The Role of Transcription Factor Krüppel-like Factor 2 in Lymphocyte Migration and Homeostasis

By

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LIST OF FIGURES

Page

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LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

4-OHT	4-hydroxytamoxifen
AML	Acute myeloid leukemia
APC	Antigen presenting cell
BCR	B cell receptor
BM	Bone marrow
BrdU	5-bromo-2’-deoxyuridine
CFSE	Carboxyfluorescein succinimidyl ester
CMTMR	Carboxytetramethylrhodamine
CCL	C-C motif ligand
CCR	C-C motif ligand
CD62E	E-selectin
CD62L	L-selectin
CD62P	P-selectin
CPD	CDC4 phosphodegron
CR	Chemokine receptor
CISH	Cytokine inducible-src homology 2 (SH2) containing protein
CD	Cluster of differentiation
CLP	Common lymphoid precursor
cNK	Conventional natural killer cell
CXCL	C-X-C motif ligand

CX3CR1	C-X-3-C motif receptor 1
CXCR	C-X-C motif receptor
DAB	3, 3’-diaminobenzidine
DC	Dendritic cell
DMEM	Dulbecco’s Modified Eagle’s Medium
EKLF/KLF 1	Erythroid Krüppel-like factor
EOMES	Eomesodermin
FTY720	Fingolimod
GSK-3	Glycogen synthase kinase-3
GVHD	Graft versus host disease
GvL	Graft versus leukemia
GPCR	G-protein coupled receptor
GlyCAM-1	Glycan-bearing cell adhesion moledule-1
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplant
HEV	High endothelial venules
HLA	Human leukocyte antigen
HMG-CoA	3-hydroxy-3-methylglutaryl CoA reductase
HR	Homing receptor
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
iNK	Immature natural killer cell

ILC	Innate-like lymphocyte
ICAM	Intracellular adhesion molecule
IFNγ	Interferon γ
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
JAK	Janus kinases
KIR	Killer cell immunoglobulin like receptor
KLF	Krüppel-like factors
KLF1/EKLF	Erythroid Krüppel-like factor
KLF2/LKLF	Krüppel-like factor 2 leukocyte functional antigen 1
LAK	Lymphokine activated-killer cell
LAMP-1/CD107a	Lysozyme-associated membrane protein-1
LCMV	Lymphocytic choriomeningitis virus
LFA-1	Leukocyte functional antigen 1
LKLF/KLF2	Lung Krüppel-like factor
LN	Lymph node
MadCAM-1	Mucosal addressin cell adhesion molecule 1
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
mNK	Mature natural killer cell
MS	Multiple sclerosis
msLN	Mesenteric lymph node

mTOR	Mammalian target of rapamycin
NK	Natural killer cell
NKP	Natural killer cell precursor
OPD	*o*-Phenylenediamine dihydrochloride
PAMP	Pathogen associated molecular patterns
PFU	Plaque forming unit
PI3K	Phosphoinositide 3-kinase
PLO	Primary lymphoid organ
PMA	Phorbol 12-myristate 13-acetate
Poly (I:C)	Polyinosinic-polycytidylic acid
PRR	Pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand-1
RAG	Recombinase activated gene
S1P	Sphingosine phosphate 1
S1P1-5	Sphingosine phosphate receptors 1-5
SDS-PAGE	Sulfate polyacrylamide gel electrophoresis
SLO	Secondary lymphoid organ
Sp1	Specificity protein 1
STAT5	Signal transducer and activator of transcription 5
SYK	Spleen tyrosine kinase
TCR	T cell receptor
TLR	Toll-like receptor

TNF	Tumor necrosis factor
TNP-OVA	2,4,6-Trinitrophenol-ovalbumin
TRAIL	Tumor necrosis factor-related (TNF) apoptosis-inducing ligand
Treg	Regulatory T cell
trNK	Tissue resident natural killer cell
VCAM-1	Vascular adhesion protein-1
VLA-4	Very late antigen-4
XCR1	X-C motif receptor

CHAPTER 1

INTRODUCTION

The immune system is charged with the task of protecting the body from pathogens and clearing apoptotic or cancerous cells. Controlled cell migration shapes lymphocyte subset development, tissue remodeling, homeostasis, and immune responses in a lineage specific manner. These migration patterns guide precursor cells through primary lymphoid organs (PLO) such as the bone marrow and thymus for development and promote crosstalk between cell types within secondary lymphoid organs (SLO) such as the lymph nodes and spleen for pathogen clearance. Under homeostatic conditions, these patterns prevent immune cells from accumulating in high numbers in peripheral or tertiary tissues in the absence of inflammation. Only in response to inflammatory cues can lymphocytes be recruited to peripheral tissues. Defects in migration within PLO result in compromised immune cell development. Likewise, breakdowns in lymphocyte recirculation within SLO lead to impaired effector responses, and accumulation of lymphocytes within tertiary tissues promote chronic disease. Therefore, understanding the mechanisms coordinating immune cell migration is essential for understanding the immune system and developing new therapeutics to promote pathogen clearance and prevent autoimmunity. The primary goals of this thesis were to understand how the transcription factor Krüppel-like factor 2 (KLF2) differentially

regulates lymphocyte migration between conventional $CD4^+CD25^-$ T cells, $CD8^+$ T cell lineages, and natural killer (NK) cells, and to assess the consequences of KLF2-deficiency on lymphocyte homeostasis and effector function.

Mechanisms of leukocyte migration

Leukocyte migration is a controlled process governed by the expression of specific repertoires of G-protein coupled chemokine receptors, adhesion molecules, and integrins collectively known as homing receptors (HR). Models of leukocyte migration have been best characterized by studying neutrophil responses to inflammation. Neutrophils circulate within the blood and are terminally differentiated to be the first responders (1, 2). In the absence of inflammation circulating neutrophils will not extravasate to tertiary tissues. When pathogens penetrate primary immune defense barriers such as the skin, macrophages phagocytose these pathogens and become activated via pattern recognition receptors (PRR) that bind conserved pathogen associated molecular patterns (PAMPs) (3). Following PRR activation, macrophages release cytokines such as tumor necrosis factor α (TNFα), interleukin-1 β (IL-1β), interleukin-6 (IL-6), interleukin-12 (IL-12), and the chemokine C-X-C motif ligand 8 (CXCL8) that cause vascular endothelial cells to secrete additional inflammatory cytokines and to recruit leukocytes to respond to the pathogen (3, 4). To traffic from the blood to peripheral tissues, neutrophils and other immune cells move through 4 classic steps: 1) rolling and tethering, 2) activation, 3) adhesion, and 4) diapedesis. Neutrophils expressing glycoprotein-attached carbohydrate sialyl-Lewis X tether to the surface of

endothelial cells through binding of E-selectin (CD62E) (1). Once tethered to the endothelial surface by selectin binding, chemokine receptors such as C-X-C motif receptor 8 (CXCR8) on the neutrophil recognize macrophage secreted CXCL8. Chemokine-induced signaling within the neutrophil then results in the rearrangement of cytoskeletal proteins such as actin and the activation of integrins, which increases the binding affinity of these receptors for their ligands (1). Chemokine receptor activation of neutrophils leads to increased activation of the integrin leukocyte functional antigen 1 (LFA-1/α_L: β_2) which binds intracellular adhesion molecule-1 (ICAM-1) to adhere tightly to the endothelial surface (1). Once the neutrophil adheres strongly to the surface of the endothelium through integrin binding it can then transmigrate across the endothelial barrier in response to chemokine gradients to inflamed tissue in a process called diapedesis. Each of these steps are facilitated through the coordinate expression of three different classes of receptor-ligand interactions between the leukocyte and endothelial surface including 1) adhesion molecules such as selectins and their carbohydrate ligands, 2) chemokines and chemokine receptors and 3) integrins and immunoglobulin superfamily molecules. In the next section I will discuss how leukocytes utilize each class of homing receptors to migrate.

Selectins

In the first steps of transendothelial migration, leukocytes roll across and attach to the endothelial surface through the expression of adhesion molecules such as selectins. Adhesion molecules may be expressed by either the migrating lymphocyte,

activated endothelial cells, or the extracellular matrix. Selectins are membrane bound glycoproteins that bind specific carbohydrate groups. There are three flavors of selectins associated with endothelial cells, platelets, and lymphocytes named E-selectin (CD62E), P-selectin (CD62P), and L-selectin (CD62L) (5). CD62E is primarily expressed on activated endothelial cells and recognizes sialylated Lewis X or Lewis A attached to glycoproteins such as P-selectin glycoprotein 1 (PSGL-1) expressed by leukocytes such as granulocytes, neutrophils, and memory T cells (5). CD62P is expressed by platelets and endothelial cells and also binds sialyl-Lewis X attached to PSGL-1 (5). In contrast CD62L is expressed on naïve T cells (5), mature NK cells (2, 6, 7), and other lymphocytes. CD62L facilitates attachment of these cells to specialized blood vessels known as high endothelial venules (HEV) associated with lymph nodes. Mice deficient in this adhesion molecule (*CD62L*$^{-/-}$) have reduced T cell numbers in SLO and defects in initiating immune responses (8). CD62L binds at least three endothelial cell ligands: glycan-bearing cell adhesion molecule-1 (GlyCAM-1), mucosal addressin cell adhesion molecule-1 (MadCAM-1), and the glycoprotein CD34 (5). Upon naïve T cell activation, CD62L is shed from the cell surface of activated T cells in a PI3K-dependent manner which prevents lymph node re-entry (9). Through these mechanisms CD62L plays key roles in maintaining homeostasis and regulating effector cell migration.

Chemokines and chemokine receptors

Chemotaxis is defined as the movement of an organism in response to a chemical gradient. Chemokines are a large family of ~50 structurally homologous small cytokines that are ~8-15kDa (10). Most chemokine contains two internal disulfide loops formed through two cysteine residues on the N-terminal domain (11). Based on these cysteine residues, chemokines can be categorized into four subtypes: 1) the CC-type comprised of CCL1-28 where the two cysteines are adjacent, 2) the CXC-type comprised of CXCL1-16 where the cysteines are separated by one amino acid, 3) the CX3C-type comprised only of CX3CL1 where the cysteines are separated by three amino acids, and 4) the XC-type comprised of XCL1-2 containing a single cysteine residue (11). Chemokines bind both G-protein coupled receptors (GPCR) and non-signaling scavenger receptors that shape chemokine gradients (10). To migrate, lymphocytes differentially express GPCR subtypes, which are named aptly where CCL proteins bind CCR1-10, CXCL proteins bind CXCR1-6, CX3CL1 binds CX3CR1, and XCL proteins bind XCR1 (10). In addition to the conventional chemokine receptors (CR), leukocytes also express a family of GPCR receptors named S1P1-5 that bind the sphingolipid sphingosine phosphate 1 (S1P) expressed in the blood and lymph (12). Chemokines may bind a single receptor or promiscuously bind multiple receptors. Chemokine-induced signaling within lymphocytes results in the rearrangement of cytoskeletal proteins such as actin and the activation of integrins, which increases the binding affinity of these receptors for their ligands through inside-out signaling.

Integrins

Integrins are a family of heterodimeric receptors composed of different alpha and beta subunits. In mammalian systems there are 18 different alpha subunits and eight different beta subunits (13). Integrins facilitate cell-cell interactions through inside-out and outside-in signaling. Activation of chemokine receptors increases the affinity of integrin binding to Ig-domain containing intercellular cell adhesion molecules (ICAMs) through inside out signaling (13). Integrins like leukocyte functional antigen 1 (LFA-1/$\alpha_L\beta_2$) expressed on the surface of leukocytes bind intracellular adhesion molecules (ICAM's) expressed on the endothelium (1). Once a leukocyte tightly adheres to the endothelial surface through integrin binding it can then transmigrate across the endothelial barrier in a process called diapedesis (1). Upon recruitment to the inflamed tissue leukocytes can respond to the pathogen. In addition to using homing receptors to respond to inflammation, leukocytes utilize these migration patterns for development and homeostasis. Successful navigation of lymphoid precursors toward and within SLO is necessary for development, effector function, and self-tolerance.

T cell migration during development

T cell migration is a tightly restricted process necessary for development, homeostasis, responses to antigens, and maintaining peripheral tolerance. During T cell development, the thymus plays a vital role in maturation. Lymphoid precursors must traffic correctly to and within the thymus for successful T cell development. Migration of thymic precursors from the bone marrow to the thymus is controlled through the

expression of CCR7 and CCR9 (14, 15). Once thymic precursors enter the thymic cortical-medullary junction and undergo TCR rearrangement they must navigate their way within the thymus to receive the correct signals for positive and negative selection. While the exact cues guiding precursors through the thymus are not well understood, these signals allow thymocytes to interact with epithelial cells and medullary thymic epithelial cells bearing major histocompatibility complex (MHC) proteins displaying self-peptides necessary for the selection of functional, self-tolerant T cells. Altered thymocyte trafficking can lead to defective T cell selection processes and autoimmunity as observed in the ICAM-1 altered model serine/threonine kinase 4 deficient (*Skt4/Mst1*$^{-/-}$*)* mice (16). Mst1 regulates the expression of ICAM-1 and thymocytes within *Mst1*$^{-/-}$ mice do not efficiently interact with autoimmune regulator (AIRE^{+}) epithelial cells expressing self-antigen necessary for negative selection and the removal of self-reactive T cells and the development of regulatory T cells (Tregs) (16). Successful recruitment of thymic precursors to thymus and thymocyte migration within this organ is critical for maintaining T cell homeostasis and tolerance.

Naïve T cell migration

Under homeostatic conditions, naïve T cells recirculate within a closed circulatory loop between the blood, lymph, and secondary lymphoid organs (SLO). This homeostatic patrolling loop is thought to optimize naïve T cell interactions with activated antigen presenting cells (APC) bearing cognate antigen and co-stimulatory receptors, while simultaneously restricting exposure to self-antigen. To enter lymph nodes from the

blood, naïve T cells must cross HEV by tethering to endothelial cells expressing CD34 and GlyCAM-1 via CD62L (Figure 1-1, top). Upon chemokine receptor activation by the CCR7 ligands CCL19 and CCL21, naïve T cells tightly adhere to HEV expressing the adhesion molecules ICAM through the binding of the integrin LFA-1($\alpha_L\beta_2$) expressed by T cells. Tightly adherent T cells can then traverse across tight junctions in the endothelial barrier and across the basement membrane in response to CCL19 and CCL21 gradients expressed in the T cell zone. Once in the lymph node, naïve T cells continually search the microenvironment for activated dendritic cells (DC) displaying their cognate antigens via MHC receptors (MHC I and MHC II).

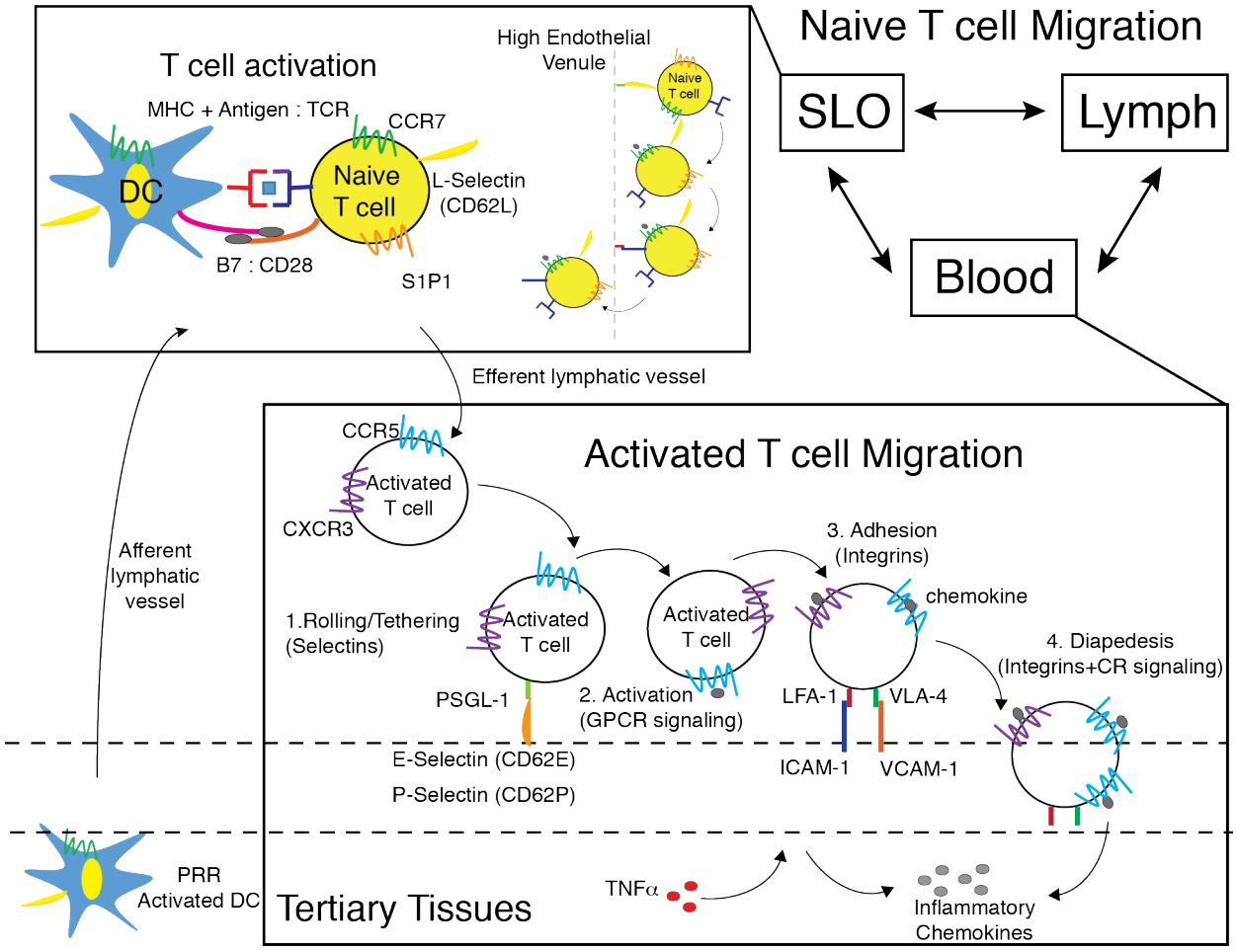


Figure 1-1. Schematic of naïve and activated T cell migration. T cell migration involves 4 classical steps: 1) Rolling/Tethering, 2) chemokine receptor activation, 3) adhesion via integrins, and 4) diapedesis through tight junctions. Naive T cells recirculate between the blood, lymph, and secondary lymphoid organs (SLO). Dendritic cells (DC) that become activated in tertiary tissues through recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRR) upregulate CCR7 and migrate to T cell rich areas of the lymph node to present antigen. Naïve T cell activation requires recognition of cognate antigen presented in major histocompatibility complexes (MHC) and co-stimulation through B7:CD28 interactions. T cell activation induces transcriptional reprograming which downregulates homeostatic homing receptors (HR) like S1P1, CCR7, and CD62L and increases expression of inflammatory HR like CXCR3 and CCR5. Altered HR expression allows activated T cells to leave the closed circulatory loop of naïve T cell migration and enter tertiary tissues.

Effector T cell migration

When a naïve T cell meets an activated APC bearing its cognate MHC:antigen complex, it becomes activated and down-regulates homeostatic receptors such as S1P, CCR7 and CD62L, and upregulates inflammatory homing receptors such as CXCR3 and CCR5 (17). These alterations in homing receptor expression allow antigen-primed T cells to transmigrate across the endothelial barrier and toward inflammatory chemokine gradients produced by inflamed tissues (Figure 1-1, bottom right). If a naïve T cell fails to find its cognate antigen, the cell will exit the lymph node via the efferent lymphatic vessel through the expression of S1P (18) and recirculate between the blood, lymph, and SLO. In the absence of antigenic stimulation, naïve T cells are unable to migrate to the periphery and their exposure to self-antigens is limited to APC, which actively migrate back to T cell zones in the SLO. We speculate that these patterns of segregated migration between lymphocyte lineages promote self-tolerance.

Therapeutics for altering T cell migration

Currently, standard therapies approved to treat autoimmune disease include steroids, cytokine inhibitors, and CR inhibitors. Prolonged steroid and anti-cytokine therapies are associated with many side effects and may compromise the ability of the patient to clear opportunistic infections. Therapeutics that modulate lymphocyte homing may be an appealing alternative to steroids. Among the available HR modulating therapeutics, S1P1, CCR5, and CXCR4 CR inhibitors (19, 20) have been approved for clinical use. Both CCR5 and CXCR4 inhibitors have been primarily approved for use to

prevent HIV-1 entry, but in 2010 the S1P1-inibitor FTY720 (Fingolimod) was the first oral-HR inhibitor approved to treat relapsing-remitting multiple sclerosis (MS) (19, 20). FTY720 is an analog of natural sphingosine. It is converted into a metabolically active form *in vivo* by sphingosine kinase 2 capable of binding receptors S1P1 and S1P3-5 (19). T cells express S1P1 to leave the thymus and lymph nodes in response to S1P gradients in the blood and lymph (21, 22). Lymphocytes which bind FTY720 are unable to exit the lymph nodes via S1P-dependent pathways and become sequestered there (23). This sequestration is believed to reduce the numbers of autoreactive T cells in the periphery of MS patients treated with FTY720. Long term studies on FTY720 treated MS patients seem to support the therapeutic benefits of this HR drug (24). In addition to pharmaceutics that directly target specific HR, statins have been demonstrated to ameliorate symptoms of autoimmunity (25-27). Statins are currently used to lower cholesterol levels in patients with cardiovascular disease by inhibiting 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, but were found to increase the transcription factor KLF2 through off-target mechanisms in endothelial cells (28, 29). Follow up studies revealed that statins can also increase KLF2 levels in T cells (25, 30), and through KLF2 these FDA approved drugs can be used to ameliorate symptoms of autoimmunity by altering T cell HR expression (26). In the next sections I will discuss the KLF transcription factor family, how KLF2 controls migration within naive $CD4^+$ T cells, and how *Klf2*-gene targeted models can be used as a tool to understand how migration impacts lymphocyte homeostasis and development. By understanding how

KLF2 directs lymphocyte migration, we may develop treatment protocols, which alter migration in a KLF2-dependent manner.

Krüppel-like transcription factors

Krüppel-like factors (KLF) are a family of 17 zinc-finger transcription factors within a larger family of specificity protein 1 (Sp1)-related transcription factors that are highly conserved in mammalian systems (31). These transcription factors play important roles in regulating proliferation, differentiation, survival, responses to external stress, and migration (31). Sp1/KLF family members contain three zinc-finger binding motifs at the C-terminus that share homology with the DNA binding domain of the *Drosophila* protein Krüppel, in which the length and spacing between each zinc-finger are preserved (31, 32). The Krüppel protein, meaning “cripple” in German, was originally found to be necessary for thoracic segmentation; therefore the related proteins were described as “Krüppel-like” (31). Erythroid Krüppel-like factor (EKLF/KLF1) was the first mammalian member of the KLF family to be identified and is necessary for erythrocyte development (32). Due to the sequence similarity of the zinc fingers of KLF1 to the protein Krüppel, KLF1 was predicted and found to bind to the CACCC element in the β-globulin promoter to promote transcription (31). Given the conserved nature of the zinc finger motifs between KLF family members, all KLF family are also predicted to bind the CACCC element. However, each KLF member differs at the N-terminal region, allowing diversity among their transcriptional binding partners. Using phylogenic analysis of protein sequences of the 17 human KLF sequences, KLF members can be categorized

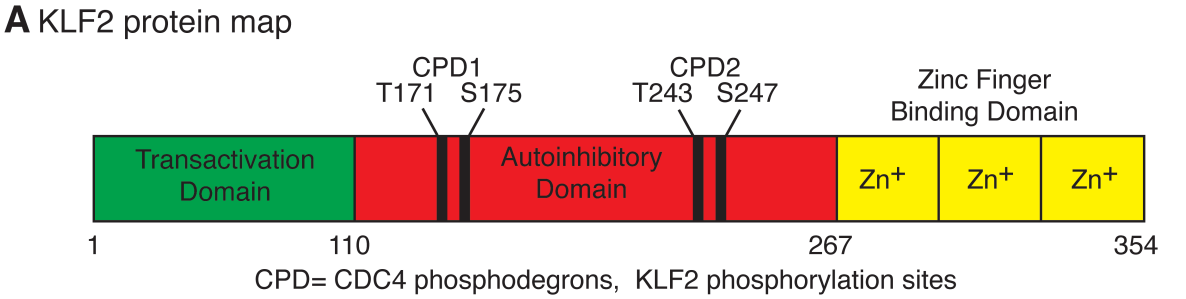
into three groups based on homology (31). Group 1 KLF members (KLF3, 8, and 12) and group 3 KLF members (KLF9, 10, 11, 13, 14 and 16) are predicted to be transcriptional repressors by interacting with the transcriptional co-repressor carboxy-terminal binding protein (CtBP) or the common transcriptional co-repressor SIN3 transcription regulator homolog A (Sin3A) (31). In contrast, group 2 KLF members (KLF1, 2, 4, 5, 6, and 7) are predicted to be transcriptional activators due to their ability to bind histone acetylases (31). Lung Krüppel-like factor (LKLF/KLF2) in contrast, contains both a transactivation domain and an auto-inhibitory domain, making it difficult to predict whether this transcription factor functions strictly as an activator or a repressor (33). Therefore, the limited knowledge available about how KLF members bind DNA is insufficient to describe the level of transcriptional regulation they exert over a wide array of biological processes. In the next section, I will discuss our current understanding of how KLF2 regulates transcription.

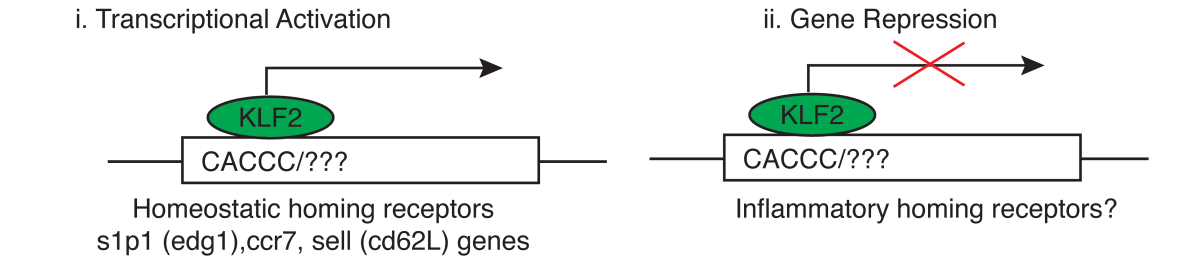
Biochemistry of KLF2

Lung Krüppel-like factor (LKLF/KLF2) was first identified in a mouse genomic library using KLF1/EKLF as a hybridization probe (34, 35). Among peripheral tissues, KLF2 is highly expressed in the lung and is necessary for lung development (36), hence its name LKLF, but was also found to be expressed in the spleen (34, 37). KLF2 is 354 amino acids long and is comprised of an N-terminal transactivation domain (amino acids 1-110), followed by an auto-inhibitory domain (amino acids 111-267), and three Cys2/His2 zinc finger motifs at the C-terminus (amino acids 268-354) (33, 34) (Figure 1-

2A). The transactivation domain is believed to promote gene expression by interacting with transcriptional binding partners; however, the auto-inhibitory domain can repress the activity of the transactivation domain (33). Like KLF1/EKLF, KLF2 is predicted to bind CACCC sequences, but its unique DNA sequence targets and binding partners are poorly understood. Depending upon KLF2's interactions with binding partners, it is possible that KLF2 can function as a (a) promoter or (b) repressor of the same gene between different cell lineages (Figure 1-2B). KLF2 protein levels and biological activity can be altered through post-translational modification in addition to its mRNA transcript. Within the auto-inhibitory domain (amino acids 111-267), KLF2 contains two conserved, phosphorylation motifs called CDC4 phosphodegrons (CPD), positioned at threonine (T) and serine (S) residues T171/S175 (CPD1) and positioned at T243/S247 (CPD2) (38). These sites may be phosphorylated by various kinases such as phosphoinositide 3-kinase (PI3K), glycogen synthase kinase-3 (GSK-3), ERK-2, and possibly others in a pathway dependent manner (30, 38, 39). KLF2 protein is ~38kDa and when resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized through immunoblotting, KLF2 and phosphorylated KLF2 can be observed as a doublet (38, 40). This phosphorylated form of KLF2 is then recognized by various E3-ubiquitin ligase subunits such as WWP1, Smurf-1, and FBW7, and ubiquitinated at lysine 121 for degradation by the 26S proteasome (38, 41, 42). Degradation of KLF2 results in decreased expression of trans-activated genes and increased expression of genes repressed by KLF2. Therefore, protein levels of KLF2 and its regulated genes can be pharmaceutically stabilized by (a) inhibiting kinases which phosphorylate KLF2 for

recognition by E3-ubiquitin ligases or by (b) inhibiting the E3-ubiquitin ligases which target KLF2 for degradation by the proteasome. Depending on whether KLF2 is functioning as an activator or repressor, altering KLF2 levels can promote or inhibit gene signatures. In the next section I will discuss how KLF2 controls the biology of lymphocytes and our current understanding of KLF2's downstream targets.





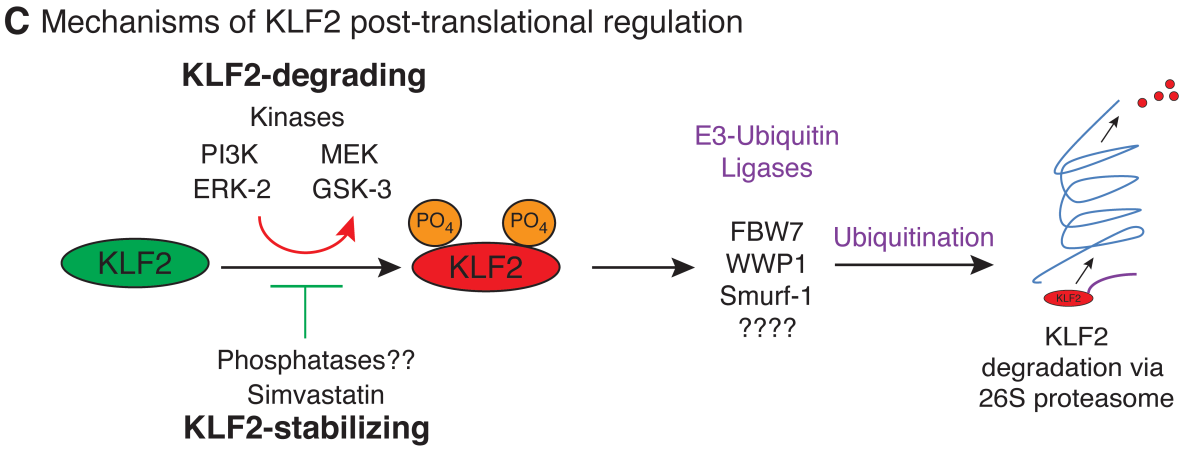


Figure 1-2. Biochemistry of KLF2. (A) Map of KLF2 protein. KLF2 is 354 amino acids long and is comprised of an N-terminal transactivation domain (amino acids 1-110), followed by an auto-inhibitory domain (amino acids 111-267), and three Cys2/His2 zinc finger motifs at the C-terminus (amino acids 268-354). The auto-inhibitory domain contains two conserved, phosphorylation motifs called CDC4 phosphodegrons (CPD), positioned at threonine (T) and serine (S) residues T171/S175 (CPD1) and positioned at T243/S247 (CPD2). (B) Mechanisms of KLF2 transcriptional regulation. (i) KLF2 is predicted to activate gene transcription by binding CACCC and unknown sequences. (ii) KLF2 may repress gene expression by binding DNA and inhibiting polymerase activity. (C) Mechanisms of KLF2 post-translational regulation. KLF2 is phosphorylated by various kinases to promote proteasomal degradation. KLF2 protein levels may be stabilized by phosphatase activity and simvastatin.

Biology of KLF2 in lymphocytes

KLF2 plays a physiologic role in B cell and T cell lineages (43-46). KLF2 is necessary for normal cardiac and lung development and knockout embryos die in utero between d12.5-14.5 due to cardiovascular abnormalities, before the T and B cell compartment can fully mature (35). To circumvent this problem and investigate the role of KLF2 within the immune system, recombination-activating-gene-2-deficient (*Rag2*$^{-/-}$) blastocysts were reconstituted with *Klf2*$^{-/-}$ embryonic stem cells to generate chimeric mice with KLF2-deficient lymphocytes (35, 37). The *Klf2*$^{-/-}$ chimeric mice were reported to have a slight accumulation of single positive T cells in the thymus, and a severe reduction of T cells within the secondary lymphoid organs in the periphery (37). Moreover, the single positive thymocytes examined in *Klf2*$^{-/-}$ chimeras displayed an activated phenotype (CD44^{hi} CD69^{hi} and CD62L^{lo}) suggesting that these T cells were spontaneously activated. Based upon these data Jeffrey Leiden's group proposed that KLF2 was necessary to prevent apoptosis via Fas ligand-dependent activation induced death. A follow-up study by this same group reported that overexpression of KLF2 in Jurkat T cells, a human T cell line, promoted T cell quiescence and limited proliferation through a c-Myc dependent pathway (47). From these studies, KLF2 was thought to function as a transcription activator that prevented spontaneous T cell activation and cell cycling. Indeed KLF2 may suppress cell cycling and clonal expansion following CD8^{+} T cell activation independent of c-Myc (48), and during pre-B cell clonal expansion (40). Despite the initial findings indicating that KLF2 was necessary for the survival of T lymphocytes, subsequent reports have refuted these claims, leading to vast

misconceptions within the field. Later studies by the Jameson-Hogquist and Sebzda groups demonstrated that KLF2 is dispensable for T cell survival and that KLF2-deficient thymocytes do not undergo spontaneous apoptosis, implying that KLF2 plays other roles in regulating lymphocyte homeostasis (43, 44).

Within the T cell compartment, it is theorized that the primary role of KLF2 in T cell homeostasis is to regulate migration and promote recirculation within SLO (43-45, 49). KLF2 is first expressed at high levels during the single positive stage of thymocyte development and facilitates the emigration of thymocytes to the periphery by promoting the expression of S1P1 so that single positive T cells may respond to S1P gradients in the lymph and blood (18, 21, 37, 43, 44). Upon leaving the thymus, naïve T cells recirculate between the blood, lymph, and SLO. KLF2 intrinsically promotes the expression of homeostatic migratory receptors such as S1P, CD62L and CCR7 by directly binding promoter regions of these genes in naïve T cells (43, 44, 50). T cell activation and subsequent PI3K signaling induces KLF2 phosphorylation and recognition by E3-ubiquitin ligase machinery for targeted proteasomal degradation (30, 37, 51). This activation induced loss in KLF2 leads to decreased expression of homeostatic receptors such as S1P1, CD62L, and CCR7 (44), an increase in inflammatory chemokine receptors and adhesion molecules such as CCR3, CCR5, CXCR3, and CD44 (43, 44). Transcriptional reprograming of HR expression prevents activated T cells from crossing HEVs and entering lymph nodes in to the absence of CD62L and CCR7, and allows them to respond to inflammatory gradients.

Within *Klf2*-gene targeted models, T cells are displaced from naïve T cell closed circulatory loop (blood, lymph, and lymph nodes) and accumulate within peripheral tissues (43-45). KLF2-deficient $CD4^+$ T cells express elevated transcripts of inflammatory chemokine receptors CCR1-9, CXCR1-7, XCR1, and CX3CR1 and decreased levels of homeostatic receptors S1P1, CD62L, CCR7, and β_7 integrin when compared to wild type controls (43, 44, 46). A recent report from the Sebzda lab indicates that KLF2-directed migration patterns within the regulatory T cell (Treg) compartment are necessary for maintaining peripheral tolerance (26). Tregs express half the level of KLF2 when compared with a naïve $CD4^+$ T cell ($CD4^+CD25^-$) and express hybrid levels of homeostatic receptors (S1P1, CD62L, and CCR7) and inflammatory receptors (CCR4, CCR6, and CCR8) at steady state (26). Targeted deletion of *Klf2* in Tregs using a *Foxp3-cre* system results in spontaneous autoimmunity *in vivo* (26). *Ex-vivo* treatment of Tregs with KLF2 stabilizing drugs such as simvastatin prior to adoptive transfer in a model of graft versus host disease increased recruitment of Tregs to SLO that helped suppress pathology (26). These data suggest that the primary function of KLF2 in naïve T cells is to restrict their homeostatic migratory pattern within the closed circulatory loop of the blood, lymph, and SLO which is necessary for T cell homeostasis and self-tolerance.

Given that $CD4^+$ T cells, $CD8^+$ T cells, and natural killer (NK) cells have well documented heterogeneous effector functions, and KLF2 differentially regulates migration between conventional $CD4^+CD25^-$ and Treg subsets (26), it is unlikely that KLF2 regulates migration between these subsets through completely identical

mechanisms. Under steady state conditions $CD4^+$ T cells, $CD8^+$ T cells, and NK cells do not express identical repertoires of homing receptors. For example, within the spleen $CD8^+$ T cells express higher levels of CCR9 than $CD4^+$ T cells under steady-state (52), and NK cells preferentially express different repertoires of homing receptors based on their maturation status (2, 53, 54). Currently, it is unknown how or if KLF2 plays a biological role in the NK cell compartment. Among $CD4^+$ and $CD8^+$ T cells KLF2 is assumed to regulate homing receptor expression identically through two mechanisms: a) intrinsically, by directly promoting homeostatic receptor expression and repressing inflammatory receptor expression (44); or b) extrinsically, by repressing excessive production of cytokines like interleukin 4 (IL-4), interferon gamma (IFNγ), and tumor necrosis factor alpha (TNFα) by $CD8^+$ T cells, that increases inflammatory homing receptor expression on bystander T cells (55). Ultimately, it is likely to be a hybrid of both mechanisms and additional mechanisms that differentially regulate HR expression between $CD4^+$ and $CD8^+$ T cell subsets through KLF2. Using *Klf2* gene targeted models as a tool, we can use this to understand how/if $CD4^+$ T cells, $CD8^+$ T cells, and if NK cells differentially regulate migration through KLF2 and determine how these migration patterns may be biologically significant. **One of the primary goals of my thesis is to identify HR differentially regulated by KLF2 in $CD4^+$ and $CD8^+$ T cells.**

Of note, NK cells do not recirculate between the blood, lymph, and lymph nodes like naïve T cells. If KLF2 regulates migration within this lymphocyte subset, it is unclear how altering NK cell migration patterns will impact NK cell homeostasis. In the next sections I will discuss the functions of natural killer cells in the immune system, how

they develop, and factors necessary for maintaining homeostasis. Data in subsequent chapters will hopefully shed light on the role of KLF2 in NK cell homeostasis.

Functions of natural killer cells

Natural killer cells are large granular lymphocytes capable of secreting cytokines and eliminating virally infected or transformed cells without prior antigen recognition. Because of their intrinsic capacity to kill, they are thought to be a first line of defense against aberrant cells and belong to the group 1 innate lymphocytes (ILC1) in the innate lymphoid cell (ILC) family (56). Once an NK cell identifies a target cell, it will either kill the target cell or secrete cytokines to activate the adaptive immune system. At steady state, NK cells do not express high protein levels of IFNγ or lytic molecules such as granzymes (A/B/C) or perforin, but instead sequester high mRNA levels of these transcripts for immediate translation upon recognition of aberrant cells (57, 58). To kill target cells, NK cells express ligands such as Fas and tumor necrosis factor-related (TNF) apoptosis-inducing ligand (TRAIL) that engage death receptors on target cells such as Fas ligand and TRAIL receptors DF4 and DF5 (59, 60), or secrete granzymes (A/B/C) through perforin-dependent mechanisms (60). NK cells also recognize and kill IgG antibody-coated targets by binding antibody Fc regions through the activating receptor CD16 (FcRIII) in a process termed antibody-dependent cell mediated cytotoxicity (ADCC). NK cells may also be primed for action by cytokines secreted by activated DC and other myeloid cells including type I interferons, IL-12, IL-15, and IL-18 (60). IL-12 and IL-18 synergistically signal NK cells to increase production of cytokines

such as IFNγ but do not promote the production of high levels of granzyme B and perforin necessary for cytotoxicity (57). High levels of IL-15 produced by DC prime NK cells to increase production of both IFNγ and granzyme B (57). NK cell priming by APCs maximizes effector cell potential by increasing the killing efficacy of NK cells, and incorporating signals from other innate immune cells to promote activation of T cells in the adaptive immune system through the secretion of IFNγ (61). Collectively, these mechanisms facilitate the clearance of virally infected or transformed cells.

In the context of health and disease, patients who are NK cell lymphopenic are susceptible to infection, particularly herpes and papilloma viral infections (62). Conversely, cancer patients with high circulating NK cells or NK cell infiltration at the tumor site correlate with good disease prognosis (62). During viral infection, NK cells can facilitate pathogen clearance by activating the adaptive branch of the immune system through secretion of IFNγ to promote Th1 priming (61), or impede specifically murine cytomegalovirus (MCMV) and lymphocytic choriomeningitis virus (LCMV) clearance by killing APC necessary for T cell priming and killing virus-specific effector T cells (63, 64). NK cells can play both beneficial and detrimental roles in autoimmune diseases. NK cells may prevent auto-reactivity by targeting APCs displaying self-antigen or through direct killing of self-antigen-specific T cells (65). In models of chronic inflammatory disease such as type I diabetes NK cells can directly target β-cell islets for destruction (66) and are increased in psoriatic plaques in psoriasis patients (67). Therefore, therapies that modulate the number of effector NK cells can be exploited within the clinic for a variety of disease scenarios.

NK cell recognition of self and non-self

NK cells distinguish between healthy "self" and virally-infected or cancerous "non-self" cells using a series of germ-line encoded inhibitory and activating receptors. Unlike B and T cells, NK cell receptors do not undergo gene rearrangement and recognize a limited repertoire of conserved antigens. Many inhibitory receptors recognize self-molecules such as MHC class I, which are often down-regulated on virally infected or transformed cells. The recognition and lysis of MHC class I^{lo} cells has been termed "Missing-self" recognition, theorized to target virally infected cells or tumor cells that evade recognition by $CD8^+$ T cells (68). In contrast, activating receptors such as Ly49H, NKp46, and NKG2D recognize specific viral proteins such as cytomegalovirus protein p157, influenza hemagglutinin, and self-stress molecules such as heat shock proteins and Rae-1 on malignant cells (69). In the presence of self-molecules like MHC class I, and the absence of stress molecules, NK cells are tolerant toward self. However, in the absence of MHC class I and recognition of specific viral antigens, NK cells readily kill their targets or secrete inflammatory cytokines such as IFNγ and TNFα to activate the adaptive arm of the immune system (60). In cases where both inhibitory and activating ligands are engaged, it is a combination of all signaling pathways that determines whether the NK cell remains quiescent or eliminates its target (60, 69). Therefore, the balance between activating and inhibitory receptors is essential for preventing the destruction of healthy self-tissues.

Conventional NK cell development

Conventional NK cell development begins within the bone marrow where hematopoietic stem cells (HSC) differentiate into the common lymphoid precursor (CLP). CLP become innate lymphocyte cell (ILC) precursors upon expression of CD122 (IL-15Rβ) (70). The expression of CD122 allows natural killer cell precursors (NKP) to respond to IL-15, which is critical for NK cell development, differentiation and survival (71-73). NK cell development is thought to occur primarily within the bone marrow (70). However, NKP can also be found scattered in the periphery at low frequencies within the spleen, liver, and lymph nodes indicating that some differentiation may occur in the periphery as well (74). In mice NKP then acquire a series of activating receptors and inhibitory receptors such as NKG2D, NK1.1, and NKp46, followed by the Ly49 family of receptors, which allow NK cells to distinguish between self and non-self. Activating receptors signal through immunoreceptor tyrosine-based activation motifs (ITAMs), which are associated with DAP10 or DAP12 adaptor proteins that promote kinase activation and subsequent effector cell functions. DAP10 signals directly through PI3K (75), and is non-covalently bound to receptors like NKG2D that recognize self-ligands such as Rae-1 or MICA expressed on stressed cells (76) and NK1.1, which is predicted to recognize self-molecules (77). Whereas DAP12 associated receptors, like viral hemagglutinin protein recognizing NKp46 and murine cytomegalovirus protein m157 recognizing Ly49H, signal indirectly through PI3K via SYK and ZAP70 tyrosine kinases (78). In contrast to activating receptors, inhibitory receptors signal through immunoreceptor tyrosine-based inhibitory motif (ITIMs). ITIMs recruit SH2-containing

protein tyrosine phosphatase-1 (SHP-1) or 2 (SHP-2) that suppress kinase activity and inhibit effector NK cell effector functions (79, 80). Repertoire acquisition of inhibitory Ly49 receptors within a single individual seems to be stochastic in nature with strain-specific tendencies, and depend upon levels of trans-presented IL-15 (81). Regardless, these receptors are germ line encoded and do not undergo recombinase activated gene rearrangement. The process in which NK cells are educated to distinguish between self- and non-self through the recognition of self-MHC ligands has been termed by Yokoyama and colleagues as “MHC licensing”(82).

MHC licensing is an education similar to thymic positive selection in T cells, where NK inhibitory receptors engage at least one self-MHC ligand, to become “licensed” NK cells capable of full effector function. β_2 microglobulin is necessary for the formation of the MHC class I heterodimer, and β_2 microglobulin deficient mice ($\beta_2 m^{-/-}$) do not express MHC class I (83). In the absence of MHC I, such as in $\beta_2 m^{-/-}$ mice, “unlicensed” NK cells display defects in IFNγ production in response to anti-NK1.1 cross-linking (82). Interference in downstream signaling of inhibitory receptors through overexpression of a dominant negative form of the SHP-1 results in hyporesponsive NK cells (84). Therefore, engagement of self-ligands and subsequent inhibitory receptor signaling is necessary for fully competent NK cell responses. MHC licensing is thought to occur primarily in the bone marrow, but recently it has been proposed to even occur multiple times within the periphery in tissues such as the liver and spleen (85, 86). Adoptive transfer of functionally licensed NK cells into MHC class I-deficient or an altered MHC environment resulted in diminished NK cell responsiveness, indicating that

this could occur multiple times throughout the lifespan of an NK cell within peripheral tissues (86, 87).

Once NK cells have gone through MHC education, these cells then undergo a homeostatic proliferative burst before differentiating into mature effector cells (2). Homeostatic proliferation is antigen-independent and requires IL-15 receptor-signaling (53, 88). It is distinct from mature clonal proliferation, which occurs during viral infection. Virally induced clonal proliferation in NK cells is IL-15-independent (89) and requires antigen-dependent activation through activating receptors like Ly49H during murine cytomegalovirus infection (MCMV), the cytokines IL-12 and IL-18 (90-92), and the transcription factor zbtb32 (90-92). Following homeostatic proliferation, mature effector cells then migrate from precursor niches into the periphery to patrol for aberrant cells.

In murine systems, NK cells can be identified using the markers NK1.1 in C57BL/6 strains, CD49b (DX5), and NKp46, and are CD3 negative. The extracellular markers CD27 and CD11b can be used to characterize NK cell development (2, 93). NK precursors are double negative for both these markers. These NKP then differentiate into $CD27^{+}CD11b^{-}$ NK cells (Stage I, immature) which give rise to $CD27^{+}CD11b^{+}$ NK cells (Stage 2, intermediate) that further mature into $CD27^{-}CD11b^{+}$ NK cells (Stage 3, mature) (Figure 1-3, left). CD11b can be used with CD43 to characterize stages in development through an alternate but similar classification system where $CD43^{-}CD11b^{-}$ (Stage 1)→ $CD43^{-}CD11b^{+}$ (Stage 2)→ $CD43^{+}CD11b^{+}$ (Stage 3) (94). Through 5-bromo-2'-deoxyuridine (BrdU) incorporation studies, $CD27^{+}CD11b^{-}$ (Stage 1) cells were found to be highly proliferative (2, 93). In contrast, $CD27^{+}CD11b^{+}$ (Stage 2) and $CD27^{-}CD11b^{+}$

(Stage 3) proliferate less and are mature effector NK cell populations. $CD27^{+}CD11b^{+}$ stage 2 are capable of producing the highest levels of IFN γ relative to the other NK cell subsets (2). $CD27^{-}CD11b^{+}$ stage 3 cells produce the highest levels of granzyme B relative to other stages in development and are necessary for the clearance of tumor cells *in vivo*. Mature stage 3 cells also express high levels of the inhibitory receptor KLRG1 (2).

In humans, effector NK cell populations within the peripheral blood are identified as $CD56^{+}CD3^{-}$ and the brightness of CD56 can be used to dissect cytokine producing and cytolytic subsets (95). The $CD56^{bright}$ subset comprising ~5-10% of peripheral blood NK cells, has high cytokine producing potential and is thought to give rise to the more mature $CD56^{dim}$ subset (96). Analogous to $CD11b^{hi}$ murine NK cell subsets, the mature $CD56^{dim}$ population constitutes the majority of circulating NK cells and expresses higher levels of perforin and granzyme (95, 96). Of note, the selective loss of mature cytolytic $CD11b^{hi}$ or $CD56^{dim}$ NK cells, without immature NK cell or T cell lymphopenia, in mice or humans respectively, leads to severely impaired anti-viral and anti-tumor responses (97-99). Therefore, alterations in NK homeostasis that lead to expansion and maintenance of this effector population are of clinical significance. In addition to conventional circulating NK cell subsets, resident populations have been recently identified in the periphery, which may play tissue-specific roles in disease. In the next section, I will discuss the current knowledge regarding tissue resident NK cells and how they differ from conventional NK cells.

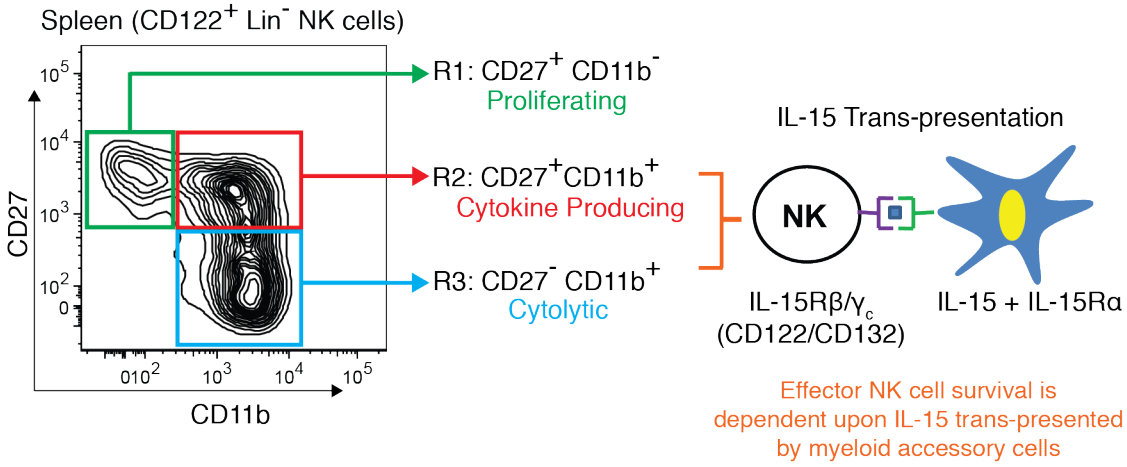


Figure 1-3. Schematic of developing NK cells and IL-15 trans-presentation. Contour plot of $CD122^+$ Lin^- (CD3, CD8, CD19, Gr-1, TCRβ) splenic NK cells co-stained for CD27 versus CD11b in a C57BL/6 mouse. $CD27^+CD11b^-$ (R1, stage 1) NK cells have the highest proliferative capacity and differentiate into $CD27^+CD11b^+$ (R2, stage 2) NK cells with the highest cytokine producing capacity. $CD27^+CD11b^+$ (R2, stage 2) NK cells can further differentiate into $CD27^-CD11b^+$ (R3, stage 3) NK cells with the highest expression of cytolytic proteins such as granzyme B. $CD11b^+$ NK cells are considered mature effector populations that depend upon trans-presented IL-15 for survival.

Tissue resident NK cells and thymic-derived ILC1

In addition to conventional NK cells (cNK), the lung, liver, uterus, peritoneal cavity, and skin contain resident NK cell populations. The vast majority of lung NK cells are of bone marrow origin and depend on basic leucine transcription factor E4BP4/Nilf3 for development (100). Recently, a significant proportion of NK cell populations within the uterus, liver, peritoneal cavity, and skin were found to develop independent of E4BP4/Nilf3, indicating a different origin for these resident cells (100, 101). Tissue resident NK populations (trNK) within the liver express extracellular markers similar to cNK cells. Until recently these cells were thought to represent an intermediate population but have since been demonstrated to be an intrinsically distinct population of fetal liver origin (102, 103). Parabiosis experiments revealed that unlike cNK cells, trNK cells do not recirculate within the periphery (101-103). Their development is distinct from both bone marrow-derived cNK cells and interleukin-7 (IL-7) dependent thymic derived ILC1 (101, 103). cNK cells, trNK cells, and thymic-derived ILC1 all express the hallmark NK cell markers NK1.1 and CD122, making them indistinguishable in most studies prior to 2013. Several studies reported in depth surface receptor and transcription factor analysis to accurately distinguish liver resident cells from conventional NK cells and thymic-derived ILC1 in murine systems. Moreover, a recent studies suggest some of these markers may be similar in humans (104, 105). Table 1-1 is a summary of characteristic markers used to distinguish between cNK, trNK, and thymic-derived ILC1.

ILC Subset	Origin	Key Transcription Factors	Effector Molecules	Phenotypic Markers
Conventional NK cells (cNK)	Bone marrow	E4BP4/Nilf3	IFNγ, granzyme B	$CD122^+$ $NK1.1^+$ TCR^- $CD49a^-CD49b^+$ $TRAIL^-$ $EOMES^{hi}$
Tissue Resident NK cells (trNK)	Fetal liver	T-bet	IFNγ, IL-2, GM-CSF, TNFα, CCL3, TRAIL, granzymes A/C	$CD122^+$ $NK1.1^+$ TCR^- $CD49a^+$ $CD49b^+$ $TRAIL^+$ $EOMES^{lo}$
Thymic-Derived ILC1	Thymus	GATA3	GM-CSF, IFNγ, TNFα	$CD122^+$ $NK1.1^+$ TCR^- $CD27^+$ $CD127^+$ $EOMES^{lo}$

Table 1-1. Characteristic features distinguishing conventional NK cells, tissue resident NK cells, and thymic-derived ILC1 populations. All three populations are lineage negative (Lin^- = CD3, CD8, Gr-1, CD19, TCRβ).

From these studies, trNK cells and cNK cells can be distinguished using the extracellular markers CD49a (α_1 integrin), CD49b (DX5/α_2 integrin), TRAIL, and the transcription factor eomesodermin (EOMES). Bone marrow-derived cNK cells can be identified as $CD122^+NK1.1^+TCR^-CD49a^-DX5^+TRAIL^-EOMES^{hi}$ (101, 103). Thymic-derived ILC1 are defined as $CD122^+NK1.1^+TCR^-CD27^+CD127^+EOMES^{lo}$, depend on the transcription factor GATA3 for their development and can be distinguished by the expression of $CD127^+$ (IL-7Rα) (101, 103, 106). Thymic-derived ILC1 also secrete cytokines like TNFα, GM-CSF, and IFNγ but are less cytolytic than cNK (106). trNK cells are defined as $CD122^+NK1.1^+TCR^-CD49a^+DX5^-TRAIL^+EOMES^{lo}$ and are intrinsically dependent upon T-bet for their development (101, 103). Anatomically, cNK cells are present in the afferent (arteries) and efferent (venules) blood of the liver, whereas trNK cells are positioned within the hepatic sinusoids (102). Functionally, trNK cells are capable of secreting a broader spectrum of cytokines including interleukin 2 (IL-2), granulocyte-macrophage colony-stimulating factor GM-CSF, tumor necrosis factor α (TNFα), the chemokine (C-C motif) ligand 3 (CCL3), and IFNγ, compared to cNK cells which primarily secrete IFNγ (101, 103). trNK cells preferentially express higher levels TRAIL and granzyme A/C compared to cNK cells which primarily express granzyme B indicating differential mechanisms for cytolysis between each population (102, 103). Moreover, trNK cells have been suggested to possess antigen-specific recall or “memory-like” capabilities in response to chemical haptens or viruses in models of skin contact hypersensitivity and viral challenge (107-109). Collectively, cNK cells and trNK cells have overlapping markers and functions, however liver resident cells may

contribute to or ameliorate pathology in organ-specific infections such as hepatitis B/C virus (110). Currently, the functional differences between cNK cells and trNK cells and its significance in the context of the immune response are not completely understood but the homeostasis and survival of both populations are dependent upon IL-15 (101). In the next section I will discuss the unique attributes of IL-15 and its role in conventional NK cell homeostasis.

IL-15 in NK cell biology

IL-15 was first identified by its ability to induce T cell proliferation in a manner similar to IL-2 (111, 112). It is part of a family of cytokines which signals through the common gamma chain CD132 (γ_c), including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, and signals through the IL-15Rβ/γ_c heterodimer complex (113). NK cells are highly dependent upon IL-15 for proliferation, differentiation, activation, and survival (71, 114, 115). *IL-15*$^{-/-}$ and $\gamma_c^{-/-}$ mice are deficient in peripheral NK cells (71, 116, 117). In vivo, IL-15 is found in two forms: as a free secreted cytokine and in complex with the high affinity IL-15Rα displayed on the cell membrane of accessory cells (118). IL-15 is unique among γ_c-cytokines in that its biological action is delivered primarily through trans-presentation and requires cell-cell contact (71, 119). Within lymphoid organs, the cytokine IL-15 is expressed by accessory cells including macrophages, dendritic cells, and neutrophils, mesenchymal stromal cells within the spleen, bone marrow, and thymus under homeostatic conditions (115, 120). Accessory cells present membrane bound high affinity IL-15Rα-IL-15 complexes in *trans* to NK cells bearing the IL-15Rβ/γ_c

heterodimer complex (Figure 1-3, right) (121). IL-15-IL-15Rα complexes have been termed “superagonists” for their ability to induce more selective expansion of NK and memory $CD8^+$ T cells, significantly longer half-life, and 150-fold greater binding affinity for IL-15Rβ/γ_c heterodimer compared to free IL-15 (122, 123). Cell-extrinsic defects that interfere with the trans-presentation of IL-15 result in global NK cell deficiencies (71, 115, 119, 124). This mechanism can be observed in *Tbet*$^{-/-}$ mice which display defects in myeloid IL-15Rα trans-presentation, Blimp-1 and KLF4 deficient models which signal upstream of Tbet, and in models of targeted myeloid cell depletion using clodronate liposomes or diphtheria-toxin induced depletion (125-127). Collectively, these data reinforce the necessity of IL-15 and the IL-15α expressing niche for maintaining NK cell homeostasis.

Activation of the IL-15Rβ/γ_c heterodimer complex induces phosphorylation and activation of Janus kinases (JAK1 and JAK3), which in turn causes activation of multiple pathways: 1) PI3K (phosphatidylinositol-3-kinase) - kinase AKT-serine-threonine kinase mTOR (mechanistic target of rapamycin), 2) STAT5 (signal transducer and activator of transcription activation) phosphorylation and dimerization, 3) phosphorylation of SRC-family kinases LCK and SYK (spleen tyrosine kinase), 4) Ras-Raf-MAPK (mitogen activated protein kinase) (113). In NK cells, low levels of IL-15 induce the activation of STAT5 and up regulate the anti-apoptotic factor Bcl-2 to maintain NK cell survival (114, 128), whereas high levels of IL-15 induce activation of the PI3K-AKT-mTOR pathways to induce cell cycling and effector priming (129, 130). Specific deletion of STAT5 and mTOR within NK cells results in defects in lineage maturation and survival (128, 129). In

the context of infection, myeloid cells become activated via pattern recognition receptors; dendritic cells load and display more high affinity IL-15-IL-15Rα complexes to induce trans-activation of NK cells (131). Super-agonist activity leads to signaling via PI3K-AKT-mTOR signaling which primes NK cells to increase effector function and expand to combat the pathogen (129, 130). Therefore, different levels of IL-15 signaling control NK cell homeostasis, survival, and effector function through a single receptor. Given the critical role of IL-15 in NK cell maintenance and effector responses, knowledge regarding IL-15 biology can potentially be applied to improving NK cell based cancer therapies. In the next section I will discuss the current approaches and hurdles for NK cell based cancer therapies.

NK cells in cancer therapy

Use of NK cells for cancer therapy has been an appealing prospect since they were first identified as lymphocytes with spontaneous cytolytic activity against tumor cells in the 1970s (132). Early clinical studies attempted to induce anti-tumor therapy through the adoptive transfer of IL-2 ex-vivo expanded peripheral blood mononuclear cells known as lymphokine activated-killer (LAK) cells (133). Despite the increased numbers and cytotoxicity achieved *in vitro*, the overall clinical benefit was minimal (134). Direct administration of high dose IL-2 to patients resulted in toxicity due to capillary leakage syndrome (134). In 2002, a study by Velardi and colleagues introduced the novel concept of adoptively transferring human leukocyte antigen (HLA)-mismatched NK cells and hematopoietic stem cells (HSC) to treat patients with acute myeloid

leukemia (AML) (135). To eradicate leukemic cells following hematopoietic stem cell transplantation (HSCT), both the HSC (NK precursor pool) and NK cells were HLA mismatched such that their inhibitory receptors termed “killer cell immunoglobulin-like receptors (KIRs)” did not recognize HLA types on the hosts leukemic cells. The matching donor bone marrow was selected to differ from the recipient only in specific KIR alleles. During NK cell licensing, NK cells express inhibitory KIRs for self-HLA and are educated to be hyporesponsive against self-cells, including leukemic cells bearing the same HLA. By transferring allogeneic NK cells bearing inhibitory receptors that do not engage HLA’s on recipient leukemic cells, patients had better graft vs leukemia (GvL) clearance and improved HSC engraftment. As an added benefit to this treatment, allogeneic NK cells do not cause graft versus host disease (GVHD). GVHD is caused by the activation and expansion of residual T cells originating from donor tissues that perceive host antigens and tissue as foreign (136). In fact, NK cells ameliorate GVHD by direct killing of antigen presenting cells that display host-antigens within the graft, improving patient outcome (135). Of note, NK cells alone do not elicit autoimmunity and only exert GvL responses following adoptive transfer. Adoptively transferred NK cells are well tolerated by patients and do not result in significant toxicity or damage to non-cancerous tissues, indicating a measure of tolerance toward healthy tissues maintained by donor NK cells (135, 137). Due to the promising benefits of allogeneic NK cell therapy observed in the treatment of AML, clinical trials are in progress to treat solid tumors and cancers without HSCT (138). Regarding allogeneic NK cell treatment, there are several hurdles to successful outcomes including: (1) generating mature cytolytic

$CD56^{dim}$ cells, (2) maintaining persistence of these cytolytic effectors, and (3) recruitment and penetration of cytolytic NK cells within solid tumors. Good clinical outcomes can be correlated with high levels of cytolytic $CD56^{dim}$ circulating donor NK cells. Understanding aspects of NK cell homeostasis that can lead to increased NK cell maturation, persistence, and altered migration are clinically significant. Data in chapters 3 and 4 will address each of these points.

Given the role of KLF2 in regulating migration within $CD4^+$ T lymphocyte lineages, I hypothesized that KLF2-directed migration is lineage specific and necessary for maintaining lymphocyte homeostasis and effector function. Therefore, the primary goals of my thesis were to

1. Determine how KLF2 differentially regulates migration between $CD4^+CD25^-$ and $CD8^+$ T cell lineages.
2. Assess the consequences of KLF2 deficiency in maintaining NK cell homeostasis and effector function.
3. Determine how KLF2 regulates migration of NK cells.

Significance

In my thesis I report that KLF2 differentially regulates migration between $CD4^+CD25^-$, $CD8^+$, and NK cell lymphocyte lineages. KLF2-directed migration patterns within the $CD4^+CD25^-$ and $CD8^+$ T cell compartment are not necessary for effector T cell function but may be necessary for maintaining peripheral tolerance. In contrast,

within the NK cell compartment KLF2 is essential for maintaining NK cell homeostasis. In NK cells KLF2 suppresses immature NK cell proliferation and controls NK cell migration to promote localization with IL-15 rich niches necessary for mature NK cell differentiation and survival. The data I report in my subsequent chapters reveal fundamental aspects of how NK cell homeostasis is regulated through KLF2. Through these novel KLF2-directed mechanisms it may be possible to increase the number and persistence of mature NK cell populations to improve NK cell based cancer therapies.

CHAPTER 2

KLF2 DIFFERENTIALLY REGULATES MIGRATION PATTERNS IN CONVENTIONAL $CD4^+CD25^-$ AND $CD8^+$ T CELLS

Introduction

KLF2-directed migration patterns promote the retention and recirculation of naïve $CD4^+CD25^-$ T cells within secondary lymphoid organs (SLO) to maintain T cell homeostasis (43-45, 49). KLF2 directly promotes the expression of homeostatic migratory receptors like CD62L, CCR7, S1P, and β_7 integrin (1-3), while repressing the expression of inflammatory receptors (43, 44). Naïve $CD4^+CD25^-$ T cells express high levels of KLF2 and TCR activation induces rapid KLF2 degradation (37, 51) and changes in the HR profile which allow effector $CD4^+CD25^-$ T cells to leave SLO and access peripheral tissues (43, 44, 51). Tregs express half the level of KLF2 when compared with a naïve $CD4^+$ T cell ($CD4^+CD25^-$) and express hybrid levels of homeostatic receptors (S1P1, CD62L, and CCR7) and inflammatory receptors (CCR4, CCR6, and CCR8) at steady state (26). A recent study by the Sebzda lab indicates that *ex-vivo* treatment of Tregs with KLF2-stabilizing drugs such as statins prior to adoptive transfer in a model of graft versus host disease increased recruitment of Tregs to SLO that ameliorates symptoms of autoimmunity (26). Although, KLF2 is assumed to control migration and homeostasis through similar mechanisms in $CD8^+$ T cells as in naïve $CD4^+CD25^-$ T cells and Tregs, this has yet to be experimentally tested. Of note,

$CD4^+CD25^-$ and $CD8^+$ T cells have well documented heterogeneous effector functions. Therefore, it is possible that KLF2 may directly promote or repress migration within $CD4^+CD25^-$ and $CD8^+$ T cells in a lineage specific manner. Moreover, it is not clear whether these KLF2-directed migratory patterns are necessary to promote pathogen clearance or maintain peripheral tolerance.

In this chapter I report that genetic ablation of *Klf2* within the T cell compartment reveals that $CD8^+$ T cells are present in lower frequencies within SLO relative to $CD4^+CD25^-$ T cells. Under steady-state conditions, $CD8^+$ T cells express a wider array of inflammatory receptors than co-stained $CD4^+CD25^-$ T cells. Likewise, KLF2 also suppresses a wider array of inflammatory receptors in $CD8^+$ T cells than $CD4^+CD25^-$ T cells and loss of KLF2 leads to a more stringent repression of CCR7 and CD62L in $CD4^+CD25^-$ T cells. These data suggest that $CD8^+$ T cells are more dependent upon the loss of KLF2 to leave SLO following T cell activation, and that $CD4^+CD25^-$ T cells may possess both KLF2-dependent and KLF2-independent pathways which maintain their presence within the draining lymph nodes following TCR activation. This is consistent with the fact that $CD8^+$ T cells primarily fulfill effector function in the periphery to facilitate viral clearance. Whereas $CD4^+CD25^-$ T cells perform effector functions within both the periphery to facilitate lymphocyte recruitment and within SLO to promote B cell class-switching. Loss of KLF2 within the T cell compartment (*Lck-cre; Klf2*$^{fl/fl}$) alone did not affect T cell effector function or result in spontaneous autoimmunity. However, loss of KLF2 in the T cell compartment in combination with a loss of CCR7 (*CCR7*$^{-/-}$*; Lck-cre; Klf2*$^{fl/fl}$) resulted in unrestrained autoimmunity and morbidity. Pathology observed in

KLF2 T cell conditional knockout mice (*Lck-cre; Klf2*$^{fl/fl}$) associated with murine norovirus, but not norovirus-free mice, was similar to pathology observed in *CCR7*$^{-/-}$*; Lck-cre; Klf2*$^{fl/fl}$ autoimmune mice. Collectively these data suggest that KLF2-directed T cell migration patterns are dispensable for pathogen clearance but contribute to peripheral tolerance.

Results

KLF2 strongly suppresses a wider array of inflammatory homing receptors in CD8$^+$ T cells relative to conventional CD4$^+$CD25$^-$ T cells

It has been previously reported that KLF2-deficiency within the T cell compartment results in reduced numbers of T cells within the SLO (37, 43, 44). To document differences in migration patterns in the absence of *Klf2* between CD4$^+$CD25$^-$ T cells and CD8$^+$ T cells I quantified the number of T cell subsets within SLO of T cell conditional knockout mice (*Lck-cre; Klf2*$^{fl/fl}$) previously generated by the Sebzda lab (Figure 2-1, A). The numbers of conventional CD4$^+$CD25$^-$ T cells and CD8$^+$ T cells within the SLO of *Lck-cre; Klf2*$^{fl/fl}$ mice were significantly reduced relative to *Klf2*$^{fl/fl}$ controls as previously reported (37, 43, 44). When I calculated the ratio of conventional CD4$^+$CD25$^-$ T cells to CD8$^+$ T cells, there were proportionately fewer CD8$^+$ T cells present in *Lck-cre; Klf2*$^{fl/fl}$ mice compared to *Klf2*$^{fl/fl}$ controls (Figure 2-1, B). These data suggest that KLF2 may more strongly regulate the retention of CD8$^+$ T cells within SLO

than in conventional $CD4^+CD25^-$ T cells, and differentially regulate migration between these two lineages.

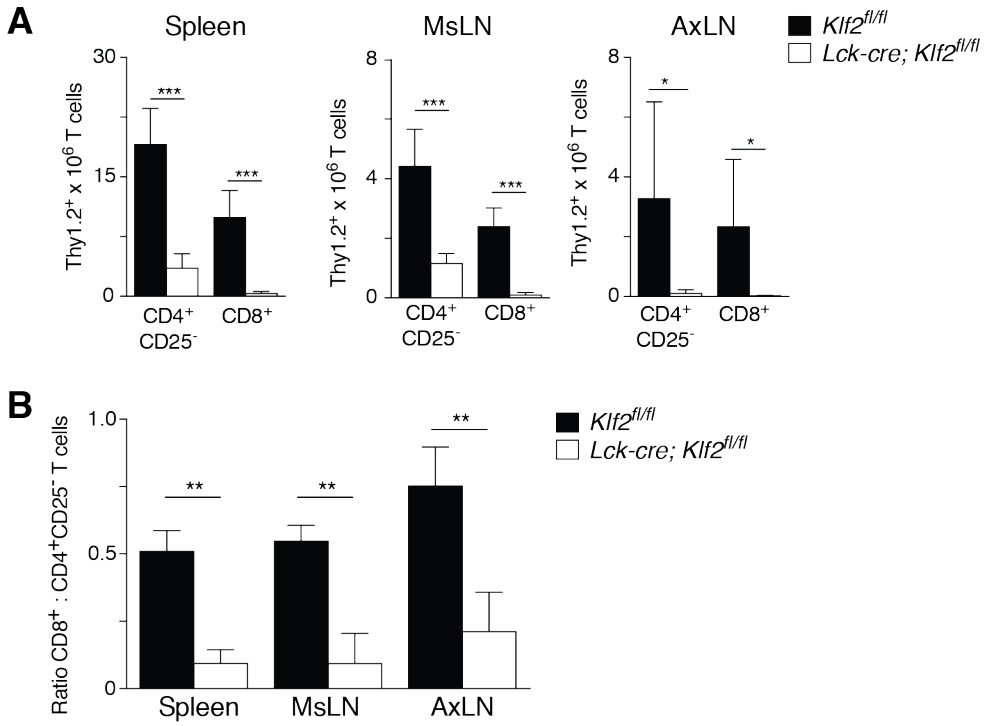


Figure 2-1. KLF2 strongly promotes the retention of $CD8^+$ T cells within secondary lymphoid organs (SLO) relative to $CD4^+CD25^-$ T cells. (A) $CD4^+CD25^-$ and $CD8^+$ T cells are reduced in the SLO of *Klf2*-gene targeted mice. Quantification of $CD4^+CD25^-$ and $CD8^+$ $Thy1.2^+$ T cells within the SLO by flow cytometry in $Klf2^{fl/fl}$ and *Lck-cre;* $Klf2^{fl/fl}$ mice. N=6 mice per cohort from two combined experiments. MsLN=mesenteric lymph node; AxLN= axillary lymph node. Data represents the mean ± SD. *P<0.05, ***P<0.001. Means were compared by untailed Student's t-test. (B) There are proportionately fewer $CD8^+$ T cells relative to $CD4^+CD25^-$ present in the SLO of *Lck-cre;* $Klf2^{fl/fl}$ mice compared to $Klf2^{fl/fl}$ controls. Ratio of $CD8^+$ to $CD4^+CD25^-$ T cells in $Klf2^{fl/fl}$ and *Lck-cre;* $Klf2^{fl/fl}$ mice. N=6 mice per cohort from two combined experiments. Data represents the mean ± SD. **P<0.01. Means were compared by a Mann-Whitney U test.

To determine whether KLF2 differentially regulates migration between these two T cell lineages, I screened for differences in homing receptor expression by flow cytometry (Table 2-1). My panel was limited by the availability of antibodies that could reliably detect extracellular levels of homing receptors. Of the screened homing receptors, only CCR7 and CD62L are expressed under steady-state conditions (Table 2-1, highlighted). The remainder of the panel were receptors upregulated on T cells under inflammatory conditions (Table 2-1, un-highlighted). As expected, *Klf2*-deficient $CD4^+CD25^-$ T cells and $CD8^+$ T cells expressed higher levels of inflammatory homing receptors (Table 2, Figure 2-2 A). In both T cell subsets, KLF2 similarly promoted the expression of homeostatic receptors CCR7 and CD62L (Table 2-1, Figure 2-2 B, C), but in *Lck-cre; Klf2*$^{fl/fl}$ animals KLF2-deficient $CD4^+CD25^-$ T cells expressed lower levels of both receptors compared to KLF2-deficient $CD8^+$ T cells (Table 2-1, Figure 2-2 B). When we focused our analysis on inflammatory homing receptors, KLF2-deficient $CD8^+$ T cells expressed higher levels of CCR2, CCR3, CCR5, CCR6, CCR8, and CCR9 relative to KLF2-deficient $CD4^+CD25^-$ T cells (Table 2, Figure 2-2 A bottom row). In contrast KLF2-deficient $CD4^+CD25^-$ T cells expressed higher levels of CCR4 (Table 2-1, Figure 2-2 A top right). Homing receptors CXCR3, CXCR5, and CD44 were similarly repressed by KLF2 in both T cell lineages (Table 2-1, Figure 2-2 A top left). These data suggest that KLF2 similarly promotes homeostatic receptor expression (CCR7, CD62L) in both T cell subsets but may differentially represses inflammatory receptor expression in $CD4^+CD25^-$ and $CD8^+$ T cells.

Following T cell activation, $CD4^+CD25^-$ T cells may exert effector functions in both the draining lymph node in which the cell was activated and the periphery. Effector $CD4^+CD25^-$ T cells promote B cell class switching within the draining lymph node and coordinate lymphocyte recruitment at peripheral sites of inflammation. KLF2-regulated loss of CD62L and CCR7 in $CD4^+CD25^-$ T cells following TCR activation may prevent these effector cells from entering non-draining SLO and promote retention within the draining lymph node in which it was activated to facilitate B cell activation and class switching. In contract to $CD4^+CD25^-$, $CD8^+$ T cell effector functions primarily occur within the periphery to promote viral clearance. Under steady state, $CD8^+$ T cells express higher levels of inflammatory receptors compared to conventional $CD4^+CD25^-$ T cells (Figure 2-2 C) and KLF2 more strongly represses a wider array of inflammatory receptors in $CD8^+$ T cells compared to $CD4^+CD25^-$ T cells (Figure 2-2 D). These data suggest that upon TCR stimulation and KLF2-degradation, $CD8^+$ T cells may be able to access the periphery more easily than conventional $CD4^+CD25^-$ T cells by expressing a wider array of inflammatory homing receptors, consistent with their effector functions. In addition, our data reveal conventional $CD4^+CD25^-$ T cells possess functionally redundant, KLF2-independent pathways that promote retention within SLO. Given that these KLF2-directed migration patterns were consistent with their effector functions, we hypothesized that KLF2 in $CD4^+CD25^-$ and $CD8^+$ T cells may be necessary to promote pathogen clearance or maintain peripheral tolerance.

Table 2-1. Homing receptor expression on splenic Thy1.2$^+$ T cell subsets by flow cytometry in *Klf2*$^{fl/fl}$ and *Lck-cre; Klf2*$^{fl/fl}$ mice

Homing Receptors (HR)	% HR$^+$ *Klf2*$^{fl/fl}$ CD4$^+$CD25$^-$	% HR$^+$ *Lck-cre; Klf2*$^{fl/fl}$ CD4$^+$CD25$^-$	%HR$^+$ *Klf2*$^{fl/fl}$ CD8$^+$	%HR$^+$ *Lck-cre; Klf2*$^{fl/fl}$ CD8$^+$	*Klf2*$^{fl/fl}$ CD4$^+$CD25$^-$ vs. *Lck-cre; Klf2*$^{fl/fl}$ CD4$^+$CD25$^-$	*Klf2*$^{fl/fl}$ CD8$^+$ vs. *Lck-cre; Klf2*$^{fl/fl}$ CD8$^+$	*Klf2*$^{fl/fl}$ CD4$^+$CD25$^-$ vs *Klf2*$^{fl/fl}$ CD8$^+$	*Lck-cre; Klf2*$^{fl/fl}$ CD4$^+$CD25$^-$ vs *Lck-cre; Klf2*$^{fl/fl}$ CD8$^+$
CCR2	6.0 ± 2.0	17.6 ± 4.6	8.5 ± 4.2	33.5 ± 10.0	***	***	N.S	**
CCR3	3.3 ± 2.0	11.8 ± 4.9	4.0 ± 2.2	30.4 ± 19.6	***	***	N.S	*
CCR4	8.9 ± 2.0	40.7 ± 13.1	3.1 ± 1.1	18.5 ± 8.3	***	***	***	**
CCR5	8.3 ± 2.2	54.0 ± 11.5	25.2 ± 5.6	79.7 ± 13.7	***	***	***	**
CCR6	2.8 ± 0.9	6.5 ± 2.5	3.8 ± 1.2	19.2 ± 13.2	***	**	*	*
CCR7	87.1 ± 4.4	16.5 ± 9.0	71.3 ± 7.9	29.2 ± 8.9	***	***	***	**
CCR8	6.1 ± 2.3	21.0 ± 8.1	10.2 ± 4.9	47.7 ± 12.1	***	***	*	***
CCR9	10.2 ± 4.2	29.8 ± 13.2	20.8 ± 4.6	45.9 ± 11.6	***	***	***	*
CXCR3	18.1 ± 5.0	83.7 ± 5.0	49.4 ± 7.5	79.8 ± 11.8	***	***	***	N.S.
CXCR5	5.9 ± 1.0	13.5 ± 1.6	8.5 ± 1.0	15.1 ± 4.6	***	*	**	N.S.
CD44	23.1 ± 4.3	93.8 ± 3.8	31.7 ± 2.9	89.9 ± 3.2	***	***	**	N.S.
CD62L	78.6 ± 2.7	8.5 ± 7.5	92.0 ± 1.2	52.7 ± 17.5	***	**	***	*

Highlighted cells represent analysis of homeostatic migratory receptors directly promoted by KLF2
Unhighlighted cells are inflammatory receptors repressed by KLF2

Splenic T cells were enriched using Thy1.2 microbeads harvested from *Klf2*$^{fl/fl}$ and *Lck-cre; Klf2*$^{fl/fl}$ mice prior to flow cytometry analysis. n=3-9 mice per cohort. This experiment was performed 1-3 times per homing receptor. Data represents the mean ± SD. Means were compared between the indicated groups. *P<0.05, **P<0.01, *P<0.001.

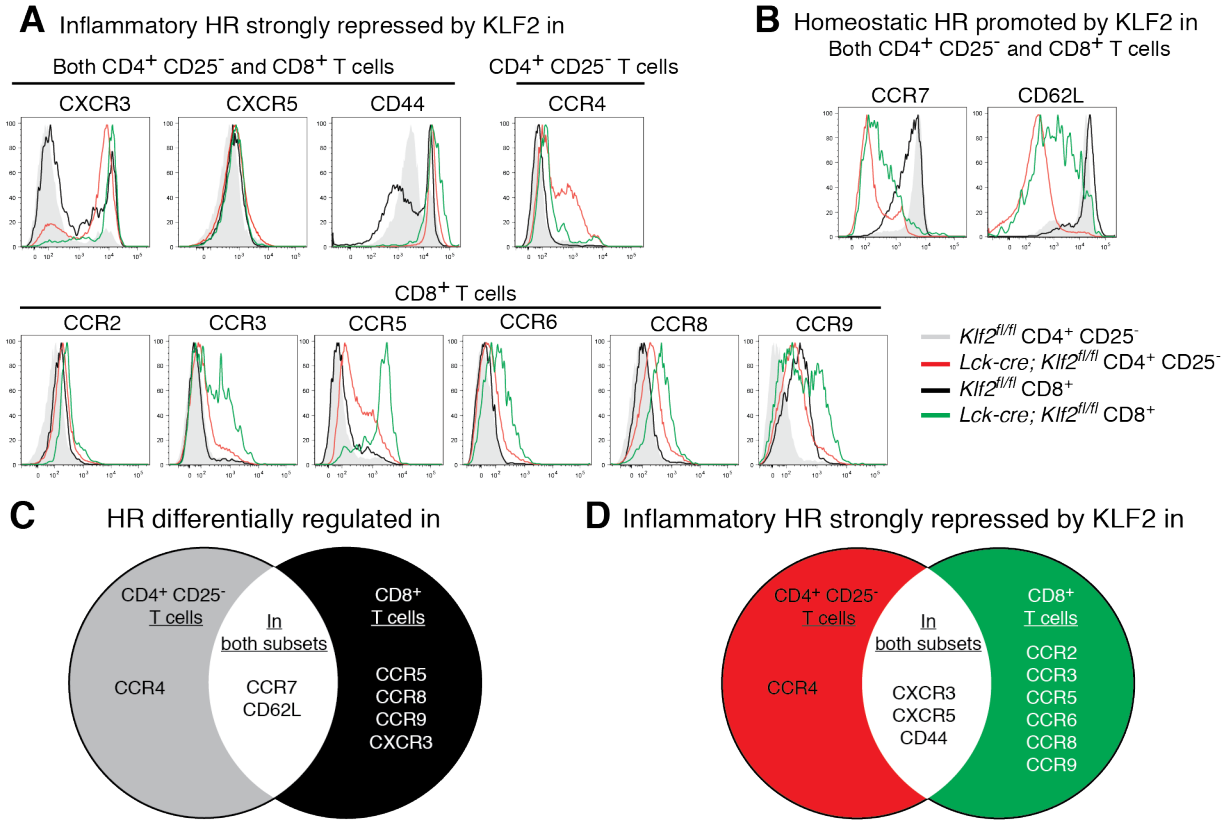


Figure 2-2. KLF2 similarly promotes homeostatic HR expression in $CD4^+CD25^-$ and $CD8^+$ T cells but differentially represses inflammatory HR expression. (A) Representative histograms of homing receptors responding to ligands expressed under inflammatory conditions repressed similarly by KLF2 in both $CD4^+CD25^-$ and $CD8^+$ T cells, robustly in $CD4^+CD25^-$ T cells, and $CD8^+$ T cells respectively. (B) Representative histograms of homing receptors responding to ligands expressed under steady-state conditions similarly promoted by KLF2 in both $CD4^+CD25^-$ and $CD8^+$ T cells. (D) Venn diagram of homing receptors differentially regulated between $CD4^+CD25^-$ and $CD8^+$ T cells under steady-state. (D) Venn diagram of inflammatory homing receptors differentially repressed by KLF2 between $CD4^+CD25^-$ and $CD8^+$ T cells.

KLF2 is dispensable for T cell effector function

Upon activation via pattern recognition receptors, antigen presenting cells process and display antigen in the context of MHC and migrate to SLO via CCR7. T cell recirculation within the SLO increases the likelihood of a naïve T cell to encounter an activated APC presenting its cognate antigen. Due to the reduced numbers of T cells within SLO in *Lck-cre; Klf2*$^{fl/fl}$ mice, we hypothesized these mice may display defects in pathogen clearance stemming from decreased APC and T cell interactions. To test our hypothesis, we infected *Lck-cre; Klf2*$^{fl/fl}$ mice and *Klf2*$^{fl/fl}$ controls with lymphocytic choriomeningitis (LCMV) and assessed their ability to clear this pathogen. LCMV clearance requires CD8$^+$ T cells and mice deficient in this population develop persistent infections (139). Wild type mice intraperitoneally infected with 10^1-10^6 pfu of LCMV typically clear the virus between 7-10 days (139). When we examined the spleens of mice infected with 10^6 pfu LCMV-Armstrong 8 days post-infection, the number of CD8$^+$ T cells in *Lck-cre; Klf2*$^{fl/fl}$ were similar to those observed in *Klf2*$^{fl/fl}$ co-infected controls, indicating that KLF2-deficient CD8$^+$ T cells are capable of responding to virus (Figure 2-3 A). Consistent with these findings, KLF2-deficient CD8$^+$ T cells were also capable of specifically killing target cells presenting viral antigen indicating that their cytolytic functions were intact (Figure 2-3 B). *Lck-cre; Klf2*$^{fl/fl}$ mice also had similar viral titers compared to *Klf2*$^{fl/fl}$ controls on day 8, and had cleared the majority of the virus by day 30 (Figure 2-3 C). To confirm whether the *Lck-cre; Klf2*$^{fl/fl}$ mice which cleared the majority of virus possessed LCMV specific CD8$^+$ T cells, we re-stimulated splenocytes from these animals with MHC class I-restricted viral peptide p33 (C57BL/6 specific) from

day 30 harvested animals. From this day 30 cohort, 2 of 4 *Lck-cre; Klf2*$^{fl/fl}$ mice displayed an increase in the frequency of CD8^{+} IFNγ^{+} T cells reflecting a prior response to LCMV (Figure 2-3 D). Of *Lck-cre; Klf2*$^{fl/fl}$ mice that did not respond p33 re-stimulation, their CD8^{+} T cells expressed higher levels of PD-1 indicating that these cells were exhausted, and had once been responsive towards stimuli (data not shown). To test whether KLF2 was necessary to for CD4^{+}CD25^{-} T cells effector functions, we tested the ability of KLF2-deficient CD4^{+}CD25^{-} T to induce B cell class-switching in *Lck-cre; Klf2*$^{fl/fl}$ mice. *Lck-cre; Klf2*$^{fl/fl}$ mice were capable of inducing class switching when stimulated and re-challenged with TNP-OVA in a manner similar to *Klf2*$^{fl/fl}$ control mice (Figure 2-4). Collectively, these data indicate that KLF2 directed migration patterns are not necessary for pathogen clearance and for preserving T cell effector functions, but may be necessary for peripheral tolerance.

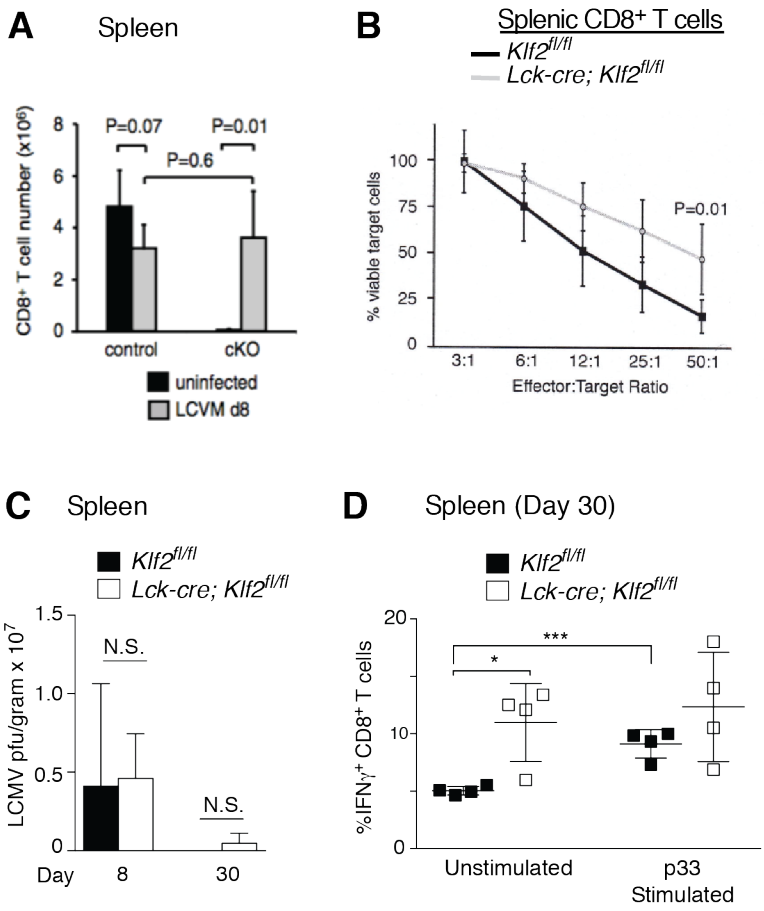


Figure 2-3. CD8$^+$ T cell effector responses are intact in *Lck-cre; Klf2$^{fl/fl}$* mice. (A) Quantification of splenic CD8$^+$ T cells in *Klf2$^{fl/fl}$* and *Lck-cre; Klf2$^{fl/fl}$* mice from cohorts of uninfected or day-8 lymphocytic choriomeningitis virus (LCMV, 10^6 pfu, i.p.) –infected mice. N=7 mice per cohort. This experiment was performed once. (B) Cytolytic effector efficiency of CD8$^+$ T cells harvested from LCMV-infected mice (day 8). CD8$^+$ T cells were co-cultured with RMA-S target cells pre-loaded with LCMV peptide p33. Target cell lysis (release of intracellular and membrane-associated dyes) was measured by flow cytometry. % Viable target cells= [(% experimental CFSE$^+$PHK26$^+$ RMA-S cells)/ (% CFSE$^+$PHK26$^+$ RMA-S cells co-cultured with naïve CD8$^+$ T cells)] x 100. Data represents the mean ± SD. P values > 0.05 except as indicated. N = 7 mice per infected group. This experiment was performed once. (C) *Lck-cre; Klf2$^{fl/fl}$* mice clear LCMV. LCMV viral titers were measured at days 8 and 30 post infection. N=4-5 mice per cohort. (D) *Klf2*-deficient LCMV-primed CD8$^+$ T cells respond to LCMV peptide p33 upon re-stimulation. Splenocytes were harvested from mice 30 days post-infection and re-stimulated with p33 for 5hrs and the frequency of IFNγ^+ CD8$^+$ T cells was quantified by flow cytometry. N=4 mice per cohort. This experiment was performed once.

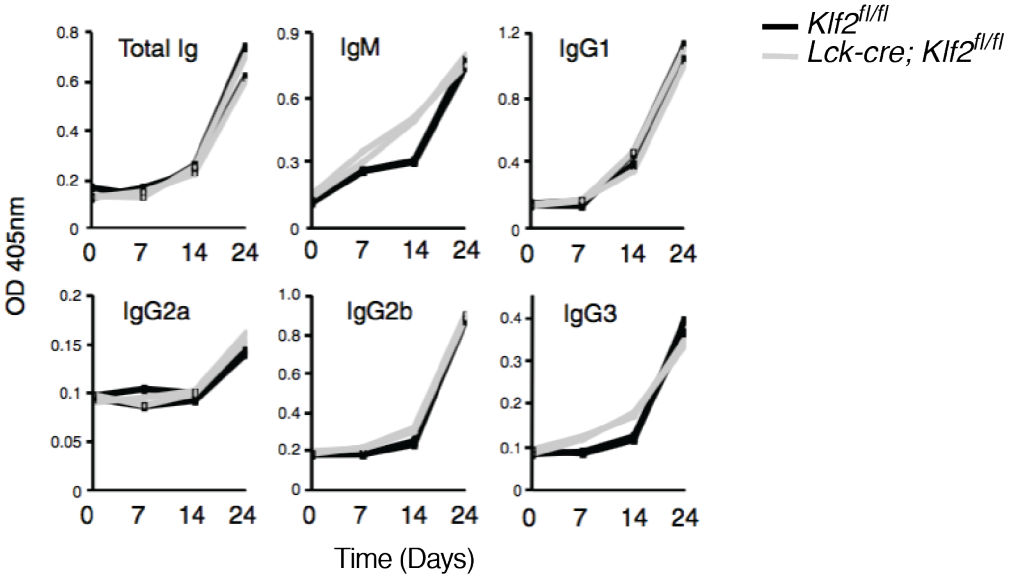


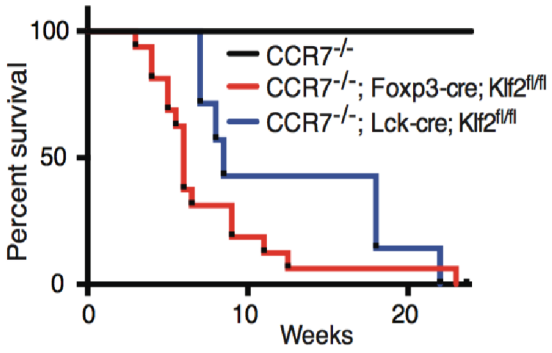
Figure 2-4. $CD4^{+}CD25^{-}$ T cell effector responses are intact in *Lck-cre; Klf2*$^{fl/fl}$ mice. T-dependent Ig class-switching. Trinotrophenyl (TNP)-specific Ig concentrations in the sera of *Klf2*$^{fl/fl}$ and *Lck-cre; Klf2*$^{fl/fl}$ mice after challenge with TNP-OVA and re-challenge with TNP-OVA at day 14. Titers were detected by ELISA using TNP-ficoll coated plates and represented as optical density (O.D.). This experiment was performed twice with one mouse per group.

KLF2-regulated migratory patterns in T cells promotes self-tolerance

The Sebzda lab has recently demonstrated that KLF2-directed migration patterns in Tregs are necessary to maintain peripheral tolerance. KLF2-directed retention of Tregs within SLO is necessary to prevent spontaneous autoimmunity. When *Klf2* is excised in a conditional knockout using a Foxp3-cre system, *Foxp3-cre; Klf2*$^{fl/fl}$ mice develop overt symptoms of autoimmunity including cachexia, alopecia, and gastrointestinal distress associated with systemic lymphocyte infiltration in multiple organs including the skin, small intestine, colon, and lung. Despite the striking phenotype observed within *Foxp3-cre; Klf2*$^{fl/fl}$ mice, *Lck-cre; Klf2*$^{fl/fl}$ mice exhibit T cell displacement but are otherwise healthy and fertile. Although T cells are drastically reduced within the SLO of *Lck-cre; Klf2*$^{fl/fl}$, there are redundant KLF2-independent mechanisms that promote a minimal amount of $CD4^+$ T cell retention within SLO relative to $CD8^+$ T cells (Figure 2-1 B). To test whether both KLF2-dependent migration patterns and KLF2-independent migration patterns within SLO promote peripheral tolerance, I crossed *Lck-cre; Klf2*$^{fl/fl}$ mice with *CCR7*$^{-/-}$ to further reduce T cell numbers within these tissues. Consistent with autoimmune phenotypes observed in *Foxp3-cre; Klf2*$^{fl/fl}$ animals, *CCR7*$^{-/-}$*; Lck-cre; Klf2*$^{fl/fl}$ mice displayed symptoms of cachexia, severe gastrointestinal inflammation, and were moribund within 24 weeks, whereas *Lck-cre; Klf2*$^{fl/fl}$ and *CCR7*$^{-/-}$ mice remained healthy for the course of this study (Figure 2-5 A). While breeding mice during the course of my thesis, I noticed that 20-30% of *Lck-cre; Klf2*$^{fl/fl}$ mice began to spontaneously succumb to severe gastrointestinal distress when they were previously asymptomatic and healthy. In cages containing large amounts of

diarrhea and irregular excrement, nearly all *Lck-cre; Klf2*$^{fl/fl}$ mice became moribund, while co-housed *Klf2*$^{fl/fl}$ mice remained healthy (Figure 2-5 B). All *Lck-cre; Klf2*$^{fl/fl}$ mice in cages with normal fecal excrement remained healthy. Necropsy of a symptomatic *Lck-cre; Klf2*$^{fl/fl}$ mouse with gastrointestinal distress revealed high numbers of lymphocytic infiltrates in the small intestine and colon similar to symptoms observed in *CCR7*$^{-/-}$*; Lck-cre; Klf2*$^{fl/fl}$. However, this mouse also tested positive for murine norovirus by PCR analysis (data not shown). Recombinase-activated gene deficient (*RAG*$^{-/-}$) mice and signal transducer and activator of transcription 1 (*STAT1*$^{-/-}$) mice are susceptible to murine norovirus and display symptoms of gastrointestinal distress including diarrhea (140). Because symptoms of murine norovirus and autoimmunity associated with KLF2-deficiency in T cells are strikingly similar, it is difficult to determine whether these *Lck-cre; Klf2*$^{fl/fl}$ mice (Figure 2-5 B) died from (a) murine norovirus or (b) an inability to shut down responses against the virus resulting in a state of chronic inflammation. In addition, because the *Lck-cre* system excises *Klf2* in both conventional $CD4^+CD25^-$ T cells and these mice lack peripheral Tregs (pTregs) (30), it is difficult to discern whether these phenotypes are due to defects in the conventional $CD4^+CD25^-$ T cells lineage or the absence of pTregs in SLO. These data reflect a strong correlation between *Lck-cre; Klf2*$^{fl/fl}$ morbidity and murine norovirus. Given that T cell effector functions in *Lck-cre; Klf2*$^{fl/fl}$ mice are intact (Figure 2-3, 2-4) and these mice lack pTregs, it more likely that these mice succumb to unrestrained effector cell associated inflammation rather than the virus itself. Collectively, these data indicate that KLF2 restricted migration patterns in conventional $CD4^+CD25^-$ and $CD8^+$ T cells promote peripheral tolerance.

A



B Pathology associated with murine norovirus

Strain	Incidence	Severity	Notes
Lck-cre; Klf2$^{fl/fl}$	7 of 7	6 of 7 moribund and euthanized to minimize pain/distress 1 recovered	Symptoms include diarrhea, dehydration, inflammation of colon and small intestine, with or without rectal prolapse
Klf2$^{fl/fl}$	0 of 9	0 of 9	Co-house with *Lck-cre; Klf2$^{fl/fl}$*

Figure 2-5. KLF2-directed T cell migration promotes self-tolerance. (A) *CCR7$^{-/-}$; Lck-cre; Klf2$^{fl/fl}$* mice (n=7) and succumb to autoimmunity whereas *Lck-cre; Klf2$^{fl/fl}$* (n=3) and *CCR7$^{-/-}$* mice (n=3) remain healthy. (B) *Lck-cre; Klf2$^{fl/fl}$* mice succumb to pathology associated with murine norovirus whereas co-housed *Klf2$^{fl/fl}$* mice remained healthy. Necropsy of a *Lck-cre; Klf2$^{fl/fl}$* mouse displaying inflammation of the small intestine and colon revealed significant neutrophilia in these organs and the presence of murine norovirus in the mesenteric lymph node by PCR. Lymphocyte infiltration observed in the small intestine and colon in sick *Lck-cre; Klf2$^{fl/fl}$* mice was similar to pathology observed in *CCR7$^{-/-}$; Lck-cre; Klf2$^{fl/fl}$* mice.

Discussion

In this chapter, I demonstrated that KLF2 differentially regulates homing receptor expression in conventional $CD4^+CD25^-$ T cells and $CD8^+$ T cells. Despite reduced numbers within SLO in *Lck-cre; Klf2$^{fl/fl}$* mice, these animals are capable of mounting $CD8^+$ dependent T cell responses to LCMV. *Lck-cre; Klf2$^{fl/fl}$* mice can efficiently clear this virus and are capable of inducing B cell class switching. Within $CD8^+$ T cells, KLF2 strongly suppresses a wider array of inflammatory receptors compared to conventional $CD4^+CD25^-$ T cells. This may allow $CD8^+$ T cells to more easily leave the KLF2 restricted niche (SLO, blood, and lymph), upon KLF2 degradation relative to $CD4^+CD25^-$ T cells. In contrast in $CD4^+CD25^-$ T cells KLF2 more controls the expression of homeostatic receptors CCR7 and CD62L necessary for entering SLO. Conventional $CD4^+CD25^-$ T cells exert their effector functions in both the periphery and SLO. KLF2-dependent loss of CCR7 and CD62L combined with KLF2-independent mechanisms that retain this population within SLO may restrict an effector $CD4^+CD25^-$ T cell to the draining lymph node in which it was activated and prevent it from accessing other SLO. This migration pattern may promote effector $CD4^+CD25^-$ T cell and B cell interactions necessary for B cell activation and class-switching. These data also explain why the ratio of $CD8^+$ T cells to $CD4^+CD25^-$ T cells is significantly lower in *Lck-cre; Klf2$^{fl/fl}$* mice compared to *Klf2$^{fl/fl}$* controls. The presence of $CD4^+CD25^-$ T cells within the SLO of *Lck-cre; Klf2$^{fl/fl}$* mice also reveals a functional redundancy that is KLF2-independent, which maintains a minimal number of T cells within the SLO and promotes tolerance. Genetic ablation of both *Klf2* and CCR7 in *CCR7$^{-/-}$; Lck-cre; Klf2$^{fl/fl}$* mice but in not *Lck-cre; Klf2$^{fl/fl}$*

mice, resulted in the development of autoimmunity indicating that KLF2 directed migration patterns in T cells contribute to peripheral tolerance. Within *Lck-cre; Klf2*$^{fl/fl}$ animals *Klf2* is excised in both conventional $CD4^+CD25^-$ T cells and Tregs, and these animals lack pTregs. Due to the technical limitation of this system and available Cre-Lox systems, we cannot distinguish whether the break in tolerance observed in *CCR7*$^{-/-}$*; Lck-cre; Klf2*$^{fl/fl}$ mice or *Lck-cre; Klf2*$^{fl/fl}$ animals associated with norovirus were due to a defect in KLF2-deficient $CD4^+CD25^-$ T cells or Tregs. However, it is likely that KLF2 in both $CD4^+CD25^-$ T cells or Tregs contributes to peripheral tolerance.

Recently, our lab has demonstrated that KLF2 stabilizing drugs such as statins, PI3K-inhibitors, MEK-inhibitors, and rapamycin can increase of Treg production (141) and the recruitment of these cells to SLO to prevent the initiation of autoimmune disease by suppressing T cell activation (26). Homing receptor analysis of $CD4^+CD25^-$ and $CD8^+$ T cells in *Lck-cre; Klf2*$^{fl/fl}$ and *Klf2*$^{fl/fl}$ mice indicates that KLF2-stabilizing drugs like statins, PI3K-inhibitors, MEK-inhibitors, and rapamycin may similarly promote SLO recruitment, however increased recruitment of $CD4^+CD25^-$ T cells but not $CD8^+$ T cells may be necessary to promote tolerance. Our data suggests that KLF2 directs inflammatory migration more stringently in $CD8^+$ T cells than conventional $CD4^+CD25^-$ T cells and a substantial reduction of $CD8^+$ T cells within the SLO of *Lck-cre; Klf2*$^{fl/fl}$ mice, does not result in overt autoimmunity. Therefore, when treating autoimmunity with KLF2 stabilizing drugs like simvastatin, it may be more important to stabilize KLF2 levels in $CD4^+$ populations including $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ Tregs than in $CD8^+$ T cells.

Like NK cells, some memory $CD8^+$ T cell populations require trans-presented IL-15 for survival (71, 115, 142), whereas other memory $CD8^+$ T cell populations are maintained through IL-15 independent mechanisms (143). Transcriptome analysis revealed NK cells are most similarly related to $CD8^+$ T cells than any other lymphocyte (58). Our data suggest that the KLF2-directed migration patterns of $CD8^+$ T cells within SLO may not be necessary to directly suppress T cell activation, like in Tregs. Alternatively, these migration patterns may allow memory $CD8^+$ T cells to access IL-15 rich niches like NK cells for which are necessary for memory $CD8^+$ T cell survival. This NK cell-specific mechanism will be discussed in depth in chapters 3 and 4. It is possible that upon loss of KLF2 within the memory $CD8^+$ T cell compartment, this population may be untethered from the IL-15 survival niche to limit their effector potential as is observed in NK cells. This theory is also consistent with original reports suggesting that KLF2 is necessary for T cell survival (37, 47). Moreover, this alternate theory could explain why the numbers of $CD8^+$ T cells are severely reduced in *Lck-cre; Klf2*$^{fl/fl}$ mice. Further studies testing whether KLF2 is necessary for the maintenance of $CD8^+$ T cells using *in vivo* tamoxifen-inducible models (*T2-cre; Klf2*$^{fl/fl}$) are needed to confirm whether KLF2 promotes self-tolerance similarly in memory $CD8^+$ T cells and NK cells. If this theory holds true, it is likely that KLF2 directed migration patterns may promote self-tolerance within all lymphocyte subsets.

CHAPTER 3

TRANSCRIPTION FACTOR KLF2 REGULATES HOMEOSTATIC NK CELL PROLIFERATION AND SURVIVAL

Introduction

Natural killer cells are a subset of group 1 ILC (ILC) that play key roles in anti-viral and anti-tumor responses by secreting cytokines to recruit other effector lymphocytes or killing virally infected or transformed cells. In the context of cancer, patients with high circulating numbers of NK cells or NK cell infiltration at the tumor site correlate with good disease prognosis (62). Conversely, deficiencies in NK cell populations are associated with chronic herpes infection and susceptibility to cancer (62). NK cells have been successfully utilized in the clinic to treat acute myeloid leukemia through the adoptive transfer of allogeneic NK cells following hematopoietic stem cell transplantation (HSCT). This approach is clinically advantageous because transferred NK cells simultaneously eliminate residual leukemic cells while preventing graft-versus-host-disease (GVHD). GVHD outcomes are improved by NK cells through killing of APC presenting self-antigens that activate grafted T cells to target the host. Moreover, these transferred allogenic NK cells are well tolerated by patients with minimal toxicity (135, 137). Patients receiving allogeneic NK cells that expand in high numbers and are maintained over longer periods post-transfer are associated with better graft-versus-leukemia (GvL) effects and disease prognosis (137). Therefore,

understanding basic mechanisms regulating NK cell homeostasis have broad clinical applications.

Conventional NK cells primarily develop within the bone marrow and following MHC licensing, patrol the blood, lung, liver, and splenic red pulp for stressed or virally infected cells. In mice NK cell development is a four step process that can be characterized using the cell surface markers CD11b and CD27. NK precursor cells are $CD27^-CD11b^-$ (DN) which give rise to $CD27^+CD11b^-$ (stage I, immature) → $CD27^+CD11b^+$ (stage 2, intermediate) → $CD27^-CD11b^+$ (stage 3, late-stage) (2, 93). With regard to peripheral NK cell homeostasis, immature DN and $CD27^+CD11b^-$ (stage 1) are associated with IL-15-dependent homeostatic proliferation (53, 88). In contrast, $CD11b^+$ subsets are functionally mature effectors capable of cytokine secretion and cytolytic activity. These NK cell populations are dependent upon IL-15 for survival (115). Manipulating these two key IL-15 dependent events may lead to increased NK cell expansion and persistence *in vivo*.

To better understand how NK cell homeostasis is regulated, we investigated the possible role of the transcription factor Krüppel-like factor 2 (KLF2) in the NK cell compartment by using *Klf2* gene targeted mice. In B and T lymphocytes, KLF2 is associated with quiescence and limiting proliferation in these subsets upon ectopic over expression (37, 40, 144), and regulates lineage specific migration patterns to maintain homeostasis (26, 43, 44, 46, 145). Based on these observations we predicted that *Klf2*-gene targeted animals would have increased numbers of mature NK cells due to unrestrained proliferation. Indeed, we find that KLF2 intrinsically suppresses

spontaneous proliferation in immature $CD27^+CD11b^-$ NK cells and under steady-state conditions immature KLF2-deficient NK cells were displaced from the red pulp and were present in increased numbers in the IL-15 poor white pulp. Contrary to our expected outcome, mature effector $CD11b^+$ NK cells in *Klf2*-gene targeted mice were absent. By co-tracking migration in mature $CD11b^+$ KLF2-sufficient and KLF2-deficient NK cells under dynamic conditions, we found that KLF2 controls late-stage NK cell survival by dictating homing toward IL-15 rich regions. Furthermore, induced excision of *Klf2* in NK cells led to increased NK cell apoptosis and subsequent loss of mature $CD11b^+$ NK cells. Collectively these data reveal novel roles for KLF2 in suppressing NK cell expansion maintenance that may be manipulated for improving NK cell based therapies.

Results

KLF2 maintains conventional NK cell homeostasis

KLF2 maintains homeostasis within T and B lymphocyte lineages (30, 37, 43, 44, 46, 145). To determine whether KLF2 plays a similar role within conventional NK cells, I measured *Klf2* mRNA and KLF2 protein levels within developing NK cell subsets under steady-state conditions. Using the developmental markers CD11b and CD27, we FACS sorted NK cell subsets during early $CD27^+CD11b^-$ (R1), intermediate $CD27^+CD11b^+$ (R2), and late $CD27^-CD11b^+$ (R3) stages of NK cell development. We confirmed that KLF2 is expressed within the NK cell lineage and both transcript and protein levels increase with maturation (Figure 3-1).

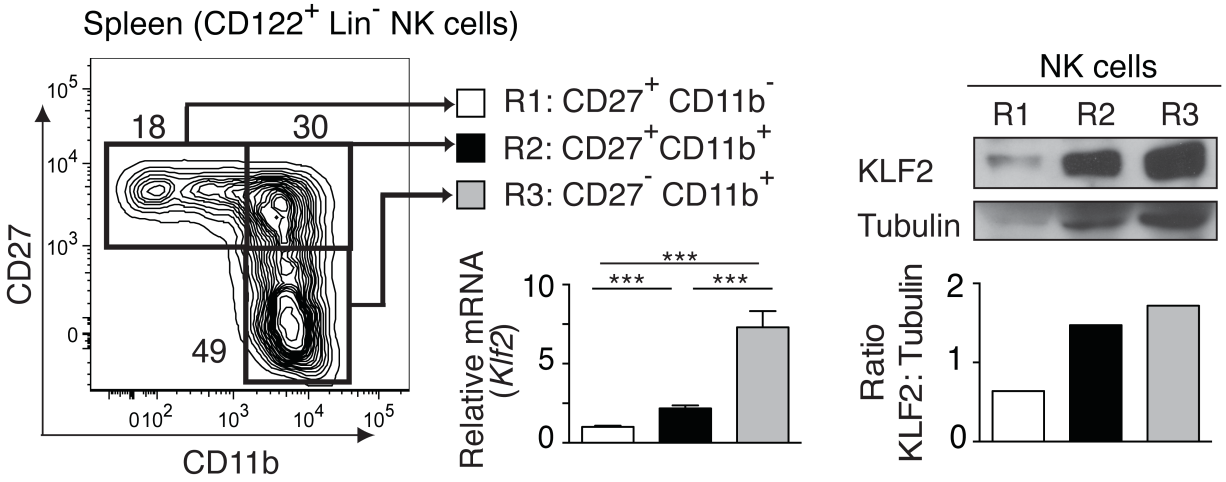


Figure 3-1. KLF2 is expressed in the NK cell lineage. (A) *Klf2* mRNA and KLF2 protein levels in NK cell subsets. Splenic $CD122^{+}Lin^{-}$ (CD3, CD8, CD19, Gr-1, TCRβ) NK cells were FACS sorted into maturing NK cell subsets (R1, $CD27^{+}CD11b^{-}$; R2, $CD27^{+}CD11b^{+}$; R3, $CD27^{-}CD11b^{+}$) from C57BL/6 mice. *Klf2* mRNA and KLF2 protein levels were normalized to *gapdh* and tubulin, respectively. This experiment was repeated twice. *Klf2* mRNA levels reflect the mean ± SD of four technical replicates from one representative sort. KLF2 protein levels reflect the densitometry levels of one representative blot. ***P<0.001.

To investigate the role of KLF2 in the NK cell compartment, *Klf2*-floxed animals were crossed with a *Vav-cre* transgenic animal to excise *Klf2* in hematopoietic stem cells (*Vav-cre; Klf2*$^{fl/fl}$). This genetic system induced efficient gene excision within the NK cell lineage (Figure 3-2 A) and allowed us to examine the role of KLF2 in early and late NK cell development. MHC licensing is a process that enables NK cells to elicit full effector function upon engagement of self-ligand. This process occurs early in NK cell development and primarily within the bone marrow. Alterations in the frequencies of activating and inhibitory receptors within the bone marrow reflect defects in MHC education. The frequencies of activating (NK1.1, NKG2D, NKp46, and Ly49H) and inhibitory (Ly49C/I, Ly49D, and Ly49G2) receptors were similar between *Vav-cre; Klf2*$^{fl/fl}$ conditional knockout mice (cKO) and littermate controls within the bone marrow, indicating that MHC licensing was intact (Figure 3-2 B). These data indicate that KLF2 is dispensable for MHC licensing.

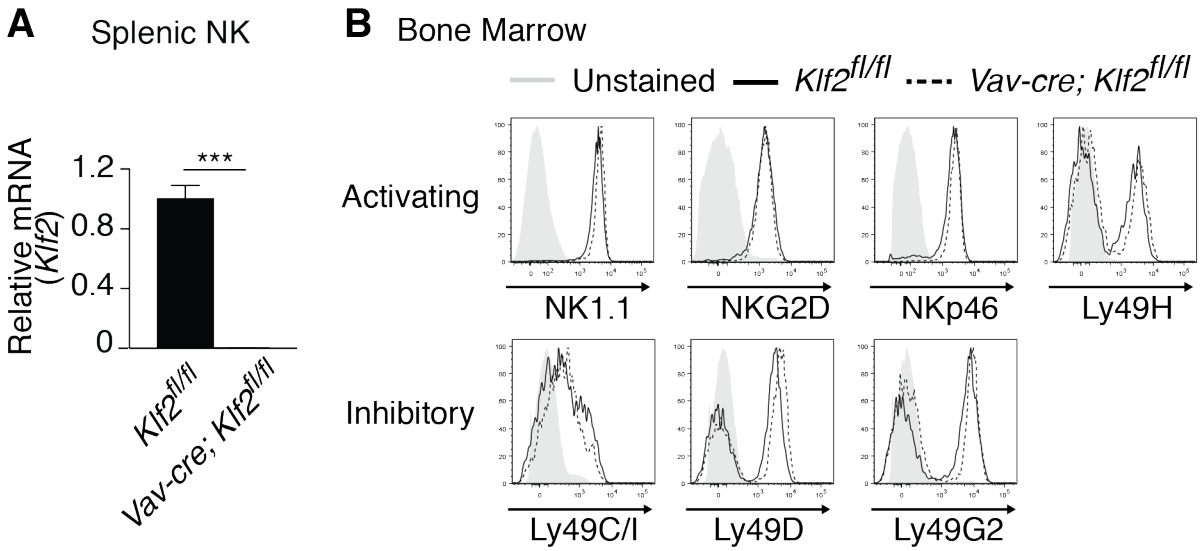


Figure 3-2. *Klf2* is efficiently excised in *Vav-cre; Klf2*$^{fl/fl}$ mice and is dispensable for MHC licensing. (A) *Klf2* mRNA levels expressed in MACS-sorted NK cells harvested from *Klf2*$^{fl/fl}$ versus *Vav-cre; Klf2*$^{fl/fl}$ mice normalized to gapdh. *Klf2* mRNA levels reflect the mean ± SD of four technical replicates. ***P<0.001. (B) Histogram overlays of activating and inhibitory receptors expressed on bone marrow NK cells ($CD122^{+}Lin^{-}NK1.1^{+}$) from *Vav-cre; Klf2*$^{fl/fl}$ versus *Klf2*$^{fl/fl}$ controls. This experiment was repeated twice.

Although MHC licensing in the bone marrow was intact, *Vav-cre; Klf2*$^{fl/fl}$ mice displayed increased frequencies of phenotypically immature CD27^{+}CD11b^{-} (R1) NK cells within all tissues except the mesenteric lymph node (Figure 3-3 A). This phenotype was accompanied by increased numbers of CD27^{+}CD11b^{-} (R1) NK cells within the bone marrow and liver, progenitor sites for both conventional NK (cNK) cells and tissue resident NK (trNK) cells respectively (Figure 3-3 A). Moreover, KLF2-deficient splenic NK cells expressed the immature NK cell marker CD51, not typically expressed by cNK cells outside of the bone marrow (Figure 3-3 C, left panel). Tissue resident NK cells (trNK) and thymic ILC1 cells express similar levels of the hallmark NK cell receptors CD122, NK1.1, and NKp46, and in addition express CD27 and CD51 (102), making them indistinguishable from immature cNK cells in most studies prior to 2013.

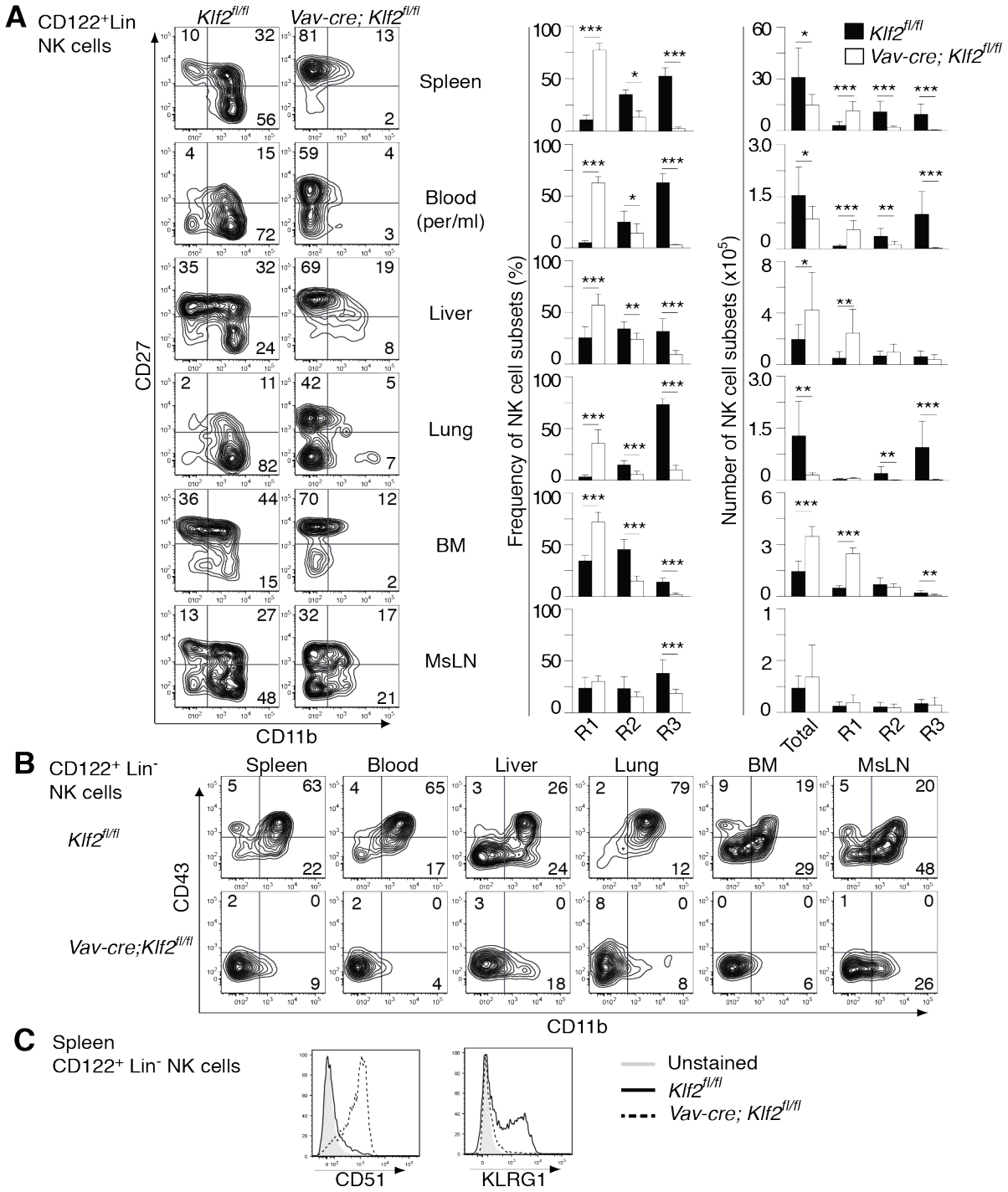


Figure 3-3. KLF2 is necessary for late stage NK cell homeostasis. Contour plots of $CD122^+$ Lin^- (CD3, CD8, CD19, Gr-1, TCRβ) NK cells co-stained for CD27 versus CD11b in *Vav-cre; Klf2*$^{fl/fl}$ and *Klf2*$^{fl/fl}$ controls (A). Frequencies and absolute cell numbers are graphed of maturing NK cell subsets (R1, $CD27^+CD11b^-$; R2, $CD27^+CD11b^+$; R3, $CD27^-CD11b^+$). BM, bone marrow; MsLN, mesenteric lymph node. Data are pooled from three independent experiments (n=10 mice per group). (B) Alternate analysis of $CD122^+Lin^-$ NK cell populations using CD11b and CD43. (n=10 mice per cohort). (C) Representative histogram overlays of early (CD51) and late-stage (KLRG1) maturation markers in splenic NK cells. This experiment was repeated twice with n=3 mice per cohort. Data represent the mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

To determine whether the increased frequencies of immature $CD27^+CD11b^-$ NK cells found within the livers and spleens of *Vav-cre; Klf2$^{fl/fl}$* mice were of cNK cell, trNK cell, or thymic ILC1 origin, we stained spleen and liver lymphocyte suspensions for CD49a, CD49b (DX5), EOMES, CD127 and TRAIL expression. Conventional bone marrow derived cells can be identified as $CD122^+Lin^-NK1.1^+TCR^-CD49a^-CD49b^+TRAIL^-EOMES^{hi}$ (101, 103). Thymic derived ILC1 are defined as $CD122^+Lin^-NK1.1^+TCR^-CD27^+EOMES^{lo}$, and can be distinguished by the expression of $CD127^+$ (101, 103, 106). Fetal liver derived trNK cells are defined as $CD122^+Lin^-NK1.1^+TCR\beta^-CD49a^+CD49b^-TRAIL^+EOMES^{lo}$ (101, 103). The majority $CD122^+Lin^-NK1.1^+$ cells found within the spleens of *Vav-cre; Klf2$^{fl/fl}$* mice were $CD127^-$ $EOMES^{hi}$ (Figure 3-4 A), and $CD49a^-CD49b^+$ (Figure 3-4 B), indicating that the cells were of cNK cell origin and not misidentified thymic derived ILC1 or trNK cells. Likewise, trNK cell numbers were unaffected by the loss of KLF2 as evidenced by the presence of $CD49b^-TRAIL^+EOMES^{lo}$ cells (Figure 3-4 C) within the livers of *Vav-cre; Klf2$^{fl/fl}$* mice. Moreover, trNK ($CD49a^+CD49b^-$) bearing an immature phenotype ($CD27^+$ $CD11b^-$, R1-like) (Figure 3-4 D, center panel) were present in normal numbers compared to *Klf2$^{fl/fl}$* littermate controls (Figure 3-4 D, bar graph). Therefore, the increased presence of immature $CD27^+CD11b^-$ (R1) NK cells arises from an expansion within the cNK cell lineage. Collectively, these data suggest that KLF2 limits cNK cell proliferation within immature $CD27^+CD11b^-$ (R1) cells, but does not affect the homeostasis of thymic derived ILC1 and trNK cells.

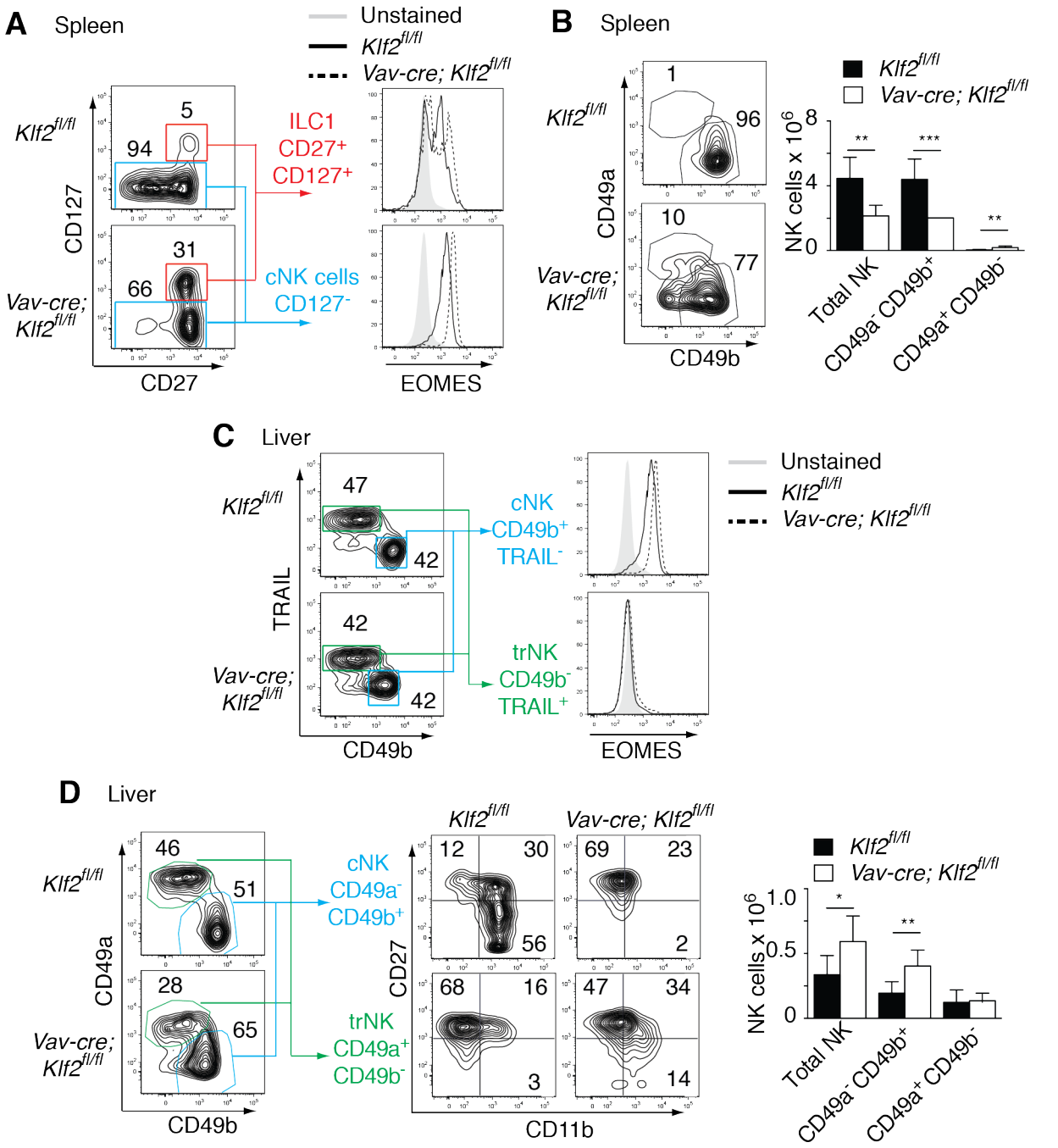


Figure 3-4 ILC1 homeostasis and liver-resident NK cell homeostasis is intact in *Vav-cre; Klf2*$^{fl/fl}$ mice. (A) CD122^{+}Lin^{-}NK1.1^{+} cells were co-stained for CD27, CD127, and EOMES to identify thymic derived ILC1 (CD27^{+}CD127^{+}EOMES^{lo}) and cNK (CD27^{+}CD127^{-}EOMES^{hi}) cells within the spleens of *Vav-cre; Klf2*$^{fl/fl}$ and littermate controls. (B) CD122^{+}Lin^{-}NK1.1^{+} cells were also co-stained with CD49a and CD49b to quantify trNK (CD49a^{+}CD49b^{-}) and cNK (CD49a^{-}CD49b^{+}) in the spleen. Data represent n=6 per cohort from two independent experiments. (C, D) The livers of *Vav-cre; Klf2*$^{fl/fl}$ mice contain normal numbers of trNK (CD49b^{-}TRAIL^{+}, CD49a^{+}CD49b^{-}) but increased numbers of cNK (CD49b^{+}TRAIL^{-}, CD49a^{-}CD49b^{+}) compared to *Klf2*$^{fl/fl}$ controls. Data represent n=6 per cohort from two independent experiments. Data represent the mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

When I expanded our analysis to examine the mature NK cell compartment, *Vav-cre; Klf2*$^{fl/fl}$ mice had significantly fewer CD27^{+}CD11b^{+} (R2, intermediate) and CD27^{-} CD11b^{+} (R3, late-stage) NK cells compared to *Klf2*$^{fl/fl}$ littermate controls in all tissues examined including the spleen, blood, liver, lung, bone marrow, and mesenteric lymph node (Figure 3-3 A). This finding was confirmed using an alternative late-stage gating strategy using CD11b versus CD43, where CD11b^{+}CD43^{-} and CD11b^{+}CD43^{+} gates represent intermediate and late-stage populations, respectively (Figure 3-3 B). Additionally, *Klf2*-deficient NK cells did not express the late-stage marker KLRG1 within the spleen (Figure 3-3 C, right panel). Of note, NK cells depend on IL-15 to maintain homeostasis and survival (71, 114, 115). The transcription factor E4BP4/Nilf3 regulates CD122 expression and confers IL-15 responsiveness necessary for conventional NK cell development (88), and E4BP4/Nilf3 conditional knockout mice are cNK deficient (88, 100). Therefore, the absence of late-stage NK cells in the periphery led me to hypothesize that KLF2 may be necessary for normal IL-15 dependent signaling events and *Klf2*-deficient NK cells would be hypo-responsive to IL-15 stimulation. The IL-15 receptor is a heterodimer comprised of CD122 (IL-15Rβ) and CD132 (γ_c), and administration of high levels of exogenous IL-15 to wild type NK cells induces downstream mTOR activation and phosphorylation of phospho-S6 (129). To determine whether the IL-15 receptor and downstream signaling was intact, I stained for the expression of CD122 and CD132 on splenic NK cells and measured phosph-S6 expression in response to IL-15 stimulation. Contrary to my hypothesis, expression of the heterodimer subunits was normal in *Vav-cre; Klf2*$^{fl/fl}$ mice compared to controls

(Figure 3-5 A), and KLF2–deficient NK cells responded normally to IL-15 stimulation (Figure 3-5 B). Immature NK cells exhibit increased baseline expression of phosphor-S6 due to their elevated metabolism (129); however, when KLF2–sufficient and deficient responses to IL-15 were compared in the immature (R1) population they were comparable (Figure 3-5 B, left panels), further negating the hypothesis that the absence of late-stage NK cells in KLF2-gene targeted mice was due to a defect in IL-15 signaling.

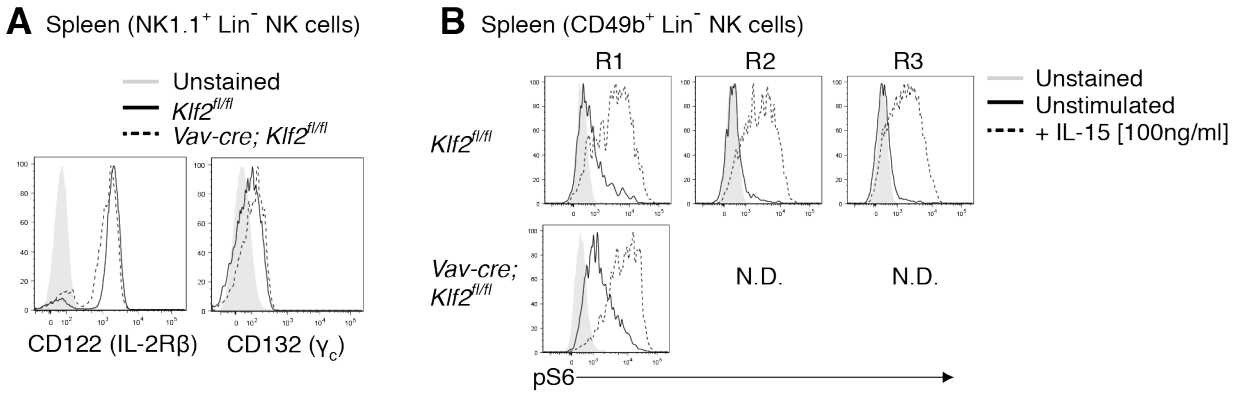


Figure 3-5. IL-15 receptor expression and signaling is intact in *Vav-cre; Klf2*$^{fl/fl}$ mice. (A) Histogram overlays of the IL-15 receptor (IL-2Rβ/γ_c heterodimer complex) in splenic NK cells from *Vav-cre;Klf2*$^{fl/fl}$ and *Klf2*$^{fl/fl}$ controls. (B) IL-15 signaling responses in *Klf2*-sufficient (top) and deficient (bottom) NK cells. NK cells were stimulated ± IL-15 [100ng/ml] for 1hr prior to intracellular staining for phospho-S6, a downstream target of mTOR. These experiments were performed three times.

In the T cell compartment, KLF2 prevents naïve T cells from entering peripheral tissues by controlling the expression of homing receptors (selectins, integrins, adhesion molecule, CR) and *Klf2*-deficient T cells are scattered throughout tertiary tissues (44). Therefore, it is possible that late-stage NK cells may be displaced within unanalyzed tissues in *Vav-cre; Klf2*$^{fl/fl}$ mice. Alternatively, KLF2 controls the expression of late stage markers CD11b (α_M integrin), CD43 (leukosialin, anti-adhesive molecule), and KLRG1, and late-stage NK cells were misidentified as immature. High levels of granzyme B expression are restricted to the most mature effector NK cells. To address the second possibility, I measured the expression of granzyme B and assessed cytolytic activity as a surrogate marker to identify late-stage NK cells. Splenic NK cells from *Vav-cre; Klf2*$^{fl/fl}$ mice expressed lower levels of granzyme B (Figure 3-6 A) and displayed a reduced ability to lyse NK-sensitive YAC-1 target cells *ex vivo* (Figure 3-6 B). To address whether the lack of mature NK cells in the spleens of *Vav-cre; Klf2*$^{fl/fl}$ mice could be attributed to displacement of this population in unanalyzed tissues, I measured the ability of *Vav-cre; Klf2*$^{fl/fl}$ mice to clear RMA-S target cells *in vivo*. RMA-S target cells are unable to stably express MHC class I at 37°C and are susceptible to NK cell lysis, whereas RMA cells are NK cell-resistant (146). When RMA-S target cells are injected intraperitoneally, they are lysed through the recruitment of NK cells to the peritoneal cavity (147). Thus, if mature NK cells are displaced in the periphery of *Vav-cre; Klf2*$^{fl/fl}$, they may be recruited in response to RMA-S cells. Compared to littermate controls, *Vav-cre; Klf2*$^{fl/fl}$ mice could efficiently recruit immature NK cells into the peritoneal cavity

(data not shown), but were unable to efficiently clear RMA-S target cells, indicating that late-stage cytolytic NK cells are absent in conditional knockout mice (Figure 3-6 C).

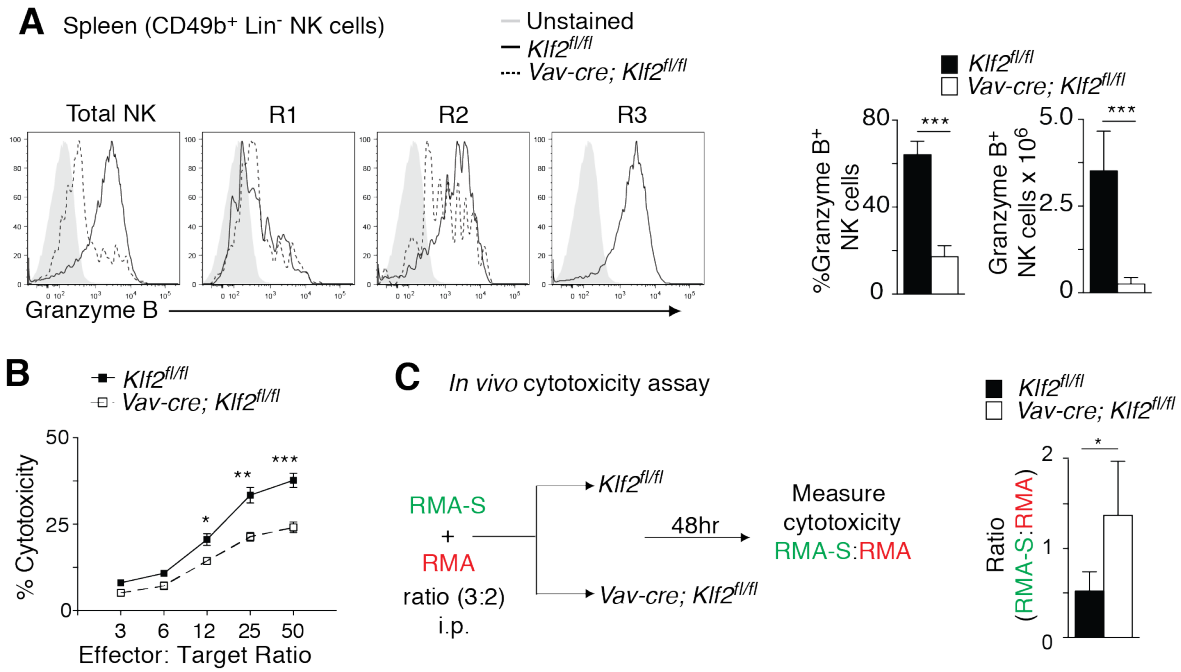


Figure 3-6. Mature cytolytic NK cells are absent in *Vav-cre; Klf2*$^{fl/fl}$ mice. (A) Histogram overlays (left) and quantification of granzyme B expression following (PMA+ionomycin)-stimulation of splenic NK cells harvested from *Klf2*$^{fl/fl}$ versus *Vav-cre; Klf2*$^{fl/fl}$ mice. Histograms display individual subsets, whereas columns are total NK cells. N=9-11 mice per cohort, polled from three independent experiments. (B) Ex vivo cytolytic activity of IL-2 primed splenocytes cultured with Yac-1 target cells for 4hrs in an LDH release assay. This experiment was performed once in quadruplicate. (C) CD107a surface expression on NK cells cultured for 6hrs±plate-bound NK1.1 antibody [pre-coated 25μg/ml]. n=6 mice per cohort, pooled from two independent experiments. (D) Labeled RMA control (Cell tracker orange) and RMA-S target cells (Cell tracker green) were co-injected intraperitoneally (i.p.) at a 3:2 *Klf2*$^{fl/fl}$ versus *Vav-cre; Klf2*$^{fl/fl}$ mice and the ratio of labeled cells was reassessed 48hrs later. This experiment as repeated twice by using three mice per cohort. Data represent the mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

In addition to possessing cytolytic potential, NK cells are also potent producers of cytokines such as IFNγ. NK cells carry out effector functions through the release of secretory vesicles in a process termed degranulation. To determine if the impaired cytolytic response observed in *Vav-cre; Klf2*$^{fl/fl}$ mice were due to defects in degranulation and whether this extended to cytokine secretion, I stained for CD107a (lysosomal associated protein 1, LAMP-1) and IFNγ expression, respectively. In response to stimulation with plate-bound anti-NK1.1 antibody, KLF2-deficient NK cells were capable of both degranulation and IFNγ secretion, as evidenced by increased expression of the degranulation marker CD107a (LAMP-1) (Figure 3-7 A) and IFNγ staining (Figure 3-7 B), respectively. Of note, cytokine and cytolytic activity are thought to be mutually exclusive functions, in part because each utilizes a separate endosomal pathway for secretion (148). Cytokine secretion utilizes recycled endosomes (RE) whereas cytolytic pathways are RE-independent (148). In addition, intermediate CD27^{+}CD11b^{+} (R2) NK cells are more potent producers of IFNγ compared to mature CD27^{+}CD11b^{-} (R3) NK cells (2, 93). The increased frequency of IFNγ producing NK cells observed in *Vav-cre;Klf2*$^{fl/fl}$ mice was likely due to increased proportion of immature NK cells with higher cytokine potential (Figure 3-7 B). Collectively these data indicate that KLF2 is does not intrinsically control degranulation and is dispensable for the production of IFNγ in immature NK cells. Therefore, I conclude that cytolytic effector NK cells are absent in *Vav-cre; Klf2*$^{fl/fl}$ mice and that KLF2 is necessary to support mature NK cell homeostasis but not immature effector function.

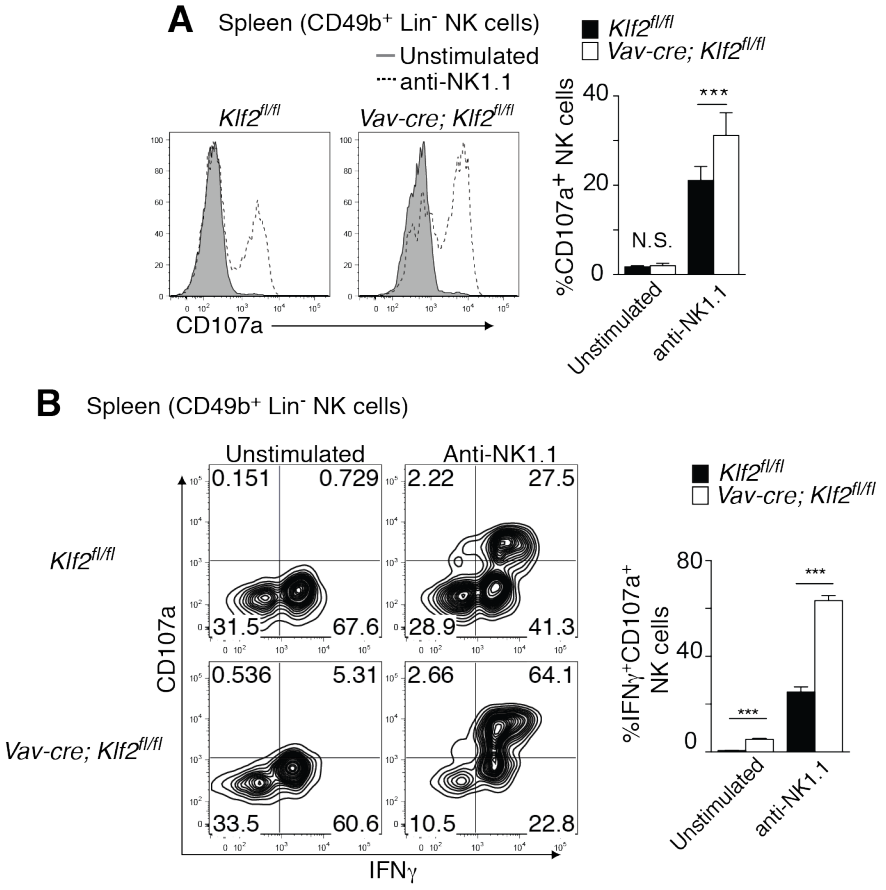


Figure 3-7. KLF2 is dispensable for degranulation and IFNγ secretion. (A) CD107a surface expression on NK cells cultured for 6hr ± plate-bound NK1.1 antibody [25μg/ml]. n=6 mice per cohort pooled from two independent experiments. (B) Representative contour of splenic NK cells stimulated as in A and quantification of $IFN\gamma^+CD107a^+$ NK cells. This experiment was performed once with n=3 mice per cohort. Data represent the mean ± SD. ***P<0.001, N.S (not significant).

KLF2-regulated NK cell homeostasis is cell intrinsic

Mature NK cell survival is dependent upon IL-15 typically presented in complex with the high affinity IL-15Rα expressed on myeloid and mesenchymal cells (71, 119-121, 125, 149). Defects in IL-15Rα expression and myeloid cells lead to deficiencies in mature NK cells (71, 119, 125). However, these defects can be rescued through the adoptive transfer of NK cells into wild type hosts capable of IL-15/IL-15Rα trans-presentation (119, 124). Because *Vav-cre; Klf2*$^{fl/fl}$ mice excise KLF2 in the entire hematopoietic compartment, including myeloid cells, NK cell homeostasis may be altered due to cell extrinsic defects in IL-15 trans-presentation. To determine if KLF2 regulates NK cell homeostasis by controlling myeloid cell trans-presentation, *Klf2* was excised within myeloid cells using a *LysM-cre* mouse model (*LysM-cre; Klf2*$^{fl/fl}$). *LysM-cre; Klf2*$^{fl/fl}$ mice had normal numbers of immature CD27^{+}CD11b^{-} (R1) and intermediate CD27^{+}CD11b^{+} (R2) NK cells in the spleen, but a slight decrease in mature CD27^{-} CD11b^{+} (R3) NK cells (Figure 3-8 A). Of note, mature CD27^{-}CD11b^{+} (R3) NK cells were still present in *LysM-cre; Klf2*$^{fl/fl}$ mice, suggesting that the major NK phenotypes observed in *Vav-cre; Klf2*$^{fl/fl}$ mice are myeloid-independent and cell intrinsic. These findings were further corroborated by the inability of a wild type cells to rescue KLF2-deficient NK cell maturation 48hrs post transfer, indicating that this defect was not dependent upon the host environment (Figure 3-8 B).

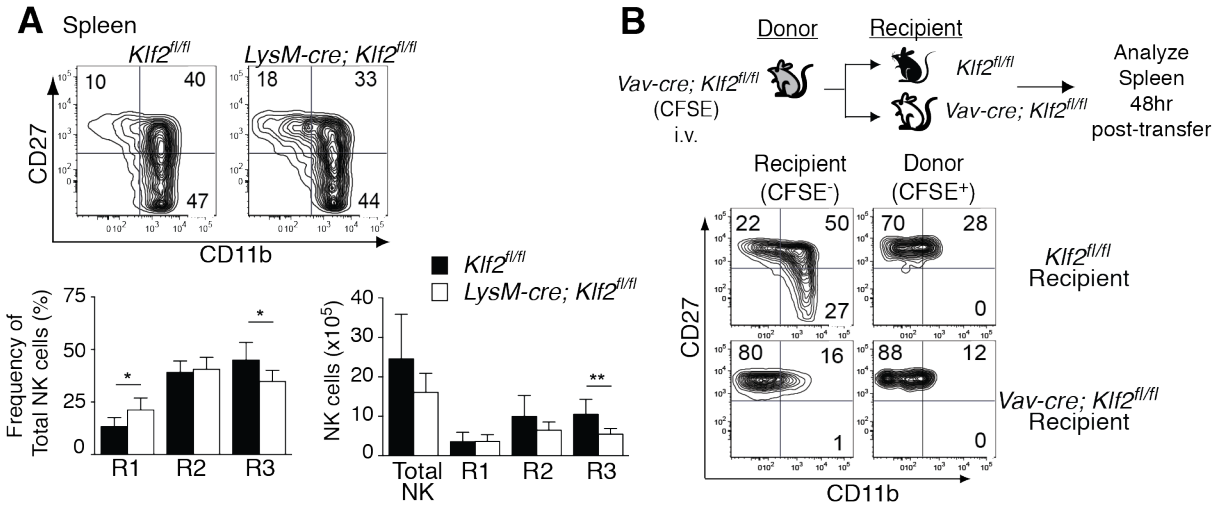


Figure 3-8. Defective NK cell homeostasis is consistent with cell-intrinsic mechanism. (A) Flow cytometric analysis of $CD122^{+}Lin^{-}$ NK cell harvested from the spleens of 8-week old *Klf2*$^{fl/fl}$ versus *LysM-cre; Klf2*$^{fl/fl}$ littermates. n=7 mice per cohort pooled from two independent experiments. (B) CFSE-labeled CD19-depleted *Vav-cre; Klf2*$^{fl/fl}$ splenocytes (2 x10^{7}) were adoptively transferred i.v. (intra-venous) into *Klf2*$^{fl/fl}$ or *Vav-cre; Klf2*$^{fl/fl}$ recipients. $CFSE^{+}CD122^{+}Lin^{-}$ NK cells were analyzed 48hr after transfer to determine whether neighboring cells could rescue KLF2-deficient NK differentiation. This experiment was repeated twice. *P<0.05, **P<0.01.

To confirm that the defect in NK cell homeostasis within *Vav-cre; Klf2*$^{fl/fl}$ mice was cell intrinsic, we generated mixed chimeras by reconstituting lethally irradiated wild type (CD45.2$^+$) hosts with an equal ratio of wild type (CD45.1$^+$) and *Vav-cre; Klf2*$^{fl/fl}$ (CD45.2$^+$) bone marrow. The chimeras were analyzed 8-weeks later to allow complete reconstitution of the NK cell compartment. In the chimeric animals, wild type (CD45.1$^+$) NK cells were primarily mature CD27$^-$CD11b$^+$ (R3) whereas KLF2-deficient (CD45.2$^+$) NK cells maintained an immature phenotype (R1, CD27$^+$CD11b$^-$), further indicating that the wild type accessory cells could not rescue development in KLF2-deficient NK cells (Figure 3-9, contour plots). Surprisingly, there were at least 3-fold more KLF2-deficient (CD45.2$^+$) NK cells than KLF2-sufficient (CD45.1$^+$) NK cells in all tissues analyzed except the lung (Figure 3-9, bar graphs). Collectively, these data indicate that KLF2 intrinsically regulates late-stage NK cell homeostasis and may suppress proliferation in immature NK cells.

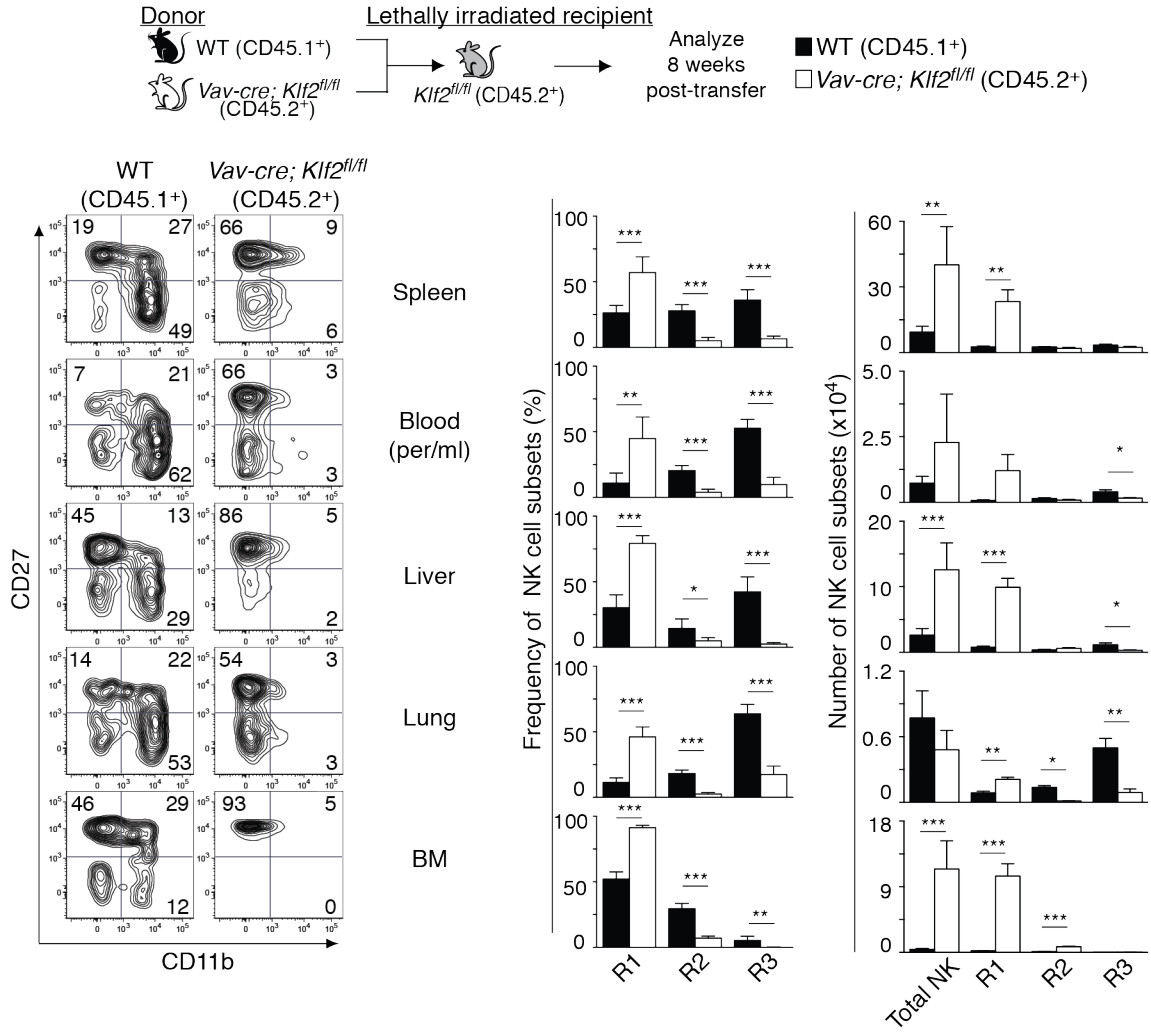


Figure 3-9. KLF2-mediated NK cell homeostasis is cell intrinsic. Analysis of mixed bone marrow chimeras that were generated by reconstituting lethally irradiated *Klf2*$^{fl/fl}$ (CD45.2$^+$) mice with wild type (CD45.1$^+$) and *Vav-cre; Klf2*$^{fl/fl}$ (CD45.2$^+$) bone marrow. Flow cytometric analysis was performed 8 weeks after transfer. Representative contour plots, frequencies, and cell numbers of gated KLF2-sufficent (CD45.1$^+$, black) and KLF2-deficient (CD45.2$^+$, white) NK cell (CD122$^+$Lin$^-$) populations. This experiment was performed once with five recipient animals. Data represents the mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

KLF2 suppresses homeostatic proliferation in NK cells.

KLF2 has been previously reported to suppress proliferation in Jurkat T cells (47, 144) and pre-B cells (40). To further investigate how KLF2 may suppress NK cell proliferation, I characterized steady-state proliferation in *Vav-cre; Klf2*$^{fl/fl}$ mice by measuring 5-bromo-2-deoxyuridine (BrdU) incorporation within a 4.5 day period. After BrdU treatment, there were significantly more immature CD27^{+}CD11b^{-} (R1) BrdU^{+} NK cells present within the bone marrow, spleen, and liver of *Vav-cre; Klf2*$^{fl/fl}$ mice compared to *Klf2*$^{fl/fl}$ control animals (Figure 3-10 A). To confirm that these immature CD27^{+}CD11b^{-} (R1) BrdU^{+} cells were actively dividing and were not simply accumulating within these tissues, I stained for the nuclear marker Ki-67. Indeed, immature KLF2-deficient NK cells exhibited increased NK cell cycling within the bone marrow, spleen, and liver, consistent with the BrdU results (Figure 3-10 B). These data indicate that KLF2-deficient NK cells are hyperproliferative *in vivo*.

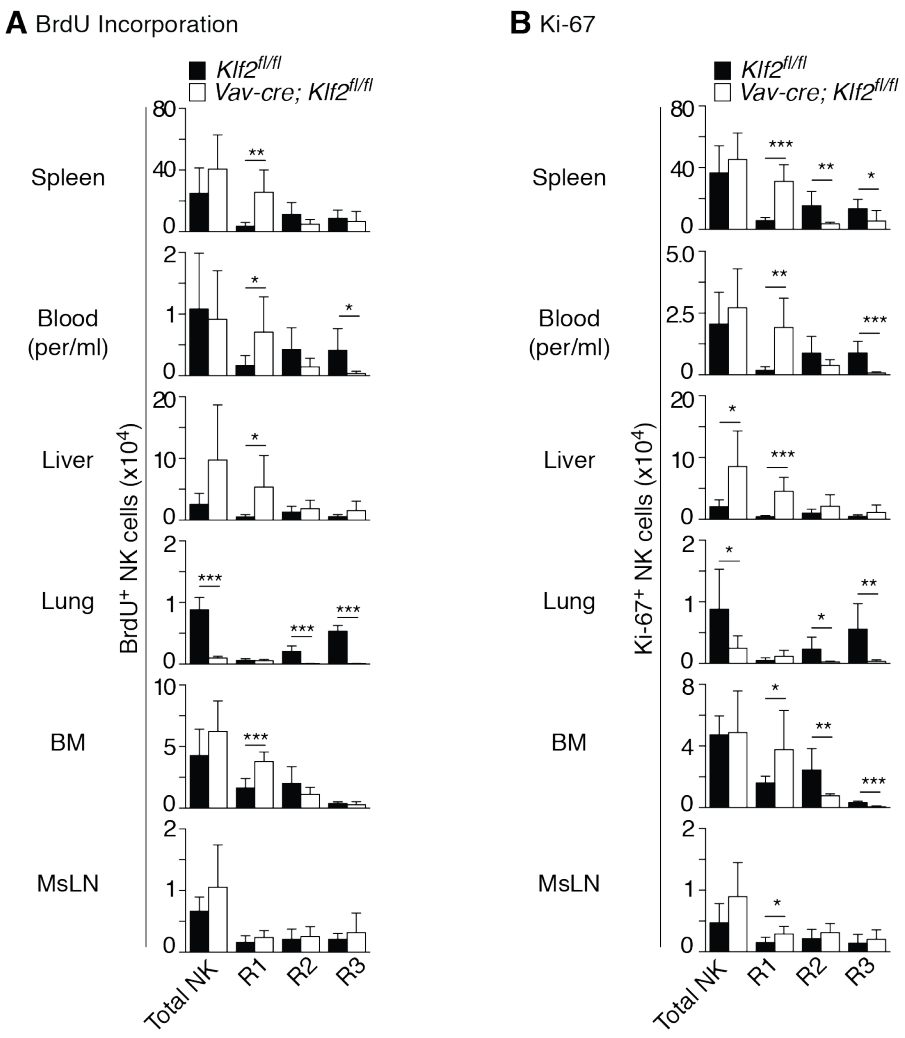


Figure 3-10. KLF2 suppresses proliferation in immature NK cells *in vivo*. (A) BrdU incorporation over 4.5 days, administered intraperitoneally every 12 hrs, was used to assess NK cell ($CD122^+Lin^-$) proliferation in various tissues harvested from *Vav-cre; Klf2*$^{fl/fl}$ and *Klf2*$^{fl/fl}$ animals. The data is pooled from two independent experiments. N=6 mice per cohort. (B) The number of actively dividing cells were quantified by intracellular staining for Ki-67. N=7 mice per cohort from two pooled experiments. The data represent the mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

Immature $CD27^+CD11b^-$ (R1) cells have a higher proliferative capacity than their mature $CD11b^+$ counterparts. In addition, KLF2-deficient T cells are capable of secreting higher levels of cytokines such as IL-2 in response to TCR stimulation (55), (Sebzda lab data not shown). Therefore, the increased numbers of immature NK cells observed in *Vav-cre; Klf2*$^{fl/fl}$ animals may be due to increased bioavailability of IL-2 or differentiation bias due to the absence of mature NK cells. To test whether KLF2 intrinsically suppresses NK cell proliferation in response to IL-2, we examined cell cycling in competition under *ex vivo* conditions. To prevent subset bias, we utilized tamoxifen inducible mice (*T2-cre; Klf2*$^{fl/fl}$) which have similar frequencies of immature $CD27^+CD11b^-$ cells when compared to wild type ($CD45.1^+$) NK cells prior to *Klf2* excision (Figure 3-11 A). MACS sorted NK cells from wild type ($CD45.1^+$) and *T2-cre; Klf2*$^{fl/fl}$ ($CD45.2^+$) animals were co-cultured (1:1 ratio) in media supplemented with IL-2 and 4-hydroxytamoxifen (4-OHT). After 5 days in culture and *Klf2* excision (Figure 3-11 B), increased numbers of KLF2-deficient ($CD45.2^+$) cells were recovered relative to KLF2-sufficient ($CD45.1^+$) controls (Figure 3-10 C, upper panel), primarily due to an expansion of immature $CD27^+CD11b^-$ (R1) NK cells. In co-culture where IL-2 was equally accessible, these immature KLF2-deficient cells also exhibited increased blast morphology compared to KLF2-sufficient controls (Figure 3-11 C, lower panel). Collectively, KLF2 intrinsically suppresses immature NK cell proliferation.

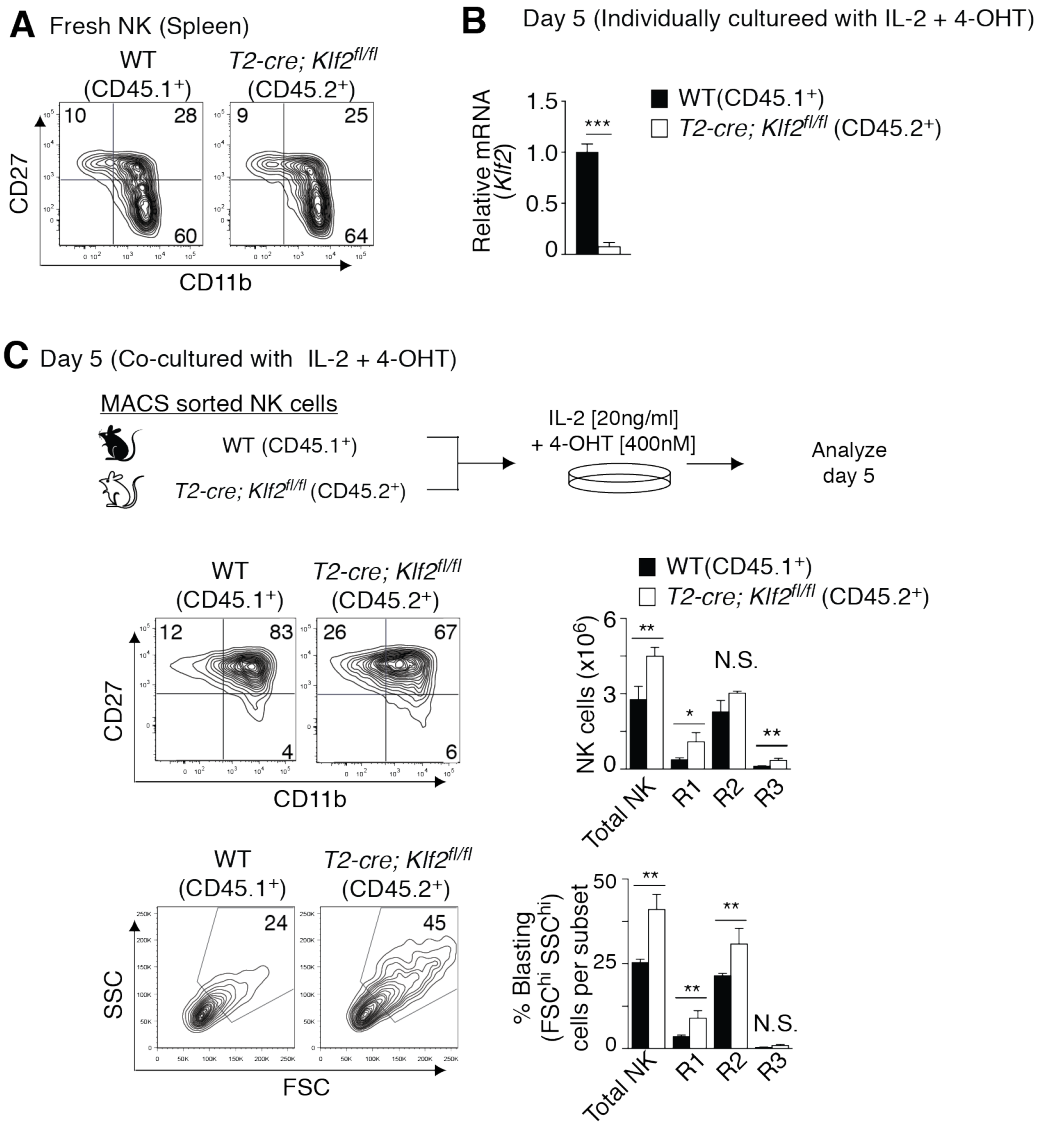


Figure 3-11. KLF2 intrinsically suppresses proliferation in immature NK cells. (A) Representative contour plots of splenic NK cells ($CD122^{+}Lin^{-}$) from wild type ($CD45.1^{+}$) and *T2-cre; Klf2*$^{fl/fl}$ ($CD45.2^{+}$) mice by CD27 vs CD11b staining before *Klf2* excision. (B) *Klf2* mRNA levels after 5 days of culture with IL-2 [20ng/ml] and 4-hydroxytamoxifen (4-OHT) [400nM] measured by RT-PCR. (C) Equal numbers of MACS-sorted splenic NK cells from wild type ($CD45.1^{+}$) and *T2-cre; Klf2*$^{fl/fl}$ ($CD45.2^{+}$) mice were co-cultured with IL-2 + 4-OHT as in (B) for 5 days and were analyzed by flow cytometry for subset numbers (CD27 vs CD11b) and blast morphology (FSC-forward scatter, SSC-side scatter) after *Klf2* excision. The data above reflects a single experiment with three biological replicates per group. This experiment was repeated twice. Bar graphs represent the mean ± SD. *P<0.05, **P<0.01, ***P<0.001, N.S. (not significant).

KLF2 controls access to trans-presented IL-15

Typically, lymphocytes must stop cycling before undergoing differentiation. Given that KLF2 suppresses immature NK cell proliferation, we hypothesized that this transcription factors function may be extended to control mature NK cell differentiation. To test this hypothesis, I performed an NK cell differentiation assay to determine whether mature NK cells could be generated from immature NK cells in the absence of KLF2. Although there are no standard NK cell differentiation protocols, treatment of NK cells with a combination of IL-15, IL-12, and IL-18 has been previously demonstrated to promote NK cell maturation from $CD27^+CD11b^+$ (R2)→ $CD27^-CD11b^+$ (R3) *ex vivo* (150). Using only immature $CD27^+CD11b^-$ (R1) NK cells cultured with IL-12, IL-15, and IL-18 we were unable to promote R1→R2→ R3 differentiation in wild type cells (data not shown), but were able to promote differentiation when whole bone marrow was cultured or stromal support cells were provided. To normalize the frequency of immature $CD27^+CD11b^-$ (R1) NK cells between both starting populations, I CD11b-depleted the bone marrow from *Vav-cre; Klf2*$^{fl/fl}$ and *Klf2*$^{fl/fl}$ mice (Figure 3-12, top row) and cultured each separately with stromal support cells (wild type $CD45.1^+$ bone marrow), supplemented with IL-15, IL-12, and IL-18. After three (Figure 3-12, middle row) and four (Figure 3-11, bottom row) days in culture, KLF2-deficient NK cells differentiated into $CD27^+CD11b^+$ (R2) and $CD27^-CD11b^+$ (R3) cells that expressed the late-stage maturation marker KLRG1, CD43 (data not shown), and granzyme B (Figure 3-12). Both KLF2-sufficient and KLF2-deficient NK cells expressed similar levels of IFNγ (data not shown). From these data I conclude that KLF2 does not intrinsically control KLRG1,

CD43, or granzyme B expression and is dispensable for late-stage NK cell differentiation.

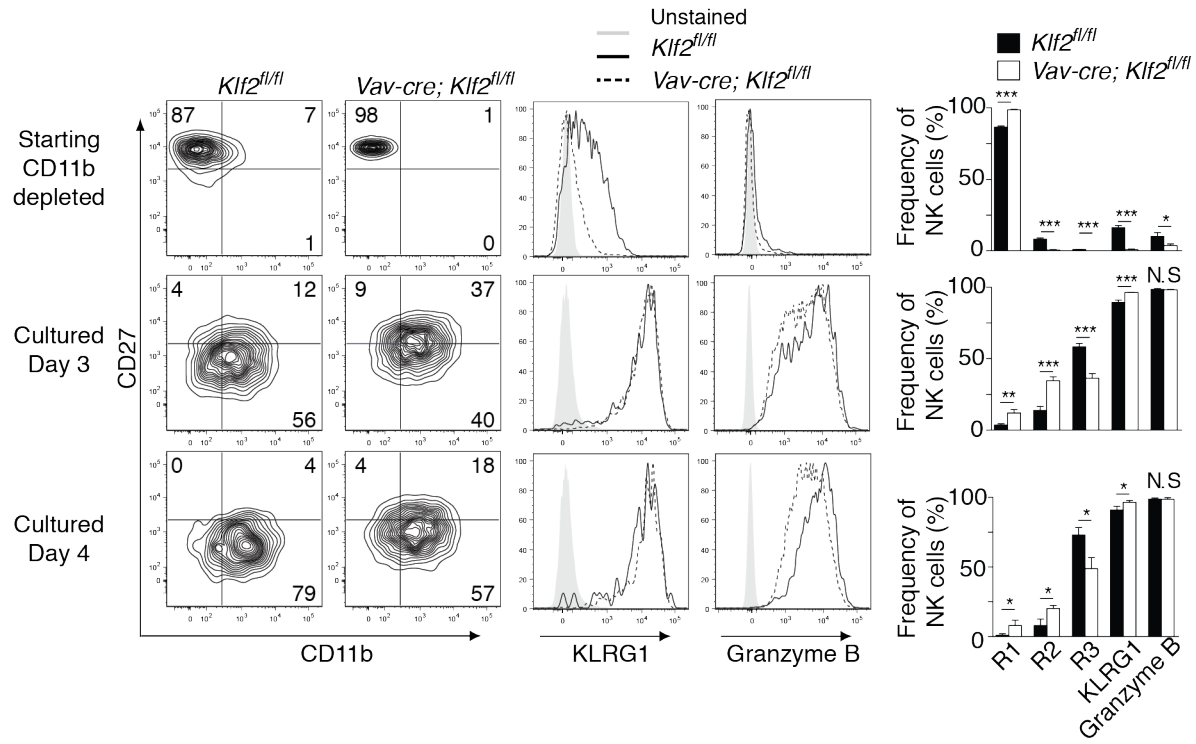


Figure 3-12. KLF2-deficient NK cell differentiation is rescued in culture. CD11b-depleted bone marrow from *Klf2*$^{fl/fl}$ and *Vav-cre; Klf2*$^{fl/fl}$ (CD45.2^{+}) mice was cultured with wild type (CD45.1^{+}) bone marrow supplemented with IL-15, IL-12, and IL-18. Gated NK cells (CD45.2^{+} CD122^{+} Lin^{-}) were analyzed before culture and after 3-4 days for NK cell differentiation (CD11b vs CD27 contour plots) and late-stage maturation markers (KLRG1, granzyme B) by flow cytometry. Differentiation experiments were performed twice using three biological replicates per-cohort, generating similar results. The data represents the mean ± SD. *P<0.05, **P<0.01, ***P<0.001, N.S. (not significant).

The finding that KLF2-deficient NK cells can fully differentiate into cytolytic effectors *ex vivo* conflicts with previous results demonstrating that there is a cell-intrinsic defect in maturation *in vivo*. Given that IL-15 is necessary for late-stage NK cell differentiation and survival (11, 20-24), and *ex vivo* conditions promote equal access to this cytokine between KLF2-deficient and KLF2-sufficient NK cells, two scenarios can account for the discrepancy observed *in vivo*: (1) IL-15 bioavailability is limiting or (2) NK cells are unable to access trans-presented IL-15. To test both these hypotheses, I documented the location of these NK cells relative to IL-15 in splenic sections from *Vav-cre; Klf2*$^{fl/fl}$ and *Klf2*$^{fl/fl}$ mice by immunohistochemistry. The anti-MOMA-1 antibody visualizes metallophilic macrophages localized at the marginal sinus surrounding the white pulp and delineates it from the red pulp. Under steady-state conditions NK cells are predominantly found within the red pulp of the spleen (151), where IL-15 is presented by myeloid cells and vascular cell adhesion molecule-1 (VCAM-1)$^{+}$ stromal cells (120, 151). As expected, in *Klf2*$^{fl/fl}$ control spleens, NK cells were found outside of the MOMA-1 ring in the red pulp (Figure 3-13 A) and in close proximity to IL-15 rich areas (Figure 3-13 B). In contrast, there were significantly more KLF2-deficient NK cells present in the T-cell-rich area of the white pulp and away from the IL-15 rich niche (Figure 3-13 A B, bar graphs), reflecting an intra-splenic migration defect *in vivo*. Moreover, the IL-15 niche was intact in *Vav-cre; Klf2*$^{fl/fl}$ indicating that the bioavailability was not limiting in these mice. IL-15 specific staining was confirmed by the absence of staining in an IL-15 knockout spleen (Figure 3-13 C).

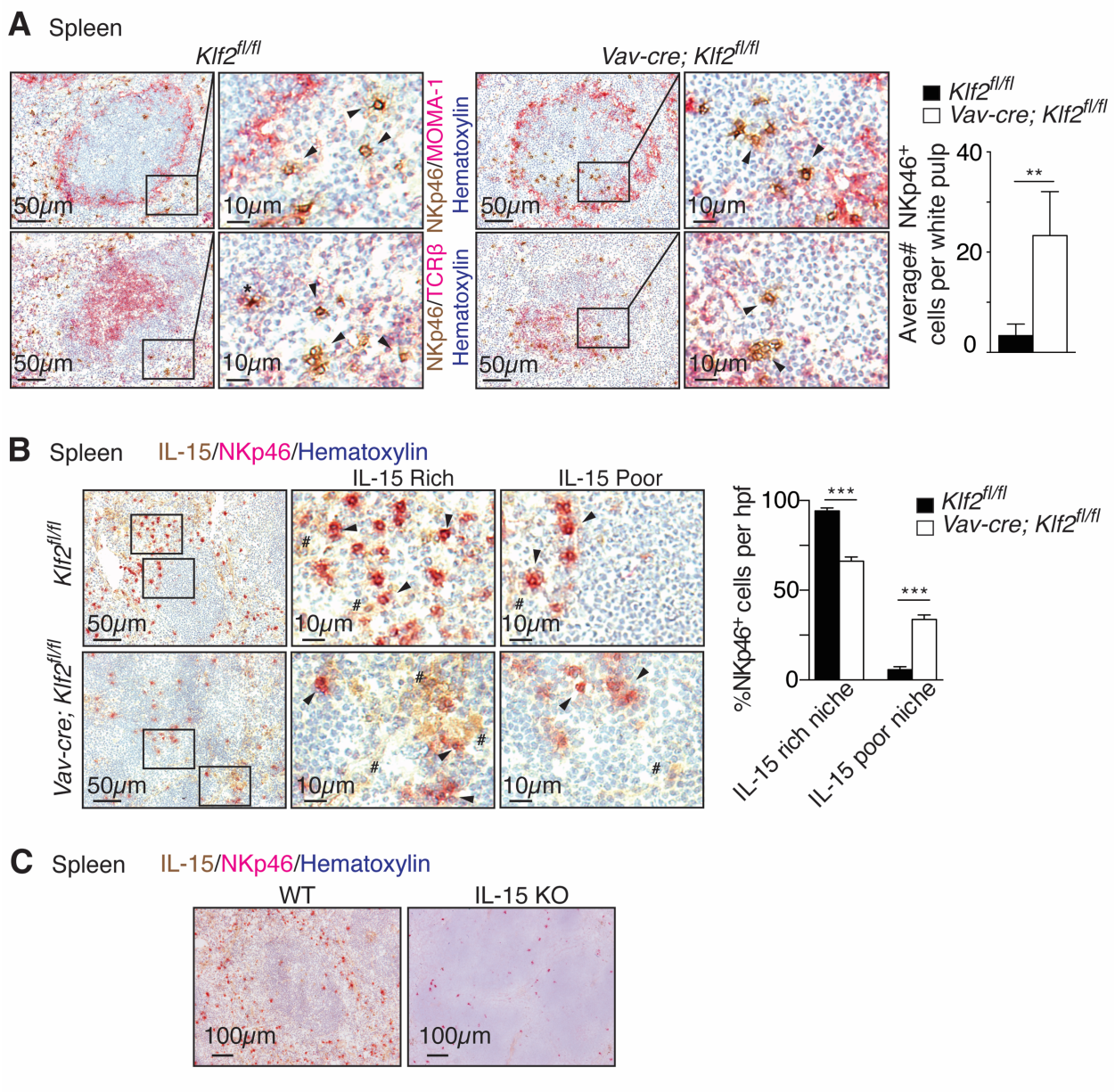


Figure 3-13. At steady-state the NK cells are displaced from the IL-15 rich niche in *Vav-cre; Klf2*$^{fl/fl}$ mice. (A) Immunohistochemistry of *Klf2*$^{fl/fl}$ and *Vav-cre; Klf2*$^{fl/fl}$ splenic serial sections co-stained for NKp46/MOMA-1 (top row) and NKp46/TCRβ (bottom row). Enlarged images that identify NK cells (arrows) and NKT cells (*). Associated bar graph shows the average number of NKp46^{+} cells within 10 similarly sized MOMA-1-encased sections per mouse. N=4 mice per cohort. (B) *Klf2*$^{fl/fl}$ (top row) and *Vav-cre; Klf2*$^{fl/fl}$ (bottom row) splenic sections co-stained for NKp46 and IL-15. Enlarged images identify IL-15-rich (middle) and IL-15-poor (right) regions in representative sections (left). NKp46^{+} (arrow) and IL-15^{+} (#) tissue are identified. Associated bar graph shows the average frequency of NK cells identified in IL-15-rich and IL-15-poor regions in five-high powered fields per mouse. N=3-4 mice per cohort. (C) The polyclonal anti-IL-15 antibody used for NKp46/IL-15 co-staining specifically binds IL-15 in wild type (WT) but not IL-15 knockout (KO) splenic sections. The data represent the mean ± SD. **P<0.01, ***P<0.001.

Developing NK cell subsets have been documented to preferentially express homing receptors based on their maturation status (54). To verify that the differences in NK cell localization in *Vav-cre; Klf2*$^{fl/fl}$ and *Klf2*$^{fl/fl}$ mice were due to an intrinsic migration defect and not maturation bias, we examined mature KLF2-deficient NK cell trafficking *in vivo*. NK cells develop normally in *T2-cre; Klf2*$^{fl/fl}$ animals (Figure 3-11 A). KLF2-deficient NK cells can be harvested from *T2-cre; Klf2*$^{fl/fl}$ mice after treatment with tamoxifen infused chow for 5 days without substantial changes to subset frequencies (Figure 3-14 A). When KLF2^{+} and KLF2^{-} NK cells were co-transferred into wild type animals, KLF2^{+} NK cells homed to the red pulp (Figure 3-14 B) and associated with F4/80^{+} myeloid cells capable of trans-presenting IL-15. In contrast, there were significantly fewer KLF2^{-} NK cells associating with F4/80^{+} cells (Figure 3-14 B) and significantly more of these cells within the white pulp (Figure 3-14 C), indicating that KLF2 promotes NK cell migration to the IL-15 rich niche for survival.

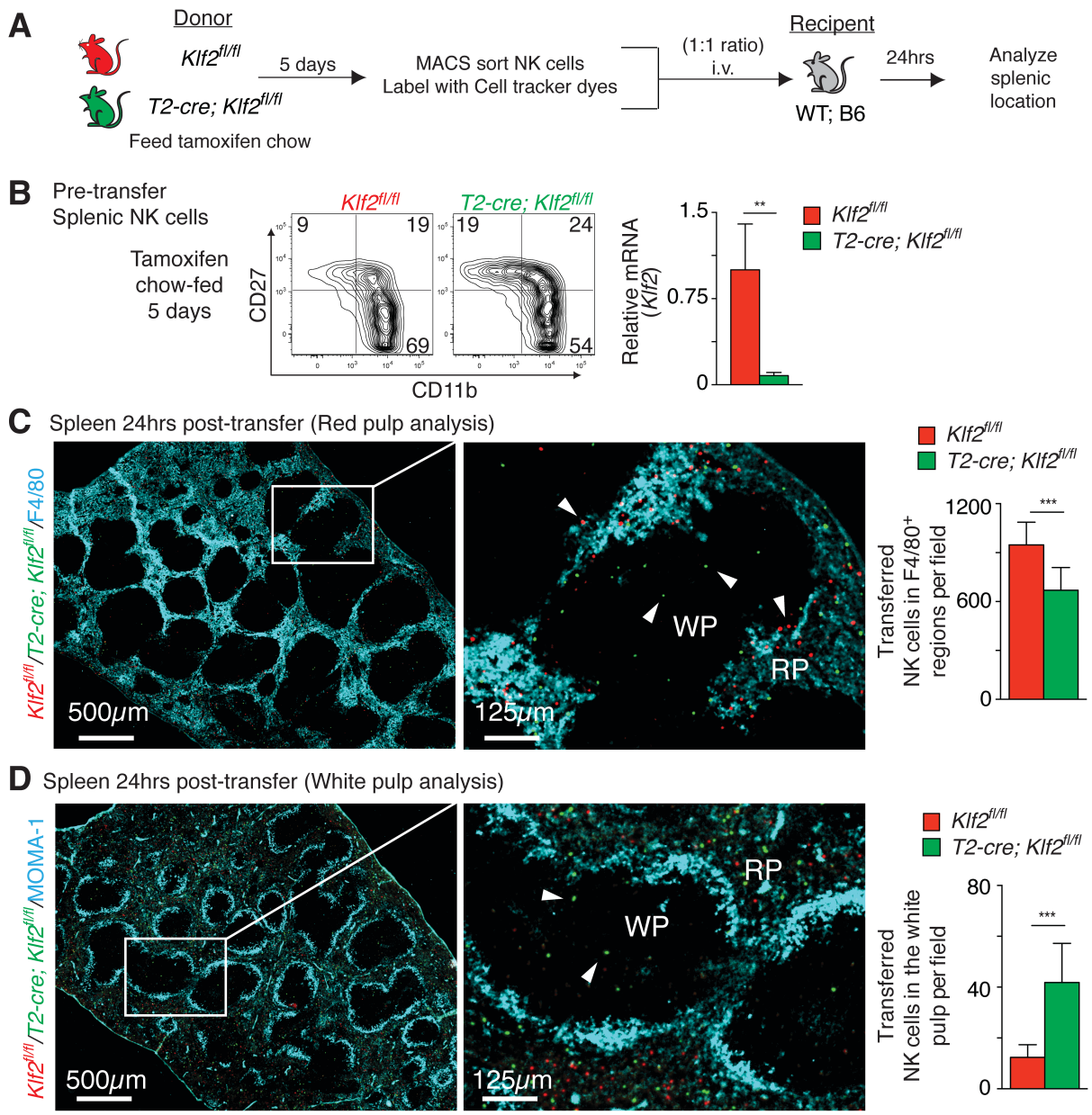


Figure 3-14. KLF2 promotes NK cell migration toward IL-15 associated regions. (A) Dynamic migration of mature NK cell populations within the spleen. Splenic NK cells were isolated from (MACS-sorted) from *Klf2*$^{fl/fl}$ and *T2-cre; Klf2*$^{fl/fl}$ fed tamoxifen-chow for 5 days and labeled with Cell tracker red or green dyes, respectively. These cells were then co-transferred (1:1 ratio) into a wild type recipient and splenic localization was assessed 24hrs later. (B) CD11b vs CD27 contour plots of gated splenic NK cells (CD122^{+}Lin^{-}) from chow fed mice in (A), and *Klf2* mRNA levels in sorted NK cells after tamoxifen treatment. (C) 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. (D) Immunohistochemistry of co-transferred NK cells in relation to the white pulp, as outlined with MOMA-1 antibody. The average number of transferred cells per field was calculated from 25 individual low-power field images. This experiment was repeated twice.

KLF2 is necessary for the maintenance of late-stage NK cells

To confirm that KLF2-directed migration is necessary for the maintenance of late-stage NK cells, we examined the frequency of mature NK cells in *T2-cre; Klf2*$^{fl/fl}$ and *Klf2*$^{fl/fl}$ mice fed tamoxifen-infused chow. Prior to day 5, there was no significant excision of *Klf2* or loss of late-stage CD27^{-}CD11b^{+} (R3) NK cells (Figure 3-15 A). After 7 days of tamoxifen-induced *Klf2* excision, there was a significant loss of late-stage CD27^{-}CD11b^{+} (R3) NK cells *in vivo*. This was accompanied by an increase in the frequency of mature apoptotic cells as reflected by Annexin V and FAM staining, which measure membrane flipping via cell surface phosphatidylserine and cleaved-caspase expression respectively, in tamoxifen-infused chow fed *T2-cre; Klf2*$^{fl/fl}$ and *Klf2*$^{fl/fl}$ mice (Figure 3-15 C). Collectively, these data indicate that under homeostatic conditions, KLF2 promotes mature NK cell migration toward the IL-15 rich niche to support late-stage NK cell survival.

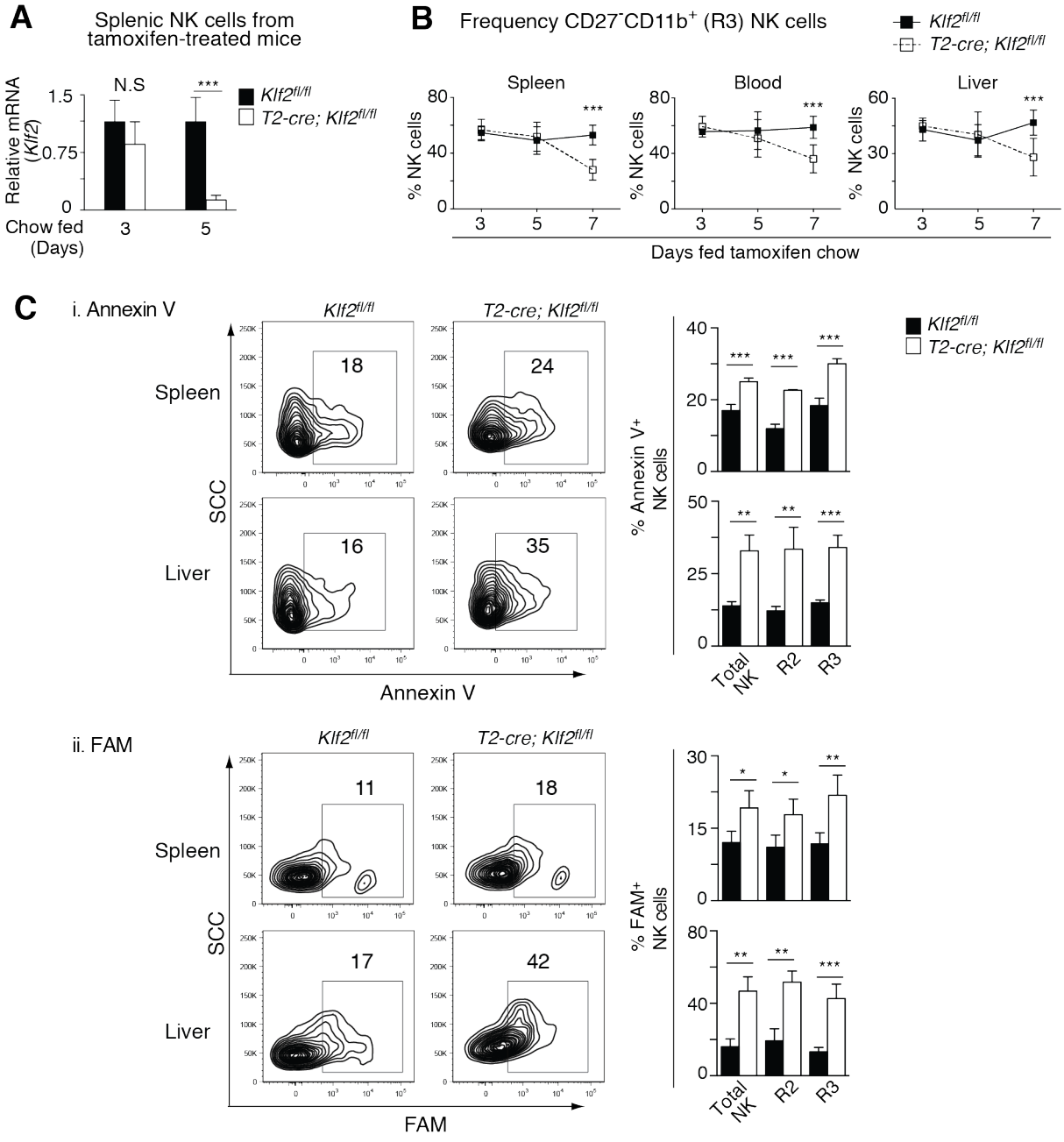


Figure 3-15. KLF2 is necessary for mature NK cell survival. (A) Klf2 mRNA levels in MACS isolated NK cells from *T2-cre; Klf2*$^{fl/fl}$ and *Klf2*$^{fl/fl}$ mice fed tamoxifen-infused chow for 3 and 5 days measured by RT-PCR. (B) Freuency of mature (CD122^{+}Lin^{-}CD27^{-}CD11b^{+}) NK cells in the spleen, blood, and liver of *T2-cre; Klf2*$^{fl/fl}$ and *Klf2*$^{fl/fl}$ mice fed tamoxifen-infused chow for the indicated time points as determined by flow cytometry. N=3-11 mice per time point. (C) NK cell apoptosis was measured by flow cytometry by staining for Annexin V (i) and using the pan-cleaved-caspase marker FAM (ii) in total and mature NK cell subsets. Contour plots represent Annexin V and FAM staining of total NK cells (CD49b^{+}Lin^{-}). Mature NK cells were defined as CD27^{+}CD11b^{+} (R2) or CD27^{-}CD11b^{+} (R3). data reflects one representative experiment with n=3-4 mice per cohort. This experiment was repeated three times. The data represents the mean ± SD. *P<0.05, **P<0.01, ***P<0.001, N.S. (not significant).

Discussion

The adoptive transfer of allogeneic NK cells is a promising strategy for cancer therapy. Successful use of NK cells against cancer depends upon the sustained GvL effect, increasing the *in vivo* expansion of transferred cells, and long term maintenance of mature allogeneic cells *in vivo*. Following hematopoietic stem cell transfer (HSCT) of allogenic bone marrow for acute myeloid leukemia (AML) treatment, patients that exhibit faster reconstitution of the mature $CD56^{dim}$ cytolytic population are associated with better clinical outcome. Therefore, improving mature $CD56^{dim}$ differentiation in patients is key to improving therapeutic approaches. Our data finds that KLF2 suppresses immature NK cell proliferation while promoting maturation and survival in mature effector cells (Figure 3-16). Under non-inflammatory conditions, excision of *Klf2* in mature $CD11b^{+}$ NK cell populations leads to altered migration patterns and subsequent apoptosis. Although a certain degree of GvL activity depends upon selection of appropriate HLA mismatched donor cells, NK cell expansion and persistence may be improved by manipulating or bypassing KLF2-directed NK cell homeostasis. To by-pass KLF2-directed homeostasis it may be necessary to supplement allogenic NK cell therapy with recombinant IL-15 or induce co-recruitment of accessory cells to cancer sites to promote *in vivo* NK cell expansion and persistence. Currently, allogeneic NK cell therapy is being optimized by co-infusion with recombinant IL-15 to improve GvL responses with some success (137).

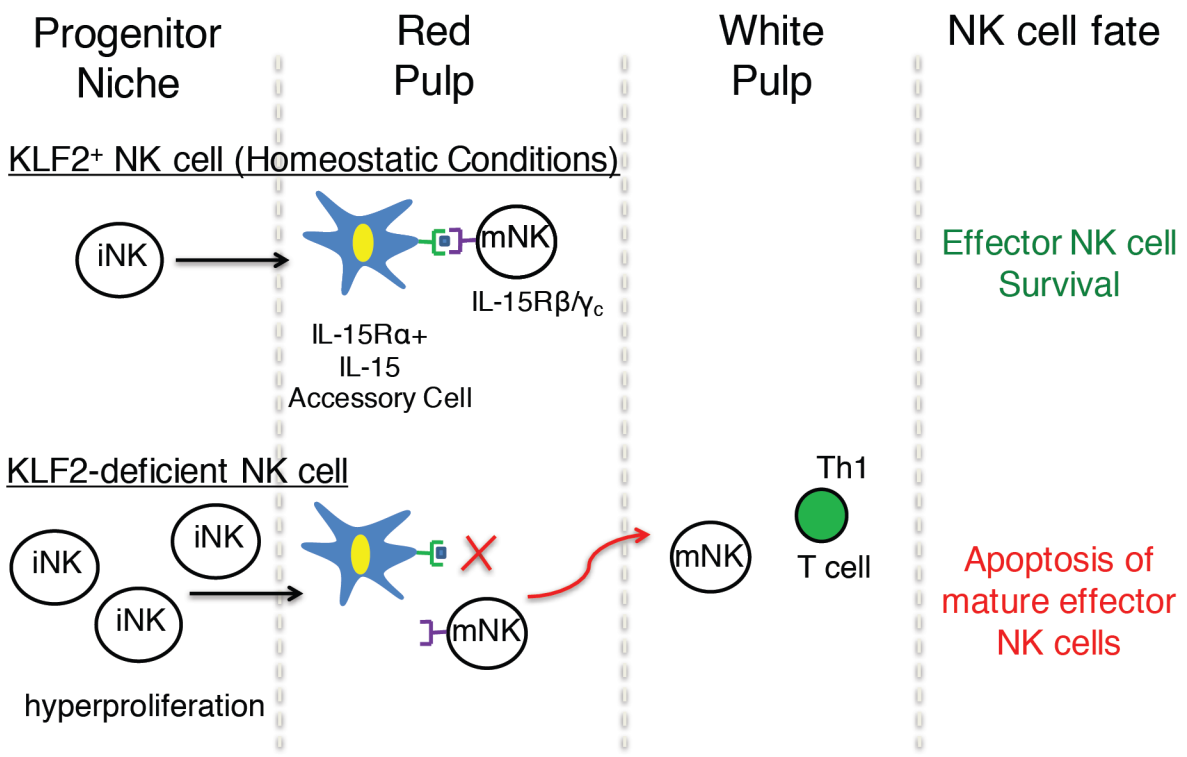


Figure 3-16. Model of KLF2-regulated NK cell homeostasis. Under homeostatic conditions KLF2 suppresses immature NK cell proliferation (iNK, $CD27^{+}CD11b^{-}$) promotes mature NK cell (mNK, $CD11b^{+}$) migration toward to IL-15 trans-presenting niches to maintain cell survival. Genetic ablation of Klf2 within the NK cell compartment results in immature NK cell hyperproliferation, increased migration toward the T cell rich white pulp, and decreased interactions with the IL-15 survival niche.

An additional hurdle to NK cell based cancer therapies includes the recruitment of NK cells to un-vascularized cancer sites necessary for solid tumor treatment. Currently, little is known about how migration is regulated in NK cells except that like in all lymphocytes it is associated with a PI3K/PTEN-signaling axis (152) and homing receptor expression is associated with maturation status (2, 93). While our data correlates KLF2 expression in NK cells with migration toward IL-15 rich niches, the mechanisms by which KLF2 controls migration and homing receptor expression have yet to be described. Of note, PI3K plays a key role in phosphorylating KLF2 for targeted proteolytic degradation in activated T cells (9, 30, 46). Given the close association of PI3K signaling with IL-15 receptor signaling and activating receptor signaling, we have yet to determine how IL-15 and PI3K-associated receptor activation may affect KLF2 levels in NK cells and possibly homing receptor expression. In my next chapter I will describe how KLF2 levels are altered in response to activating stimuli and varying degrees of IL-15 signaling, and how this is essential to limiting NK cell responses during inflammation and possibly maintaining self-tolerance.

CHAPTER 4

KLF2 CONTROLS HOMEOSTATIC BUT NOT INFLAMMATORY MIGRATION IN MATURE NK CELLS

Introduction

Natural killer cells are innate lymphocytes capable of excreting high amounts of cytokine and killing virally infected or transformed cells without prior recognition. Although NK cells alone appear to be unable to initiate autoimmunity, high numbers of NK cells have been implicated in exacerbating pathology in disease models associated with chronic inflammation such as type I diabetes (66) and psoriasis (67). In the context of viral infection, NK cells are necessary for controlling viral titers in the early stages of disease. NK cells are thought to control viral titers by directly lysing virally infected cells and secreting IFNγ to promote effector T cell responses. Conversely, during chronic viral infection, like in murine cytomegalovirus infection, NK cells may dampen T cell effector responses by targeting APCs presenting viral antigen to effector cells (63) and by directly lysing activated $CD4^+$ and $CD8^+$ T cells expressing NKG2D and TRAIL ligands (153-156). Therefore, maximizing NK cell potential during the acute phase of an immune response and minimizing NK effector function during chronic disease states may optimize immune function.

In the previous chapter we demonstrated that KLF2 promotes NK cell homeostasis and survival by (a) suppressing proliferation in immature NK cells and (b)

guiding mature NK cells toward IL-15 rich sustaining niches. Within naive T cells, KLF2 regulates migration patterns by promoting the expression of homeostatic homing receptors while simultaneously suppressing inflammatory homing receptors (43, 44). When, KLF2 is degraded in naïve T cells following PI3K-dependent activation, HR repertoires are altered to grant effector T cells access to peripheral tissues. Based on these findings I predicted that KLF2 would also be degraded within the NK cell compartment in a similar manner and HR expression would be altered. Indeed, KLF2 is similarly degraded upon activation via PI3K-associated activating receptors such as NKG2D and NK1.1 and upon exposure to high levels of IL-15, but is surprisingly spontaneously degraded when removed from the homeostatic niche. Using tamoxifen inducible *Klf2*-gene targeted mice, loss of KLF2 results in a downregulation of homing receptors which respond to constitutively expressed ligands but not homing receptors corresponding to ligands expressed under inflammatory conditions. In contrast to T cells, loss of *Klf2* within NK cells does not enhance recruitment to peripheral tissues but instead induces migration patterns that promote apoptosis under non-inflammatory conditions. These data reveal a fundamental difference in how KLF2 regulates migration between T cells and NK cells, and tailors NK cell migration patterns to optimize innate responses. In this chapter we report that KLF2 functions as a molecular sensor within NK cells to suppress homeostatic migration following NK cell activation. These regulated migratory patterns may represent a novel form of NK cell regulation that promotes optimal adaptive responses and self-tolerance.

Results

KLF2 is degraded upon NK cell activation

Within naïve T cells, KLF2 regulates the expression of homeostatic receptors that promotes recirculation within secondary lymphoid organs and prevents migration to the periphery (44). Upon TCR activation, T cells rapidly degrade KLF2 and alter their homing receptor repertoire to respond to inflammatory gradients. Therefore we sought to determine whether KLF2 is similarly regulated following activation to alter migration in NK cells (9, 37, 44). First, we assessed whether KLF2 was degraded in response to activating stimuli in NK cells. C57BL/6 NK cells were stimulated by cross-linking activating receptors NK1.1 and NKG2D with plate-bound antibody. The ligand to NK1.1 is unknown, however NKG2D recognizes ligands expressed on stressed cells such as virally infected cells, cancer cells, and activated T cells. As previously observed in T cells, NK cells downregulate *Klf2* mRNA within four hours (Figure 4-1 A) and KLF2 protein levels within three hours of receptor cross-linking (Figure 4-1B) relative to unstimulated controls. A similar loss of *Klf2* mRNA (Figure 3-1A) and KLF2 protein was also observed in response to phorbol 12-myristate 13-acetate and ionomycin (PMA/I) induced NK cell activation (Figure 4-2 C). The Sebzda lab has previously demonstrated that KLF2 protein levels in T cells are primarily regulated through post-translational modification, and upon T cell activation, KLF2 is rapidly phosphorylated and recognized by E3-ubiquitin ligases for proteasomal degradation (30). Following PMA/I stimulation, NK cells also rapidly phosphorylate KLF2 within 10 minutes and degrade KLF2 within

60 minutes (Figure 4-2 C). Moreover, when PMI/I activated NK cells are treated with the proteasome inhibitor MG132, the phosphorylated KLF2 moiety (pKLF2) accumulates, indicating that degradation is an active process requiring the proteasome (Figure 4-2 D). These data indicate that following NK cell activation via activating receptors, KLF2 is phosphorylated and degraded by the proteasome.

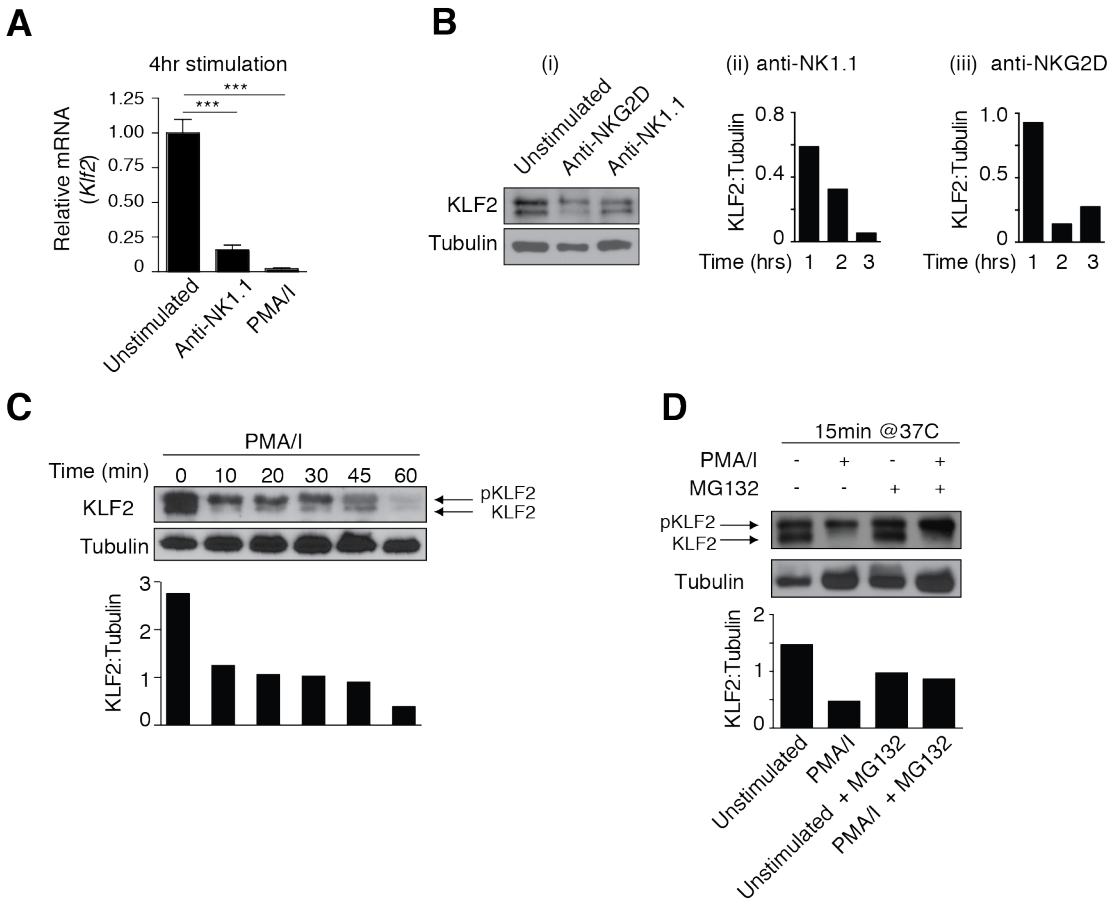


Figure 4-1. *Klf2* mRNA and KLF2 protein are downregulated upon NK cell activation. (A) *Klf2* mRNA and KLF2 protein levels are reduced following NK cell activation. (i) *Klf2* mRNA levels were measured in MACS sorted NK cells from C57BL/6 mice were left unstimulated, stimulated with plate bound anti-NK1.1 [25ug/ml], or phorbol 12-myristate 13-acetate and ionomycin (PMA/I, 50 and 500ng/ml respectively) for 4hrs. This experiment was repeated three times. (ii) Representative KLF2 immunoblots and densitometry of KLF2 relative to tubulin in NK cells stimulated with PMA/I as in (i) at indicated time points. This experiment was repeated twice. (B) KLF2 is phosphorylated prior to degradation by the 26s proteasome in NK cells. NK cells isolated and stimulated ± PMA/I for 15min as in (A) in the presence or absence of the 26s proteasome inhibitor MG132 [10μM]. Arrows indicate the unmodified (KLF2) and phosphorylated (pKLF2) moieties of KLF2 observed in the immunoblot. (C) Engagement of activating receptors promotes KLF2 degradation. Representative KLF2 immunoblot at 3hrs (i) and densitometry plots of KLF2 relative to tubulin following stimulation of MACS-sorted NK cells with (ii) anti-NK1.1 [15μg/ml] or (iii) anti-NKG2D [10μg/ml] plate bound antibody. This experiment was repeated twice with similar results.

KLF2 is stabilized within the homeostatic niche by low but not high levels of IL-15

When determining the kinetics of KLF2 degradation following NK cell activation, we observed that KLF2 protein was spontaneously degraded in unstimulated NK cells within four hours (Figure 4-2 A). This phenomenon was surprising considering that the NK cells were isolated through negative selection, leaving their activating receptors untouched, and NK cells cultured *ex-vivo* for four hours are not spontaneously activated as evidenced by IFNγ and CD107a staining in unstimulated wild type controls (Chapter 3, Figure 3-7 B, $Klf2^{fl/fl}$ unstimulated control). Purifying NK cells by default removes them from their homeostatic niche and NK cells cultured *ex-vivo* in the absence of IL-15 undergo apoptosis within four hours (157). In addition, inhibitory receptors that recognize self-ligands recruit phosphatases in order to prevent NK cell activation, and NK cell isolation may inadvertently remove these inhibitory signals provided by the homeostatic niche. Under steady state, NK cells are sequestered within the red pulp of the spleen in close proximity to IL-15 trans-presenting myeloid cells (151). From these observations, I hypothesized that disrupting the homeostatic microenvironment may lead to spontaneous KLF2 degradation in *ex-vivo* cultured NK cells due to the absence of phosphatase promoting signaling events. To accomplish this, I disrupted the NK microenvironment by generating single cell-suspensions of spleens harvested from C57BL/6 mice, and culturing them for four hours in media alone or with PMA/I as a positive control for KLF2 degradation. Following stimulation, I isolated NK cells from these suspensions by negative selection (MACS isolation) and assessed KLF2 levels in the NK and non-NK cell fractions by immunoblotting. As expected, in the non-NK cell

fractions including B and T cells, the media alone treated cells expressed KLF2 and the PMA/I stimulated cells did not (Figure 4-2 B). In contrast, both NK cell suspensions failed to express KLF2 four hours of stimulation, verifying that an intact microenvironment is necessary for maintaining KLF2 protein levels in NK cells *ex vivo*.

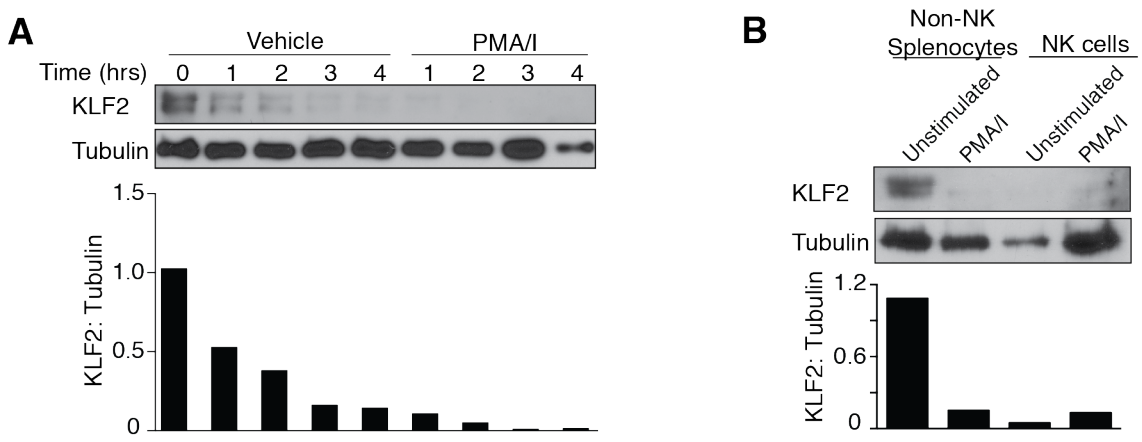


Figure 4-2. KLF2 is degraded outside of the homeostatic niche. (A) KLF2 spontaneously degrades in NK cells upon disruption of the splenic microenvironment. Single cell suspensions of whole splenocytes were stimulated ± PMA/I [50 and 500ng/ml, respectively] ex-vivo for 4hrs. NK cells were isolated from stimulated suspensions through negative selection. KLF2 protein levels were measured in the non-NK cell splenocyte fractions and isolated NK cells through immunoblotting (top panel) and densitometry (lower panel). This experiment was performed three times. (B) KLF2 spontaneously degrades *ex vivo* within 4 hrs in NK cells cultured alone. KLF2 protein levels were measured in ± PMA/I stimulated NK cells at the indicated time points. This experiment was performed once.

Due to the robust level of KLF2 degradation observed in NK cells removed from their homeostatic niche, I utilized this phenomenon as a tool to identify kinase-associated pathways leading to KLF2 phosphorylation and subsequent degradation by culturing NK cells outside of the homeostatic niche in the presence of pathway inhibitors. MACS isolated NK cells were cultured in the presence of the proteasome inhibitor MG132, the pan-PI3K inhibitor LY294002, MEK inhibitor PD98059, the KLF2-stabilizer simvastatin, a STAT5 inhibitor, and the mammalian target of rapamycin (mTOR) inhibitor rapamycin (Figure 4-3 A). LY294002, rapamycin, and simvastatin impaired KLF2 degradation in NK cells, implying that PI3K and mTOR pathways phosphorylate KLF2 in NK cells and these drugs can be potentially used to stabilize KLF2 levels in NK cells. Both PI3K and mTOR signal downstream of the IL-15 receptor complex (IL-15Rβ/γ_c) in the presence of high but not low levels of IL-15 (129, 130). High levels of IL-15 induce NK cells proliferation, which is suppressed by KLF2 (Chapter 3). Moreover, the IL-15Rβ/γ_c complex is negatively regulated by a phosphatase containing cytokine inducible-src homology 2 (SH2) containing protein (CISH) in response to IL-2 (158, 159) and IL-15 (160). Therefore, varying levels of IL-15Rβ/γ_c complex signaling may have different effects on KLF2 stability due to differences in KLF2-phosphorylating kinase activity. Indeed, when KLF2 levels were characterized following stimulation with different concentrations of IL-15, KLF2 levels were stabilized in the presence of low (0.2ng/ml) but not high (20ng/ml) levels of IL-15 stimulation relative to unstimulated and freshly isolated NK cell controls (Figure 4-3 B). Collectively these data indicate that

KLF2 levels are stabilized within the homeostatic niche by low but not high levels of IL-15.

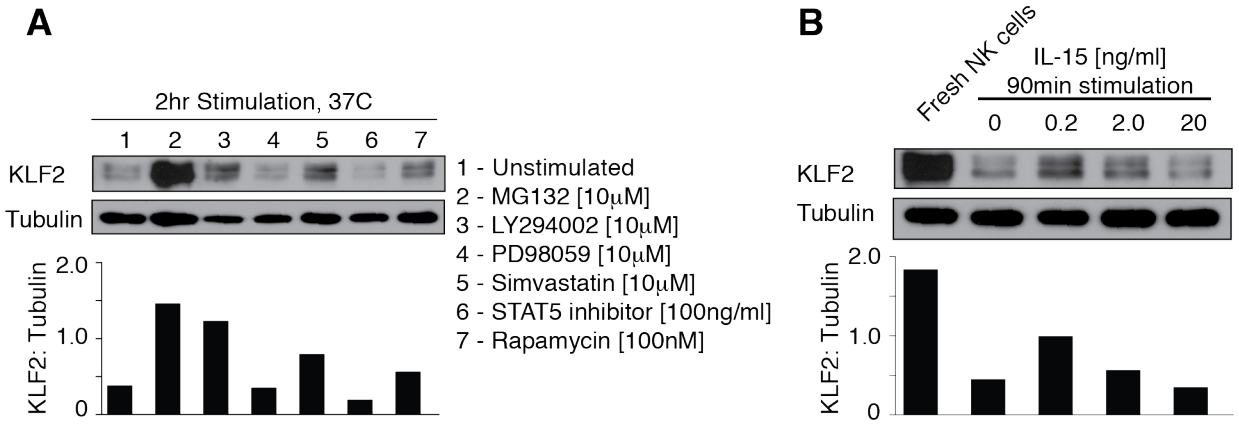


Figure 4-3. Characterization of pathways that promote and inhibit spontaneous KLF2 degradation. (A) KLF2 is degraded via PI3K and mTOR dependent pathways and stabilized by simvastatin. Spontaneous degradation of KLF2 was assessed in the presence of a proteasome inhibitor (MG132), PI3K-inibitor (LY294002), MEK-inhibitor (PD98059), KLF2-stablizer (simvastatin), STAT5 inhibitor, mTOR inhibitor (rapamycin). MACS sorted NK cells were cultured in the presence of the indicated inhibitors for 2hrs. This experiment was performed twice. (B) Spontaneous degradation of KLF2 is inhibited by low [0.2ng/ml] but not high levels [2.0, 20 ng/ml] of IL-15. MACS sorted NK cells were stimulated with the indicated concentrations of IL-15 for 90min. KLF2 protein levels were assessed by immunoblotting (top panels) and densitometry (lower panels). This experiment was performed twice.

KLF2 regulates homeostatic HR in mature NK cells

My data suggests KLF2 may be degraded in mature NK cells (i) following NK cell activation via self-ligands signaling through PI3K-associated receptors like NKG2D, (ii) in tertiary tissues with no available IL-15, (iii) in response to high levels of trans-presented IL-15 by activated myeloid cells, or (iv) in the absence of phosphatase-associated inhibitory receptor signaling provided by the homeostatic niche. Degrading KLF2 in NK cells may serve two major functions: (1) To alter migration patterns in mature NK cells and (2) To lift KLF2-suppressed proliferation in immature NK cells exposed to high levels of IL-15. Consistent with previous findings indicating that KLF2 suppresses homeostatic proliferation (Chapter 3), *ex vivo* IL-15 expanded NK cells did not express detectable levels of KLF2 by immunoblotting (data not shown). To address whether KLF2 alters migration patterns in mature NK cells, I utilized a tamoxifen inducible system to assess the consequences of *Klf2* ablation on NK cell trafficking and homing receptor expression *in vivo*. Under steady-state conditions, NK cells readily express HR that recognize ligands expressed under non-inflammatory (CD62L, CX_3CR_1, $S1P_5$) and inflammatory (CCR2, CCR5, CXCR3) conditions (54), enabling these cells to instantly respond to inflammatory gradients without prior activation. Furthermore, developing NK cells have been documented to preferentially express homing receptors based on their maturation status (54). To circumvent maturation bias, I compared expression between KLF2-sufficient and KLF2-deficient animals by gating on similar $CD27^+CD11b^+$ (R2), and $CD27^-CD11b^+$ (R3) populations. Upon gene excision, mature $CD11b^+$ KLF2-deficient NK cells expressed lower levels of homeostatic

homing receptors CD62L by flow cytometry (Figure 4-4 A) and lower levels of Cx3cr1, and Edg8 (s1p5) as measured by RT-PCR, (Figure 4-4 B), but did not significantly alter expression of inflammatory receptors. Loss of homeostatic receptor expression upon *Klf2* excision preceded loss of mature CD11b^{+} NK cells caused by decreased association with IL-15 sustaining microenvironment (Figure 3-15). In the previous chapter, I demonstrated that KLF2-directed homing patterns maintain late-stage NK cell homeostasis and survival by promoting co-localization with IL-15 associated niches. S1P5 is necessary for NK cells to populate peripheral tissues including the spleen, liver, and lung in normal numbers, but *S1P5*$^{-/-}$ mice do not have altered subset frequencies (161). Upon *Klf2* excision, *T2-cre; Klf2*$^{fl/fl}$ mice exhibit a loss of NK cells in peripheral tissues (Figure 3-15 B), consistent with decreased S1P5 expression but also exhibit altered subset frequencies not observed in *S1P5*$^{-/-}$ mice, indicating that expression of this receptor and possibly others may collectively promote NK cell homeostasis. The expression of CCR7 by naïve T cells is essential for efficient entry into secondary lymphoid organs (162), and in response to inflammation NK cells are recruited from the blood to T cell rich areas in the lymph nodes to promote Th1-differentiation through the secretion of IFNγ (61). Consistent with the increased number of KLF2-deficient NK cells found in the T cell rich white pulp under steady-state in *Vav-cre; Klf2*$^{fl/fl}$ animals (Figure 3-13) and following adoptive transfer (Figure 3-14), tamoxifen treated *T2-cre; Klf2*$^{fl/fl}$ mice had a higher frequency of CCR7^{+} NK cells compared to *Klf2*$^{fl/fl}$ control mice (Figure 4-4 A, top panel). These data indicate that KLF2 controls the expression of homing receptors that respond to homeostatic but not inflammatory ligands. Under non-

inflammatory conditions, ablating *Klf2* in mature NK cells results in altered migration patterns which precedes a subsequent loss of late-stage effector cells and increased migration to the white pulp. These migratory patterns may serve to shut down innate NK cell-mediated immune responses by dissociating effector NK cells from survival niches upon (i) PI3K-associated receptor activation (ii) in the absence of phosphatase associated inhibitory receptor signaling (iii) or in response to strong IL-15-dependent myeloid priming, while simultaneously promoting NK cell recruitment to T cell rich areas via CCR7 to initiate adaptive immune responses.

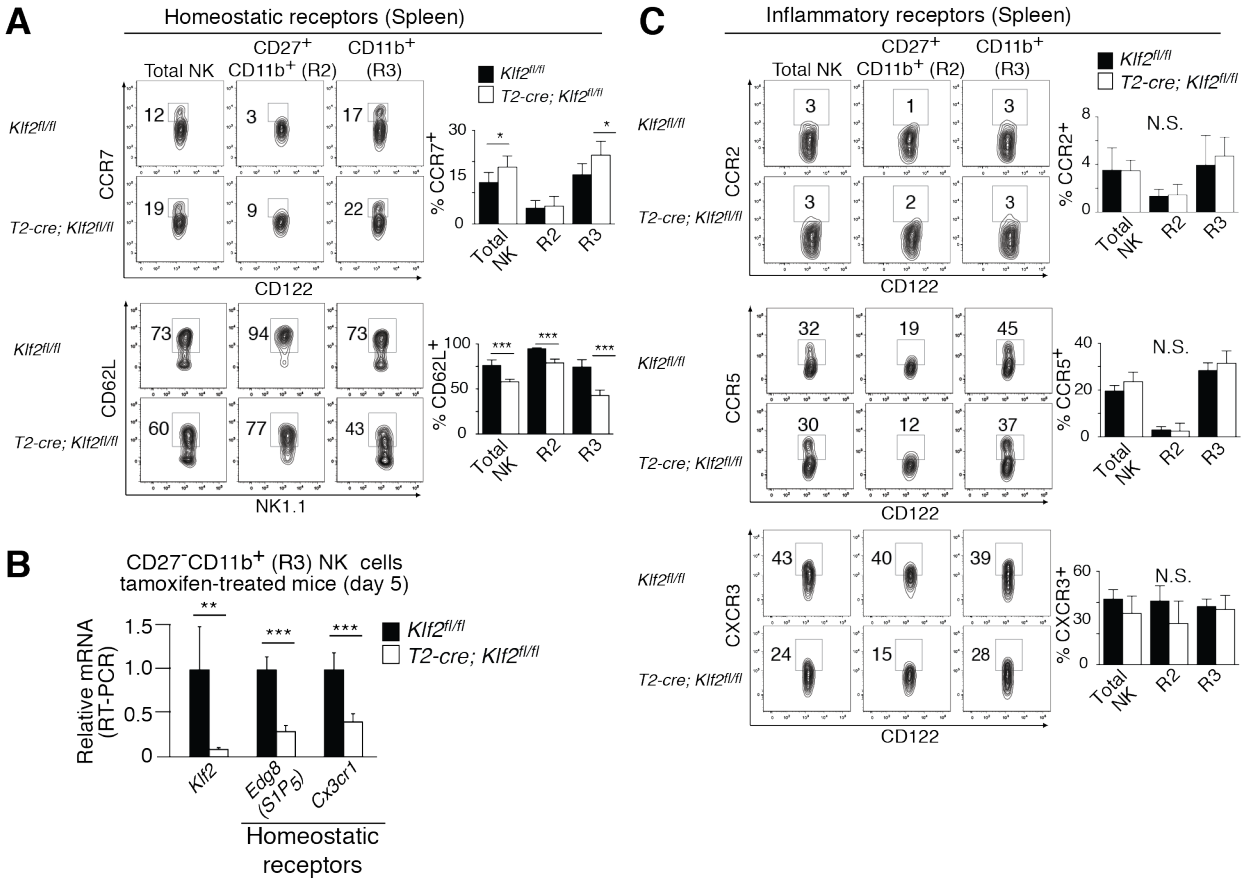


Figure 4-4. KLF2 regulates expression of homeostatic homing receptors in NK cells. (A-C) *T2-cre; Klf2*$^{fl/fl}$ and *Klf2*$^{fl/fl}$ mice were placed on tamoxifen-infused chow for 5-6 days and then analyzed for expression of homing receptors that respond to constitutively expressed ligands (A and B) or inflammatory chemokines (C). (A) Surface expression of CCR7 and CD62L on CD122$^+$ Lin$^-$ NK cells (R2= CD27$^+$CD11b$^+$; R3= CD27$^-$ CD11b$^+$), by flow cytometry. CCR7 (n=6-7/group, day 5), CD62L (n=6-8 per group, day 6). (B) Relative mRNA expression of *Klf2*, *Edg8* (*S1p5*), and *Cx3cr1* in FACS sorted CD122$^+$ Lin$^-$ CD27$^-$ CD11b$^+$ (R3) splenic NK cells. This experiment was repeated twice. (C) Surface expression of CCR2, CCR5, and CXCR3 on CD122$^+$ Lin$^-$ NK cells by flow cytometry. CCR2 (n=11-13 per group, day 6), CCR5 (n=5-6 per group, day 5), CXCR3 (n=5-6 per group, day 5). CCR2 (n=11-13 per group, day 6), CCR5 (n=5-6 per group, day 5), CXCR3 (n=5-6 per group, day 5). Data represents the mean ± SD. This experiment was performed 2-3 times per homing receptor. *P<0.05, **P<0.01, ***P<0.001.

KLF2-regulated HR are altered upon NK cell activation

Under steady-state conditions, IL-15 trans-presenting cells such as dendritic cells express low levels of IL-15 (131). In response to pattern recognition receptor activation through lipopolysaccharide (LPS) via toll like receptor 4 (TLR4) or polyinosinic-polycytidylic acid [poly (I:C)] via toll-like receptor 3 (TLR3), DC's present higher levels of superagonist IL-15-IL-15Rα complexes, which in turn activate NK cells *in vivo* (131). To verify homing receptors are altered upon NK cell activation and to assess how closely *Klf2* excision replicated homing receptor regulation following NK cell activation I briefly activated NK cells *in vitro* with PMA/I and *in vivo* by challenging mice with poly (I:C). By using an *in vivo* system, I was able to avoid spontaneous degradation observed in *ex-vivo* conditions, which could alter the interpretation of results. Wild-type C57BL/6 mice were challenged with poly (I:C) and then examined for KLF2 protein levels and differential receptor expression 16 hours post-injection. Following poly (I:C) challenge, KLF2 levels are reduced in splenic NK cells (Figure 4-5 A), consistent with previous experiments demonstrating that KLF2 is degraded in activated NK cells (Figure 4-1). Poly (I:C) has been previously reported to induce splenic NK cell displacement into the liver (163), and intra-splenic migration from the red pulp to the white pulp (164). As a control for activation, I verified these recruitment patterns by monitoring the frequency of mature $CD27^{-}CD11b^{+}$ NK cells following poly (I:C) treatment (Figure 4-5 B) and staining for CCR7 expression (Figure 4-5 C), respectively. As observed in KLF2-deficient NK cells, poly (I:C) challenged NK cells similarly altered surface levels of CCR7 and CD62L (Figure 4-5 C) and PMA/I-activated $CD27^{-}CD11b^{+}$ NK cells expressed lower levels of

Edg8 mRNA (Figure 4-5 D). In contrast, poly (I:C) treatment increased NK cell expression of inflammatory homing receptors CCR2, CCR5, and CXCR3 that was not observed following tamoxifen induced *Klf2* excision, indicating that these receptors are regulated through KLF2-independent mechanisms. This method of NK cell activation did not result in loss of late-stage NK cells as observed upon tamoxifen induced excision of *Klf2*. Moreover, increased levels of trans-presented IL-15 following poly (I:C) treatment were more likely to promote immature proliferation or prolong NK cell effector functions. Therefore, under conditions in which high levels of IL-15 are present, KLF2 may be degraded to promote homeostatic proliferation in immature NK cells and NK cell effector functions may be prolonged; however, primed cells may no longer be able to return to the homeostatic niche in the absence of KLF2. From these results I conclude that KLF2 regulated homeostatic receptors are altered upon NK cell activation, but only lead to apoptosis under non-inflammatory conditions.

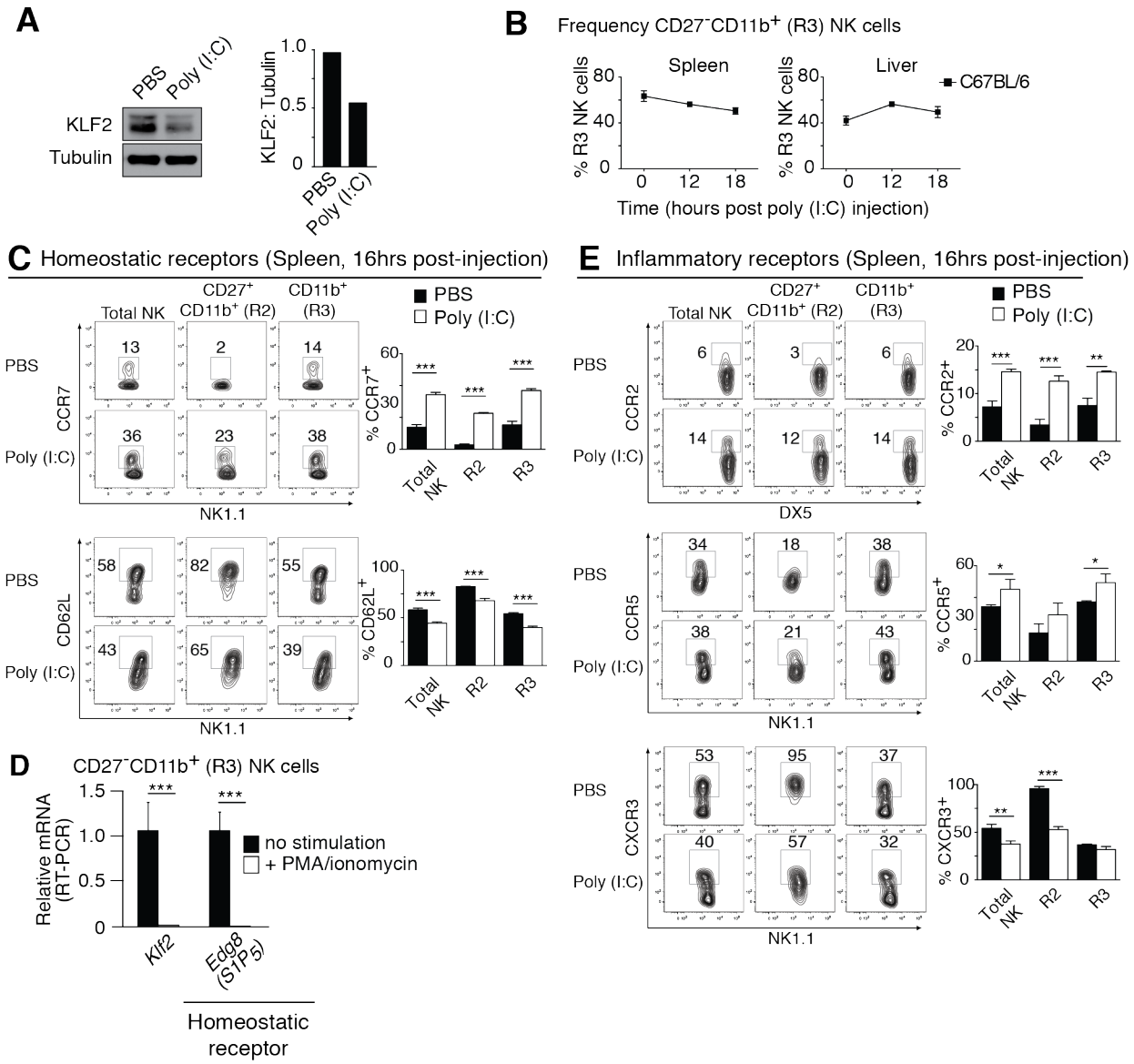


Figure 4-5. KLF2-regulated homeostatic but not inflammatory HR are altered upon NK cell activation. (A-C) C57BL/6 mice were treated with control (PBS) or poly (I:C) (200μg, i.p.) and then analyzed 16hrs post-treatment for expression of KLF2 (A) and homing receptors that respond to constitutively expressed ligands (C, D) or inflammatory chemokines (E). (A) KLF2 immunoblot (left panel) and densitometry (right panel) of MACS sorted splenic NK cells harvested from control (PBS) and poly (I:C)-treated mice. This experiment was repeated twice. (B) Frequency of mature (R3=CD27$^-$CD11b$^+$R3) NK cells in the spleen and liver of C57BL/6 mice following poly (I:C)-treatment. n=3 mice per time point. (C) CD49b$^+$Lin$^-$ NK cell subsets (R2=CD27$^+$CD11b$^+$; R3=CD27$^-$CD11b$^+$) were examined for surface expressions of CCR7 and CD62L. N=3 mice per group, repeated twice. (D) Relative mRNA expression of *Klf2* and *Edg8* (*S1p5*) following 4hrs PMA/I stimulation of FACS-sorted CD27$^-$CD11b$^+$ splenic NK cells harvested from C57BL/6 mice. This experiment was performed once in triplicate. (E) CD49b$^+$Lin$^-$ NK cell subsets were examined for surface expressions of CCR2, CCR5, and CXCR3. N=3 mice per group, repeated twice. Data represents the mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

Discussion

Understanding how NK cell responses expand and contract in response to activation and inflammation are critical to devising strategies to prolong their effector responses for cancer therapy and resolving chronic inflammation. Under steady-state conditions, KLF2 maintains NK cell survival by promoting the expression of homeostatic receptors that restrict NK cell migration within myeloid rich IL-15 presenting niches. The homeostatic niche provides low levels of IL-15 that promotes NK cell survival and likely promotes the expression of the phosphatase CISH, which in turn stabilizes KLF2 by preventing its phosphorylation by PI3K and mTOR (Figure 4-6). In addition, KLF2 may also be stabilized by the engagement of ITAM-associated receptors via unknown self-ligands on healthy cells within the homeostatic niche, which induces downstream phosphatase activity via associated SHP-1 or SHP-2 adaptor proteins. Therefore, KLF2 restricts NK cell migration within the microenvironment through a positive feedback loop maintained by the homeostatic environment. Our data indicate that KLF2 is degraded in NK cells (i) following activation via PI3K-associated activating receptors like NK1.1 in mice and NKG2D in both mice and humans, (ii) following activation via high levels of IL-15 and (iii) in the absence of feedback from the homeostatic niche. These mechanisms promoting KLF2 degradation may serve to 1) promote homeostatic proliferation in response to high levels of IL-15Rα/γ_c signaling cytokines such as IL-2 and IL-15, 2) to alter NK cell homing receptor patterns which induce IL-15 starvation and subsequent apoptosis, and 3) promote NK cell migration toward T cell rich areas to activate the adaptive immune system via IFNγ secretion (Figure 4-7). Over the course of viral

infection, it has been observed that NK cells rapidly expand within the first 5 days of infection following activation via type I interferons and then begin to contract by day 10, allowing T and B cells of the adaptive immune system to take over (165). These data provide an explanation of how NK cells may contract following an immune response. By restricting the ability of NK cells to access survival niches, KLF2 negatively regulates NK cell associated inflammation upon activation, immediately pushing the system to return to homeostasis following PI3K/mTOR-associated signaling events which may be IL-15 dependent or independent.

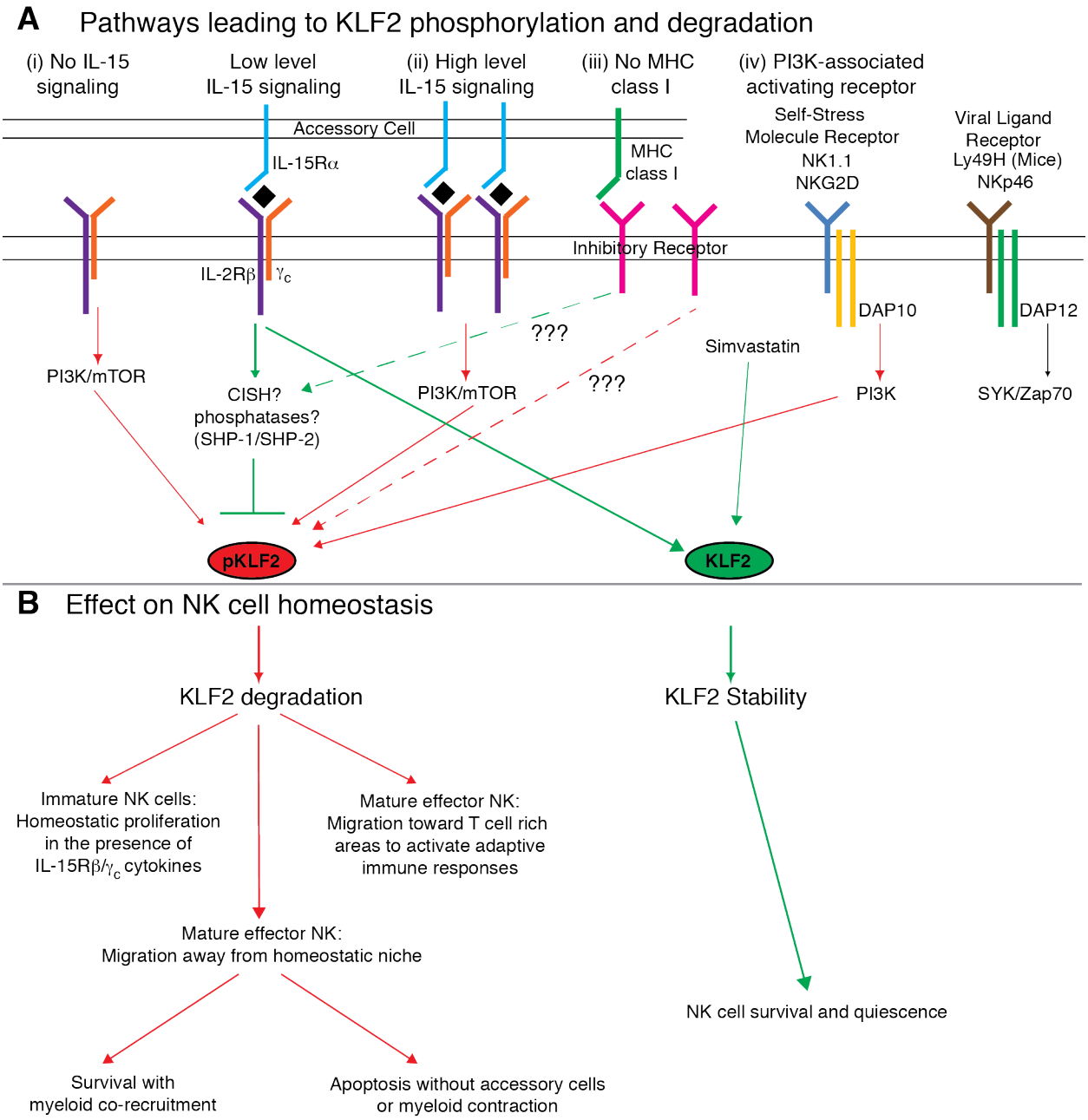


Figure 4-6. Model of signaling pathways leading to KLF2 phosphorylation and is effect on NK cell homeostasis. (A) Signaling pathways detailing how different levels of IL-15 signaling, receptor activation, and simvastatin impact KLF2 protein expression in NK cells. (B) Consequences of pathway dependent KLF2 degradation and stabilization on NK cell homeostasis.

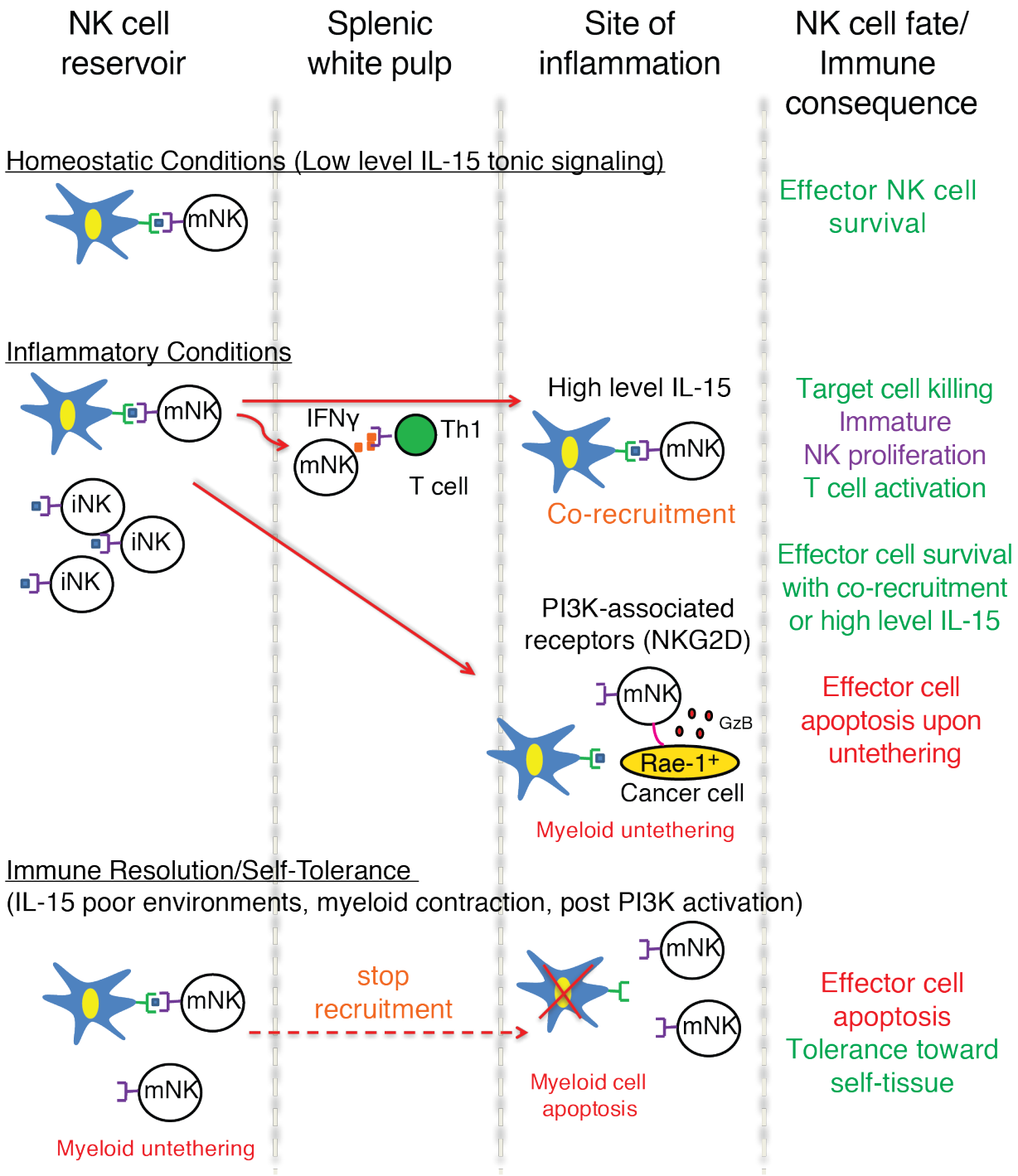


Figure 4-7. Model of KLF2 directed migration under homeostatic conditions, inflammatory conditions, and following resolution of an immune response. mNK= mature $CD11b^{+}$ NK cell; iNK= immature $CD27^{+}$ $CD11b^{-}$ NK cells.

Regarding IL-15 independent signaling events, NK cells may be activated through activating receptors that signal through DAP10 or DAP12 adaptor proteins. DAP10 associated receptors like NKG2D recognizes self-ligands such as Rae-1 or MICA expressed on stressed cells (76), and NK1.1 is predicted to recognize self-molecules (77). DAP10 associated receptors, like self-antigen recognizing NKG2D-L (NKG2D long isoform) expressed on resting NK cell, signal directly through PI3K (75). DAP12 associated receptors, like viral hemagglutinin protein recognizing NKp46 and murine cytomegalovirus protein m157 recognizing Ly49H, signal through SYK and ZAP70 tyrosine kinases (78). Therefore, I hypothesize that within the homeostatic niche, self-recognizing DAP10 associated receptors will degrade KLF2 more efficiently, and induce late-stage NK cell migration away from self-sustaining niches more quickly than virally recognizing DAP12 associated receptors as a form of self-tolerance. This theory is supported by the fact that upon MCMV infection in mice, $Ly49H^+$ NK cell populations do not readily decline following recognition of infected cells and mature $Ly49H^+$ cells clonally expand (90, 92). However, our data do not rule out a role for KLF2 in regulating virally induced mature NK cell clonal expansion and would need to be experimentally tested to verify this hypothesis. This model of KLF2-regulated migration may explain why mice and humans are seemingly devoid of NK cell-initiated autoimmunity, despite the fact that NK cells readily recognize and kill self-targets. Collectively these data indicate that KLF2 may function as a molecular sensor to ensure self-tolerance and resolve inflammation by regulating homeostatic migration patterns.

CHAPTER 5

DISCUSSION

Summary

In chapters 2, 3, and 4, I sought to determine how KLF2 differentially regulates migration in conventional $CD4^+CD25^-$ T cells, $CD8^+$ T cells, and NK cells, and understand how these migration patterns affect effector function and self-tolerance. My data indicate that KLF2 differentially controls lymphocyte homing in conventional $CD4^+CD25^-$ T cells, $CD8^+$ T cells, and NK cells, but these migration patterns may similarly promote self-tolerance. We observe that loss of *Klf2* using *Lck-cre; Klf2*$^{fl/fl}$ mice leads to a more substantial loss of $CD8^+$ T cells than $CD4^+CD25^-$ T cells within SLO. HR screening assays in chapter 2 indicate that within conventional $CD4^+CD25^-$ T cells and $CD8^+$ T cells KLF2 similarly promotes the expression of homeostatic receptors and represses inflammatory HR to promote T cell retention within SLO and restrict access to tertiary tissues. Of significance, KLF2 does not equally repress expression of inflammatory HR between $CD4^+CD25^-$ and $CD8^+$ T cell lineages. Within $CD8^+$ T cells, KLF2 strongly represses a wider array of inflammatory HR compared to $CD4^+CD25^-$ T cells. These data indicate that $CD8^+$ T cell retention within SLO is primarily dependent upon KLF2, but $CD4^+CD25^-$ T cells utilize KLF2-dependent and KLF2-independent mechanisms for SLO retention. In addition, it may be possible that memory $CD8^+$ T cell populations within SLO are maintained by KLF2-directed migration patterns which

promote localization toward IL-15 rich niches for survival, like in NK cells (Chapter 3). Our data suggests that these T cell migration patterns are not necessary for pathogen clearance but may be necessary for peripheral tolerance. Indeed, KLF2-deficient $CD8^+$ T cells are capable of specifically killing cells presenting viral antigen, and KLF2-deficient $CD4^+$ T cells are capable of inducing B cell class-switching, reflecting intact effector T cell responses. Under non-infectious conditions, Klf2 deficiency in T cells alone in *Lck-cre; Klf2$^{fl/fl}$* mice does not result in spontaneous autoimmunity. However, when *Klf2* is ablated within the T cell compartment and redundancies in SLO retention are further eliminated by CCR7 deficiency, *CCR7$^{-/-}$; Lck-cre; Klf2$^{fl/fl}$* mice develop spontaneous autoimmunity. In addition, I observed a strong correlation between murine norovirus and morbidity in *Lck-cre; Klf2$^{fl/fl}$* mice. *Lck-cre; Klf2$^{fl/fl}$* mice associated with murine norovirus develop inflammatory symptoms similar to those observed in *CCR7$^{-/-}$; Lck-cre; Klf2$^{fl/fl}$* mice. These data indicate that these KLF2 migration patterns contribute to maintaining tolerance, and may prevent unrestrained T cell activation under inflammatory conditions within SLO and could possibly limit $CD8^+$ T cell activity via IL-15 tethering.

In chapters 3 and 4, I identified novel roles for KLF2 in NK cell biology that influence the expansion, persistence, and mobilization of these cells *in vivo*. Ablation of *Klf2* in the NK cell compartment results in 1) the expansion of immature NK cells which are displaced from the red pulp to the white pulp in the spleen, and 2) the absence of late-stage effector NK cells necessary for tumor clearance *in vivo*. Within the NK cell compartment, KLF2 (a) suppresses immature NK cell proliferation and (b) controls

homing receptor expression to promote the migration of NK cells toward IL-15 rich niches for survival. KLF2 may be degraded in NK cells following (i) activation via PI3K-associated activating receptors like NKG2D by self-ligands on stressed cancer cells or activated T cells, (ii) activation via high levels of trans-presented IL-15 by myeloid cells, (iii) in the absence of IL-15, or (iv) in the absence of inhibitory receptor signaling. Loss of KLF2 in NK cells may serve to (a) lift KLF2 directed repression over proliferation in response to high levels of IL-15 in immature cells, (b) restrict access to IL-15 rich niches necessary for survival, possibly to limit NK cell effector responses, and (c) promote NK recruitment to T cell rich areas within SLO to facilitate Th1 adaptive responses. Within NK cells and possibly $CD8^+$ T cells, KLF2 functions as a molecular sensor that may limit effector cell activity in response to PI3K-associated activation and changes in the homeostatic niche. Feedback from the immune environment following an inflammatory response via KLF2 may facilitate contraction of NK cell effector responses to prevent chronic inflammation and promote self-tolerance through migration. Data within my thesis provides fundamental mechanisms about NK cell biology that may be targeted through FDA approved KLF2 modifying drugs or bypassed through IL-15 treatment, to increase the number, persistence and recruitment of NK cells to benefit cancer therapy. Understanding NK cell biology through KLF2 may lead to the development of better NK cell based therapeutics.

New Insights About NK cell Migration

By comparing KLF2-controlled migration patterns in T cells and NK cells, we can use this as a tool to discover new insights about lymphocyte migration and the impact of migration on health and disease (Figure 5-1). Under non-inflammatory conditions, naive T cells ($KLF2^+$) recirculate between the blood, lymph, and SLO (Figure 5-1 A, right panel), and are restricted from entering tertiary tissues (liver, skin, skeletal muscle, etc.) (Figure 5-1A, left panel). In contrast to T cells, mature $KLF2^+$ $CD11b^+$ NK cells recirculate between the blood, splenic red pulp, liver arterioles and venules, and lung and comprise between 2-10% of lymphocytes within these tissues (Figure 5-1 B, left panel). $KLF2^+$ $CD11b^+$ NK cells are notably absent or reduced (<1%) under non-inflammatory conditions within the splenic white pulp, lymph node cortex, liver sinusoids, and bone marrow (Figure 5-1B, right panel). NK cells can only accumulate within these restricted tissues upon loss of KLF2. Of these KLF2-restricted tissues, the lymph node cortex, splenic white pulp, and bone marrow are regions associated with proliferation and T cell activation. Even under inflammatory conditions or KLF2-deficiency, only a limited number of NK cells are recruited to the lymph node or T cell rich white pulp through increased CCR7 expression (Figure 4-4 A, Figure 4-5 C). Proliferating cells expressing self-stress ligands like Rae-1 and MICA, such as cancer cells or activated T cells, are susceptible to NK cell lysis. Areas where NK cells and T cells co-circulate like the blood, do not exhibit high levels of basal proliferation. Therefore, segregating T cell and NK cell migration may serve to minimize killing of dividing cells and activated T cells within tissues like the bone marrow and lymph node

(Figure 5-1B, right panel). By restraining NK cell entry to these areas under homeostatic and inflammatory conditions, this may prevent unrestricted killing of APC and effector T cells necessary for efficient adaptive immune responses.

Based on this model and the proportions of cytolytic NK cells within KLF2-permissive and KLF2-restricted niches at steady state, I would predict that NK cells would be very efficient at patrolling and eliminating cancer cells within the KLF2-permissive niche (Figure 5-1B, left panel), but would be less effective at patrolling tissues like the bone and lymph node in the KLF2-restricted niche (Figure 5-B, right panel). This may be another mechanism explaining strong metastasis bias toward the bone marrow and lymph node in cancer patients, which has not been previously described in the literature. Cancer itself induces inflammation that will readily recruit NK cells expressing inflammatory HR. If NK cells can be efficiently recruited to KLF2-restricted regions like the lymph node and kill transformed cells in a relatively short time period, it is likely that they can prevent metastasis within these tissues. However, if there are high numbers of transformed cells within KLF2-restricted niches which cannot be efficiently cleared with the limited numbers of NK cells recruited, it is possible that (a) recruited NK cells may eliminate APC presenting cancer antigens and cancer-specific T cells, (b) the prolonged persistence of self-ligand expressing cancer cells may induce NK cell MHC re-education and tolerance towards cancerous cells. It may be possible to test these theories by comparing the ability of NK cells to clear tumor cells *in vivo* administered intravenously (KLF2-permissive) or intra-lymphatically (KLF2-restricted).

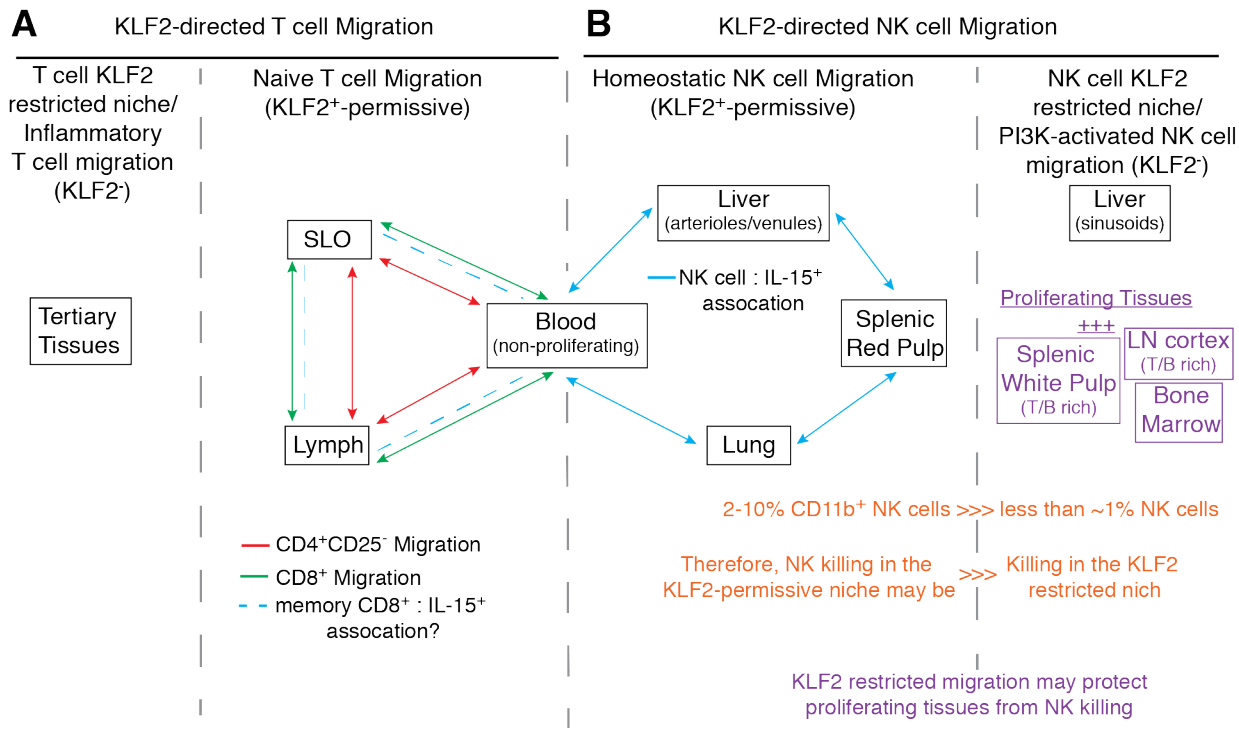


Figure 5-1. Model of KLF2-directed T cell and NK cell migration. (A) KLF2 promotes recirculation and retention of $CD4^+CD25^-$ and $CD8^+$ T cells within the blood, lymph, and SLO while restricting access to tertiary tissues by repressing inflammatory HR expression. (B) KLF2 controls the maintenance of mature $CD11b^+$ NK cells and possibly memory $CD8^+$ T cells by promoting localization toward IL-15 rich niches. In the absence of KLF2 NK cells accumulate in KLF2-restricted tissues such the liver sinusoids and areas associated with high levels of basal proliferation like the bone marrow, splenic white pulp, and lymph node cortex. Mature cytolytic $CD11b^+$ NK cells are much more abundant in KLF2-permissive areas than KLF2-restricted areas at steady state.

Based on the segregated migration patterns of T cells and NK cells, caution should be used when utilizing NK cell based and T cell based therapies. It may be necessary to apply an approach that has a bias favoring an NK cell or T cell response for engineering effective anti-cancer therapies. To date, NK cell based therapies include the transfer of allogeneic NK cells (138) and treatment with IL-15 (137). NK cell based therapies may be more useful during early stages of cancer, if cancers are present in the NK cell KLF2-directed niche like the blood and lung, or in situations where T cells are absent like in allogeneic NK therapy following HSCT. Likewise, patients receiving NK cell based therapies should be treated as if their T cell responses are impaired, in case the increased circulating NK cells suppress T cell mediated responses against pathogens. Conversely, it may be more effective to use T cell based therapies in cancers that have metastasized to NK cell restricted regions like the lymph node and bone marrow. Moreover, if patients are receiving cancer treatment using T cell based therapies like CAR therapy, circulating NK cells may dampen T cell specific responses, and depleting NK cells may increase treatment efficacy. Whether cancer or pathogen, NK cell or T cell based therapies should be selected upon the location and persistence (time) of the transformed cells. Further studies are required to confirm these theories.

Future Directions

There are still several outstanding questions regarding the role of KLF2 in lymphocyte migration and function within the $CD8^+$ T cell and NK cell compartment and its impact on the immune response.

a) Does KLF2 direct $CD8^+$ T cell migration toward IL-15 rich niches for survival and to promote self-tolerance?

b) Which additional ligands and pathways induce KLF2 degradation or promote KLF2 stability in NK cells?

c) How does KLF2 transcriptionally suppress immature NK cell proliferation and does it play a role in mature NK cell clonal expansion in response to viral infection?

d) Is KLF2 necessary for the generation of NK cell memory responses?

e) Can NK cells re-express KLF2 following PI3K-induced activation? And is re-expression necessary for the generation of NK cell memory?

Memory $CD8^+$ T cells are a heterogeneous subset which are reportedly maintained through IL-15 dependent (115, 121, 142) and IL-15 independent mechanisms (143). A possible way to determine whether KLF2 promotes $CD8^+$ T cell survival through IL-15 association would be to utilize the tamoxifen model (*T2-cre; Klf2*$^{fl/fl}$) to track memory $CD8^+$ T cell survival and proximity to IL-15 rich niches upon *Klf2* excision by flow cytometry and immunohistochemistry, respectively. Given that quiescent $CD8^+$ T cell subsets express heterogeneous levels of KLF2 and are high in effector memory (T_{EM}) but are low in tissue resident memory (T_{RM}) under steady state

conditions (166), and many markers used to identify memory $CD8^+$ T cell subsets are controlled by KLF2, it is unlikely we will be able to identify specific subsets which may utilize this pathway for survival. However, data obtained from these experiments would provide fundamental knowledge of how $CD8^+$ T cell populations could possibly contract following an immune response.

Within T and B cells KLF2 is degraded following PI3K-associated TCR or BCR activation (37, 145). My data indicate that in NK cells KLF2 may be degraded in response to PI3K/mTOR-associated activating receptor engagement or in the absence of phosphatase promoting signals from the homeostatic niche; however, specific repertoires of receptors are still undefined. *Ex vivo* culture of primary NK cells results in degradation of KLF2 within 4hrs, indicating that almost all *ex vivo* NK experiments reported in the literature are not physiologic because they lack KLF2. Developing technologies that stabilize KLF2 under *ex vivo* conditions may improve our tools for understanding NK cell signaling and function. In addition, identifying receptors and pathways that induce KLF2 degradation or promote KLF2 stability will be necessary to understand how NK cells restrict effector responses to virally infected or transformed cells and maintain self-tolerance. These data may provide additional insight to indicate whether KLF2 plays a role in viral infection or memory that we have yet to explore.

Accumulating evidence indicates that NK cells can generate memory in response to a limited repertoire of antigens recognized by germ line encoded receptors (107, 109, 167). Memory NK cells exhibit faster recall responses than naïve NK cells when re-challenged with the same antigen and can convey protective immunity (109). During

viral infection like with herpes related MCMV, NK cells undergo mature clonal proliferation that is necessary for early viral control and the generation of long-lived NK cell memory (109). Murine NK cells express the activating receptor Ly49H that recognizes MCMV protein m157. Upon MCMV infection, there is a rapid increase in the number of Ly49H+ cells. This NK cell specific clonal response is an IL-15 independent event (89) that requires cytokines IL-12 (90) and IL-18 (91), and the transcription factor zbtb32 (92). In *IL-12*$^{-/-}$, *IL-18*$^{-/-}$, and NK-specific *zbtb32*$^{-/-}$ mice, NK cells fail to expand upon infection with MCMV (89-92). In the absence of Ly49H^{+} clonal expansion, mice fail to develop long-lived memory NK cells (90). By determining whether KLF2 is necessary for mature NK cell clonal expansion we can predict whether KLF2 is also necessary for the generation of NK cell memory. To test both of these hypotheses, *Klf2* conditional knockout mice would have to be crossed with NKp46-cre mice (151), to excise KLF2 in the NK cell compartment and eliminate NK-extrinsic effects. *NKp46-cre; Klf2*$^{fl/fl}$ mice could then be challenged with MCMV to determine if KLF2-deficient Ly49H^{+} NK cells clonally expand and then re-challenged to determine if these mice are capable of generating long-lived memory NK cells. If KLF2-deficient NK cells are capable of undergoing clonal expansion, but are unable to develop memory NK cells, it may be likely that KLF2 expression or re-expression may be necessary to promote memory NK cell survival.

Based on the data in my thesis, we have been unable to determine whether NK cells may re-express KLF2 following PI3K-activation. Re-expression of KLF2 by NK cells post activation may explain why the majority of NK cells do not undergo apoptosis

following poly (I:C) challenge. *Klf2* mRNA levels are robustly downregulated upon activation with PMA/ionomycin and anti-NK1.1 crosslinking (Figure 4-1 A) but are not altered by anti-NKG2D, anti-Ly49H crosslinking, or changes in IL-15 levels under *ex vivo* conditions (data not shown). Following PI3K-associated KLF2 degradation, it is likely that NK cells become untethered from the IL-15 survival niche and undergo apoptosis. If KLF2 is re-expressed in a small fraction of NK cells this may facilitate re-association with IL-15 rich accessory cells and the generation of long-lived memory cells. To determine whether *Klf2* is re-expressed in NK cells to facilitate re-association with the IL-15 rich niche, it may be necessary to use KLF2 reporter mice as a tool to assess KLF2 levels *in vivo* (166). NK cells stimulated *ex vivo* via PI3K-activation ($KLF2^{lo}$), unstimulated control ($KLF2^{hi}$, positive control) NK cells, or KLF2-deficient (negative control) NK cells could be transferred into the peritoneal cavity of wild type hosts and assessed for their ability to return to the KLF2-permissive niche and stably express KLF2. The peritoneal cavity is an area which does not support large numbers of mature $CD27^{-}CD11b^{+}$ NK cells under steady state (101). If PI3K-activated ($KLF2^{lo}$) NK cells are able to return to KLF2-permissive areas like the blood and re-express KLF2, this would suggest that KLF2 re-expression may facilitate NK cell survival post-activation. Future studies utilizing models with NK cell specific deletion of *Klf2* are necessary for determining the possible significance of KLF2 in infection and memory.

CHAPTER 6

MATERIALS AND METHODS

Mice. *Vav-cre; Klf2*$^{fl/lf}$, *Lck-cre; Klf2*$^{fl/fl}$, and *T2-cre; Klf2*$^{fl/fl}$ mice were generated as previously described (30, 44, 168); LysM-cre and B6.SJL (CD45.1^{+}) mice were purchased from Jackson Laboratories. *IL-15*$^{-/-}$ mice were purchased from Taconic. Mice were housed in specific pathogen-free conditions in accordance with IACUC at Vanderbilt University.

Cell Isolation. Single cell suspensions were made from spleen, liver, lung, bone marrow, mesenteric lymph node, and blood for flow cytometry analysis. All organs isolated were mechanically dissociated and put through a 70 µm cell strainer. Single cell suspensions were resuspended in 40% Percoll buffered in phosphate buffered saline (PBS) and centrifuged for 12min at 680g at room temperature to pellet the leukocyte fraction. Blood was collected in PBS supplemented with 2mM EDTA through cardiac puncture. Lungs were minced with a razor blade and digested with collagenase IV (1mg/ml, Worthington) in HBSS (Gibco) supplemented with 5 mM $CaCl_2$ for 1hr at 37°C. Following digestion, lungs crushed through a 70 µm cell strainer. Bone marrow was isolated from a single femur flushed with a 25-gauge needle. Erythrocytes were lysed using ACK lysis buffer (150mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA) or water. Cells were counted using a hemocytometer and trypan blue or 3% acetic acid with methyl

blue (Stem Cell Technologies, Catalog#07060) to exclude dead cells.

Flow Cytometry. Cell sorting and data was acquisition was performed on a five-laser BD Bioscences Life Science Research II flow cytometer and analyzed with FlowJo (TreeStar) software. Cells were sorted to a purity >99%.

Flow Cytometry Antibodies and Staining Procedures. The following antibodies were purchased from BD Biosciences: Ly49A (A1), Ly49C/I (5E6), Ly49D (4E5), Ly49G2 (4D11), NK1.1 (PK136), CD3e (145-2C11), CD8 (53-6.7), CD11b (M1/70), CD49a (Ha31/8), CCR6 (polyclonal, 557976), CXCR5 (2G8), Gr-1 (RB6-8C5), TCRβ (H57-597). The following antibodies were purchased eBiosciences: EOMES (Dan11mag), IFNγ (XMG1.2), Ki-67 (SolA15), KLRG1 (2F1), Ly49H (3D10), NKG2D (A10), CD19 (1D3), CD27 (LG3A.10), CD49b (DX5), CD90.2 (53-2.1), CD127 (A7R34), CD132 (TUGm2). The following antibodies were purchased from Biolegend: CD45.1 (A20), CD45.2 (104), CD107a (1D4B), CCR3 (J073E5), CCR4 (2G12), CCR5 (HM-CCR5), CCR7 (4B12), CCR9 (CW1.2), CXCR3 (CXCR3-173); R&D, NKp46 (AF2225). The following antibodies were purchased from Tonbo: CD4 (GK1.5), CD25 (PC61.5), CD62L (MEL-14). The following antibodies were purchased from Cell Signaling Technologies: p-STAT5, p-S6, p-mTOR, p-AKT. CCR2 (E68) antibody was purchased from AbCam. Granzyme B (FGB12) was purchased from Life Technologies. CCR8 (polyclonal, orb 15269) was purchased from Biorbyt. Purified rabbit anti-CCR2 was recognized using chicken anti-rabbit IgG (H+L) (Life Technologies, A2441). Purified goat anti-NKp46 was

recognized using donkey anti-goat IgG (H+L) (Jackson Immuno, 705-606-147) in 2% donkey sera to avoid bovine cross reactivity. Intracellular and surface staining was performed at 4°C using standard procedures unless indicated. All chemokine receptors where stained in in the presence of 0.05% sodium azide and fixed with 4% paraformaldehyde to prevent receptor internalization. Chemokine receptors CCR4, CCR5, CCR7, and CXCR3 were stained in at 37°C for 40 min to enhance staining.

Mixed Bone Marrow Chimeras. *Klf2*$^{fl/fl}$ (CD45.2^{+}) hosts receiving 500cGy irradiation were reconstituted with 1 x 10^6 whole bone marrow cells (1:1 wild type CD45.1^{+}/ *Vav-cre; Klf2*$^{fl/fl}$ CD45.2^{+}), injected retro-orbitally. After 8 weeks, the mice were sacrificed and analyzed for NK cell frequencies and numbers by flow cytometry.

Cell Culture and MACS Sorting. For functional assays, cells were cultured in Iscove's Modified Dulbecco's Medium supplemented with 10% (vol/vol) FBS (Gibco), 1x antibiotic/antimycotic (Gibco), 5μM β-mercaptoethanol, and 1x nonessential amino acids (Sigma). Recombinant murine IL-2, IL-12, and IL-15 were purchased from Peprotech. Recombinant murine IL-18 was purchased from MBL. MACS-sorted NK cells were isolated by using the NK cell Isolation Kit II (Miltenyi Biotech). T cells were enriched from spleens by positive selection using anti-Thy1.2 (CD90.2) microbeads for chemokine receptor staining (Miltenyi Biotech). For some experiments CD11b^{+} cells were depleted from the bone marrow using anti-CD11b microbeads (Miltenyi Biotech).

NK Cell Apoptosis. *Klf2*$^{fl/fl}$ and *T2-cre; Klf2*$^{fl/fl}$ littermates were placed on tamoxifen chow for 5 days, killed 4 days 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.

***Ex vivo* NK cell Functional Assays.** NK cell degranulation and granzyme B expression were measured by flow cytometry following 2hr of priming cells with plate-bound anti-NK1.1 (10 μg/mL) or PMA/ionomycin (50 ng/mL + 500 ng/mL) and 4hr incubation of Golgi Plug/Stop (BD Biosciences)-treated cells to accumulate signal. NK cell cytotoxicity assays were performed by using IL-2–primed splenocytes (20 ng/mL, 24hr) that were co-cultured with 2×10^4 YAC-1 target cells for 4hrs at 37 °C. Cytotoxicity was assessed by using a CytoTox Non-Radioactive Cytotoxicity Assay kit (Promega). Data is expressed as: % Cytotoxicity= 100 x (Experimental LDH release)/ (Maximum LDH release). Proliferation following *ex vivo Klf2* excision was examined by co-culturing MACS-sorted NK cells (Miltenyi Biotech) harvested from *Klf2*$^{fl/fl}$ versus *T2-cre; Klf2*$^{fl/fl}$ littermates (1:1 ratio, 5×10^5/mL) in complete media supplemented with IL-2 (20 ng/mL) and 4-OHT (400 nM) for 5 days. *Klf2* excision was >95% as determined by RT-PCR. To examine *ex vivo* NK cell differentiation, CD11b-depleted bone marrow from *Klf2*$^{fl/fl}$ versus *Vav-cre; Klf2*$^{fl/fl}$ mice was co-cultured on stromal support (CD45.1$^+$ bone marrow) in media supplemented with IL-15 (20 ng/mL), IL-12 (20 ng/mL), and IL-18 (50 ng/mL) for 3–4 days, then analyzed by flow cytometry.

***In vivo* NK cell Functional Assays.** NK cell cytotoxicity was measured by co-injecting 4×10^5 RMA and 6×10^5 RMA-S cells [labeled with carboxytetramethylrhodamine (CTMR) and carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes), respectively] into mice i.p., performing a peritoneal lavage 48hrs later, then analyzing the ratio of target (RMA-S) to control (RMA) cells by flow cytometry. NK cell proliferation was assessed following i.p. injection of BrdU (1 mg/mL, every 12hrs for 4.5 days) according to manufacturer's instructions (BrdU Flow Kit; BD Biosciences). Alternatively, Ki-67 staining (eBioscience) was used to detect cell cycling.

Lymphocytic Choriomeningitis Assays (LCMV). *Klf2*$^{fl/fl}$ and *Lck-cre; Klf2*$^{fl/fl}$ mice were infected i.p. with 10^6 pfu LCMV and analyze at days 8 and 30 post-infection. To measure viral titers, spleens from infected mice were weighed and homogenized in 0.5ml of complete IMDM. $3.5x10^5$ C57SV target cells were seeded in 24-well plates with serial dilutions of homogenate supernatant in a total volume of 0.4ml Dulbecco's Modified Eagle's Medium (DMEM, Gibco) and allowed to adhere for 6hrs. Following cell adherence, 0.3ml of 2% methylcellulose diluted in DMEM was added over the target cells. After 48hrs, the cultures were fixed for 20min with 4% paraformaldehyde. Fixed cultures were incubated with rat-anti VLA-4 antibody for 1hr at room temperature followed by a 1hr incubation goat anti-rat HRP (Jackson Immuno). Plaques were visualized using *o*-Phenylenediamine dihydrochloride (OPD, Sigma). Viral titers were determined by counting the number of plaque forming units per gram of tissue homogenate. Cytolytic efficiency was accessed splenic $CD8^+$ T cells harvested from

Klf2$^{fl/fl}$ and *Lck-cre; Klf2*$^{fl/fl}$ mice infected 8 days prior. RMA-S target cells were loaded with LCMV peptide p33 by culturing cells and peptide for 4hrs at room temperature. Following peptide loading, MACS sorted CD8$^+$ T cells from LCMV-primed mice were co-cultured with peptide loaded RMA-S target cells. Target cell lysis (release of intracellular and membrane-associated dyes) was measured by flow cytometry. Percent viable target cells = (% experimental CFSE$^+$PHK26$^+$ RMA-S cells/% CFSE$^+$PHK26$^+$ RMA-S cells co-cultured with naïve CD8$^+$ T cells) × 100. The frequency of LCMV specific CD8$^+$ T cells was determined by re-stimulating LCMV-primed splenocytes with p33 peptide for 6hrs and measuring IFNγ expression by intracellular staining and flow cytometry.

Class-switching assays. To test the ability of T cells in *Klf2*$^{fl/fl}$ and *Lck-cre; Klf2*$^{fl/fl}$ mice to induce B cell class-switching, animals were injected with 200ug TNP-Ovalbumin (TNP-OVA, Biosearch Technologies Inc.) intraperitoneally. TNP-OVA prepared by reconstituted lyophilized protein in PBS at 4mg/ml and mixing this solution in a 1:1 ratio with Imject Alum (Thermo Fisher, Catalog No. 77161). Animals were then re-challenged with TNP-OVA on day 14. TNP specific antibody isotypes in the serum of immunized animals were detected using ELISA plates pre-coated with TNP [5μg/ml] and the SBA Clonotyping System-HRP (Southern Biotech, Catalog No. 5300-05).

Immunohistochemistry. Before to cryosectioning, spleens were embedded and snap frozen in OCT (Sakura) and stored at -80° C. 5μM serial cryo-sections were fixed in 100% acetone for 10min, air dried for 10min, rehydrated in TBS, and blocked in 10%

donkey sera with 50% avidin solution for 30min, followed by 10% donkey sera with 50% biotin solution for 30min using the Avidin/Biotin Blocking kit (Vector labs) to block endogenous avidin and biotin activity before staining. For NKp46/TCRβ and NKp46/MOMA-1 co-staining, sections were incubated with titrated amounts of polyclonal goat anti-NKp46 (R&D Systems, AF2225) and biotinylated anti-TCRβ (BD Biosciences, H57-597) or biotinylated anti-MOMA1 (Cedarlane) overnight at 4°C or 2hrs at RT. MOMA-1 and TCRβ biotinylated antibodies were visualized using the VECTASTAIN ABC-AP (STANDARD) and VECTOR Red Alkaline Phosphatase (AP) substrate kit (Vector Labs) supplemented with levamisole (Vector Labs) to block endogenous phosphatase activity, according to the manufacturer's instructions. Sections were then subsequently incubated with 1.8% hydrogen peroxide and 0.2% sodium azide to quench endogenous peroxidases for 1hr, and re-blocked using the Avidin/Biotin Blocking kit to block biotinylated sites of the first antibody. The anti-NKp46 antibody was visualized by incubating with secondary biotinylated $F(ab')_2$ Frag donkey anti-goat IgG (H+L) (Jackson Immuno, 705-066-147), followed VECTASTAIN Elite ABC Kit (Standard), and developed using the DAB Peroxidase (HRP) Substrate Kit. Nuclei were counterstained using Meyer's hematoxylin solution (Sigma). Sections were dried overnight and cleared with xylene for 30min before permanent mounting with Permount (Fisher). For dual staining for IL-15 and NKp46, because both anti-NKp46 and anti-IL-15 (R&D Systems, AF447-SP) are goat polyclonal antibodies, sections were first stained for NKp46 using alkaline phosphatase/Vector Red to visualize NK cells and then subsequently incubated with biotinylated anti-IL-15, without additional permeablization,

and then visualized using HRP/DAB according to the procedures above. Specificity was determined using similar titrations of biotinylated isotype controls for monoclonal antibodies, or secondary antibody staining alone for polyclonal antibodies on sections stained in tandem, and by staining *IL-15*$^{-/-}$ spleens. Images were acquired using the Nikon AZ 100 (Nikon) and NIS-Elements (Nikon) software.

Dynamic Migration Assay. MACS-sorted NK cells were harvested from *Klf2*$^{fl/fl}$ and *T2-cre; Klf2*$^{fl/fl}$ littermates placed on tamoxifen-infused chow for 5 d. Before cell sorting, *Klf2*$^{fl/fl}$ and *T2-cre; Klf2*$^{fl/fl}$ splenocytes were labeled with cell tracker CMRA [10 μM] and CMFDA [10 μM] (Molecular Probes), respectively, for 40 min at 4 °C. Equal numbers of labeled NK cells (6×10^6) were injected (i.v.) into a wild-type recipient. The recipient spleen was harvested 24 h after transfer and fixed in PBS containing 4% (wt/vol) paraformaldehyde and 30% (wt/vol) sucrose before cryoembedding to preserve cell tracker dyes before immunohistochemical analysis. 8μm cryosections were then avidin/biotin/normal serum-blocked as described above before being incubated with biotinylated anti–MOMA-1 and anti-F4/80 antibodies. Biotinylated antibodies were visualized with streptavidin BV421 (BD Biosciences), and slides were mounted in fluorescence mounting media (DAKO). Fluorescent images were overlayed by using ImageJ software

Homing Receptor Regulation. Wild-type mice were challenged i.p. with poly(I:C)-LMW (200 μg; InvivoGen) and killed 16 h later. Alternatively, *Klf2*$^{fl/fl}$ and *T2-cre; Klf2*$^{fl/fl}$ mice

were placed on tamoxifen-infused chow (250 mg/kg; Harlan Teklad) for 5 d. In both cases, surface expression of homing receptors was assessed by flow cytometry in a subset-specific manner or transcription levels of homing receptors present in $CD27^{-}CD11b^{+}$ FACS-sorted NK cells was determined by RT-PCR

NK Cell Stimulation and Western Blots. NK cells were stimulated with plate-bound NKG2D (25μg/mL) or NK1.1 (25μg/mL) antibody for select time points at 37°C. These plates were supplemented with plate-bound fibronectin (10μg/mL) and low-dose IL-15 (0.5 ng/mL) to improve cell viability. Alternatively, NK cells were labeled with biotinylated anti-NK1.1 (10μg/mL) or anti-NKG2D (10μg/mL) at 4°C before cross-linking with streptavidin PE (10μg/mL) at 37°C for 1–3 hrs. For indicated experiments NK cells were stimulated with PMA/ionomycin (50ng/ml + 500ng/ml) for select time points at 37°C. To inhibit proteasome, PI3K, or MEK1 activity, NK cells were cultured with MG132 (10 μM), LY294002 (10μM), or PD98059 (10μM), respectively. Following stimulation, NK cells were washed with PBS and lysed in PBS-RIPA buffer (1.37M NaCl, 27mM KCl, 100mM Na_2HPO_4, 18mM KH_2PO_4, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 2mM EDTA, 2mM EGTA) with protease inhibitor mixture 2 (Sigma) for 15 min at 4°C before pelleting debris. Lysates were resolved on a 10% SDS/PAGE and probed by using anti-mouse KLF2 (Millipore) and anti–α-tubulin (Santa Cruz Biotechnology). Densitometry was measured by using ImageJ software

Real-Time PCR. RNA was extracted from sorted NK cells using the RNeasy Mini or Micro Kits (Qiagen) depending on cell number. cDNA was generated using the iScript cDNA Synthesis Kit (Bio-Rad) and real-time reactions were performed in quadruplicate using the iQ Sybr Green Supermix (Bio-Rad) and iCycler iQ Real-Time PCR Detection System (Bio-Rad**).** Data is expressed as: 2^ [(CT for Klf2-deficient experimental gene – CT for Klf2-deficient gapdh) – (CT for control experimental gene – CT for control gapdh)].

Klf2-F: 5'-GCG GCA AGA CCT ACA CCA AGA G-3'

Klf2-R: 5'-CTT TCG GTA GTG GCG GGT AAG C-3'

Gapdh-F: 5'-GCC TTC CGT GTT CCT ACC-3'

Gapdh-R: 5'-GCC TGC TTC ACC ACC TTC-3'

Cx3cr1-F: 5'-CGA CAT TGT GGC CTT TGG AAC CAT-3'

Cx3cr1-R: 5'-AGA TGT CAG TGA TGC TCT TGG GCT-3'

Edge 8 ($s1p_5$)-F: 5'-CTT GCT ATT ACT GGA TGT CGC-3'

Edge 8 ($s1p_5$)-R: 5'-GTT GGA GGA GTC TTG GTT GC-3'

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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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.

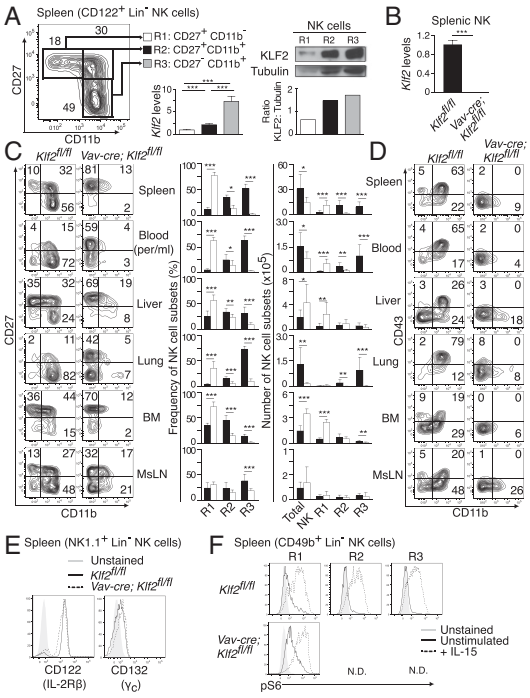

Fig. 1. KLF2 is necessary for NK cell homeostasis. (*A*) *Klf2* mRNA and KLF2 protein levels in NK cell subsets. Splenic CD122^{+}Lin^{-} (CD3, CD8, CD19, Gr-1, TCRβ) NK cells were FACS sorted into maturing NK cell subsets (R1, CD27^{+}CD11b^{-}; R2, CD27^{+}CD11b^{+}; R3, CD27^{-}CD11b^{+}) from C57BL/6 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.

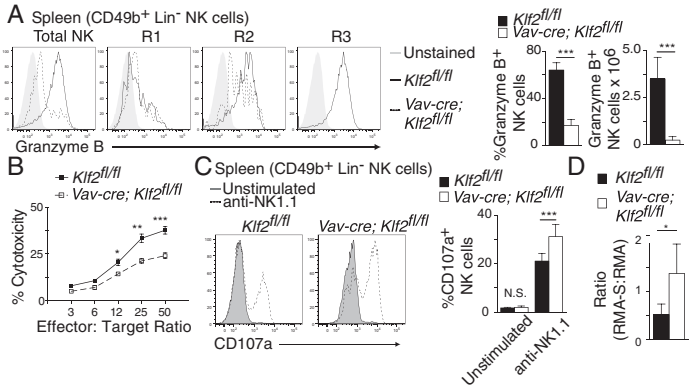

Fig. 2. Mature cytolytic NK cells are absent in *Vav-cre; Klf2$^{fl/fl}$* mice. (*A*) Histogram overlays (*Left*) and quantification (*Right*) of granzyme B expression following (PMA + ionomycin)-simulation of splenic NK cells harvested from *Klf2$^{fl/fl}$* versus *Vav-cre; Klf2$^{fl/fl}$* mice. Histograms 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$^+$

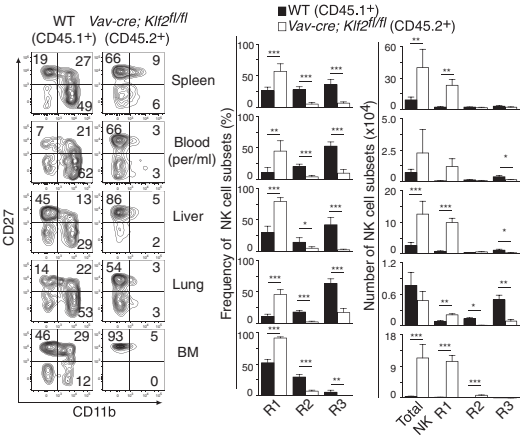

Fig. 3. KLF2-mediated NK cell homeostasis is cell intrinsic. Analysis of mixed bone marrow chimeras that were generated by reconstituting lethally irradiated *Klf2$^{fl/fl}$* (CD45.2$^+$) mice with wild-type (CD45.1$^+$) and *Vav-cre; Klf2$^{fl/fl}$* (CD45.2$^+$) bone marrow. Flow cytometric analysis 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.

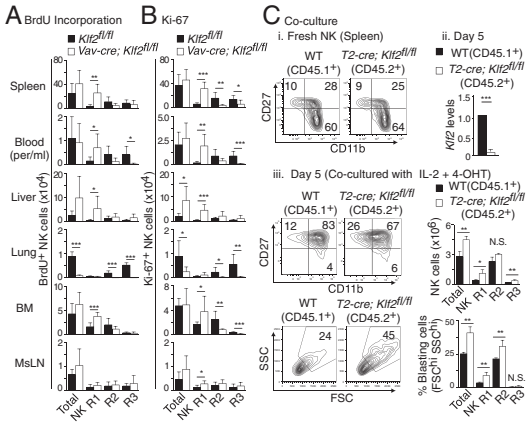

Fig. 4. KLF2 suppresses proliferation in immature NK cells. (*A*) BrdU incorporation over 5 d was used to assess NK cell ($CD122^+Lin^-$) proliferation in various tissues harvested from *Klf2*$^{fl/fl}$ versus *Vav-cre; Klf2*$^{fl/fl}$ littermates. n = 6 mice per cohort, pooled from two independent experiments. (*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

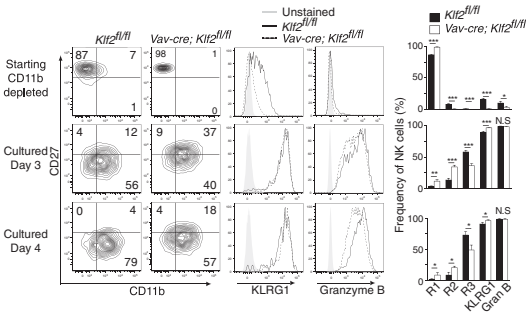

Fig. 5. KLF2-deficient NK cell differentiation is reestablished in culture. CD11b-depleted bone marrow from *Klf2*$^{fl/fl}$ and *Vav-cre; Klf2*$^{fl/fl}$ mice was plated on wild-type bone marrow ($CD45.1^+$) supplemented with IL-15, IL-12, and IL-18. Starting material and cells cultured for 3–4 d were 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.

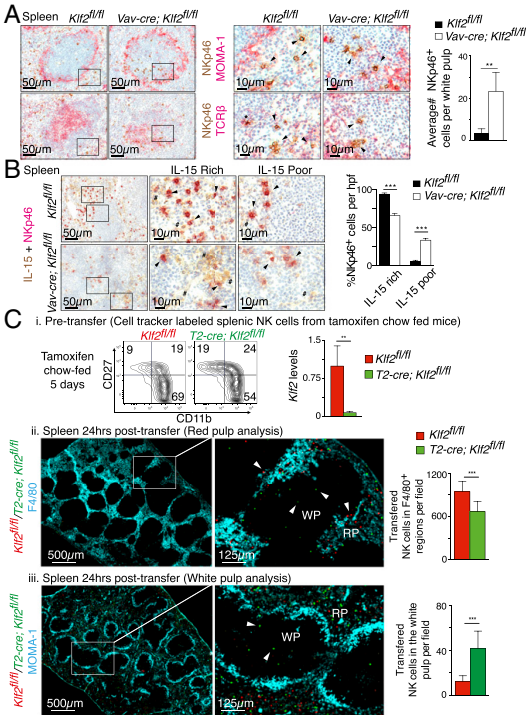

Fig. 6. KLF2 promotes NK cell migration toward IL-15–rich niches. (*A*) Immunohistochemistry of *Klf2*$^{fl/fl}$ and *Vav-cre; Klf2*$^{fl/fl}$ splenic serial sections costained for NKp46/MOMA-1 (*Top*) and NKp46/TCRβ (*Bottom*). Enlarged images that identify NK cells (arrows) and NKT cells (*) are shown 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

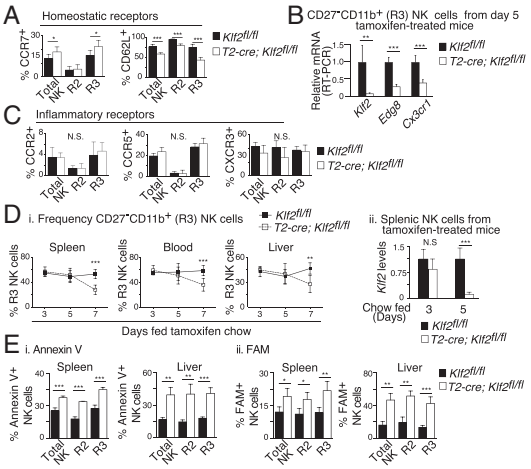

Fig. 7. KLF2 supports mature NK cell survival by regulating expression of homeostatic homing receptors. (*A*–*C*) *Klf2*$^{fl/fl}$ and *T2-cre; Klf2*$^{fl/fl}$ mice were placed on tamoxifen-infused chow for 5 d, then analyzed for expression of homing receptors that respond to constitutively expressed 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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Supporting Information

Rabacal et al. 10.1073/pnas.1521491113

SI Materials and Methods

Cell Isolation. Single-cell suspensions were made from spleen, liver, lung, bone marrow, mesenteric lymph node, and blood for flow cytometry analysis. All organs isolated were mechanically dissociated and put through a 70-μm cell strainer. Single-cell suspensions in complete media were resuspended in 40% (vol/vol) Percoll and centrifuged for 12 min at 680 × *g* at room temperature to pellet the leukocyte fraction. Blood was collected in PBS supplemented with 2 mM EDTA via cardiac puncture. Lungs were minced with a razor blade and digested with collagenase IV (1 mg/mL; Worthington) in HBSS (Gibco), supplemented with 5 mM $CaCl_2$ for 1 h at 37 °C. Following digestion, lungs were passed through a 70-μm cell strainer. Bone marrow was isolated from a single femur flushed with a 25-gauge needle. Erythrocytes were lysed by using ammonium-chloride-potassium lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). Cells were counted by using a hemocytometer and trypan blue to exclude dead cells.

Antibody Staining. Anti-Ly49A (A1), anti-Ly49C/I (5E6), anti-Ly49D (4E5), anti-Ly49G2 (4D11), anti-NK1.1 (PK136), anti-CD3ε (145-2C11), anti-CD8 (53-6.7) anti-CD11b (M1/70), anti-CD49a (Ha31/8), anti–Gr-1 (RB6-8C5), and anti-TCRβ (H57-597) were purchased from BD Biosciences. Anti-EOMES (Dan11mag), anti–Ki-67 (SolA15), anti-KLRG1 (2F1), anti-Ly49H (3D10), anti-NKG2D (A10), anti-CD19 (1D3), anti-CD27 (LG3A.10), anti-CD49b (DX5), anti-CD127 (A7R34), and anti-CD132 (TUGm2) were purchased from eBioscience. Anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD107a (1D4B), anti-CCR5 (HM-CCR5), anti-CCR7 (4B12), and anti-CXCR3 (CXCR3-173) were purchased from Biolegend. Anti-NKp46 (AF2225) and anti–IL-15 (AF447-SP) were purchased from R&D Systems. Anti-CD62L (MEL-14) and anti-F4/80 were purchased from Tonbo Biosciences. Anti-phosophoS6 was purchased from Cell Signaling Technologies. Anti-CCR2 (E68) was purchased from Abcam. Anti-granzyme B (FGB12) and chicken anti-rabbit IgG (H+L) that was used to recognize purified rabbit anti-CCR2 were purchased from Life Technologies. Purified goat anti-NKp46 was recognized by using donkey anti-goat IgG (H+L) (Jackson Immunoresearch; 705-606-147) in 2% (vol/vol) donkey sera to avoid bovine cross-reactivity. Intracellular and surface staining was performed by using standard procedures except CCR5, CCR7, and CXCR3 stains, which were done at 37 °C for 40 min in the presence of 0.05% sodium azide to enhance staining, then fixed with 4% (wt/vol) paraformaldehyde to prevent receptor internalization.

Cell Culture and MACS Sorting. For functional assays, cells were cultured in Iscove's Modified Dulbecco's Medium supplemented with 10% (vol/vol) FBS (Gibco), 1× antibiotic/antimycotic (Gibco), 5 μM β-mercaptoethanol, and 1× nonessential amino acids (Sigma). IL-2, IL-12, and IL-15 were purchased from Peprotech. IL-18 was purchased from MBL. MACS-sorted NK cells were isolated by using the NK cell Isolation KitII (Miltenyi Biotech).

Ex Vivo Functional Assays. NK cell degranulation and granzyme B expression were measured by flow cytometry following 2 h of priming cells with plate-bound anti-NK1.1 (10 μg/mL) or PMA/ionomycin (50 ng/mL + 500 ng/mL) and 4-h incubation of Golgi Plug/Stop (BD Biosciences)-treated cells to accumulate signal. NK cell cytotoxicity assays were performed by using IL-2–primed splenocytes (20 ng/mL, 24 h) that were cocultured with 2×10^4 YAC-1 target cells for 4 h at 37 °C. Cytotoxicity was assessed by using a CytoTox Non-Radioactive Cytotoxicity Assay kit (Promega). Proliferation following ex vivo *Klf2* excision was examined by coculturing MACS-sorted NK cells (Miltenyi Biotec) harvested from $Klf2^{fl/fl}$ versus *T2-cre;* $Klf2^{fl/fl}$ littermates (1:1 ratio, 5×10^5/mL) in Complete media supplemented with IL-2 (20 ng/mL) and 4-OHT (400 nM) for 5 d. *Klf2* excision was >95% as determined by RT-PCR. To examine ex vivo NK cell differentiation, CD11b-depleted bone marrow from $Klf2^{fl/fl}$ versus *Vav-cre;* $Klf2^{fl/fl}$ mice was cocultured on stromal support ($CD45.1^+$ bone marrow) in media supplemented with IL-15 (20 ng/mL), IL-12 (20 ng/mL), and IL-18 (50 ng/mL) for 3–4 d, then analyzed by flow cytometry.

In Vivo Functional Assays. NK cell cytotoxicity was measured by coinjecting 4×10^5 RMA and 6×10^5 RMA-S cells [labeled with carboxytetramethylrhodamine (CTMR) and carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes), respectively] into mice i.p., performing a peritoneal lavage 48 h later, then analyzing the ratio of target (RMA-S) to control (RMA) cells by flow cytometry. NK cell proliferation was assessed following i.p. injection of BrdU (1 mg/mL, every 12 h for 4.5 d) according to manufacturer's instructions (BrdU Flow Kit; BD Biosciences). Alternatively, Ki-67 staining (eBioscience) was used to detect cell cycling.

Immunohistochemistry. Before cryosectioning, spleens were embedded and snap frozen in OCT (Sakura) and stored at −80 °C. Five-micrometer serial cryosections were fixed in 100% acetone for 10 min, air dried for 10 min, rehydrated in TBS, and blocked in 10% (vol/vol) donkey sera with 50% (vol/vol) avidin solution for 30 min, followed by 10% (vol/vol) donkey sera with 50% (vol/vol) biotin solution for 30 min using the Avidin/Biotin Blocking kit (Vector Laboratories) to block endogenous avidin and biotin before staining. For NKp46/TCRβ and NKp46/MOMA-1 costaining, sections were incubated with titrated amounts of polyclonal goat anti-NKp46 and biotinylated anti-TCRβ or biotinylated anti-MOMA1 (Cedarlane) overnight at 4 °C or 2 h at room temperature. MOMA-1 and TCR-β biotinylated antibodies were visualized by using the VECTASTAIN ABC-AP (STANDARD) and VECTOR Red Alkaline Phosphatase (AP) substrate kit (Vector Labs) supplemented with levamisole (Vector Labs) to block endogenous phosphatase activity. Sections were then incubated with 1.8% (vol/vol) hydrogen peroxide and 0.2% sodium azide to quench endogenous peroxidases for 1 h, and reblocked by using the Avidin/Biotin Blocking kit to block biotinylated sites of the first antibody. The anti-NKp46 antibody was visualized by incubating with secondary biotinylated $F(ab')_2$ Frag donkey anti-goat IgG (H+L), then VECTASTAIN Elite ABC Kit (Standard), followed by development using the DAB Peroxidase (HRP) Substrate Kit. Nuclei were counterstained by using Meyer's hematoxylin solution (Sigma). Sections were dried overnight and cleared with xylene for 30 min before permanent mounting with Permount (Fisher). For dual staining for IL-15 and NKp46, sections were first stained for NKp46 by using alkaline phosphatase/Vector Red, then serially incubated with biotinylated anti-IL-15 and HRP/DAB as described. Specificity was determined by using similar titrations of biotinylated isotype controls for monoclonal antibodies, secondary antibody staining alone for polyclonal antibodies on sections stained in tandem, or IL-15 gene-deficient (KO) sections when appropriate.

Homing Receptor Regulation. Wild-type mice were challenged i.p. with poly(I:C)-LMW (200 μg; InvivoGen) and killed 16 h later. Alternatively, $Klf2^{fl/fl}$ and *T2-cre;* $Klf2^{fl/fl}$ mice were placed on tamoxifen-infused chow (250 mg/kg; Harlan Teklad) for 5 d. In both cases, surface expression of homing receptors was assessed by flow cytometry in a subset-specific manner or transcription

levels of homing receptors present in $CD27^{-}CD11b^{+}$ FACS-sorted NK cells was determined by RT-PCR.

Dynamic Migration Assay. MACS-sorted NK cells were harvested from *Klf2*$^{fl/fl}$ and *T2-cre; Klf2*$^{fl/fl}$ littermates placed on tamoxifen-infused chow for 5 d. Before cell sorting, *Klf2*$^{fl/fl}$ and *T2-cre; Klf2*$^{fl/fl}$ splenocytes were labeled with cell tracker CMRA [10 μM] and CMFDA [10 μM] (Molecular Probes), respectively, for 40 min at 4 °C. Equal numbers of labeled NK cells (6×10^6) were injected (i.v.) into a wild-type recipient. The recipient spleen was harvested 24 h after transfer and fixed in PBS containing 4% (wt/vol) paraformaldehyde and 30% (wt/vol) sucrose before cryoembedding to preserve cell tracker dyes before immunohistochemical analysis. Eight-micrometer cryosections were then avidin/biotin/normal serum-blocked as described above before being incubated with biotinylated anti–MOMA-1 and anti-F4/80 antibodies. Biotinylated antibodies were visualized with streptavidin BV421 (BD Biosciences), and slides were mounted in fluorescence mounting media (DAKO). Fluorescent images were overlayed by using ImageJ software.

NK Cell Stimulation and Western Blots. NK cells were stimulated with plate-bound NKG2D (25 μg/mL) or NK1.1 (25 μg/mL) antibody for select time points at 37 °C. These plates were supplemented with plate-bound fibronectin (10 μg/mL) and low-dose IL-15 (0.5 ng/mL) to improve cell viability. Alternatively, NK cells were labeled with biotinylated anti-NK1.1 (10 μg/mL) or anti-NKG2D (10 μg/mL) at 4 °C before cross-linking with streptavidin PE (10 μg/mL) at 37 °C for 1–3 h. To inhibit proteasome, PI3K, or MEK1 activity, NK cells were cultured with MG132 (10 μM), LY294002 (10 μM), or PD98059 (10 μM), respectively. Following stimulation, NK cells were washed with PBS and lysed in PBS-RIPA buffer (1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 2 mM EDTA, 2 mM EGTA) with protease inhibitor mixture 2 (Sigma) for 15 min at 4 °C before pelleting debris. Lysates were resolved on a 10% SDS/PAGE and probed by using anti-mouse KLF2 (Millipore) and anti–α-tubulin (Santa Cruz Biotechnology). Densitometry was measured by using ImageJ software.

Real-Time PCR Primer Sequences. RNA was extracted by using the RNeasy Micro Kit (Qiagen), and cDNA was generated by using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time reactions were performed by the iQ Sybr Green Supermix (Bio-Rad) and iCycler eQ Real-Time PCR Detection System (Bio-Rad). Data were expressed as 2^[(CT KLF2-deficient experimental gene – CT KLF2-deficient Gapdh) – (CT control experimental gene – CT control Gapdh)].

Klf2-F/R: GCGGCAAGACCTACACCAAGAG, CTTTCGGTAGTGGCGGGTAAGC

Gapdh-F/R: GCCTTCCGTGTTCCTACC, GCCTGCTTCACCACCTTC

Cx3cr1-F/R: CGACATTGTGGCCTTTGGAACCAT, AGATGTCAGTGATGCTCTTGGGCT

Edg8-F/R: CTTGCTATTACTGGATGTCGC, GTTGGAGGAGTCTTGGTTGC

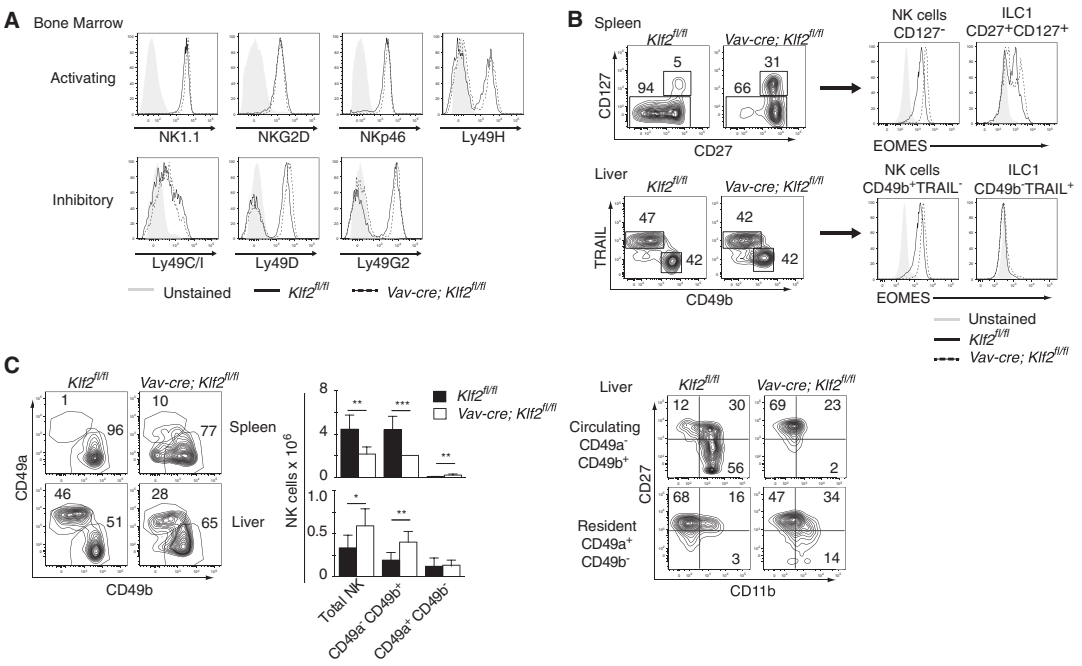

Fig. S1. NK cell MHC licensing, ILC1 homeostasis, and liver-resident NK cell homeostasis is intact in *Vav-cre; Klf2$^{fl/fl}$* mice. (*A*) Histogram overlays of activating and inhibitory receptors expressed on the surface of NK cells (CD122$^+$ Lin$^-$ NK1.1$^+$) isolated from the bone marrow of *Klf2$^{fl/fl}$* versus *Vav-cre; Klf2$^{fl/fl}$* animals. This experiment was repeated twice. (*B*) Conventional lineage markers were used to identify ILC1 cells in the spleen (CD27$^+$ CD127$^+$) and liver (CD49b$^-$ TRAIL$^+$) of *Klf2$^{fl/fl}$* and *Vav-cre; Klf2$^{fl/fl}$* mice after gating on CD122$^+$ Lin$^-$ NK1.1$^+$ cells. Consistent with the literature (24), EOMES$^+$ ILC1 cells are confined to the spleen. (*C*) Liver-resident NK cells, which are distinct from conventional NK cells (25) were found at normal numbers in *Vav-cre; Klf2$^{fl/fl}$* mice. CD49a$^+$ CD49b$^-$ NK cells (gated on CD122$^+$ Lin$^-$ NK1.1$^+$ cells) were primarily confined to the liver in both sets of animals. Increased CD49a$^-$ CD49b$^+$ NK cells found in the liver of *Vav-cre; Klf2$^{fl/fl}$* mice were CD27$^+$CD11b$^-$ (R1). This experiment was repeated twice by using three mice per cohort. *P < 0.05; **P < 0.01; ***P < 0.001.

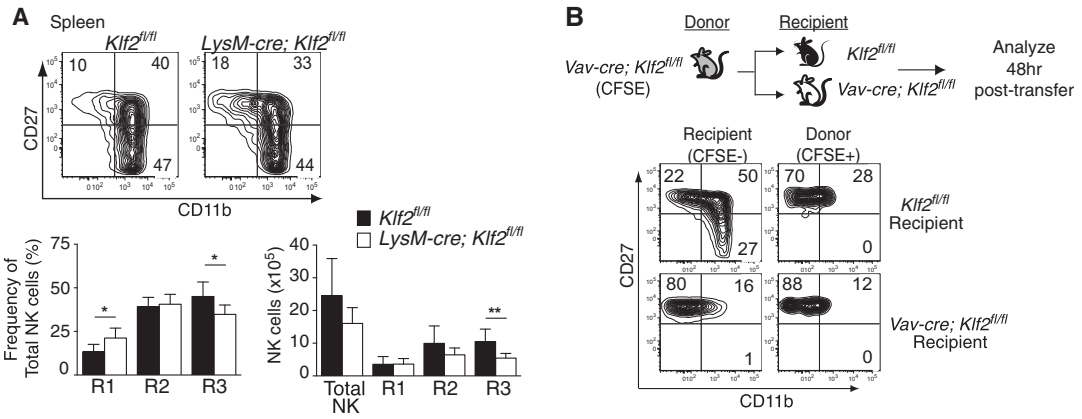

Fig. S2. Defective NK cell homeostasis is consistent with a cell-intrinsic mechanism. (*A*) Flow cytometric analysis of CD122$^+$ Lin$^-$ NK cells harvested from the spleens of 8-wk-old *Klf2$^{fl/fl}$* versus *LysM-cre; Klf2$^{fl/fl}$* littermates. n = 7 mice per cohort. (*B*) CFSE-labeled CD19-depleted *Vav-cre; Klf2$^{fl/fl}$* splenocytes (2.5 × 10^7) were adoptively transferred into *Klf2$^{fl/fl}$* or *Vav-cre; Klf2$^{fl/fl}$* recipients. CFSE$^+$ CD122$^+$ Lin$^-$ NK cells were analyzed 48 h after transfer to determine whether neighboring cells could rescue KLF2-deficient NK cell differentiation. This experiment was repeated twice. *P < 0.05; **P < 0.01.

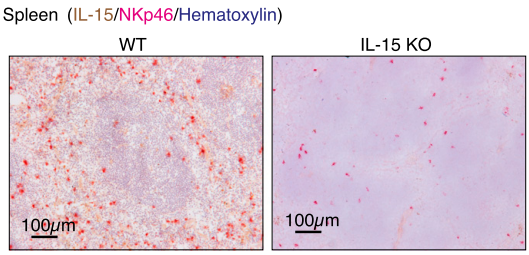

Fig. S3. Spleens harvested from wild-type (WT) and IL-15–deficient (IL-15 KO) mice were costained for NKp46 (pink), hematoxylin (purple), and IL-15 (brown) to verify IL-15 antibody specificity.

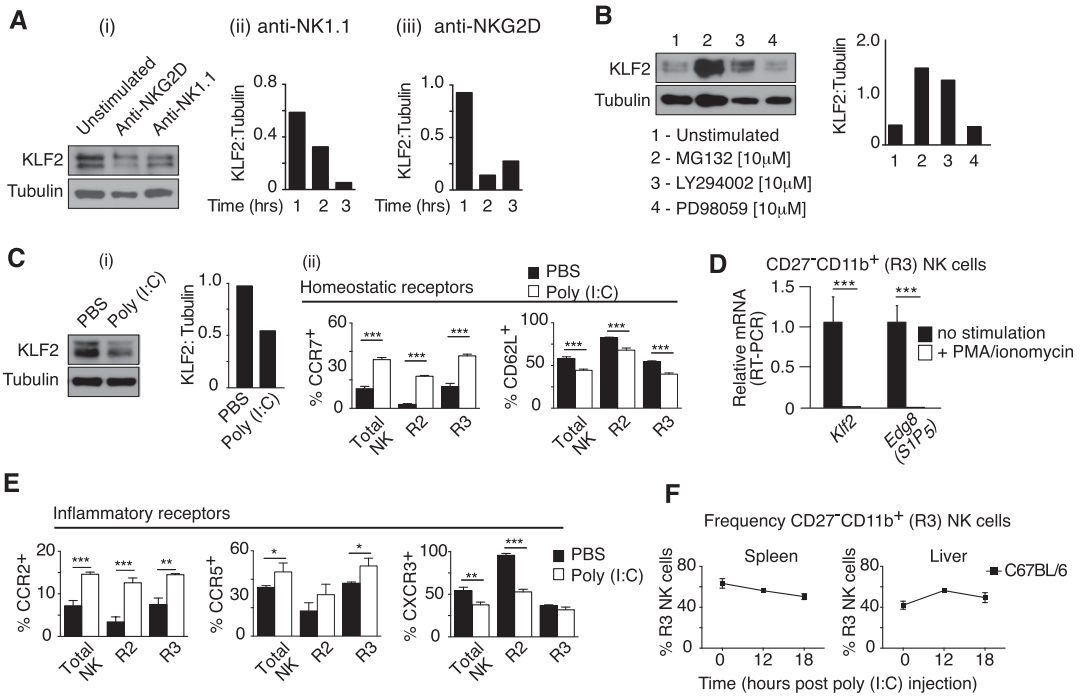

Fig. S4. Activated NK cells degrade KLF2 and alter homeostatic and inflammatory homing receptors. (*A*) Engagement of activation receptors promotes KLF2 degradation. Representative KLF2 immunoblot at 3 h (*i*) and densitometry plots of KLF2 relative to tubulin following stimulation of MACS-sorted NK cells with NK1.1 (*i*) or NKG2D (*iii*) antibody. This experiment was repeated twice. (*B*) KLF2 immunoblot of MACS-sorted NK cells cultured for 4 h ± MG132 (proteasome inhibitor), LY294002 (PI3K inhibitor), or PD98059 (MEK1 inhibitor). This experiment was repeated twice. (*C*) In vivo activation of NK cells promotes KLF2 degradation and altered expression of homeostatic homing receptors. (*i*) KLF2 immunoblot and densitometry of splenic NK cells harvested from control (PBS) versus poly(I:C)-treated mice 16 h after injection. This experiment was repeated twice. (*ii*) Flow cytometric analysis of splenocytes harvested from wild-type mice treated with PBS (black) versus poly(I:C) (white). Sixteen hours after injection, CD49b$^+$Lin$^-$ NK cell subsets (R2, CD27$^+$CD11b$^+$; R3, CD27$^-$CD11b$^+$) were examined for surface expression of homing receptors that recognize constitutively expressed ligands. $n = 3$ mice per group, repeated twice. (*D*) Relative mRNA expression of *Klf2* and *Edg8* (S1P5) following (PMA + ionomycin) stimulation of FACS-sorted CD27$^-$CD11b$^+$ NK cells (4 h) harvested from wild-type mice. This experiment was performed once in triplicate. (*E*) Same as *C*(*ii*), except cytometric analysis focused on homing receptors that recognize chemokines associated with inflammation. (*F*) Frequency of mature (CD27$^-$CD11b$^+$) NK cells in the spleen and liver of wild-type mice over time following poly(I:C)-treatment. $n = 3$ mice per time point. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.