine response. *PLoS ONE* 4(12):e8508.
36. O'Connell RM, Chaudhuri AA, Rao DS, Baltimore D (2009) Inositol phosphatase SHIP1 is a primary target of miR-155. *Proc Natl Acad Sci USA* 106(17):7113–7118.
37. Bezman NA, et al.; Immunological Genome Project Consortium (2012) Molecular definition of the identity and activation of natural killer cells. *Nat Immunol* 13(10): 1000–1009.
38. Sun JC, Lopez-Verges S, Kim CC, DeRisi JL, Lanier LL (2011) NK cells and immune "memory." *J Immunol* 186(4):1891–1897.
39. Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP (2011) A ceRNA hypothesis: The Rosetta Stone of a hidden RNA language? *Cell* 146(3):353–358.
40. Sun JC, Lanier LL (2008) Tolerance of NK cells encountering their viral ligand during development. *J Exp Med* 205(8):1819–1828.