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Summary: The important role of microRNAs in directing immune responses has become increasingly clear. Here, we highlight discoveries uncovering the role of specific microRNAs in regulating the development and function of natural killer (NK) cells. Furthermore, we discuss the impact of NK cells on the entire immune system during global and specific microRNA ablation in the settings of inflammation, infection, and immune dysregulation.

Keywords: natural killer, NK, microRNA, development, cytotoxicity

General introduction

Natural killer (NK) cells are critical mediators of host immunity to malignancy and infection. Humans (and mice) lacking functional NK cells exhibit increased susceptibility to a variety of viral pathogens, especially herpesviruses including cytomegalovirus, Epstein–Barr virus, varicella zoster virus, and herpes simplex virus (1–6). Similarly, NK cells mediate potent anti-tumor responses in mice (7–10) and, as evidenced in a growing number of clinical studies, in humans (7, 11–19).

NK-cell function is controlled by germ-line encoded surface receptors, which can be broadly categorized as either activating or inhibitory. Activating receptors, such as NKG2D and Ly49H in mice or KIR2DL and CD94-NKG2C in humans, recognize self- or virally encoded molecules expressed on infected or malignant cells and transduce signals that drive cytotoxic responses (20–23). In contrast, many of the inhibitory receptors bind to self-major histocompatibility complex class I molecules expressed on healthy cells and are important for restraining NK-cell function (22, 24–26). In addition, mature NK cells express receptors for and are highly responsive to proinflammatory and prosurvival cytokines such as interleukin-12 (IL-12), IL-18, IL-2, Type I interferons (IFNs), and IL-15, which can activate NK cells alone or synergize with signals downstream of activating receptors (20, 27–33).

Productive NK-cell activation leads to cytotoxicity, cytokine secretion, and in specific cases, the robust proliferation

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of antigen-specific effector cells (34). NK-cell-mediated cytotoxicity, which involves the targeted release of lytic molecules such as perforin and granzyme B, results in apoptosis of virus-infected, malignant, or diseased host cells. In addition, NK-cell-derived chemokines and cytokines, most notably IFN- γ , can exert potent regulatory effects on target cells, as well as recruit and activate other cells of the immune system, including macrophages and dendritic cells, and T and B lymphocytes, which mediate further antiviral and anti-tumor immunity (35, 36).

In addition to cytotoxicity and cytokine secretion, certain stimuli (e.g. specific viral proteins) are able to trigger robust clonal proliferation in NK cells. This response shares many features with CD8⁺ T-cell responses, including antigen specificity and dependency, defined proliferation and contraction phases, and the development of a small pool of long-lived 'memory' cells, which exhibit heightened functionality upon secondary antigen exposure (34, 37–40). For example, certain mouse strains (such as C57BL/6) harbor a subset of NK cells that express the Ly49H-activating receptor (6, 41, 42). This receptor can recognize the m157 glycoprotein encoded by the mouse cytomegalovirus (MCMV). During MCMV infection in C57BL/6 mice, binding of the Ly49H receptor to the m157 viral protein expressed on infected cells triggers a robust, clonal expansion of Ly49H⁺ NK cells (43–45). A similar clonal-like expansion has been observed for human NK cells expressing the activating receptor, CD94-NKG2C, during human cytomegalovirus infection, suggesting a conserved mechanism of antigen-driven NK-cell expansion (46).

MicroRNAs (miRNAs) comprise a large family of small, non-coding regulatory RNAs that act as post-transcriptional repressors of protein-coding target messenger RNA (mRNA) species (47). miRNAs are initially generated as long primary transcripts (pri-mRNAs) that fold into a hairpin secondary structure (48). Pri-mRNAs are then processed by a nuclear enzyme complex containing the RNase III-like enzyme, Drosha, and its double-stranded RNA (dsRNA)-binding co-factor, DiGeorge syndrome critical region 8 protein (Dcgr8), into 70–80 nucleotide dsRNA precursors (known as pre-miRNAs) (49–51). Following export to the cytoplasm via the nuclear transporter Exportin V (52, 53), pre-mRNAs undergo further enzymatic trimming by a second RNase III-like enzyme, Dicer, yielding 16–25 nucleotide length RNA duplexes that contain the mature miRNA (54). One strand of the mature miRNA duplex interacts with an Argonaute family protein (55), of which there are four known in mammals (Ago1–Ago4), to form a miRNA-induced

silencing complex (miRISC) (47). Regulation is achieved by binding of the miRNA (as part of the larger miRISC) to imperfect complementary 'seed' sequences often located in the 3'-untranslated region (3'-UTR) of the target mRNA. Formation of the miRNA:mRNA complex promotes degradation and/or prevents translation of the target mRNA, thus silencing protein expression of targeted genes (47, 56–58).

miRNAs in higher vertebrates are often highly conserved and can each target hundreds of distinct protein-coding mRNA targets (59–62). Although complete elimination of any one target is rarely achieved by miRNAs in mammalian cells, simultaneous downmodulation of many genes provides a mechanism by which a single miRNA, or several miRNAs in concert, can drastically modify the global expression profile of a cell (62, 63). For this reason, miRNAs are important regulators of diverse aspects of cellular development and function. Here, we summarize recent findings on the role of miRNAs in NK-cell biology in the context of development, inflammation, tumor surveillance, and viral infection.

miRNAs in NK-cell development and maturation

Like T and B cells, NK cells develop from pluripotent common lymphoid progenitor (CLP) cells in a multistage process that occurs primarily in the bone marrow in adults (although peripheral sites of development have been described) (34, 64–67). Discrete developmental stages can be identified by the sequential acquisition of distinct surface receptors. Very early NK-cell progenitors (pre-NK cells) in mice express CD122, the shared γ -chain of the IL-2 and IL-15 receptors. Studies in IL-15- and IL-15R-deficient mice, as well as *in vitro* models of human and mouse NK-cell differentiation, have shown that IL-15 is required for the survival of NK cells past the progenitor stage (68–71). As development progresses, pre-NKs acquire expression of the NK-cell-lineage-defining receptors that will ultimately control their activation and effector function; examples include NK1.1 (in C57BL/6 and SJL mouse strains), CD94-NKG2A, NKG2D, CD49b, and natural cytotoxicity receptors, along with the Ly49 receptors in mice and NKG2C, NKG2A, and KIR in humans. These immature NK cells then acquire the final vestiges of cytotoxic and proinflammatory functionality: expression of preformed transcripts for granzyme B, perforin, and IFN- γ , the translation of which is rapidly initiated upon NK-cell activation in the periphery. These functional but still immature NK cells emigrate from the bone marrow and undergo further differentiation and maturation in peripheral tissues such as the spleen and liver. In mice, terminal maturation in the periphery is associated with downregulation of

CD27 and upregulation of CD11b expression, as well as acquisition of full cytolytic and cytokine secretion potential.

Recent work in animals lacking specific or global miRNAs, in combination with comprehensive miRNA expression profiling studies, has allowed investigators to explore the mechanisms by which miRNAs regulate NK-cell development. Microarray studies revealed the expression of nearly 200 unique miRNAs in human and mouse primary NK cells (72). Among these, 80% of those identified in human NK cells could be found in their mouse counterparts, and 59% of the miRNAs present in mouse NK cells were also found in human NK cells, indicating significant interspecies overlap (72). Among the most highly expressed miRNAs in both mouse and human NK cells were miR-150, miR-23b, miR-29a, miR-23a, miR-16, miR-21, let-7a, let-7f, miR-24, miR-15b, miR-720, let-7g, miR-103, and miR-26a (Fig. 1). Similar results were generated in separate studies that used next-generation sequencing to identify >400 miRNAs in human (73) and >300 in mouse NK cells (74). In the latter study, the top 10 most highly expressed in NK cells accounted for approximately 65% of the total miRNA pool (74).

Early studies addressing the importance of miRNAs in NK-cell development took advantage of the universal role of Dicer and Dgcr8 in miRNA biogenesis. To circumvent the embryonic requirement for Dicer and Dgcr8, Bezman et al. (72) studied the impact of global miRNA deficiency on NK-cell development using genetically modified mice expressing either loxP-flanked ('floxed') *Dicer1* or *Dgcr8* alleles and a chimeric Cre recombinase that could be specifically activated by exogenous tamoxifen treatment. In this system, drug-induced deletion of *Dicer1* or *Dgcr8* led to a significant decrease in the number of splenic and liver NK cells, and this defect was shown to stem from impaired survival and proliferation in these cells (72). In addition, both *Dicer*- and *Dgcr8*-deficient mice harbored a relative deficit of mature (CD27^{lo}CD11b^{hi}) NK cells and a relative surplus of immature (CD27^{hi}CD11b^{lo}) NK cells, highlighting a role for miRNAs in NK-cell maturation (72). Although *Dicer* is required for the biogenesis of other small RNAs (including siRNA, shRNA, and snoRNAs), the fact that deletion of *Dgcr8*, which is involved only in miRNA synthesis, similarly impaired NK-cell development suggested that loss of miRNAs was specifically responsible for the observed defect in both animal cohorts (72). Sullivan et al. (75) further showed that the requirement for miRNAs in NK-cell development was cell intrinsic because deletion of *Dicer1* only in developing lymphocytes (via

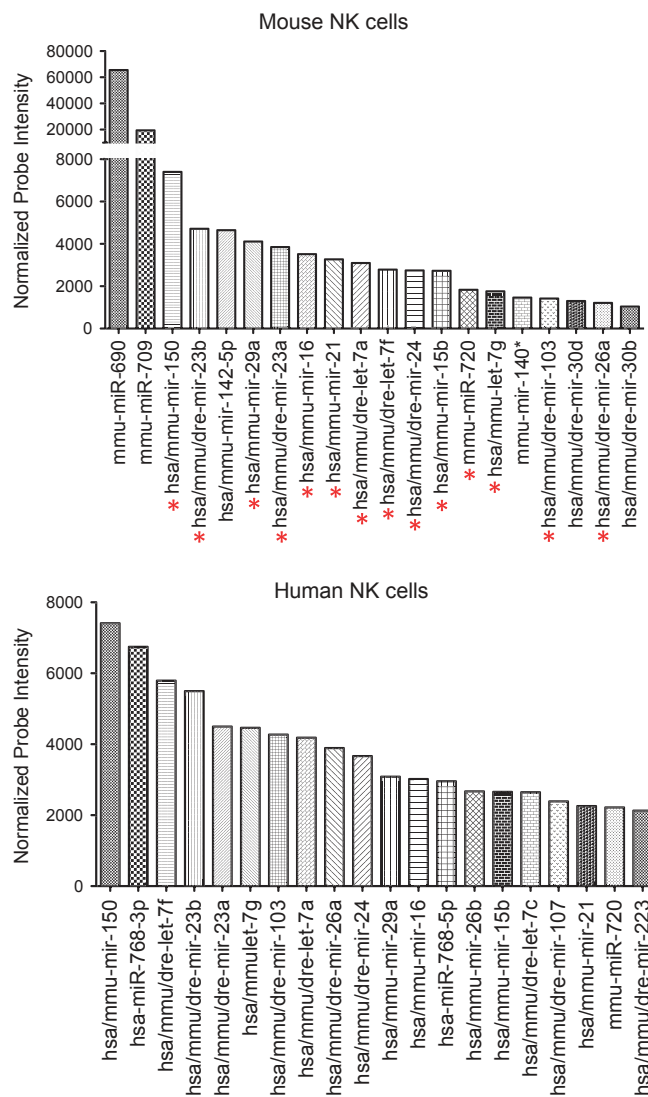


Fig. 1. Top 20 most highly expressed miRNAs in resting mouse and human natural killer (NK) cells by microarray. Total RNA was extracted from sorted mouse splenic NK cells (NK1.1⁺ TCRβ⁻) and human peripheral blood NK cells (CD56⁺CD3⁻). Expression of individual miRNAs was assessed by microarray. After normalization, 170 oligonucleotide probes gave mean fluorescence values above background. Shown are the normalized expression values (in arbitrary units) of the top 20 most highly expressed miRNAs in mouse (top) and human (bottom) NK cells. miRNAs common to both groups are indicated with a red asterisk below the top graph.

transgenic expression of a human CD2 promoter-driven Cre cassette) also led to a reduced frequency and impaired maturation of NK cells.

Although global miRNA deficiency impairs NK-cell homeostasis and maturation, recent work by Thomas et al. (76) suggests that an overabundance of miRNAs may similarly have a negative impact on NK-cell development. Mice lacking *Eri1*, a 3'-to-5' exoribonuclease that suppresses small RNA function in mammalian cells, have a global and

sequence-independent increase in miRNA abundance. Interestingly, these mice also exhibited a substantial reduction and aberrant maturation of peripheral NK cells, defined by delayed acquisition of Ly49 receptors in the bone marrow and a reduction in cells expressing Ly49D and Ly49H in the periphery (76). Using mixed bone marrow chimeric animals, the requirement for Eri1 in developing NK cells was found to be cell intrinsic. Thus, either elevation or reduction in the cellular levels of miRNA expression has a negative impact on NK-cell homeostasis.

Although informative, modulation of global miRNA levels, as occurs in *Dicer-*, *Dcgr8-*, and *Eri1-*deficient mice, does not allow for delineation of which miRNAs regulate NK-cell development and their individual mechanisms of action. Rather, experimental modulation of individual miRNAs has been necessary to understand their specific roles in the context of NK-cell biology. miR-150, for example, is important for B- and T-cell development and function (77–80) and, given its high expression in both mouse and human resting NK cells (72) (Fig. 1), it was identified as a potential regulator of NK-cell function. Indeed, genetic deletion of miR-150 led to a significant reduction in the number of peripheral, mature NK cells in mice (81). This reduction was attributed to a developmental blockade in NK-cell maturation, as evidenced by the accumulation of immature NK cells in both the bone marrow and the peripheral organs of *Mir150^{-/-}* animals. Reciprocally, transgene-mediated overexpression of miR-150 increased the number and frequency of NK cells in mice and led to the development of NK cells with a more mature phenotype (81). The developmental phenotype of *Mir150^{-/-}* NK cells was attributed in part to increased expression of *c-Myb*, a known miR-150 target (78) and a direct transcriptional activator of *Myc* (82, 83) and *Bcl-2* (84), genes with important pro-proliferative and prosurvival functions, respectively, in developing lymphocytes. *Mir150^{-/-}* NK cells exhibited elevated levels of *c-Myb* and its downstream targets, *c-Myc* and *Bcl-2*, supporting this hypothesis (81). Moreover, mice lacking one *Myb* allele exhibited a phenotype that closely mirrored that of miR-150 overexpression, i.e. an increase in the number and frequency of mature NK cells (81). Thus, miR-150, on its own, is critical for NK-cell development and maturation in part because of its function as a regulator of *c-Myb* expression (81).

Another miRNA, miR-181, was recently reported to be upregulated in developing human NK cells. Consistent with its known role in T- and B-cell development (85, 86), knockdown of miR-181 in human CD34⁺ hematopoietic

progenitor cells dramatically blocked their ability to develop into NK cells *in vitro* (87). Mechanistically, miR-181 was found to be required for the repression of nemo-like kinase (NLK) (87), a known inhibitor of Notch signaling [which is important for NK-cell development (88, 89)]. Knockdown of miR-181 led to an increase in NLK protein, which correlated with impaired NK-cell development, whereas either NLK deficiency or miR-181 overexpression enhanced development (87). The signals that regulate miR-181 expression in developing NK cells are not fully understood. However, miR-181 was recently shown to be induced by and act downstream of the T-cell receptor (TCR) in mouse T cells (85). In this context, miR-181 acted as a modulator of TCR signal strength by repressing the protein tyrosine phosphatases, *Ptpn22*, *Shp2*, *Dusp5*, and *Dusp6*, and thereby boosting basal TCR signals. Given that all of these phosphatases are expressed in mouse NK cells (90, 91), it has been suggested that activating NK-cell receptors may similarly utilize miR-181 to modulate signal strength downstream of receptor engagement in NK cells (72). Thus, both global and specific ablation of miRNAs results in the dysregulation of NK-cell development.

miRNAs in NK-cell homeostasis and cancer

In addition to acting as regulators of developmental processes, studies in NK-cell lymphomas have implicated miRNAs as important repressors of oncogenic transformation during homeostasis. Overexpression of *c-Myc*, a classic oncogene implicated in the pathogenesis of a wide variety of cancers, was recently shown to repress a number of anti-proliferative miRNAs during tumorigenesis (92). In keeping with a putative tumor suppressor function, a number of miRNAs (e.g. miR-150, miR-342-5p, miR-22*, miR-181a-2*, miR-101, miR-26a, miR-26b, miR-28-5, miR-363, and miR-146) have been found to be downregulated in human NK-/T-cell lymphomas (NKTLs) (93–95). Moreover, downregulation of at least one of these, miR-146a, was shown to independently predict poor clinical outcomes in NKTL patients (93). Additional evidence that miRNAs may function as tumor suppressors comes from experimental data showing that ectopic re-expression of many of these downregulated miRNAs, including miR-146a and miR-150, was sufficient to reduce the growth rate and/or increase the incidence of apoptosis in NK cell lines (93–95). Computational prediction of the mRNAs targeted by these miRNAs revealed an enrichment of genes involved in cell-cycle regulation, p53 signaling, and mitogen-activated protein kinase (MAPK) signaling, pathways with critical regulatory roles in

cellular transformation. Several of the predicted targets, including *STMN1*, *IRF4*, *PRDM11*, and *BCL2*, were verified experimentally to be overexpressed in NKTL tumors (94).

Specific examples of miRNAs that regulate oncogenic potential in NK cells include miR-150, miR-146a, and miR-30b. As mentioned above, re-expression of miR-150 in miR-150^{lo} NKTL cell lines can promote apoptosis and reduce cellular proliferation, as well as dampen phosphoinositide-3-kinase-(PI3K)-AKT activation (95), a pathway that has been implicated in NK-/T-cell lymphomagenesis (96). These activities were linked to the ability of miR-150 to repress *DKC1*, a protein involved in telomere maintenance (97), and *AKT2*, a PI3K-AKT-associated kinase with putative oncogenic functions in human hepatocellular carcinomas and colorectal cancers (98, 99). Re-expression of miR-150 in human NKTL cell lines reduced *DKC1* and *AKT2* levels, lowered telomerase activity, and increased levels of tumor suppressor proteins such as *Bim* and *p53*, thereby promoting cellular senescence and apoptosis, respectively (95). Like miR-150, re-introduction of miR-146a in miR-146^{lo} NKTL cell lines was shown to suppress cellular proliferation and promote apoptosis (93). This was attributed to the ability of miR-146a to repress *TRAF6*, an activator of NF κ B signaling, and thereby reduce expression of the anti-apoptotic factor, *BCL-2*, which is upregulated downstream of the NF κ B pathway (93). And lastly, miR-30b was shown in NKTL cells to directly target the 3'-UTR of *BLIMP1* (94), an important lineage-specification factor associated with B-cell lymphomas in patients. Thus, miRNAs are important for promoting cell-cycle regulation, cellular senescence, and apoptosis, processes that result in immortalization and aberrant proliferation during miRNA dysregulation.

Although some may act as tumor suppressors, other miRNAs may promote cell proliferation and survival, features that can also be dysregulated in transformed cells to exacerbate oncogenic traits. miR-21 and miR-155, for example, have been shown to be overexpressed in NKTL cells and knockdown of either resulted in increased expression of phosphatase and tensin homolog (*PTEN*), programmed cell death 4 (*PDCD4*), and *SHIP1*, factors that dampen NK-cell proliferation and function (96). In addition to promoting the expression of negative regulatory factors, knockdown of miR-21 or miR-155 decreased expression of the pro-proliferative factor, *pAKT*. Conversely, overexpression of miR-21 or miR-155 had the opposite effect on the expression levels of these genes (96).

Thus, miRNAs regulate processes associated with basal homeostasis, cell turnover, and survival at steady-state

(i.e. in the absence of inflammation, infection, or disease), and dysregulated miRNA expression in immune cells, including NK cells, can result in diseases such as cancer (Fig. 2). miRNAs will undoubtedly be discovered to play specific roles in NK-cell homeostasis. In addition to promoting transformation of NK cells themselves, miRNA-specific loss of NK-cell function could lead to diminished tumor immunosurveillance by this arm of the immune system, resulting in miRNA-unrelated but NK-cell-related transformation and disease.

miRNAs in NK-cell effector responses

Evidence that miRNAs regulate not only NK-cell development but also effector function is rooted in early studies evaluating the effects of disrupting *Dicer* or *Dcgr8* in mouse NK cells. Although these studies reported conflicting details of exactly how miRNAs regulate NK-cell function, they nevertheless agree that global loss of miRNAs has a significant impact on NK-cell activation and effector function. Bezman et al. (72) showed that tamoxifen-induced global deletion of either *Dicer1* or *Dcgr8* in adult mice impaired the ability of NK cells to degranulate or produce IFN- γ in response to activation through activating receptors, including NK1.1, NKp46, and Ly49H. Interestingly, this defect was not observed when the cells were stimulated with the pro-inflammatory cytokines, IL-12 and IL-18 (72). In addition to cytokine production, miRNA deficiency in these animals also impaired the ability of antigen-specific Ly49H⁺ NK cells to

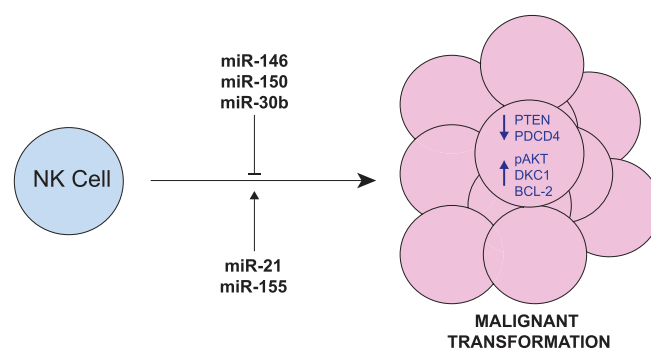


Fig. 2. miRNAs have oncogene and tumor suppressor activity in natural killer (NK) cells. miR-150 and miR-21 are often overexpressed in natural killer/T-cell lymphomas (NKTLs) and have been shown to promote oncogenic transformation by repressing tumor suppressor factors (e.g. *PTEN* and *PDCD4*) and by promoting pro-proliferative pathways (e.g. the PI3K-AKT cascade). In contrast, miR-146, miR-150, and miR-30b, which are often underexpressed in NKTLs, have been attributed with tumor suppressor activity, owing in part to their ability to repress pro-proliferative and prosurvival factors, such as *AKT2*, *DKC1*, and, indirectly, *BCL-2*.

expand in response to MCMV challenge *in vivo*. This defect was attributed to a requirement for miRNAs in the survival of activated NK cells (72). Contradictory results were obtained in an independent study by Sullivan et al. (75), where it was shown that deletion of *Dicer1* in developing NK cells, using human CD2 promoter-controlled Cre expression, actually enhanced IFN- γ production and degranulation by NK cells following activation via proinflammatory cytokines, stimulation through the NK1.1 receptor, or incubation with YAC-1 target cells, an NK-sensitive tumor cell line. Similarly, *Dicer*-deficient NK cells in this study produced significantly higher levels of IFN- γ in response to MCMV infection *in vivo*. The conflicting results of these two studies are likely due to differences in the systems used to delete *Dicer* in NK cells. In contrast to miRNA ablation, increasing global levels of miRNAs through deletion of *Eri1* (a negative regulator of miRNAs) had no impact on NK-cell degranulation, IFN- γ production, or cytotoxicity against target cells *in vitro* (76). However, *Eri1*^{-/-} NK cells were limited in their ability to proliferate in response to MCMV infection *in vivo*, and this defect correlated with a substantial increase in viral titers in *Eri1*^{-/-} mice. Collectively, the studies in *Dicer*-, *Dcgr8*-, and *Eri1*-deficient animals highlight a critical role for miRNAs in regulating NK-cell activation and effector function.

Beyond global perturbation of miRNA levels, studies on individual miRNAs have further refined our understanding of how specific miRNAs regulate NK-cell activation. Several groups have identified unique miRNAs that regulate IFN- γ production in resting and activated NK cells. For example, either cytokine-induced or transgene-mediated upregulation of miR-155 enhanced IFN- γ production by activated human NK cells, whereas miR-155 knockdown impaired this activity (73, 100). Mechanistically, miR-155 promoted NK-cell responses by targeting and suppressing SHIP-1, a potent-negative regulator of NK-cell effector function. Similar defects in IFN- γ production were observed in NK cells from mice lacking *Bic*, the gene encoding miR-155, suggesting an evolutionarily conserved role for this miRNA (100). Like miR-155, miR-150 is also required for optimal IFN- γ production by mouse NK cells. Compared with wildtype NK cells, fewer *Mir150*^{-/-} NK cells produce IFN- γ after stimulation *in vitro*, whereas transgenic overexpression of miR-150 in mice was shown to enhance IFN- γ production by activated NK cells (81).

Beyond IFN- γ production, miRNAs also regulate other NK-cell effector responses, including cytotoxicity and the secretion of other proinflammatory cytokines, such as TNF α . In human NK cells, miR-30c-1* was recently shown to act

downstream of the activating NK-cell receptor, CD226, to promote cytotoxic functionality and TNF- α production (101). This function was attributed to the ability of miR-30c-1* to target HMBOX1, a transcription factor shown to inhibit NK-cell activation.

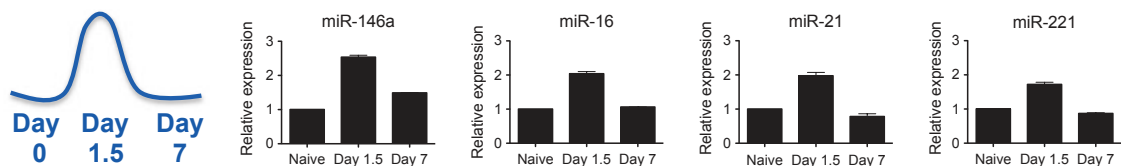
Rather than promote effector responses, some miRNAs may function to repress the translation of mRNA for effector molecules, such as granzyme B and IFN γ , which are present at high levels in resting NK cells, but remain untranslated in the absence of activation stimuli. For example, mir-29 and members of the miR-15/16 family (i.e. miR-15a/15b/16) restrict IFN- γ production by NK cells by directly targeting the IFN- γ 3'-UTR (75, 102). Consistent with the presumed role of these miRNAs in restraining NK-cell function until activating signals are received, stimulation of mouse NK cells *in vitro* led to a reduction in miR-29 or miR-15/16 expression and a concomitant increase in IFN- γ production. Ma et al. (102) further showed that NK cells in mice expressing a miR-29 'sponge' construct (which competes with endogenous miR-29 targets and reduces the amount of 'free' miR-29) produced exaggerated levels of IFN- γ following activation *in vitro* or infection with *Listeria monocytogenes* *in vivo*. This latter response correlated with enhanced clearance of bacterial burden, indicating that miR-29 may act during infection to suppress protective immune responses. miRNAs also regulate untimely cytotoxic activity in resting NK cells. miR-223, for example, directly targets and represses the 3'-UTR of granzyme B at steady state, whereas stimulation with IL-15 causes a decrease in miR-223 expression and a corresponding increase in granzyme B levels (74). Comparable roles for miR-27a*, miR-30e, and miR-378 have also been described. miR-27a* was found to directly target the 3'-UTR of perforin and granzyme B transcripts in human NK cells and miR-27a* knockdown substantially increased NK-cell lysis of target cells *in vitro* (103). Similarly, miR-378 and miR-30e, two miRNAs abundantly expressed in resting human NK cells, were found to target granzyme B and perforin transcripts, respectively (104). Like miR-223, cytokine stimulation reduced miR-378 and miR-30e expression and thereby augmented activation-induced cytotoxicity by human NK cells.

To further understand how miRNAs might regulate effector responses in antigen-specific NK cells responding to viral infection, we sorted Ly49H⁺ NK cells from MCMV-infected animals on days 0, 1.5, and 7 postinfection and used quantitative real-time PCR (qRT-PCR) to assess expression of 7 miRNAs with known or suspected roles in NK-cell biology (miR-146a, miR-16, miR-21, miR-221, miR-182, let-7a,

and miR-140), most of which have been discussed above. The observed expression patterns could be broadly grouped into three categories (Fig. 3): (i) miRNAs transiently upregulated only at day 1.5 postinfection, or early response miRNAs (e.g. miR-146a, miR-16, miR-21, and miR-221); (ii) miRNAs upregulated (e.g. miR-182) or downregulated (e.g. let-7a) only on day 7 postinfection, or late-response miRNAs; and (iii) miRNAs whose expression remained unchanged at the assessed time points. Early response miRNAs would be predicted to act downstream of signals from proinflammatory cytokines, which are particularly abundant in the first 24–72 h postinfection (27). As such, these miRNAs might be predicted to regulate ‘innate’ NK-cell functions, such as IFN- γ secretion and cytotoxicity, or shape the clonal proliferation of antigen-specific Ly49H⁺ NK cells that is initiated during this time frame. In keeping with this hypothesis, roles for miR-16 in controlling IFN- γ production (75) and for miR-21 in promoting proliferation (96) in NK cells have already been discussed. Future studies will be necessary to more fully understand how and if the other early-response miRNAs identified in our study regulate NK-cell function.

Given the delayed expression of late-response miRNAs, it is less likely that they regulate cytotoxicity or IFN- γ production. Instead, these miRNAs may be important instructors of the antigen-driven clonal expansion (or contraction) of Ly49H⁺ NK cells, which peaks at day 7 postinfection, then slowly declines over the following weeks until a small pool of long-lasting ‘memory’ NK cells remains. One of these miRNAs, miR-182, has been shown to act downstream of the IL-2 receptor (IL-2R) in T-helper cells to promote clonal expansion by regulating Foxo1 transcripts (105). Given that NK cells also require IL-2R signaling for their development and maturation, we used *Mir182*^{-/-} mice to investigate the extent to which miR-182 was necessary for NK-cell expansion during MCMV infection. Normal NK-cell numbers were found in *Mir182*^{-/-} mice (data not shown), indicating that miR-182 was not required for NK-cell maturation or homeostasis at steady state (i.e. in the absence of inflammation). *Mir182*^{-/-} NK cells also exhibited normal expression of markers associated with maturation and activation, including CD27, CD11b, KLRG1, and CD69 expression (data not shown), further underscoring unperturbed NK-cell development in these animals. To address the role of

Early response miRNAs



Late response miRNAs



Unchanged miRNAs

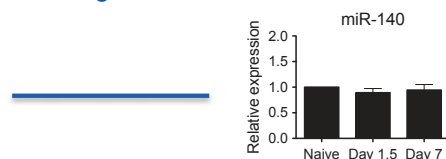


Fig. 3. miRNA profile of natural killer (NK) cells during mouse cytomegalovirus (MCMV) infection. Expression of select miRNAs in splenic Ly49H⁺ NK cells sorted from animals infected with Smith strain MCMV, as assessed by qRT-PCR. Levels of mature miRNAs were normalized to sno-202. Data are shown as the fold change at day 1.5 and day 7 versus day 0 (naive). miRNAs transiently upregulated on day 1.5 postinfection (i.e. Early response miRNAs) include miR-146a, miR-16, miR-21, and miR-221. miRNAs upregulated or downregulated on day 7 postinfection (i.e. Late response miRNAs) include miR-182 and let-7a. miR-140 levels remained constant on the assessed time points.

miR-182 in NK-cell expansion during infection, we co-transferred equal numbers of wildtype (WT) and *Mir182*^{-/-} Ly49H⁺ NK cells into Ly49H-deficient hosts and evaluated their ability to expand following MCMV challenge. We observed a similar expansion of WT and *Mir182*^{-/-} Ly49H⁺ NK cells at day 7 and equal percentages of memory NK cells at day 28 postinfection (Fig. 4A). The absence of a defect in NK-cell expansion and memory formation in *Mir182*^{-/-} animals may be due to functional compensation by other miRNAs. Indeed, mir-183/96/182 cluster family members, which along with miR-182 are all expressed from a single pri-miR transcript, all share a nearly identical seed sequence (Fig. 4B), indicating that these miRNAs may target an overlapping suite of mRNAs. Additional studies would be necessary to address whether deletion of the whole mir-183/96/182 cluster would impact NK-cell responses during MCMV infection. Alternatively, upregulation of miR-182 on day 7 postinfection may be an inconsequential after effect of the extensive changes in gene expression that occur during MCMV infection. However, the observation that the expression of mir-183/96/182 cluster miRNAs was also significantly elevated in primary human fibroblasts infected with human CMV suggests that there

might be a common mechanism connecting mir-183/96/182 cluster family members and CMV (106).

In summary, an increasing number of studies indicate that specific miRNAs can regulate both early and late responses of NK cells against viral infection, and disruption of many of these individual miRNAs can result in susceptibility to infection. Future studies, in particular those using conditional ablation of individual miRNAs in genetically engineered mice (107), will undoubtedly identify additional miRNAs with key regulatory roles in NK-cell responses to viral infection.

miRNAs as common regulators of NK and CD8⁺ T-cell programs

NK cells have long been appreciated to share many common features with CD8⁺ T cells, most notably their shared ability to kill infected or malignant target cells through direct cytotoxic mechanisms. Developmental parallels also exist between NK and CD8⁺ T cells, including shared origin from CLP cells, a reliance on common γ -chain-dependent cytokines (e.g. IL-15 and IL-7) for survival during development and homeostasis, and analogous education processes during development (i.e. 'licensing' or 'disarming' for NK cells and thymic selection for T cells) (34). Activation is similarly dependent on engagement of activating receptors (i.e. the TCR for CD8⁺ T cells and receptors such as NKG2D or Ly49H for NK cells) and proinflammatory cytokines (e.g. IL-12, IL-18, and Type I interferons). Moreover, it is now appreciated that antigen-specific engagement of certain activating receptors on NK cells can drive a strong proliferative response and the generation of long-lived 'memory' cells with enhanced functionality in response to antigen re-exposure. The kinetics and nature of this response is highly reminiscent of antigen-specific CD8⁺ T-cell responses against pathogens (34). Given these parallels, specific miRNAs may function in both cell types to regulate common pathways of development, homeostasis, and effector function. Consistent with this hypothesis, both NK cells and naïve CD8⁺ T cells are reduced in frequency and number in *Dicer*- and *Dgcr8*-deficient mice (72, 75, 108, 109). Moreover, a survey of published miRNA profiling studies reveals substantial overlap in the specific miRNAs expressed by NK cells and CD8⁺ T cells. Examples include miR-142-3p, miR-150, miR-16, miR-23a, miR-15b, miR-29a, miR-30b, miR-146, and miR-26a (72–74, 110, 111). To more precisely compare miRNAs expressed by naïve NK cells and naïve and effector CD8⁺ T cells, we used qRT-PCR to evaluate the relative expression of 35 unique miRNAs in the three cell

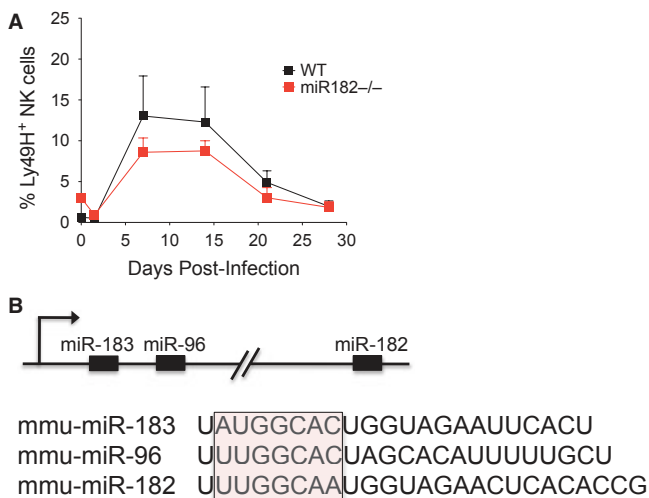


Fig. 4. miR-182 is dispensable for natural killer (NK)-cell responses to mouse cytomegalovirus (MCMV) infection. (A) Equal numbers of WT (CD45.1⁺) and *Mir182*^{-/-} (CD45.2⁺) Ly49H⁺ NK cells were co-transferred into Ly49H-deficient hosts. Following infection with MCMV, the percentage of adoptively transferred WT (black) and *Mir182*^{-/-} (red) Ly49H⁺ NK cells within the total NK-cell population in blood was assessed at the indicated time points postinfection. Error bars show SEM (n = 3). Data are representative of three independent experiments. (B) Schematic representation of the miR-183/96/182 cluster. miR-183, miR-96, and miR-182 share sequence homology, particularly within the seed sequence used to bind target mRNAs (pink box).

populations. Several broad patterns of expression were especially notable: (i) miRNAs highly expressed in all three cell subsets, (ii) miRNAs with moderate-to-high expression in NK cells and effector CD8⁺ T cells, and (iii) miRNAs uniquely upregulated in NK cells as compared with either CD8⁺ T-cell population (Fig. 5).

Among those highly expressed in all three subsets (e.g. miR-150, miR-142-3p, miR-16, miR-15b, miR-93, and let-7d), several have described roles in lymphocyte biology. For example, miR-150 promotes NK development (as described above) (81) and, at least in the context of overexpression, represses invariant natural killer T (iNKT)- (81) and B-cell development (79). Similarly, miR-16 and miR-15b, which repress IFN- γ transcript in NK cells (75), have also been identified as tumor suppressor factors in B-cell leukemias, where they repress expression of oncogenes such as BCL2, MCL1, CCND1, and WNT3a (112). Although no role has yet to be described for miR-142-3p in NK or CD8⁺ T cells, it is known to regulate the cyclic AMP (cAMP) pathway in CD4⁺ T cells (113). Future work will provide insight into whether these miRNAs indeed have overlapping roles in CD8⁺ T cells

and NK cells, perhaps as regulators of development or homeostasis.

In terms of the miRNAs highly expressed only by effector CD8⁺ T cells and NK cells, including miR-21, miR-23a, miR-24, let-7c, and miR-103, it is tempting to speculate that these may function to control effector functions common to both cell types, such as IFN- γ secretion and cytotoxicity. Studies in mice have hinted at such a role for miR-21, which is often upregulated in B and T cells from human systemic lupus erythematosus patients and from genetically defective mouse strains with lupus-like disease symptoms (114–116). Indeed, silencing of miR-21 in 'lupus-prone' mice was shown to reverse symptoms of autoimmunity, including splenomegaly and aberrant PDCD4 expression and disease-associated alteration in the CD4⁺/CD8⁺ T-cell ratio, suggesting that miR-21 may function as an important regulator of autoimmune lymphocyte responses in lupus (114). Altogether, the evidence strongly suggests that parallel pathways of regulation are at work in NK and CD8⁺ T cells, where individual miRNA expression is modulated to enhance or repress specific cellular processes and functions.

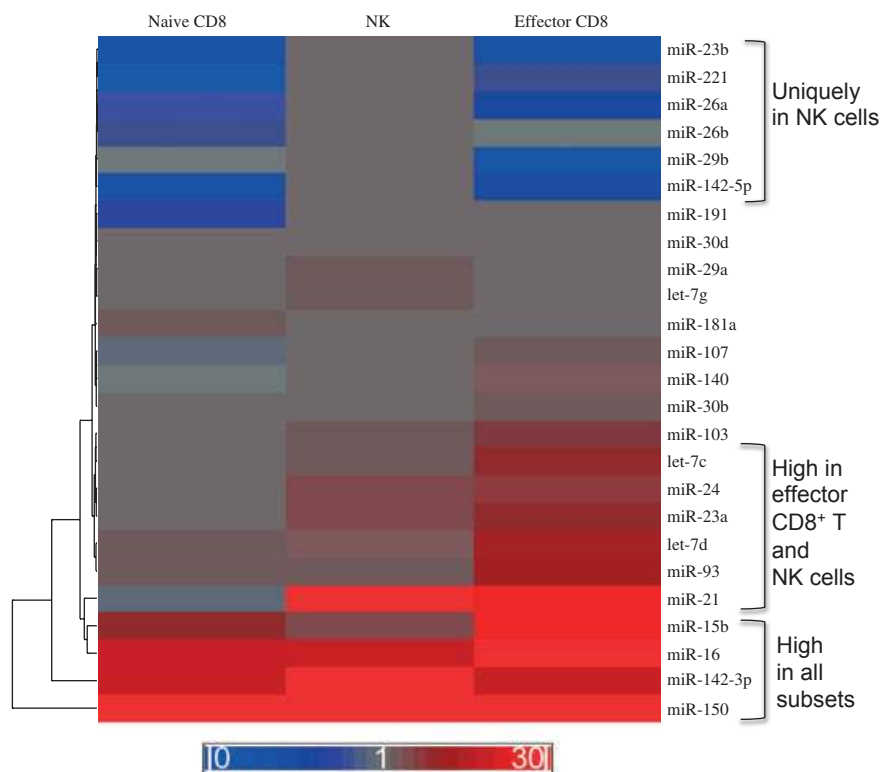


Fig. 5. Common miRNA expression programs between natural killer (NK) and CD8⁺ T-cell populations. miRNAs expressed in resting NK cells as compared with resting (naive) CD8⁺ T cells or effector CD8⁺ T cells. NK1.1⁺ TCR β ⁻ NK cells were sorted from C57BL/6 mice. Naive CD8⁺ T cells were sorted from CD45.1⁺CD45.2⁺ P14 mice bearing the D^gGP33-specific TCR. Effector CD8⁺ T cells were sorted from chimeric mice generated by adoptive transfer of P14 T cells into C57BL/6 (CD45.2⁺) recipients that were infected intraperitoneally with 2×10^5 plaque forming units (PFU) of LCMV Armstrong. Levels of mature miRNAs were measured by qRT-PCR and normalized to sno202. Fold expression relative to sno-202 is depicted as a heat map. Three patterns emerging from the data are highlighted.

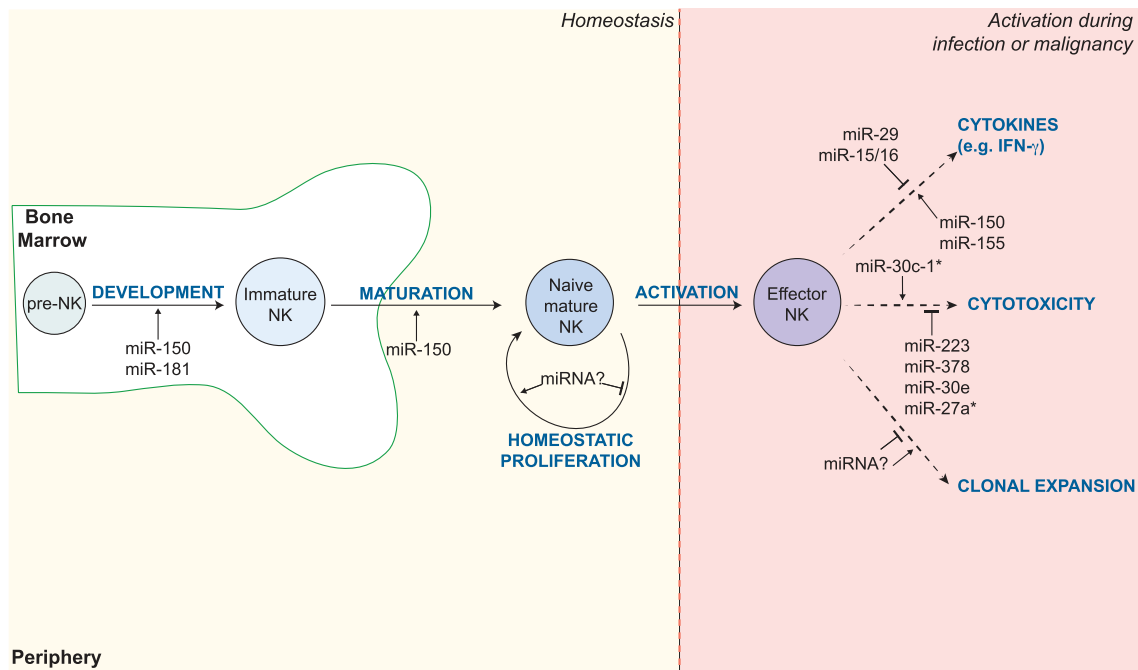


Fig. 6. miRNAs regulate the development, maturation, and effector function of natural killer (NK) cells. A summary of individual miRNAs known to regulate NK-cell development and function at steady state and in the context of activation (e.g. during infection or malignancy). Examples include miR-150 and miR-181, which are required for NK-cell development and maturation in the bone marrow; miR-150, miR-155, miR-29, and miR-15/16, which modulate cytokine production by activated NK cells; and miR-30c-1*, miR-223, miR-378, miR-30e, miR-27a*, which control expression of cytotoxic molecules, such as granzyme B and perforin.

Conclusions and future directions

Mounting evidence underscores the critical role of miRNAs as regulators of development, maturation, proliferation, differentiation, and activation of NK cells (Fig. 6). Within the context of complex transcriptional networks, the control of gene expression has been historically attributed to transcription factors. However, a growing number of studies have shown that miRNAs are equally critical regulators of cellular mRNA and protein levels during both homeostasis and disease. Whereas transcription factors function in many respects as ‘on-off’ switches of gene expression, miRNAs act more like rheostats, finely tuning the level of translatable mRNAs in response to specific developmental or stimulatory cues and changing cellular requirements. This rapid and reversible mechanism of action makes them well suited to

control NK- and T-cell effector responses, such as cytokine production and cytotoxicity, which must be rapidly mounted in the face of viral or malignant assaults, but then rapidly disengaged with equal potency to prevent pathogenic destruction of healthy, bystander cells.

Future studies are required to appreciate the full spectrum of NK-cell activities controlled by miRNAs, as well as to understand the activating and developmental signals that orchestrate their expression during homeostasis, infection, and cancer. High-throughput and genome-wide technologies that match individual miRNAs to their cognate target transcripts, such as Ago-CLIP (117, 118), will be particularly useful in dissecting the specific cellular pathways controlled by individual miRNAs in NK cells *in vivo*.

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