Stage-specific requirement for Eomes in mature NK cell homeostasis and cytotoxicity

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Stage-Specific Requirement for Eomes in Mature NK Cell Homeostasis and Cytotoxicity

Highlights
- Induced Eomes deletion results in a rapid decrease in NK cell numbers
- Eomes-deleted stage III NK cells exhibit increased apoptosis
- Eomes-deleted stage II and III NK cells exhibit differentiation defects
- Induced Eomes deletion compromises NK cytotoxicity and MHCI$^{-/-}$ rejection in vivo

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In Brief
The transcription factor Eomes is important for early natural killer (NK) cell development. Wagner et al. utilize an inducible, type 1 ILC-specific cre model to demonstrate a stage-specific role for Eomes in NK cell survival and homeostasis as well as a persistent requirement for Eomes in promoting NK cell cytotoxicity.

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INTRODUCTION

Natural killer (NK) cells are the founding members of a family of innate lymphoid cells (ILCs) (Colonna, 2018) that also includes helper ILC1s, ILC2s, and ILC3s (Cherrier et al., 2018). NK cells protect against infection and mediate antitumor responses via two primary effector functions: production of immunomodulatory cytokines and direct cytotoxicity (Caligiuri, 2008; Vivier et al., 2008). NK cells recognize target cells based on the integration of signals from numerous germline DNA-encoded activating and inhibitory receptors. In general, activating receptors recognize cell stress ligands, while inhibitory receptors recognize major histocompatibility complex (MHC) class I molecules (Lanier, 2005). In addition, NK cells constitutively express cytokine receptors that support their development and tune their function by ligating activating or inhibitory signals in the surrounding environment (Romee et al., 2014; Yang et al., 2010).

The developmental and transcription factor programs of NK cells are distinct from those of helper ILCs. ILC development begins in the bone marrow (BM) from the common lymphoid progenitor (CLP), which gives rise to both the NK and helper ILC lineages. Thereafter, most terminal NK cell maturation takes place in peripheral tissues (Cherrier et al., 2018; Yu et al., 2013). Numerous transcription factors are required to regulate ILC development and promote the different lineages (Ishizuka et al., 2016; Leong et al., 2017). NK cells and ILC1s both express T-bet, but of type-1 ILCs, only NK cells express Eomesodermin (Eomes), another T-box transcription factor critical for their development, and mice with hematopoietic- or constitutive NK-cell-specific Eomes deletion display a severe phenotype (Russ et al., 2000). An important role for Eomes in NK cell biology has been demonstrated in CD8 T cell biology; it promotes the development of central memory CD8 T cells as well as the cytotoxic T cell effector program by positively regulating interferon γ (IFN-γ), perforin, and granzyme B (Banerjee et al., 2010; Intlekofer et al., 2005; Pearce et al., 2003). Eomes is also critical for NK cell development, and mice with hematopoietic-or constitutive NK-cell-specific Eomes deletion display a severe phenotype (Banerjee et al., 2010; Intlekofer et al., 2005; Pearce et al., 2003). Eomes is also critical for NK cell development, and mice with hematopoietic-or constitutive NK-cell-specific Eomes deletion display a severe phenotype.
Figure 1. Tamoxifen Induces Robust and Type-1-ILC-Specific cre Activity in Mice Harboring the Ncr1-iCreERT2 Knockin Locus

(A) Schematic depicting the Ncr1-iCreERT2 locus and mouse model.

(B) Mean ± SEM type 1 ILC (NK1.1+CD3-) and T cell (CD3+) percent YFP+ in the blood (Bl), spleen (Sp), bone marrow (BM), lymph node (LN), and liver (Liv) of Ncr1-iCreERT2 Rosa26YFP<sup>LSL</sup> mice 2 days following three consecutive daily doses of 3 mg tamoxifen in corn oil (or no tamoxifen [No Tam]). Representative flow cytometry data from the spleen of a tamoxifen-treated mouse shown to the right. n = 2–3 mice per group, two independent experiments.

(D) 3mg tamoxifen via oral gavage

Day 1 Day 2 Day 3 Day 5-6 Day 9

Tam-3d Tam-6d Tam-9d

(E) Eomes WT Eomes ΔΔ

% Eomes+ YFP+ NKp46+ cells

(F) Sp BM

Legend continued on next page
and global decrease in conventional NK cell numbers but preserved ILC1s (Daussy et al., 2014; Gordon et al., 2012; Pikovskaya et al., 2016). Eomes is expressed by NK cells of all maturation stages, with highest expression in the relatively immature stage II and III populations (Gordon et al., 2012). NK cell terminal maturation is thought to rely on T-bet, whose expression increases in terminally mature (stage IV) NK cells, opposite to the decrease in Eomes (Daussy et al., 2014; Townsend et al., 2004). Functionally, Eomes expression correlates with NK cell IFN-γ production in vivo (Gill et al., 2012) and Prf1 transcription (Pearce et al., 2003), but T-bet has also been shown to regulate NK cell cytotoxic protein expression (Townsend et al., 2004). Thus, the importance of Eomes in mature NK cell homeostasis and function remains unclear.

Studies of Eomes in NK cell homeostasis and function have been limited by a lack of appropriate inducible genetic models. In the constitutive Ncr1-cre models available (and similarly for Vav1-cre), cre-driven Eomes deletion in immature BM NK cells results in abortion of NK cell development and thus precludes the study of Eomes in mature NK cell biology (Gordon et al., 2012; Pikovskaya et al., 2016). Indeed, what were once thought to be Eomes-negative NK cells in such constitutive models are now better understood to have been type 1 ILCs (Gordon et al., 2012). To overcome this limitation, we developed a type-1-ILC-specific tamoxifen-inducible cre mouse model and confirmed its properties using a Rosa26-YFP reporter allele. Eomes-floxed mice were crossed to this model in order to elucidate the role of Eomes in mature NK cell biology. Here, we studied the impact of induced Eomes deletion on mature NK cell homeostasis and function. We demonstrate that there are Eomes-dependent (stage II/III) and independent (stage IV) murine NK cell subsets and that loss of Eomes does not reverse NK cell maturation, as has been previously suggested (Gordon et al., 2012). In addition, we demonstrate a requirement of ongoing Eomes expression for NK cell cytotoxicity and in vivo responses to MHC-I-deficient target cells.

RESULTS

The Ncr1-iCreERT2 Tamoxifen-Inducible cre Model Specifically Activates cre within Type 1 ILCs

Mouse models with constitutive type 1 ILC-specific cre expression utilizing Ncr1 regulatory elements (Eckelhart et al., 2011; Nanni-Mancinelli et al., 2011) have limitations. In these models, cre expression initiates with normal Ncr1 gene expression in immature BM stage I NK cells (Walzer et al., 2007). Hence, loxP-directed excision events occur early in NK cell development, precluding the study of developmentally critical genes in mature NK cells. To overcome this barrier, we developed a type-1-ILC-specific, tamoxifen-inducible cre mouse (Figure 1A) generated by genetic targeting of a tamoxifen-responsive iCreERT2 cassette into the Ncr1 locus. This cassette is linked to Nkpp46 C-terminal translation via a P2A ribosomal skip site. This Ncr1-iCreERT2 model was crossed to a reporter allele consisting of a lox-stop-lox (LSL)-flanked YFP cassette genetically targeted to the Rosa26 locus in order to track cre nuclear activity (Srinivas et al., 2001). To test the timing of cre expression in this model, mice underwent oral gavage with 3 mg tamoxifen for 3 consecutive days (Heger et al., 2014; Herold et al., 2014), and 3 days later, YFP expression was analyzed in various tissues (Figure 1B). YFP expression was observed in Nkp46+ cells of the blood, spleen, and liver (∼90% YFP+) as well as BM and lymph node (LN) (∼80% YFP+). YFP expression was restricted to Nkp46+ cells and not expressed by other hematopoietic lineages, including T cells (Figure 1B; data not shown). Similar to other iCreERT2 models (Kristianto et al., 2017; Maurel et al., 2019), mature (8- to 12-week-old) Ncr1-iCreERT2 mice had small background levels of cre nuclear localization (∼5%–10%) in Nkp46+ cells in the absence of tamoxifen that increased slowly over time (Figures 1B and S1). Therefore, in this report, experiments were performed in 8- to 12-week-old mice unless otherwise noted.

This model is also useful to assess cellular dynamics within the Nkp46+ ILC compartment. NK cells and tissue-resident ILC1s were compared in a 3-day tamoxifen “pulse” YFP “chase” experiment. Here, we treated Ncr1-iCreERT2 x Rosa26YFP-LSL mice with tamoxifen and then assessed splenic and liver NK cells as well as liver ILC1s for YFP positivity over the next 5 weeks. The proportion of YFP+ liver ILC1s remained high (∼90%), consistent with replenishment from NKp46+ tissue-resident populations and/or less cellular attrition. In contrast, splenic and liver NK cells displayed considerable turnover, and YFP positivity returned to near-baseline levels by the end of the 5-week period (Figure 1C). Thus, Ncr1-iCreERT2 x Rosa26YFP-LSL mice exhibited robust and specific cre activity in Nkp46+ ILCs after tamoxifen administration, which was tracked in subsequent experiments using YFP. For the remainder of the study, experiments were performed at three time points relative to tamoxifen administration: Tam-3d, Tam-6d, and Tam-9d (Figure 1D).

Tamoxifen Rapidly Eliminates Eomes in Nkp46+ Cells of Ncr1-iCreERT2 x Eomes<sup>Cre</sup> Mice

We next crossed Ncr1-iCreERT2 x Rosa26YFP-LSL mice to mice with floxed Eomes alleles (Zhu et al., 2010). Eomes allelic excision was confirmed in splenocytes of Ncr1-iCreERT2<sup>Ki/wt</sup> x Rosa26YFP-LSL x Eomes<sup>fl/d</sup> (henceforth ILC-Eomes<sup>Δ/Δ</sup>) mice
Figure 2. Induced Eomes Deletion Results in a Significant Decrease in Global NK Cell Numbers, with a Particularly Profound Loss of Stage III NK Cells

(A) Mean ± SEM, YFP⁺ NKp46⁺ cell percentage of lymphocytes and number in the blood (Bl; per mL), spleen (Sp), BM (per femur), LN (per inguinal LN), or liver (Liv) of ILC-Eomes⁻/⁻ versus WT mice following the Tam-6d regimen.

B) Spleen

Blood

BM

LN

Liver

C

Liver

D

Normalized T-bet MFI

YFP⁺ NKp46⁺ Cells

B) Spleen

Blood

BM

LN

Liv

Eomes WT

Eomes Δ/Δ
after the Tam-3d regimen, as PCR analysis of the Eomes locus revealed excision in flow-sorted YFP+ NK cells but not YFP- T cells (Figure 1E). This was verified at the protein level after the Tam-6d regimen using flow cytometry in ILC-EomesΔ/Δ compared to control NK cells (Figure 1F). Thus, tamoxifen-induced cre efficiently translocated to the nucleus and excised Eomes in mature NK cells within 2 days.

**Induced Eomes Deletion Results in a Rapid Loss of NK Cells, Most Prominently Stage III**

To assess the impact of induced Eomes deletion on the NK cell compartment, we treated ILC-EomesΔ/Δ and control mice with the Tam-6d regimen and then assessed NK cell numbers and maturation. We observed a significant decrease in global YFP+ NK cell numbers in ILC-EomesΔ/Δ compared to wild-type (WT) control mice in all tissues examined (blood, spleen, BM, LN, and liver; Figure 2A). Notably, induced Eomes deletion had a particularly profound effect on less mature stage II (CD27+/CD11b+) and stage III (CD27+/CD11b+) NK cells. Stage III NK cells, in particular, were significantly decreased in number and percentage in all tissues analyzed (Figure 2B). While stage IV (CD27−CD11b+) NK cell numbers were reduced in the blood, BM, and LN in ILC-EomesΔ/Δ mice, their relative proportion increased in all tissues except the liver, where it was unchanged. As expected, Eomes-dependent NK cell numbers were decreased in both the proportion of YFP+ NKP46+ cells and absolute number in the liver, while the proportion of Eomes-independent ILC1s increased, but numbers remained unchanged (Figure 2C) (Sojka et al., 2014). Despite evidence that Eomes and T-bet negatively cross-regulate one another (Daussy et al., 2014), we did not observe increased T-bet protein levels in ILC-EomesΔ/Δ NK cells (Figure 2D). Thus, induced Eomes deletion negatively affects NK cell numbers and homeostasis, particularly those of the stage III subset.

**Loss of Stage III NK Cells in ILC-EomesΔ/Δ Mice Results from Apoptosis and Altered Cell-Cycling Dynamics**

We hypothesized that the decrease in NK cell numbers observed following Eomes deletion could be the result of enhanced apoptosis or altered cell cycling. This was tested at an earlier time point after tamoxifen gavage (Tam-3d), when cells undergoing apoptosis or attempting to proliferate may still be quantifiable. At this time point, >70% of splenic ILC-EomesΔ/Δ and control NK cells were YFP+, Eomes protein was nearly absent, and reductions in NK cells had started but were more modest compared to the Tam-6d time point (Figures 3A and 3B). A significantly greater fraction of apoptotic (Annexin V+) stage III NK cells were present in the spleen and BM of ILC-EomesΔ/Δ compared to WT mice (Figure 3C). This was associated with a significantly increased caspase activation in splenic stage III NK cells of ILC-EomesΔ/Δ compared to control mice (Figure 3D). To further understand this apoptosis phenotype, we performed unbiased RNA sequencing (RNA-seq) of flow-sorted stage II, III, and IV ILC-EomesΔ/Δ and WT splenic NK cells. Gene set enrichment analysis (GSEA) of the RNA-seq data revealed significant enrichment of the Hallmark Apoptosis Pathway in ILC-EomesΔ/Δ compared to WT, stage II NK cells (Figure 3E). This enrichment was not observed in stage III or IV NK cell comparisons (Figure S2). These data are consistent with the observed loss of stage III NK cells, as they suggest that induced Eomes deletion activates apoptotic machinery in stage II NK cells that die at stage III.

ILC-EomesΔ/Δ and WT NK cells were also co-stained for Ki67 (a marker of proliferation) and DNA content to assess cell-cycle dynamics (Figures 3F, 3G, and S3A). The cell-cycle distribution of bulk, stage II, and stage IV NK cells was identical between WT and ILC-EomesΔ/Δ mice in the spleen and BM (Figure S3B). However, a significantly greater percentage of stage III NK cells were in G1 in ILC-EomesΔ/Δ compared to WT mice (Figure 3F). No cells progressed to S/G2/M, however, even when analyzed an additional day after the Tam-3d regimen (Figure 3G). This suggests that induced Eomes deletion promotes a G0 to G1 transition in stage III NK cells, yet the cell cycle is unable to progress beyond that point, possibly due to cell-cycle-arrest-related apoptosis. Eomes-deleted NK cells are capable of cell division, however, as demonstrated via transfer experiments into a homeostatic proliferation environment (Figure S4). Here, both ILC-EomesΔ/Δ and Eomes WT YFP+ splenic NK cells showed robust and comparable proliferation 14 days post-transfer into Rag2−/−;γc−/− recipient mice (Figure S4B). While restrictions on our flow panel did not allow for stage-specific analyses, given the deleterious effect of Eomes on stage III NK cell numbers, we hypothesize that the proliferated ILC-EomesΔ/Δ NK cells were largely stage II and IV. Short-term proliferation of ILC-EomesΔ/Δ and WT YFP+ NK cells after 4 days in vivo was also similar but significantly reduced compared to cre-negative NK cells, suggesting that induction of cre concurrent with a strong proliferation signal may alter normal cellular proliferation (Figure S4A) (Loonstra et al., 2001). Collectively, these findings suggest that Eomes loss promotes stage III apoptosis either by directly activating apoptotic genes in precursor stage II NK cells or as a result of cell-cycle arrest.

**IL-15 Signaling Is Altered in Stage II and III NK Cells as a Result of Induced Eomes Deletion but Does Not Account for Stage III NK Cell Loss**

Interleukin-15 (IL-15) is the major cytokine involved in NK cell development, survival, and proliferation. Three major pathways are activated downstream of the IL-15 receptor (IL-15R): JAK/STAT (JAK1/3 and STAT3/5), mitogen-activated protein kinase (MAPK) (JAK1/3 and STAT1/3), and the Janus-activated kinase (JAK) signaling pathway. To assess the impact of induced Eomes deletion on IL-15 signaling, we performed bulk RNA sequencing in ILC-EomesΔ/Δ and WT NK cells. This demonstrated a significant decrease in the expression of the IL-15Rα and IL-15Rβ chains in ILC-EomesΔ/Δ compared to WT mice, indicating a decrease in the level of IL-15R signaling. However, this decrease was not sufficient to account for the observed loss of stage III NK cells, suggesting that additional factors contribute to the loss of stage III NK cells.

(B) Summary data show mean ± SEM. YFP+ NK1.1+ cell stage-specific percentages and numbers in ILC-EomesΔ/Δ or Eomes WT mice in the five different tissues. ILC1s are included in liver gating. Representative flow cytometry data are shown for the spleen.

(C) Summary data show mean ± SEM. YFP+ NK cell (CD49a+/DX5+) versus ILC1 (CD49a−/DX5−) percentage and numbers in the liver. n = 9 ILC-EomesΔ/Δ mice, 5 Eomes WT mice, two independent experiments.

(D) Summary data show mean ± SEM. T-bet MFI in YFP+ ILC-EomesΔ/Δ and WT NKp46+ cells across the type 1 ILC compartment, normalized to the mean T-bet MFI of ILC-EomesΔ/Δ NKp46+ cells in each tissue. n = 9 ILC-EomesΔ/Δ mice, 5 Eomes WT mice, two independent experiments. Data were compared using false-discovery-rate-corrected t tests.

*p < 0.05, **p < 0.01, ***p < 0.001.
and phosphatidylinositol 3-kinase (PI3K) (Mishra et al., 2014). STAT5, in particular, is known to be critical for NK cell survival, since it promotes the transcription of anti-apoptotic molecules like Bcl-2 and Mcl-1 (Huntington et al., 2007; Ranson et al., 2003). Since Eomes is known to regulate the β chain of the IL-15R (CD122) (Intlekofer et al., 2005), we hypothesized that induced Eomes deletion resulted in decreased expression of the intermediate-affinity (β/γ) IL-15R and thus decreased IL-15 signaling and STAT5 phosphorylation in vivo, leading to stage III NK cell apoptosis. Indeed, a significant decrease in CD122 expression on both stage II/III and stage IV Tam-3d-treated ILC-Eomes<sup>D</sup>/D compared to WT NK cells was observed (Figure 4A). We used phospho-flow cytometry to interrogate activation of downstream signaling pathways in response to low-dose (10 ng/mL) and high-dose (100 ng/mL) IL-15 in NK cells from WT and ILC-Eomes<sup>D</sup>/D mice treated with the Tam-3d regimen (Figure 4B). Unexpectedly, we observed no defect in pSTAT5 activation at either IL-15 concentration tested and, accordingly, no

**Figure 3. Induced Eomes Deletion Results in Increased Stage III NK Cell Apoptosis and Altered Cell-Cycling Dynamics**

ILC-Eomes<sup>β/κ</sup> and Eomes WT mice were treated with the Tam-3d regimen, and then apoptosis and cell cycling dynamics were assessed.

(A) YFP expression, percentage, and number of splenic ILC-Eomes<sup>β/κ</sup> and Eomes WT NK cells. n = 5–6 mice per group, two independent experiments.

(B) Mean ± SEM percentage of Eomes<sup>+</sup> splenic NK cells. Representative flow histogram shows Eomes protein in splenic Eomes WT (shaded blue line) and ILC-Eomes<sup>β/κ</sup> NK cells (empty blue line), and NK1.1- CD3- lymphocytes (background, shaded gray). n = 8 ILC-Eomes<sup>β/κ</sup> mice, 4 Eomes WT mice, two independent experiments.

(C) Mean ± SEM. Annexin V<sup>+</sup> ILC-Eomes<sup>β/κ</sup> or Eomes WT NK cells by stage in the BM and spleen. n = 12–17 mice per group, four to seven independent experiments.

(D) Mean ± SEM percent of ILC-Eomes<sup>β/κ</sup> or Eomes WT splenic stage II or III NK cells with activated poly-caspase expression. n = 5–6 mice per group, two independent experiments.

(E) GSEA of RNA-seq data from ILC-Eomes<sup>β/κ</sup> and Eomes WT stage II NK cells. An enrichment plot for the hallmark apoptosis pathway is shown to the left, with all significantly enriched or downregulated pathways shown to the right.

(F) Mean ± SEM proportion of G0, G1, and S/G2/M stage III Eomes WT and ILC-Eomes<sup>β/κ</sup> NK cells in the spleen and BM, with representative flow plots shown. n = 9–12 mice per group, three or four independent experiments.

(G) Mean ± SEM proportion of G0, G1, and S/G2/M stage III ILC-Eomes<sup>β/κ</sup> and Eomes WT NK cells in the spleen and BM one day after the Tam-3d regimen. n = 6 mice per group, two independent experiments. Data were compared using t tests with false discovery rate correction where appropriate.

*p < 0.05, **p < 0.01, ***p < 0.001.
difference in Bcl-2 protein expression between ILC-EomesD/- and WT NK cells (Figure S5A). In contrast, pERK and pAkt median fluorescence intensity (MFI) was decreased in ILC-EomesD/- compared to WT stage II/III NK cells in response to low-dose, but not high-dose, IL-15. This is consistent with higher levels of IL-15 being required to activate these pathways, compared to STAT5, in murine NK cells (Marcàis et al., 2014). While they are not considered to be the principal regulators of NK cell apoptosis, the PI3K/Akt/mTOR and MAPK pathways have been shown to play important roles in promoting NK cell survival (Huntington et al., 2007). However, stage III NK cells were not rescued in mice that received exogenous IL-15 concomitantly with tamoxifen (Figures S5B–S5D). Thus, the negative effect of Eomes deletion on stage III NK cell survival is likely not the result of impaired IL-15 signaling.

**Induced Eomes Deletion Impairs NK Cell Maturation**

**In Vivo**

The PI3K and MAPK pathways are involved in other aspects of NK cell homeostasis, including maturation (Tassi et al., 2007). We hypothesized that the stage III NK cell defect might also be due to impaired maturation from precursor stage II cells. To test this hypothesis, we treated ILC-EomesD/- and WT mice with the Tam-3d regimen then sorted YFP+CD27+ (stage II/III) or YFP+CD27−CD11b+ (stage IV) NK cells, transferred them into congenic recipients, and allowed them to mature for 2 weeks *in vivo*. N ≥ 3 pooled mice per group, three independent experiments. Data were compared using t tests.


different in Bcl-2 protein expression between ILC-EomesD/- and WT NK cells (Figure S5A). In contrast, pERK and pAkt median fluorescence intensity (MFI) was decreased in ILC-EomesD/- compared to WT stage II/III NK cells in response to low-dose, but not high-dose, IL-15. This is consistent with higher levels of IL-15 being required to activate these pathways, compared to STAT5, in murine NK cells (Marcàis et al., 2014). While they are not considered to be the principal regulators of NK cell apoptosis, the PI3K/Akt/mTOR and MAPK pathways have been shown to play important roles in promoting NK cell survival (Huntington et al., 2007). However, stage III NK cells were not rescued in mice that received exogenous IL-15 concomitantly with tamoxifen (Figures S5B–S5D). Thus, the negative effect of Eomes deletion on stage III NK cell survival is likely not the result of impaired IL-15 signaling.

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*p < 0.05, **p < 0.01, ***p < 0.001.

**Figure 5. Eomes Is Required for Normal NK Cell Maturation**

(A) ILC-EomesD/- and Eomes WT mice were treated with the Tam-3d regimen. Splenic NK cells were sorted to YFP+CD27+ (stage II/III) or YFP+CD27−CD11b+ (stage IV) and transferred into CD45.2 WT recipients. 2 weeks later, YFP+ cell maturation was assessed.

(B) Stage distribution of ILC-EomesD/- or Eomes WT sorted stage II/III or IV NK cells before (light blue) or 2 weeks after transfer (dark blue).

(C) Representative flow cytometry data showing maturation of stage II/III ILC-EomesD/- or Eomes WT NK cells after 2 weeks *in vivo*. N ≥ 3 pooled mice per group, three independent experiments. Data were compared using t tests.

*p < 0.05, **p < 0.01, ***p < 0.001.
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Diagram A: % Specific Killing

- Eomes WT
- Eomes ΔΔ

Effects: 1:1, 5:1

Diagram B: % NK Cells (CD45+)

- Eomes WT
- Eomes ΔΔ

Diagram C: % Granzyme B+

- WT Pre
- WT Post
- ΔΔ Pre
- ΔΔ Post

Diagram D:

- Tam-3d
- Tam-6d
- Tam-9d

- % IFN-γ
- % CD107a+

- Unstim, IL12/15, YAC-1, NK1.1

Diagram E:

- Tam-3d
- Tam-6d
- Tam-9d

- % IFN-γ
- % CD107a+

- Unstim, IL12/15, YAC-1, NK1.1

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Eomes Deletion Impairs NK Cell Cytotoxicity, but Not Cytokine Production

ILC-Eomes<sup>Δ/Δ</sup> mice were next used to assess the impact of Eomes deletion on mature NK cell function. To assess ex vivo cytotoxic responses, we treated ILC-Eomes<sup>Δ/Δ</sup> and WT mice with the Tam-3d regimen, and on the 2nd day of tamoxifen also administered poly(I:C), a synthetic Toll-like receptor 3 (TLR3) agonist that activates NK cells (Fehniger et al., 2007). 24 h later, splenocytes were harvested, and enriched NK cells were used as effectors in flow-based killing assays against MHC-class I-deficient RMA-S leukemia targets. We observed a significant decrease in RMA-S killing by ILC-Eomes<sup>Δ/Δ</sup> NK cells at both effector-to-target ratios examined (Figure 6A), consistent with prior reports showing robust granzyme B protein levels in Eomes-deficient ILCs (Gordon et al., 2012). However, RNA-seq analysis revealed a ≥2-fold decrease in perforin mRNA in stage II and III ILC-Eomes<sup>Δ/Δ</sup> relative to WT NK cells (Figure S7A), consistent with prior reports of NK cell perforin regulation by Eomes (Intlekofer et al., 2005). Indeed, we performed Eomes chromatin immunoprecipitation sequencing (ChIP-seq) on primary, IL-15-expanded murine NK cells and confirmed the Prf1 transcription start site to be a direct target of Eomes (Table S1). In addition, since inducible Eomes deletion might impact the NK cell granular exocytosis pathway more globally (Salandrini et al., 2013), we performed GSEA of our RNA-seq data for the Kyoto Encyclopedia of Genes and Genomes (KEGG) NK-cell-mediated cytotoxicity pathway and observed significant downregulation of this pathway in ILC-Eomes<sup>Δ/Δ</sup> compared to WT NK cells of all stages (Figure S7B). Thus, induced Eomes deletion appears to affect several components of the NK cell cytotoxic program, including perforin.

To complement cytotoxicity assessments, we examined in vitro NK cell cytokine and degranulation (surface CD107a) responses after stimulation. A time course of ILC-Eomes<sup>Δ/Δ</sup> versus WT NK cell function following the Tam-3d, Tam-6d, or Tam-9d regimens was utilized (Figures 6D and 6E). At each time point, we observed no differences in ILC-Eomes<sup>Δ/Δ</sup> versus WT NK cell degranulation or IFN-γ production in response to cytokine (IL-12/15) stimulation, YAC-1 lymphoma, or activating receptor NK1.1 ligation (Figure 6D). We also investigated stage-specific ILC-Eomes<sup>Δ/Δ</sup> versus WT NK cell function, and similarly, there were few substantial differences. The most consistent was a modest deficit in ILC-Eomes<sup>Δ/Δ</sup> stage II NK cell functional responses to anti-NK1.1 ligation (Figure 6E). Thus, in vitro NK cell cytokine and degranulation responses were largely preserved upon induced Eomes deletion, despite reduced ex vivo cytotoxicity.

Induced Eomes Deletion Impairs Rejection of MHC-Class-I-Deficient Splenocytes In Vivo

To investigate the importance of induced Eomes deletion on NK cell function in vivo, we assessed the ability of NK cells to reject MHC-class I-deficient targets (Bix et al., 1991). ILC-Eomes<sup>Δ/Δ</sup> and WT mice were treated with the Tam-3d regimen and injected intravenously (i.v.) on day 3 with a 50:50 mix of WT and i2 m<sup>b2m</sup>/C0 (MHC-class-I-deficient) splenocytes. 18–20 h later, mice were sacrificed and the WT:il2 m<sup>b2m</sup>/C0 ratio of transferred cells in the spleen assessed (Figure 7). i2 m<sup>b2m</sup>/C0 cells were efficiently rejected in WT mice, but no rejection occurred in ILC-Eomes<sup>Δ/Δ</sup> mice (Figures 7A–7C). Indeed, the WT:il2 m<sup>b2m</sup>/C0 ratio of transferred cells in the spleen after 18–20 h was the same for ILC-Eomes<sup>Δ/Δ</sup> mice as it was for negative control mice that had been NK cell depleted. Alterations in the NK cell Ly49 repertoire could underlie this finding, and prior publications have demonstrated that Eomes is required for the generation of a diverse Ly49 repertoire, but not its maintenance (Gordon et al., 2012). However, there were no substantial differences in Ly49 receptor expression between ILC-Eomes<sup>Δ/Δ</sup> and WT NK cells (Figure 7D), suggesting that Ly49 alterations were unlikely to account for the impaired in vivo rejection phenotype. Thus, the impaired ex vivo cytotoxicity of ILC-Eomes<sup>Δ/Δ</sup> NK cells was manifest in vivo, as the cells showed no ability to reject MHC-class-I-deficient targets.

DISCUSSION

The role of Eomes in mature NK cell homeostasis and function has been incompletely understood, in large part due to a lack of inducible, conditional genetic models. Constitutive Ncr1-cre and Vav-cre models have conclusively demonstrated that DX5<sup>+</sup> NK cells fail to develop in the absence of Eomes (Gordon et al., 2012; Pikovskaya et al., 2016). While some studies attempted to investigate the functional and phenotypic properties of cells that did develop in these models, particularly in the liver, these results are challenging to interpret in light of our current understanding that these were ILC1s and not Eomes-deficient NK cells.
cells. Since constitutive Ncr1-cre and Vav-cre models delete genes early in NK cell development (stage I or earlier), they preclude the study of genes required for normal NK cell ontogeny (like Eomes). In addition, global inducible models are complicated by cell-extrinsic effects and the requirement for adoptive transfer experiments (Madera et al., 2018). To overcome these limitations, we developed a highly penetrant, tamoxifen-inducible type-1-ILC-specific cre mouse. A transgenic inducible model that expressed cre in a small minority of NKP46+ cells was previously reported but lacked highly penetrant type 1 ILC-specific gene targeting (Nabekura and Lanier, 2016). The Ncr1-creER12 model reported here results in near-complete Eomes protein loss throughout the NK cell compartment in 2 days and permitted the demonstration of a persistent requirement for Eomes in mature NK cell homeostasis and function. Like most cre-loxP systems, our Ncr1-creER12 model has limitations that must be considered during experimental design, including small background levels of cre expression in older mice and potentially altered proliferation kinetics. These limitations reinforce the importance of having a reporter for cre activity in experimental mice.

Eomes deletion negatively affected NK cell homeostasis, with a particularly profound effect on stage III NK cells. We determined that the stage III deficit arose from several mechanisms, including increased apoptosis (likely caspase dependent), altered cell cycling, and impaired maturation from stage II NK cells. IL-15-induced activation of PI3K/Akt/mTOR and MAPK was impaired in response to low-dose IL-15 in ILC-EomesΔΔ NK cells, yet exogenous IL-15 failed to rescue stage III NK cells in vivo. Thus, Eomes appears to regulate NK cell survival via mechanisms that are not rescued by exogenous IL-15. Indeed, we observed enrichment of apoptosis-related genes specifically in ILC-EomesΔΔ compared to WT stage II NK cells, which likely undergo cell death as they transition to stage III. These data reveal a role for Eomes in regulating hematopoietic cell apoptosis, a greater understanding of which could potentially lead to novel ways of supporting NK cells in the setting of adoptive transfer. The deficit in PI3K and MAPK activation by IL-15 at physiologic cytokine levels could, however, contribute to the maturation defect observed in stage II ILC-EomesΔΔ NK cells (Tassi et al., 2007). Importantly, and in contrast to prior models that induced Eomes deletion ex vivo in mature NK cells (Gordon et al., 2012), we demonstrate that mature (stage IV) NK cells are largely Eomes independent and do not revert to an immature state if Eomes is lost. In addition, although we saw no overall deficits in NK cell homeostatic proliferation upon induced Eomes deletion, stage-specific analyses as well as investigations of activation or inflammation-driven proliferation were not performed and will be important to fully appreciate the impact of Eomes deletion on NK cell proliferation. For the last decade, NK cell maturation has largely been classified based on CD27 expression in ILC-EomesΔΔ NK cells.
suggest that an alternate way of characterizing NK cell maturation is based on Eomes dependence. As such, stage II/III NK cells are Eomes dependent, while stage IV NK cells are predominantly Eomes independent. Terminally mature NK cells downregulate Eomes and upregulate T-bet, and so we hypothesize that stage IV NK cell homeostasis is T-bet dependent. Additional studies utilizing the inducible cre model with both T-bet and Eomes floxed alleles will clarify these T-box transcription factor requirements.

Functionally, we observed that induced Eomes deletion impaired NK cell killing, as evidenced by both in vivo rejection of MHC-class-I-deficient splenocytes and ex vivo killing of tumor target cells. While the decreased NK cell number arising from induced Eomes deletion could be a contributing factor, the killing defect in vivo was disproportionate to the NK cell deficit, and the ex vivo killing assay was performed with equal NK cell numbers. Thus, Eomes deletion impairs NK cell killing in vivo and ex vivo via NK-cell-intrinsic mechanisms. NK cells kill via exocytosis of cytotoxic-protein-containing granules and via the ligation of death receptors on target cells. Ongoing Eomes-regulated transcription is not required for short-term granzyme B protein expression, as we observed no reduction in granzyme B protein levels in ILC-EomesΔ/Δ compared to WT NK cells. In contrast, RNA-seq revealed a significant reduction in perforin mRNA in ILC-EomesΔ/Δ stage II and III NK cells compared to WT, and Eomes ChIP-seq confirmed Prf1 to be an NK cell target of Eomes. While we were unable to quantify perforin protein due to technical limitations of available flow-based antibodies, decreases in perforin protein are likely to have occurred given the perforin sensitivity of RMA-S cells (Wallin et al., 2003). However, perforin mRNA was unchanged by induced Eomes deletion in stage IV NK cells, and since these cells are major NK cytotoxic mediators, this suggested that other components of the NK cell cytotoxic machinery may have been altered by induced Eomes deletion. Indeed, we observed downregulation of the KEGG NK-cell-mediated cytotoxicity pathway in ILC-EomesΔ/Δ NK cells of all stages, demonstrating a broader role for Eomes in regulating NK cell cytotoxicity. It is also possible that alterations in death receptor ligand expression contributed to the killing defects observed. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), in particular, is expressed on tissue-resident, Eomes-negative ILC1s, so it is possible that it is upregulated upon Eomes deletion. However, RMA-S cells are relatively TRAIL and FasL resistant in short-term killing assays (Screpanti et al., 2001; Wallin et al., 2003). Thus, while it will be important to investigate death receptor ligand expression and function on ILC-EomesΔ/Δ NK cells in the future, they likely did not contribute substantially to our cytotoxicity phenotype.

Unlike the cytotoxicity results, short-term NK cell degranulation and IFN-γ production were largely unaltered by induced Eomes deletion. It has been suggested that Eomes may be important for sustained NK cell functional responses and that Eomes downregulation, for example in cells infiltrating a tumor, might be a sign of NK cell exhaustion (Gill et al., 2012). As such, it will be informative to perform studies of sustained NK cell function (such as infection with murine cytomegalovirus [MCMV] or tumors) using our inducible cre system to assess the role of Eomes in these types of NK cell responses. Intriguingly, our ChIP-seq data, performed on IL-15-expanded NK cells, revealed a weak binding peak near the granzyme B promoter (Table S1). We speculate that in settings of sustained activation, Eomes may play a role in regulating the expression of this cytotoxic protein as well and that this may be a mechanism through which Eomes loss contributes to NK cell exhaustion. A greater understanding of how Eomes deletion affects the entire NK-cell-activating (e.g., NKG2D and NKp30) and inhibitory (e.g., NKG2A) repertoire will also be important to fully understand virus and tumor cell responses.

This conditional, inducible cre model has many potential applications beyond the study of developmentally critical genes, which will make it important for addressing other outstanding questions in the type 1 ILC field. For example, it could be used to study NK cell populations that arise only after specific physiologic triggers, such as MCMV-specific innate memory or cytolytic-induced memory-like NK cells. In addition, since we demonstrated that ILC1s regenerate at a much slower rate than NK cells, or from Nkp46+ precursors, this model may be useful to selectively ablate genes within ILC1s, thereby permitting studies of the in vivo functions of these distinct but related cell types. Thus, this study provides a critical tool for the field to address new questions and avenues of investigation in type 1 ILC biology.

Limitations
Submission of this manuscript occurred during the SARS-CoV-2 pandemic, limiting our ability to perform experiments to address all issues raised during peer review. Some experiments, while of great interest, were beyond the feasible scope of the manuscript in light of restrictions imposed to contain the pandemic. These issues will be addressed in future studies, including (1) whether death receptor ligands (TRAIL and FasL) are differentially expressed on ILC-EomesΔ/Δ versus control NK cells and whether they contribute significantly to ILC-EomesΔ/Δ NK cell cytotoxicity; (2) whether induced Eomes deletion alters sustained NK cell function, as determined by an in vivo virus or tumor model; (3) if the expression of other activating and inhibitory receptors (e.g., NKG2D, NKp30, and NKG2A) differs between ILC-EomesΔ/Δ and control NK cells; (4) evaluation of perforin protein in addition to decreased mRNA in stage II and III ILC-EomesΔ/Δ NK cells; and (5) a comprehensive assessment of the impact of Eomes deletion on NK cell proliferation.

In summary, we developed a type-1-ILC-specific inducible cre mouse to identify a stage-specific requirement for Eomes in regulating NK cell homeostasis and cytotoxicity. In addition to elucidating the role of Eomes in mature NK cell biology, this study highlights the importance of complementing studies of constitutive cre with inducible models in order to elucidate the biological roles of genes at all stages of cellular development. As such, we anticipate that the Ncr1-ΔCreERT2 model will provide important insights into the nascent field of type 1 ILC biology.

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Detailed methods are provided in the online version of this paper and include the following:

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Supplemental Information can be found online at https://doi.org/10.1016/j.cellrep.2020.107720.

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AUTHOR CONTRIBUTIONS
J.A.W., P.W., and T.A.F. conceived and designed the study. All authors collected, analyzed, or assembled the data. J.A.W., P.W., and T.A.F. wrote the manuscript. All authors reviewed the data and edited and approved the final version of the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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REFERENCES


# STAR Methods

## Key Resources Table

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RESOURCES AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Todd A. Fehniger (tfehnige@wustl.edu).

Materials Availability
The Ncr1-iCreERT2 mouse generated in this study will be made available to qualified investigators by the Lead Contact, Todd A. Fehniger (tfehnige@wustl.edu) upon request.

Data and Code Availability
The accession numbers for the RNaseq and CHIPseq data reported in this paper are GEO:GSE132942 (RNaseq) and GEO:GSE133048 (CHIPseq).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
Ncr1-iCreERT2 mice were developed in the Fehniger lab on the C57BL/6J background. Mice were bred to Rosa26 YFP LSL knock-in mice (The Jackson Laboratory, stock no. 006148) as well as Eomes6/6 mice (The Jackson Laboratory, stock no. 017293). For all experiments, ILC-EomesD/D refers to Ncr1-iCreERT2 KI/WT x Rosa26YFP LSL x Eomes6/6 mice. Ncr1-iCreERT2 KI/WT x Rosa26YFP LSL x EomesW/W mice were used as WT controls in Figures 1E, 2, 3A, 3B, 3D, 3F, 3G, 5, 6A, and 6B, as well as Figures S1 and S3–S5. In the remaining figures, if these mice were limiting, Ncr1-iCreERT2 WT/WT x Rosa26YFP LSL x Eomes6/6 mice as well as CD45.1 and CD45.2 C57BL/6J mice (The Jackson Laboratory, nos. 002014 and 000664) were also utilized. CD45.1 C57BL/6J mice were utilized as WT controls in Figure S6 and as cre-negative controls in Figure S4. Rag2−/−,γc−/− mice (Taconic, no. 4111) were used as recipients for in vivo homeostatic proliferation assays (Figure S4). All mice (Eomes WT and ILC-EomesD/D) were treated with identical tamoxifen regimens for all experiments. Mice were orally gavaged with 3mg tamoxifen (Sigma) in corn oil (Sigma) for 2-3 days as described. Mice were bred and maintained in specific pathogen-free housing, and experiments were conducted in accordance with the guidelines of and with the approval of the Washington University Animal Studies Committee. Experiments were performed on 8-12 week old male and female mice.

Cell Lines
RMA-S and YAC-1 cell lines were maintained at 37°C in 5% CO2 in RPMI-1640 plus 10% FBS and supplements (10mM HEPES, 1% NEAA, 1% sodium pyruvate, 1% L-glutamine, 1X penicillin and streptomycin) following ATCC guidelines and kept in continuous culture for < 2 months.

METHOD DETAILS

Organ isolation and processing
Mice were sacrificed by CO2 asphyxiation and organs harvested immediately. Blood was harvested by cardiac puncture prior to removal of other organs. Spleens and inguinal nodes were isolated and crushed through a 70μm filter to generate a single cell suspension. Bone marrow was harvested by flushing femurs with a 23-gauge needle. Whole livers were desiccated with a tissue grinder then lymphocytes were isolated using a Percoll gradient. All tissues were ammonium-chloride-potassium lysed. Cell counts were obtained using propium iodide exclusion with a Cellometer counter (Nexcelcom).

Flow cytometry and cell sorting
Surface antibody staining was performed for 15 minutes at 4°C. Intracellular staining was performed in permeabilization buffer following fixation/permeabilization with kits from BD Biosciences (for IFN-γ staining) or Invitrogen (for Eomes, Granzyme B, Bcl2, and Ki67 staining). EomesΔ/Δ NK cells (and cre-positive WT cells) were gated on YFP+ events whenever possible. Since Invitrogen fixation/permeabilization quenches YFP, cells were pre-fixed in 1% PFA for 2 minutes at room temperature prior to fixation/permeabilization in order to preserve YFP when intracellular proteins were assessed after the Tam-6d regimen (Figures 1E and 2). However, pre-fixation was unable to preserve YFP after the Tam-3d regimen, so intracellular analyses were performed on bulk NK cells (which were nearly 80% YFP+, Figure 3A) at this time point. For phosphoflow cytometry assays, splenocytes were stimulated with recombinant mouse IL-15 (Peprotech) at 10ng/mL or 100ng/mL for 15 minutes (pSTAT5) or 1 hour (pERK, pAkt). Cells were then fixed with 1% PFA for 10 minutes at room temperature. Next, cells were permeabilized in ice cold methanol for 30 minutes at 4°C, then washed and stained overnight with phosphoflow antibodies. Flow cytometry data were collected on a Gallios flow cytometer (Beckman Coulter) and analyzed using FlowJo (Treestar) software. Cell sorting was performed on a BD FACS Aria II to greater than 95% purity.
Cell cycle and viability assays
Cell cycle was assessed using Ki67 and FxCycle Violet (Invitrogen) staining following Invitrogen Fixation/Permeabilization. For viability assays, freshly-isolated splenocytes were incubated at 37°C for 2-3 hours then assayed for poly-caspase activation (Immunochemistry Technologies) or surface Annexin V (performed in Annexin V buffer, both BD Biosciences).

**In vivo maturation experiments**
In Figure 5, ILC-EomesΔ/Δ and Eomes WT mice were treated with the Tam-3d regimen. Next, NK cells were purified via negative selection (StemCell Technologies, Inc.) from pooled ILC-EomesΔ/Δ or Eomes WT spleens and sorted to YFP+ stage II/III (CD27+) or stage IV (CD27- CD11b+). Cells were injected into separate male CD45.2 WT recipients. 2 weeks later, mice were sacrificed and the stage distribution of YFP+ splenocytes analyzed via flow cytometry. In Figure S6, NK cells were purified from pooled ILC-EomesΔ/Δ or CD45.1 WT spleens, labeled with CTV (Invitrogen) and sorted to stage II/III (CD27+) or stage IV (CD27- CD11b+) prior to tamoxifen administration. ILC-EomesΔ/Δ and Eomes WT cells of the same stage were pooled and injected into male CD45.2 WT mice, who then received 3 daily doses of 3mg tamoxifen in corn oil via oral gavage. 2 weeks later, recipient mice were sacrificed and the stage of CTV+YFP+ (ILC-EomesΔ/Δ) and CTV+CD45.1+ (WT) cells analyzed by flow cytometry.

**In vivo homeostatic proliferation**
ILC-EomesΔ/Δ and Eomes WT mice along with Cre- CD45.1 WT controls were treated with the Tam-3d regimen. Splenocytes were CTV-labeled, then NK cells were purified from pooled splenocytes via negative selection (StemCell Technologies, Inc.) and sorted to NK1.1+CD3-YFP+ (ILC-EomesΔ/Δ and WT) or NK1.1+CD3- (CD45.1WT). Sorted cells were injected in CD45.2 Rag2−/−c−/− recipients and CTV dilution of YFP+ (ILC-EomesΔ/Δ and WT) or CD45.1+ cells (CD45.1 WT) was assessed 4 or 14 days post-transfer via flow cytometry.

**ChIP sequencing**
In 3 separate experiments, purified NK cells from pooled CD45.2 Eomes WT splenocytes were expanded _in vitro_ for 4 days with 100ng/mL IL-15. Cells were then harvested and approximately 10 × 10⁶ cells were used per ChIP experiment. Chromatin immunoprecipitation was performed using the MAGnify Chromatin Immunoprecipitation System (Thermo Fisher) as per manufacturer’s instructions with a ChIP-grade anti-Eomes antibody from Abcam (ab23345). Non-immunoprecipitated DNA was used as an input control. ChIP DNA was blunt ended, had addition of “A” base to 3’ end, and had sequencing adapters ligated to the ends. The fragments were size selected to 200-600 base pairs, and underwent amplification for 15 cycles with primers incorporating p5 and p7 sequences and a unique index tag for multiplexing. The resulting libraries were sequenced using the Illumina HiSeq3000 as single reads extending 50 bases. The raw data was demultiplexed and aligned to the reference genome using Novoalign. MACS was used to call peaks. For downstream analysis, gene-associated peaks that had greater than 5-fold enrichment over input and were present in all three replicates (∆500 base pairs from each other) were identified (Table S1).

**RNA sequencing and Gene Set Enrichment Analysis**
In 3 separate experiments, ILC-EomesΔ/Δ or CD45.2 Eomes WT mice were treated with the Tam-3d regimen. Next, NK cells were purified from pooled ILC-EomesΔ/Δ or Eomes WT splenocytes and sorted to stage II (CD27+CD11b-), stage III (CD27+CD11b+), or stage IV (CD27-CD11b+). KO cells were also YFP+. Cells were stored in Trizol at −80°C until RNA isolation using the Direct-zol RNA MicroPrep kit (Zymo Research). NextGen RNA sequencing was performed using an Illumina HiSeq 2500 sequencer. RNA-Seq reads were aligned to the Ensembl release 76 top-level assembly with STAR version 2.4.0b. Gene counts were derived from the number of uniquely aligned unambiguous reads by Subread:featureCount version 1.4.5. Differential expression analysis was performed to analyze for differences between conditions and the results were filtered for only those genes with Benjamini-Hochberg false-discovery rate adjusted p values less than or equal to 0.05.

We utilized Gene Set Enrichment Analysis (GSEA) with the Molecular Signatures Database (MSigDB) (Subramanian et al., 2005; Mootha et al., 2003) developed by the Broad Institute and UC San Diego on the RNA sequencing results of sorted stage II, III, and IV ILC-EomesΔ/Δ and Eomes WT NK cells to identify biological pathways that were enriched or downregulated in ILC-EomesΔ/Δ versus Eomes WT NK cells in respective maturation stages. Pathways were considered significantly enriched or downregulated if p < 0.05 and FDR < 0.25.

**In vivo β2 m− rejection**
ILC-EomesΔ/Δ and WT mice were orally gavaged with 3mg tamoxifen in corn oil for 3 consecutive days. On the third day, mice also received 4x6 splenocytes that were a 50/50 mix of CD45.2 WT and β2 m− (a gift from Wayne Yokoyama). Transferred splenocytes were differentially labeled with CTV for discrimination. In addition, on the first day of tamoxifen administration, some control mice received 200 μg anti-NK1.1 intraperitoneally (i.p.) to control for non NK-specific killing. 18-20 hours after splenocyte transfer, mice were sacrificed and the ratio of CTVhi (WT) to CTVlow (β2 m−) splenocytes was assessed. Percent rejection was calculated as: \(1 - \frac{|\text{Ratio WT:} \beta2 \text{ m}^-/\text{Experimental}|}{\text{Ratio WT:} \beta2 \text{ m}^-/\text{NK depleted}}\) × 100 (Parikh et al., 2015).
In vitro cytotoxicity (FLoKA) assays
ILC-EomesΔ/Δ and WT mice were administered 3mg tamoxifen in corn oil via oral gavage for 2 consecutive days. On the second day, mice were also administered 300 μg of poly I:C (Invivogen) i.p. 20-24 hours post-poly I:C, splenocytes were pooled from 2-3 mice per group and NK cells were enriched via negative selection (Stem Cell Technologies). NK-enriched splenocytes were used in killing assays against CTV-labeled RMA-S targets at the indicated E:T ratios for 4 hours. Dead cells were identified via 7-AAD staining and specific killing calculated as: % 7-AAD+effector+target - % 7-AAD+target alone. Mice were cheek bled immediately prior to poly I:C administration for a baseline NK cell granzyme B readout, and splenic NK cell granzyme B was also assessed at the time of the killing assay.

In vitro functional assays
ILC-EomesΔ/Δ and Eomes WT mice were treated with the Tam-3d, Tam-6d, and Tam-9d regimens. At each time point, functional assays were performed on splenocytes. Splenocytes were either unstimulated (media alone) or stimulated with cytokines (10ng/mL IL-12 + 10ng/mL IL-15, both Peprotech), YAC-1 lymphoma targets (E:T = 10:1), or plate-bound purified anti-NK1.1 for 6 hours. Anti-CD107a (LAMP-1) antibody was added at the start of the assay to detect degranulation, and brefeldin A and monensin (BD Biosciences) were added for the final 5 hours. Cells were then surface stained, fixed/permeabilized (BD Biosciences), and intracellularly stained for IFN-γ, followed by flow cytometry analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical analyses were performed using GraphPad Prism 7 software. Tests utilized are indicated in figure legends. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.
Supplemental Information

Stage-Specific Requirement for Eomes in Mature NK Cell Homeostasis and Cytotoxicity

Figure S1. Background cre expression in Ncr1-iCreER<sup>T2</sup> mice. Related to Figure 1. Ncr1-iCreER<sup>T2</sup> x Rosa<sup>26</sup>YFP<sup>LSL</sup> mice were cheek bled every 2 weeks from 4-18 weeks of age and splenic NK cell YFP positivity assessed by flow cytometry. N=2-5 mice per group, 1 experiment.
Figure S2. RNA sequencing reveals stage-specific changes in ILC-Eomes\textsuperscript{ΔΔ} vs. Eomes WT splenic NK cells. Related to Figure 3. (A) Hallmark GSEA of ILC-Eomes\textsuperscript{ΔΔ} vs. Eomes WT stage III and IV splenic NK cells.
Figure S3. Induced Eomes deletion does not alter bulk, stage II, or stage IV NK cell cycling dynamics. Related to Figure 3. ILC-EomesΔ/Δ and WT mice were treated with the Tam-3d regimen then cell cycling dynamics were assessed. (A) Schematic of G0 vs. G1 vs. S/G2/M gating. (B) Mean ± SEM proportion of G0, G1, and S/G2/M in bulk, stage II, and stage IV ILC-EomesΔ/Δ vs. Eomes WT NK cells from the spleen and BM. Data were compared using false discovery rate-corrected t-tests.
Figure S4. Ncr1-iCreER^{T2} mice have delayed homeostatic proliferation kinetics that are not altered by induced Eomes deletion. Related to Figure 3. CD45.1 WT (Cre-), ILC-Eomes^{ΔΔ} (Δ/Δ), and Ncr1-iCreER^{T2+} Eomes WT (WT) mice were treated with the Tam-3d regimen. On day 3, splenocytes were CTV-labeled, NK cells purified, and cells were sorted as NK1.1+CD3- (Cre-) or NK1.1+CD3-YFP+ (WT and Δ/Δ). Sorted cells were transferred into CD45.2 Rag^{−/−}γc^{−/−} recipients, and homeostatic proliferation (CTV dilution) was measured 4 or 14 days post-transfer. (A) Mean ± SEM percent proliferated Cre-, WT, or Δ/Δ NK cells at the Day 4 time point. Representative flow histograms shown to the left. (B) Mean ± SEM percent proliferated WT or Δ/Δ NK cells at the Day 14 time point. Representative flow data shown to the left (Cre- shown at Day 4). N = 3-4 mice per group, 1-2 independent experiments. Data were compared using a one-way ANOVA with Tukey’s multiple comparisons testing.
Figure S5. Bcl-2 protein levels are not altered by induced Eomes deletion and high-dose IL-15 does not rescue ILC-EomesΔ/Δ stage III NK cells. Related to Figure 4. (A) ILC-EomesΔ/Δ and WT mice were treated with the Tam-3d regimen. Summary data show mean ± SEM splenic NK cell Bcl-2 positivity and MFI overall and by stage (stage II, III, IV). N = 4-6 mice per group, 1 experiment. Data were compared using false discovery rate-corrected t-tests. (B-D) ILC-EomesΔ/Δ and WT mice were treated with the Tam-3d regimen and half of the mice received 5mg IL-15 i.p. along with each tamoxifen dose. (B) Mean ± SEM YFP+ NK cell percent of lymphocytes. (C) Mean ± SEM NK cell percentage by stage. (D) Representative flow data of NK cell stage distribution. N= 2 mice per group, 1 experiment.
Figure S6. Eomes is required for normal NK cell maturation in vivo. Related to Figure 5. (A) NK cells from ILC-EomesΔ/Δ (Δ/Δ) and CD45.1 WT mice were CTV-labeled and sorted to stage II/III or IV. Δ/Δ and WT cells of the same stage were pooled and co-transferred into CD45.2 WT recipients. Recipient mice received 3 doses of tamoxifen and maturation of transferred cells was assessed 2 weeks later. (B) Stage distribution of Δ/Δ or WT sorted stage II/III or IV NK cells pre- (light blue) or 2 weeks post-transfer (dark blue). (C) Representative flow cytometry data showing maturation of stage II/III Δ/Δ or WT NK cells after 2 weeks in vivo. A modestly increased proportion of stage IV NK cells developed when tamoxifen was administered post- compared to pre-transfer (Figure 5), likely attributable to experimental variability combined with the potential bias introduced by sorting on YFP+ NK cells that have likely already initiated apoptosis. N >3 mice per group, 2-3 independent experiments. Data were compared using t-tests.
Figure S7. Inducible Eomes deletion results in global decreases in the NK cell cytotoxic program. Related to Figures 6 & 7. (A) RNA-Seq data of Prf1 mRNA fold change in ILC-EomesΔ/Δ compared to WT stage II and stage III splenic NK cells. (B) GSEA of the KEGG “Natural Killer Cell Mediated Cytotoxicity” pathway performed on RNA-Seq data from ILC-EomesΔ/Δ vs. Eomes WT NK cells of different stages