11-9-2023

T-bet and Eomes Are Required for Mature NK Cell Homeostasis and Function

Pamela Wong
Washington University in St. Louis

WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences
Immunology

Dissertation Examination Committee:
Todd Fehniger, Chair
Megan Cooper
Anthony French
Jacqueline Payton
Wayne Yokoyama

T-bet and Eomes Are Required for Mature NK Cell Homeostasis and Function

by
Pamela Wong

A dissertation presented to
Washington University in St. Louis
in partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

December 2023
St. Louis, Missouri

Table of Contents

List of Figures

Acknowledgments

I would like to first and foremost thank my thesis committee members Drs. Tony French, Wayne Yokoyama, Megan Cooper, and Jacqueline Payton for their support on my thesis work. Tony, Wayne, Megan, Jackie and their labs were invaluable in helping me develop my thesis project that spans cellular and molecular aspects of NK cell biology.

I would like to thank the fun, crazy, top-notch lab that is the Fehniger Lab. It was a privilege to be able to work in such a collaborative and vibrant team. I would like to thank my PI Todd Fehniger for his mentorship over the past 6 years. Todd was always kind, generous, and empathetic. I especially appreciated the trust Todd had in me to let me work at my own pace. This was a great deal of freedom, which was sometimes daunting but most of the time much appreciated, but Todd was always only one slack message away when I needed him.

I would like to thank our lab manager Timothy Schappe, who was possibly the very best at his job. Tim meticulously managed our lab's mouse colony, where every single mouse was accounted for, and knew exactly where everything is in the lab. Tim was always calm and collected no matter what the situation was. Even when I thought the world is ending because I could not find that one reagent I ordered, Tim was always there to let me know that he put it away, labeled with my initials, in my deli fridge area. I would like to thank the student cohort over the years in the Fehniger Lab—Julia Wagner, Margery Gang, Celia Cubitt, Jenn Tran, Sadia Afrin, and Lyra Morina for making our lab a fun place. I am thankful that together we made sure every birthday in the lab gets celebrated and made sure the candy drawer was always stocked. I

would like to give an extra shout out to Celia Cubitt, the person I spend the most time with other than my spouse Chris as we work the most similar (strange) schedules in the lab. Having the ultimate lab partner and great friend like Celia was critical for my sanity and survival in grad school. Thank you Celia for always being supportive, kind, and never getting annoyed at me for having to look at my cheat sheets to understand your latest CD8 signaling theory. I would like to thank the two pillars of the Fehniger Lab: Melissa Berrien-Elliott and Michelle Becker-Hapak for making sure everybody on the 6th and 7th floor of our lab knows what we were doing, and for designing, running, and analyzing all the clinical trials of our lab. I want to thank the most wonderful staff scientists and technicians Nancy Marin, Lynne Marsala, and Mark Foster for being the ultimate team players who were always willing to help everyone. I am thankful for the MD/PhD fellows that were always there when I needed unofficial medical advice. I would like to thank super technician Carly Neal for being my trusty mouse harvest partner. I would like to thank single cell RNA-sequencing guru Jennifer Foltz for teaching me how to play around and get lost, in a good way, in Seurat data analysis on my own. And finally, I would also like to thank the many undergrads I have had the privilege to work with over the years—Lily, Allie, and Kim—for letting me give my best attempt at teaching and mentoring.

I have come a long way since I first started in science, and I am grateful for the many mentors I have since my undergraduate days at Emory University who provided me the tools and mentorship I needed to get here. I would like to thank Dr. Steven Baker, Dr. Nitya Jacob, Dr. Elizabeth Gleim and for teaching me the fundamental concepts of biology and genetics that piqued my interest in biological research. I would like to especially thank Dr. Elizabeth Gleim for letting me work with her on her research on tick born diseases in my sophomore year, my

first research experience ever! I would like to thank Dr. Linheng Li and his lab at the Stowers Institute, for letting me to get a taste of what it is like to immerse myself in scientific research full time for a summer after my sophomore year. I would like to thank undergraduate thesis committee member and my first immunology teacher Dr. Rustom Antia. Beyond introducing me to key immunological concepts, Dr. Antia taught me importance of understanding the big picture and being able to explain the science without jargon. I also would like to thank him for instilling confidence in me that I was a capable scientist and for writing recommendation letters for me for grad school applications.

I had no hesitation in choosing my path to pursing a PhD because of my experience in Dr. Lawrence Boise's lab at the Winship Cancer Institute where I worked in my junior and senior year as an undergraduate student. I would like to thank Dr. Boise for giving me the opportunity to work in his lab, where I met some of the most influential mentors and friends in my science career. Dr. Boise and his lab allowed me to be independent, letting me troubleshoot and figure things out on my own, but they were also there to provide guidance and sympathy when I get stuck. I would like to especially thank Dr. Shannon Matulis for being my main mentor in the Boise Lab from the start. Shannon taught me how to do cell culture, western blots, co-ips, flow, many of these techniques I still do today. I would like to thank the fun graduate students in the Boise Lab at the time–Cathy, Shardule, Katie, Jason, Tyler, and Dave—who made me forward to going to lab every day. Beyond basic things like cloning and transfection, they also taught me some wisdoms on scientific research as a grad student 1. "high hopes, low expectations", 2. how to look for seminars with free food, and 3. most importantly how to be kind and supportive labmates. I was well-prepared for many aspects of grad school because the Boise Lab allowed

me to integrate into their community even though I was just an undergraduate student. I especially want to thank Cathy and Shardule who had continued to mentor me in my career even many years after we have left the Boise Lab. As I had the chance to mentor undergraduates myself over my own grad school years, it only made me appreciate my time in the Boise lab more. I thank the Boise lab for setting the gold-standard for how I strive to mentor and for the positive community I will strive to foster in any of my future positions.

I would like to thank my friends from college--Jessica, Elaine, Amelia, and Mandy—and friends from the immunology program, especially Maria Cimpean and Stephanie Crowley, for being there for me all these years despite my slow message response rate and how I talk about lab 80% of the time. Thank you all for being a community I could count on to have my back and to tell me to take a break and relax whenever I most needed that.

Most of all, I would like to thank my family for their unconditional support. My parents had always let me pursue whatever I wanted, even a career like science which they are completely unfamiliar with. I am incredibly grateful for everything they have done for me. Finally, I would like to thank my husband Chris for making coffee every morning, taking care of our sweet kitties, and taking care of me.

Pamela Wong

Washington University in St. Louis

December 2023

ABSTRACT OF THE DISSERTATION

T-bet and Eomes Are Required for Mature NK Cell Homeostasis and Function

by

Pamela Wong

Doctor of Philosophy in Biology and Biomedical Sciences

Immunology

Washington University in St. Louis, 2023

Professor Todd Fehniger, Chair

Natural Killer (NK) cells are innate lymphocytes known for their role in recognizing and killing virus-infected or tumor cells without prior sensitization. NK cells express a variety of inhibitory and activating receptors that control their effector functions. Once activated by target cells, cytokines, or activating receptor ligation, NK cells are capable of potent IFN-γ secretion in addition to direct cytotoxicity mediated by perforin and granzymes. Because of their anti-tumor function, NK cells are currently utilized in promising immunotherapy approaches.

NK cells develop in the bone marrow and arise from the common lymphoid progenitor, which also gives rise to T, B, and innate lymphoid cell (ILC) lineages. ILCs and NK cells share a closer progenitor that further gives rise to a NK progenitor that differentiates into mature NK cells. Most NK cells circulate in the blood and throughout the lymphoid organs while ILCs are tissue-resident. A main differentiating factor that distinguishes an NK cell from other ILCs is that NK cells express both Eomes and T-bet while ILCs only express either Eomes or T-bet, depending on the ILC type.

Eomes and T-bet are transcription factors in the T-box family, defined by their T-box DNA binding domains. Eomes and T-bet are the only two T-box family transcription factors

expressed by lymphocytes. T-box family transcription factors, including Eomes, are important for embryogenesis and early development. Eomes and T-bet specifically have been shown to regulate NK cell development. Nevertheless, while well-studied in their role in regulating NK development in mice, the impact of Eomes and T-bet deletion on mature NK cells is currently unknown. Furthermore, while there have been various mouse models studying Eomes and T-bet in NK cells, they used hematopoietic or constitutive knockout models that led to NK cell developmental defects, which precluded genetic loss-of-function studies on mature NK cells. As studies investigating the impact of gene deletion on mature NK cells have been limited by a lack of appropriate models, there is a gap in our knowledge about Eomes and T-bet's role in mature murine NK cell homeostasis and function, especially in human NK cells.

We hypothesized that Eomes and T-bet expression are required for mature NK cells to retain their homeostasis and function. To investigate this, we utilized a tamoxifen-inducible, NK cell specific mouse model that overcome previous mouse models' limitations to study the impact of lacking either one or both of Eomes and T-bet on mature murine NK cells. In addition, we used CRISPR/Cas9 to genetically delete Eomes and T-bet individually and together from mature human NK cells. In both mouse and human settings, we demonstrated that persistent T-bet and Eomes expression are required to maintain mature NK cell homeostasis and function. Particularly in primary human NK cells, T-bet and Eomes expression are critical to maintain NK cell identity. This study contributes to a deeper understanding of mechanisms governing NK cell homeostasis and function, which will ultimately allow for identification of novel ways to enhance NK cell immunotherapies.

Chapter 1: Introduction

1.1 NK cells

NK cells are innate lymphoid cells important for immune responses against pathogens and malignant cells(1–3). NK cells develop in the bone marrow and arise from the common lymphoid progenitor, which also gives rise to T, B, and other innate lymphoid cell (ILC) lineages(1). ILCs and NK cells share a closer progenitor, the common ILC progenitor that further gives rise to a NK progenitor that differentiates into NK cells and a common helper ILC progenitor that gives rise to ILC-1, ILC-2, and ILC-3 (4–6). Most NK cells circulate in the blood and throughout the lymphoid organs while ILCs are generally tissue-resident (7,8). Non-NK cell ILC lineages—ILC1, ILC-2 and ILC-3—are known for their production of cytokines analogous to their helper T cell counterparts(9,10).

1.1.1 NK Cell Function

Like other ILCs, NK cells can modulate immune responses through the production of cytokines like IFN-γ, but in addition to that NK cells can also directly kill diseased target cells via cytotoxic granules and death receptors (2,3,11). NK cell responses are determined by the integration of signals NK cells receive through germline-encoded, stochastically expressed activating and inhibitory receptors(12). Tumor or infected cells upregulate stress ligands that can recognized by activating receptors on NK cells like NKG2D(13). Another activating receptor on mouse NK cells, Ly49H recognizes the murine cytomegalovirus (MCMV) encoded ligand m157 that can be expressed by MCMV-infected cells(14,15). Furthermore, MHC class I-deficient tumor cells or infected cells are particularly sensitive to NK cell killing because of NK cells'

ability to recognize their lack of MHC-I (2,3). This “missing-self” recognition and rejection by NK cells is thought to be mediated through NK cell inhibitory receptors (16–18). During their development, NK cells acquire the ability to become sensitive to the loss of MHC-I signals through their inhibitory receptors, allowing the NK cells to be “licensed” or “educated” to kill cells that do not express MHC-I and maintain tolerance of cells that express MHC-I (17).

1.1.2 NK Cell Development and Maturation

After committed NK progenitors differentiate into NK cells in the bone marrow, defined by their expression of NK1.1 and NKp46, these NK cells further undergo functional maturation in the periphery to acquire their full functional capacity, NK cells mature from more proliferative and mainly cytokine-producing immature NK cell subsets into more highly cytotoxic mature NK cell subsets. In the mice, NK cells maturation is commonly identified with their surface expression pattern of CD27 and CD11b (Figure 1.1) (19). Stage I NK cells are the most immature and are $CD27^{-}CD11b^{-}$. Then they gain CD27 expression first and become $CD27^{+}CD11b^{-}$ Stage II NK cells that are highly proliferative in the bone marrow. Next CD11b expression is acquired and $CD27^{+}CD11b^{+}$ NK cells begin to express Ly49 receptors. Finally, the most mature subset Stage IV NK cells are $CD27^{-}CD11b^{+}$. Stage IV NK cells have a full arsenal of cytotoxic effectors like granzymes and perforin and are the majority of NK cells in the periphery.

Human NK cells are defined by their expression of CD56 and are commonly divided into two maturation subsets—the immature $CD56^{bright}$($CD56^{high}CD16^{low/-}$) and $CD56^{dim}$ ($CD56^{low}CD16^{high}$) (Figure 1.1). Similar to in mice, the majority of NK cells in the periphery is composed of $CD56^{dim}$ NK cells whereas $CD56^{bright}$ NK cells are primarily in the bone marrow and secondary lymphoid organs(2,20,21). Also similar Stage II and Stage III NK cells in the

mice, $CD56^{bright}$ NK cells are less cytotoxic, but are more proliferative and better that producing cytokines, than the more mature $CD56^{dim}$ NK cells at baseline(22). The process of NK cell development and maturation in both human and mice are critically controlled by T-box transcription factors Eomes and T-bet(23,24).

1.2 T-box Transcription Factors: T-bet and Eomes

T-box transcription factors are a family of transcription factors first discovered for their role in embryonic development(25). T-box transcription factors are defined by their DNA-binding motif named the T-box domain. T-box transcription factors have also been suggested to act as pioneer factors that can modulate chromatin accessibility beyond directly regulation of transcription (26–29). Eomesodermin (Eomes) was first discovered as an important regulator of *Xenopus* mesoderm formation and soon after reported to be similarly important for murine embryogenesis and mesoderm formation(30,31). Since then, Eomes has been demonstrated to be important for effector T cell function and memory differentiation (32,33). T-bet is also implicated in T cell function, first described as critical for differentiating Th1 from helper T cell precursors and promoting IFN-γ expression(34).

At the genomic level, Tbx21 (gene that encodes T-bet) and Eomes are highly homologous in their T-box DNA binding domains but the rest of the gene is very different, suggesting potentially redundant targets but also at the same time capacity to interact with different partners (34). Among the 20+ members for the T-box transcription factor family, T-bet and Eomes are the only T-box transcription factors expressed by lymphocytes. NK cells specifically are characterized by their high expression of both Eomes and T-bet at homeostasis (35).

1.2.1 T-bet and Eomes in Mouse NK cells

Expression Pattern of T-bet and Eomes in NK cells

Studies in mice have shown that T-box transcription factors, EOMES and T-BET are required for NK cell development, and their expressions persist in mature murine and human NK cells after development (35,36). Tbx21 transcript is lowly expressed in NK progenitor but is upregulated as NK cells mature (29) (Figure 1.1). Unlike low T-bet expression in bone marrow immature NK cells, Eomes is most highly expressed in Stage II and Stage III NK cells ($CD27^{+}CD11b^{-/+}$) and modestly lower in the most mature subset($CD27^{-}$, $CD11b^{+}$) (Figure 1.1) (37,38).

Regulation of T-bet and Eomes Expression

Eomes and T-bet expression are differentially regulated by different signals and transcription factors. The transcription factor Nfil3, important for the development of all innate lymphocytes, contributes to the induction of Eomes, but not T-bet, in developing NK cells (39–41). Unlike Eomes, T-bet expression is actively suppressed in NK cells in the bone marrow and thought to be induced as NK cells migrate out into the periphery(37,42). It is still currently unclear what induces and maintains the low, but still present, T-bet expression in bone marrow developing NK cell. In in the context of cytokine stimulation, in vitro studies with murine NK cells show that IL-12 and IL-15 can induce *Tbx21* mRNA expression(26,29,43). Furthermore, IL-18 and Ly49D engagement in combination with IL-12, but not without IL-12, can further increase Tbx21 mRNA (29). Additionally, IL-12 signaling through STAT4 can induce expression of T-bet, but not Eomes, during MCMV infection (44). Interestingly in CD4 T cells, T-bet, which is important for the development of Th1 subsets, does not depend on STAT4(45,46). Indeed, NK

cells and Th1 cells rely on different enhancer elements to induce T-bet expression(47). These studies indicate that T-box transcription factor expression regulation is cell-type and context-dependent. Thus, it should not be assumed that results from T cell studies of these transcription factors will be the same in NK cells, and likewise the role of T-box transcription factors at homeostasis may be different than their role during active immune responses.

Requirement of T-bet and Eomes for NK cell development

The requirement of T-bet and Eomes in NK cell development has been extensively demonstrated in various genetic loss-of-function studies using global knock-out (KO) mice as well constitutive, cell type specific KO mice. Globally T-bet deficient mice had decreased number of NK cells in the periphery (blood, spleen, liver) but have been observed to have increased NK cells in the bone marrow and lymph node(29,48,49). This accumulation in the bone marrow corresponds with increased of immature/progenitor-associated markers like cKit, alpha-5 integrin, and B220 in NK cells of global T-bet-/- mice(29). IL-15 is a key cytokines that regulate multiple facets of NK cell biology including NK cell development, survival, proliferation, and function (50–52). The critical requirement for IL-15 for NK cell differentiation from NK precursor was demonstrated in IL-15 and IL-15 receptor deficient mouse models, where NK cells are largely absent despite normal NK precursor numbers(53,54). Nevertheless, T-bet-/- mice do not have decreased expression of the IL-15 receptor component CD122, indicating that the loss of NK cells in T-bet-/- mice is not due to the inability to respond to IL-15 (29).

Unlike in T-bet deficiency, the expression of CD122 is decreased in Eomes deficient models (42). The dampened IL-15 signals in Eomes deficient NK cells is perhaps one explanation why

Eomes deficiency affect the NK cell compartment more than T-bet deficiency(48). When comparing the loss of just one of the two T-box transcription factors, the disruption of the NK cell compartment is more evident when Eomes is loss compared to the more modest NK cell loss in T-bet deficient mice(29,48,49). Global, constitutive Eomes KO is embryonic lethal, and thus initial studies of Eomes deficient NK cells were performed with *Vav*-Cre x Eomes$^{fl/fl}$ mice, where Eomes expression is abrogated after early embryonic development and specifically in the hematopoietic compartment(31,48). In this model, NK cells numbers were reduced in the periphery as well as in the bone marrow, though more modestly(48). Comparing global T-bet$^{-/-}$ and *Vav*-Cre x Eomes$^{fl/fl}$ mice side by side, Gordon et al. showed that compared to control wild type mice, NK cell frequency was 50% lower in T-bet$^{-/-}$ mice and 86% lower in *Vav*-Cre x Eomes$^{fl/fl}$ spleen; in the bone marrow, T-bet-mice had a 30% decrease in T-bet-/- while *Vav*-Cre x Eomes$^{fl/fl}$ mice had a 73% decrease in NK cell frequency(48). More recent studies that utilized constitutive, *Ncr1*-Cre to specifically knock-out of EOMES or T-BET occurring at early stages of NK cell development resulted in decreased NK cell numbers in the periphery, indicating that Eomes and T-bet promote NK cell development in a NK cell-intrinsic manner(38,55).

It has been suggested that since T-BET and EOMES have highly homologous DNA binding domains, deletion of one could be compensated by the other (24,33). This corresponds with the observation that global T-bet-/- mice NK cells have higher expression of Eomes(48). Indeed, when both T-bet and Eomes are deleted in a model where the global T-bet$^{-/-}$ mouse was crossed to *Vav*-Cre x Eomes$^{fl/fl}$ mouse, there is a striking, complete loss of NK cells in the bone marrow and the periphery(48).

NK cell functions regulated by T-bet and Eomes

Both T-bet and Eomes have been demonstrated to directly regulate the expression of hallmark NK cell effector function molecules, like IFN-γ, perforin and granzyme B(34,38,56,57). The decreased expression of these molecules by Eomes or T-bet deficient NK cells corresponds similarly decreased capacity of their functional responses. NK cells from Ncr1-Cre Eomes-/- have impaired killing of YAC-1 but IFN-γ response is in tact in vitro(38). There has been conflicting reports on whether NK cells from T-bet-/- mice can kill target cells efficiently, but it has been corroborated in multiple studies that NK cells from T-bet-/- NK cells have impaired IFN-γ response in vitro (29,38). In vivo, T-bet-/- mice have impaired control of NK cell dependent cancer models, including B16-F10 melanoma and CT-26 colorectal cancer(58,59). These tumor studies have not been performed in Eomes deficiency model, perhaps because of the obvious expected outcome with the highly reduced NK compartment in the various Eomes deficient mouse models.

T-bet and Eomes in Mature Murine NK cells

As these two transcription factors are clearly important for NK development, these T-bet and Eomes deficiency models, in which their expressions are abrogated before NK cell complete development, precluded the studies of mature NK cells. A study by Madera et al. attempted to bypass the developmental defect of these previous models by transferring T-bet$^{fl/fl}$x *Ub*-Cre and Eomes$^{fl/fl}$ x *Ub*-Cre NK cells into recipient mice which they then treated with tamoxifen after engraftment to induce T-bet and Eomes deletion(44). Their experiments with this adoptive transfer model concluded that Eomes and T-bet are both dispensable for NK cell homeostasis, which differs from a more physiological system that our lab has generated—a tamoxifen inducible, Ncr1-specific Cre mouse to perform loss-of-function experiments in an intact NK

compartment instead of during NK cell development(60). We generated an inducible, NKp46-sepcific Eomes deletion model with this mouse and revealed that EOMES was required for regulating mature mouse NK cells homeostasis and function in a stage-specific fashion (60). These discrepancies between previously published studies and our model could be due to how a such complex adoptive transfer model is not optimal for recapitulating normal physiology. My thesis continues the work of studying T-box transcription factor requirement in mature NK cells using this inducible deletion model, with a focus on inducible T-bet deletion, simultaneous inducible T-bet and Eomes deletion, and comparing inducible T-bet vs Eomes deletion in mature murine NK cells.

1.2.2 T-bet and Eomes in Human NK cells

T-bet and Eomes follow a similar expression pattern in human NK cell as in mouse NK cells, where the more immature subset($CD56^{bright}$) express higher level of Eomes than the more mature subset ($CD56^{dim}$), and vice versa for T-bet (Figure 1.1) (61). Recently, a single case of a patient with inherited T-BET deficiency was reported, with the patient exhibiting a defect in the NK cell compartment, and a separate study overexpressed EOMES and T-BET in NK progenitors cells thereby promoting NK cell differentiation (62,63). These studies suggest that the T-box transcription factors can promote human NK cell development similar to in mice.

Nevertheless, most mechanistic studies of transcription factors in human NK cells to date have been performed in cell lines, NK cells differentiated in vitro from $CD34^{+}$ derived HSC, or NK cells that have been ex vivo expanded (62,64,65). While these models provide a starting point for study of mature human NK cell biology, the requirement for T-bet and Eomes has not been

studied in unexpanded primary human NK cells, which most closely recapitulate normal NK cell physiology.

1.3 Figures

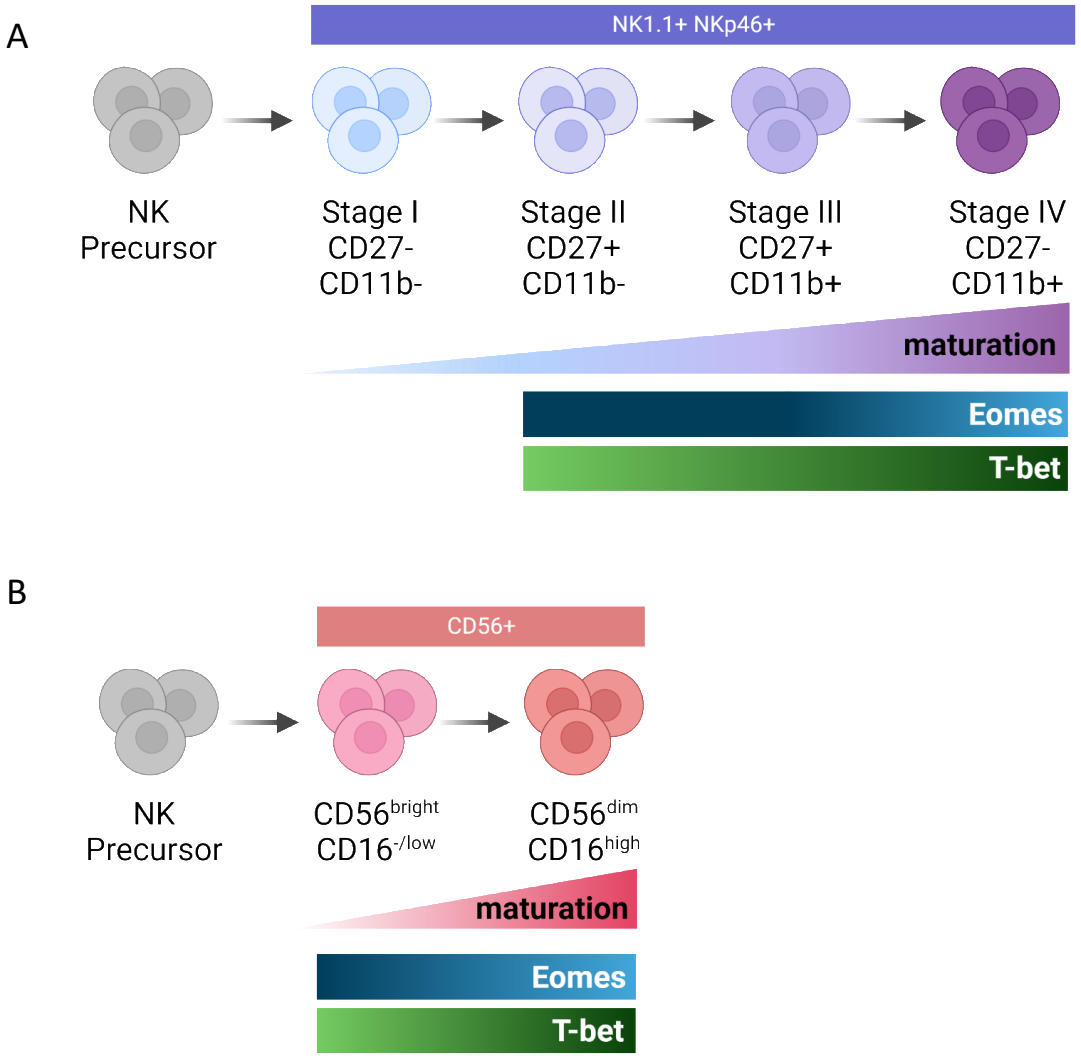

Figure 1.1. Maturation stages and relative T-box transcription factor expression in (A) mouse NK cells and (b) human NK cells.

Chapter 2: T-bet and Eomes sustain mature human NK cell identity and anti-tumor function

Published in the *Journal of Clinical Investigations* (66).

2.1 Summary

Since T-box transcription factors (TFs) T-BET and EOMES are necessary for initiation of NK cell development, their ongoing requirement for mature NK cell homeostasis, function, and molecular programming remains unclear. To address this, T-BET and EOMES were deleted in unexpanded primary human NK cells using CRISPR/Cas9. Deleting these TFs compromised in vivo anti-tumor response of human NK cells. Mechanistically, T-BET and EOMES were required for normal NK cell proliferation and persistence in vivo. NK cells lacking T-BET and EOMES also exhibited defective responses to cytokine stimulation. Single-cell RNA-sequencing revealed a specific T-box transcriptional program in human NK cells, which was rapidly lost following T-BET and EOMES deletion. Further, T-BET and EOMES deleted $CD56^{bright}$ NK cells acquired an ILCP-like profile with increased expression of ILC-3-associated TFs *RORC* and *AHR*, revealing a role of T-box TF in maintaining mature NK cell phenotypes and an unexpected role of suppressing alternative ILC lineages. Our study reveals the critical importance of sustained EOMES and T-BET expression to orchestrate mature NK cell function and identity.

2.2 Introduction

Natural Killer (NK) cells are innate lymphoid cells important for responses against pathogens and malignant cells. They direct the immune response through production of cytokines and

directly kill diseased target cells via cytotoxic granules and death receptors (2,3). In recent years, NK cell therapies have emerged as a promising option for treating cancers because of their low toxicity profile and their potent ability to drive anti-tumor responses (67–69). NK cell products can be sourced from peripheral blood, cord-blood, as well as differentiated from cord-blood or induced pluripotent stem cells (70–72). Additional strategies to enhance NK cell function such like inducing memory-like NK differentiation with cytokines (67,69,73–75), introducing a tumor-targeting chimeric antigen receptors (76), or using NK cells in combination with tumor targeting antibodies or inhibitory check point blockades are now being tested in multiple clinical trials with promising results (70). Thus, understanding the fundamentals of mature NK cell biology will inform the nascent field of NK cell immunotherapy.

Studies in mice have shown that T-box transcription factors (TFs), EOMES and T-BET are required for initiating NK cell development, and their expressions persist in mature murine and human NK cells after development (35,36). Constitutive, NK cell-specific knock-out of EOMES or T-BET occurring at early stages of development results in a deficiency of mature NK cells (29,48). In addition, T-BET also regulates mouse NK cell trafficking out of the bone marrow (49). However, because these models lack mature NK cells, the study of EOMES and T-BET in fully mature NK cells has not been feasible, and thus their ongoing importance to NK cell biology during maturity remains an open question in the field. Employing a tamoxifen-inducible NK cell-specific Cre mouse model revealed that EOMES was required for regulating mature mouse NK cells homeostasis and function, in a murine stage-specific fashion (60). Consistent with this, EOMES-deletion in murine NK cells also impacted NK cell homeostatic turnover (77). Thus, while data related to EOMES and T-BET deficiency are emerging from inducible,

conditional mouse models, our understanding of the importance of T-box transcription factors for mature human NK cell programs remains limited.

Most mechanistic studies of transcription factors in human NK cells to date have been performed in cell lines, NK cells differentiated in vitro from $CD34^+$ derived HSC, or NK cells that have been ex vivo expanded (62,64,65). While these models provide a starting point for study of mature human NK cell biology, these approaches do not recapitulate normal NK cell physiology. Recently, a single case of a patient with inherited T-BET deficiency was reported, with the patient exhibiting a defect in the NK cell compartment, and a separate study overexpressed EOMES and T-BET in NK progenitors cells thereby promoting NK cell differentiation (62,63). While these studies suggest EOMES and T-BET play a role in human NK cell development, evaluation of EOMES or T-BET genetic loss-of-function in mature human NK cells has not been reported. Further, since EOMES and T-BET have similar DNA binding motifs and may play redundant roles, examination of mature NK cells with combined EOMES and T-BET deficiency is needed to address this gap in our knowledge and elucidate T-box TFs' contribution to maintain NK cell molecular programs.

To address the importance and regulation of mature NK cell programs by T-box TFs, we utilized CRISPR-Cas9 to genetically delete EOMES and T-BET expression in unexpanded, primary human NK cells. We hypothesized that beyond their roles in regulating NK development, EOMES and T-BET are critical for maintaining the NK cell functional programs that define NK cells, including proliferation, survival, cytotoxicity, and cytokine production. To define molecular mechanisms, we also employed single-cell RNA-sequencing (scRNA-seq) and Assay for Transposase-Accessible Chromatin-sequencing (ATAC-seq) to uncover key transcriptomic

and chromatin changes in mature human NK cells with deletion of EOMES and T-BET. These findings revealed a profound dependency on T-box TFs to maintain mature human NK cell function and identity.

2.3 Results

CRISPR-editing deletes T-BET and EOMES in unexpanded primary human NK cells

Genetic manipulation of primary human NK cells has been a challenge in the field, limiting our ability to use loss- or gain-of-function to mechanistically understand human NK cell biology. Peripheral blood NK cells were freshly isolated from healthy donors, purified to >95% by negative enrichment, cultured in low dose IL-15 (required for NK cell survival), and electroporated with Cas9 mRNA along with sgRNA targeting *EOMES* or *TBX21* using the Maxcyte GT system (73). This approach achieved consistent deletion of EOMES and T-BET protein expression in both the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets without the need to expand them with high dose cytokines or feeder cells (Figure 2.1).

Since T-BET and EOMES have highly homologous DNA binding domains, deletion of one could result in compensation by the other (24,33). To address their redundancy, both EOMES and T-BET were CRISPR-edited by simultaneous electroporation of *TBX21* and *EOMES* gRNAs, which successfully abrogated both EOMES and T-BET protein expression to generate double knock-out (DKO) NK cells (Figure 2.2, A-C). To discern between all cells that received the CRISPR gRNAs and the subset that are deficient for T-BET and EOMES at the protein level, "*T+E* edited" and "DKO" will be used, respectively. We observed no significant changes in *T+E* edited NK cell survival in vitro, compared to control cells that were CRISPR-edited with *TRAC* gRNA used as control, indicating the two T-box TFs are not absolutely required for NK cell survival (Figure 2.2D). This approach of simultaneous T-BET and EOMES deletion then allows

investigation of the functional impact of naturally developed and mature primary human NK cells lacking these two T-box TFs.

EOMES and T-BET deletion does not affect short-term in vitro killing but impairs long-term in vitro killing.

One hallmark of NK cell function is their ability to eliminate MHC class I-deficient tumor cells (2,3). To assess the impact of T-box TF deficiency on NK cell's cytotoxic ability in a short-term assay, we sorted $CD56^{bright}$ and $CD56^{dim}$ NK cells 5 days after CRISPR-editing, rested them overnight in low-dose IL-15, and then co-cultured them with the MHC-I-deficient cell line K562 for 4-6 hours. In this short-term killing assay, there was no significant effect of T-box TF deficiency on NK cells' ability to kill K562 targets (Figure 2.3A). This was expected due to the presence of granzyme B and perforin protein within preformed granules in the NK cells that result in immediate cytotoxicity. To test the effect of T-box TF deficiency in a longer-term in vitro cytotoxicity assay, we utilized the IncuCyte Imaging System to monitor NK cell control of the ovarian cancer cell line SKOV-3 over the course of 6 days (Figure 2.3B). We similarly observed no difference between control NK cells and *T+E* edited NK cells at early time points but over the course of 6 days, *T+E* edited NK cells are unable to control SKOV-3 cells as effectively as control NK cells (Figure 2.3B). This is consistent with the need to replenish cytotoxic effector proteins after initial pre-formed granules are depleted.

EOMES and T-BET are required for tumor control in vivo

To assess the impact of T-box TF deficiency on NK cell's ability to control tumor in vivo, we engrafted *TRAC* gRNA-CRISPR edited (control NK), *TBX21* edited, *EOMES* edited or *T+E* edited NK cells into NOD-*scid* $IL2Rg^{null}$ (NSG) mice, which lack T, B, or NK cells and thus

allow for xenograft of human cells (Figure 2.2E) (78). Recombinant human (rh) IL-15 was administered 3x per week to support human NK cell survival. The NK cells were engrafted and allowed 4 days for TBET and EOMES protein to be downregulated in vivo. The mice were then challenged with MHC-I-deficient K562-luciferase (K562-luc) tumor cells. Mice that received control NK cells had minimal bioluminescent imaging (BLI) signals on Day 7 and Day 10 post-tumor challenge, while mice that received single *TBX21* or *EOMES* edited, and *T+E* edited NK cells have significantly higher tumor burden than those that received control NK cells (Figure 2.2, F-G), indicating reduced NK cell anti-tumor response in the absence of T-BET and/or EOMES. While mice that received single *TBX21* or *EOMES* edited NK cells still have significantly reduced tumor burden compared to mice that received no NK cells, mice that received *T+E* edited NK cells had an average tumor burden that was not significantly different than mice the did not receive NK cells (Figure 2.2G).

EOMES and T-BET are required for NK cell persistence and proliferation in vivo

We hypothesized that the significant defect in tumor control by *T+E* edited NK cells was due to reduced persistence, proliferation or decreased functionality of the DKO NK cells. To address these possibilities, the ability of NK cells to undergo homeostatic proliferation and persist in vivo without EOMES and T-BET was evaluated. *T+E* edited NK cells were engrafted into NSG mice, and 2-3 weeks later the number of NK cells in various tissues was determined (Figure 2.4A and 2.5A). Notably, on average $\geq$ 75% fewer *T+E*-edited NK cells were recovered compared to control NK cells in all three tissues investigated: spleen, blood, and liver (Figure 2.4B-D). Since *T+E* editing is not completely efficient, we expected the small fraction of T-BET^{+}EOMES^{+} wild-type (WT) NK cells to have an expansion advantage in vivo over this time course. Consistent with this, intracellular flow cytometry staining revealed that the number of *T+E* edited NK cells

that were deficient for both T-BET and EOMES (DKO) at the protein level were significantly reduced in frequency after in vivo proliferation for 2-3 weeks, compared to the in vitro Day 7 expression (Figure 2.4E). While NK cells expressing WT level of EOMES and T-BET within the *T+E* edited group were only a minority after 7 days in vitro, these WT NK cells became a majority of the NK cells recovered from mice that received *T+E* edited NK cells when assessed after 2-3 weeks (Figure 2.4E), providing further evidence that the DKO NK cells had a competitive disadvantage.

Since cell death was minimally impacted in *T+E* edited NK cells (Figure 2.2D), we hypothesized that EOMES and T-BET are required for NK cell homeostatic proliferation, and this mechanism explains the lower number of *T+E* edited NK cells recovered following engraftment into NSG mice. To assess in vivo proliferation, *T+E* edited NK cells were labeled with CellTrace Violet dye, transferred into NSG mice, and dye dilution was quantified by flow cytometry after 1.5-2 weeks (Figure 2.4F). The numbers of divisions that flow-gated T-BET and EOMES WT, single KO (ΔT-BET and ΔEOMES), and DKO NK cells had underwent were assessed. While single T-BET or EOMES deletion resulted in impaired proliferation, deleting both T-BET and EOMES profoundly reduced proliferation (Figure 2.4, G-H). In fact, a majority of DKO NK cells did not proliferate, in contrast to almost all WT NK cells having divided at least once by this time (Figure 2.4H). This proliferation defect observed in DKO NK cells is consistent with the low numbers of NK cells recovered and the increase in the frequency of WT NK cells in the NK compartment from mice that received *T+E* edited NK cells (Figure 2.4, A-E).

Since it was previously reported in a murine NK cell study that NK cell proliferation can be regulated differentially at different states of maturation (38), we further categorized the human NK cells harvested from the NSG mice into human maturation stages: $CD57^{-}NKG2A^{+}$,

$CD57^+NKG2A^+$, $CD57^+NKG2A^-$ and assessed the effect of EOMES and T-BET deficiency on the proliferation of these specific subset (Figure 2.5B). DKO of T-BET and EOMES significantly reduced proliferation of all three subsets, while single deletion of T-BET or EOMES only reduced proliferation in the more mature $CD57^+$ subsets (Figure 2.5B).

EOMES and T-BET deletion impair NK cell cytokine production

We next assessed the functionality of NK cells after they were CRISPR-edited to abrogate T-BET and EOMES expression. Unlike in vivo transferred NK cells where nearly all human NK cells recovered are $CD56^{dim}$, both $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets are readily discernable in vitro (Figure 2.1A and 2.5A). Based on the different functional characteristics of these subsets (79,80), $CD56^{bright}$ and $CD56^{dim}$ NK cells were analyzed separately. CRISPR-edited NK cells were stimulated with K562 or cytokines (IL-12 and IL-15) and assessed for their ability to degranulate (surface CD107a) or produce immunomodulatory cytokines (IFN-γ and TNF), compared to control NK cells (Figure 2.6, A-D and Figure 2.7, A-F). *TBX21* editing alone did not significantly affect NK cell function in this assay (Figure 2.6A and Figure 2.7, A and B). *EOMES* editing alone only reduced $CD56^{bright}$, but not $CD56^{dim}$, NK cell IFN-γ production after stimulation by cytokines or K562 (Figure 2.6B and Figure 2.7, C and D). Simultaneous deletion of EOMES and T-BET led to marked reduction of IFN-γ and TNF production in both $CD56^{bright}$ and $CD56^{dim}$ NK cells, even when the potent stimulating cytokine combination of IL-12, IL-15, and IL-18 was utilized (Figure 2.6, C and D, and Figure 2.7, E and F). Notably, degranulation in response to K562 cells was not affected by genetic deletion (Figure 2.7, E and F).

To allow the effect of T-BET and EOMES deletion to have more time to manifest prior to assessing these functions, we engrafted NSG mice with NK cells CRISPR-edited with *T+E*

gRNAs and 1.5-2 weeks later assessed functionality ex vivo with isolated splenocytes. Here, intracellular flow-gating was used to separately analyze NK cells that were ΔT-BET or ΔEOMES single KO or DKO at the protein level (Figure 2.6, E-H). ΔT-BET and ΔEOMES single KO NK cells have significantly impaired IFN-γ and TNF production in response to cytokine stimulation compared to WT NK cells (Figure 2.6, F-G). However, like the in vitro setting, degranulation was not significantly impacted with single T-BET and EOMES deletion alone (Figure 2.6H). DKO of both T-BET and EOMES resulted in marked reduction of NK cell function ex vivo: DKO NK cells have minimal production IFN-γ and TNF in response to the potent combined cytokine stimulation of IL-12+IL-15+IL-18 (Figure 2.6F-G). In contrast to the in vitro setting, NK cells lacking both EOMES and T-BET also have marked impairment of K562-induced degranulation (Figure 2.6H). These data demonstrate that both EOMES and T-BET are critical for primary NK cell degranulation and cytokines production upon stimulation with cellular targets or pro-inflammatory cytokines.

We also tested whether T-BET and EOMES DKO NK cells can produce similar amounts of IFN-γ compared to control NK cells if we bypass receptor signaling completely by using PMA and ionomycin to stimulate. Similar to responses following K562 or cytokine stimulation, PMA and ionomycin-stimulated T-BET and EOMES DKO NK cells were only able to produce minimal IFN-γ (Figure 2.6I).

EOMES and T-BET deletion impairs NK cell numbers and effector molecules expression at tumor sites

To assess the in vivo responses of EOMES and T-BET deficient NK cells, we followed the same experimental approach as in Figure 2.2E, but sacrificed K562-luc tumor-bearing mice 3 days after tumor injection to assess numbers and phenotypes of control vs T-BET and EOMES DKO

NK cells (Figure 2.8). At this time point, while *T+E* edited NK cells are able to traffic to the tumor infiltrated lung and liver, *T+E* edited NK cells percentages and absolute numbers are reduced compared to control NK cells (Figure 2.8, A-C). In addition, T-BET and EOMES DKO NK cells harvested from tumor-bearing organs have reduced IFN-γ, GRANZYME B, and PERFORIN compared to WT NK cells (Figure 2.8D).

IL-15 and IL-12 receptor signaling responses are impacted by lack of T-BET and EOMES

Multiple signaling pathways downstream of the IL-15 receptor (IL-15R) impact NK cell functions, including survival, proliferation, cytokine production, and cytotoxicity (81). We hypothesized that signaling downstream of cytokine receptors may be defective in NK cells that lack T-BET and EOMES, thereby explaining their proliferative and functional defects. The NK cell response to IL-15R stimulation was assessed by measuring phosphorylation of signaling intermediate proteins in pathways downstream of IL-15R (STAT5, ERK1/2 (MAPK), and AKT pathways) by flow cytometry upon IL-15 stimulation (Figure 2.9, A and B) (81). In both $CD56^{bright}$ and $CD56^{dim}$ NK cells, phosphorylation of STAT5 was unaltered, even in NK cells that lack both T-BET and EOMES, consistent with their intact survival. However, phosphorylated (p)ERK was reduced in *TBX21* or *EOMES* single edited NK cells, and this defect was markedly more evident in *T+E* edited NK cells (Figure 2.9, A and B). pAKT was only significantly decreased in *T+E* edited NK cells in both $CD56^{bright}$ and $CD56^{dim}$ subsets (Figure 2.9, A and B). The responsiveness of *TBX21*, *EOMES* and *T+E* edited NK cells to IL-12R signaling was assessed by STAT4 phosphorylation (Figure 2.9C). pSTAT4 of $CD56^{bright}$ NK cells was significantly impacted by deletion of one or both T-box TFs. pSTAT4 of $CD56^{dim}$ NK cells followed the same trend, with statistically significant reduction in *TBX21* edited and *T+E* edited NK cells, and a non-significant trend (p=0.06) in *EOMES* edited NK cells (Figure 2.9C).

Western blots of control, *TBX21* edited, *EOMES* edited, and *T+E* edited NK cell lysates show that total AKT, ERK1/2, and STAT4 proteins were not affected by T-BET or EOMES deletion, indicating that the differences in phosphorylated-proteins observed were not due to decreases in total protein level of the specific signaling protein (Figure 2.10A-B). We also assessed the expression of the IL-15R subunit CD122 and found no significant differences between control and *T+E* edited NK cells maintained in vitro at the time point when the phosphorylation assay was performed on 6 days post-electroporation (Figure 2.10C).

EOMES and T-BET are required to sustain the NK cell transcriptional program

As T-BET and EOMES primarily orchestrate gene transcription, we evaluated NK cell transcriptomes following T-BET and EOMES deletion. Single-cell RNA-sequencing (scRNA-seq) (10X Genomics) was performed on *TRAC*-edited (control) and *T+E* edited NK cells. CRISPR-edited NK cells from each donor were transferred into NSG mice, followed by rhIL-15 support 3x per week for survival. After 1 week, splenocytes of the NSG mice were isolated and human NK cells were purified by flow sorting ($mCD45^-$, $hCD45^+$, $hCD3^-$, $hCD56^+$, >98% purity; 3 donors in vivo). To provide an in vitro comparison to account for in vivo phenotypic changes, and to allow analysis of both $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets, CRISPR-edited NK cells were cultured for 1 week in vitro in parallel (5 donors total in vitro). In vitro and in vivo data sets were analyzed separately, contrasting control edited and *T+E* edited NK cells (Figure 2.11A). This approach revealed distinct clusters of NK cells in uniform manifold approximation and projection (UMAP) space (Figure 2.11, B and C).

The single cell sequencing approach allowed us to distinguish KO cells within the heterogeneous population of edited cells in the analysis. We first assigned in vitro clusters to be either $CD56^{bright}$ or $CD56^{dim}$ NK cells based on established scRNA-seq expression patterns of markers

for each subset (82,83). Almost all NK cells recovered 1 week following transfer in vivo were $CD56^{dim}$ NK cells, since all clusters express the $CD56^{dim}$ subset marker *FCGR3A* (CD16) (Figure 2.11C). While EOMES and T-BET protein are expressed by all conventional NK cells, their transcript levels are known to be relatively low and difficult to detect due to limitations in existing scRNA-seq technology. Thus, we used the sample origin proportion of each cluster to identify the cells that are most likely to be T-BET and EOMES KO as those cells will be unique to *T+E* edited samples (Figure 2.11B-C, See Methods). Notably, the majority of cells belonging to cycling clusters from both in vitro and in vivo originate from control samples, which is consistent with our experimental data demonstrating impaired proliferation of KO NK cells (Figure 2.11, B and C, and Figure 2.4, F-H). Finally, we re-clustered only the non-cycling control and KO clusters for visualization (Figure 2.12).

Differential expression analysis identified numerous genes that are downregulated in KO clusters, with many that are consistently downregulated across the three subsets: in vivo, in vitro $CD56^{bright}$ and in vitro $CD56^{dim}$ NK cells (Figures 2.12-2.14). Genes responsible for various NK cell functions were altered in KO cells (Figures 2.12 and 2.13). For example, genes that encode several cytotoxic granzymes and chemokines, including CCL5, secreted by NK cells to recruit other cells during an immune response were also downregulated (84–86). Many NK cell trafficking regulators are downregulated in KO clusters as well, for example *S1PR5*, known to promote NK cell migration (49,87). The gene expression of granule protein NKG7 which regulates degranulation in NK cells was also reduced in KO clusters (88). The KO clusters did have significantly higher expression of some notable genes compared to control clusters, including *TNFRSF18* (GITR), which has been shown to negatively regulate NK proliferation and activation (89–91). Increased expression of integrin *ITGB7* was also observed in all three

settings. We also observed a decrease in IL12RB2 transcript expression in KO clusters in vitro CD56^{bright} and CD56^{dim} NK cells and in vivo (CD56^{dim}), which may mechanistically contribute to the decreased of pSTAT4 in response to IL-12 stimulation in NK cells that lack EOMES and T-BET (Figures 2.13A and Figure 2.9C).

We performed gene set enrichment analysis (GSEA) to assess KEGG pathways enrichment on control vs KO NK cell clusters, and in concordance with the individual genes we observed to be downregulated, the "Natural Killer cell mediated cytotoxicity" pathway is negatively enriched in KO cells from the in vitro as well as the in vivo samples (Figure 2.13, C-D). In agreement with the downregulated *PRF1* and *GZMB* transcripts, a significant decrease in their protein expression was observed in DKO NK cells by flow cytometry (Figure 2.15). These data reveal that T-BET and EOMES exert a sustained and ongoing control of key human NK cell effector function mediator genes, and when deleted, these programs are rapidly curtailed.

Single knock-out of T-BET or EOMES has modest effect on human NK cell transcriptional profiles

To investigate the individual contribution of EOMES and T-BET to these changes, control, *TBX21* edited, *EOMES* edited, and *T+E* edited NK cells generated in vitro were compared. Control, *TBX21*, and *EOMES* edited samples had similar UMAP clustering, indicating that single *TBX21* or *EOMES* editing had minimal effect on major NK cell transcriptional profiles (Figure 2.16, A-D). Comparing single and double T-box TFs edited NK cells against control samples within CD56^{bright} and CD56^{dim} clusters, *T+E* edited NK cells have more differentially expressed genes (DEGs) than single edited NK cells compared to control (Figure 2.16, E-K). In this analysis, 375 out of 527 DEGs identified of all comparisons belong to the T+E edited samples

compared against control and were not significant in single edited sample comparisons, for example, *KLRD1* and *IL2RG* in CD56^{bright} clusters and *PRF1, SLAMF7* in CD56^{dim} clusters (Figure 2.16, I-K). Some genes like *NKG7* (CD56^{bright}), *GZMB* (both CD56^{bright} and CD56^{dim}), and *S1PR5* (CD56^{dim}), were downregulated by single deletion of either *TBX21* or *EOMES*, but the fold change over control was greater when both TFs are edited. *TBX21* single edited NK cells had the fewest transcriptional changes (31 and 29 DEGs in CD56^{bright} and CD56^{dim}, respectively). While more genes, for example *TNRFSF18* (CD56$^{bright)}$ and *LYST* (CD56^{dim}), were differentially expressed in EOMES edited (113 and 48 DEGs in CD56^{bright} and CD56^{dim}, respectively) and T+E edited (336 and 304 DEGs in CD56^{bright} and CD56^{dim}, respectively) but not in NK cells that were only *TBX21* edited (Figure 2.16, I-K). Since the effect of single TBX21 or EOMES single CRISPR-editing was minimal on the transcriptional profile of NK cells, we focused subsequent analyses on control vs *T+E* edited NK cells.

T-BET and EOMES regulate expression of other transcription factors

Among the significantly differentially expressed genes in the KO vs control clusters of *T+E* edited vs control NK cell samples, we identified TF genes implicated in lymphocyte and NK cell function and identity (Figure 2.17). Expression of *ZEB2*, a known target of T-BET required for mouse NK cell terminal maturation is significantly decreased in all three settings of in vitro CD56^{bright}, CD56^{dim}, and in vivo KO compared to control clusters (Figure 2.17) (92). RUNX3, critical for ILC lineage and function, and BHLHE40 (a cofactor of T-BET) that promotes *IFNG* expression in lymphocytes are downregulated in the KO clusters of in vitro CD56^{dim} and the in vivo set compared to control clusters (57,93–96). Two notable TF genes that are altered in KO NK cells in the in vivo setting are *NFATC2* and *KLF2* (Figure 2.18A). NFAT is induced upon activation of NK cells and promotes transcription of *IFNG* in NK cells (97). KLF2, a negative

regulator of NK cell proliferation, is increased in KO clusters in vivo (98) (Figure 2.17). Interestingly *KLF2* expression is lower in the in vitro CD56^{bright} KO cluster compared to control (Figure 2.17C). This demonstrates that the expression and regulatory contributions of TFs downstream of T-BET and EOMES are both NK subset and context dependent.

In both in vitro CD56^{bright} and CD56^{dim} NK cells, *ETS1*, known for its essential role in NK cell development and in promoting *IFNG* expression, have reduced expression in the KO clusters (Figure 2.17B-C) (99–101). *BCL11B*, which is a key transcription factors critical for human NK cell to differentiate from CD56^{bright} to CD56^{dim} human NK cells is also decreased in CD56^{dim} KO clusters (102).

Loss of T-box TFs results in ILC-3-biased ILC progenitor cells

NK cells are categorized as group 1 ILCs, along with ILC-1s. There are two other lineages of innate lymphocytes (ILC-2s and ILC-3s) that produce cytokines analogous to their helper T cell counterparts (9,10). Accompanying the decreased expression of the T-box TFs that promote NK cell maturation, we observed an increased expression of TFs associated with ILC-3 identity in the KO cluster of in vitro CD56^{bright} NK cells. The expression of ILC-3-defining TFs *AHR* and *RORC* are significantly higher in the KO compared to the control cluster, consistent across donors (Figure 2.18A)(10,103–105). This is in concordance with the increased expression of immature NK cell/innate lymphocyte markers *KIT* and *IL23R* in these CD56^{bright} KO clusters (Figure 2.18A)(9,10). *IKZF3* (AIOLOS) expression, which is normally suppressed in ILC-3s but expressed by NK cells, was also downregulated in CD56^{bright} KO clusters(106,107). To investigate whether these markers are altered in all versus a subset of cells within the CD56^{bright} KO cluster, clustering and UMAP plotting were performed with only the CD56^{bright} KO and control clusters identified in Figure 2.12A (Figure 2.18 B-E). This revealed a cluster (Cluster 4),

predominantly comprised of cells from the *T+E* edited samples, that highly expresses ILC-3-associated markers (Figure 2.18, D and E). The other cluster predominantly comprised of the *T+E* edited NK cells (Cluster 1) shares similar high expression of *KIT* and decreased expression *KLRD1* and *KLRF1* as Cluster 4. Taking into consideration that the NK cells used in this study are peripheral blood (PB) derived, Cluster 1 and Cluster 4 (which expresses ILC-3 transcripts) appears most similar to $CD117^{+}$ $CD56^{+}$ ILC precursors (ILCPs) that can give rise to both NK cells and ILC-3s, since fully mature ILC-3s are predominantly tissue-resident (108,109). We validated increased protein expression of CD117, encoded by *KIT*, as well as decreased protein expression of CD94, NKp80, and NKG2D in DKO $CD56^{bright}$ NK cells by flow cytometry consistent with the expression patterns of these molecules on ILCP stage of NK development (Figure 2.18F) (108). These data suggest that T-BET and EOMES are required to actively suppress alternative ILC lineage defining programs in $CD56^{bright}$ NK cells, and when these T-box TFs are removed, NK cells become ILCP-like and can acquire genes associated with a different ILC lineage.

T-BET and EOMES regulate chromatin accessibility in human NK cells

As T-box TFs have been suggested to act as pioneer factors that can modulate chromatin accessibility, we hypothesized that loss of T-BET and EOMES in mature NK cell subsets consequently results in epigenetic remodeling (26–29). Assay for transposase-accessible chromatin with sequencing (ATAC-seq) was performed on control and *T+E* edited NK cells, to elucidate the impact of T-BET and EOMES deletion on chromatin accessibility. Consistent with our hypothesis, chromatin accessibility was decreased in many genomic regions in the *T+E* edited, compared to control NK cells (Figure 2.19A). Motif analysis reveals significant enrichment of T-box family motifs within loci that had reduced accessibility in *T+E* edited NK

cells. Further, ETS and Runx motifs were also enriched in these regions, suggesting that T-BET, EOMES, RUNX3, and ETS1 together likely coordinate a large component of the human NK cell molecular program (Figure 2.19B). Differentially accessible regions (DARs) identified are distributed mainly in introns (44.6%), distal intergenic regions (26.3%), and promoter regions (21.6%) (Figure 2.19C). Many DARs that are less accessible in *T+E* edited NK cells are in or near DEGs that are decreased in KO clusters ($CD56^{bright}$ and/or $CD56^{dim}$) identified from the scRNA-seq data (Figure 2.13 and Figure 2.19D). The 100 DARs (annotated to 82 unique genes) that overlap with DEGs are distributed similarly in genomic regions as all DARs identified (Figure 2.19E). As examples, decreased peak signals were identified in putative regulatory regions and promoter regions of *PRF1*, *S1PR5*, and *GZMM* whose transcript expression levels were decreased in KO clusters of the scRNA-seq experiment (Figure 2.13, 2.14 and 2.19F). This suggests that T-BET and EOMES maintain accessibility of critical regulatory regions of genes, thereby sustaining the NK cell transcriptional program.

2.4 Discussion

In this paper, we report that ongoing transcriptional regulation by T-BET and EOMES is required for proper function of mature human NK cells. Deletion of both EOMES and T-BET resulted in reduced ability of human NK cells to control tumor targets in vivo. Mechanistically, this is explained by reduced proliferation, impaired cytokine-receptor signaling, and defective NK cell effector functions. Cytokine production was reduced when NK cells lack one or both transcription factors. T-BET and EOMES DKO cells produced almost no IFN-γ and had compromised IL-15 and IL-12 receptor signaling. NK cells lacking both T-BET and EOMES also had reduced degranulation to tumor targets after a more prolonged time period following the

CRISPR deletion. Further, T-BET and EOMES single deletion had differential effect on the function of $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets, suggesting both redundant and unique regulatory roles in an NK cell subset specific fashion. Finally, by scRNA-seq analysis, NK cells demonstrated a profound loss of the NK cell functional and identity-defining transcriptional programs, as well as the unexpected emergence of an ILC-3-biased ILCP molecular program. Collectively, these findings demonstrate that EOMES and T-BET are required for fully developed NK cell to properly respond to stimuli and, as well as to maintain NK cell identity.

The role of EOMES and T-BET for initiation of NK cell development is well-established in mouse models. T-BET global KO mice lack mature NK cells in the periphery(29,48). Similarly, in hematopoietic compartment-specific and constitutive NK cell-specific EOMES KO mouse models, mature NK cell numbers are markedly reduced (48,110). Likewise, the importance of T-BET in human NK cell development was evidenced by a patient with T-BET deficiency, who had an impaired group 1 ILC compartment (62). Forced overexpression of T-BET and EOMES can accelerate in vitro NK cell differentiation from cord-blood, but these studies do not inform their requirement for maintenance of mature NK cell function or molecular program (63). Collectively, these published studies have defined the clear requirement for T-box TFs to *initiate* NK cell molecular programs, as they develop from multi-potential progenitors. However, these models are generally not sufficient to understand the ongoing importance and genetic programs sustained by T-box TFs, which dynamically alter expression levels during NK cell maturation (38).

Recently, our group developed an inducible NK-specific EOMES KO mouse model that revealed the requirement of sustained EOMES expression for proper murine NK cell effector functions

and the homeostasis of stage II and III murine NK cells (60). This contrasts the EOMES-deleted human NK cells reported here, where human NK cell subset-specific functions are minimally affected, without impacting NK cell survival. Based on these observations, the study of T-box TFs within human NK cells is required to understand their role in human NK cell molecular programs. Here, we show that single deletion had modest impact on human NK cell functions and transcriptional profile compared to double T-BET and EOMES deletion. These findings contrast to mouse studies showing functional defects and widespread transcriptional changes in T-BET or EOMES single knock-out NK cells. This again highlights distinctions between murine and human NK cell transcriptional control (60,111). Moreover, there have been no murine studies of simultaneous T-BET and EOMES conditional and inducible deletion to date. Our data demonstrate that T-BET and EOMES exhibit redundancy in human NK cells in terms of functional regulation as well as transcriptional regulation. Thus, our finding that EOMES and T-BET are of profound and critical importance for ongoing human NK cell functions and identity provides important new insights into the NK cell molecular program, including key downstream TFs impacted by EOMES and T-BET regulation.

ScRNA-seq analysis of CRISPR-edited primary human cells revealed EOMES and T-BET regulated key NK cell functional pathway genes, including cytotoxic effector molecules, NK cell receptors, and trafficking and migration regulators (e.g. chemokines and chemokine receptors). Using a loss-of-function approach, several transcription factors were discovered to be directly regulated by T-BET and EOMES, many of which have roles in regulating *IFNG* transcription in other cell types. For example, in both $CD56^{bright}$ and $CD56^{dim}$ T-BET and EOMES KO human NK cells, a reduced expression of ZEB2 was observed, which is a direct target of T-BET in mouse NK cells and required for mouse NK cells to mature and acquire optimal function (92).

Moreover, T-BET and EOMES KO cells have reduction of RUNX3, which has been shown to be critical for ILC lineage initiation, cytotoxic molecule expression, and IL-15-induced proliferation (57,93,94). Further, BHLHE40, a cofactor of T-BET that normally promotes *IFNG* expression in lymphocytes, also had markedly decreased expression in DKO human NK cells (95,96). The downregulation of ETS1 in DKO NK clusters likely contributed to the reduced IFN-γ upon IL-12 stimulation in *T+E* edited NK cells (99–101). Collectively, the approach of T-box TF deletion followed by analysis of RNA expression in single cells reveals critical links between T-BET, EOMES and these transcriptional regulators, providing evidence of their ongoing regulation by T-box TFs in human NK cells.

In the more immature $CD56^{bright}$ NK cell compartment, we observed a reversion of NK cells to have ILC precursor (ILCP)-like marker expression pattern and an ILC-3-biased ILCP population that was specifically enriched in the T-BET and EOMES KO samples. This cluster resembles the previously described $CD56^{+}$ ILCPs in its high expression of CD117 and IL23R(108). Consistent with the description that the $CD56^{+}$ ILCPs can give rise to group 1 and group 3 ILCs, this ILCP-like cluster observed in our study seems to be biased towards the ILC-3 lineage with its high expression of ILC-3 associated transcription factors RORC and AHR and low expression of IKZF (106,109). This observation is also consistent with functional plasticity between ILC groups, in this case mature human NK cells and ILCP, governed by expression levels of ILC group-specific transcription factors (1,106,110). Our data suggest that EOMES and T-BET suppresses non-NK ILC lineages, with a bias specifically against the ILC-3 lineage, and when EOMES and T-BET are deleted, NK cells acquire a transcriptional signature that match that of an ILC-3-biased ILCP.

This study also revealed that T-BET and EOMES not only regulate transcript expression in NK cells, but also participate in maintaining chromatin accessibility. Abrogation of T-BET and EOMES led to decreased chromatin accessibility near the *PRF1* and *S1PR5* loci, both of which had decreased transcript expression in *T+E* edited NK cells, among many other NK cell effector function-related gene loci that were also transcriptionally affected. The reduced chromatin accessibility upon T-box TF deletion indicates an active role in maintaining the NK cell state, and reveals a new layer of regulation that T-BET and EOMES beyond transcriptional regulators, as they are critical for NK cell chromatin states.

NK cellular therapy is a promising cancer immunotherapy, since tumor cells commonly express NK-activating ligands as well as downregulate MHC Class I molecules, which render them sensitive to NK cell-mediated clearance (67,68). Expression of T-BET and EOMES is negatively regulated by immunosuppressive cytokines like TGF-β in tumors (112,113). In a mouse adoptive transfer model, reduced EOMES and T-BET expression over time in NK cells post-adoptive transfer correlated with reduced IFN-γ production and impaired long term tumor control (114). Our loss-of-function study showed that T-BET and EOMES in mature NK cells are indeed required for NK cell function, and thus reduced T-BET box transcription factors directly limit NK cell functional capacity. As our study focused on conventional, peripheral blood NK cells, future work should investigate the expression and importance of T-BET and EOMES in various NK therapeutic approaches such as cord-blood derived NK cells, iPSC-derived NK cells, cytokine-induced memory-like NK cells, and NK cells transduced with chimeric antigen receptors (69,76,115–118). The expression levels and kinetics of T-BET and EOMES in those

settings could be potentially utilized as a measure of NK cell identity integrity and optimal function.

In summary, this study reveals that EOMES and T-BET are required for sustaining mature NK cell identity and functional activity. The deletion of EOMES and T-BET led to functional, proliferative, and signaling defects that resulted in an impaired response against tumor cells in vivo. This illustrates the importance of maintaining T-BET and EOMES expression for optimal NK cell anti-tumor responses. Moreover, deletion of these NK cell identity TFs results in emergence of an ILC-3-biased ILCP program that may represent a default developmental pathway. Future studies that interrogate the role of genes that are directly regulated by T-BET and EOMES revealed in our study, and which are indirectly regulated through other transcription factors regulated by T-BET and EOMES, are important steps to further elucidate how NK cells orchestrate a transcription network responsible for mature NK cell responses.

2.5 Methods

Human NK cell isolation and culture

Healthy donor NK cells were isolated from leukapheresis chamber from platelet donors using RosetteSep (STEMCELL Technologies) (routinely >95% CD56+, CD3-) followed by Ficoll-Paque PLUS (GE Health) centrifugation. NK cells were maintained in low dose (1-3 ng/mL) IL-15 in RPMI 1640 + 10% heat-inactivated human AB serum + 10mM HEPES + 1X penicillin/streptomycin + 1% of non-essential amino acids, sodium pyruvate, and L-glutamine.as previously described(73), with media changes every other day.

Mice

NOD-*scid* IL2Rg^{null} (NSG) were purchased from the Jackson laboratory (RRID:IMSR_JAX:005557). Mice were then 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 7-16 week old male and female mice. Within each experiment, mice were age and gender matched.

Cell Lines

K562 cells were obtained from ATCC and authenticated in 2015 by SNP analysis. K562 cells were cultured in RPMI 1640 medium + 10% Heat-inactivated FBS + 10mM HEPES + 1X penicillin/streptomycin + 1% of non-essential amino acids, sodium pyruvate, and L-glutamine.

CRISPR-editing of human NK cells

Freshly isolated NK cells were rested in low dose IL-15 (1-3 ng/mL) overnight. The next day, NK cells were harvested, washed with PBS, and resuspended in Maxcyte EP Buffer at concentrations recommended by the manufacturer. Cas9 mRNA and gRNA were introduced into the NK cells by electroporation using the protocol WUSTL-2 on the Maxcyte GT electroporation machine. Cells were incubated for 10 minutes at 37 ^{o}C immediately after electroporation. Low dose IL-15 media was added after the incubation. Media changes were performed every 2-3 days. Synthetic sgRNAs were produced by Synthego with modifications (2'-O-Methyl at first 3 and last bases and 3' phosphorothioate bonds between first 3 and last 2 bases). The sgRNA sequences are as follows: *EOMES*: AACCAGTATTAGGAGACTCT, *TBX21*: CACCACTGGCGGTACCAGAG, *TRAC*: GAGAATCAAAATCGGTGAAT.

Apoptosis Assessment

On Day 6/7 after CRISPR electroporation, NK cells were harvested and assessed for apoptosis using Annexin V and 7-AAD (Sigma-Aldrich) in 1X Annexin V Binding Buffer (Thermo Fisher) after surface marker staining for flow cytometry analysis.

In vitro Flow-Based killing Assay

CRIPSR-edited NK cells were sorted into $CD56^{bright}$ ($CD56^{high}$ CD16-) and $CD56^{low}$($CD56^{low}$ CD16+) 5 days after CRISPR and rested in 3 ng/mL IL-15. The next day, NK cells were incubated with CFSE-labeled K562 target cells for 4-6 hours in the presence of 3ng/mL IL-15. 7-AAD staining was performed at the end of the incubation and assessed by flow cytometry to determine % specific lysis, as previously described(73).

Incucyte Killing Assay

GFP-expressing SKOV-3 cells (ATCC) were cultured in McCoy 5A+ 10% FBS + 1% Pen/Strep and plated at 10,000 cells/well at least 1 hour prior to addition of NK cells. CRISPR-edited NK cells were incubated with the plated target cells at indicated effector to target ratios in the presence of 1ng/mL IL-15 for 6 days. SKOV-3 numbers (Green object count) were monitored over the course of 6 days on the Incucyte Live-Cell Analysis system (Sartorius).

NSG Xenograft and Tumor Model

The next day after CRISPR electroporation, 1 x 10^6 NK cells were washed with PBS and injected into NSG mice i.v. and supported with 1 µg rhIL-15 i.p. 3x per week. For K562 tumor

challenge experiments, ~1.5 x 10^6 luciferase-expressing K562 cells were injected i.v. 4 days after NK cell injection and 100 ng/mouse rhIL-15 was used to support NK cells from for the duration of the experiment after tumor injection. Bioluminescent imaging was performed twice a week on the AMI imager 10 minutes after i.p. injection of 150 mg/kg D-luciferin. Quantification of BLI signals were performed using the Aura software.

For proliferation assessment, NK cells were washed with PBS and incubated with 1:2000 CellTrace Violet following manufacturer's protocol for labeling prior to injection into the mice. Dye dilution was tracked at time of mouse harvest by flow cytometry.

For experiments assessing persistence, proliferation, and ex vivo functionality, NK cells were maintained with 1µg/mouse recombinant human IL-15 i.p. 3x per week for the entire course of the study.

Flow cytometry

Surface marker staining was performed in PBS + 1 mM EDTA + 2% heat-inactivated FBS at 4^{o}C in the presence of heat-inactivated goat serum (Sigma-Aldrich). Mouse F-Block was also used in in NSG mice experiments. Intracellular staining was performed with the eBioscience FoxP3 staining kit following manufacturer's protocol. Data were acquired on Beckman Coulter Gallios and Thermo Fisher Attune flow cytometers and analyzed using FlowJo(Tree STAR).

The following antibody clones and reagents from indicated manufacturers were used:

Beckman Coulter: CD56 (N901), CD3 (UCHT1), CD45 (J33); Life Technologies: CD16 (3G8); Biolegend: T-BET (4B10), IFN-γ (4S.B3), PERFORIN (dG9), TNF (Mab11), CD107a (H4A3),

GZMB (GB12), CD117 (104D2), CD94(DX22), CD122 (TU27); eBioscience: EOMES (WD-1928); BD: Annexin V, AKTpS473 (M89-61), STAT4pY693 (38/p-Stat4), ERK1/2 pT202/pY204 (20A), mouse CD45 (30-F11), mouse FcBlock; R&D systems: NKp80 (239127); Invitrogen: NKG2D (1D11).

Phospho-signaling Assessment

On Day 6/7 after CRISPR electroporation, NK cells were harvested from tissue culture plate and cytokine-containing media was washed off and replaced with cytokine-free media. NK cells were rested in cytokine-free media for 30 minutes up to 2 hours. In each experiment equal number of NK cells, up to 200,000 NK cells, were plated for each condition. Then, IL-15 and IL-12 were used to stimulate NK cells (timing and concentration indicated in figure legend). At the end of incubation, NK cells were fixed with pre-warmed 1% PFA and permeabilized using ice cold methanol. Cells were washed 3 times with FACS buffer prior to staining with surface markers and phospho-antibodies overnight at 4^{o}C.

Western Blotting

CRISPR-edited NK cells 1 week after electroporation were spundown and resuspended in 1X RIPA Buffer (CST) supplemented with 1X Protease Inhibitor Cocktail (CST). Lysates were ran on NuPAGE™ 4 to 12% Bis-Tris Mini Protein Gel (Thermo Fisher) along with Precision Plus Protein™ WesternC™ Blotting Standards (Biorad), and transferred using the iBlot2 Western Blot Transfer System to nitrocellulose membrane(Thermo Fisher). Membrane was blocked in 5% Nonfat dry milk (Biorad) prior to blocking with primary antibody. The following antibodies purchased from Cell Signaling Technology were used: STAT44 (C46B10) Rabbit mAb, AKT

(pan) (C67E7) Rabbit mAB, p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb, β-Actin (8H10D10) Mouse mAb, Anti-rabbit IgG HRP-linked Antibody, Anti-mouse IgG HRP-linked Antibody. Blots were incubated in SuperSignal West Pico Plus ECL substrate (34577) prior to imaging on the Chemidoc MP system (Biorad). When necessary, Restore™ PLUS Western Blot Stripping Buffer (Thermo Fisher) was used following manufacturer's protocol. Quantification of bands were performed using ImageJ.

Assessment of degranulation and cytokine production

For in vitro maintained cells, unless otherwise indicated, on Day 6/7 after electroporation up to 200,000 NK cells were plated in one well of a 96-well U bottom plate for each condition. In each independent experiment, equal number of NK cells were plated for all samples. Stimulation conditions were as follows: K562 at the ratio of 5 NK to 1 K562; 5ng/mL IL-12 + 25 ng/mL IL-15; 5ng/mL IL-12 + 25 ng/mL IL-15 + 5ng/mL IL-18. Immediately after stimulation began, anti-CD107a antibody was added to all wells of the assay. After 1 hour, GolgiPlug and GolgiStop were added and the assay was incubated for 5 hours more, for a total of 6 hours. Then flow cytometry staining was performed as described above to assess intracellular IFN-γ and TNF.

Ex vivo assessment was performed similarly but with isolated splenocytes 1.5-2 week post-injection of NK cells into NSG mice. Stimulation conditions were as follows: K562 at the ratio of 10 splenocytes to 1 K562; 20ng/mL IL-12 + 100ng/mL IL-15; 20ng/mL IL-12 + 100ng/mL IL-15 + 20ng/mL IL-18.

For PMA/ionomycin stimulation experiments, NK cells were stimulated with 1X eBioscience™ Cell Stimulation Cocktail. After 2 hours, GolgiPlug and GolgiStop were added and the assay was

incubated for 4 hours more, for a total of 4 hours. Then flow cytometry staining was performed as described above to assess intracellular IFN-γ.

Single-Cell RNA-Sequencing

For each donor, live human NK cells (Zombie$^-$mCD45$^-$hCD45$^+$hCD3$^-$CD56$^+$) were sorted from splenocytes of NSG mice 7 days after injection using the BD FACSAria Cell Sorter (>98% purity). In parallel, NK cells that were injected into NSG mice was also cultured in vitro in 1ng/mL IL-15, harvested and subjected to flow sorting at the same time as the in vivo-maintained human NK cells followed by 10x Genomics scRNA-seq (5' v2). The resulting data were analyzed as previously described using CellRanger (v6.0) with genome alignment to GRCh38; downstream analyses was performed using Seurat v4 (74,75). For each of the 2 donor samples in Fig. S7, the 4 experimental conditions were stained with hashtag oligonucleotide-tagged (HTO) antibodies, pooled together, and ran as one run for CITE-seq andfastq files were aligned as above with the addition of a feature barcoding reference file for hashtagging alignment and count as previously described(75). Experimental conditions were demultiplexed in Seurat (v4) by first removing dead cells (percent mitochondrial < 10%), and cells with extremely high or low gene counts (nFeature_RNA > 500; nCount_RNA < 25000 (Dnr1) or nCount_RNA < 40000 (Dnr2); nCount_HTO < 8000), followed by normalizing antibody reads using centered log ratio by feature followed by HTODemux (quantile = 0.99) retaining all cells positively assigned to one antibody (Hashtag-2: dTRAC; Hashtag-3: dTbet; Hashtag-4: dEomes; Hashtag-5: DKO). The 2 donors' control and T+E edited samples were then pooled with additional 3 donor in vitro samples for analyses in Figures 2.12-2.18. For the 3 additional donors used in scRNA-seq in Figures 2.12-2.18, Low quality and dead cells were filtered using the

following thresholds: RNA features > 200 & percent mitochondrial reads < 10 to 12.5 (sample dependent). Significant principle components were chosen by JackStraw Analysis and Elbow Plot followed by louvain unsupervised clustering and UMAP (119). Batch correction was performed grouped by donor using Harmony R package (120).

Resulting UMAP clusters were first assigned to be $CD56^{bright}$ or $CD56^{dim}$ and cycling clusters where cells have high expression of S phase and G2M phase associated genes. Then clusters were grouped into the following--"KO": non-cycling clusters where >75% of cells within the cluster originate from a *T+E* edited samples, "Control": non-cycling clusters where <75% of cells are *T+E* edited samples. "Control" and "KO" clusters were then re-clustered for visualization. Differential gene expression analysis was performed using Wilcoxon Rank-Sum test implemented in the Seurat FindMarkers function (parameters: logfc.threshold = 0.25, min.pct = 0.1). Gene Set Enrichment Analysis was performed using the R package clusterProfiler (121). Transcription factor genes presented were only those included in the "Collection of known and likely human TFs" by Lambert et al(122).

Assay for Transposase-Accessible Chromatin (ATAC) sequencing

CRISPR-edited NK cells were harvested from tissue culture plate on Day 7 and Day 10 post CRISPR electroporation. Nuclei and libraries were prepared for ATAC sequencing following established protocol using the Illumina kits(123). The samples from the two time points were treated as technical replicates in the analysis using standard ATAC-seq analysis pipeline and tools. Briefly, paired-end reads were trimmed for adaptors and low-quality reads were removed using Cutadapt (v3.2). Trimmed reads were aligned to the Homo sapiens genome assembly hg38

using Bowtie2 (v2.4.1). Samtools (v1.3.1) was used to filter reads by alignment score and remove mitochondrial reads and PCR duplicates were removed using Picard (v2.25.0). Peaks calling was performed using Genrich (v0.6) ATAC-seq mode (-j -d 100). Differential accessibility analysis was determined by DESEQ2 using the DiffBind (v4.2) and annotated using Chipseeker(v1.28.3) R packages. De novo motif enrichment analysis on all differentially accessible genomic regions was performed using HOMER (v4.11) using findMotifsGenome.pl with automatically generated background by HOMER.

Data availability

The scRNA-seq (GSE227636) and ATAC-seq (GSE227878) data are deposited to Gene Expression Omnibus (GEO). No new code was generated in this study; all analyses were performed using existing packages.

Statistics

Statistical comparisons were performed as indicated in each figure using GraphPad Prism(v9) software or in R. Data represented are mean±SEM and all significance testing comparisons are two-sided. The specific statistical tests and the sample size are indicated in respective figure legend. * = $p < 0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

Study Approval

All animal studies were IACUC approved (20170129) and experiments were conducted in accordance with the guidelines of and approval by the Washington University Animal Studies Committee.

2.6 Acknowledgements

We would like to acknowledge the Siteman Flow Cytometry Core, the Genome Technology Access Center at the McDonnell Genome Institute, and the Genome Engineering and IPSC Center at Washington University School of Medicine. Schema were created using Biorender.com

2.7 Figures

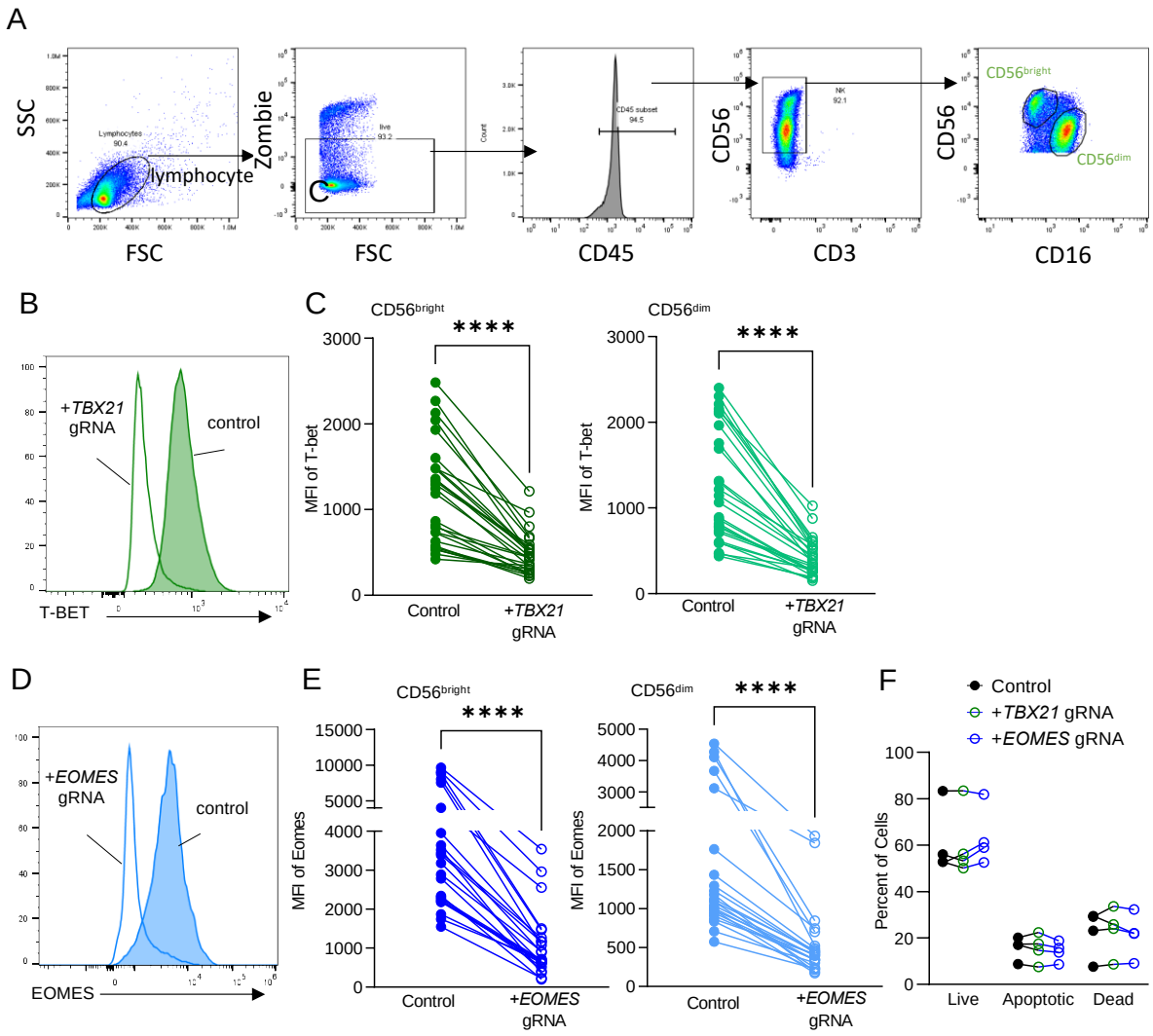

Figure 2.1. Electroporation of *EOMES*/*Tbx21* gRNA and Cas9 mRNA successfully abrogates EOMES and T-BET protein expression in unexpanded primary human NK cells. (A) Example flow gating of human NK cells maintained in vitro. (B-E) NK cells were subjected to electroporation to deliver Cas9 mRNA with respective sgRNA using the Maxcyte GT system. NK cells were cultured in low dose IL-15, and on D6/7 T-BET and EOMES protein expression was assessed by flow cytometry. (B) Representative flow histogram plot and (C) summary data

of T-BET protein expression in NK cells electroporated with Cas9 mRNA and gRNA targeting *TBX21* locus in $CD56^{bright}$ and $CD56^{dim}$ NK cells identify by flow gating. (D) Representative histogram plot and (E) summary data of EOMES protein expression in NK cells electroporated with Cas9 mRNA and gRNA targeting *EOMES* genomic locus in $CD56^{bright}$ and $CD56^{dim}$ NK cells gated by flow. (B-E) n=23-27 healthy donors, 18-24 independent experiments. (F) NK cells were cultured in low dose IL-15 then harvested on Day 6/7, stained for Annexin V and 7-AAD, and analyzed by flow cytometry. n=4 donors, 4 independent experiments. (C,E) Data were compared using ratio paired t-tests. (F) Data were compared with 2 way ANOVA with Holm-Šídák multiple comparison test.

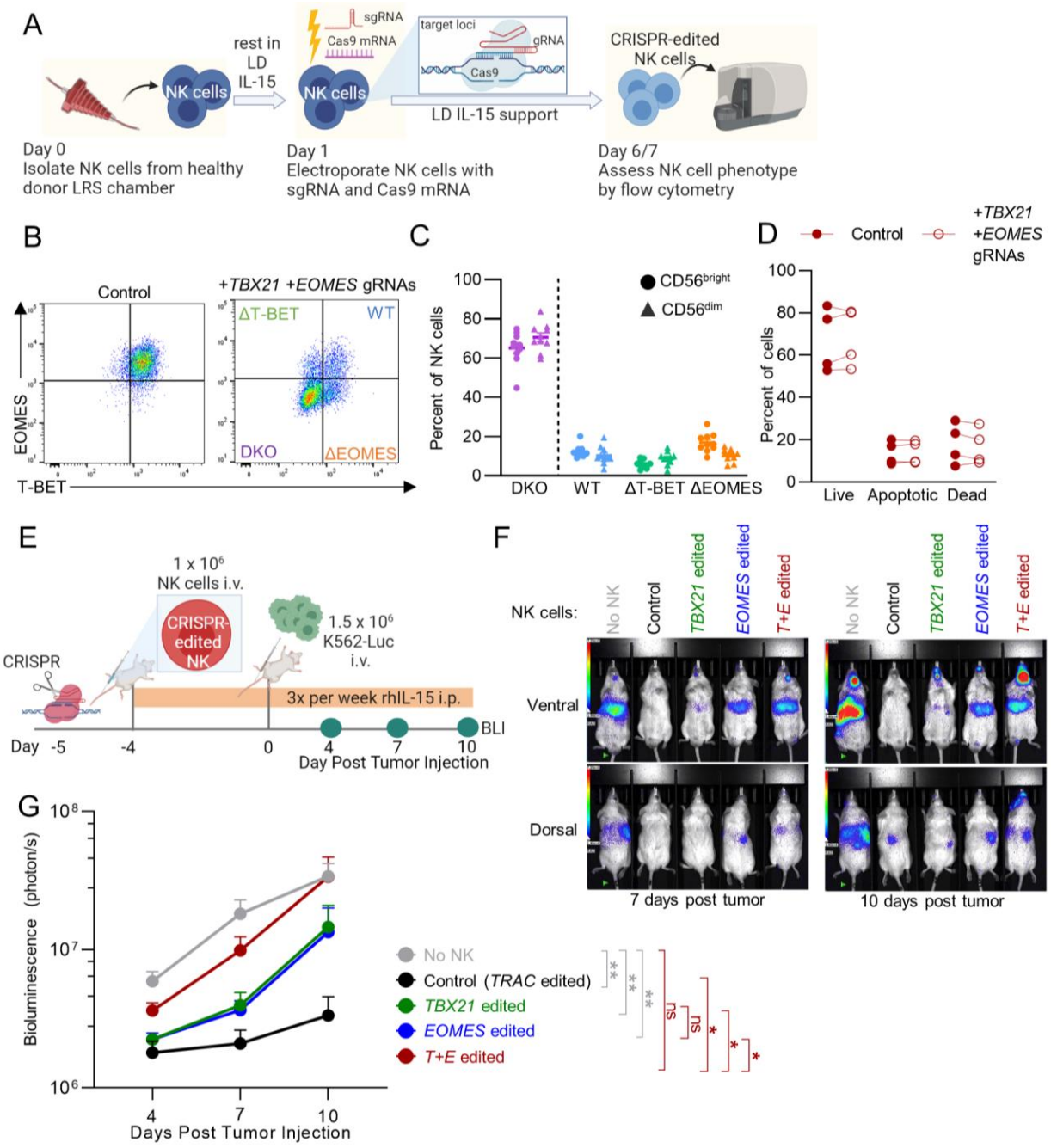

Figure 2.2. EOMES and T-BET are required for optimal tumor control in vivo.

(A) NK cells were subjected to electroporation to deliver Cas9 mRNA and sgRNAs. NK cells were cultured in low dose (LD) IL-15, and on D6/7 T-BET and EOMES protein expression were assessed by flow cytometry. **(B)** Representative flow plot of EOMES and T-BET protein

expression in control NK cells and NK cells targeted with *TBX21* and *EOMES* gRNAs (*T+E* edited) simultaneously. **(C)** Summary data of DKO efficiency in *T+E* edited NK cells. n=10 donors, 9 independent experiments **(D)** *T+E* edited NK cells were cultured in LD IL-15 then harvested on Day 6/7, stained for Annexin V and 7-AAD, and analyzed by flow cytometry. n=4 donors, 4 independent experiment. **(E)** NK cells from either *TRAC* gRNA-edited (control), *TBX21* edited, *EOMES* edited, or *T+E* edited group were injected i.v. into NSG mice. 4 days later, mice were challenged with luciferase-expressing K562 tumor cells. Injections of rhIL-15 i.p. were performed 3x a week to support the human NK cells. **(F)** Representative BLI imaging of tumor burden in NSG mice that received no NK, control NK or TBX21 and EOMES edited NK cells. **(G)** Summary data of tumor burden measured by BLI imaging. 2 outliers in control NK group were identified by ROUT (Q = 0.1%) and excluded from the analysis. n = 6-9 mice each group, from 5 donors, 5 independent experiments. Data were compared using 2-way ANOVA in (**C-D**), and Mixed-effect analysis with Holm-Sidak's multiple comparisons test in (**G**).

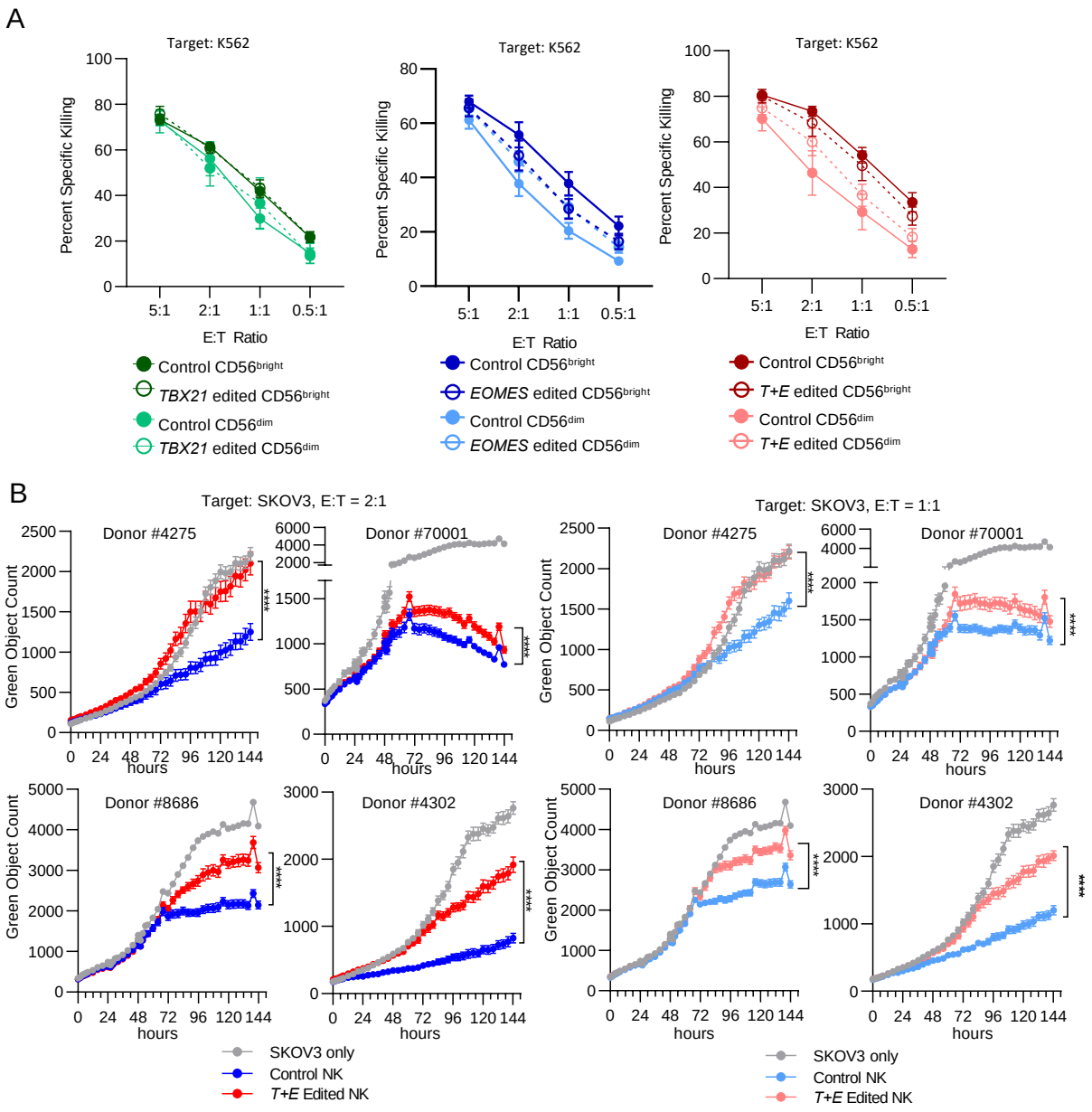

Figure 2.3. EOMES and T-BET are not required for short-term killing of target cells but are required for long-term killing. (A) Sorted CD56^{bright} and CD56^{dim} primary human NK cells were incubated with CFSE-labeled K562 targets at the indicated effector:target (E:T) ratios 5 days after electroporation with Cas9 mRNA. 4-6 hours after, cells were harvested and stained with 7-AAD to determine percent specific killing of CFSE+ targets by NK cells. (B) Incucyte long-term killing against the ovarian cancer cell line SKOV3. Six days after electroporation,

T+E edited or control NK cells were co-cultured with GFP-expressing SKOV3 at the indicated E:T ratio. Green object counts(SKOV3) were monitored over 6 days. Data were compared using 2-way ANOVA.

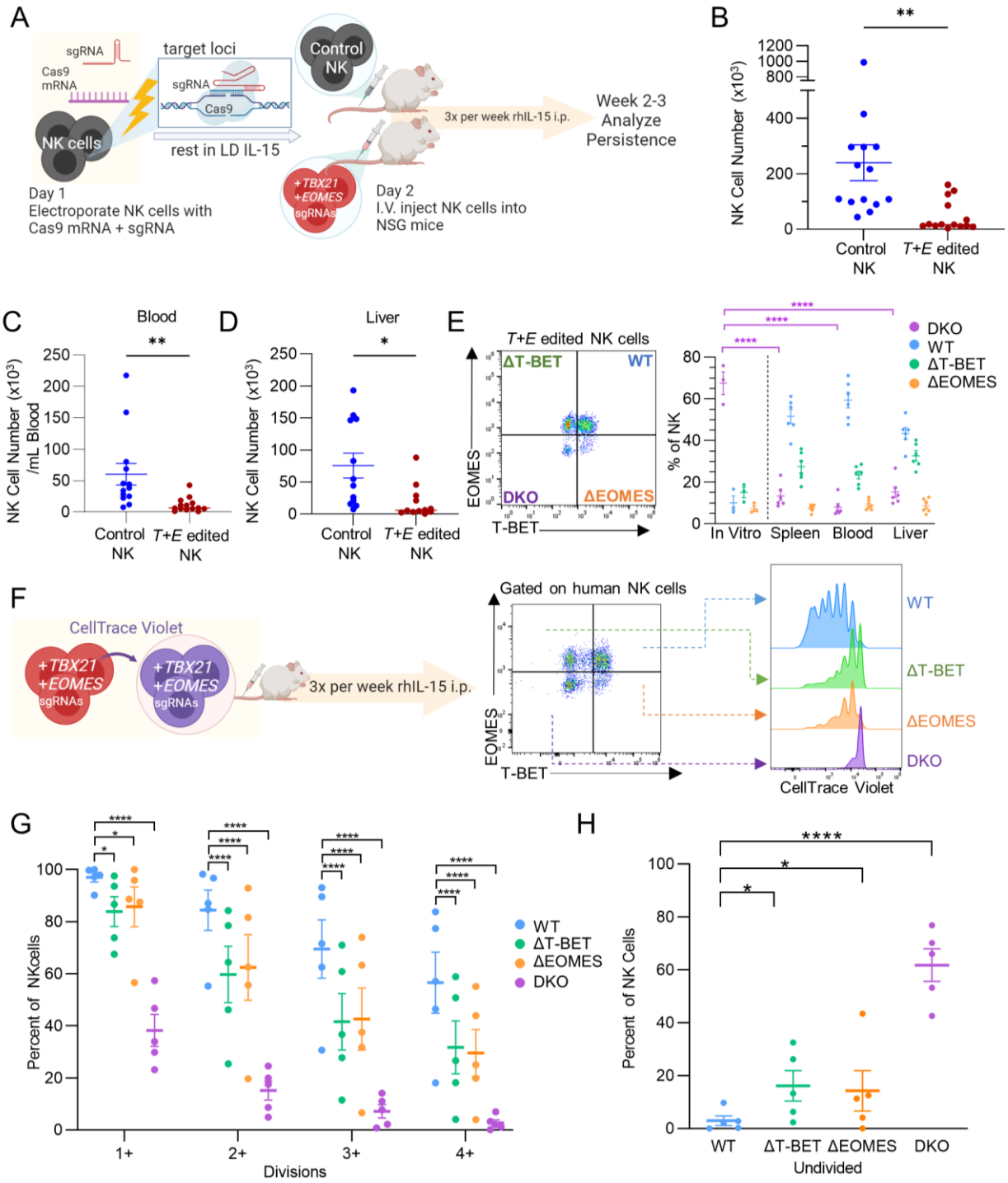

Figure 2.4. EOMES and T-BET are required for NK cell persistence and proliferation in vivo. (A) Experimental Schema for (B-E). **(B-D)** Summary data of NK cell numbers recovered from indicated tissues of NSG mice that received control NK cells or *T+E* edited NK. n=12-15

mice per condition, 6 donors, 5 independent experiments. **(E)** Wildtype (WT) ($TBET^{+}EOMES^{+}$), ΔT-BET ($T\text{-}BET^{-}EOMES^{+}$), ΔEOMES($T\text{-}BET^{+}EOMES^{-}$), and DKO ($T\text{-}BET^{-}EOMES^{-}$) cells were identified by flow cytometry. Left: Representative flow plot and right: summary data of the percentage of each population within the NK compartment of indicated tissues of mice that received *T*+*E* edited NK cells. In Vitro percentages were assessed ~1 week post-electroporation. n=3 donors, 3 independent experiments. **(F)** Schema for proliferation study. *T*+*E* edited NK cells were labeled with CellTrace Violet (CTV) dye prior to injection into NSG mice. 1.5-2 weeks later, percentage of NK cells that have **(G)** undergone indicated number of divisions and (H) those that have not divided were assessed by CTV dye dilution by flow cytometry. n=3 donors, 5 mice, 3 independent experiments. Data were compared using unpaired t-test in **(B-D)** and 2-way ANOVA with Holm-Šídák multiple comparison test in **(E, G, H).**

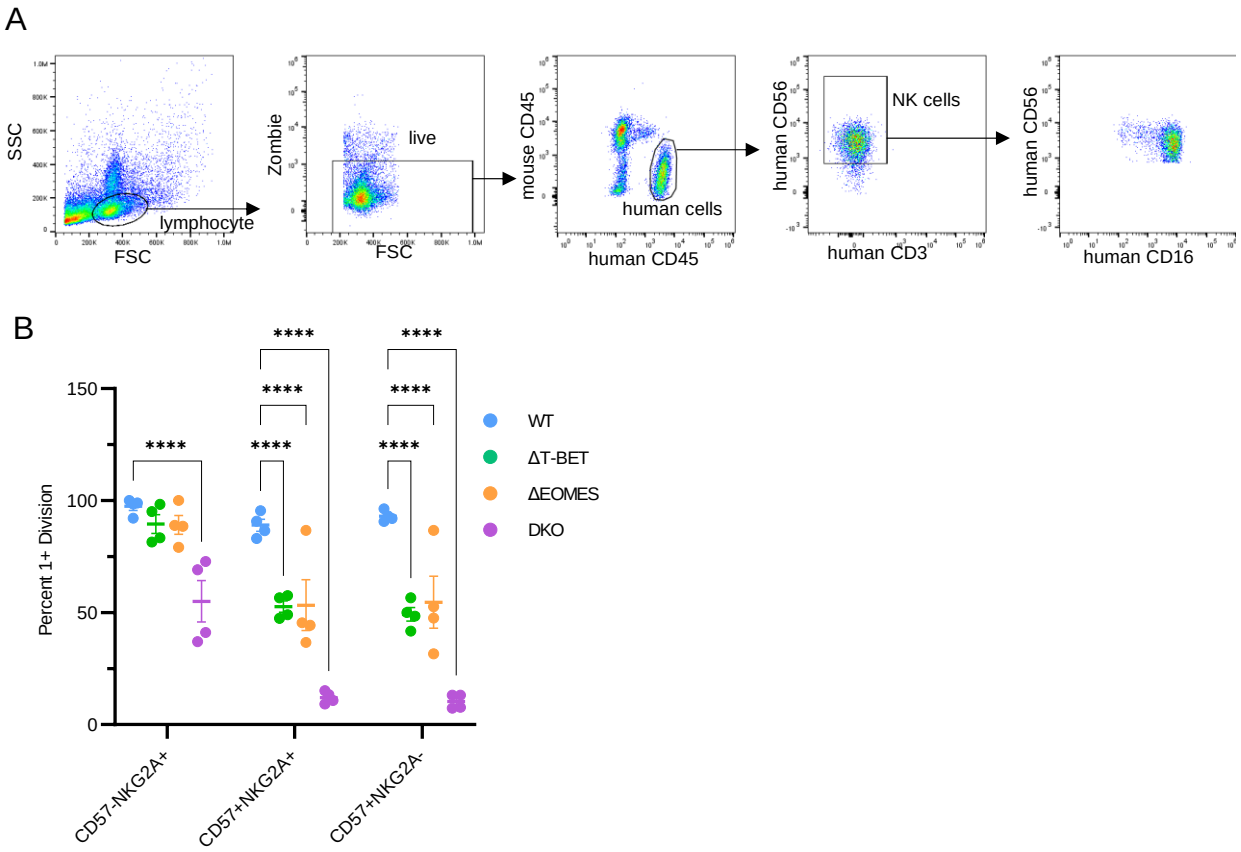

Figure 2.5. EOMES and T-BET are required for NK cell proliferation in vivo especially in less mature subsets of NK cells. (A) Example flow gating of human NK cells maintained in vivo in NSG mice. (B) *T+E* edited NK cells were labeled with CellTrace Violet(CTV) dye prior to injection into NSG mice. At 1.5-2 weeks after injection, NK cells were harvested and CTV dilution was analyzed by flow cytometry. Summary data of percentage of NK cells that have divided at least once. N=4 mice from 3 independent experiments. Data were compared using 2-way ANOVA with Holm-Šídák multiple comparison test.

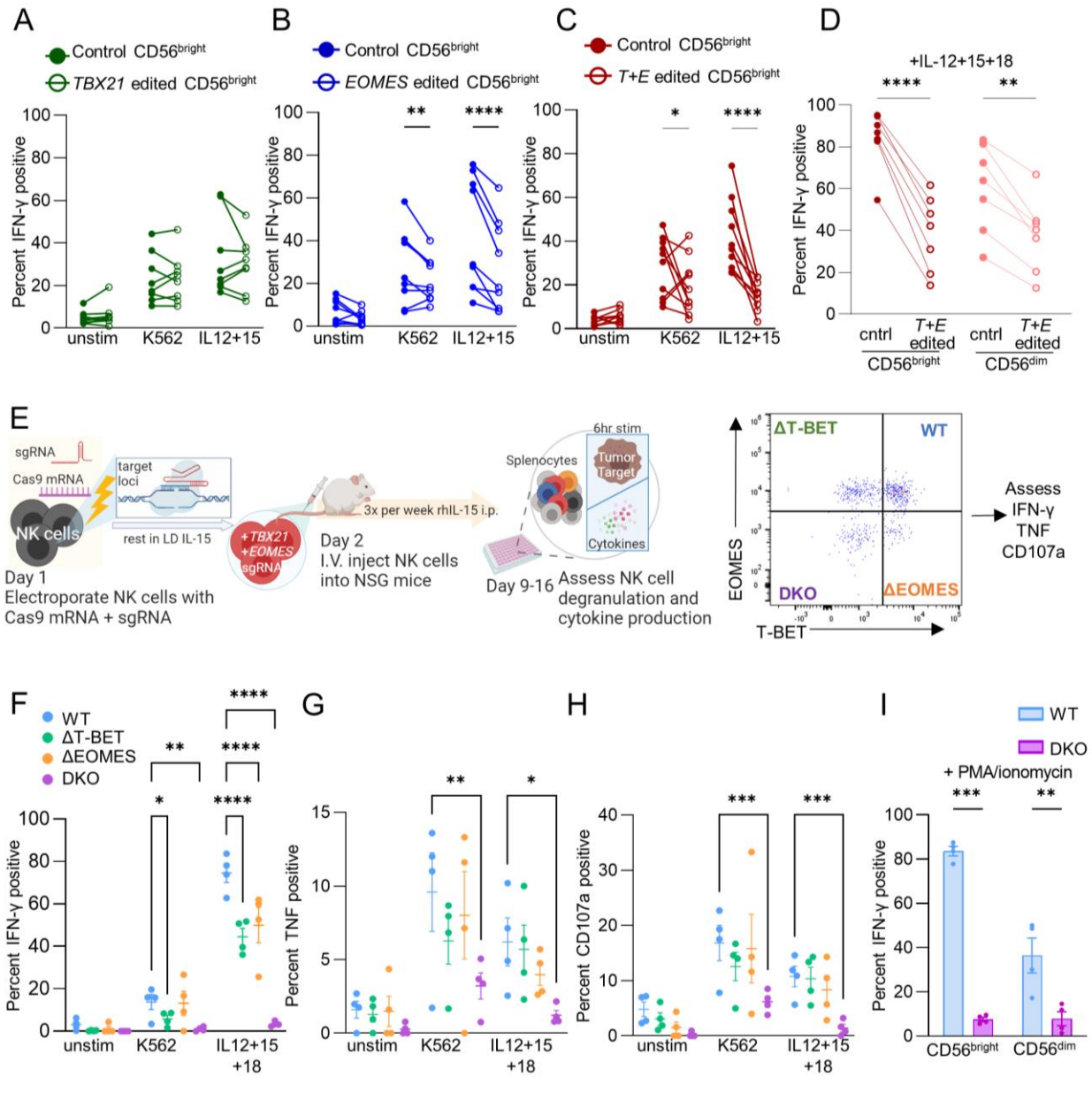

Figure 2.6. EOMES and T-BET deletion impaired NK cell cytokine response. (A-D) in vitro functional assessment. Day 6/7 after CRISPR-electroporation, NK cells were stimulated with K562 and IL-12+15. Degranulation (CD107a) and IFN-γ production were quantified by intracellular flow staining. Summary data of IFN-γ response by **(A)** *TBX21* edited, **(B)** *EOMES*-edited, **(C)** *T+E* edited CD56^{bright} NK cells. **(D)** Summary data of IFN-γ response control (cntrl) and *T+E* edited NK cells stimulated with IL-12+15+18. n=7-10 donors, 4-7 independent

experiments in A-D. **(E)** Schema of ex vivo functional assessment experiment. 1.5-2 weeks after NK cell injection, splenocytes were harvested and stimulated with K562, IL-12+15 and IL-12+15+18 for 6 hours. Summary data of **(F)** IFN-γ, **(G)** TNF, and **(H)** CD107a in human NK cells within indicated T-BET/EOMES flow gate. n = 2 donors, 4 mice, 3 independent experiments. **(I)** 1.5 weeks after CRIPSR-editing, in vitro maintained NK cells were stimulated with PMA/ionomycin for 6 hours. IFN-γ production by NK cells by flow gated $CD56^{bright}$ and $CD56^{dim}$ subsets are shown. n = 4 donors, 2 independent experiments. Data were compared by 2-way ANOVA with Holm-Šídák multiple comparison test.

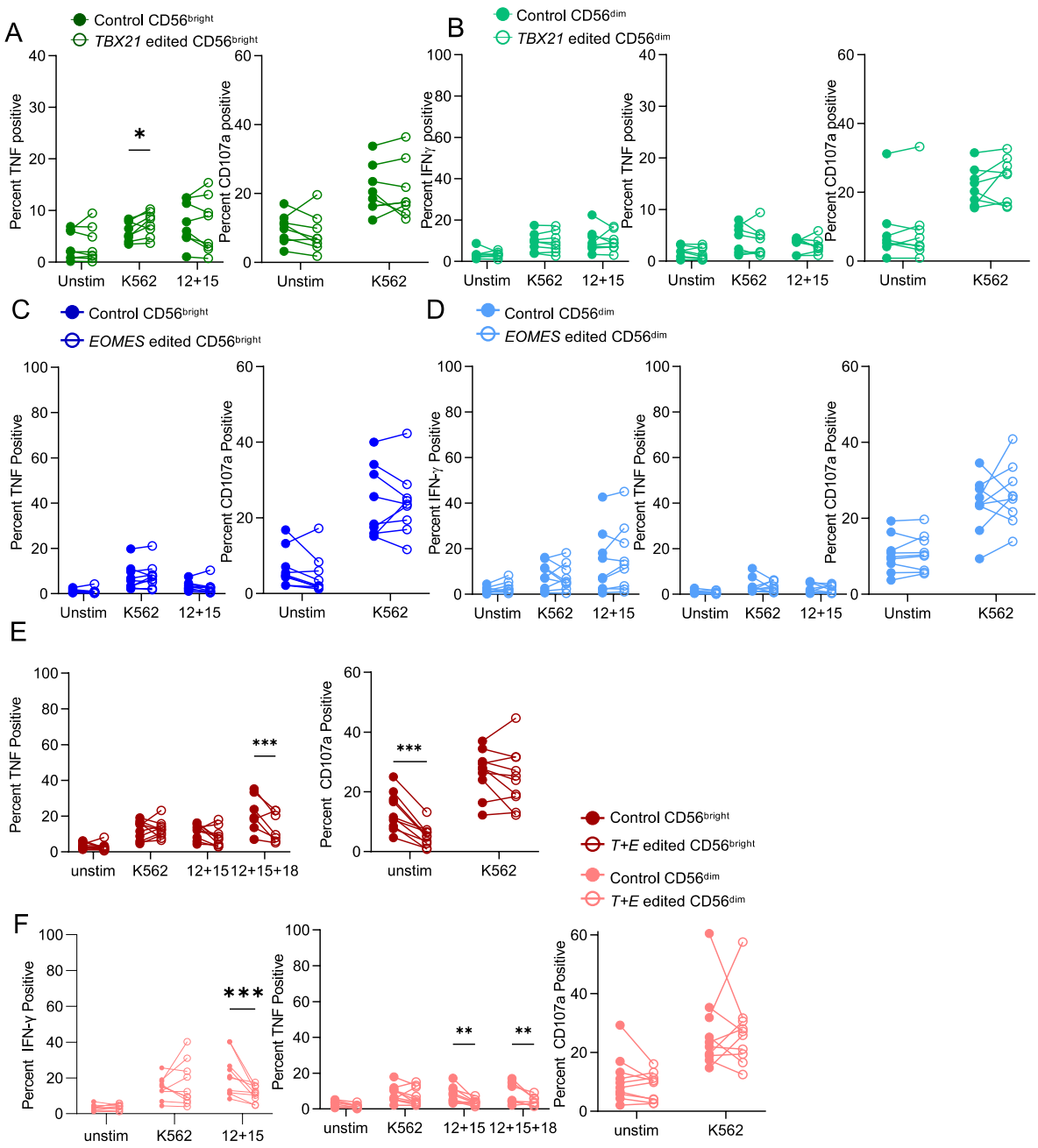

Figure 2.7. **NK cell in vitro effector functions are profoundly impaired in *T+E* edited NK cells.** In vitro functional assessment on Day 6/7 after CRISPR-editing, NK cells were stimulated with K562 and IL12+15. Degranulation (CD107a) and cytokine production were quantified by intracellular flow staining in (A-B) *TBX21* edited, (C-D) *EOMES* edited, and (E-F) *T+E* edited

NK cells flow gated to be $CD56^{bright}$ or $CD56^{dim}$. n=7-10 donors, 4-7 independent experiments. Data were compared by 2-way ANOVA with Holm-Šídák multiple comparison test.

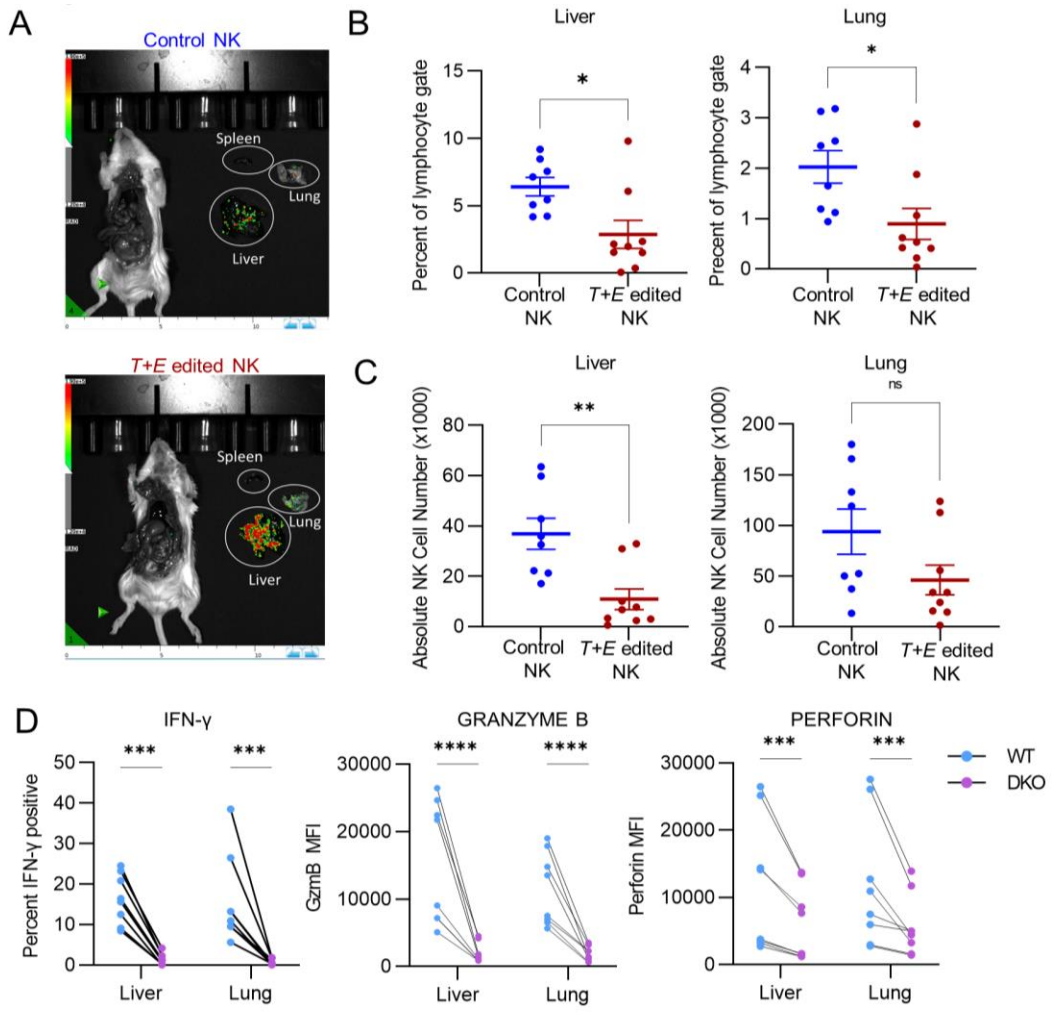

Figure 2.8. Deletion of EOMES and T-BET impairs NK cell numbers and effector molecules expression in K562 tumor bearing mice. NK cells from either *TRAC* gRNA-edited (control) NK group or *T+E* edited NK group were injected i.v. into NSG mice the day after electroporation and i.p. injections of rhIL-15 were performed 3x a week to support the human NK cells. 4 days after NK injection, mice were challenged with 1.5±0.1 x 10^6 luciferase-expression K562 tumor cells i.v. 3 days after tumor injection, mice were imaged and tumor-bearing tissues were harvested and assessed. **(A)** Representative BLI image of tumor signals. **(B)** Percent of lymphocyte gate (FSC by SSC) and **(C)** Absolute NK cell number in the livers and

lungs of tumor-bearing mice. **(D)** Flow cytometry assessment of IFN-γ, Granzyme B, and Perforin expression in gated WT and DKO NK cells from the liver and lung of tumor bearing mice that received *T+E* edited NK cells. Data were compared with Welch's t-test in (B-C), and 2-way ANOVA with Holm-Šídák multiple comparison test in **(D)**. n=8-9 mice per group, 4 donors, 4 independent experiments

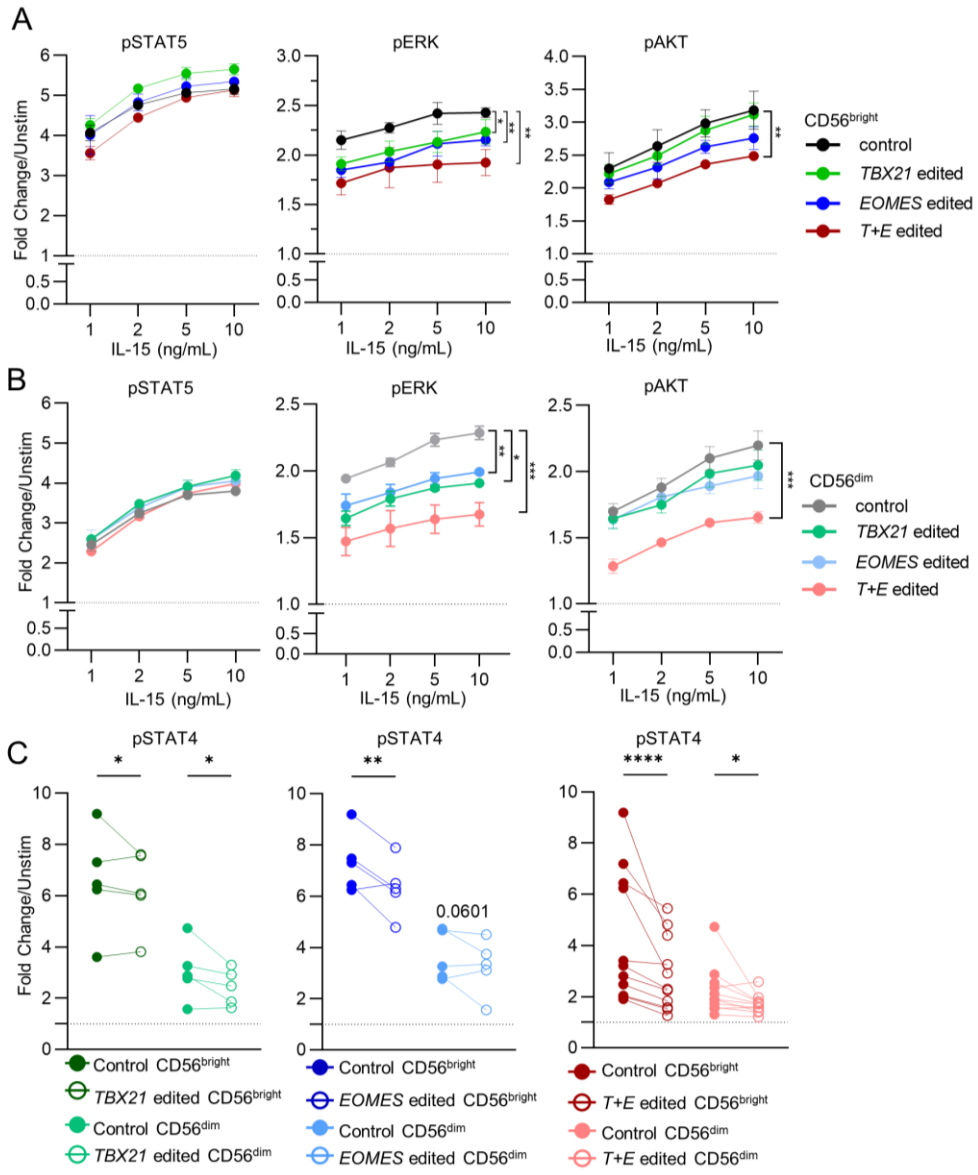

Figure 2.9. Deletion of EOMES and T-BET impairs phosphorylation of ERK and AKT downstream of IL-15 signaling. NK cells were rested in cytokine-free media for 1 hour prior to stimulation with the indicated concentrations of IL-15. Phosphorylated-(p) STAT5 was assessed in cells stimulated for 15 minutes while pERK and pAKT were assessed at 1 hour. Summary data of MFI fold change of **(A)** CD56^{bright} and **(B)** CD56^{dim} NK cells. n=3 donors, 3 independent experiments. **(C)** Summary data of STAT4 phosphorylation upon IL-12 stimulation. pSTAT4

was assessed in cells stimulated with IL-12 for 1 hour. n = 5-8 donors, 3-6 independent experiment. Data were compared with 2-way ANOVA with Holm-Šídák multiple comparison test.

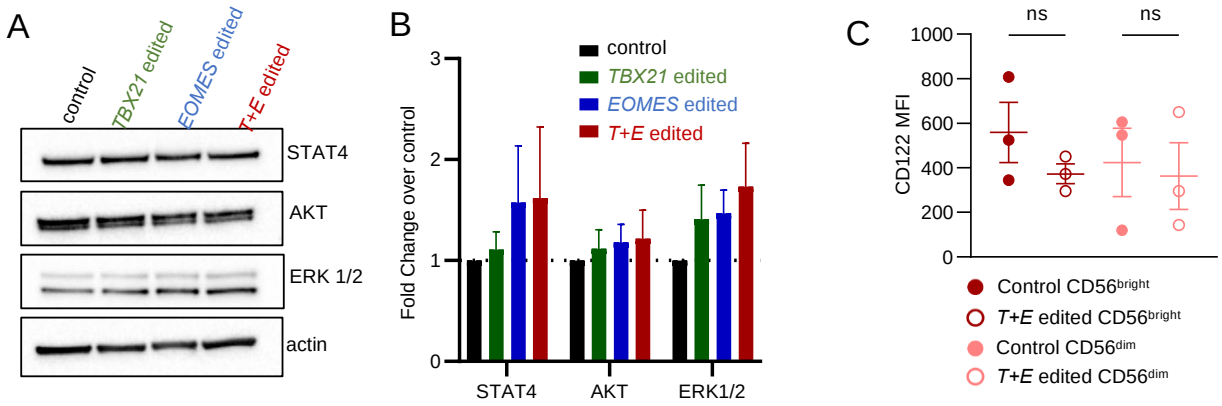

Figure 2.10. Total signaling proteins downstream of IL-15 and IL-15 receptor subunit CD122 protein expression are not affected in *T+E* edited NK cells. (A) Representative western blot and (B) summary data of total STAT4, AKT, and ERK1/2 proteins within CRISPR-edited samples 1 week after electroporation. n=4 donors from 4 independent experiments. No statistical significance was found by 2-way ANOVA with Holm-Šídák multiple comparison test. (C) Expression of CD122 6 days after electroporation of NK cells cultured vitro. n = 3 donors from 3 independent experiments. No statistical significance was found by t-test.

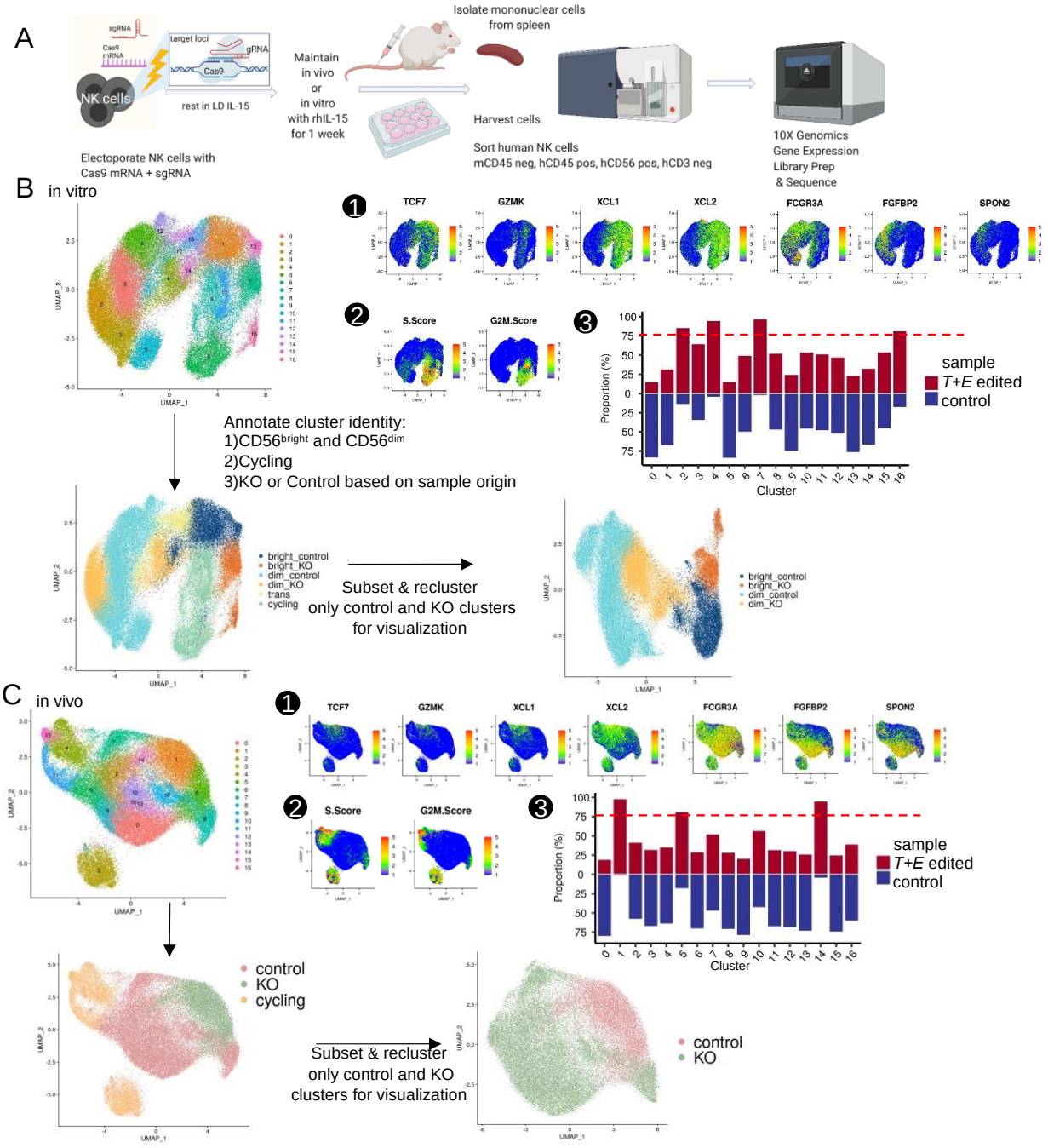

Figure 2.11. UMAP plots of *TRAC*-edited(control) and *T+E* edited scRNA-seq samples show distinct clusters. (A) Schema of single cell RNA-sequencing experiment. The day after electroporation, *TRAC*-edited (control) or *T+E* edited NK cells were injected into NSG mice or maintained in vitro. After 1 week, splenocytes isolated from NSG mice and in vitro maintained

NK cell were FACS sorted (live,hCD45+mCD45-CD56+CD3-). (B) UMAP of in vitro and (C) in vivo maintained samples. Clusters were first assigned to be $CD56^{bright}$ or $CD56^{dim}$ clusters based on known markers for each respective subset($CD56^{bright}$:TCF7, GZMK, XCL1, XCL2; $CD56^{dim}$: FCGR3A, FCGBP2, SPON2). Cycling clusters were them identified by G2M and S scores. Then clusters were grouped into the following-"KO": non-cycling clusters where >75% of cells within the cluster originate from a *T+E* edited samples, "Control": non-cycling clusters where <75% of cells are T+E edited samples. Finally, "Control" and "KO" clusters were reclustered for visualization. n=5 donors, 5 independent experiments in vitro and n=3 , 3 independent experiments in vivo.

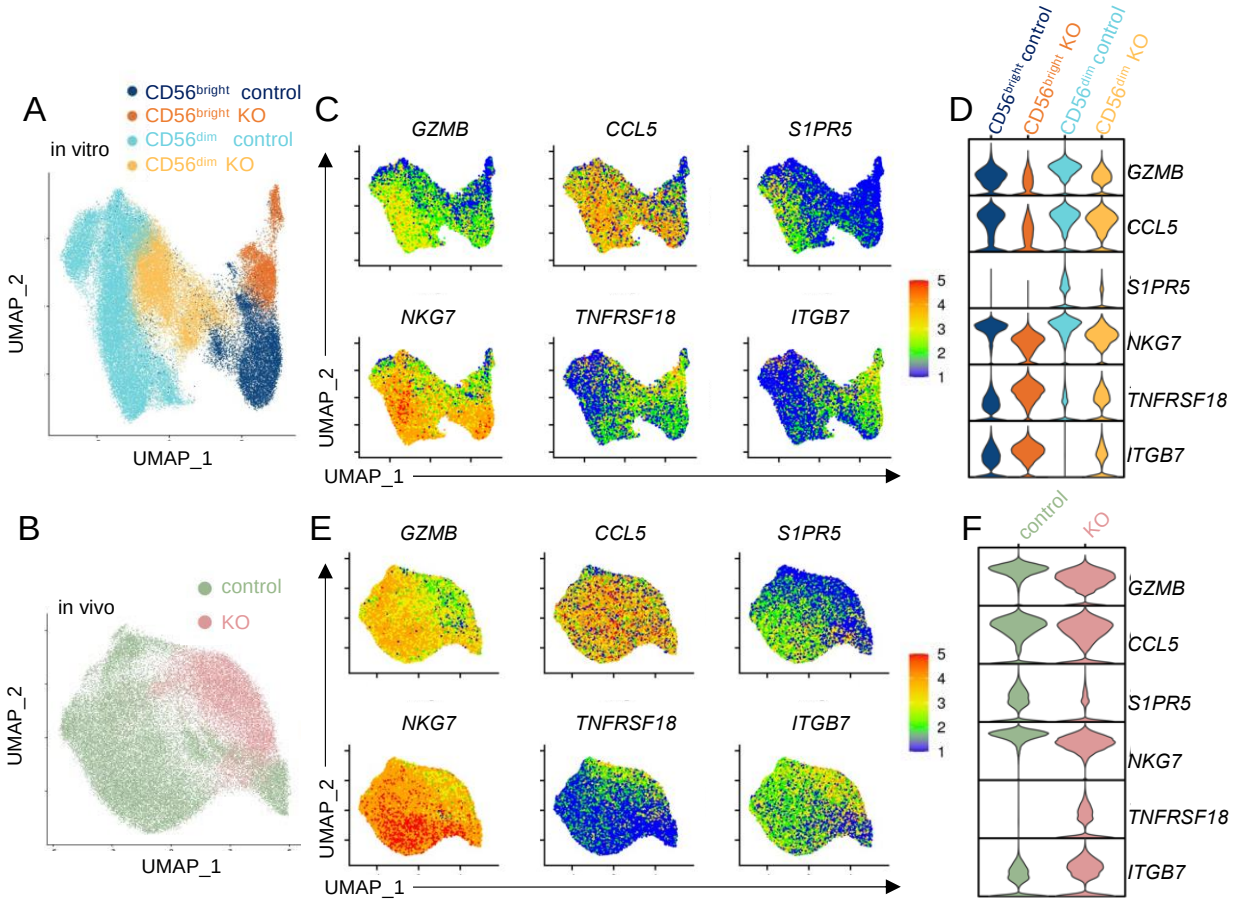

Figure 2.12. Single-cell RNA-sequencing reveals transcriptional profile regulated by EOMES and T-BET in human NK cells. In vitro and in vivo-maintained CRISPR-edited human NK cells were subjected to scRNA-sequencing. Data shown is pooled analysis of n=3-5 donors from 3-5 independent experiments. UMAP of NK cells maintained **(A)** in vitro and **(B)** in vivo, with control and KO clusters identified (see Method and Figure 2.11). **(C, E)** Expression level of selected DEGs overlayed on UMAP. **(D,F)** Violin plots of expression of selected DEGs in control and KO clusters. Wilcoxon Rank-Sum test was used for differential analysis with a threshold of p_val_adj < 0.05.

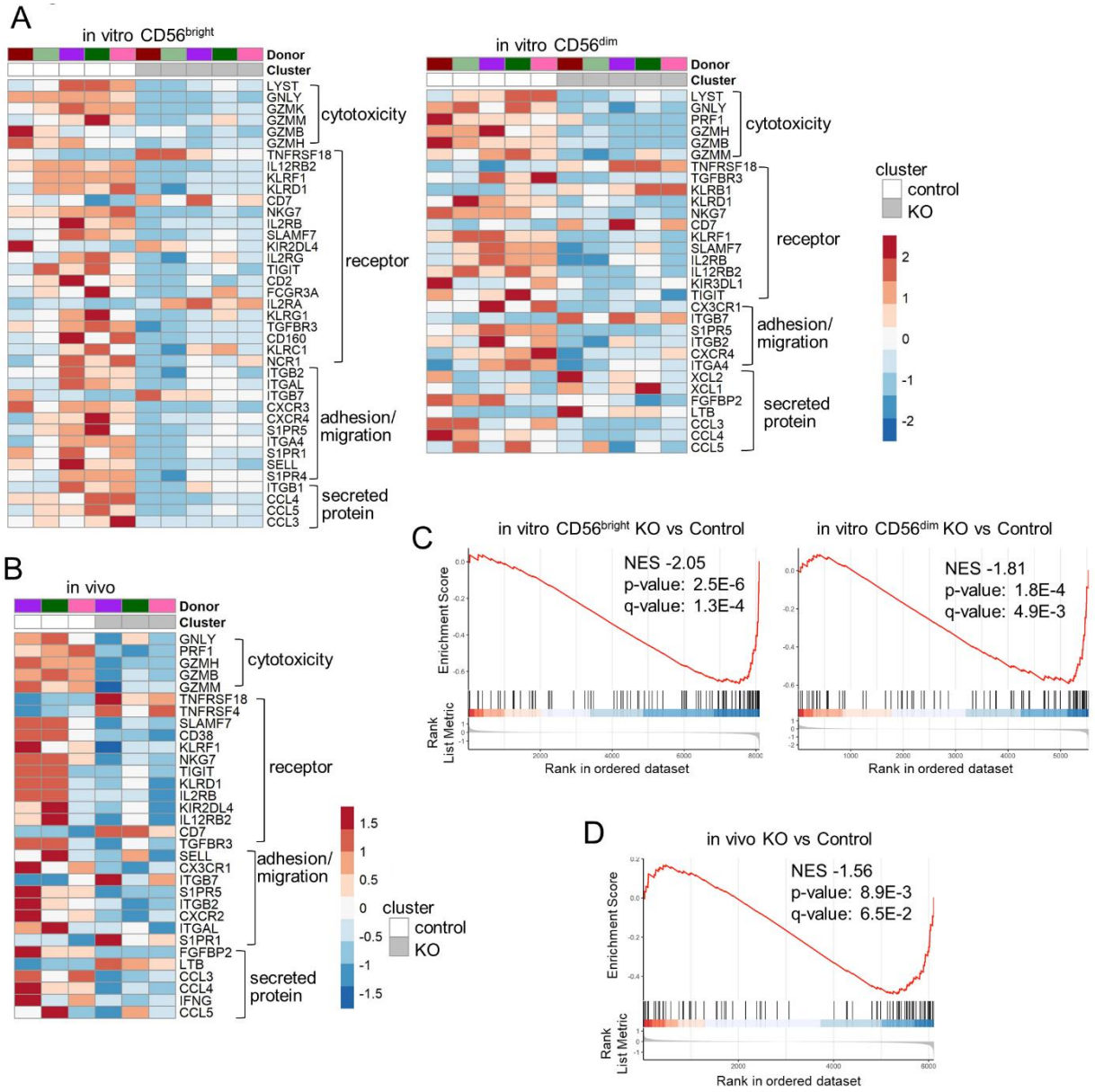

Figure 2.13. EOMES and T-BET are required to sustain the NK cell transcriptional program. (A-B) Heatmap of selected DEGs within each subset identified by scRNA-seq analysis in of control and *T+E* edited NK cells in Figure 6. Wilcoxon Rank-Sum test was used for differential analysis with a threshold of p_val_adj < 0.05. (**C-D**) Gene set enrichment analysis of the "NK cell medicated cytotoxicity pathway" from KEGG data base in KO vs control clusters.

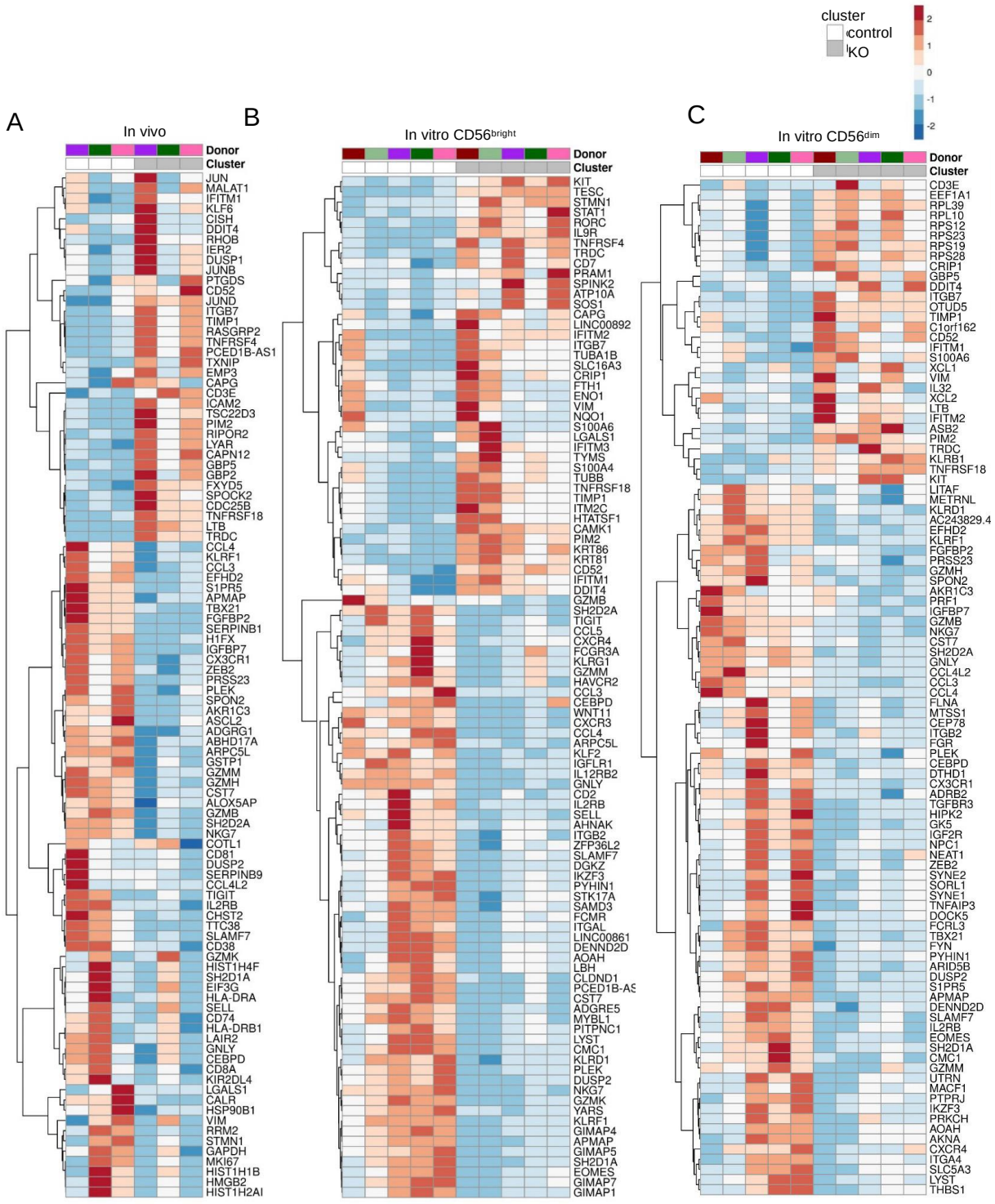

Figure 2.14. scRNA-seq reveals transcriptional profile regulated by T-BET and EOMES in human NK cells. Heatmaps of average expression of Top 100(by Fold Change) differentially

expressed genes of KO vs control clusters from (A) in vivo, (B) in vitro $CD56^{bright}$, and (C) in vitro $CD56^{dim}$ NK cells. n=5 donors, 5 independent experiments in vitro and n=3, 3 independent experiments in vivo.

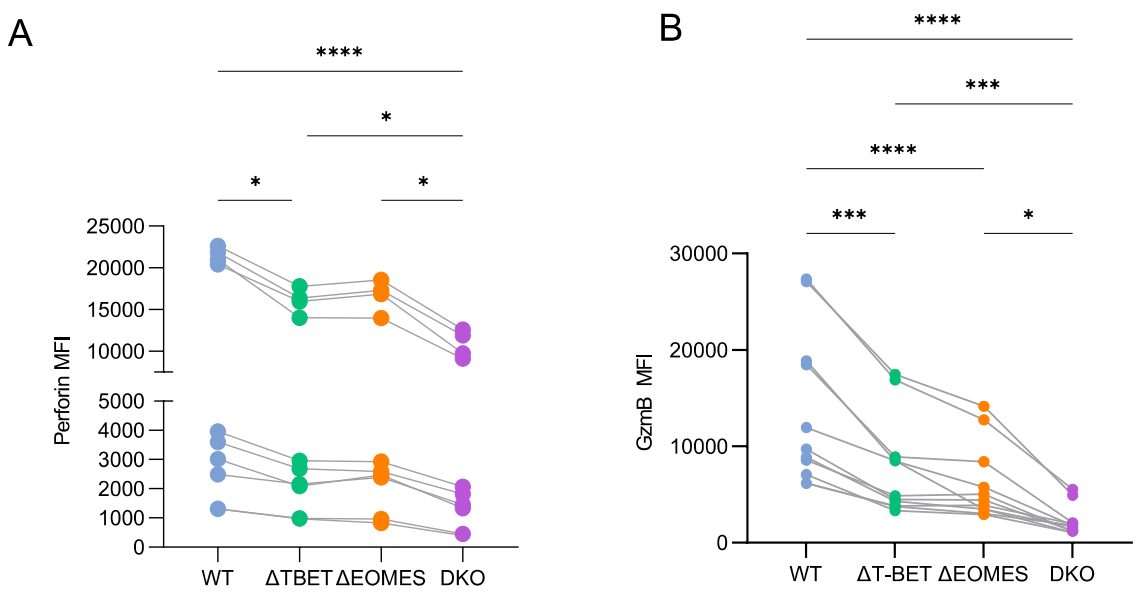

Figure 2.15. T-BET and EOMES regulate protein expression of NK cell cytotoxic effector molecules. Expression of (A) PERFORIN and (B) GRANZYME B in *T+E* edited NK cells from spleens of NSG mice 1 week post NK cell injection. Data were compared using one way ANOVA with Tukey's multiple comparison test. n=5-6 donors, 10-11 mice total, 5 independent experiments.

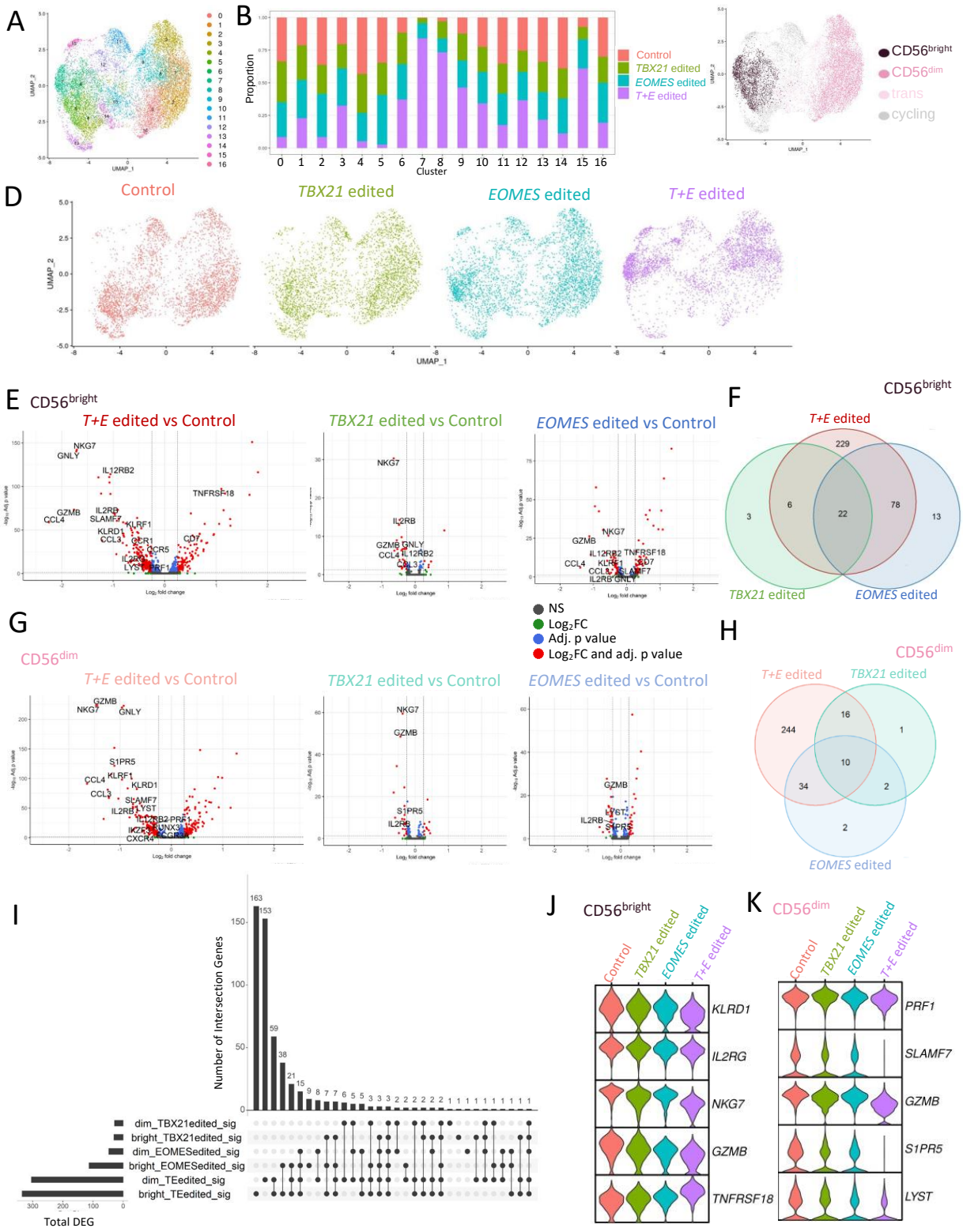

Figure 2.16. T-BET and EOMES single knock-out have minimal effect on human NK cell transcriptional profiles. 8 days after CRISPR electroporation, NK cells were FACS sorted

(live,hCD45+mCD45-CD56+CD3-) and subjected to single cell RNA-sequencing (10X Genomics). (A) UMAP of control, *TBX21* edited, *EOMES* edited, and *T+E* edited NK cells. (B) Distribution of sample groups in each cluster identified by FindClusters function in Seurat. (C) UMAP indicating seurat clusters assigned to be $CD56^{bright}$, transitioning, $CD56^{dim}$, and cycling based on known markers for each respective subset(Fig. S5) (D) Splited UMAPs showing control, *TBX21* edited, *EOMES* edited, and *T+E* edited samples separately. (E-K) Differential expression analysis was performed within $CD56^{bright}$ or $CD56^{dim}$ clusters. T*BX21* edited, *EOMES* edited, or *T+E* edited NK cells were compared against control samples within the respective clusters (E) Volcano plots of DEGs($|log2FC| > 0.25$, adj. p vale < 0.05), and (F) venn diagram depicting unique and overlapping DEGs in comparisons within $CD56^{bright}$ cell clusters. (G) Volcano plots of DEGs and (H) venn diagram depicting unique and overlapping DEGs in comparisons within $CD56^{dim}$ cell clusters. (I) Upset plot depicting intersections of genes with all 6 comparisons in $CD56^{bright}$ and $CD56^{dim}$ clusters. (J-K) Violin plots of example DEGs. n=2 donors from 2 independent experiments.

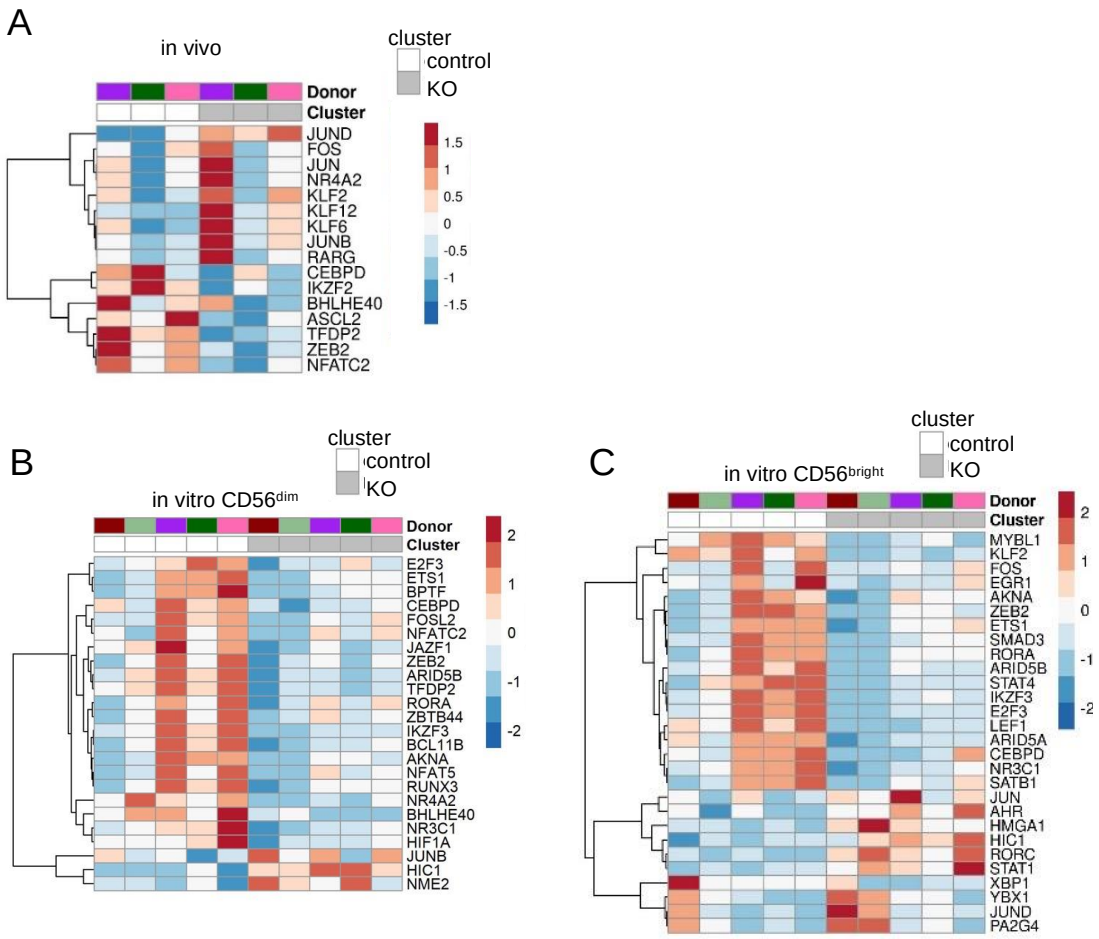

Figure 2.17. T-BET and EOMES regulate expression of other transcription factors.

Heatmaps of average expression of transcription factor genes in control and KO clusters of (A) in vivo, (B) in vitro CD56^{dim}, (C) in vitro CD56^{bright} NK cells in scRNA-seq experiment of control and *T+E* edited NK cells.

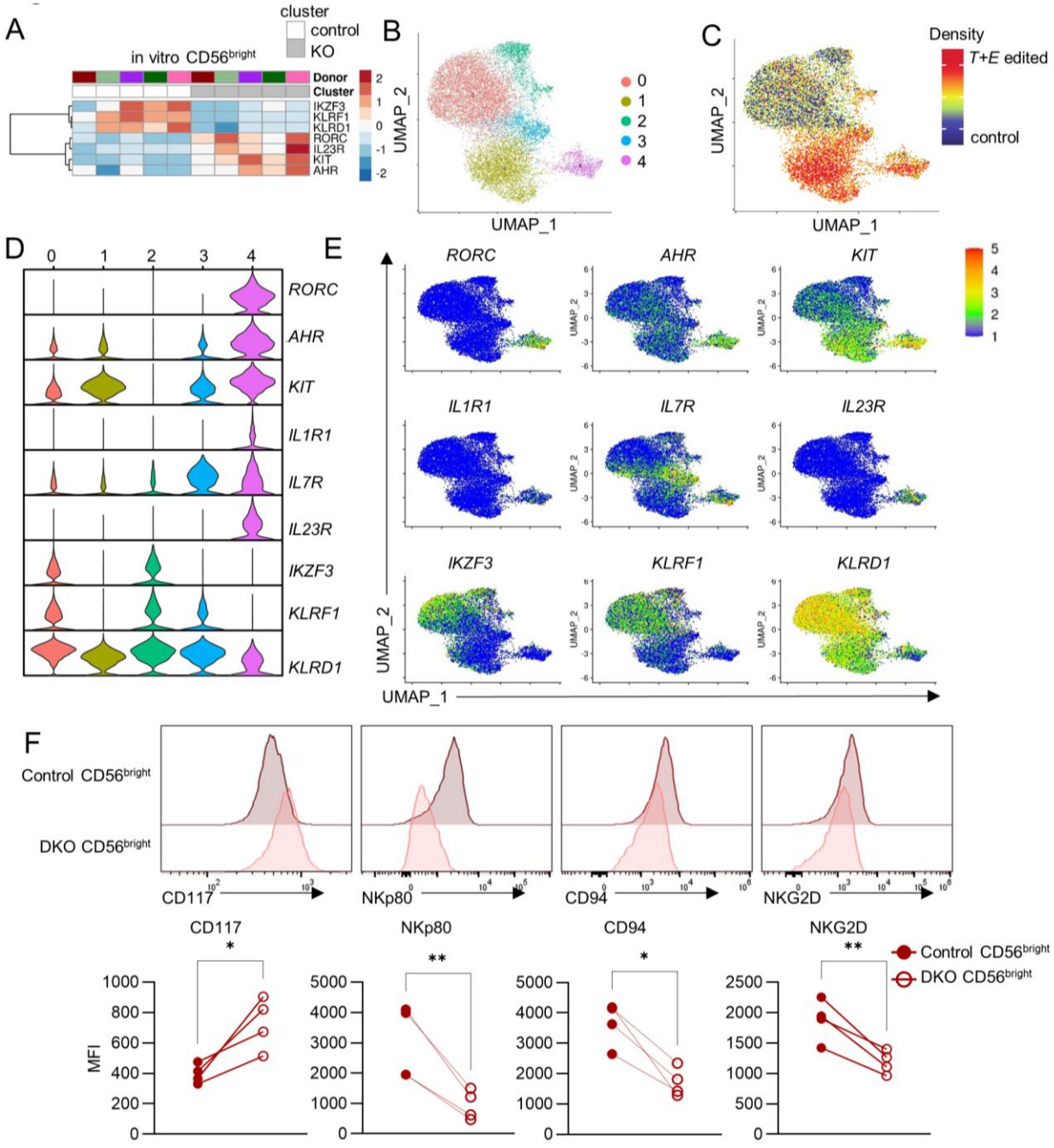

Figure 2.18. Loss of T-box TFs in NK cells results in ILC-3-biased ILC progenitor cell phenotype. (A) Heatmap of differentially expressed ILCP-associated marker genes expressed by in vitro CD56^{bright} KO vs control clusters. **(B)** UMAP of in vitro CD56^{bright} NK cells only. **(C)** UMAP overlayed with density of cells originating from control samples and *T+E* edited samples. **(D)** Violin plot of ILCP related markers within clusters identified in (B). **(E)** Expression

of indicated ILCP related markers overlaid on UMAP space. DEGs determined using Wilcoxon Rank-Sum test and adjusted p-value of < 0.05. **(F)** Protein expression of CD117. NKp80, CD94 and NKG2D of in vitro-maintained *TRAC* edited (control) and gated T-BET EOMES DKO cells 8 day post CRISPR electroporation quantified by flow cytometry. n = 4 donors, 2 independent experiments. Data were compared with ratio paired t-test.

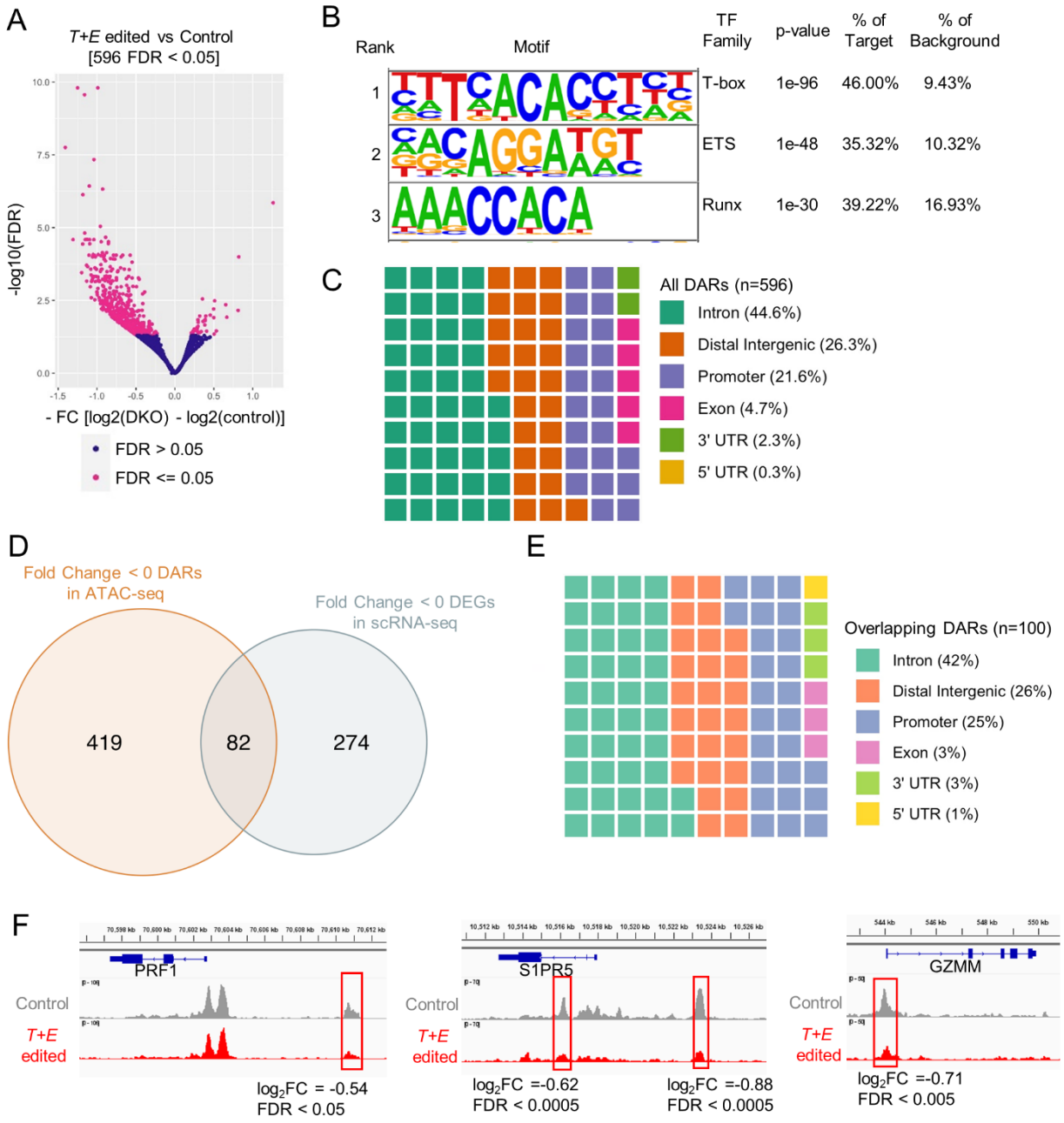

Figure 2.19. EOMES and T-BET maintain NK cell chromatin accessibility. (A) Differentially accessible regions (DARs) identified (blue; FDR > 0.05; pink; FDR <=0.05) by DESEQ2. **(B)** Homer de novo motif enrichment analysis on all DAR using findMotifsGenome.pl with automatically generated background by HOMER **(C)** Square Pie chart of genomic region

distribution of all DARs annotating using Chipseeker annotatePeak function. **(D)** Venn Diagram of overlapping genes annotated as downregulated DARs in ATAC-seq and genes identified as downregulated DEG in $CD56^{bright}$ or $CD56^{dim}$KO clusters in scRNA-seq. **(E)** Genomic region distribution of DARs(100 regions total, annotated to 82 unique genes) that are annotated to be in/near DEGs. **(F)** Representative peaks in *PRF1*, *S1PR5*, and GZMM, regions showing loss of accessibility in *T*+*E* edited NK cells. n = 2 donors, 2 independent experiments.

Chapter 3: The Requirement of T-bet in Murine NK cells and ILC-1s

3.1 Summary

T-box transcription factors Eomes and T-bet are best known for their requirement in NK cell development. As such, mature NK cells are absent in previous global and constitutive T-bet deletion mouse model and thus precluded the study for mature NK cell-intrinsic requirement for T-bet. Here in this study, we generated *Ncr1*-CreER^{T2} x *Tbx21*$^{fl/fl}$ mice that allowed for the study of inducible T-bet deletion in an intact NK cell and ILC-1 compartments. We found that mature NK cells in the periphery and ILC-1s in the liver require T-bet expression to persist. Furthermore, direct comparison of inducible T-bet deletion with inducible Eomes deletion mice demonstrate differential requirement for the two transcription factors to maintain protective response against MCMV infection.

3.2 Introduction

Natural Killer (NK) cells are innate lymphoid cells that are known for their role in recognizing and killing virus-infected or tumor cells(1,2). Once activated by target cells or cytokines, NK cells are capable of potent IFN-γ secretion in addition to direct cytotoxicity(1,2). NK cell activation is directed by integrating signals from a variety of inhibitory and activating receptors that control their effector functions(124,125). NK cells are characterized by their expression of two T-box transcription factors, T-bet and Eomes(3,24,48,61). Our group has recently reported in human NK cells that sustained expression of Eomes and T-bet is required for human NK cell function and identity(66). However, the in vivo study of human NK cells lacking these

transcription factors were limited to xenograft transfers using NSG mice, which precludes a comprehensive study of the transferred NK cells in a species-matched environment.

In mouse NK cell models, these two transcription factors have been extensively demonstrated to be critical for NK cell development(29,37,110). Nevertheless, these previous models used hematopoietic or constitutive knockout models that lead to NK cell developmental defects, which precludes genetic loss-of-function studies on mature NK cells(29,37,38,48). To address that, our lab generated a tamoxifen-inducible *Ncr1*-CreER^{T2} mouse model that allowed the study of inducible gene deletion in a fully developed, intact NK cell and ILC-1 compartment(60). Our previous study using *Ncr1*-CreER^{T2} x Eomes$^{f/f}$ mice showed that Eomes continues to be important for NK cell function and homeostasis beyond promoting NK cell development, which contradicts published studies using less specific mouse models that has impaired NK development or ex vivo or adoptive transfer models(44,48,60,110). Thus the study of T-bet using an inducible model is also warranted to uncover the requirement of T-bet alone and T-bet and Eomes together in the mature murine NK cell compartment in vivo.

While T-bet and Eomes do share some redundant targets at the genomic level due to their highly homologous T-box DNA binding domain, they have also been demonstrated to have unique functions(24,44,126,127). In NK cell development and maturation, T-bet but not Eomes has been suggested to be most important for terminal NK cell maturation where as Eomes is more important for early NK maturation(38,48). Furthermore, T-bet has also been implicated in regulating NK cell egression from the bone marrow into the periphery(49,111). Here in this study, we generated *Ncr1*-CreER^{T2} x *Tbx21*$^{f/f}$ mice that allowed for the study of inducible T-bet deletion in an intact NK compartment with fully developed and mature NK cells as well as liver ILC-1s, which are all absent in previous global and constitutive T-bet deletion mouse

models(29,37,48). Furthermore, we directly compared *Ncr1*-CreER^{T2} x *Tbx21* $^{f/f}$ and *Ncr1*-CreER^{T2} x Eomes$^{fl/fl}$ mice to uncover the differential requirement for T-bet and Eomes in NK cells for MCMV protection.

3.3 Results

Induced T-bet deletion results in alteration of NK cell compartment maturation stage distribution

To assess the requirement of persistent T-bet in an fully-developed, mature NK cell compartment, we crossed our previously generated *Ncr1*-iCreER^{T2} x Rosa-YFP^{LSL} mice with *Tbx21*$^{fl/fl}$ mice (T-bet FKO) (60,128) . As T-bet has been demonstrated to be critical for NK cell development and especially terminal maturation, this approach allows us to induce the deletion of T-bet in a fully intact NK cell compartment. To determine the impact of inducible T-bet deletion in NK cells, we treated T-bet FKO mice with tamoxifen for 3 days by oral gavage (Day 1-3), then on day 6 we assessed NK cell numbers and maturation (Figure 3.2). The inducible *Ncr1*-CreER^{T2} activity was highly effective with 90% of NK1.1+ NKp46+ cells expressing YFP at this time point, and T-bet protein expression was abrogated in YFP+ NK cells in the T-bet FKO mice across various organs (Figure 3.2, B-C). Inducible T-bet deletion had different impact on NK cell maturation across the different tissues assessed (Figure 3.2D). The NK cell numbers in the spleen, lymph nodes and bone marrow were not significantly different between T-bet WT and T-bet FKO mice (Figure 3.2D). In contrast, in the blood and the lung, NK cell numbers were significantly decreased in T-bet FKO mice.

After murine NK cells differentiate from the NK precursor, they undergo sequential maturation stages that can be identified based on surface expression of CD27 and CD11b(19). Stage I NK cells (CD27^{-}CD11b^{-}) are the most immature subset. Stage II NK cells (CD27^{+}CD11b^{-}) are highly

proliferative but have less robust effector functions than the mature Stage III ($CD27^{+}CD11b^{+}$) and Stage IV ($CD27^{-}CD11b^{+}$) NK cells are mature NK cells with Stage IV being the highest functional capacity(19). Thus, we next assessed the contribution of T-bet in maintaining the distribution of NK cells in the various maturation stage (Figure 3.3). In the spleen and lymph nodes, there was a significant increase in Stage III NK cells. However in the blood and lung, Stage III NK cell numbers were intact but there was a significant loss of Stage IV NK cells in the T-bet FKO mice. In the bone marrow, there was an trending but not statistically significant increase of Stage II NK cells (Figure 3.3).

IFN-γ response is preserved in inducible T-bet deletion but cytotoxicity is impaired

Next we assessed whether T-bet expression was required for IFN-γ production by NK cells in response to stimulation target cells, cytokines, and activating receptor (NK1.1) engagement for 6 hours (Figure 3.4). The ability for NK cells to produce IFN-γ was minimally impacted by inducible T-bet deletion overall (Figure 3.4A). However, when looking specifically within maturation stages, T-bet FKO Stage II NK cells had significantly decreased IFN-γamma production in response to IL-12 and IL-15, and in contrast IFN-γ responses were intact in T-bet FKO Stage III and Stage IV NK cells. This indicates that Stage III and Stage IV NK cells do not require T-bet for this response (Figure 3.4B).

In contrast to the minimal impact on IFN-γ production, NK cell cytotoxicity was significantly impaired when T-bet expression is abrogated (Figure 3.4C-E). Degranulation upon stimulation by YAC-1 target cells was significantly lower in NK cells from T-bet FKO mice. We next tested whether this is true in in vivo setting. In an in vivo cytotoxicity assay with B2M-/- splenocytes,

T-bet FKO mice had decreased ability to reject MHC class I deficient splenocytes compared to WT mice (Figure 3.4D).

It was previously reported that global T-bet$^{-/-}$ mice had impaired control of B16F10 lung metastasis and it was suggested to be a NK cell-dependent phenomenon. Indeed in T-bet FKO mice, we observed impaired control of B16F10 lung metastases compared to control mice (Figure 3.4E). This is interesting because they T-bet FKO mice have a mostly intact NK compartment, except for the loss of mature NK cells in the blood and lung (Figure 3.2-3.3). This demonstrates that the loss of Stage IV NK cells in the blood and lung is sufficient for the loss of B10F10 metastasis control.

T-bet is critically required to maintain ILC-1 in the liver

Next, we investigated whether T-bet expression is required to maintain the ILC-1 compartment in the liver. Previous studies with T-bet global KO mice that T-bet is required for the fetal development of ILC-1s in the liver and also other NKp46+ ILCs in the intestines, but whether developed ILC-1s still require T-bet to persist is not known(41,48). Using the inducible Ncr1-T-bet KO mice, we found a dramatic, significant loss of ILC-1 in the livers upon inducible T-bet deletion. In contrast, NK cell numbers are mostly intact (Figure 3.5A). This loss of ILC-1s is observed as early as Day 4 of the tamoxifen regiment, demonstrating that not only do ILC-1s require T-bet during development, ILC-1s in the liver require T-bet to persist (Figure 3.5B).

Expression of T-bet and Eomes are absolutely required for NK cell survival in the periphery

It has been previously suggested that T-bet and Eomes have redundant function. Indeed in a previous study where they crossed the *Vav*-Cre x Eomes$^{fl/fl}$ mice to the global T-bet-/- mice,

there was a complete absence of NK cells despite intermediate phenotype in the parental single Eomes/T-bet knock-out strains. Here, we crossed the inducible *Ncr1*-iCreER^{T2} mice to mice to both Tbx21 and Eomes floxed alleles. Upon induced simultaneous deletion of T-bet and Eomes, there was a drastic loss of NK cells that was observed as early as Day 4 in the spleen and blood. However, overall NK cell numbers remain unaltered in the bone marrow up to Day 9 when looking at the bulk NK population (Figure 3.6A). For most tissues, it seems that NK cells in different maturations were all affected by the loss of Tbet and Eomes, with most significant effect in Stage IV NK cells which are a majority of NK cells in the spleen and blood. Interestingly in the bone marrow, Stage III and Stage IV NK cell numbers remain the same even when both Eomes and T-bet are abroagated, but Stage II NK cell numbers are significantly decreased (Figure 3.6B-C).

Individual contribution of T-bet and Eomes in MCMV infection

As T-bet and Eomes have been previously to be demonstrated to have different effects in MCMV infections using adoptive transfer models to permit study of downregulation of T-bet and Eomes in existing NK cells, here we investigated their individual contribution in our NK cell specific inducible model. Previous studies have showed that T-bet expression is increased during MCMV infection and is important for the expansion of NK cells in an adoptive transfer model. Here we treat our T-bet and Eomes FKO mice with tamoxifen chow for 3 days, then we infected them with 5e4pfu MCMV (Figure 3.7A). All mice lost weight during the course of infection but Eomes FKO mice were unable to regain weight, suggesting a suboptimal NK cell response against the infection (Figure 3.7B). This is in concordance with significantly higher viral titer in

the Eomes FKO mice compared to control mice which was not observed in T-bet FKO mice (Figure 3.7C).

We next assessed NK cell activation and MCMV-induced expansion of Ly49H+ NK cells in inducible T-bet and Eomes KO mice. NK cells that had T-bet and Eomes deleted had same level of KLRG1 expression as control NK cells (Figure 3.7D). The percent of them expressing Ly49H, the receptor for m157, were also not significantly different (Figure 3.7E). Nevertheless, the absolute number of NK cells on day 7 post infection was significantly decreased in Eomes FKO NK cells that was concordant with increased apoptosis in Eomes FKO NK cells on 7dpi (Figure 3.7 F-G). Finally, assessing individual maturation stages, we observed significant decreases in Stage IV T-bet and Eomes FKO NK cells compared to control NK cells (Figure 3.7H). In Madera et al., they concluded in their adoptive transfer model that T-bet deletion will revert the population to a more immature phenotype with increased Stage III cells by percentage. However, here assessing absolute numbers, inducible T-bet deletion mice had a loss of Stage IV NK cells and did not have increased numbers of immature subsets (Figure 3.7H). These results altogether indicates that despite the loss of Stage IV NK cells in T-bet observed in the spleen by 7 days post infection, T-bet FKO mice were still able to mount a comparable response MCMV compared to control mice to protect the host against. However, Eomes FKO mice have a dramatic defect in their NK compartment, making them unable to produce an effective, protective MCMV response.

3.4 Discussion

This study demonstrates that T-bet is required to maintain the mature NK cells in the periphery beyond NK cell development and trafficking. Inducible deletion of T-bet in NKp46-expressing

cells led to a rapid decrease of Stage IV NK cells in the periphery and a dramatic loss of ILC-1s in the liver. Our study also reveals that despite the loss of the most mature NK cells in the periphery and ILC-1s, T-bet FKO mice are able to effectively control MCMV infection until Eomes FKO mice, demonstrating a differential requirement for these transcription factors for MCMV response.

NK cells in the blood and lung are particularly affected by acute abrogation of T-bet, specifically the predominant Stage IV subsets in those locations. However, in the bone marrow and lymph nodes, where more immature subsets preside, induced T-bet deletion in the NK cells there did not have significant impact on the most mature subset. Instead, T-bet deletion led to an increase of Stage III NK cells in the lymph node, perhaps suggesting a trafficking defect as T-bet as been shown to be important for in global KO models(49). We further show that there is a certain level of redundancy with T-bet and Eomes cooperating to maintain mature NK cells as when both are simultaneously deleted , there was a rapid loss of NK cell numbers across various tissues. While it has been shown before that T-bet and Eomes double deletion will preclude development, our model is the first to show that murine NK cells, across all maturation subsets, still require T-bet and Eomes to persist(48).

Functionally, T-bet is dispensable for IFN-γamma production by the most mature NK cells, but it is required for Stage II NK cells to respond to IL-12 and IL-15 to produce IFN-γ. NK cell cytotoxicity against MHC-I deficient targets and lung metastasis however required T-bet expression in NK cells, this is perhaps due to the loss of NK cells in the blood observed in the T-bet FKO mice. Interestingly, despite this loss of NK cells in the blood and the loss of ILC-1s,

MCMV response was mostly intact in T-bet FKO mice, unlike Eomes FKO mice. Eomes FKO mice were unable to mount an effective protective response against sublethal dose of MCMV, while T-bet FKO mice were protected like control mice.

Previously in an attempt to study NK cell specific T-box transcription factor deletion, Madera et al. utilized an adoptive transfer model with T-bet $^{fl/fl}$ x Ub-Cre NK cells and concluded that T-bet is dispensable for NK homeostasis but critical for MCMV response, which contradicts our lab's data here using the NKp46-specific, inducible T-bet deletion model. These discrepancies between published studies and our model could be due to how a such complex adoptive transfer model is not optimal for recapitulating normal physiology.

It was surprising to observe in our study that NK cells in the bone marrow can survive even after inducible loss of Eomes and T-bet expression, contrasting previous studies with T-bet$^{-/-}$ x Vav-Cre x Eomes$^{fl/fl}$ mice where without both T-bet and Eomes, there was a complete loss of NK cells development even in the bone marrow(48). Perhaps the bone marrow niche has sufficient survival signals and growth factors that promote ant-apoptotic pathways in NK cells in a T-bet and Eomes-independent manner, but once NK cells exit the bone marrow, they require T-bet and Eomes to survive. In recent years, NK cells have emerged as a promising immunotherapy option to treat diseases like cancer (68). Thus, a comprehensive understanding of the fundamental mechanisms of NK cell biology can provide insights to potential ways to enhance NK cell therapy.

3.5 Methods

Mice

Ncr1-iCreERT2 x Rosa26 YFPLSL knock-in mice were generated by our lab as reported previously (Wagner et al) and were crossed to Tbx21fl/fl mice (The Jackson Laboratory, RRID:IMSR_JAX:022741). For all experiments, *Ncr1*-iCreER^{T2} KI/WT or KI/KI x *Rosa26*-YFP^{LSL} x Tbx21 $^{WT/WT}$ Eomes$^{WT/WT}$ mice were used as WT controls unless otherwise indicated. Mice in each experiment were treated with identical tamoxifen regimens. Tamoxifen was administered in corn oil (Sigma) for, 1mg per day up to 3 days as described. For MCMV studies, tamoxifen chow was used instead of oral gavage. 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 8-16 week old male and female mice.

Cell Lines

YAC-1 cells were maintained at 37ºC in 5% CO_2 in RPMI-1640 plus 10% FBS and supplements; B16-F10 cells were maintained in DMEM plus 10% FBS and supplements following ATCC guidelines and kept in continuous culture for < 2 months.

Organ isolation and processing

Mice were sacrificed by CO_2 asphyxiation and organs harvested immediately. Cardiac puncture was performed to collect blood prior to removal of other organs when appropriate. Spleens and inguinal nodes were isolated and crushed through a 70□m filter to generate a single cell suspension. Bone marrow was harvested by flushing one femur from each mouse with a 23-

gauge needle. Percoll gradient was used to isolate lymphocytes on homogenoized livers. All tissues were ACK lysed to remove red blood cells.

Flow cytometry and cell sorting

Surface antibody staining was performed as previously described(60). Intracellular staining was performed in permeabilization buffer following fixation/permeabilization kits from BD Biosciences (for IFN-γ staining) or Invitrogen eBioscience Fix/Perm kit. When using the eBioscience Fix/Perm kit quenches YFP, after surface staining cells were briefly fixed in 1% PFA for 2 minutes at room temperature prior to fixation/permeabilization in order to preserve YFP signal when intracellular proteins were assessed. For T-bet staining, cells were incubated in eBioscience 1X Fix/Perm overnight at 4 degrees in the dark, then the next day proceeded to staining overnight in 1X Perm Buffer at 4 degrees in the dark. Flow cytometry data were collected on Gallios, Attune Nxt, or Cytek Northern Light flow cytometers, and analyzed using FlowJo (Treestar) software.

In vivo B2m$^{-/-}$ rejection

Mice were treated with tamoxifen in corn oil by oral gavage for 2 or 3 consecutive days. On the day after the last dose of tamoxifen, mice also received 2-4 x 10^6 splenocytes that were a 1:1 mix from WT and B2m$^{-/-}$ mice (JAX). Prior to transfer, the splenoctytes were labled with CFSE and differentially labelled with CTV. Some mice received 200ug anti-NK1.1 intraperitoneally (i.p.) the day before transfer. 18-20 hours after splenocyte transfer, mice were sacrificed and the ratio of CTV^{hi} (WT) to CTV^{low} (B2m$^{-/-}$) splenocytes was assessed. Percent rejection was calculated as: (1-[(Ratio B2m$^{-/-}$: WT)$_{experimental}$/(Ratio B2m$^{-/-}$: WT)$_{NK\ depleted}$])*100 (129).

In vitro functional assays

Splenocytes were either unstimulated (media with low dose IL-15) or stimulated with additional cytokines (10ng/mL IL-12 + additional 10ng/mL IL-15, both Peprotech), YAC-1 lymphoma targets (E:T = 10:1), or 2.5ug plate-bound purified anti-NK1.1 for 6 hours. Anti-CD107a (LAMP-1) antibody was added at the start of the assay, and brefeldin A and monensin (BD Biosciences) were added after the first hour. Cells were then stained for surface antigens, fixed/permeabilized using the BD Biosciences Fix/Perm kit, and intracellularly stained for IFN-γ to be analyze by flow cytometry.

MCMV infection

Age and gender-matched mice were infected with indicated plaque forming units (pfu) of Smith strain MCMV via intraperitoneal (i.p) injections as previously described (cooper lab papers). Mice were monitored daily after infection for weight loss. Sub-lethal MCMV challenge was performed by infecting mice with 5×10^4 pfu i.p. and sacrificing the animals at the indicated time points post infection for viral titers by qPCR and flow cytometric assessment as previously described.

Quantification and Statistical Analysis

Statistical analyses were performed using GraphPad Prism software. Tests utilized are indicated in figure legends. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

3.6 Figures

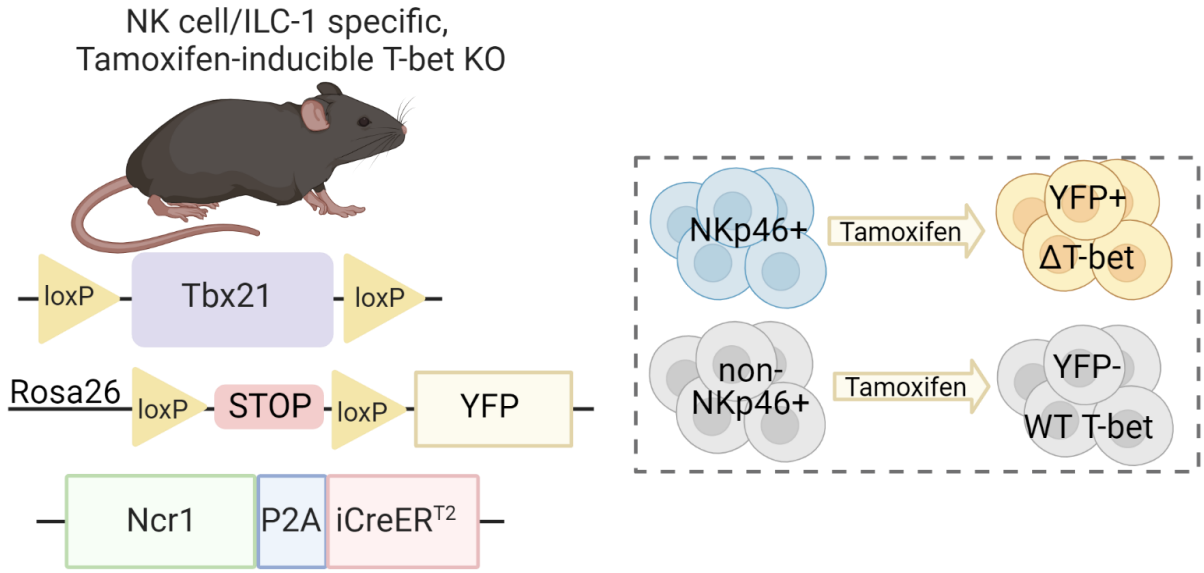

Figure 3.1. *Ncr1*-iCreER^{T2} x *Rosa26*-YFP^{LSL} x Tbx21$^{fl/fl}$ (T-bet FKO) mouse schema

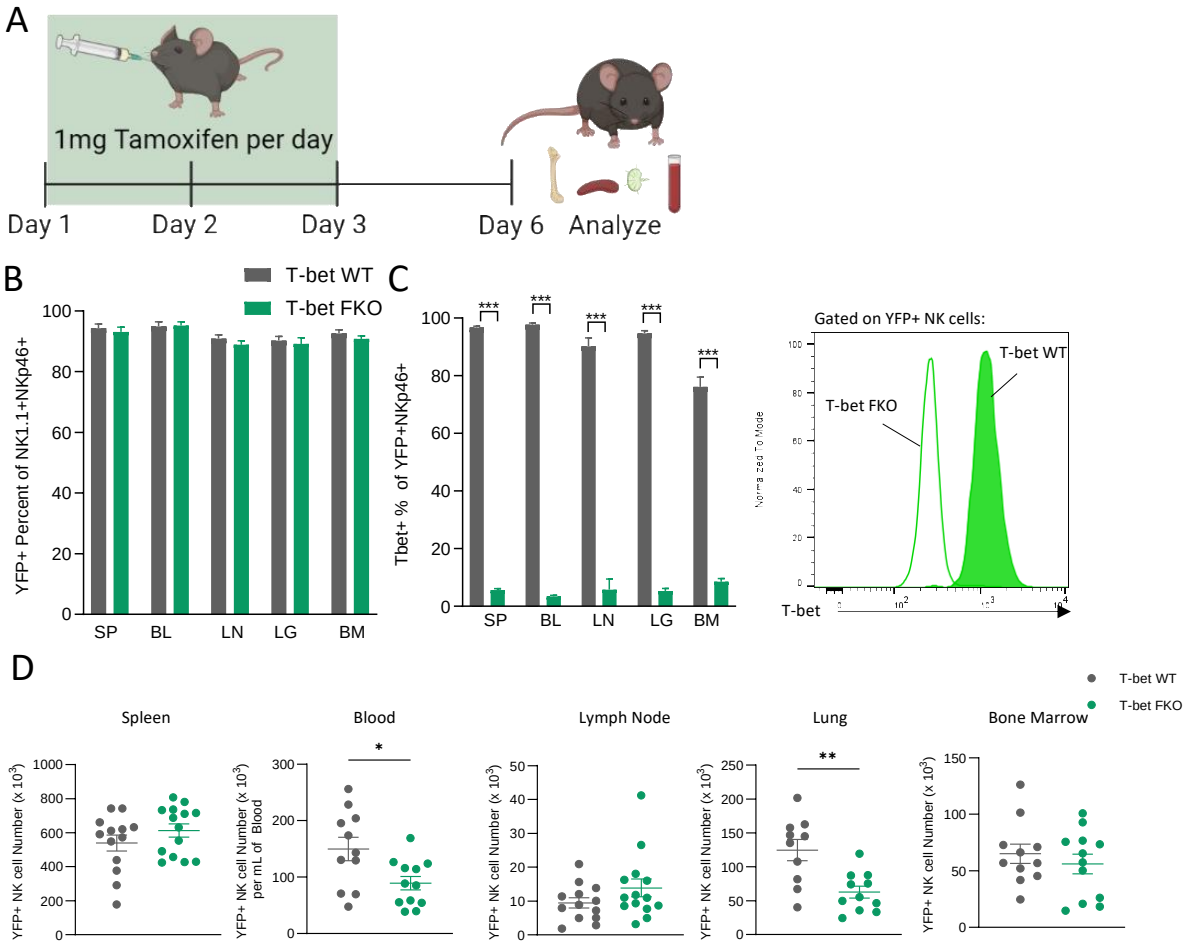

Figure 3.2. Induced T-bet deletion results in alteration of NK cell numbers. (A) *Ncr1*-iCreER^{T2} x *Tbx21*$^{fl/fl}$ (T-bet FKO) mice and *Ncr1*-iCreER^{T2} x *Tbx21*$^{WT/WT}$ (T-bet WT) mice were treated with 1mg tamoxifen per day by oral gavage for 3 days (Day 1-3), then on Day 6, mice were sacrificed for analysis. **(B)** YFP+ percentage of NK cells (CD3^{-}NK1.1^{+} NKp46^{+}) in indicated organs: spleen (sp), blood (Bl – per mL), lymph nodes (LN –inguinal LNs), bone marrow (BM – per femur). **(C)** Summary data of T-bet^{+} percentage within YFP+ NK cells, left. Representative histogram of T-bet expression, right. **(D)** Summary data of all YFP+ NK cell numbers in Ncr1-T-bet$^{D/D}$ or T-bet WT mice in the 5 different tissues. Statistical significance was determined by t-test.

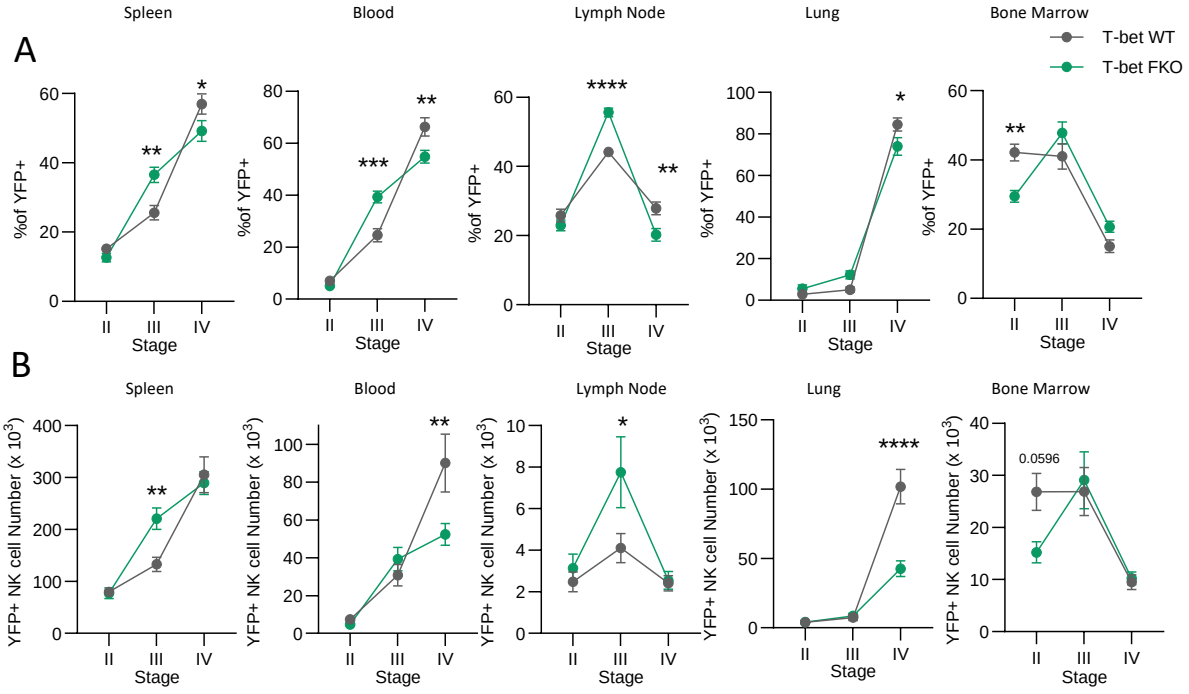

Figure 3.3: Induced T-bet deletion results in alteration of NK cell compartment maturation stage distribution. *Ncr1*-iCreER^{T2} x *Tbx21*$^{fl/fl}$ mice (T-bet FKO) and *Ncr1*-iCreER^{T2} x *Tbx21*$^{WT/WT}$ (T-bet WT) were treated with 1mg tamoxifen by oral gavage for 3 days (Day 1-3), then on Day 6, mice were sacrificed for analysis. Summary data of **(A)** percentage and **(B)** absolute numbers of YFP^{+} NK cell within each indicated maturation stage. Statistical significance was determined by 2-way ANOVA.

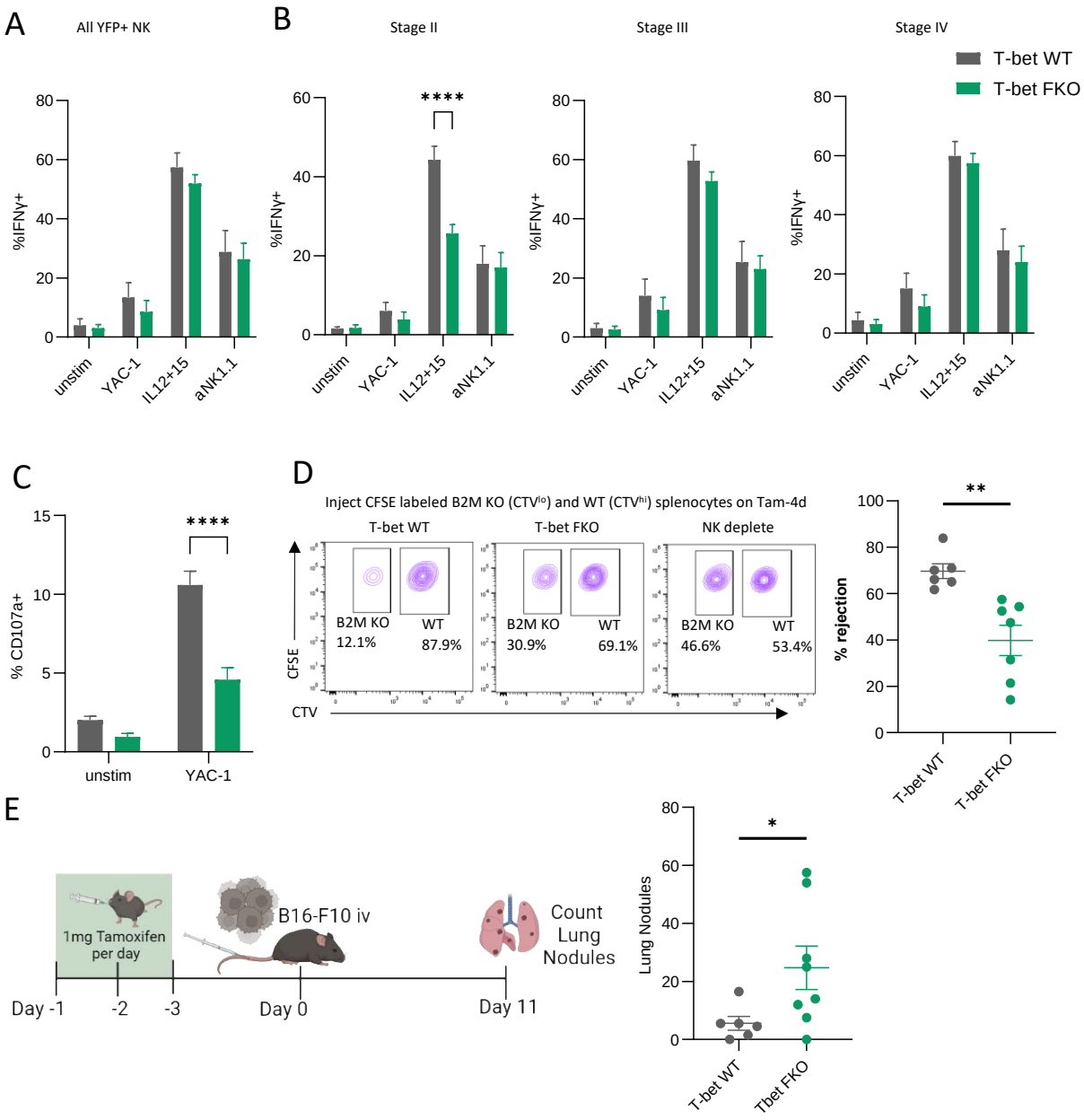

Figure 3.4. IFN-γ response is preserved in T-bet FKO NK cells but in vivo cytotoxicity is impaired. (A-C) T-bet FKO mice were treated with 1mg tamoxifen by oral gavage for 3 days (Day 1-3), then on Day 6, splenocytes were harvested for ex vivo functional assessment. **(A)** IFN-γ production by all YFP$^+$ NK cells (CD3$^-$NK1.1$^+$ NKp46$^+$) and **(B)** IFNg production by all YFP$^+$ NK cells within indicated maturation stage in response to YAC-1, IL-12+15 and plate

bound anti-NK1.1 stimulation. **(C)** Summary data of CD107a+ percentage within YFP+ NK cells in response to YAC-1 stimulation **(D)** T-bet FKO and T-bet WT mice were treated with 1mg tamoxifen by oral gavage for 2-3 days, then the day after the last dose of Tamoxifen, labeled WT and B2M KO splenocytes were transferred into the recipient mice at a 1:1 ratio. Left, representative flow plots showing selective rejection of B2M KO splenocytes and right, summary data of percent B2M KO rejection. **(E)** T-bet FKO and T-bet WT mice were treated with 1mg tamoxifen by oral gavage for 3 days, on Day 4, 2.5×10^5 B16-F10 cells were injected i.v.. Eleven days later, lungs were harvested, and lung nodules were counted. Statistical significance was determined by 2-way ANOVA (A-C) and t-tests (D-E).

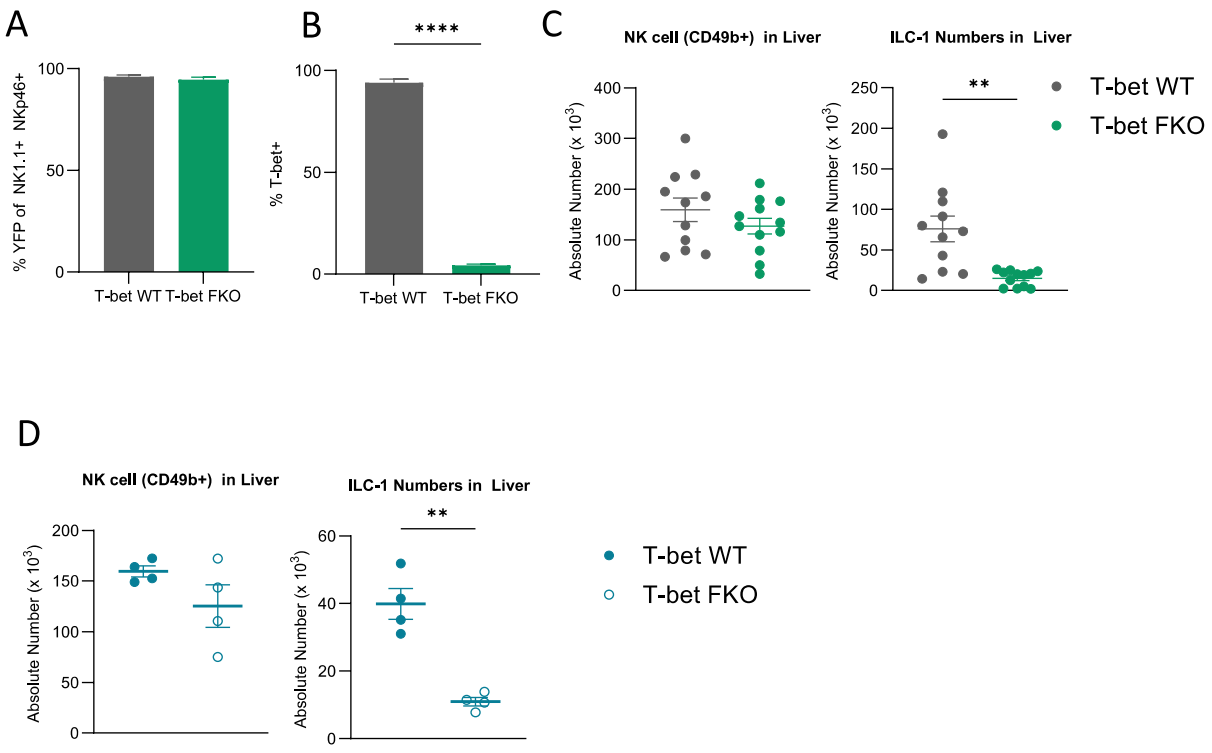

Figure 3.5. ILC-1 requires sustained expression of T-bet to persist. T-bet FKO mice were treated with 1mg tamoxifen per day by oral gavage for 3 days (Day 1-3), then on Day 6, mice were sacrificed for analysis. **(A)** Summary data of YFP^+ percentage within $NK1.1^+$ $NKp46^+$ cells in the liver. **(B)** Summary data of percentage of $T\text{-}bet^+$ within YFP+ NK1.1+ NKp46+ cells in the liver. **(C)** Absolute numbers of NK cells ($CD49a^-CD49b^+$) and YFP^+ ILC-1s ($CD49a^+CD49b^-$) in the liver. **(D)** Mice were sacrifice on Day 4 for analysis of Absolute numbers of YFP+ NK cells and YFP+ ILC-1s in the liver. Statistical significance was determined by t-test.

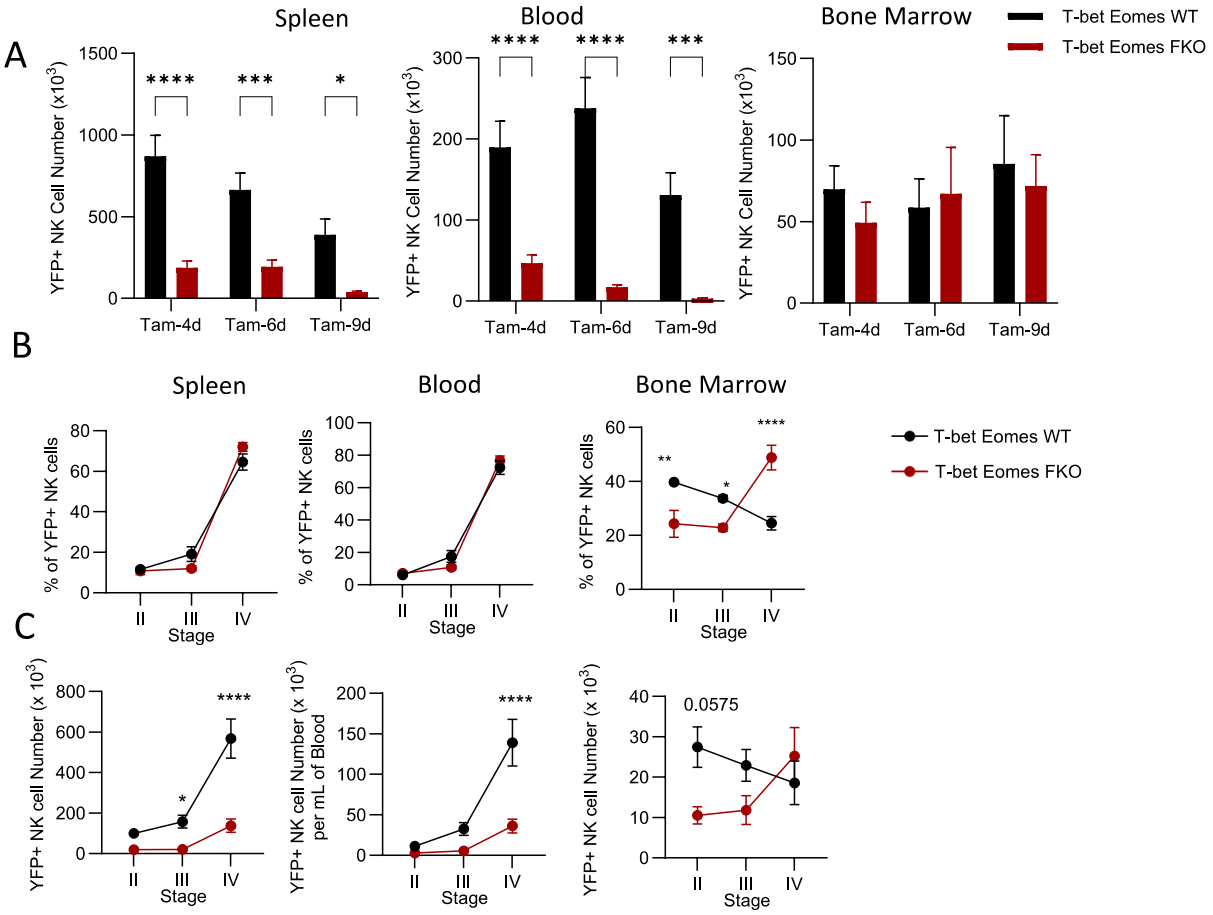

Figure 3.6. Expression of T-bet and Eomes are required for murine NK cells in the spleen and blood. *Ncr1*-iCreER^{T2} x *Tbx21*$^{fl/fl}$ x Eomes$^{fl/fl}$ (T-bet Eomes FKO) mice were treated with 1mg tamoxifen per day by oral gavage for 3 days (Day 1-3), then on Days 4,6, and 9 mice were sacrificed for analysis. **(A)** Summary data of all YFP+ NK cell numbers in the spleen, blood, and bone marrow. Summary data of **(B)** percentage and **(C)** absolute numbers of YFP^{+} NK cell within each indicated maturation stage on Day 4. Statistical significance was determined by 2-way ANOVA.

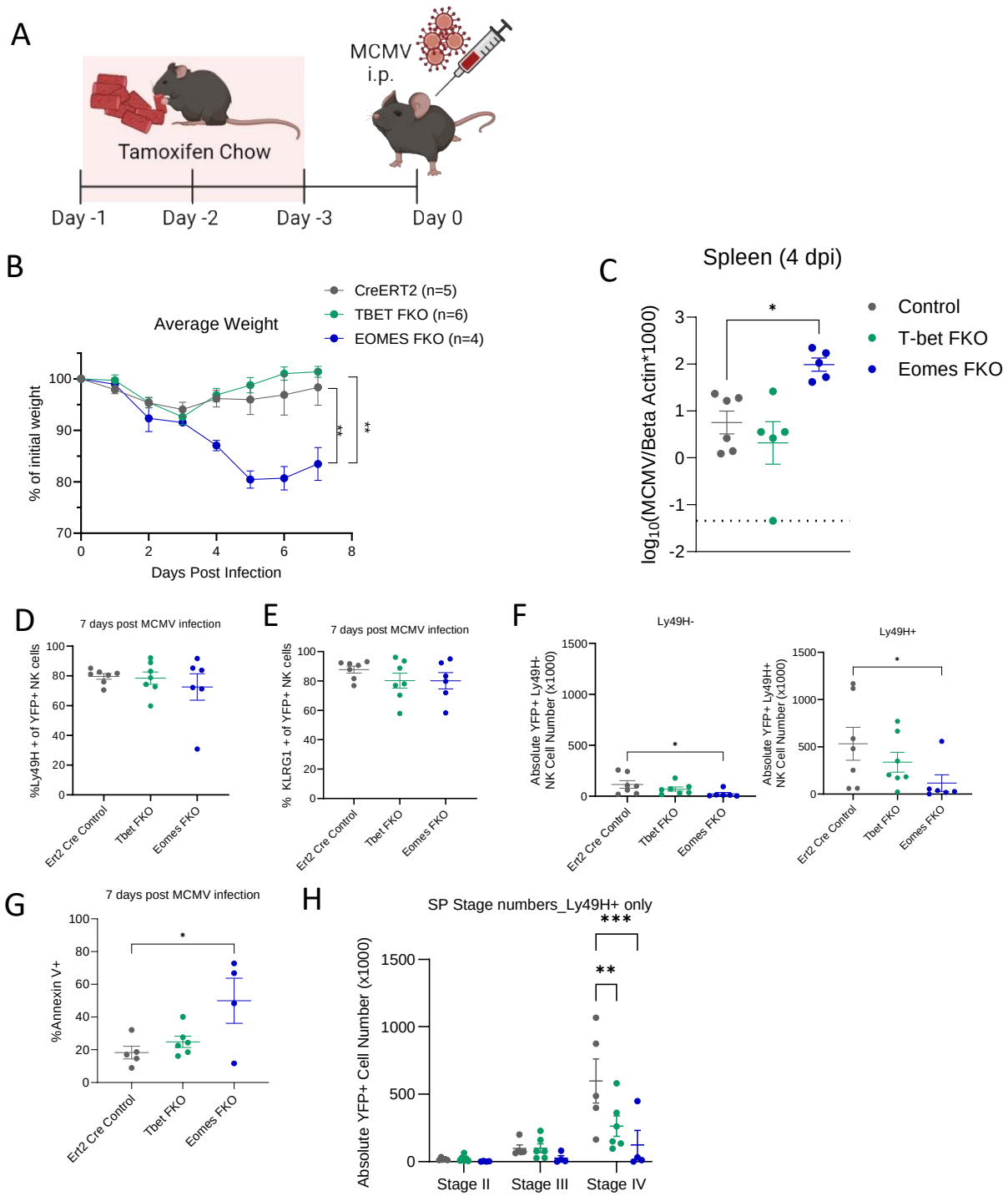

Figure 3.7. T-bet expression in NK cells is dispensable while Eomes expression is required for protective MCMV response. (A) Schema. *Ncr1*-iCreER^{T2} x *Tbx21*$^{fl/fl}$ (T-bet FKO), x *Ncr1*-iCreER^{T2} x *Eomes*$^{fl/fl}$ (Eomes FKO) and control mice were fed Tamoxifen chow for 3 days. The

day after tamoxifen chow was removed, the mice were infected with a sublethal MCMV dose of 5e4 pfu. **(B)** Summary weigh loss data. **(C)** Summary data of MCMV titer measured as IE1 copy number per Beta actin *1000 in the spleen on 4 days post infection. **(D)** Ly49H+ and **(E)** KRLG1+ percentage within YFP+ NK cells on 7 days post infection. **(F)** Absolute numbers of Ly49H- and Ly49H+ NK cells in the spleen 7 days post infection. **(G)** Ex vivo assessment of Annexin V expression within YFP+ NK cells 7 days post infection **(H)** Absolute YFP+ Ly49H+ NK cell numbers within indicated maturation stages in the spleen 7 days post infection.

Chapter 4: Future Directions

Mechanism of Disappearance of NK cells in the blood and ILC-1s in inducible T-bet FKO mouse model

In the inducible *Ncr1*-T-bet$^{fl/fl}$ (T-bet FKO) model, we observed rapid, dramatic loss of NK cells in the blood. Because mature NK cells are not highly proliferative in the periphery at homeostasis, it seems unlikely that a possible proliferative impact by loss of T-bet is the major driver of this loss of blood NK cells(130,131). Another possible explanation is a trafficking defect caused by loss of T-bet as It has been reported that T-bet is important for the egression of NK cells from the bone marrow(49). Nevertheless, we did not observe significant accumulation of NK cells in the bone marrow in the same time frame where we already observed a significant loss of NK cells in the blood. Thus, the most likely explanation for this loss of blood NK cells is a rapid increase of cell death in T-bet FKO NK cells in the blood. To test the hypothesis that inducible T-bet deletion in NK cells increases cell death, we could assess the percentage of T-bet FKO NK cells undergoing cell death by ex vivo Annexin V or activated caspase staining at an early time point before the loss of NK cell is fully manifested. With similar reasoning, the rapid loss of ILC-1 in the liver is also most likely due to inducible loss of T-bet causing ILC-1 to be unable to survive. Thus, cell death analysis at early time points, before the loss of ILC-1s in the liver is fully manifested, should also be performed.

Intestinal innate lymphocytes

While NK cells and ILC-1 are the majority of innate lymphoid cells that express NKp46 and T-bet and thus were impacted by the inducible Ncr1-specific inducible T-bet knock-out model, a subset of ILC3s, predominantly in the small intestines are also NKp46^{+} and T-bet^{+} and thus could have been affected by our T-bet FKO model. ILC3s are characterized by their expression of the transcription factor RORgt (132,133). Analogous to Th17 cells, ILC3s produce type 3 cytokines like IL-22 and IL17 in response to activation by IL-1B and IL-23 and are important for gut immunity. NKp46+ ILC-3 subset also expresses T-bet (but not Eomes) and can produce IFN-γamma like NK cells and ILC-1s(134). This Nkp46+T-bet+ ILC3 subset is not present in the global T-bet-/- mouse, indicating that T-bet is important for their development, similar to NK cells, iNKT cells, and ILC-1s. Thus, it would be interesting to study whether these NKp46+ ILC-3s persist in our inducible Ncr1-specific T-bet deletion model, whether the loss of T-bet will convert them to be NKp46- ILC-3s, and the functional implication of the resulting phenotype.

NK cells in the Bone Marrow Niche

In our study of the inducible NKp46-specific T-bet deletion model, out of all the tissues assessed, the NK cells in the bone marrow were the least affected by the loss of T-bet. This could be potentially explained by how T-bet expression has been demonstrated to be actively suppressed in the bone marrow, and thus NK cells in the bone marrow were perhaps already conditioned to persist with low T-bet levels in the bone marrow(37). Surprisingly, in our inducible T-bet and Eomes simultaneous knock-out model that the NK cells in the bone marrow were not significantly affected. The bone marrow microenvironment is a nurturing niche for hematopoietic stem cells, precursors cells, and long-lived immune cells(135–138). Perhaps the bone marrow niche has sufficient survival signals and growth factors that promote ant-apoptotic

pathways in NK cells in a T-bet and Eomes-independent manner, but once NK cells exit the bone marrow, they require T-bet and Eomes to reinforce pro-survival, anti-apoptotic pathways. It would be informative to compare the expression of apoptosis pathway proteins expression in bone marrow and peripheral NK cell. In addition, the overall functional capacity of bone marrow NK cells vs peripheral NK cells has also not been investigated. The low expression of T-bet in the bone marrow suggests a hypothesis of bone marrow NK cells being less functional than NK cells in the periphery even when comparing bone marrow and periphery NK cells that belong to the same maturation stage by the conventional CD27 and CD11b identification. Direct comparison of bone marrow NK cells and peripheral NK cells in these multiple facets could contribute to better understanding of host NK cell function in hematological malignancies that manifest in the bone marrow and provide insights to improve NK cell therapy strategies.

The persistence of NK cells in the bone marrow even when both T-bet and Eomes are inducibly deleted contrast previous studies with T-bet$^{-/-}$ x Vav-Cre x Eomes$^{fl/fl}$ mice where without both T-bet and Eomes, there was a complete loss of NK cells development even in the bone marrow(48). This further highlights the utility of our inducible deletion model where we could perform loss-of-function studies on developmentally critical transcription factors in mature NK cells, as the requirement for these transcription factors could be context dependent like we observed here. We could extend our study to investigate other transcription factors important for NK cell development like, Ets1, Id2 and Tcf1, to unveil whether their expression are continuously required in mature NK cells (139–141)

Context-dependent targets of Eomes and T-bet

As our lab's studies here and many other previous studies have demonstrated differentiation maturation stage-, tissue-, and inflammation- dependent requirement of T-bet and Eomes, it would be informative to study the target DNA loci of these T-box transcription factors in these various contexts instead of only at homeostasis. Zhang et al. recently assessed Eomes and T-bet targets side by side using Chromatin immunoprecipitation sequencing (ChIP-seq), but it was only performed in bulk, resting, splenic murine NK Cells (38). ChIP seq studies are traditionally challenging to perform with primary cells because of the large number of cells required. However, with recent advances in techniques like Cut&Run that can assess transcription factor binding sites using much lower cell input compared to traditional ChIP sequencing, it is now more accessible to perform side by side comparison of the targets Eomes and T-bet in specific NK maturation stage as well as in NK cells from different tissue(142). It would also be interesting to investigate in an infection setting like MCMV or in response to cytokine stimulation, what the T-bet and Eomes targets are compared to resting NK cells. Finally, Cut&Run experiments using NK Cells from our inducible NKp46-specific T-bet/Eomes FKO mice can directly elucidate the extent to which T-bet and Eomes can compensating for each other at the level of DNA target loci binding. We could assess whether the T-bet or Eomes would bind to loci that normally the one inducibly deleted would normally occupy.

Mechanisms of Gene Regulation by T-bet and Eomes

T-bet and Eomes are master regulators of NK cell identity and function, as previous studies have demonstrated in developing NK cells and as we have demonstrated here in mature NK cells, where their deficiency leads to drastic transcriptional changes(66). Nevertheless, there are still

gaps in the understanding of the layers of the transcriptional network downstream of T-bet and Eomes. What genes are regulated by T-bet and/or Eomes directly, what genes are regulated by T-bet and/or Eomes in concert with cofactors, and what genes are indirectly regulated by T-bet and/or Eomes? We observed in our study that T-bet and Eomes regulate the expression of many transcription factors with known function in lymphocytes. It would be informative to figure out which of these transcription factors are directly responsible for genes that are being regulated by T-bet and Eomes indirectly through these downstream transcription factors. Perhaps we can get a clue by analyzing the differentially expressed genes in our scRNA-seq data of T-bet and Eomes DKO human NK cells, specifically looking for genes that do not contain have T-box DNA binding site, and investigate transcription factors regulated by T-bet and Eomes that have consensus site at these genes. We can then apply our established CRISPR-Cas9 system for human NK cells and inducible *Ncr1*-Cre-lox deletion model for mouse NK cells to uncover this network of transcription factors that are directed by the master regulators T-bet and Eomes to maintain mature NK cell identity and function.

In recent years, immunotherapies targeting and/or utilizing NK cells have emerged as promising strategies for treating cancers because of NK cells' low toxicity profile, their natural ability to drive anti-tumor responses, and multitude of ways that NK cells can be engineered to have even more enhanced function (73,117,143). Thus, a comprehensive understanding of the fundamental mechanisms of NK cell biology is important to inform more safe and more efficacious ways to improving NK cell therapy.

References

1. Colonna M. Innate Lymphoid Cells: Diversity, Plasticity, and Unique Functions in Immunity. Vol. 48, Immunity. 2018. p. 1104–17.

2. Caligiuri MA. Human natural killer cells. Blood. 2008 Aug 1;112(3):461–9.

3. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nature Immunology. 2008 May 18;9(5):503–10.

4. Cherrier DE, Serafini N, Di Santo JP. Innate Lymphoid Cell Development: A T Cell Perspective. Vol. 48, Immunity. 2018. p. 1091–103.

5. Ishizuka IE, Constantinides MG, Gudjonson H, Bendelac A. The Innate Lymphoid Cell Precursor. Annual Review of Immunology. 2016;34(1):299–316.

6. Scoville SD, Freud AG, Caligiuri MA. Modeling human natural killer cell development in the era of innate lymphoid cells. Frontiers in Immunology. 2017;8(MAR).

7. Spits H, Bernink JH, Lanier L. NK cells and type 1 innate lymphoid cells: Partners in host defense. Vol. 17, Nature Immunology. 2016. p. 758–64.

8. Gasteiger G, Fan X, Dikiy S, Lee SY, Alexander Y, Program I, et al. Tissue residency of innate lymphoid cells in lymphoid and non- lymphoid organs. Science. 2015;350(6263):981–5.

9. Mjösberg J, Spits H. Human innate lymphoid cells. Journal of Allergy and Clinical Immunology. 2016;138(5):1265–76.

10. Huang Q, Seillet C, Belz GT. Shaping Innate Lymphoid Cell Diversity. Frontiers in Immunology. 2017;8(November):1–9.

11. Orange JS. Formation and function of the lytic NK-cell immunological synapse. Nat Rev Immunol. 2008 Sep;8(9):713–25.

12. Long EO, Sik Kim H, Liu D, Peterson ME, Rajagopalan S. Controlling Natural Killer Cell Responses: Integration of Signals for Activation and Inhibition. Annual Review of Immunology. 2013 Mar;31(1):227–58.

13. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. Nature Immunology. 2008 May;9(5):495–502.

14. Arase H, Mocarski ES, Campbell AE, Hill AB, Lanier LL. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. Science (New York, NY). 2002 May 17;296(5571):1323–6.

15. Smith HRC, Heusel JW, Mehta IK, Kim S, Dorner BG, Naidenko O V., et al. Recognition of a virus-encoded ligand by a natural killer cell activation receptor. Proceedings of the National Academy of Sciences of the United States of America. 2002 Jun 25;99(13):8826–31.

16. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature. 1986 Feb;319(6055):675–8.

17. Kim S, Poursine-Laurent J, Truscott SM, Lybarger L, Song YJJ, Yang L, et al. Licensing of natural killer cells by host major histocompatibility complex class I molecules. Nature. 2005 Aug;436(7051):709–13.

18. Kärre K. Natural killer cell recognition of missing self. Nat Immunol. 2008 May;9(5):477–80.

19. Chiossone L, Chaix J, Fuseri N, Roth C, Vivier E, Walzer T. Maturation of mouse NK cells is a 4-stage developmental program. Blood. 2009 May;113(22):5488–96.

20. Freud AG, Yokohama A, Becknell B, Lee MT, Mao HC, Ferketich AK, et al. Evidence for discrete stages of human natural killer cell differentiation in vivo. The Journal of experimental medicine. 2006 Apr;203(4):1033–43.

21. Freud AG, Caligiuri MA. Human natural killer cell development. Immunol Rev. 2006 Dec;214(1):56–72.

22. Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghaheri B a, Ghayur T, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood. 2001 May;97(10):3146–51.

23. Simonetta F, Pradier A, Roosnek E. T-bet and Eomesodermin in NK Cell Development, Maturation, and Function. Frontiers in Immunology. 2016;7(June):1–6.

24. Zhang J, Marotel M, Fauteux-Daniel S, Mathieu AL, Viel S, Marçais A, et al. T-bet and Eomes govern differentiation and function of mouse and human NK cells and ILC1. European Journal of Immunology. 2018 May;48(5):738–50.

25. Papaioannou VE. The T-box gene family: emerging roles in development, stem cells and cancer. Development. 2014 Oct;141(20):3819–33.

26. Agarwal P, Raghavan A, Nandiwada SL, Curtsinger JM, Bohjanen PR, Mueller DL, et al. Gene Regulation and Chromatin Remodeling by IL-12 and Type I IFN in Programming for CD8 T Cell Effector Function and Memory. The Journal of Immunology. 2009 Aug 1;183(3):1695–704.

27. Istaces N, Splittgerber M, Lima Silva V, Nguyen M, Thomas S, Le A, et al. EOMES interacts with RUNX3 and BRG1 to promote innate memory cell formation through epigenetic reprogramming. Nature Communications. 2019 Jul 24;10(1):1–17.

28. Lewis MD, Miller SA, Miazgowicz MM, Beima KM, Weinmann AS. T-bet's Ability To Regulate Individual Target Genes Requires the Conserved T-Box Domain To Recruit Histone Methyltransferase Activity and a Separate Family Member-Specific Transactivation Domain. Molecular and Cellular Biology. 2007 Dec 15;27(24):8510–21.

29. Townsend MJ, Weinmann AS, Matsuda JL, Salomon R, Farnham PJ, Biron CA, et al. T-bet regulates the terminal maturation and homeostasis of NK and Vα14i NKT cells. Immunity. 2004 Apr;20(4):477–94.

30. Ryan K, Garrett N, Mitchell A, Gurdon JB. Eomesodermin, a key early gene in Xenopus mesoderm differentiation. Cell. 1996 Dec 13;87(6):989–1000.

31. Russ AP, Wattler S, Colledge WH, Aparicio SAJR, Carlton MBL, Pearce JJ, et al. Eomesodermin is required for mouse trophoblast development and mesoderm formation. Nature. 2000;404(6773):95–9.

32. Llaó-Cid L, Roessner PM, Chapaprieta V, Öztürk S, Roider T, Bordas M, et al. EOMES is essential for antitumor activity of CD8+ T cells in chronic lymphocytic leukemia. Leukemia. 2021 Nov;35(11):3152–62.

33. Yang Y, Xu J, Niu Y, Bromberg JS, Ding Y. T-bet and Eomesodermin Play Critical Roles in Directing T Cell Differentiation to Th1 versus Th17. Journal of immunology (Baltimore, Md : 1950). 2008 Dec 15;181(12):8700.

34. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell. 2000 Mar 17;100(6):655–69.

35. Knox JJ, Cosma GL, Betts MR, McLane LM. Characterization of T-bet and Eomes in peripheral human immune cells. Frontiers in Immunology. 2014;5(MAY):1–13.

36. Simonetta F, Pradier A, Roosnek E. T-bet and Eomesodermin in NK Cell Development, Maturation, and Function. Frontiers in Immunology. 2016;7(June):1–6.

37. Daussy C, Faure F, Mayol K, Viel S, Gasteiger G, Charrier E, et al. T-bet and Eomes instruct the development of two distinct natural killer cell lineages in the liver and in the bone marrow. The Journal of experimental medicine. 2014 Mar;211(3):563–77.

38. Zhang J, Le Gras S, Pouxvielh K, Faure F, Fallone L, Kern N, et al. Sequential actions of EOMES and T-BET promote stepwise maturation of natural killer cells. Nature Communications 2021 12:1. 2021 Sep;12(1):1–17.

39. Seillet C, Huntington ND, Gangatirkar P, Axelsson E, Minnich M, Brady HJM, et al. Differential Requirement for Nfil3 during NK Cell Development. The Journal of Immunology. 2014 Feb;192(6):2667–76.

40. Seillet C, Rankin LC, Groom JR, Mielke L a., Tellier J, Chopin M, et al. Nfil3 is required for the development of all innate lymphoid cell subsets. Journal of Experimental Medicine. 2014;211(9):jem.20140145-.

41. Rankin LC, Groom JR, Chopin M, Herold MJ, Walker JA, Mielke LA, et al. The transcription factor T-bet is essential for the development of NKp46+ innate lymphocytes via the Notch pathway. Nat Immunol. 2013 Apr;14(4):389–95.

42. Intlekofer AM, Takemoto N, Wherry EJ, Longworth S a, Northrup JT, Palanivel VR, et al. Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. Nature immunology. 2005 Dec;6(12):1236–44.

43. Klose CSN, Flach M, Möhle L, Rogell L, Hoyler T, Ebert K, et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. Cell. 2014;157(2):340–56.

44. Madera S, Geary CD, Lau CM, Pikovskaya O, Reiner SL, Sun JC. Cutting Edge: Divergent Requirement of T-Box Transcription Factors in Effector and Memory NK Cells. The Journal of Immunology. 2018;200(6):1977–81.

45. Afkarian M, Sedy JR, Yang J, Jacobson NG, Cereb N, Yang SY, et al. T-bet is a STAT1-induced regulator of IL-12R expression in naïve CD4+ T cells. Nat Immunol. 2002 Jun;3(6):549–57.

46. Mullen AC, High FA, Hutchins AS, Lee HW, Villarino AV, Livingston DM, et al. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. Science. 2001 Jun 8;292(5523):1907–10.

47. Fang D, Cui K, Cao Y, Zheng M, Kawabe T, Hu G, et al. Differential regulation of transcription factor T-bet induction during NK cell development and T helper-1 cell differentiation. Immunity. 2022 Apr 12;55(4):639-655.e7.

48. Gordon SM, Chaix J, Rupp LJ, Wu J, Madera S, Sun JC, et al. The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation. Immunity. 2012 Jan;36(1):55–67.

49. Jenne CN, Enders A, Rivera R, Watson SR, Bankovich AJ, Pereira JP, et al. T-bet-dependent S1P5 expression in NK cells promotes egress from lymph nodes and bone marrow. The Journal of experimental medicine. 2009 Oct;206(11):2469–81.

50. Waldmann TA. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. Nat Rev Immunol. 2006 Aug;6(8):595–601.

51. Cooper MA, Bush JE, Fehniger TA, Vandeusen JB, Waite RE, Liu Y, et al. In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells. Blood. 2002;100(10):3633–8.

52. Ranson T, Vosshenrich CAJ, Corcuff E, Richard O, Mu W, Santo JPD. IL-15 is an essential mediator of peripheral NK-cell homeostasis. Blood. 2003;101(12):4887–93.

53. Kennedy MK, Glaccum M, Brown SN, Butz E a, Viney JL, Embers M, et al. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. The Journal of experimental medicine. 2000;191(5):771–80.

54. Vosshenrich CAJ, Ranson T, Samson SI, Corcuff E, Colucci F, Rosmaraki EE, et al. Roles for Common Cytokine Receptor γ-Chain-Dependent Cytokines in the Generation, Differentiation, and Maturation of NK Cell Precursors and Peripheral NK Cells in Vivo1. The Journal of Immunology. 2005 Feb 1;174(3):1213–21.

55. Cuff AO, Male V. Conventional NK cells and ILC1 are partially ablated in the livers of Ncr1iCreTbx21fl/fl mice. Wellcome Open Research. 2017;2:39.

56. Pearce EL, Mullen AC, Martins G a, Krawczyk CM, Hutchins AS, Zediak VP, et al. Control of effector CD8+ T cell function by the transcription factor Eomesodermin. Science (New York, NY). 2003;302(5647):1041–3.

57. Cruz-Guilloty F, Pipkin ME, Djuretic IM, Levanon D, Lotem J, Lichtenheld MG, et al. Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs. The Journal of experimental medicine. 2009;206(1):51–9.

58. Werneck MBF, Lugo-Villarino G, Hwang ES, Cantor H, Glimcher LH. T-Bet Plays a Key Role in NK-Mediated Control of Melanoma Metastatic Disease. The Journal of Immunology. 2008 Jun 15;180(12):8004–10.

59. Malaisé M, Rovira J. KLRG1+ NK Cells Protect T-bet–Deficient Mice from Pulmonary Metastatic Colorectal Carcinoma. The Journal of \ldots [Internet]. 2014; Available from: http://www.jimmunol.org/content/192/4/1954.short

60. Wagner JA, Wong P, Schappe T, Berrien-Elliott MM, Cubitt C, Jaeger N, et al. Stage-Specific Requirement for Eomes in Mature NK Cell Homeostasis and Cytotoxicity. Cell Reports. 2020 Jun;31(9):107720.

61. Collins A, Rothman N, Liu K, Reiner SL. Eomesodermin and T-bet mark developmentally distinct human natural killer cells. JCI Insight [Internet]. 2017;2(5). Available from: https://insight.jci.org/articles/view/90063

62. Yang R, Mele F, Worley L, Langlais D, Rosain J, Benhsaien I, et al. Human T-bet Governs Innate and Innate-like Adaptive IFN-γ Immunity against Mycobacteria. Cell. 2020 Dec 23;183(7):1826-1847.e31.

63. Kiekens L, Van Loocke W, Taveirne S, Wahlen S, Persyn E, Van Ammel E, et al. T-BET and EOMES Accelerate and Enhance Functional Differentiation of Human Natural Killer Cells. Frontiers in Immunology. 2021;0:3741.

64. Putz EM, Guillerey C, Kos K, Stannard K, Miles K, Delconte RB, et al. Targeting cytokine signaling checkpoint CIS activates NK cells to protect from tumor initiation and metastasis. OncoImmunology. 2017;6(2):e1267892.

65. RS H, HA S, MC L, YJ C, S L. Enhanced NK-92 Cytotoxicity by CRISPR Genome Engineering Using Cas9 Ribonucleoproteins. Frontiers in immunology. 2020 May 22;11.

66. Wong P, Foltz JA, Chang L, Neal CC, Yao T, Cubitt CC, et al. T-BET and EOMES sustain mature human NK cell identity and antitumor function. Journal of Clinical Investigations. 2023 Jul 3;133(13).

67. Gang M, Wong P, Berrien-Elliott MM, Fehniger TA. Memory-like natural killer cells for cancer immunotherapy. Seminars in Hematology. 2020 Oct;57(4):185–93.

68. Shimasaki N, Jain A, Campana D. NK cells for cancer immunotherapy. Nature Reviews Drug Discovery. 2020 Mar 6;19(3):200–18.

69. Romee R, Rosario M, Berrien-Elliott MM, Wagner JA, Jewell BA, Schappe T, et al. Cytokine-induced memory-like natural killer cells exhibit enhanced responses against myeloid leukemia. Science translational medicine. 2016 Sep 21;8(357):357ra123.

70. Berrien-Elliott MM, Jacobs MT, Fehniger TA. Allogeneic natural killer cell therapy. Blood. 2023 Nov;141(8):856–68.

71. Cichocki F, van der Stegen SJC, Miller JS. Engineered and banked iPSCs for advanced NK- and T-cell immunotherapies. Blood. 2023 Feb;141(8):846–55.

72. Mehta RS, Shpall EJ, Rezvani K. Cord Blood as a Source of Natural Killer Cells. Frontiers in Medicine. 2016 Jan 5;2(JAN):1–10.

73. Berrien-Elliott MM, Cashen AF, Cubitt CC, Neal CC, Wong P, Wagner JA, et al. Multidimensional Analyses of Donor Memory-Like NK Cells Reveal New Associations with Response after Adoptive Immunotherapy for Leukemia. Cancer Discovery. 2020 Dec 21;10(12):1854–71.

74. Bednarski JJ, Zimmerman C, Berrien-Elliott MM, Foltz JA, Becker-Hapak M, Neal CC, et al. Donor memory-like NK cells persist and induce remissions in pediatric patients with relapsed AML after transplant. Blood. 2021/12/07 ed. 2022 Mar 17;139(11):1670–83.

75. Berrien-Elliott MM, Foltz JA, Russler-Germain DA, Neal CC, Tran J, Gang M, et al. Hematopoietic Cell Transplantation Donor-derived Memory-Like NK Cells Functionally Persist after Transfer into Patients with Leukemia. Science translational medicine. 2022 Feb 23;14(633):eabm1375.

76. Liu E, Marin D, Banerjee P, Macapinlac HA, Thompson P, Basar R, et al. Use of CAR-transduced natural killer cells in CD19-positive lymphoid tumors. The New England journal of medicine. 2020 Feb 6;382(6):545–53.

77. Adams NM, Diaz-Salazar C, Dang C, Lanier LL, Sun JC. Cutting Edge: Heterogeneity in Cell Age Contributes to Functional Diversity of NK Cells. The Journal of Immunology. 2021 Feb 1;206(3):465–70.

78. Shultz LD, Lyons BL, Burzenski LM, Gott B, Chen X, Chaleff S, et al. Human Lymphoid and Myeloid Cell Development in NOD/LtSz-scid IL2R null Mice Engrafted with Mobilized Human Hemopoietic Stem Cells. The Journal of Immunology. 2005;174(10):6477–89.

79. Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghaheri B a, Ghayur T, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood. 2001 May 15;97(10):3146–51.

80. Nagler A, Lanier LL, Cwirla S, Phillips JH. Comparative studies of human FcRIII-positive and negative natural killer cells. Journal of immunology (Baltimore, Md : 1950). 1989 Nov 15;143(10):3183–91.

81. Mishra A, Sullivan L, Caligiuri MA. Molecular pathways: Interleukin-15 signaling in health and in cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2014 Apr 15;20(8):2044–50.

82. Smith SL, Kennedy PR, Stacey KB, Worboys JD, Yarwood A, Seo S, et al. Diversity of peripheral blood human NK cells identified by single-cell RNA sequencing. Blood Advances. 2020 Apr 14;4(7):1388–406.

83. Crinier A, Milpied P, Escalière B, Piperoglou C, Galluso J, Balsamo A, et al. High-Dimensional Single-Cell Analysis Identifies Organ-Specific Signatures and Conserved NK Cell Subsets in Humans and Mice. Immunity. 2018 Nov 20;49(5):971-986.e5.

84. Trapani J a. Granzymes: a family of lymphocyte granule serine proteases. Genome biology. 2001;2(12):REVIEWS3014.

85. Böttcher JP, Bonavita E, Chakravarty P, Blees H, Cabeza-Cabrerizo M, Sammicheli S, et al. NK Cells Stimulate Recruitment of cDC1 into the Tumor Microenvironment Promoting Cancer Immune Control. Cell. 2018;172(5):1022-1037.e14.

86. Robertson MJ. Role of chemokines in the biology of natural killer cells. Journal of Leukocyte Biology. 2002 Feb 1;71(2):173–83.

87. Drouillard A, Mathieu AL, Marçais A, Belot A, Viel S, Mingueneau M, et al. S1PR5 is essential for human natural killer cell migration toward sphingosine-1 phosphate. Journal of Allergy and Clinical Immunology. 2018 Jun 1;141(6):2265-2268.e1.

88. Ng SS, De Labastida Rivera F, Yan J, Corvino D, Das I, Zhang P, et al. The NK cell granule protein NKG7 regulates cytotoxic granule exocytosis and inflammation. Nature Immunology 2020 21:10. 2020 Aug 24;21(10):1205–18.

89. Buechele C, Baessler T, Wirths S, Schmohl JU, Schmiedel BJ, Salih HR. Glucocorticoid-induced TNFR-related protein (GITR) ligand modulates cytokine release and NK cell reactivity in chronic lymphocytic leukemia (CLL). Leukemia 2012 26:5. 2011 Nov 8;26(5):991–1000.

90. Liu B, Li Z, Mahesh SP, Pantanelli S, Hwang FS, Siu WO, et al. Glucocorticoid-induced Tumor Necrosis Factor Receptor Negatively Regulates Activation of Human Primary Natural Killer (NK) Cells by Blocking Proliferative Signals and Increasing NK Cell Apoptosis. The Journal of Biological Chemistry. 2008 Mar 28;283(13):8202.

91. Baltz KM, Krusch M, Bringmann A, Brossart P, Mayer F, Kloss M, et al. Cancer immunoediting by GITR (glucocorticoid-induced TNF-related protein) ligand in humans: NK cell/tumor cell interactions. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2007 Aug;21(10):2442–54.

92. van Helden MJ, Goossens S, Daussy C, Mathieu AL, Faure F, Marçais A, et al. Terminal NK cell maturation is controlled by concerted actions of T-bet and Zeb2 and is essential for melanoma rejection. The Journal of experimental medicine. 2015;212(12):2015–25.

93. Ebihara T, Song C, Ryu SH, Plougastel-Douglas B, Yang L, Levanon D, et al. Runx3 specifies lineage commitment of innate lymphoid cells. Nature Immunology. 2015;16(September):1–11.

94. Levanon D, Negreanu V, Lotem J, Bone KR, Brenner O, Leshkowitz D, et al. Transcription Factor Runx3 Regulates Interleukin-15-Dependent Natural Killer Cell Activation. Molecular and Cellular Biology. 2014 Jan;34(6):1158–69.

95. Kanda M, Yamanaka H, Kojo S, Usui Y, Honda H, Sotomaru Y, et al. Transcriptional regulator Bhlhe40 works as a cofactor of T-bet in the regulation of IFN-γ production in iNKT cells. Proceedings of the National Academy of Sciences of the United States of America. 2016 Jun 14;113(24):E3394–402.

96. Uyeda MJ, Freeborn RA, Cieniewicz B, Romano R, Chen P, Liu JMH, et al. BHLHE40 Regulates IL-10 and IFN-γ Production in T Cells but Does Not Interfere With Human Type 1 Regulatory T Cell Differentiation. Frontiers in Immunology. 2021 Jul 7;12:2705.

97. Tassi I, Cella M, Presti R, Colucci A, Gilfillan S, Littman DR, et al. NK cell-activating receptors require PKC-theta for sustained signaling, transcriptional activation, and IFN-gamma secretion. Blood. 2008 Nov 15;112(10):4109–16.

98. Rabacal W, Pabbisetty SK, Hoek KL, Cendron D, Guo Y, Maseda D, et al. Transcription factor KLF2 regulates homeostatic NK cell proliferation and survival. Proceedings of the National Academy of Sciences of the United States of America. 2016 May 10;113(19):5370–5.

99. Barton K, Muthusamy N, Fischer C, Ting CN, Walunas TL, Lanier LL, et al. The Ets-1 transcription factor is required for the development of natural killer cells in mice. Immunity. 1998 Oct;9(4):555–63.

100. Taveirne S, Wahlen S, van Loocke W, Kiekens L, Persyn E, van Ammel E, et al. The transcription factor ETS1 is an important regulator of human NK cell development and terminal differentiation. Blood. 2020 Jul 16;136(3):288–98.

101. Grenningloh R, Bok YK, Ho IC. Ets-1, a functional cofactor of T-bet, is essential for Th1 inflammatory responses. Journal of Experimental Medicine. 2005 Feb 21;201(4):615–26.

102. Holmes TD, Pandey RV, Helm EY, Schlums H, Han H, Campbell TM, et al. The transcription factor Bcl11b promotes both canonical and adaptive NK cell differentiation. Science Immunology. 2021;6(57):eabc9801.

103. Hughes T, Briercheck EL, Freud AG, Trotta R, McClory S, Scoville SD, et al. The Transcription Factor AHR Prevents the Differentiation of a Stage 3 Innate Lymphoid Cell Subset to Natural Killer Cells. Cell Reports. 2014 Jul 10;8(1):150–62.

104. Mjösberg J, Bernink J, Peters C, Spits H. Transcriptional control of innate lymphoid cells. European journal of immunology. 2012 Aug;42(8):1916–23.

105. Scoville SD, Mundy-Bosse BL, Zhang MH, Chen LL, Zhang X, Keller KA, et al. A Progenitor Cell Expressing Transcription Factor RORγt Generates All Human Innate Lymphoid Cell Subsets. Immunity. 2016;44(5):1140–50.

106. Cella M, Gamini R, Sécca C, Collins PL, Zhao S, Peng V, et al. Subsets of ILC3−ILC1-like cells generate a diversity spectrum of innate lymphoid cells in human mucosal tissues. Nature Immunology 2019 20:8. 2019 Jun 17;20(8):980–91.

107. Holmes ML, Huntington ND, Thong RP, Brady J, Hayakawa Y, Andoniou CE, et al. Peripheral natural killer cell maturation depends on the transcription factor Aiolos. The EMBO Journal. 2014 Nov;33(22):2721.

108. Chen L, Youssef Y, Robinson C, Ernst GF, Carson MY, Young KA, et al. CD56 Expression Marks Human Group 2 Innate Lymphoid Cell Divergence from a Shared NK Cell and Group 3 Innate Lymphoid Cell Developmental Pathway. Immunity. 2018 Sep 18;49(3):464-476.e4.

109. Björklund ÅK, Forkel M, Picelli S, Konya V, Theorell J, Friberg D, et al. The heterogeneity of human CD127(+) innate lymphoid cells revealed by single-cell RNA sequencing. Nature immunology. 2016 Apr 15;17(4):451–60.

110. Pikovskaya O, Chaix J, Rothman NJ, Collins A, Chen YH, Scipioni AM, et al. Cutting Edge: Eomesodermin Is Sufficient To Direct Type 1 Innate Lymphocyte Development into the Conventional NK Lineage. Journal of immunology (Baltimore, Md : 1950). 2016;196(4):1449–54.

111. Cuff AO, Perchet T, Dertschnig S, Golub R, Male V. Tbet promotes CXCR6 expression in immature natural killer cells and natural killer cell egress from the bone marrow. Immunology. 2020 Sep 1;161(1):28–38.

112. Ghiringhelli F, Ménard C, Terme M, Flament C, Taieb J, Chaput N, et al. CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. The Journal of experimental medicine. 2005;202(8):1075–85.

113. Viel S, Marçais A, Guimaraes FS fonseca, Loftus R, Rabilloud J, Grau M, et al. TGF- b inhibits the activation and functions of NK cells by repressing the mTOR pathway. 2016;9(415):1–14.

114. Gill S, Vasey AE, De Souza A, Baker J, Smith AT, Kohrt HE, et al. Rapid development of exhaustion and down-regulation of eomesodermin limit the antitumor activity of adoptively transferred murine natural killer cells. Blood. 2012 Jun 14;119(24):5758–68.

115. Miller JS, Soignier Y, Panoskaltsis-Mortari A, McNearney SA, Yun GH, Fautsch SK, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Blood. 2005 Apr 15;105(8):3051–7.

116. Cichocki F, Wu CY, Zhang B, Felices M, Tesi B, Tuininga K, et al. ARID5B regulates metabolic programming in human adaptive NK cells. Journal of Experimental Medicine. 2018;215(9):2379–95.

117. Gang M, Marin NDD, Wong P, Neal CCC, Marsala L, Foster M, et al. CAR-modified memory-like NK cells exhibit potent responses to NK-resistant lymphomas. Blood. 2020 Nov 12;136(20):2308–18.

118. Zhu H, Blum RH, Bernareggi D, Ask EH, Wu Z, Hoel HJ, et al. Metabolic Reprograming via Deletion of CISH in Human iPSC-Derived NK Cells Promotes In Vivo Persistence and Enhances Anti-tumor Activity. Cell Stem Cell. 2020 Aug 6;27(2):224-237.e6.

119. Hao Y, Hao S, Andersen-Nissen E, Mauck WM, Zheng S, Butler A, et al. Integrated analysis of multimodal single-cell data. Cell. 2021 Jun;184(13):3573-3587.e29.

120. Korsunsky I, Millard N, Fan J, Slowikowski K, Zhang F, Wei K, et al. Fast, sensitive and accurate integration of single-cell data with Harmony. Nature Methods 2019 16:12. 2019 Nov 18;16(12):1289–96.

121. Yu G, Wang LG, Han Y, He QY. ClusterProfiler: An R package for comparing biological themes among gene clusters. OMICS A Journal of Integrative Biology. 2012 May 1;16(5):284–7.

122. Lambert SA, Jolma A, Campitelli LF, Das PK, Yin Y, Albu M, et al. The Human Transcription Factors. Cell. 2018 Feb 8;172(4):650–65.

123. Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. Nature methods. 2013 Dec;10(12):1213–8.

124. Lanier LL. NK cell recognition. Ann Rev Immunol. 2005 Jan;23(1):225–74.

125. Sivori S, Vacca P, Del Zotto G, Munari E, Mingari MC, Moretta L. Human NK cells: surface receptors, inhibitory checkpoints, and translational applications. Cellular & Molecular Immunology. 2019 May;16(5):430–41.

126. McLane LM, Banerjee PP, Cosma GL, Makedonas G, Wherry EJ, Orange JS, et al. Differential Localization of T-bet and Eomes in CD8 T Cell Memory Populations. The Journal of Immunology. 2013;190(7):3207–15.

127. Fixemer J, Hummel JF, Arnold F, Klose CSN, Hofherr A, Weissert K, et al. Eomes cannot replace its paralog T-bet during expansion and differentiation of CD8 effector T cells. PLoS Pathog. 2020 Sep;16(9):e1008870.

128. Intlekofer AM, Banerjee A, Takemoto N, Gordon SM, Dejong CS, Shin H, et al. Anomalous type 17 response to viral infection by CD8+ T cells lacking T-bet and eomesodermin. Science (New York, NY). 2008;321(5887):408–11.

129. Parikh BA, Piersma SJ, Pak-Wittel MA, Yang L, Schreiber RD, Yokoyama WM. Dual Requirement of Cytokine and Activation Receptor Triggering for Cytotoxic Control of Murine Cytomegalovirus by NK Cells. PLoS Pathogens. 2015;11(12).

130. Kim S, Iizuka K, Kang HSP, Dokun A, French AR, Greco S, et al. In vivo developmental stages in murine natural killer cell maturation. Nat Immunol. 2002 Jun;3(6):523–8.

131. Rabacal W, Pabbisetty SK, Hoek KL, Cendron D, Guo Y, Maseda D, et al. Transcription factor KLF2 regulates homeostatic NK cell proliferation and survival. Proceedings of the National Academy of Sciences of the United States of America. 2016 May;113(19):5370–5.

132. Melo-Gonzalez F, Hepworth MR. Functional and phenotypic heterogeneity of group 3 innate lymphoid cells. Immunology. 2017 Mar;150(3):265–75.

133. Schroeder JH, Howard JK, Lord GM. Transcription factor-driven regulation of ILC1 and ILC3. Trends in Immunology. 2022 Jul 1;43(7):564–79.

134. Klose CSN, Kiss EA, Schwierzeck V, Ebert K, Hoyler T, D'Hargues Y, et al. A T-bet gradient controls the fate and function of CCR6-RORγt + innate lymphoid cells. Nature. 2013;494(7436):261–5.

135. Mendelson A, Frenette PS. Hematopoietic stem cell niche maintenance during homeostasis and regeneration. Nat Med. 2014 Aug;20(8):833–46.

136. Slamanig SA, Nolte MA. The Bone Marrow as Sanctuary for Plasma Cells and Memory T-Cells: Implications for Adaptive Immunity and Vaccinology. Cells. 2021 Jun 15;10(6):1508.

137. Stabile H, Fionda C, Santoni A, Gismondi A. Impact of bone marrow-derived signals on NK cell development and functional maturation. Cytokine & Growth Factor Reviews. 2018 Aug 1;42:13–9.

138. van Helden MJG, de Graaf N, Boog CJP, Topham DJ, Zaiss DMW, Sijts AJAM. The Bone Marrow Functions as the Central Site of Proliferation for Long-Lived NK Cells. The Journal of Immunology. 2012 Sep 1;189(5):2333–7.

139. Ramirez K, Chandler KJ, Spaulding C, Zandi S, Sigvardsson M, Graves BJ, et al. Gene deregulation and chronic activation in natural killer cells deficient in the transcription factor ETS1. Immunity. 2012 Jun;36(6):921–32.

140. Delconte RB, Shi W, Sathe P, Ushiki T, Seillet C, Minnich M, et al. The Helix-Loop-Helix Protein ID2 Governs NK Cell Fate by Tuning Their Sensitivity to Interleukin-15. Immunity. 2016;44(1):103–15.

141. Yang Q, Li F, Harly C, Xing S, Ye L, Xia X, et al. TCF-1 upregulation identifies early innate lymphoid progenitors in the bone marrow. Nature Immunology. 2015;16(10):1044–50.

142. Skene PJ, Henikoff S. An efficient targeted nuclease strategy for high-resolution mapping of DNA binding sites. eLife. 2017 Jan;6.

143. Jacobs MT, Wong P, Zhou AY, Becker-Hapak M, Marin ND, Marsala L, et al. Memory-like differentiation, tumor targeting monoclonal antibodies, and chimeric antigen receptors enhance natural killer cell responses to head and neck cancer. Clinical Cancer Research. 2023 Aug 9;CCR-23-0156.