Transcription factor KLF2 regulates homeostatic NK cell proliferation and survival

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Natural killer (NK) cells are innate lymphocytes that recognize and lyse virally infected or transformed cells. This latter property is being pursued in clinics to treat leukemia with the hope that further breakthroughs in NK cell biology can extend treatments to other cancers. At issue is the ability to expand transferred NK cells and prolong their functionality within the context of a tumor. In terms of NK cell expansion and survival, we now report that Kruppel-like factor 2 (KLF2) is a key transcription factor that underpins both of these events. Excision of *Klf2* using gene-targeted mouse models promotes spontaneous proliferation of immature NK cells in peripheral tissues, a phenotype that is replicated under ex vivo conditions. Moreover, KLF2 imprints a homeostatic migration pattern on mature NK cells that allows these cells to access IL-15–rich microenvironments. KLF2 accomplishes this feat within the mature NK cell lineage via regulation of a subset of homing receptors that respond to homeostatic ligands while leaving constitutively expressed receptors that recognize inflammatory cytokines unperturbed. Under steady-state conditions, KLF2-deficient NK cells alter their expression of homeostatic homing receptors and subsequently undergo apoptosis due to IL-15 starvation. This novel mechanism has implications regarding NK cell contraction following the termination of immune responses including the possibility that retention of an IL-15 transpresenting support system is key to extending NK cell activity in a tumor environment.

NK cell | KLF2 | NK cell proliferation | NK cell homeostasis | IL-15

Natural killer (NK) cells are a subset of group 1 innate lymphoid cells (ILCs) that participate in viral and tumor clearance by directly lysing stressed cells and producing cytokines that recruit and activate effector leukocytes (1). Humans and mice that lack NK cells have increased incidence of cancer (2), and clinical trials have demonstrated that adoptively transferred allogeneic NK cells can improve patient outcome without contributing to graft-versus-host disease (3). Moreover, in vivo expansion and persistence of donor NK cells correlates with tumor clearance (4), which suggests that therapeutic efficacy can be enhanced by augmenting NK cell survival. Therefore, understanding basic mechanisms that support NK cell homeostasis has clinical implications in terms of cancer therapy.

Following the establishment of a diverse NK cell receptor repertoire, NK cells exit the bone marrow and circulate throughout peripheral tissues including the lungs, liver, gut, lymph nodes, blood, and splenic red pulp (5, 6). In mice, peripheral NK cell differentiation is further described in relation to CD11b and CD27 surface expression, progressing in maturity from $CD27^+CD11b^-$ (stage 1) to $CD27^+CD11b^+$ (stage 2) to $CD27^-CD11b^+$ (stage 3) (7). With regard to peripheral homeostasis, early $CD27^+$ NK cell stages are associated with IL-15–dependent proliferation (8, 9), whereas later $CD11b^+$ stages require IL-15 for survival (10). As such, these two IL-15–dependent events are prime targets for controlling NK cell expansion and in vivo persistence.

To better understand how NK cell homeostasis is regulated, we investigated the potential role of transcription factor Kruppel-like factor 2 (KLF2) within the NK cell compartment by using *Klf2* gene-targeted mice. The rational for this study was threefold: (*i*) KLF2 maintains homeostasis in other lymphocyte compartments, including quiescent B (11–13) and T cells (14, 15); (*ii*) NK cell proliferation is regulated by a P13K-PDK1-Akt-mTOR signaling pathway (9, 16–18), which terminates KLF2 expression in other lymphocyte populations (19, 20; and (*iii*) Foxo1, which regulates *Klf2* transcription in T cells (21, 22), inhibits late stage NK cell differentiation (23). Based on these reports, we predicted that *Klf2* gene-targeted mice would exhibit mature NK cell hyperplasia because of dysregulated proliferation and relaxed maturation checkpoints. Indeed, *Klf2* excision promoted $CD27^+$ NK cell cycling in a cell-intrinsic manner. However, instead of a preponderance of late-stage NK cells, we found that KLF2 was necessary for $CD11b^+$ effector cell survival. Under steady-state conditions, KLF2-deficient NK cells altered expression of homeostatic homing receptors, thereby preventing these cells from accessing IL-15–rich microenvironments. Importantly, aberrant migration proceeded KLF2-deficient NK cell death, which was confined to an in vivo setting. Therefore, we conclude that KLF2 regulates mature NK cell homeostasis by limiting production of newly differentiated effector cells while simultaneously supporting their survival by guiding these cells toward transpresented IL-15. This latter event may represent a novel form of tolerance that terminates unwarranted NK cell activity.

Results

KLF2 Is Necessary for Conventional NK Cell Homeostasis. KLF2 is necessary to maintain B and T-cell homeostasis (11–15). To determine whether this transcription factor played a similar role in NK cells, we first verified that KLF2 was expressed under steady-state conditions. Following lineage commitment and initial development in the bone marrow, NK cells home to peripheral tissues, where they continue a differentiation program that is characterized by the surface expression of CD27 and CD11b (7). Isolating individual populations ($CD27^+CD11b^-$, $CD27^+CD11b^+$, and $CD27^-CD11b^+$),

Significance

Adoptive transfer of allogeneic natural killer (NK) cells into leukemia patients can lead to remission; however, therapies are hindered by inefficient expansion and limited persistence of these lymphocytes. We now report that Kruppel-like factor 2 (KLF2) regulates both NK cell proliferation and survival. KLF2 limits homeostatic expansion of NK cells in a cell-intrinsic manner. In addition, KLF2 instructs mature NK cells to home to IL-15–rich niches, which is necessary for continued survival under homeostatic conditions. Therefore, targeting KLF2 while providing rate-limiting survival factors such as transpresented IL-15 may improve NK cell engraftment and sustainability in cancer patients.

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mRNA and protein analysis revealed that KLF2 is expressed early during NK cell development and increases with maturation (Fig. 1*A*). Next, we assessed NK cell homeostasis in *Klf2* gene-targeted mice. To ensure KLF2 was depleted from the entire NK cell compartment (Fig. 1*B*), *Vav-cre* transgenic animals were used to excise floxed alleles of *Klf2* (*Klf2*$^{fl/fl}$) in hematopoietic stem cells. Early NK cell development and MHC licensing was intact in *Vav-cre; Klf2*$^{fl/fl}$ animals, as reflected by normal frequencies of bone marrow-derived NK cells expressing activating (NK1.1, NKG2D, NKp46, Ly49H) and inhibitory (Ly49C/I, Ly49D, Ly49G2) receptors (Fig. S1*A*). In contrast, loss of KLF2 resulted in increased CD27^{+}CD11b^{-} NK cell frequencies in all tissues, with the exception of mesenteric lymph nodes (Fig. 1*C*). Absolute CD27^{+}CD11b^{-} NK cell numbers were also increased in the spleen, liver, and bone marrow, the latter two tissues being sites that preferentially harbor immature NK cell populations. These cells expressed high levels of transcription factor EOMES and were either CD127^{-} (spleen) or CD49b^{+}TRAIL^{-} (liver), indicating that they were NK cells and not misidentified NK1.1^{+} ILC1 cells (24) (Fig. S1*B*). Instead, ILC1 numbers remained constant in *Vav-cre; Klf2*$^{fl/fl}$ animals. Likewise, loss of KLF2 did not affect CD49a^{+}CD49b^{-} tissue-resident NK cells in the liver (25) (Fig. S1*C*). Collectively, these data suggest that KLF2 limits proliferation associated with CD27^{+}CD11b^{-} NK cell differentiation but does not affect homeostasis of neighboring lineages, including ILC1 cells.

Despite the increase in early stage NK cells, *Vav-cre; Klf2*$^{fl/fl}$ mice had significantly fewer CD27^{+}CD11b^{+} and CD27^{-}CD11b^{+} NK cells relative to littermate controls (Fig. 1*C*). Costaining for CD43^{+} and CD11b^{+} (markers for late-stage NK cells) confirmed that KLF2 gene-targeted mice lacked mature CD43^{+}CD11b^{+} populations in all examined tissues (Fig. 1*D*). Given that IL-15 is essential for NK cell survival (26–28), we hypothesized that KLF2-deficient NK cells were unable to respond to this cytokine. However, KLF2-deficient NK cells expressed normal surface levels of CD122 (IL-2Rβ) and CD132 (γ_c) (Fig. 1*E*) that conveyed IL-15–mediated signaling events when stimulated ex vivo (Fig. 1*F*), which suggested that defective IL-15R signaling was not responsible for mature NK cell depletion. [Increased baseline expression of phospho-S6 in immature NK cells reflects elevated metabolism, as noted (17).] Instead, it was possible that mature NK cells were present in *Vav-cre; Klf2*$^{fl/fl}$ mice but that they were either misidentified (i.e., KLF2 regulates CD11b and CD43 expression) or located in alternate tissues. To address the former option, we used cytolytic activity as a surrogate marker to identify mature KLF2-deficient NK cells. Reflective of their immature status, total NK cells harvested from *Vav-cre; Klf2*$^{fl/fl}$ mice expressed low levels of granzyme B (Fig. 2*A*) and had a reduced ability to lyse YAC-1 target cells ex vivo (Fig. 2*B*). At the same time, KLF2-deficient NK cells responded to anti-NK1.1 stimulation by increasing surface expression of the degranulation marker CD107a (Fig. 2*C*), which indicated that cell-intrinsic effector functions were not directly regulated by KLF2. Together, these data suggest that mature NK cells are not present in the spleens of *Vav-cre; Klf2*$^{fl/fl}$ mice. To extend this finding to additional tissues, in vivo tumor clearance was analyzed in animal cohorts following coinjection of NK cell-sensitive (RMA-S) and NK cell-resistant (RMA) tumor cells. Compared with littermate controls, *Vav-cre; Klf2*$^{fl/fl}$ animals were unable to effectively clear RMA-S target cells (Fig. 2*D*). Therefore, we conclude that effector NK cells are absent in *Vav-cre; Klf2*$^{fl/fl}$ mice and that KLF2 is necessary to support mature NK cell homeostasis.

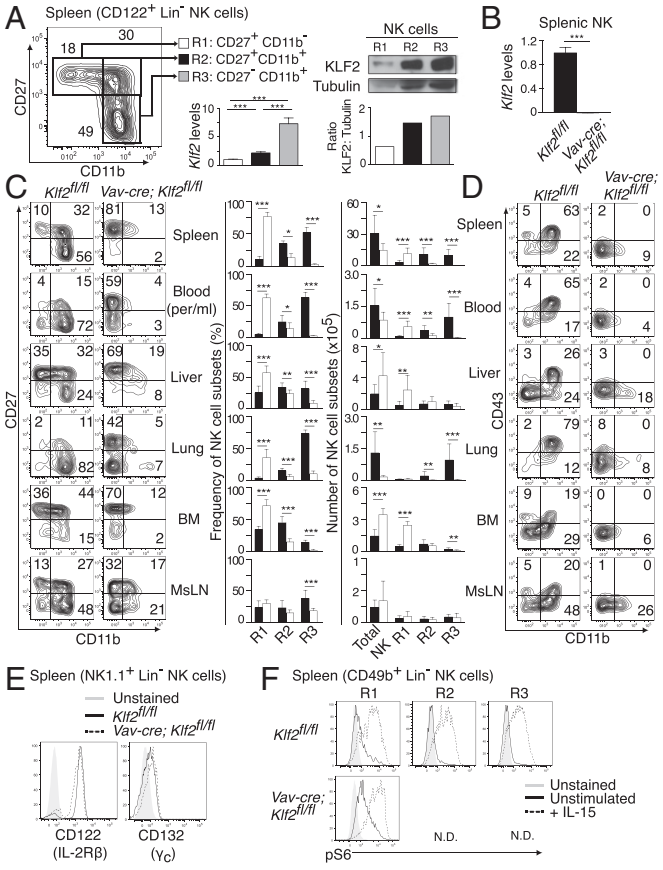

Fig. 1. KLF2 is necessary for NK cell homeostasis. (*A*) *Klf2* mRNA and KLF2 protein levels in NK cell subsets. Splenic CD122^{+}Lin^{-} (CD3, CD8, CD19, Gr-1, TCRβ) NK cells were FACS sorted into maturing NK cell subsets (R1, CD27^{+}CD11b^{-}; R2, CD27^{+}CD11b^{+}; R3, CD27^{-}CD11b^{+}) from C57BL/6 mice. *Klf2* mRNA and KLF2 protein levels were normalized to *gapdh* and tubulin, respectively. This experiment was repeated twice. (*B*) *Klf2* mRNA levels expressed in MACS-sorted NK cells harvested from *Klf2*$^{fl/fl}$ versus *Vav-cre; Klf2*$^{fl/fl}$ mice (normalized to *gapdh*). (*C*) Contour plots of CD122^{+}Lin^{-} NK cell populations harvested from *Klf2*$^{fl/fl}$ (black) versus *Vav-cre; Klf2*$^{fl/fl}$ (white) littermates. Frequencies and absolute cell numbers are graphed. BM, bone marrow; MsLN, mesenteric lymph nodes. Data are pooled from three independent experiments (n = 10 mice per group). (*D*) Alternate analysis of CD122^{+}Lin^{-} NK cell populations, using CD43 and CD11b as maturity markers. n = 10 mice per cohort. (*E*) IL-15R surface expression on splenic NK cells harvested from *Klf2*$^{fl/fl}$ versus *Vav-cre; Klf2*$^{fl/fl}$ mice. n = 3 experiments. (*F*) IL-15R signaling capacity of control (*Top*) versus KLF2-deficient NK cell populations (*Lower*). NK cells were cultured ± IL-15 (1 h) before intracellular staining for phosphorylated S6, a downstream target of mTOR activity. This experiment was repeated three times. *P < 0.05; **P < 0.01; ***P < 0.001.

KLF2-Regulated NK Cell Homeostasis Is Cell Intrinsic. Mature NK cell survival depends on IL-15 that is typically transpresented as an IL-15/IL-15Rα complex on the surface of mesenchymal and myeloid cells (28–31). Because *Vav-cre; Klf2*$^{fl/fl}$ mice excised *Klf2* within the myeloid compartment, NK cell homeostasis may have been altered because of compromised IL-15 presentation. To test this hypothesis, NK cell populations were analyzed in *LysM-cre; Klf2*$^{fl/fl}$ mice that excised *Klf2* in a myeloid-specific manner (Fig. S2*A*). Using this genetic model, we found normal numbers of CD27^{+}CD11b^{-} and CD11b^{+}CD27^{+} cells, and a slight but statistical decrease in CD27^{-}CD11b^{+} NK cells. These results suggest that major NK cell phenotypes observed in *Vav-cre; Klf2*$^{fl/fl}$ mice were independent of myeloid cells. Additionally, a prior study demonstrated that transferring mutant NK cells into wild-type animals could overcome myeloid-specific defects in NK cell development (32); however, KLF2-deficient NK cell survival was not rescued under these circumstances (Fig. S2*B*). To confirm that KLF2 intrinsically regulates NK cell homeostasis, lethally irradiated wild-type CD45.2^{+} mice were reconstituted with a 1:1 ratio of wild type (CD45.1^{+}) versus *Vav-cre; Klf2*$^{fl/fl}$ (CD45.2^{+}) bone marrow and analyzed 8 wk later. As shown in Fig. 3, CD27^{+}CD11b^{+} and CD27^{-}CD11b^{+} NK cells were primarily derived from wild-type recipients, whereas the majority of CD27^{+}CD11b^{-} NK cells were KLF2-deficient. Collectively, these data indicate that KLF2 maintains late-stage NK cell homeostasis in a cell-intrinsic manner and may a play a role in suppressing early stage NK cell proliferation.

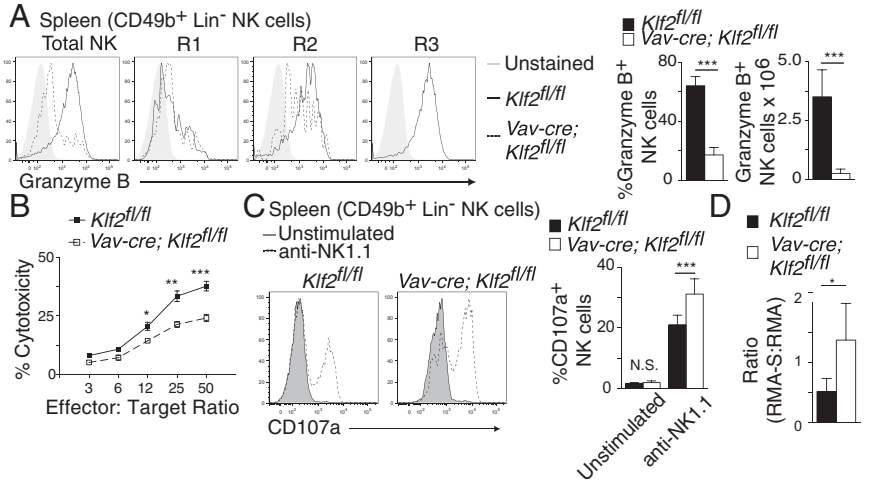

Fig. 2. Mature cytolytic NK cells are absent in *Vav-cre; Klf2$^{fl/fl}$* mice. (*A*) Histogram overlays (*Left*) and quantification (*Right*) of granzyme B expression following (PMA + ionomycin)-simulation of splenic NK cells harvested from *Klf2$^{fl/fl}$* versus *Vav-cre; Klf2$^{fl/fl}$* mice. Histograms display individual subsets, whereas columns are total NK cells. n = 9–11 mice per cohort, pooled from three independent experiments. (*B*) Ex vivo cytolytic activity of IL-2–primed splenocytes cultured with Yac-1 target cells for 4 h in an LDH release assay. This experiment was performed once in quadruplicate. (*C*) CD107a surface expression on NK cells cultured for 6 h ± plate-bound NK1.1 antibody. n = 6 mice per cohort, pooled from two independent experiments. (*D*) RMA control and RMA-S target cells were coinjected at a 1:1.5 ratio into *Klf2$^{fl/fl}$* versus *Vav-cre; Klf2$^{fl/fl}$* mice and RMA/RMA-S survival was assessed 48 h later. This experiment was repeated twice by using three mice per group. *P < 0.05; **P < 0.01; ***P < 0.001.

KLF2 Suppresses Homeostatic Proliferation of NK Cells. KLF2 was originally reported to prevent the spontaneous proliferation of quiescent T cells (33, 34); however, subsequent work demonstrated that this event was not a cell-intrinsic effect (14, 15). To better characterize KLF2's role in early stage NK cell cycling, 5-bromo-2′-deoxyuridine (BrdU) incorporation was quantified as a measure of steady-state NK cell proliferation (Fig. 4*A*). Compared with control littermates, significantly more BrdU$^+$ NK cells were present in the bone marrow of *Vav-cre; Klf2$^{fl/fl}$* animals, the majority of which were CD27$^+$CD11b$^-$. Nuclear staining for Ki-67, which identifies proliferating cells, confirmed that immature NK cells were undergoing increased cell cycling in *Vav-cre; Klf2$^{fl/fl}$* mice (Fig. 4*B*). To determine whether KLF2-deficient NK cells had increased access to proliferation-inducing factors in vivo, NK cell cycling was examined under controlled ex vivo conditions. Using a tamoxifen-inducible Cre system, NK cells harvested from *T2-cre; Klf2$^{fl/fl}$* versus littermate control mice were cocultured (1:1 ratio) in media supplemented with 4-hydroxytamoxifen (4-OHT) and IL-2, the latter cytokine added to support cell cycling. After 5 d in culture, increased numbers of CD45.2$^+$ cells from *T2-cre; Klf2$^{fl/fl}$* mice were recovered relative to CD45.1$^+$ control cells (Fig. 4*C*), primarily due to an expansion of CD27$^+$CD11b$^-$ NK cells. Flow cytometric analysis also demonstrated that more KLF2-deficient NK cells exhibited a blast morphology compared with KLF2-sufficent cells, consistent with KLF2 limiting early stage NK cell proliferation.

Mature NK Cells Require KLF2 To Access Transpresented IL-15. Lymphocyte proliferation and differentiation are typically exclusionary events, which raised the possibility that KLF2 was necessary for mature NK cell differentiation. To test this hypothesis, we performed ex vivo differentiation assays by using NK cells harvested from control versus *Vav-cre; Klf2$^{fl/fl}$* mice. To aid in our analysis, input populations were depleted of CD11b$^+$ cells to offset the increased frequency of mature NK cells present in wild-type animals. Following 72 h and 96 h in stromal culture supplemented with IL-15, IL-12, and IL-18, KLF2-deficient NK cells differentiated into CD27$^+$CD11b$^+$ (R2) and CD27$^-$CD11b$^+$ (R3) cells that expressed the maturity markers, KLRG1 and granzyme B (Fig. 5). From this result, we conclude that KLF2 is not necessary for late-stage NK cell differentiation.

Given that KLF2-deficient NK cells can fully differentiate ex vivo but not in vivo, we hypothesized that NK cells were unable to access a limited survival niche in *Vav-cre; Klf2$^{fl/fl}$* mice. Transpresented IL-15 is necessary for late-stage NK cell survival, which is rate limiting under steady-state conditions (35). For this reason, we performed immunohistochemistry to document NK cell localization relative to IL-15 in the spleens of *Vav-cre; Klf2$^{fl/fl}$* versus littermate controls. Using a metallophilic macrophage antibody (MOMA-1) to delineate the marginal sinus that forms a ring around the white pulp, we found that NK cells predominately localized in the red pulp of *Klf2$^{fl/fl}$* control mice (Fig. 6*A*). In contrast, significantly more KLF2-deficient NK cells were present in the T-cell–rich area of the white pulp. These KLF2-deficient NKp46$^+$ cells stained negatively for TCRβ, thus confirming that they were NK cells and not NKT cells. Within the spleen, IL-15 is typically presented by myeloid cells and VCAM-1$^+$ stromal cells (36) located in the red pulp. Consistent with a lack of mature NK cells in *Vav-cre; Klf2$^{fl/fl}$* mice, KLF2-deficient NK cells were preferentially located in IL-15–depleted areas of the white and red pulp (Fig. 6*B* and Fig. S3). To verify that differences in localization were directly attributable to KLF2-regulated NK cell migration and not maturation-associated homing patterns (5), we examined how similarly differentiated KLF2-replete and KLF2-deficient NK cells trafficked in vivo. In this instance, KLF2-deficient NK cells were harvested from tamoxifen-treated *T2-cre; Klf2$^{fl/fl}$* mice before alterations in subset frequencies (Fig. 6*C*, *i*). Following cotransfer into wild-type animals, we found that KLF2$^+$

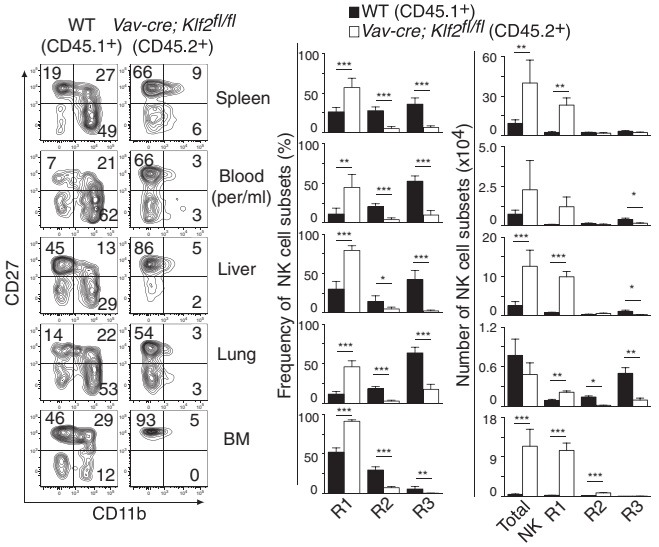

Fig. 3. KLF2-mediated NK cell homeostasis is cell intrinsic. Analysis of mixed bone marrow chimeras that were generated by reconstituting lethally irradiated *Klf2$^{fl/fl}$* (CD45.2$^+$) mice with wild-type (CD45.1$^+$) and *Vav-cre; Klf2$^{fl/fl}$* (CD45.2$^+$) bone marrow. Flow cytometric analysis was performed 8 wk after transfer. Representative contour plots, frequencies, and cell numbers of gated (CD122$^+$Lin$^-$) KLF2-sufficient (black) and KLF2-deficient (white) populations are shown. This experiment was performed once by using five recipient animals. *P < 0.05; **P < 0.01; ***P < 0.001.

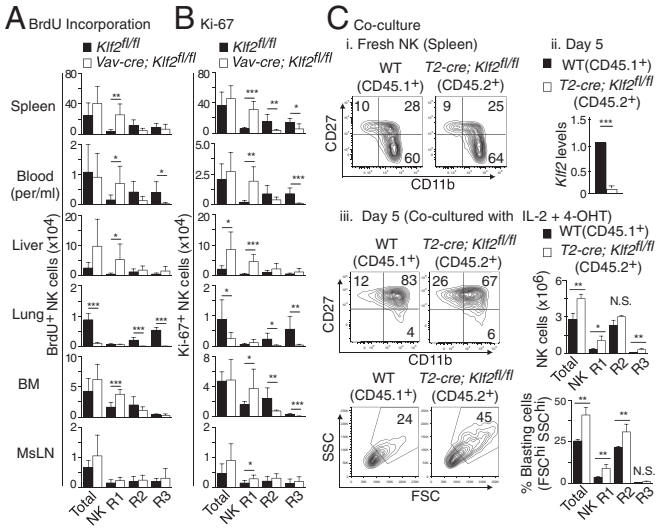

Fig. 4. KLF2 suppresses proliferation in immature NK cells. (*A*) BrdU incorporation over 5 d was used to assess NK cell ($CD122^+Lin^-$) proliferation in various tissues harvested from $Klf2^{fl/fl}$ versus *Vav-cre; Klf2*$^{fl/fl}$ littermates. n = 6 mice per cohort, pooled from two independent experiments. (*B*) The percentage of $CD122^+Lin^-$ NK cells actively proliferating was quantified by Ki-67 expression. n = 7 mice per group (two pooled experiments). (*C*) Equal numbers of MACS-sorted NK cells from wild type ($CD45.1^+$) versus *T2-cre; Klf2*$^{fl/fl}$ ($CD45.2^+$) mice were cocultured in 4-OHT and IL-2 to induce *Klf2* excision and support proliferation, respectively. NK cells were analyzed by flow cytometry before (*i*) and after (*iii*) excision and *Klf2* expression was assessed by RT-PCR at day 5 (*ii*). This experiment was repeated twice by using three biological replicates per group. *P < 0.05; **P < 0.01; ***P < 0.001; N.S., not significant.

NK cells primarily homed to the red pulp and associated with $F4/80^+$ myeloid cells (Fig. 6*C, ii*), a cell type that transpresents IL-15 (10). In contrast, a significant number of KLF2-deficient NK cells migrated to the white pulp, as defined by MOMA-1 staining (Fig. 6*C, iii*), and were distal to myeloid cells. Therefore, we conclude that KLF2 directs NK cell trafficking under noninflammatory conditions, the implication being that this migration pattern supports late-stage NK cell survival.

KLF2 Regulates Mature NK Cell Homing Receptor Expression and Survival. Mature NK cells circulate throughout the vasculature and quickly respond to inflammatory chemokine gradients without prior activation. As such, NK cells need to constitutively express a hybrid array of homing receptors that recognize ligands present under steady-state (e.g., CD62L, CX_3CR1, $S1P_5$) and inflammatory conditions (e.g., CCR2, CCR5, CXCR3). To determine whether any of these receptors were modified in response to *Klf2* excision, mature NK cell populations harvested from tamoxifen-treated *T2-cre; Klf2*$^{fl/fl}$ mice were examined by flow cytometry (Fig. 7*A*) or by RT-PCR when antibodies were not available (Fig. 7*B*). Compared with mature NK cells from tamoxifen-treated littermate controls, KLF2-deficient cells expressed significantly less CD62L, *Cx*$_3$*cr1*, and *Edg8*. Surprisingly, CCR7 was up-regulated following *Klf2* excision, which suggested that a chemokine receptor typically associated with naïve T-cell trafficking was actively repressed by KLF2 in mature NK cells. Previous studies have noted that NK cells increase CCR7 expression and decrease CD62L surface levels following NK cell activation (37) or PI3K activity (16), respectively, and because KLF2 is degraded in a PI3K-associated manner following T-cell activation (19, 20), we were curious whether a similar process existed within the NK cell compartment. Indeed, stimulation of NK cells via activating receptors (NK1.1, NKG2D) promoted KLF2 proteolysis (Fig. S4*A*). Moreover, NK cells cultured in the presence of a PI3K-inhibitor impaired KLF2 degradation (Fig. S4*B*), the implication being that this signaling pathway regulates KLF2 levels. To assess how closely *Klf2* excision replicated homing receptor regulation following NK cell activation, wild-type NK cells were stimulated with the toll-like receptor 3 agonist, polyinosinic-polycytidylic acid [poly(I:C)], then examined for differential receptor expression. Similar to KLF2-deficient cells, poly(I:C)-treated NK cells altered their surface levels of CCR7 and CD62L (Fig. S4*C*) and (PMA/ionomycin)-activated $CD27^-CD11b^+$ NK cells expressed less *Edg8* mRNA (Fig. S4*D*). Of note, elevated CCR7 expression is consistent with observed NK cell entry into splenic white pulp following poly(I:C) challenge (38). Poly(I:C)-activated NK cells also altered their surface expression of CCR2, CCR5, and CXCR3—homing receptors that respond to inflammatory cytokines (Fig. S4*E*). In contrast, *Klf2* excision did not appreciably affect the expression of these inflammatory homing receptors (Fig. 7*C*). From these studies, we conclude that KLF2 regulates expression of homing receptors that respond to homeostatic ligands; however, this transcription factor does not impact inflammatory chemokine receptors within the NK cell compartment.

With regard to how this alteration in homing receptors impacted the NK cell compartment, we found that mature NK cell numbers decreased over time when *Klf2* was excised under noninflammatory conditions (Fig. 7*D*). Unlike poly(I:C) treatment that causes splenic NK cell displacement into the liver (ref. 39; Fig. S4*F*), *Klf2* excision did not cause an accumulation of mature NK cells in any observed tissues. Instead, analysis of NK cells harvested from tamoxifen-treated *Klf2*$^{fl/fl}$ versus *T2-cre; Klf2*$^{fl/fl}$ littermates revealed that this reduction was due to increased apoptosis of mature NK cells (Fig. 7*E*). Importantly, KLF2-deficient NK cell apoptosis (d7 onwards) occurred after alterations in homing receptors (d5; Fig. 7 *A*–*C*) and aberrant migration (d5; Fig. 6*C*). From these results, we conclude that KLF2 controls NK cell migration via regulation of homeostatic homing receptors, the alteration of which does not support NK cell survival under steady-state conditions.

Discussion

Adoptive transfer of allogeneic NK cells is a promising cancer therapy (40, 41); however, to maximize its potential in the clinic, it is important to devise new ways of increasing NK cell numbers and prolonging effector functions in vivo. Results from this study indicate that low levels of KLF2 limit antigen-independent NK cell proliferation in all tissues and removal of this factor expands the proliferative burst associated with $CD27^+CD11b^-$ NK cells. These

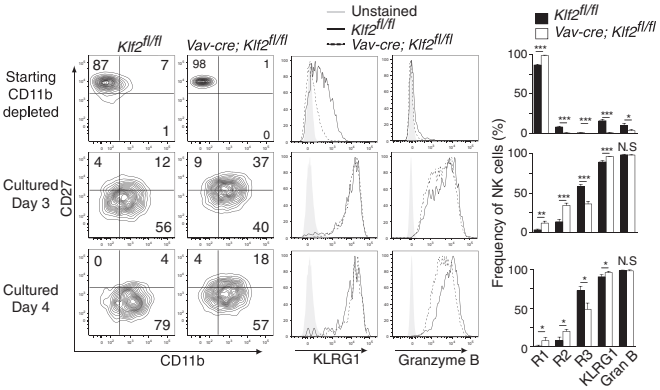

Fig. 5. KLF2-deficient NK cell differentiation is reestablished in culture. CD11b-depleted bone marrow from *Klf2*$^{fl/fl}$ and *Vav-cre; Klf2*$^{fl/fl}$ mice was plated on wild-type bone marrow ($CD45.1^+$) supplemented with IL-15, IL-12, and IL-18. Starting material and cells cultured for 3–4 d were initially gated ($CD45.2^+CD122^+Lin^-$) then analyzed for NK cell differentiation (CD27, CD11b contour plots) and maturity markers (KLRG1, granzyme B) by flow cytometry. Differentiation experiments were performed twice in triplicate, generating similar results. *P < 0.05; **P < 0.01; ***P < 0.001; N.S., not significant.

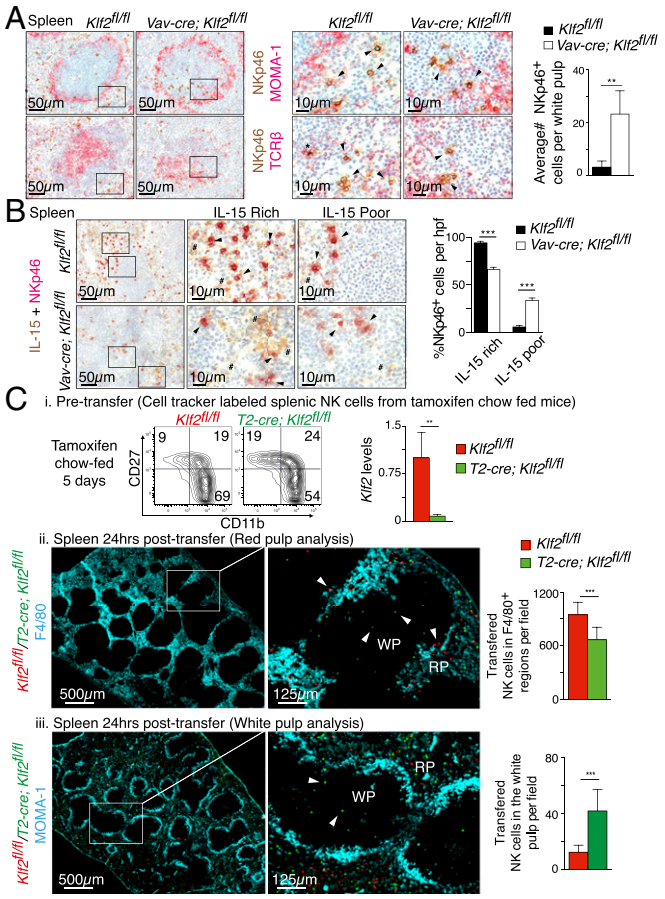

Fig. 6. KLF2 promotes NK cell migration toward IL-15–rich niches. (*A*) Immunohistochemistry of *Klf2*$^{fl/fl}$ and *Vav-cre; Klf2*$^{fl/fl}$ splenic serial sections costained for NKp46/MOMA-1 (*Top*) and NKp46/TCRβ (*Bottom*). Enlarged images that identify NK cells (arrows) and NKT cells (*) are shown at *Right*. Associated bar graph shows the average number of NKp46$^+$ NK cells within 10 similarly sized MOMA-1–encased sections per mouse. $n = 4$ mice per cohort. (*B*) *Klf2*$^{fl/fl}$ (*Top*) and *Vav-Cre; Klf2*$^{fl/fl}$ (*Bottom*) splenic sections costained for NKp46 and IL-15. *Middle* and *Right* show enlarged areas of IL-15–rich and IL-15–poor splenic sections, respectively. NKp46$^+$ cells (arrow) and IL-15$^+$ tissue (#) are identified. Associated bar graph shows the average frequency of NK cells (five high-powered fields per mouse) identified in IL-15–rich and –poor niches. $n = 3$–4 mice per cohort. (*C*) Dynamic migration of mature NK cell populations within the spleen. Splenic NK cells were isolated (MACS-sorted) from tamoxifen-treated (5 d) *Klf2*$^{fl/fl}$ versus *T2-cre; Klf2*$^{fl/fl}$ mice then labeled with red or green cell tracker dyes, respectively. These cells were subsequently cotransferred into a wild-type recipient and splenic localization was assessed 24 h later. This experiment was repeated three times. (*i*) NK cell subset frequencies (contour plots) and degree of *Klf2* excision (RT-PCR) within isolated NK cell populations before transfer. (*ii*) Immunohistochemistry of KLF2-sufficient (red) versus KLF2-deficient (green) NK cells in relation to F4/80$^+$ myeloid cells. Transferred NK cell numbers were quantified from 10 individual low-power field images. (*iii*) Immunohistochemistry of cotransferred NK cell populations in relation to the white pulp, as outlined with MOMA-1 antibody. Average number of transferred cells per field was calculated from 25 individual images. $^{**}P < 0.01$; $^{***}P < 0.001$.

findings are consistent with previous reports demonstrating that the PI3K-PDK1-mTOR signaling pathway promotes NK cell cycling (9, 17, 18, 42, 43), because we and others have shown that signaling receptors that activate the PI3K pathway suppress KLF2 expression in T cells (19, 20) and B cells (12). Surprisingly, Foxo1, which is negatively regulated by PI3K signaling and directly promotes *Klf2* transcription in T cells (21, 22), does not appear to link PI3K-mediated activating events with KLF2 expression in NK cells. This disconnect between transcription factors is evidenced by an inverse expression pattern (in contrast to KLF2, Foxo1 expression decreases from stage 1→stage 3) and increased frequencies of CD27$^-$CD11b$^+$ NK cells in *Foxo1* gene-targeted animals (23). This heretofore association between these two molecules raises the question as to what factors directly promote *Klf2* transcription in NK cells. Addressing this issue may provide a therapeutic means of increasing NK cell numbers by suppressing KLF2 expression in a lineage-specific manner.

In addition to controlling NK cell cycling, KLF2 is necessary for late-stage NK cell survival under steady-state conditions. More specifically, KLF2 regulates homeostatic homing receptors so that these cells gain access to transpresented IL-15. In contrast, activated NK cells degrade KLF2 and alter their homing receptor expression patterns accordingly. Both quiescent and activated NK cells rely on IL-15 for survival (44–46), which implies that activated NK cells have access to IL-15 that is not available to KLF2-deficient NK cells under noninflammatory conditions. We propose that during an innate immune response that activates NK cells, myeloid cells that are capable of IL-15 transpresentation are likewise activated and recruited to inflammatory sites, thereby maintaining NK cell effector functions. In the absence of this IL-15–dependent support system, NK cell effector activity is quickly terminated. Such an event might occur at the conclusion of a productive NK cell immune response when myeloid cells contract. Likewise, inappropriate NK cell

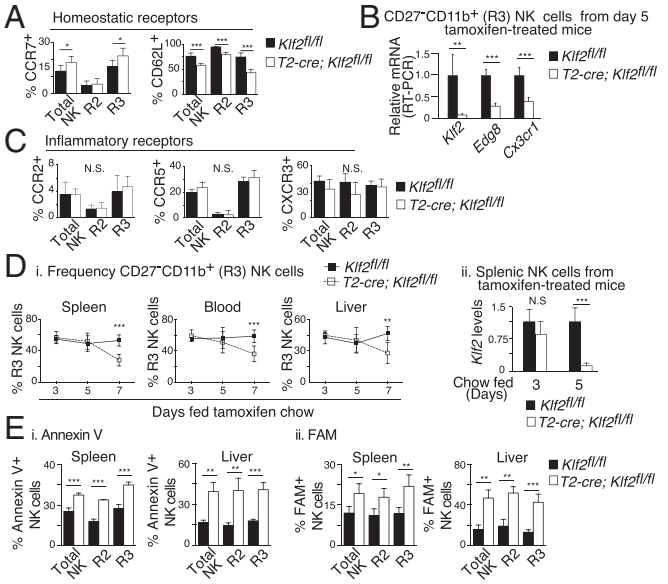

Fig. 7. KLF2 supports mature NK cell survival by regulating expression of homeostatic homing receptors. (*A*–*C*) *Klf2*$^{fl/fl}$ and *T2-cre; Klf2*$^{fl/fl}$ mice were placed on tamoxifen-infused chow for 5 d, then analyzed for expression of homing receptors that respond to constitutively expressed ligands (*A* and *B*) or inflammatory chemokines (*C*). (*A*) Surface expression of CCR7 and CD62L on CD122$^+$Lin$^-$ NK cells (R2 = CD27$^+$CD11b$^+$, R3 = CD27$^-$CD11b$^+$), as determined by flow cytometry. This experiment was repeated three times with three mice per cohort. (*B*) Relative mRNA expression of *Klf2*, *Edg8* ($S1P_5$), and *Cx3cr1* in FACS-sorted CD27$^-$CD11b$^+$ NK cells. This experiment was repeated twice. (*C*) Surface expression of CCR2, CCR5, and CXCR3 on CD122$^+$Lin$^-$ NK cells. This experiment was repeated two to three times by using a minimum of three mice per group. (*D*, *i*) Frequency of mature (CD122$^+$Lin$^-$CD27$^-$CD11b$^+$) NK cells in the spleen, blood, and liver of *Klf2*$^{fl/fl}$ versus *T2-cre; Klf2*$^{fl/fl}$ mice placed on tamoxifen-infused chow for the indicated time points, as determined by flow cytometry. $n = 3$–11 mice per time point. (*ii*) *Klf2* excision within MACS-sorted splenic NK cells was assessed at days 3 and 5 by RT-PCR. (*E*) KLF2 is necessary for mature NK cell survival under noninflammatory conditions. Frequency of Annexin V$^+$ (*i*) and FAM$^+$ (caspase active) (*ii*) NK cells isolated from *Klf2*$^{fl/fl}$ versus *T2-cre; Klf2*$^{fl/fl}$ mice placed on tamoxifen chow (d9). Mature NK cells (CD49b$^+$Lin$^-$) were defined as CD27$^+$CD11b$^+$ (R2) or CD27$^-$CD11b$^+$ (R3). This experiment was repeated three times by using three mice per cohort. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; N.S., not significant.

activation (e.g., an immune response directed against self via NKG2D) is predicted to starve these cells of IL-15 and maintain tolerance. This model may explain why mice and humans are devoid of NK cell-initiated autoimmunity, despite the fact that NK cells recognize self-ligands (e.g., stress molecules exhibited by tumor cells) (47) and do not require additional cells to become activated (48). Therefore, linking activated NK cell survival to colocalized IL-15 transpresentation may constitute a fundamental mechanism to ensure self-tolerance. Conversely, cancers may co-opt this tolerance mechanism to evade NK cell-mediated tumor surveillance. If this model of IL-15 dependence proves to be the case, then recruiting IL-15 transpresenting cells to environments that elicit NK cell activation may prevent NK cell exhaustion and restore antitumor immunity.

Materials and Methods

Mice. *Vav-cre; Klf2$^{fl/fl}$* and *T2-cre; Klf2$^{fl/fl}$* mice were generated as described (15, 19); LysM-cre and B6.SJL (CD45.1^{+}) mice were purchased from Jackson Laboratories. IL-15$^{-/-}$ mice were purchased from Taconic. Mice were housed in pathogen-free conditions in accordance with the Institutional Animal Care and Use Committee at Vanderbilt University.

Flow Cytometry. Standard flow cytometric procedures were used to acquire data on a 5-laser LSRII (BD Biosciences); analysis was performed by using FlowJo (TreeStar) software.

Bone Marrow Chimeras. Irradiated (2 × 500 cGy) *Klf2$^{fl/fl}$* (CD45.2^{+}) mice were reconstituted with 10^6 bone marrow cells (1:1 CD45.1^{+} versus *Vav-cre; Klf2$^{fl/fl}$* CD45.2^{+}), then analyzed 8 wk later by FACS.

Immunohistochemistry. Cryosections were stained with the following antibodies: polyclonal goat NKp46 (R&D Systems), biotin-TCRβ (BD Biosciences), biotin-MOMA1 (Cedarlane), biotin-F4/80 (Tonbo), and biotin-IL-15 (R&D Systems). The anti-NKp46 antibody was visualized with secondary biotinylated F(ab')$_2$ Frag donkey anti-goat IgG (H+L) (Jackson Immunoresearch). Nuclei were counterstained by using Meyer's hematoxylin solution (Sigma). Images were acquired by using Nikon AZ 100 (Nikon) and NIS-Elements (Nikon) software. Additional staining information is provided in *SI Materials and Methods*.

NK Cell Apoptosis. *Klf2$^{fl/fl}$* and *T2-cre; Klf2$^{fl/fl}$* littermates were placed on tamoxifen chow for 5 d, killed 4 d later, and NK cell populations were examined by flow cytometry. Annexin V surface expression (BD Biosciences) and caspase cleavage (Vibrant FAM kit; Life Technologies) were used as readouts for apoptosis based on manufacturers' instructions.

Statistical Analysis. Data were analyzed by using a two-tailed Student *t* test and displayed as the mean ± SD: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ unless otherwise indicated.

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