Natural killer (NK) cells can have potent antileukemic activity following haplo-mismatched, T cell-depleted stem cell transplantations for the treatment of acute myeloid leukemia (AML), but they are not successful in eradicating de novo AML. Here, we have used a mouse model of de novo AML to elucidate the mechanisms by which AML evades NK cell surveillance. NK cells in leukemic mice displayed a marked reduction in the cytolytic granules perforin and granzyme B. Further, as AML progressed, we noted the selective loss of an immature subset of NK cells in leukemic mice and in AML patients. This absence was not due to elimination by cell death or selective reduction in proliferation, but rather to the result of a block in NK cell differentiation. Indeed, NK cells from leukemic mice and humans with AML showed lower levels of TBET and EOMES, transcription factors that are critical for terminal NK cell differentiation. Further, the microRNA miR-29b, a regulator of T-bet and EOMES, was elevated in leukemic NK cells. Finally, deletion of miR-29b in NK cells reversed the depletion of this NK cell subset in leukemic mice. These results indicate that leukemic evasion of NK cell surveillance occurs through miR-mediated dysregulation of lymphocyte development, representing an additional mechanism of immune escape in cancer.

Introduction

Acute myeloid leukemia (AML) is one of the most common types of leukemia diagnosed in adults. While many advancements have been made in the understanding of the genetic components of AML, the 5-year survival rate for all types of AML is still less than 25% (1). There is a clear need for improved therapeutics and a more complete understanding of how AML escapes our natural defenses and renders the immune system incapable of controlling outgrowth of AML stem cells and blasts (12–16). While the mechanisms of immune evasion are in operation allowing for successful outgrowth of AML stem cells and blasts (12–16), the mechanisms of innate immune evasion to date have involved circumventing mature cytolytic NK cells (5–10), assessment of NK cell population is subdivided into 4 stages of NK cell development and proceeds through a discrete 5-stage development pathway, culminating in mature NK cells that lack CD3 and have low-density expression of the adhesion molecule CD56 (CD56dim) (2, 19, 20). The CD3+CD56+ subset of NK cells represent a less mature population in lymphoid tissue and blood, in that they produce higher levels of inflammatory cytokines with little or no natural cytotoxicity (2).
Rag2–/–/PTD/ITD mice were crossed with mice) develop de novo AML with 100% penetrance (28). These PTD/ITD/Rag GC KO mice; all died from leukemia as validat –

Immune cells mediate AML progression

Primary mice harboring both the mixed-lineage leukemia (MLL) partial tandem duplication (MLL-PTD) and the FMS-like tyrosine kinase 3 (FLT3) internal tandem duplication (FLT3-ITD) mutations (termed PTD/ITD mice) develop de novo AML with 100% penetrance (28). These PTD/ITD mice were crossed with Rag2–II2rg– mice to produce PTD/ITD/Rag GC KO mice; all died from leukemia as validat-
ed by elevated wbc count and/or histological confirmation by a pathologist (data not shown). PTD/ITD/Rag GC KO mice had significantly reduced survival times as compared with PTD/ITD mice (Figure 1A; P < 0.0001), suggesting the immune system plays a role in the progression of AML in the PTD/ITD murine model. Since the Rag GC KO mice lack T, B, and NK cells, we next sought to determine whether NK cells, specifically, could slow AML progression. We adoptively transferred WT NK cells with CD45.2+ leukemic blasts into irradiated WT CD45.1+ congenic recipient mice. The same absolute number of AML (CD45.2+) blasts were infused into both sets of recipient mice, and the percentage of circulating AML blasts in mice that received WT NK and AML blasts was compared with that in mice that received only AML blasts. There was a significant reduction in circulating AML blasts at 21 days after transfer in the mice that received NK cells along with the AML blasts, as compared with mice that received AML blasts only (Figure 1B; P = 0.012). Intriguingly, these mice had no overall improvement in survival time, indicating that while the NK cells are initially effective at slowing progression, as indicated by reduced CD45.2+ circulating AML blasts, the leukemic environment may support innate immune cell evasion as the disease progresses. Like blood, examination of the bone marrow also showed reduced AML blast infiltration, while the spleen did not (not shown). Thus, it is also possible that persistent disease in certain organ sites could account for similar overall survival curves.

NK cell function and homeostasis are altered in leukemic mice.

After determining that NK cells were initially effective at modulating AML outgrowth but did not improve overall survival, we evaluated NK cell activity in the setting of AML. A hallmark of NK cell function is the ability to recognize and lyse tumor targets; thus, both autologous blasts and tumor cell lines were used as targets to assess the cytotoxicity of NK cells from leukemic mice. Autologous leukemic blasts were transplanted into congenic CD45.1+ recipients with or without WT NK cells. The percentage of circulating blasts (CD45.2+) was evaluated by flow cytometry (P = 0.012; n = 4/group, 2 independent studies with a representative study depicted; 2-sample t test). *P = 0.012.

While the phenotypic surface marker expression varies, the process of NK cell development in both humans and mice is tightly controlled by transcription factors that can be activated in response to cytokine stimulation and/or additional activating signals. Two key transcriptional regulators of NK cell development are T-box–related TBX21 (TBET) and eomesodermin (EOMES); these 2 transcription factors work in concert to control the final stages of NK cell differentiation in humans and mice (21, 22). Indeed, mice that are genetically altered to inhibit expression of both TBET and EOMES lack mature NK cells (22). Recently, microRNAs (miRs) have also emerged as important regulators of immune cell development and function (23–26), and the modulation of TBET and EOMES has been linked to miR-29b in T cell studies (23, 27). To date, the importance of miR regulation of NK cell development in the setting of cancer has not been evaluated.

In the current study we show that AML is impeded early in the disease process by NK cells. With progression of AML there is a significant reduction in NK cell perforin, and a selective loss of an immature NK cell subset secondary to a deregulation of a microRNA that regulates NK cell development.

Results

Immune cells mediate AML progression

Primary mice harboring both the mixed-lineage leukemia (MLL) partial tandem duplication (MLL-PTD) and the FMS-like tyrosine kinase 3 (FLT3) internal tandem duplication (FLT3-ITD) mutations (termed PTD/ITD mice) develop de novo AML with 100% penetrance (28). These PTD/ITD mice were crossed with Rag2–II2rg– mice to produce PTD/ITD/Rag GC KO mice; all died from leukemia as validat-

Figure 1. Role of the immune system in PTD/ITD model of AML. (A) Survival of PTD/ITD primary mice compared with PTD/ITD/Rag GC KO (***P < 0.0001; n = 12 PTD/ITD, n = 5 PTD/ITD/Rag GC KO). A log-rank test was used for statistical analysis. (B) CD45.2+ AML blasts were transplanted into congenic CD45.1+ recipients with or without WT NK cells. The percentage of circulating blasts (CD45.2+) was evaluated by flow cytometry (P = 0.012; n = 4/group, 2 independent studies with a representative study depicted; 2-sample t test). *P = 0.012.

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what impact the leukemic environment might have on NK cell homeostasis. While NK cell percentages were reduced in leukemic bone marrow, lymph nodes, spleen, and blood (data not shown), the absolute numbers of NK cells differed between compartments. Compared with nonleukemic mice, absolute numbers were increased in the blood, not significantly different in the spleen, although greatly reduced in the bone marrow and lymph nodes of leukemic mice (Figure 2F and data not shown).

NK cell maturation is altered in leukemic mice. Using the transplant model, we next assessed the NK cell developmental subsets found in leukemic mice and compared them with those in nonleukemic mice. Previous studies established a progression of NK maturation based on the surface expression of CD27 and CD11b among the total pool of CD3−NK1.1+ NK cells, as reviewed above (17, 18). We noted a striking and highly significant reduction in the CD27 and CD11b double-positive (DP) splenic NK cell subset in the leukemic mice compared with the nonleukemic mice (Figure 3, A and B; ***P < 0.0001). In contrast, we noted the leukemic mice had a significant increase in the less mature CD27+CD11b− NK cell subsets (Figure 3, A and B; **P = 0.0009), while the most mature CD27−CD11b+ showed no significant change. There was also a significant reduc-

cells from nonleukemic mice (Figure 2C; P = 0.004). Soluble IFN-γ production was also evaluated in NK cells cultured ex vivo from nonleukemic and leukemic mice with and without stimulation with IL-12 and IL-18 for 24 hours. Interestingly, NK cells isolated from leukemic mice had significantly elevated IFN-γ upon stimulation as compared with NK cells from nonleukemic mice (Supplemental Figure 2). Splenic NK cell proliferation was next evaluated in nonleukemic and leukemic mice by flow cytometry. NK cells from leukemic mice exhibited significantly less proliferation as compared with NK cells harvested from nonleukemic mice (Figure 2D; P < 0.0001). To determine whether NK cells were responsive to external proliferative stimulation, IL-15 was injected into nonleukemic or leukemic mice, and cells were evaluated for proliferation 24 hours later. In all organs evaluated, proliferation was significantly decreased in the NK cells from leukemic mice as compared with NK cells in the nonleukemic mice (Figure 2E; P < 0.01, all organs). Overall these results suggest that NK cell activity is reduced in mice with progressive leukemia, with the exception of cytokine-stimulated NK cell proliferation of IFN-γ.

Once it was determined that NK cells in leukemic mice exhibited alterations in phenotype and function, we then evaluated what impact the leukemic environment might have on NK cell homeostasis. While NK cell percentages were reduced in leukemic bone marrow, lymph nodes, spleen, and blood (data not shown), the absolute numbers of NK cells differed between compartments. Compared with nonleukemic mice, absolute numbers were increased in the blood, not significantly different in the spleen, although greatly reduced in the bone marrow and lymph nodes of leukemic mice (Figure 2F and data not shown).

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In vivo NK cell adoptive transfer results in selective loss of the DP NK cell subset. The above-noted studies evaluated defects in endogenous NK cells that had developed during the progression of AML. When we evaluated the critical cytolytic granule perforin in both the DP and the more mature CD27+CD11b− NK cell subsets found in leukemic mice, there was a reduction in both the percentage of cells expressing perforin and the amount expressed per cell as measured by mean fluorescence intensity, when compared with identical NK cell subsets from nonleukemic mice (Figure 4A and refs. 32, 33). To validate that the selective loss in CD27+CD11b+ DP NK cells was not limited to the spleen in the transplant and de novo AML mouse models, we evaluated bone, blood marrow, and lymph node samples in both models. All organs had significantly reduced percentages of DP NK cells (Figure 3C; P < 0.0001, all organs). When blood was sampled at multiple time points after AML transplant, it was discovered that the DP NK cell population decreased as the AML burden increased in the mice. By 28 days, all leukemic mice had WBC counts over 100,000/μl and a highly significant difference in the DP NK cell subset when compared with nonleukemic mice (Figure 3D; ***P = 0.0005). Intriguingly, when the levels of EOMES were evaluated in the developmental NK cell subsets, there was reduced EOMES overall in the NK cells of leukemic mice, but there was also a reduction in the fraction of cells expressing EOMES within each developmental NK cell subset after the acquisition of CD27 in leukemic mice as compared with identical developmental NK cell subsets from nonleukemic mice (Figure 3E; ***P < 0.001, ***P < 0.01, ***P < 0.001; and Supplemental Figure 5A). The levels of both EOMES and TBX21 (T-bet) protein expression, as measured by the mean fluorescence intensity, were significantly decreased in bulk NK cells from leukemic mice and in each of their subsets except the most immature CD27+CD11b− subset (data not shown and Supplemental Figure 5B; **P < 0.01, ***P < 0.001). Interestingly, the most mature CD27+CD11b+ NK cell subset in leukemic mice was still present at levels seen in nonleukemic mice. When we evaluated the critical cytolytic granule perforin in both the DP and the more mature CD27+CD11b+ NK cell subsets found in leukemic mice, there was a reduction in both the percentage of cells expressing perforin and the amount expressed per cell as measured by mean fluorescence intensity, when compared with identical NK subsets found in nonleukemic mice (Figure 4A and B; *P = 0.04, **P = 0.004, ***P < 0.001).

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The percentage of perforin in splenic DP or CD27−CD11b+ NK cells was determined (**P = 0.004, ***P < 0.001; n = 5/group, 2 independent studies with a representative study depicted). Level of protein expression of perforin in splenic DP or CD27−CD11b+ NK cells, expressed as MFI (*P = 0.04, ***P < 0.001; n = 5 mice per group; 2 independent studies with a representative study depicted). Student’s t test was used for single comparisons.

Figure 4. Cytolytic perforin expression is decreased in CD11b+ NK cell subsets in leukemic mice. (A) The percentage of perforin in splenic DP or CD27−CD11b+ NK cells was determined (**P = 0.004, ***P < 0.001; n = 5/group, 2 independent studies with a representative study depicted). (B) Level of protein expression of perforin in splenic DP or CD27−CD11b+ NK cells, expressed as MFI (*P = 0.04, ***P < 0.001; n = 5 mice per group; 2 independent studies with a representative study depicted). Student’s t test was used for single comparisons.

of AML. We next sought to determine whether we would see the same selective depletion of the DP NK cell subset when naïve WT NK cells were transferred into leukemic mice. Bulk NK1.1+CD3− NK cells isolated to greater than 98% purity by FACS were transferred into either leukemic or nonleukemic controls. Within 1 week, mice had a significant and selective loss in the DP NK cell subset (Figure 5A; P = 0.031). To determine whether this loss was due to an increase in apoptosis, the experiment was repeated and the mice were evaluated for in vivo apoptosis in leukemic and nonleukemic mice. In fact, at 48 hours there was a significantly lower fraction of DP NK cells undergoing apoptosis in the leukemic mice compared with the nonleukemic mice (Figure 5B; P = 0.005). The CD27−CD11b+ DP NK cell population was also isolated by FACS and adoptively transferred into either leukemic or nonleukemic control mice for in vivo evaluation of apoptosis. In agreement with the results of the bulk NK cell transfer, there was a small but significant reduction in apoptosis in the DP NK cells that were transplanted into the leukemic mice compared with the nonleukemic mice (Supplemental Figure 6). Since a reduction in proliferation was previously observed with bulk NK cells in leukemic mice, we also evaluated the proliferative capacity of transplanted DP NK cells. WT DP NK cells transferred into leukemic mice had significantly lower levels of proliferation 48 hours after adoptive transfer as compared with WT DP NK cells transferred into nonleukemic mice (P = 0.0004; Figure 5C), indicating that this defect occurs quickly upon NK cell transfer into leukemic mice. However, the proliferative capacity of the DP NK cell subset alone was not significantly different from that seen among total NK1.1+CD3− NK cells in leukemic mice (Figure 2D), indicating that this process did not significantly contribute to the selective depletion of this NK cell subset.

Transfer of NK cell developmental intermediates identifies a block in maturation. After determining that the endogenous DP NK cells were selectively reduced in leukemic mice and exogenous DP NK cells were selectively depleted after adoptive transfer into leukemic mice without a selective increase in apoptosis or decrease in proliferation, we sought additional explanations for the reduction. While leukemic mice exhibited a significant reduction in CD27−CD11b+ DP NK cells, they also had significantly elevated levels of the CD27−CD11b immature NK cell subset, the upstream NK cell precursor population (Figure 3B; P = 0.0009). We hypothesized that NK cells were blocked at the CD27−CD11b+ stage of NK cell maturation in the leukemic mice. To test this, we isolated only CD27−CD11b+ NK cells, as indicated in the red box on the flow cytometry dot plot (Figure 5D, left), and transplanted these precursor NK cells into leukemic or nonleukemic mice. Importantly, we saw an elevated percentage of the CD27−CD11b+ NK cell precursor in the leukemic mice as compared with the nonleukemic mice within 1 week of the adoptive transfer (Figure 5D; P < 0.0001, all populations). Further, we saw a significantly lower fraction of CD27−CD11b+ DP NK cells emerge in the leukemic mice compared with the nonleukemic mice (Figure 5D; P < 0.0001). These results indicate a blockage in maturation at the CD27−CD11b+ stage of NK cell maturation in leukemic mice.

Forced overexpression of T-bet and EOMES via the genetic deletion of Mir29b restores CD27−CD11b+ NK cell population in vivo. After identifying a developmental block in NK cell development found in the PTD/ITD AML mouse model, we next sought to determine what mechanism might be responsible for this selective depletion of the developmental intermediate (DP) NK cell population. With the data demonstrating reduced T-bet and EOMES expression in NK cells taken from leukemic mice, we hypothesized that a single regulator of both transcription factors may be the target in the leukemic environment, thereby preventing normal NK cell maturation. The microRNA miR-29b has been previously demonstrated to target and regulate both T-bet and EOMES directly in T cells (23, 27). The level of Mir29b was measured by quantitative real-time RT-PCR and found to be significantly elevated in bulk NK cells and CD11b+ NK cell subsets taken from leukemic mice as compared with NK cells taken from nonleukemic mice (Figure 6A; P = 0.009; and Supplemental Figure 7). We therefore wanted to determine whether genetic disruption of the miR-29b loci would increase expression of T-bet and EOMES in the NK cell compartment, and whether the adoptive transfer of miR-29b−deficient NK cells could rescue the DP NK cell subset that is selectively depleted in leukemic mice. NK1.1+CD3− NK cells from C57BL/6 Mir29ab1−/− mice (hereafter referred to as miR-29b KO mice) or WT littermate control mice were FACS sorted to greater than 99% purity; the former showed near-complete absence of miR-29b expression (data not shown) and higher expression of both T-bet and EOMES when compared with their WT controls by quantitative real-time RT-PCR before transfer into the recipient mice (Figure 6B). It is still possible that the relationship between miR-29b and T-bet and EOMES in NK cells occurs via an indirect mechanism; however, in this model we do see lower levels of both transcription factors in the presence of elevated miR-29b in the NK cells of leukemic mice.
Next, equal numbers of FACS-sorted NK1.1+CD3+ NK cells from WT littermate control or miR-29b KO mice were adoptively transferred into WT or leukemic recipient mice. Consistent with our previous experiments, the DP population of NK cells from WT donor mice was reduced in recipient leukemic mice compared with nonleukemic mice after 7 days (Figure 6C, left; \(P = 0.02\)). In contrast, the proportion of the DP donor NK cells isolated from miR-29b KO mice was the same regardless of recipient status (leukemic or nonleukemic) (Figure 6C, right; \(P = NS\)). In addition, while there was no noticeable change in T-bet expression, there was a significant increase in the level of EOMES protein expression in the miR-29b KO NK cells in the leukemic mice after transfer as compared with the transferred WT littermate NK cells (Figure 6D; \(P = 0.01\)). Thus, miR-29b KO NK cells were resistant to the loss of the CD27+CD11b+ DP NK cell population in leukemic mice in vivo. To determine whether miR-29b KO NK cells could slow AML progression, we adoptively transferred NK cells from miR-29b KO mice along with AML blasts. There was a trend toward reduced circulating AML blasts at 21 days after transfer and a significant reduction in wbc count in the mice that received miR-29b KO NK cells along with the AML blasts, as compared with mice who received WT NK cells along with the AML blasts (Supplemental Figure 8, A and B; \(P = 0.02\)). In addition, there was a trend to improved overall survival in the leukemic mice that received miR-29b KO NK cells as compared with the leukemic mice that received WT NK cells (Supplemental Figure 8C; \(P = 0.06\)). Together, these data suggest that miR-29b in NK cells may play a role in modulating AML progression.

Reduction of an NK cell intermediate in AML patients. We next sought to determine whether a similar blockade in NK cell development occurs in patients with AML. Untreated patients with newly diagnosed AML provided blood samples that were compared with normal donor samples matched for age and sex. Importantly, we discovered that AML patients have a significant reduction in the less mature, CD56bright NK cell subset, i.e., the Lin–CD56brightCD94+CD16– NK cell subset, when compared with normal donors (Supplemental Figure 9; \(P < 0.0001\), all populations; \(n = 4\) mice per group, 2 independent studies). Student’s t test was used for single comparisons; linear mixed effects models with Holm’s adjustment were used for multiple comparisons within the same mice.

Figure 5. NK cells exhibit block in NK cell maturation. NK1.1+CD3+ NK cells (3 × 10^5) were isolated from spleens of WT congenic CD45.1+ mice and adoptively transferred into nonleukemic or leukemic mice. At various time points, spleens were harvested, and cells were labeled with anti-NK1.1, -CD3, -CD45.1, -CD27, and -CD11b and evaluated by flow cytometry. (A) Transplanted NK1.1+CD3+ NK cells (population highlighted in dot plot in red box) were harvested after 7 days and evaluated for NK maturation by flow cytometry (**\(P = 0.031\); \(n = 4\) mice per group, 2 independent studies with a representative study depicted). (B) CD27+CD11b+ splenic NK cells were harvested 3 days after adoptive transfer of NK1.1+CD3+ NK cells and evaluated for apoptosis using FLIVO in vivo imaging dye (**\(P = 0.005\); \(n = 3\) mice per group). (C) NK1.1+CD3+ NK cells were labeled with Cell Proliferation Dye eFluor 670 before adoptive transfer. Seven days after adoptive transfer, the percentage of proliferating splenic CD27+CD11b+ NK cells was determined by flow cytometry, gating on peaks indicating cell division (**\(P = 0.0004\); \(n = 4\) mice per group, 2 independent studies). (D) NK1.1+CD3+CD27–CD11b+ NK cells (2 × 10^5, as indicated in the red box) were isolated from spleens of WT congenic CD45.1+ mice and adoptively transplanted into nonleukemic or leukemic mice. After 7 days, spleens were harvested, and cells were labeled with anti-NK1.1, -CD3, -CD45.1, -CD27, and -CD11b and evaluated by flow cytometry (**\(P < 0.0001\), all populations; \(n = 4\) mice per group, 2 independent studies). Student’s t test was used for single comparisons; linear mixed effects models with Holm’s adjustment were used for multiple comparisons within the same mice.
was measured by quantitative real-time RT-PCR. There was a significant increase in Mir29b expression in the CD56 bright NK cell population in AML patients as compared with normal donor controls (P = 0.006; Figure 7D). These results suggest that a mechanism of innate immune evasion comparable to that discovered in our mouse model of AML may occur in AML patients.

Discussion

Multiple mechanisms that enable AML to evade mature NK cell surveillance have been previously identified (5–10, 34). NK cells in AML patients can have a reduction in NKP30-, NKP44-, and NKP46-activating receptors that can lead to decreased NK cell cytotoxicity against AML blasts (8, 29, 30, 35). The inhibitory receptor NKG2A has also been demonstrated to be upregulated on NK cells from AML patients, and is associated with failure to reach remission (34). Additionally, AML blasts can evade NK cells by shedding activating stress ligands such as MICA and MICB, which, once soluble, can activate NK cell activity away from the actual tumor cell and lead to innate immune exhaustion (9, 36). Interestingly, it was noted by Costello et al. that even when autologous NK cells or normal allogeneic NK cells maintained expression of these receptors and other natural cytotoxicity receptors (NCRs), the NK cells were still unable to effectively lyse primary tumor targets ex vivo, indicating that additional mechanisms of inherent immune evasion are operative in the AML blasts (29). Despite understanding of several mechanisms of mature NK cell dysfunction during the progression of AML, a complete understanding of this process has not yet been attained. Further defining the mechanisms of innate immune evasion in the leukemic environment will be critical to developing effective treatment strategies that address these defects and favorably alter the outcome for AML patients.

In this report, we first asked whether NK cells could delay the onset or progression of AML by using an immunocompetent mouse model of de novo AML. Through observation, cross-breeding, and adoptive transfer experiments we learned that AML occurs earlier and has a much more rapidly fatal course in the absence of T, B, and NK cells. Further, we showed that the NK cell itself has a significant role in delaying the early progression of this disease. However, ultimately, the barrier provided by the NK cell to delay progression of AML is not sustained in vivo, such that no survival advantage is achieved by its presence. These data suggest that AML targets the NK cell early during disease progression to effectively evade its immune surveillance. Indeed, in both
An NK cell developmental intermediate population described as cell maturation that was associated with the progression of AML. We discovered a block in NK expression of EOMES in the CD27+CD11b+ DP NK cell intermediate and EOMES. Genetic disruption of the miR-29b loci restored the transcription factors that are critical for NK cell maturation: T-bet and EOMES. We discovered a significant upregulation of miR-29b in the CD56bright NK cell population in leukemic mice compared with nonleukemic mice and a significant reduction of T-bet in NK cells from AML patients.

Figure 7. AML patients exhibit altered NK cell development. Peripheral blood was obtained from untreated newly diagnosed AML patients or age- and sex-matched normal donors. NK cells were enriched by negative selection, labeled with anti-CD94, -CD56, -CD16, -CD3, -CD15, -CD64, -CD13, -CD3, -CD14, and -CD20, and then evaluated using highly selective gating by flow cytometry. "Bright" NK cells are defined as LinCD56+CD94+CD16+ cells. (A) Flow cytometry dot plot highlighting Bright NK cells in red rectangle from normal healthy donor and AML patient donor. (B) Percentage of Bright NK cells within the total NK cell population was evaluated in age-matched normal donors and AML patients. (C) Additional samples of Bright NK cells taken from AML patients and normal donors were evaluated for T-bet expression by intracellular flow cytometry. Levels of Mir29b were evaluated by real-time RT-PCR. (D) LinCD56brightCD94+CD16+ cells were FACs-isolated from newly diagnosed untreated AML patients and normal controls. A linear mixed modeling was used to determine the statistical difference between normal and AML patient samples.

The murine model and AML patients, we discovered a block in NK cell maturation that was associated with the progression of AML. An NK cell developmental intermediate population described as CD27−CD11b+, or DP, is nearly absent in leukemic mice, while NK cells accumulate at the earlier CD27 single-positive stage of maturation. We discovered a significant upregulation of miR-29b in the NK cells of leukemic mice, which downregulates expression of 2 transcription factors that are critical for NK cell maturation: T-bet and EOMES. Genetic disruption of the miR-29b loci restored the expression of EOMES in the CD27−CD11b+ DP NK cell intermediate population that was adoptively transferred and led to a reversal in the leukemic-mediated depletion of this immature NK cell population. Importantly, untreated AML patients also exhibited a reduction in an intermediate LinCD56dimCD94+CD16− NK cell population, along with a decrease in T-bet and EOMES, and increased miR-29b expression in the CD56bright NK cell population. Notably, in both the mouse AML model and AML patients, we did still detect relatively normal percentages of terminally differentiated NK cells that are normally fully cytolytic; however, we showed that the content of perforin is depleted in mature NK cells in leukemic mice compared with nonleukemic mice and in some but not all of the few patients’ mature CD56dim NK cells that we were able to measure. This suggests a 2-pronged approach that AML is using to evade the NK cell’s innate immune surveil-
a significant reduction of these transcription factors, rather than a complete loss, may allow for some final differentiation, and/or that alternate pathways of NK cell maturation may exist. In support of both possibilities, we used the murine AML model to show that the CD27\textsuperscript{+}CD11b\textsuperscript{−} developmental intermediary NK cell population preceding the nearly absent DP intermediary NK cell population was unable to proceed through normal differentiation in vivo. When our studies were expanded to untreated AML patients, the reduction in T-bet and EOMES protein expression was again observed within the less mature Lin CD56\textsuperscript{hi}CD94\textsuperscript{−}CD16\textsuperscript{−} NK cell population. While the reduction of T-bet and EOMES in AML patients was more modest than the reduction of these transcription factors in the AML mouse model, the AML mouse model represents a homogenous system while human AML is a heterogeneous disease. All untreated patients with the diagnosis of primary AML were included in this analysis. As the Lin CD56\textsuperscript{hi}CD94\textsuperscript{−}CD16\textsuperscript{−} NK cell population is the earliest precursor observed in appreciable numbers within human peripheral blood, we do not yet have the exact comparator in the human pathway to that which has been described in mice. The expression of CD27 and of CD11b was also evaluated on NK cells in AML patients, but no significant difference was found in these populations in the patient samples (Supplemental Figure 10). Additional studies are ongoing to determine the impact of AML on the development of NK cell precursors obtained from secondary lymphoid tissues where human NK cells develop (42). It would also be of interest to evaluate patients with myelodysplastic syndrome (MDS) to determine whether the Lin CD56\textsuperscript{hi}CD94\textsuperscript{−}CD16\textsuperscript{−} NK cell depletion precedes the development of overt AML in the MDS cases in which this progression to AML occurs.

To explore the mechanism(s) responsible for the reduction of both T-bet and EOMES expression, we evaluated common regulators of both transcription factors, miR-29b has been recently described as a direct regulator of both T-bet and EOMES in CD4\textsuperscript{+} T cells (23, 27), but to our knowledge has not previously been evaluated in NK cells. We hypothesized that miR-29b may be altered by the leukemic environment and drive the changes in T-bet and EOMES in NK cells in leukemic mice. Indeed, we observed a significant upregulation of miR-29b expression in NK cells from leukemic mice. This established a possible mechanism for the reduced DP NK cell population in vivo. Consistent with this model, when we adoptively transferred miR-29b KO NK cells into leukemic mice, unlike what was observed with WT NK cell transfer into leukemic mice, there was no reduction in the proportion of DP NK cells in comparison with nonleukemic recipient mice injected with the same cells. We also validated that both T-bet and EOMES expression was increased in the cells that were transplanted as compared with the WT littermate controls.

It is also interesting to note that before cell transfer there were more DP NK cell intermediates and fewer mature CD27 CD11b\textsuperscript{−} NK cells in the miR-29 KO mice (Supplemental Figure 11). Likewise, this disproportion was again noted following adoptive transfer into leukemic mice (Figure 6C and not shown). This may be in part due to the higher EOMES expression in DP NK cells compared with the most mature CD27 CD11b\textsuperscript{−} NK cell subset under normal homeostatic conditions (Figure 3E), or is more likely related to the inability of the AML cells to target an absent miR-29b following an infusion of miR-29b KO DP NK cells. Further studies would be required to determine what role genetic disruption of miR-29b has in achieving terminal NK cell differentiation in vivo, and how this reversal of the NK cell phenotype in vivo affects progression of AML and/or overall survival. We would speculate that survival would not be altered significantly in such an aggressive and high-penetration model of AML, given the multitude of mechanisms exhibited by AML to evade both the innate and antigen-specific components of the immune system, as discussed earlier. Indeed we do have data to suggest that while AML progression may be slowed by adoptive transfer of miR-29b KO NK cells in this model, this did not lead to a significant improvement in overall survival in preliminary studies (Supplemental Figure 8). This could be due to an exogenous source of miR-29b interacting with the transferred miR-29b KO NK cells, or alterations in homing of NK cells with altered miR-29b expression. Further studies will be needed to determine the precise role of miR-29b in modulating NK cell immunity in AML.

The finding of increased miR-29b in the NK cell compartment was unexpected, as low levels of miR-29b in the AML blast population have been previously associated with aggressive disease (43–45). Elevated levels in NK cells could be due in part to release of excessive miR-29b from AML blasts in the form of exosomes or vesicles, as this mechanism of cell-to-cell miR exchange has been reported in chronic leukemia (46, 47). Previous studies have established a role for extracellular vesicle trafficking in immune suppression and shedding of negative regulators of tumor cell growth or progression (46, 48–51). Interestingly, a recent study by Yeh et al. demonstrated that while there was no reduction in miR-29b expression between normal B cells and primary chronic lymphocytic leukemia (CLL) cells, there was a significant increase in miR-29b expression in exosomes derived from these primary CLL cells as compared with normal B cells (47). Indeed, preliminary studies in AML patients demonstrate the presence of miR-29b in the exosomes of patients with primary AML. Further, coculture of NK cells with plasma exosomes from patients with AML increases miR-29b in NK cells, compared with NK cells cocultured with plasma from normal individuals (data not shown). Alternatively, other soluble factors produced by either the leukemic blasts or the leukemic microenvironment could be regulating miR-29b expression in NK cells as well as other immune populations. For example, Smith et al. demonstrated that IFN-γ, which was upregulated in cytokine-stimulated NK cells from leukemic mice ex vivo, has the ability to induce miR-29b in T cells (27). It is therefore possible that IFN-γ is functioning as a negative-feedback loop to control NK cell development and alter mature NK cells in vivo, potentially skewing NK cells toward cell exhaustion and eventual hyporesponsiveness with chronic exposure to the leukemic microenvironment. Further studies would be required to determine what is specifically driving the overexpression of miR-29b in NK cells. One limitation of this work is the inability to evaluate miR-29b in the developmental NK cell precursor populations in human AML patients. NK cell sampling could be obtained prior to the disappearance of the DP NK cell population in the mouse model of AML.

As noted earlier, the presence of terminally differentiated mature NK cells in the mouse model of de novo AML and in AML patients requires additional investigation to determine what mechanisms allow for their persistence and/or generation despite the near absence of their developmental precursors. It could be that
alternate pathways of NK cell maturation are operative when develop-
mental intermediates are selectively targeted for elimination. In
support of this hypothesis, recent data presented by Wu et al.
using genetic bar coding of CD34+ progenitor populations in rhesus
macaques indicated that CD56<sup>high</sup> and CD56<sup>dim</sup> NK cells may arise
from distinct progenitors (52). While there are ample data validat-
ing the maturation pathways established in both mice and humans
(17, 18, 30, 53–56), it is possible if not likely that an additional undis-
covered precursor exists, and the leukemic environment is shifting
the balance that is seen under normal homeostatic conditions.
Future studies would be warranted to determine whether the ter-
minal differentiated NK cells as seen in our leukemic mice and
patients arise from a noncanonical/distinct pathway.

Finally, and not inconsistent with the above, it could be that the
developmental DP NK cell intermediate in the mouse has a criti-
cal as-yet unknown function distinct from killing that keeps AML
in check, and the successful emergence of AML requires the elimi-
nation of this specific subset of innate immune effector cells in the
same way that HIV selectively attacks the CD4 T helper cell subset.
The consistency of our observation in both mice and humans would
support this possibility. Indeed, unlike the cytolytic CD56<sup>dim</sup> NK
cell found almost exclusively in peripheral blood, the less mature
CD56<sup>high</sup> human NK cell lives predominantly in the parafollicular T
cell-rich region of secondary lymphoid tissue (19, 57). The CD56<sup>high</sup>
NK cell is a potant producer of cytokines, receiving signals for acti-
vation from dendritic cells residing in secondary lymphoid tissue
and in turn priming other antigen-presenting cells with IFN-γ, likely
important for the antigen-specific arm of the immune system (57).
As such, its selective absence in AML may have more to do with
disarming T cell responsiveness to tumor-associated antigens than
with NK cell maturation. Further investigation into defects in T cells
or other immune subsets has yet to be explored in our murine model
of AML but would be interesting given our findings with NK cell
developmental alterations as AML progresses.

In summary, these studies have identified previously unknown
NK cell defects in AML, and described a novel mechanism where-
by miR dysregulation drives immune evasion through alterations
in NK cell development. While the murine model uses the MLL-PTD
and FLT3-ITD mutations to generate de novo AML, the alterations
in NK cell maturation appear to occur across multiple different sub-
types of AML, as our patients’ molecular defects were not limited to
MLL-PTD and/or FLT3-ITD disease. This suggests that the defects
uncovered with these studies may represent a broad mechanism of
targeting NK cell development to succeed with innate immune
cell evasion in AML. Additional studies are underway to target this
defect therapeutically and restore normal NK cell homeostasis. Fur-
ther understanding of the forces driving this defect is also needed
to effectively salvage NK cell development, and potentially improve
NK cell–based therapies with the goal of preventing AML relapse.

Methods

Mouse strains. All mice were bred and maintained in pathogen-free con-
ditions, and all studies were approved by the Ohio State University Insti-
tutional Animal Care and Use Committee (IACUC). Mll<sup>PTD/WT</sup> Flt3<sup>ITD/WT</sup>
(PTD/ITD) mice have been previously described (58). Congenic
C57BL/6 Rag<sup>2<sup>−/−</sup></sup> Il2rg<sup>−/−</sup> mice were purchased from Taconic Biosciences
and were crossed with Mll<sup>PTD/WT</sup> Flt3<sup>ITD/WT</sup> mice to create Rag2<sup>−/−</sup> Il2rg<sup>−/−</sup>–
Mll<sup>PTD/WT</sup> Flt3<sup>ITD/WT</sup> (PTD/ITD/Rag GC KO) mice. C57BL/6 Mir29ab1<sup>−/−</sup>
mice were generated in house by S. Costinean and C.M. Croce (27, 59).
Congenic CD45.1<sup>+</sup> C57BL/6 mice for transplant studies were obtained from
Jackson Laboratory. All mice used in these studies were sex-
matched and 6–12 weeks of age unless otherwise noted.

AML transplantation. For leukemic transplantation, CD45.2<sup>+</sup> leu-
kemic cells were obtained from the spleens of primary leukemic mice
(58). Recipient CD45.1<sup>+</sup> congenic mice were lethally irradiated (8 cGy)
using an x-ray irradiator, and rescue bone marrow (1 × 10<sup>6</sup> cells per
mouse) was provided at the time of leukemic transplantation. CD45.2<sup>+</sup>
cells were injected i.v. into the irradiated recipients, and AML devel-
opment was monitored by flow cytometry analysis of CD45.2 versus
CD45.1 percentage positivity in the blood, as well as by wbc counts
(Hemavet, Drew Scientific).

NK cell isolation. NK1.1–CD3<sup>+</sup> cells were isolated from spleens as
previously described (60). NK cells were negatively enriched by mag-
netic microbead isolation (Miltenyi Biotec), followed by FACS using a
BD FACSAria II (BD Biosciences) to greater than 99% purity. NK cell
subsets were isolated in the same manner, and purity was validated on
each subset after sorting.

Cell lines. Yac-1 murine lymphoma and C1498 murine acute
myeloid leukemia tumor cell lines were obtained from American Type
Culture Collection. Both lines were maintained in RPMI 1640 with
10% FBS (Corning) and GlutaMAX (Gibco).

Primary patient samples. All human studies were conducted under
an approved Ohio State University Institutional Review Board Protocol
(2009C0019). Fresh peripheral blood samples from newly diagnosed,
untreated AML patient samples were obtained through the Ohio State
University Leukemia Tissue Bank. All newly diagnosed, untreated
AML patients were accepted for study regardless of AML subtype.
Normal donor peripheral blood was obtained through the American
Red Cross (Columbus, Ohio, USA) or ZenBio. NK cells were enriched
by negative selection with NK Rosette, containing anti-CD3, -CD4,
-CD19, -CD36, -CD66b, and -CD123 antibodies, following the manu-
facturer’s recommendations (Stem Cell Technologies). The blood was
then layered over Ficoll-Paque PLUS (GE Healthcare) and centrifuged
at 754 g for 20 minutes at room temperature. The NK cells were then
collected from the enriched mononuclear layer. Red blood cells were
lysed with ammonium chloride lysis buffer (Stem Cell Technologies),
and single-cell suspensions were obtained. NK cells were labeled for
immunophenotypic analysis as described below.

Immunophenotypic analysis. For multiparameter flow cytometry
conducted on murine cells, single-cell suspensions were obtained from
spleen, bone marrow, lymph node, or blood as previously described (58,
61). Briefly, organs were dissociated manually and washed with RPMI
1640 supplemented with 10% FBS and 0.5% EDTA, and rbc were lysed
with ammonium chloride rbc lysis buffer (Stem Cell Technologies).
Cells were resuspended in PBS supplemented with 2% FBS, labeled
with the indicated antibodies for 30 minutes at 4°C, washed, and then
analyzed on a BD LSRII flow cytometer (BD Biosciences). Human NK
cells were labeled in the same manner. For intracellular staining, cells
were labeled first with antibodies against cell surface antigens, then
permeabilized with Cytofix/Cytoperm (BD Biosciences), stained with
antibodies against intracellular antigens, and suspended in 1% formal-
lin before flow cytometry analyses. Data analysis was conducted with
Flowjo version 7.6.5 software (Tree Star). A list of all antibody clones
used in these studies can be found in Supplemental Table 1.
In *vivo* cell proliferation assay. Murine NK cells were obtained from spleens as described above, isolated by FACS, and labeled with 10 μM Cell Proliferation Dye eFluor 670 (eBioscience) according to the manufacturer’s protocol. Proliferation was calculated by taking gating on peaks indicating cell division within the total Cell Proliferation Dye–positive population. NK cells were transplanted into mice or placed in culture to control for flow cytometry evaluation after mouse harvesting. NK cells in culture were maintained in RPMI 1640 supplemented with 20% FBS (Corning), 1% antibiotic/antimycotic (Life Technologies), 55 μM 2-mercaptoethanol (Life Technologies), and IL-2 (100 ng/ml; Peprotech).

In *vivo* apoptosis assay. The commercially available FAM–FLIVO in vivo apoptosis kit was used to determine level of apoptosis in leukemic and WT mice (ImmunoChemistry Technologies). The manufacturer’s instructions were followed. Briefly, mice were injected i.v. with 8 μg/mouse FAM-FLIVO reagent. After 60 minutes, mice were harvested and cells were isolated for flow cytometry analysis. Additional cell surface antigens were evaluated to determine the level of apoptosis in specific NK cell populations.

Adoptive transfer experiments. Murine NK cells or NK cell subsets were isolated as described, sorted by FACS to greater than 99% purity, and injected i.v. into nonleukemic or leukemic recipient mice. Cells were labeled with Cell Proliferation Dye eFluor 670 (eBioscience) for short-term studies. Purity was validated before mouse injection.

**IFN-γ production.** freshly isolated splenocytes were obtained from leukemic and nonleukemic mice and incubated with plate-bound anti-NK1.1 (15 μg/ml) or isotype control for 6 hours in the presence of GolgiStop (BD Biosciences); labeled with anti-NK1.1, -CD3, and -CD45.1 surface antibodies; then fixed, permeabilized, and labeled with anti–IFN-γ antibody and evaluated by intracellular flow cytometry. For soluble IFN-γ production, murine NK cells were obtained from spleens of nonleukemic or leukemic mice and isolated by FACS sorting to purity greater than 99%, and were placed into culture. Cells were left unstimulated or stimulated with IL-12 (20 ng/ml; Genetics Institute Inc.) and GraphPad Prism software were used for analysis.

Cytotoxicity assays. Yac-1, C1498, or autologous blasts were used as targets where indicated in a standard 4-hour chromium release assay with previously described (60).

Quantitative real-time RT-PCR. RNA was isolated from NK cells using the Total RNA Purification Kit Plus (Norgen Biotek). cDNA was then obtained using the SuperScript VILO Master Mix (Life Technologies). Quantitative PCR was performed using either predesigned TaqMan probes or primers with SYBR Green Master Mix (Life Technologies) using a Viia 7 real-time PCR system (Life Technologies). Thx2I, Eomes, and Gapdh (internal control) SYBR Green primers were obtained from Sigma-Aldrich as previously described (62), and microRNA-29b (000413 murine and human miR-29b) and U6 control (001973 U6 for mouse; 001094 RNU44 for human) TaqMan primer/probes were purchased commercially (Life Technologies). Gene expression was normalized to an internal control (ΔCT = Ct gene of interest – Ct internal control). Relative mRNA expression for each gene tested was calculated as 2^−ΔΔCT. For miR-29b expression in patients and normal controls, the average ΔCT was calculated for the normal control samples. Relative mRNA expression for each patient and normal donor was calculated based on this average ΔCT and reported as relative fold change.

Statistics. Statistical significance was determined with Student’s t test, 2-tailed, for single comparisons, or linear mixed effects models were used for multiple comparisons to take account of correlations among observations from the same mouse. Holm’s procedure was used to adjust for multiple comparisons. After adjustment for multiple comparisons, P values less than 0.05 were considered significant. Bar graphs were presented as mean ± SEM unless otherwise noted. Log-rank test analysis was used for survival comparisons in vivo. SAS 9.3 (SAS Institute Inc.) and GraphPad Prism software were used for analysis.

Study approval. All murine studies were conducted under the approved Ohio State IACUC protocol 2009A0033-R2. For all human studies, written informed consent was received from participants prior to inclusion in the study where required. All samples were obtained under the approved IRB protocol 2009CO019.

Author contributions BLM, with the help of SDS, JY, AGF, and MAC, designed experiments, analyzed the data, and wrote the manuscript. BLM, LC, KM, EHA, NZ, JC, and SH performed experiments and analyzed data. XZ helped with statistical design and analysis. SC and CMC provided expertise with miR-29b murine studies and participated in the preparation of the manuscript. KL and JCB assisted with human miR-29b studies and participated in the preparation of the manuscript. HCM helped with flow cytometry design and analysis. SV and WB procured AML patient samples and participated in the preparation of the manuscript.

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13. Ahmad SM, Svane IM, Andersen MH. The stimu-


