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Direct Reprogramming of fibroblasts to NK cells

Diogo Filipe Pértiga Cabral

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

Direct Reprogramming of fibroblasts to NK cells

by

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Dissertation submitted to School of Medicine and Biomedical Sciences of Porto for the degree of Master in Oncology

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Abstract:

Natural killer (NK) cells are innate lymphocytes with remarkable cytotoxic abilities that control cancer and viral infections independent of antigen specificity. Indeed, NK cells are the first induced pluripotent stem cell (iPSCs)-derived hematopoietic cells to be tested in clinical trials against hematological tumors. However, limited persistence *in vivo* and complexity of differentiation protocols, pose significant obstacles to widespread NK-based therapeutics. We hypothesize that direct cell reprogramming mediated by cell type-specific transcription factors (TFs) can be employed to generate NK lymphocytes from somatic cells.

To define combinations of TF that induce NK cell identity we tested a list of 19 candidate TFs for their ability to activate a NK-specific reporter in mouse embryonic fibroblasts (MEFs). We identified Ets1, Nfil3, T-bet, Eomes (TENE) that activated a NCR1-driven reporter and induce NK progenitor and mature NK global gene expression programs as assessed by single-cell mRNA seq. To evaluate species conservation, we transduced human fibroblasts with 4 TFs and assessed activation of NK-specific markers by flow cytometry. We show that CD34 and CD56 expression is induced by enforced expression of TENE, suggesting that this combination of TFs to induce NK cell identity are conserved in human. CD34 expression increased with time in culture and was enhanced by the addition of cytokines important for lymphocyte development, suggesting cell expansion. Finally, we employed a barcoded TF approach coupled with single-cell mRNA-seq to inform the specification of progenitor versus mature programs. We screened 48 TFs and first confirmed the involvement of Ets1 in immature NK development, T-bet and Eomes in mature NK, and Nfil3 in both stages. We also suggest Runx family TFs and Ikzf1 as novel regulators of NK development.

Taken together, our results contribute to the understanding of the transcriptional network driving NK lineage commitment and pave the way for the generation of patient-specific NK cells by direct cell reprogramming approaches.

Keywords: NK cell; Cell reprogramming; Cancer immunotherapy; Transcription factor; NK-based therapies; ILCs

Resumo:

As células *Natural Killer* (NK) são linfócitos inatos que apresentam características citotóxicas notáveis para controlar o desenvolvimento de cancro e infeções virais, sendo a sua atividade independente da apresentação específica de antigénios. De facto, as NK são as primeiras células hematopoiéticas derivadas de células estaminais pluripotentes a serem testadas em ensaios clínicos contra tumores hematológicos. No entanto, a sua persistência *in vivo* ainda é limitada e os protocolos usados na sua diferenciação são bastante complexos. Por esta razão, o uso generalizado das NK na terapia ainda enfrenta muitos obstáculos. Aqui propõe-se aplicação de reprogramação celular direta mediada por fatores de transcrição (FTs) para gerar células NK partindo de células somáticas.

Para definir combinações de FTs com a capacidade de induzir a mesma identidade de células NK, testaram-se 19 FTs como candidatos para ativar um repórter específico de NK em fibroblastos embrionários de ratinho. Inicialmente, identificou-se Ets1, Nfil3, T-bet, Eomes (TENE) que ativam o reporter NCR1 específico de NK e consegue induzir programas de expressão genético de progenitores NK e NK confirmado por *single-cell mRNA-seq*. Para testar a conservação entre espécies, transduziram-se fibroblastos humanos com os 4 FTs e avaliou-se a presença de marcadores específicos de NK por citometria de fluxo. Neste trabalho, demonstra-se que a expressão de CD34 e CD56 é induzida pela expressão ectópica de TENE, sugerindo a indução de identidade de células NK por ação destes TFs é conservada em humanos. A expressão de CD34 aumenta com o tempo e com a adição de citocinas importantes para o desenvolvimento de linfócitos, indicando expansão em cultura. Finalmente, empregaram-se estratégias de associação de FTs com sequências de 8 pares de base (barcodes) acopladas com *single-cell mRNA-seq* para obter informação sobre programas de maturação versus progenitor. Testaram-se 48 FTs e confirmou-se o envolvimento de Ets1 com células NK imaturas, T-bet e Eomes com células NK maduras e Nfil3 com os dois níveis de desenvolvimento. Sugere-se ainda Runx TFs e Ikzf1 como novos reguladores do desenvolvimento das NK.

Coletivamente, os resultados contribuem para estender o conhecimento sobre a rede transcricional que dá origem à linhagem das NK e ajuda no desenvolvimento linfoide. Adicionalmente, são fornecidas as primeiras provas para gerar células NK específicas para paciente aplicando reprogramação celular direta.

Palavras-chave: Células NK; Reprogramação celular; Imunoterapia contra cancro; Fator de transcrição; Terapias baseadas em NK; ILCs

Acknowledgments

A sublime journey full of remarkable learnings, unforgettable experiences, and admirable people. This is how I define my passage through the Masters in Oncology and in particular my dissertation year. It was a path of amazing growth that culminated in the project I most enjoyed doing. I thank everyone who helped me along the way.

First of all, I would like to thank Dr. Filipe Pereira for giving the opportunity to work on one of the topics that piqued my interest and for sharing the vast knowledge held in the topic. I'm also very grateful for all the help provided by Inês Caiado, that persistently taught me every step for the project realization while maintaining a friendly and supportive environment. I sincerely appreciate the encouragement and good moments shared with the scientists and friends at Pereira Lab team. Thanks to all of you, during this year I had the chance to explore different scientific perspectives and expand my knowledge in the most diverse areas.

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The best memories in life are built from moments where a Yes can change everything.

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Abbreviations:

ADCC – antibody-dependent cell cytotoxicity

BCMA – B-cell maturation antigen

BM – Bone marrow

CAR – Chimeric antigen receptor

CDS – Coding sequence

CHILP – Common helper innate lymphoid progenitor

CILP – Common innate lymphoid progenitor

CLP – Common lymphoid progenitor

CRS – Cytokine release syndrome

DC – Dendritic cell

DMSO – Dimethyl Sulphoxide

Dox – Doxycycline

DPBS – Dulbecco's phosphate-buffered saline

E4bp4 – E4 promoter-binding protein 4

FACS – Fluorescence-activated cell sorting

FBS – Fetal bovine serum

Flt3L – FMS-like tyrosine kinase 3 ligand

GM-CSF – Granulocyte-macrophage colony-stimulating factor

HEF – Human embryonic fibroblast

HEK – Human embryonic kidney

HSC – Hematopoietic stem cell

Id – DNA-binding protein inhibitor

IFN-γ – Interferon-γ

IL - Interleukin

ILC – Innate lymphoid cell

iNK – Induced NK

iPSC – Induced Pluripotent stem cell

LTi - Lymphoid tissue inducer

MACS – Magnetic-activated cell sorting

MAD – Median absolute deviations

MEF – Mouse embryonic fibroblast

Mef – myocyte enhancer factor

MHC-I – Major histocompability complex class I

mNK – mature NK

Myod1 – Myogenic differentiation 1

NCR1 – Natural cytotoxicity triggering receptor 1

Nfil3 – Nuclear factor interleukin 3

NK – Natural killer

NKP – Natural killer progenitor

NKR – Natural killer receptor

NKT cell – Natural killer T cell

OV – oncolytic virus

PBMC – peripheral blood mononuclear cell

PCR – Polymerase chain reaction

PIB – Pu.1, Irf8, and Batf3

PLB - polybrene

PLZF – promyelocytic leukemia zinc finger

ROR – RAR-related orphan receptor

SCF – Stem cell factor

scRNA-seq – Single-cell mRNA-sequencing

SFFV – Splenic focus forming virus

SOC - Super optimal broth with catabolite repression

TAA – tumor-associated antigen

TdT – TdTomato

TENE – T-bet, Ets1, Nfil3, Eomes

TF – Transcription factor

TLL – Tumor-infiltrating lymphocytes

TLR – Toll-like receptor

TME – Tumor microenvironment

TNF-α – Tumor necrosis factor α

UCB – Umbilical cord blood

Chapter 1

1 Introduction

1.1 Natural Killer Cells

NK cells are key innate lymphoid cells (ILCs) that control viral infections and cancer development. NK cells are found throughout lymphoid and nonlymphoid tissues, being most abundant in peripheral blood, spleen, and lymph nodes (1). However, a high percentage of NK cells is also found in the lung and liver (2). The function of NK cell is regulated by a set of mechanisms that allow them to act against physiologically stressed cells without harming healthy tissues. NK cells display a repertoire of receptors that regulate their activation or inhibition (Figure 1). Several receptors are involved in the recognition of stress-induced ligands that confer specificity to NK activity. For instance, C-type lectin-like receptor, NKG2D, binds to stress-ligands expressed by the self, while toll-like receptors (TLRs) allow to identify nonself ligands. The ligand-receptor interaction promotes higher production of interferon-γ (IFN-γ) and enhanced cytotoxicity important for NK activity (3). Major histocompatibility complex I (MHC-I) receptors also take part in other crucial regulatory mechanism. Ly49 receptor (in mice), Killer Ig-Like receptor (KIR) (in human) and CD94-NKG2A (both species) bind to MHC-I receptor expressed by healthy cells and inhibits NK cells. However, due to DNA damage response or tumor suppressor genes, MHC-I is downregulated by infected and cancer cells, which leads to NK activation (4). Since there are several types of receptors on the surface of NK cells, NK activity is regulated by a balance of facilitating and inhibiting signals. Thus, when self-recognition receptors are downregulated, such as MHC-I, activation stimuli exceed inhibitory patterns and the NK cells become active. The NK cell function relies on activating/inhibiting receptors that specifically drive their activity in an antigen-independent manner.

NKs are potent cytotoxic cells as they produce both cytolytic molecules (Granzyme B and perforins) and cytokines, such as IFN-γ, tumor necrosis factor (TNF)-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-10 and IL-13. This cytokine secretion profile contributes not only to NK own immune response, but also to enhance the response of other immune cells. For instance, NK cells produce IFN-γ and TNF-α that specifically promote maturation and activation of dendritic cells (DCs) and macrophages (5–7). In the human, mature NKs can be divided in two subsets with distinct cytotoxicity and cytokine secretion profiles: $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$. $CD56^{dim}CD16^{+}$ cells are highly cytotoxic perforin-producing cells and account for 90% of NK cells in blood and spleen. The CD16 receptor expressed by this subset of NK cells enables them to identify antibody-coated target cells and specifically exert antibody-dependent cell cytotoxicity (ADCC) (3). On the other hand, NKs $CD56^{bright}CD16^{-}$ are cytokine-producing

cells mainly found lymph nodes and tonsils (8). In mice, NK cell subsets characterized by the expression of CD127 were found to correspond to human subsets. $CD127^{+}$ NK cells develop in the thymus have high cytokine production and low cytotoxicity properties, alike the human $CD16^{-}$ subset. While $CD127^{-}$ produced in bone marrow (BM) correlate with $CD16^{+}$ human subset and therefore more cytotoxic. Therefore, bone marrow and thymic developmental pathways in mouse originate subsets similar to human, suggesting conservation of development and functional properties (9).

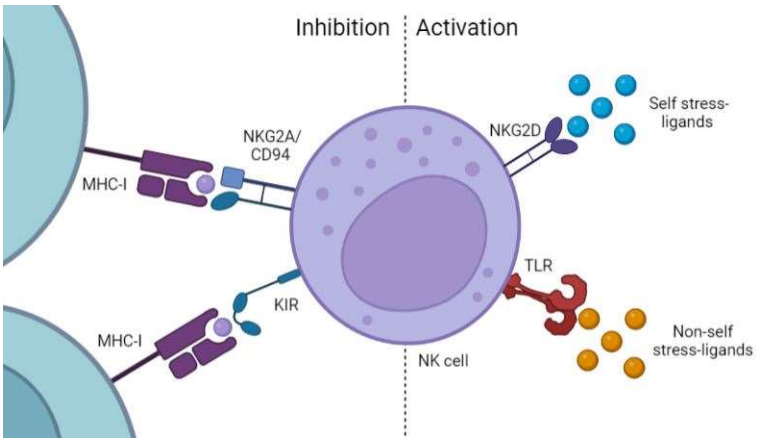


Figure 1 - Natural Killer cells activation and inhibition mechanisms. NK cell function depends on the activation and/or inhibition of several receptors. NK cell inhibition (left side panel) is mediated by NKG2A/CD94 and Killer Ig-like receptors (KIRs) that recognize major histocompatibility class I (MHC-I) molecules expressed on healthy cells, preventing NK cytotoxic activity against self. In contrast, NK cells are activated (right side panel) by stress-induced ligands. While stress-ligands expressed by self are recognized by NKG2D, Toll-like receptors (TLRs) allows the identification of nonself ligands.

1.2 NK cells Ontology and Development

NK cells are classified as ILCs, which are commonly divided into five groups (NKs, ILC1s, ILC2s, ILC3s, and Lymphoid tissue inducer (LTi)) (10). NKs display important cytotoxic characteristics and can secrete several cytokines. ILC1s are mainly cytokine-producing cells that help modulate immune responses. ILC2s express IL-5 and IL-13 crucial for immunity against helminths, whereas ILC3s are known to maintain epithelial integrity and tissue homeostasis, releasing cytokines such as IL-17 and IL-22. LTi cells are involved in the formation of embryonic lymph nodes (10,11). The origin and developmental relationship between NK cells and other ILCs is still elusive (12). For instance, NKs and ILC1s share several similarities, such as dependence on the transcription factor (TF) T-bet for IFN-γ production and common cell surface markers such as NK1.1, NKp44, and NKp46.

However, NK cells are cytotoxic cells with high production of apoptosis-inducing perforins, while ILC1s are considered as helper cells expressing chemotactic molecules that modulate immune responses. Furthermore, NK cells development depends on the TF Eomes, while ILC1s only rely on T-bet (13). Similarly, PLZF, RAR-related orphan receptor alpha (RORα), and RORγt are essential for the development of ILCs subsets, but do not support NK cell differentiation (11,14–16). Hence, NK cells are distinct from all other ILCs and therefore represent a separate branch of innate lymphoid lineage.

The development pathway of NK cells highlights their distinction from other ILCs. NKs and the remaining ILCs differentiate from a common innate lymphoid progenitor (CILP) that originate from CLPs (common lymphoid progenitors). However, at the CILP stage an early divergence of NK cell commitment occurs. CILP differentiate into NK progenitor (NKP) cells or into CHILPs (common helper innate lymphoid progenitors), which later give rise to either NKs or ILC1, ILC2, ILC3 and LTi.

Despite several transcription factors have been described to plays an important role during NK development their exact role is not fully known (Figure 2) (17). For instance, nuclear factor interleukin-3 (Nfil3, also known as E4bp4) is a basic region leucine zipper TF with an important role in multiple steps of immune function. Several studies indicate that Nfil3 is not only important for the cytolytic activity in NK cells, but also regulates the expression of other TFs involved in NK development, such as Gata-3, Eomes (18), and Inhibitor of DNA-binding 2 (Id2). In addition, the cooperation of winged helix-turn-helix proteins of the ETS family with other TFs regulating the gene expression profile in the NK lineage should also be considered. Within the ETS-family, myeloid Elf-1 like factor (Mef) deficient mice are implicated in low expression of cytotoxic molecules in NK cells (19), while lack of Ets-1 is associated with low degranulation activity due to inactivation of several NK cell receptors (NKRs). Ets-1 also regulates the expression of T-bet and Id2 (20,21). Consequently, Nfil3 and Ets-1 are therefore thought to jointly regulate Id2 gene transcription (17,22,23). Id TFs are helix-loop-helix proteins that take a role in cellular development by repressing E protein-induced transcriptional patterns (24). Expression of Id2 is observed in all types of ILCs (25), and is commonly associated with inhibition of T and B cell fate (15), which is an important step to force CLP towards innate lymphoid lineage. This lineage breakpoint is also important to define the differences between NK and NKT cells, usually misclassified due to their similar cytotoxic functionality. In fact, NKT cells represent a specific type of $CD4^{+}CD8^{+}$ T cells restricted by CD1d–lipid antigen complexes that do not contain cytoplasmatic granules. NKT cells rely on the TF promyelocytic leukemia zinc finger (PLZF; known as Zbtb16) for their development and antigen presentation (26). Although, PLZF is expressed in some ILCs, NK development does not depend on this factor (14,15).

Specific TFs have been described to play an important role for NK commitment at progenitor stage. For instance, Nfil3 and Ets1 expression specifically in CLP stage appears to drive NKP generation, as knockout mice for these TFs do not have NK cells (17). The expression of CD122 marks the acquisition of NKP signature both in mice and human. In fact, T-bet and Eomes cooperate to drive cytotoxic function by promoting the expression of CD122. These TFs are important to regulate the final stages of NK cell maturation (27,28). Loss of function assays in mice showed that: Ets1, Stat5, Ikaros (Ikzf1), Aiolos (Ikzf3), Mef, Nfil3, T-bet, Eomes and Irf2 are required for the generation of mature NK cells and their cytotoxic activity (27–30). Additionally, in the absence of Gata-3, Blimp-1 and Runx3 NK cell development is inefficient as the number of mature NK (mNK) cells is reduced compared to wild-type mice (17). Interestingly, Gata3 have been associated with the production of thymic NK cells but is dispensable for NK generation in BM, the main site of NK development in the adult (31). Although multiple TFs have been implicated in NK cell biology, the minimal transcription factor network essential for NK lineage commitment is still not know.

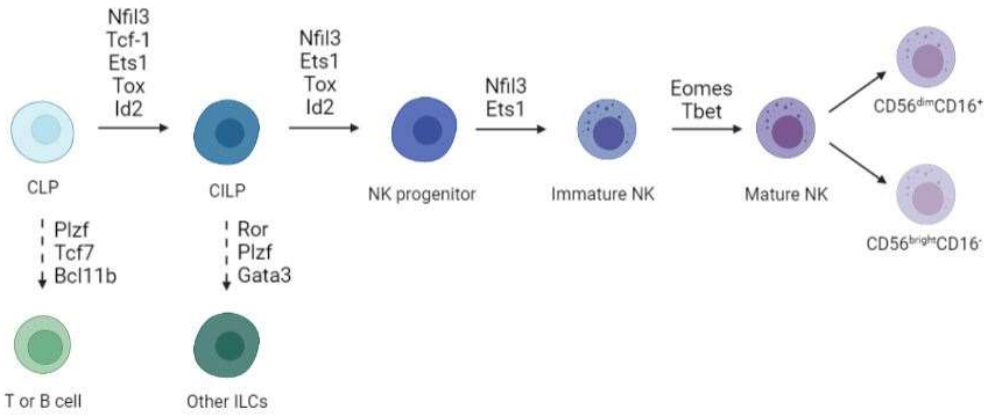


Figure 2 - Stages of NK development. Common lymphoid progenitor (CLP) give rise to different lymphoid cells (such as, T and B cells) and to a common innate lymphoid progenitor (CLIP). CLIP originate all type of innate lymphoid cells (ILCs) including NK cells. NK cells progress to a stage of NK progenitors that generate immature NK. In the human, immature NK develop to mature NK that can be divided in two different subsets: $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$. Each differentiation stage is regulated by specific transcription factors. (Adapted from Vivier et al, 2018)

1.3 Natural Killer cells in Cancer Immunotherapy

One hallmark of cancer is the ability of tumor cells to escape and evade the immune system (32). Due to their adaptability and complexity, cancer cells hijack the immune system and establishing an immunosuppressive tumor microenvironment (TME) (33). In recent years, efforts to circumvent this hallmark of cancer gave rise to Cancer Immunotherapy, i.e. strategies that use the body's own immune system to fight cancer and reinitiate the cancer-immunity cycle (34).

Several immunotherapeutic approaches have been developed and tested in the clinic, including non-personalised therapies such as oncolytic viruses (OVs), which target cancer cells by inducing tumor lysis and promoting the release of tumor antigens, and immune checkpoint inhibitors, which prevent negative regulation of T cell by blocking immune checkpoint molecules expressed on the surface of cancer cell and antigen-presenting cells (35–40). More personalised approaches include cancer vaccines and adoptive T or NK cell transfer (Figure 3). Cancer vaccines aim to restore T-cell specific immune responses against tumor-associated antigens (TAAs) by promoting antigen presentation. These include mRNA of TAAs or irradiated tumor cells vaccines and dendritic cell (DC) vaccines (41–43). As for lymphocytes, the first attempt at immunotherapy was based on T cells specifically prepared to recognize cancer antigens, referred to as tumor-infiltrating lymphocytes (TLLs) (44). Later, the association of chimeric antigen receptors (CAR) with T cells was another innovation in the field. T cells targeting CD19 and B-cell maturation antigen (BCMA) successfully help in the treatment of B-cell hematologic tumors and advanced myeloma, respectively (45,46). However, these approaches are still associated with high cytotoxicity, as the cytokine production profile of T lymphocytes is often related to the onset of cytokine release syndrome (CRS) (47). On the other hand, NK cells response is independent of antigen-specification and their cytokine secretion profile differs from T lymphocytes. Moreover, cancer cells downregulate MHC-I to escape T cell immunosurveillance, but MHC-I negative cancer cells become a target for NK lymphocytes. In fact, stress-induced MHC-I downregulation on cancer cells leads to NK activation. NK cells can be highly specific against cancer, without affecting MHC-I positive healthy tissues, avoiding the risk of graft-vs-host disease (48). In combination with their remarkable cytotoxic abilities NK cells are a promising target for cancer immunotherapy. For instance, adoptative transfer of NK cells can be used therapeutically against hematopoietic cancers, such as acute myeloid leukaemia and acute lymphoblastic leukaemia (49–51). In solid tumors, several efforts have been made to increase NK cell infiltration by associating CAR with NK cell to enhance antitumor activity. Despite their efficacy, allogenic NK cells can still be

eliminated by the recipient's immune system, limiting their persistence *in vivo* and decreasing their antitumor activity (48). Hence, a large number of NK cells is required for clinical applications.

Currently, NK cells can be isolated from peripheral blood mononuclear cells (PBMCs) or differentiated from hematopoietic stem cells (HSCs). Starting from PBMCs, NK cells can be isolated by Magnetic-Activated Cell Sorting (MACS) (52). Tipically, 5-20% of NK cells can be recovered from the initial mixture (53). Recovered NK cells can be expanded using feeder cell line systems or feeder-free bioreactors and further purified before infusion on patients. On the other hand, $CD34^+$ HSCs from umbilical cord blood (UCB) can be cultured and differentiated into NKs by stimulation with a specific cytokine combination. However, UCB-derived NK cells exhibit incomplete maturation and weaker cytotoxicity. In general, these NK cell sources are time-consuming and provide low cell yields (53). Additionally, NK cells can be differentiated from induced pluripotent stem cells (iPSCs) (54). iPSCs are first differentiated in hematopoietic progenitor cells (HPCs) using feeder layers cultured with mouse bone marrow stromal cells (OP9) and specific cytokine mixture (stem cell factor (SCF), VEGF, and BMP4) that potentiate HPCs expansion. This is followed by NK differentiation with co-culture of OP9-DL1 cell line and SCF, IL-15, IL-7, Fms related receptor tyrosine kinase 3 ligand (Flt3L), IL-3 cytokines. Allogenic iPSC-derived CAR NK cells were demonstrated to prevent tumor growth in an ovarian xenograft model (48). Indeed, NK cells are the first iPSCs-derived hematopoietic cells to be tested in clinical trials (50,51,55). Nevertheless, iPSCs-derived NK cells require a stepwise differentiation with further purification steps before clinical application. Altogether, mature cells can only be obtain after two months (54). Despite the efforts, NK cell differentiation from iPSCs remains a complex, time-consuming, and highly costly process. Therefore, new sources of NK cells are needed.

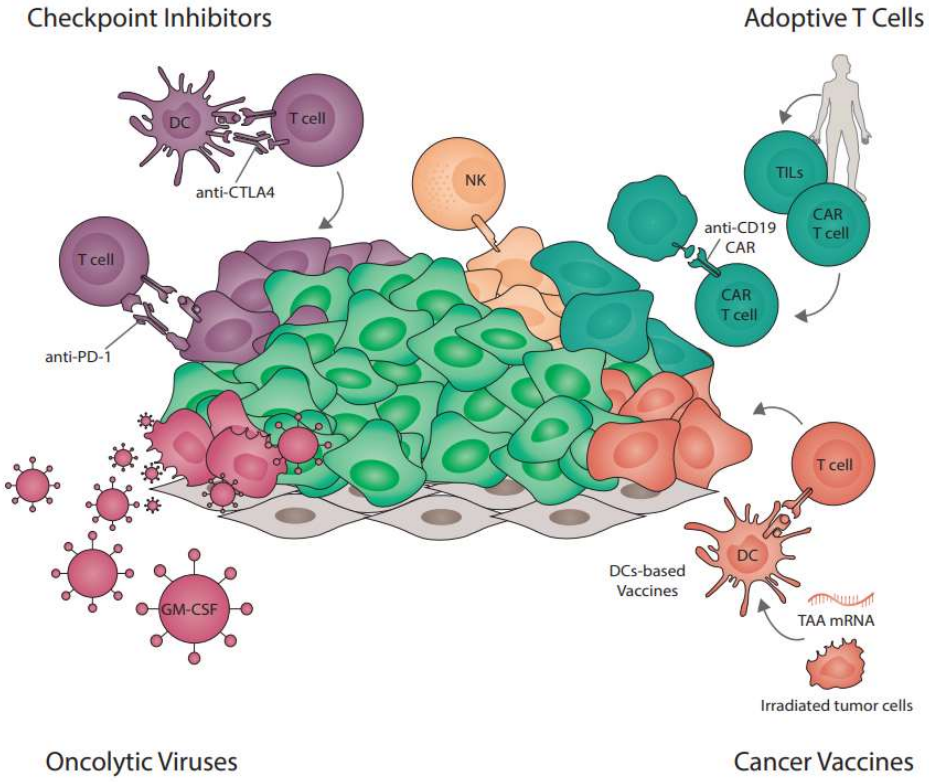


Figure 3 - Strategies for cancer immunotherapy. Overview of different approaches used in cancer immunotherapy, including immune checkpoint inhibitors, adoptive T-cell therapy, oncolytic viruses, and cancer vaccines. Immune checkpoint inhibitors (top left panel) are antibodies that target several negative regulators of T-cell activation: cytotoxic T-lymphocyte antigen (CTLA-4), anti-programmed cell death protein (PD-1) and PD-1 ligand (PD-L1). Adoptative T-cell transfer (top right panel) harboring Chimeric Antigen Receptor (CAR) are currently being used to target CD19. Additionally, tumor infiltrated lymphocytes (TILs) expanded ex vivo and NK cells associated with CAR are currently in clinical trials. Oncolytic virus (bottom left panel) specifically targets tumor cells with feeble antiviral mechanisms, inducing tumor lysis and tumor-associated antigens (TAA) release. Cancer vaccines (bottom right panel) recover T-cell specific immune responses against TAAs suppressed by tumor cells. Multiple strategies are applied to cancer vaccines: preloading of dendritic cells (DCs) with TAAs, in vivo delivery of mRNA encoding multiple tumor antigens, and administration of irradiated tumor cells expressing GM-CSF. (Adapted from Zimmernova et al, 2021).

1.4 Cell reprogramming

In the past, cell fates were assumed to be ineluctable and to occur unidirectionally. However, various cell fate reprogramming approaches have demonstrated that somatic cells can be reversed into pluripotent entities or even driven into completely different somatic cells by modulating epigenetic and transcriptional landscapes. Therefore, lineage commitment is now considered to be less permanent than previously thought.

Cell reprogramming refers to the possibility of converting a fully defined cell entity into a new cell type that has different function, morphology, and molecular features. Overall, cell

identity can be altered through mainly three different approaches: nuclear transfer, cell fusion, or enforced expression of specific factors. Nuclear transfer is explained by the isolation of an adult somatic nucleus that is transferred into an enucleated oocyte (56). The experiments in which a nucleus was transferred from a differentiated cell to originate another kind of cell or tissue, prove that the genetic information was kept after the differentiation process. A non-pluripotent cell nucleus can even be used to generate a completely new organism, as demonstrated in mammals by *Wilmut et al* (57,58). This proves that even after epigenetic silencing, gene expression programs can be re-established. Cell fusion experiments have also been ground-breaking in revealing transcription mechanism that directs cell fate into specific directions. In this case, the purpose is to fuse two or more different cells to generate a multinuclear transient cell entity (heterokaryon). The epigenetic landscapes carried by these adult somatic nuclei allowed to show which functions are active or repressed in those type of cells. In this way, specific epigenetic factors were found to play a specific role on gene activation or repression. For instance, the existence of tumor suppressor genes was demonstrated when hybrids harboring malignant and non-cancerous cells suffered repression of the malignant state at first sight but recovered the proliferation state later. As the proliferation profile re-emerged, the possibility that oncogenes were loss during cell fusion was rapidly excluded, alternatively malignancy was regulated by overexpression of tumor suppressing genes (59,60). Finally, the use of specific factors that coordinate cell fate was put in consideration. Even though the idea that different factors are crucial to push cell development by regulating genetic expression, enforced expression of transcription factors allowed to understand better how these modulators influence cell differentiation. Overexpression of lineage specifying TF contribute to understand how specific TFs engage chromatin to promote gene transcription and facilitate DNA binding of other key players. For instance, reports on overexpression of the TF myoblast determination protein 1 (MYOD1) in mouse fibroblasts, show that was possible to convert this cell into myogenic lineage (61). Another game changer happen when the generation of iPSCs from somatic cells by ectopically expressing 4 specific TFs (Myc, Oct3/4, Sox2 and Klf4) confirmed that is possible to convert a cell type to other using a set of epigenetic regulators paving the way for a new factor-based cell reprogramming approaches (62). In fact, this combination of TFs confers iPSCs plasticity and a pluripotent state that later can be used to originate almost every type of cell in the organism, including immune cells. Nevertheless, the oncogenic potential associated to iPSC reprogramming push cell reprogramming to search different procedures (60,63).

The ability of generating mature cell states from a different somatic cell type is referred as direct lineage reprogramming. For instance, reprogramming using a combination of TFs

towards mature cardiomyocytes, hepatocytes and neurons was previously shown for regenerative medicine (64,65). In hematopoietic system, macrophage-like cells induced with Pu.1 and C/EBPalpha from fibroblast cells was already described (66). Recently, our group generated DCs from fibroblasts through a combination of three transcription factors PU.1, Irf8 and Batf3 (PIB). Induced DCs showed cross-presentation antigen activity, activating T cells (67,68). This study was the first to demonstrate that is possibly to generate immune cells with ability to modulate immune responses by direct reprogramming, opening avenues for reprogramming-based immunotherapies. However, direct reprogramming of lymphocytes using specifying TFs was not yet described (Figure 4). Programmed NK cells will be an exciting tool for cancer immunotherapy and pave the way for a better understanding of the transcriptional network of NK cells and other ILC subsets.

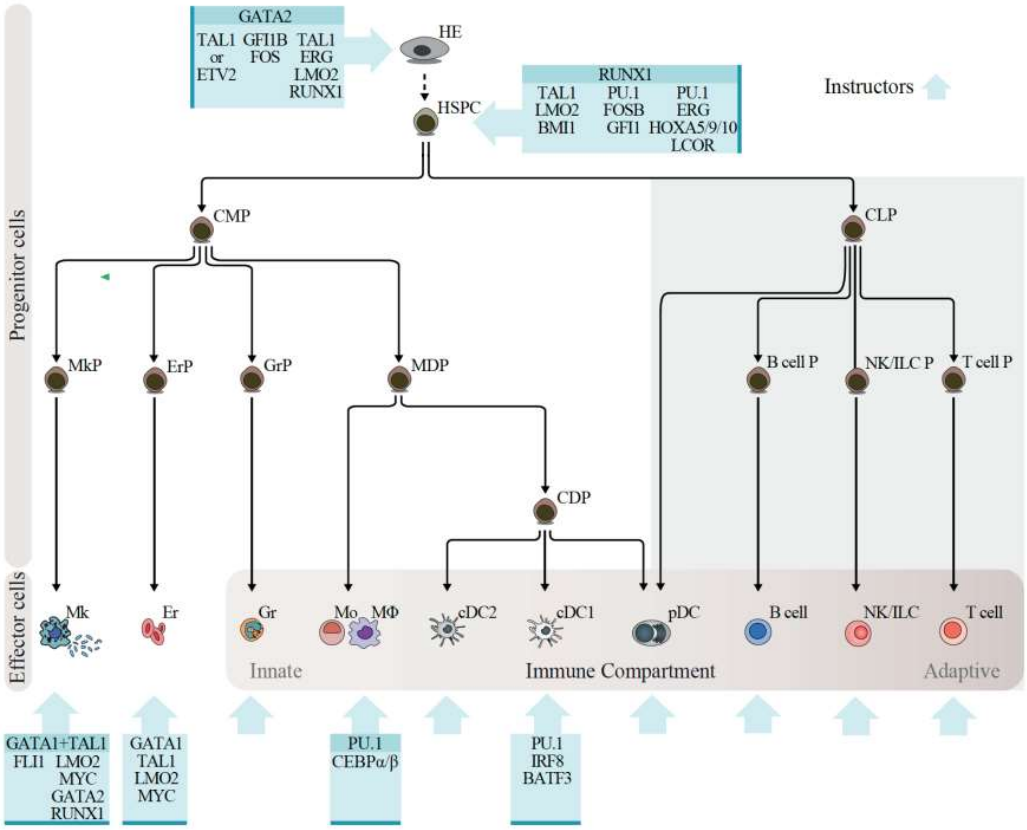


Figure 4 - Instructor TFs for hematopoietic cells reprogramming. Overview of the lineage-specifying Transcription factors within hematopoietic system. Combination of TFs used to reprogram non-hematopoietic cells in hematopoietic cells are presented in blue boxes. Abbreviations: HE, hemogenic endothelium; HSPC, hematopoietic stem and progenitor cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; Mk, megakaryocyte; Er, erythrocyte; Gr, granulocyte; Mo, monocyte; Mϕ, macrophage; cDC2, conventional dendritic cell type 2; cDC1, conventional dendritic cell type 1; pDC, plasmacytoid dendritic cell; NK, natural killer; ILC, innate lymphoid cell. (Adapted from Pires et al, 2019)

1.5 Aim

NK cells are critical players that successfully identify and eliminate virus infected and tumor cells without prior antigen sensitization or expansion. In addition, NK cells also promote adaptative immune system responses by producing cytokines and other chemotactic molecules. NK cell-based immunotherapies have been clinically applied in the treatment of cancer patients providing evidence that these cells present clinical potential. Indeed, NK lymphocytes are the first iPSC-derived hematopoietic cells being tested in clinical trials. Despite of recent success of NK lymphocytes in cancer immunotherapy, the protocol to differentiate NK cells and generate lymphoid cells from iPSCs remains time-consuming, highly costly, and very complex.

The main role of NK cells as regulators of cancer and infectious pathogens, together with their potential for cancer immunotherapy strategies motivates the search for a better understanding of NK minimal gene regulatory networks and how epigenetic landscapes are set in motion during NK cell commitment. Although some advances on comprehension NK cell developmental mechanisms, the instructor TFs that conduct NK cell fate are yet to be described.

The project focus on uncovering minimal combination of transcription factors to induce NK cells from fibroblasts. Our group have identified a combination of 4 TFs - Ets1, Nfil3, T-bet and Eomes - that activate NK-cell specific reporter in mouse embryonic fibroblasts (MEFs). The aim of this project is to optimize the reprogramming of NK-like cells, while discovering the key players required for NK cell lineage commitment. The project conducted specifically involves the following aims:

a) Identification of NK lineage transcriptional codes employing barcoding strategies at single cell level
b) Optimization of NK cell reprogramming applying identified TF combination in mouse system to human fibroblasts to ensure NK-specifying processes conservation.
c) Characterization of induced NK cell phenotype and transcriptome

The conducted studies will allow greater understanding of the mechanisms controlling NK cell specification, development, and function. Ultimately, this project will contribute to understand transcription regulation during lymphoid development and pave the way to establish a new source of NK cells for cancer immunotherapy.

Chapter 2

2 Material & Methods

2.1 Cloning

2.1.1 Oligo preparation

To identify additional TFs that instruct NK cell fate and define different maturation stages throughout NK lineage, 32 candidate TFs were cloned into SFFV vector containing a specific oligonucleotide sequence of 8 nucleotides (barcode). Each oligonucleotide contained a vector specific binding region (5' – ATCAAGCTGTACCTTCTAGA - 3', 5' - TTGTAAGTCATTGGTCTAGA - 3'; forward and reverse, respectively) allowing the cloning to the desired vector and a specific barcode sequence (5' - tgtaca-BARCODE-agcgct - 3', 5' - agcgct-BARCODE-tgtaca - 3'; forward and reverse, respectively). Each TF was associated with a unique barcode. Forward and reverse oligonucleotides annealing was performed. Annealing stock solution containing 1M Tris Buffer (Invitrogen), 5M NaCl (Invitrogen), and 0.5M EDTA (Invitrogen) was individually prepared. A volume of 1µL of each oligonucleotide (forward and reverse) was mixed with 50µL of annealing buffer and incubated at 95°C for 5min. Samples were cooled down at room temperature overnight.

2.1.2 Polymerase Chain Reaction (PCR)

Transcription Factors coding sequences (CDS) were inserted into two vectors, a constitutive splenic foci forming virus (SFFV) promoter and a doxycycline (Dox)-inducible TetO system (FUW-TetO). TFs CDS from Group's library were amplified by PCR in 20µL reactions using a proof-reading polymerase (Thermo Scientific Phusion High-Fidelity 2X PCR Master Mix).

PCR amplification protocol consisted of three different steps: initial denaturation (98°C, 30s); 35 cycles of denaturation (98°C, 10s), annealing (62-65°C, 30s) and extension (72°C, 1-2min); and final extension (72°C, 10min). PCR products differentiation was made by electrophoresis in a 1% agarose gel, followed by U.V. light image acquisition. DNA extraction from PCR-products bands was performed using NucleoSpin Gel and PCR clean-up (Macherey-Nagel).

2.1.2.1 Barcoded TFs

Individual TFs CDS were inserted into barcoded SFFV promoters (Figure 5). Forward and reverse primers were built harboring first ~20 nucleotides of TFs CDS or its reverse

complementary sequence, respectively. Restriction sites for BstBI (upstream, 5' - TT^CGAA - 3') and AgeI (downstream, 5' - A^CCGGT - 3'), and vector specific binding region (5' - CGCTAGCCCGCGG - 3', 5' – GCCCGGGGGATCCG - 3'; forward and reverse, respectively) were added to later perform one-step in-fusion reaction.

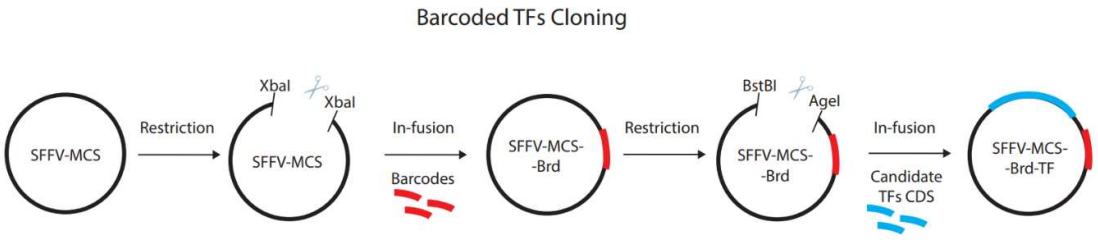


Figure 5 - Cloning strategy for Barcoded Transcription factors. Barcoded TFs Cloning with first restriction of SFFV-MCS followed by insertion of barcodes. Barcodes inserted in SFFV performing In-fusion. Second restriction to clone TFs CDS into barcoded vector by In-fusion.

2.1.2.2 Individual TFs

To identify TFs that induced NK cell fate, candidate TFs CDS were cloned into FUW-TetO. Forward and reverse primers were built with ~20 nucleotides of TFs CDS. Restriction sites for XbaI (upstream, 5' - T^CTAGA - 3') and BstBI (downstream, 5' - TT^CGAA - 3') and vector specific region (5' - CCGAATTCACCGGTTCTAGA - 3', 5' - TTATCGATAAGCTTGATATC - 3'; forward and reverse, respectively) were also added.

Nfil3, Ets1, T-bet and Eomes CDS were individually cloned into SFFV without barcode to test promoter efficiency. Primer design consisted in ~20 nucleotides of TFs CDS, restriction sites for BstBI (upstream, 5´ - TT^CGAA - 3') and AgeI (downstream, 5' - A^CCGGT - 3'), and vector specific binding region (5' – CGCTAGCCCGCGG - 3', 5' – GCCCGGGGGATCCG - 3'; forward and reverse, respectively).

Individual TFs were cloned into Fuw-TetO and SFFV promoters performing one-step in-fusion reaction.

2.1.2.3 Polycistronic constructs

TFs CDS of Nfil3, Ets1, T-bet, and Eomes were clone into Fuw-TetO as polycistronics (Figure 6). Bicistronics inserts containing TFs CDS separated by cleavage sites (P2A, E2A,

T2A) were previously ordered (Twist Bioscience). Each bicistronic harbors CDS of two different TFs. Twist bicistronics inserts were cloned into FUW-TetO in pairs to perform polycistronic cloning. Polycistronic constructs harbor CDS of 4 different TFs. The following orders were prepared:

Table 1 - Fuw-TetO polycistronic cloning from Twist Bicistronics.

TFs	Twist Bicistronic	Fuw-TetO Polycistronic
Ets1, Nfil3, Eomes, T-bet	Ets1-P2A-Nfil3-T2A + Eomes-E2A-T-bet	Ets1-P2A-Nfil3-T2A-Eomes-E2A-T-bet
Nfil3, Ets1, Eomes, T-bet	Nfil3-P2A-Ets1-T2A + Eomes-E2A-T-bet	Nfil3-P2A-Ets1-T2A-Eomes-E2A-T-bet

Forward and reverse primers were designed containing ~20 nucleotides of Twist bicistronics inserts and specific restriction sites. For Ets1-P2A-Nfil3-T2A and Nfil3-P2A-Ets1-T2A, EcoRI (5' - G^AATTC - 3') and BamHI (5' - G^GATCC - 3') restriction sites were added to forward and reverse primers, respectively. For Eomes-E2A-T-bet, BamHI and NheI (5' - G^CTAGC - 3') sites were used for forward and reverse primers. After PCR amplification, products were restricted and inserted in FUW-TetO promoter using a DNA ligase.

As SFFV polycistronic constructs were not successfully cloned, we chose to prepare bicistronics constructs (Figure 6). For that, the following Twist bicistronics were inserted into SFFV promoter:

Table 2 - SFFV bicistronic cloning harboring specific TFs.

TFs	SFFV Bicistronic
Ets1, Nfil3	Ets1-P2A-Nfil3-T2A
Nfil3, Ets1	Nfil3-P2A-Ets1-T2A
Eomes, T-bet	Eomes-E2A-T-bet

The same strategy was applied for primer design, with ~20 nucleotides of original bicistronic inserts and restriction sites. For Ets1-P2A-Nfil3-T2A and Nfil3-P2A-Ets1-T2A, SalI (5'-G^TCGAC-3') and NheI sites were added to forward and reverse primers, respectively. SalI and SacII (5'-CCGC^GG-3') sites were used for Eomes-E2A-T-bet forward and reverse primers, respectively. After PCR amplification, products were restricted and inserted in SFFV promoter using DNA ligase.

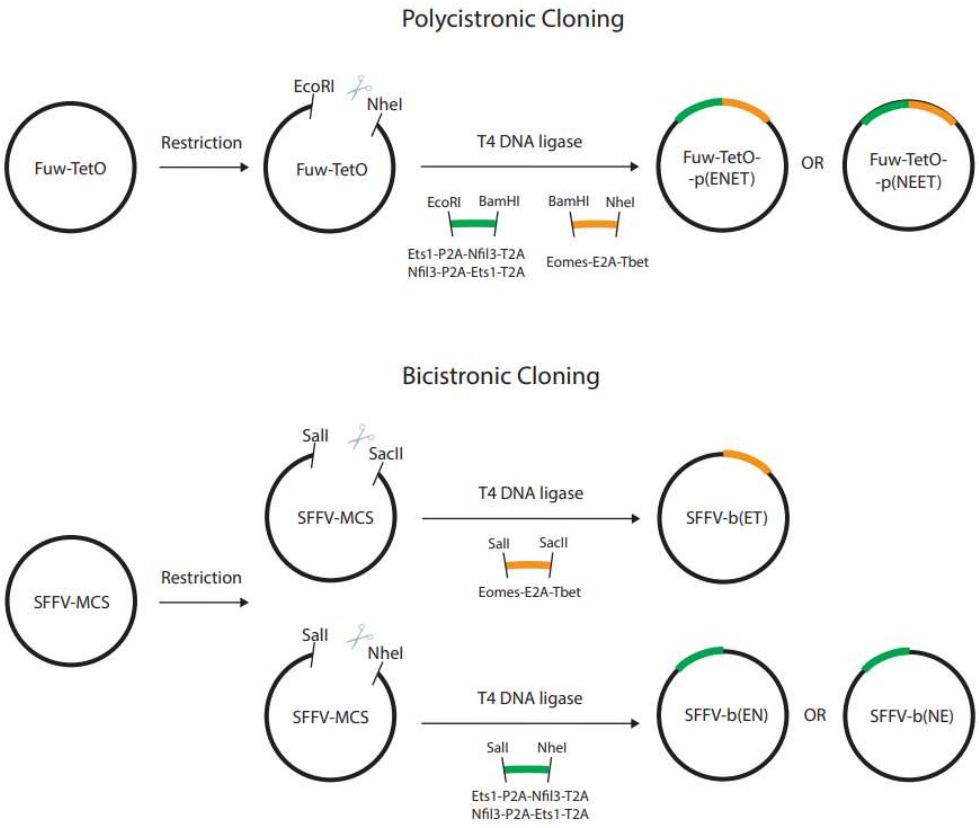


Figure 6 - Cloning strategy for Fuw-TetO and SFFV-MCS constructs. Polycistronic Cloning (top panel) with restriction of both Fuw-TetO vector and PCR products followed by insertion of two bicistronic in the same vector. Two different orders were obtained – Fuw-TetO-p(ENET) (Order: Ets1, Nfil3, Eomes, T-bet) and Fuw-TetO-p(NEET) (Order: Nfil3, Ets1, Eomes, T-bet). Bicistronic Cloning (bottom panel) with restriction of SFFV-MCS followed by cloning of different bicistronics. Three different bicistronics were obtained regarding the TFs and order – SFFV-b(EN) (Order: Ets1, Nfil3), SFFV-b(NE) (Order: Nfil3, Ets1), and SFFV-b(ET) (Order: Eomes, T-bet).

2.1.3 Enzymatic Restriction

For oligonucleotide insertion, SFFV promoter was restricted with XbaI (Thermo Fisher) that recognizes 5'-T^CTAGA-3' restriction site. While for TFs CDS insertion, SFFV promoter - containing a specific barcode - was restricted with BstBI (upstream) and AgeI (downstream) (Thermo Fisher). FUW-TetO promoter was restricted with XbaI (upstream) and BstBI (downstream) for individual TFs CDS cloning.

For polycistronic insertion, EcoRI (upstream) and NheI (downstream) (Thermo Fisher) restriction was performed both in PCR products and in the FUW-TetO promoter. PCR products were also restricted with BamHI (Thermo Scientific) for bicistronic ligation. To

clone bicistronic constructs, SFFV promoter and PCR products were restricted using SalI and SacII (Thermo Fisher).

We used 40µL reaction and samples were incubated for 30min at 37°C (optimal restriction conditions), followed by enzymatic heat-inactivation. Restriction products were separated from non-restricted products by electrophoresis in 1% agarose gel and purified using NucleoSpin Gel and PCR clean-up (Macherey-Nagel).

2.1.4 In-fusion Cloning

Barcoded TFs constructs were cloned into the restricted SFFV vector using the In-Fusion enzyme of In-Fusion HD Cloning kit (Takara) according to manufacturer's conditions. To improve cloning efficiency, for oligonucleotide 15:1 ratio of barcode versus promoter was used, while for TFs CDS 2:1 ratio was enough based on DNA sequence length. Note that oligonucleotide in-fusion required preparation of different pools containing 10 barcodes each (4 pools were prepared). TFs CDS were specifically inserted in a vector with known barcode (confirmed by sequencing).

After PCR amplification, Nfil3, Ets1, T-bet, and Eomes CDS were cloned into the restricted SFFV (without barcode) using In-fusion enzyme. A 2:1 ratio of insert versus promoter was used to maximize cloning efficiency.

Candidate TFs cloned into Fuw-TetO followed the same strategy using In-fusion enzyme. The same ratio of 2:1 was used.

After in-fusion, products were transformed using competent bacteria.

2.1.5 T4 DNA ligase Cloning

Bicistronics and Polycistronics were cloned into SFFV and FUW-TetO, respectively, after restriction. Cloning was performed using T4 DNA ligase (Thermo Scientific). Bicistronics used to build polycistronic constructs were added in the same reaction. PCR products were added to 100ng of restricted vector in 2:1 ratio. The mixture was incubated overnight at room temperature, before performing bacterial transformation.

2.1.6 Heat-shock Bacterial Transformation

All constructs were transformed using Stellar Competent Cells (Takara). Stellar Competent Cells, storage at -80°C, were thaw on ice and used for heat-shock bacterial transformation. A volume of 2.5µL transformed DNA was mixed with 50µL competent bacteria and incubated on ice for 30min, followed by heat-shock at 42°C for 45s. Super optimal broth with catabolite repression (SOC, Takara) medium was added to mixture and samples were grown at 37°C 200rpm for 1hr, before being plated in ampicillin (100µg/mL; G Biosciences) enriched LB agar (40g/L, Sigma-Aldrich) plates for antibiotic selection. Plates were kept at room temperature to total liquid absorption and then incubated 16hrs at 37°C.

2.1.7 Miniprep and Colony PCR

1-5 colonies were picked and grown for 16hrs at 37°C in 3mL of LB Broth medium (40g/L, MP Biomedicals) enriched with ampicillin. After incubation, plasmid DNA in culture (1.5mL) was extracted using GeneJET Plasmid Miniprep Kit (Thermo Scientific). Screening of positive colonies was performed by PCR amplification using 2X PCR Master Mix (Thermo Fisher Scientific). Colony PCR was prepared with 10µL reaction volume following the cycling program: initial denaturation (95°C, 1min); 30 cycles of denaturation (95°C, 30s), annealing (65°C, 30s), and extension (72°C, 1min30s); and final extension (72°C, 10min). PCR products were analyzed by electrophoresis in a 1% agarose gel and U.V. light image acquisition.

2.1.8 Maxiprep and Sanger Sequencing

After PCR screening, cultures from colonies considered positive were incubated with a ratio of 1 to 1000 in 200mL of LB Broth medium combined with ampicillin at 37°C for 16hrs. For plasmid DNA purification, we used GenElute HP Plasmid Maxiprep Kit (Sigma Aldrich) and DNA sequences were confirmed by Sanger Sequencing (Eurofins Genomics) using both forward and reverse promoter-specific primers.

2.2 Cell culture

2.2.1 MEFs isolation

To assess candidate TFs ability for NK reprogramming, mouse embryonic fibroblasts (MEFs) harboring Natural cytotoxicity triggering receptor 1 (NCR1)-tdTomato (tdT) NK-specific reporter were isolated in collaboration with Ewa Sitnicka Quinn (Lund University). MEFs were isolated from 13.5-d pregnant female mice. After euthanizing the animal by cervical dislocation, the isolated uterus was briefly washed with Dulbecco's phosphate buffered saline (DPBS, Sigma-Aldrich) and transferred into a petri dish covered with DPBS. Thereafter, embryos were separated from their placenta and surrounding membranes using forceps. Embryos head, visceral tissues and gonads were removed, and the remaining body was hashed out with a pair of scissors. The samples were transferred into a 50-mL tube containing 0.12% trypsin/0.1mM EDTA solution (3mL per each embryo) and incubated at 37°C for 20min. The previous step was repeated. After incubation, 6mL per each embryo of DMEM Complete media (Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco), 2mM GlutaMAX (Gibco), 100U/mL penicillin and 10µg/mL streptomycin (Pen-Strep) (Sigma-Aldrich) was added, followed by up and down pipetting to promote tissue dissociation. The mixture was kept for 5min at room temperature, the supernatant formed transferred into a new 50-mL tube and centrifuged 5min at 1200g. The pellet was resuspended in fresh medium and cell concentration was adjusted to $1x10^6$ cells/mL after cell counting. Cells were plated according to cell culture conditions.

2.2.2 Cell culture conditions

MEFs, human embryonic fibroblasts (HEFs) and human embryonic kidney cell (HEK) 293T cells were individually cultured and maintained in DMEM Complete media in 5% (v/v) CO_2 at 37°C. Cells were passaged after reaching ~80% confluence according to the following steps. Cells were washed with DPBS and incubated with TrypLE Express (Gibco) at 37°C. After 10min, cells were resuspended with addition of DMEM Complete media and centrifuged at 350g for 5min. Cells were then resuspended and plated 1:6 in DMEM Complete media. For fibroblasts, plates were pre-coated with 0.1% porcine-derived gelatin and incubated for 20min at 37°C.

Cells not passaged were frozen in FBS supplemented with 10% dimethyl sulphoxide (DMSO).

2.2.3 Lentiviral production

Lentiviral particles encoding TFs CDS and polycistronics constructs were produced using a 2nd generation system harboring a packaging plasmid (psPAX2), an envelope plasmid (pMD2) and a lentiviral transfer plasmid (i.e., plasmid of interest). HEK 293T cell line was expanded in DMEM Complete media for lentiviral production.

At 50-70% confluence, culture media was replaced by DMEM media without supplements and HEK 293T cells were transfected in 150mm plates with a mix of 7.5µg pPAX2, 2.5µg pMD2 and 10µg transfer plasmid added to 60µL 1mg/mL polyethylenimine combinative reagent (PEI; Sigma Aldrich) and 2mL Opti-MEM reduced serum media (Gibco). The following values of transfer plasmid were mixed for each condition: 10µg of individual barcoded TFs (prepared individually), 2.5µg of each TFs (4 in total – Nfil3, Ets1, T-bet, Eomes) for individual TFs condition, 5µg of each bicistronics (2 in total) for bicistronics condition, and 10µg of polycistronic. PEI and Opti-MEM were added to transfection mix once again.

Transfection mix was incubated at room temperature for 30min prior evenly distribution in HEK 293T cell plates. After 4-6hrs of incubation at 37°C, culture media was replaced by DMEM Complete media and placed again at 37°C. Culture media was collected at 48, 60 and 72 hours. Collection media was kept at 4°C between collections.

After last collection, supernatant containing the lentiviral particles was filtered (0,45µm) and mixed with 25% (v/v) of Lenti-X Concentrator (Takara). After incubating 45min at 4°C, samples were centrifuge at 1500g for 45min at 4°C. Viral pellet was resuspended in DMEM Complete and storage at -80°C.

2.2.4 Viral transduction and reprogramming

For cell reprogramming, HEFs were plated in 0.1% gelatin-coated (Sigma) 6-well plates at $2x10^5$ HEFs/plate of cell density and MEFs at $2.5x10^5$ MEFs/plate. At 80% of confluency, fibroblasts were transduced with 20 viral particles of each barcoded TF per cell (in different mixtures). For the other conditions cells were transduced with 20µL of viral mix. Transduction mixtures were combined with 8µg/mL of polybrene (PLB), which improves the process efficiency by reducing negative charge repulsion between cell membrane and viral particles. After 12hrs, culture media was replaced with DMEM Complete media and every 2 days for experiment time.

FUW-TetO promoter is a lentiviral vector containing the reverse tetracycline transactivator (M2rtTA), therefore, in these condition cells were co-transduced with M2rtTA in the same volume as viral mix. Culture media was also replaced by DMEM Complete media supplemented with 1 µg/ml doxycycline (Sigma).

Cytokine conditions were also tested by supplementing a growth media with IL-3 (5ng/mL), IL-7 (5ng/mL), IL-15 (10ng/mL), Flt3L (10ng/mL), and SCF (10ng/mL).

2.3 Viral RNA isolation and Virus titration (qRT-PCR)

Viral RNA was extracted using NucleoSpin RNA virus kit (Macherey-Nagel) and titrated using Quant-X One-step qRT-PCR TB Green kit (Takara). Firstly, genomic viral RNA was treated with DNAse I to remove all DNA residues left from HEK293T cells packaging. After DNAse I purification, viral samples were serially diluted 4 times and added to PCR-grade 8-well strips in ice. Simultaneously, a 5-time serial dilution of the Lenti-X RNA control template was prepared in ice for standard curve generation. Samples and controls were placed in duplicate in a 96-well PCR plate prepared with qRT-PCR mastermix containing the provided RNAse-free water, Quant-X Buffer, Lenti-X Forward and Reverse primer, ROX Reference Dye LMP, Quant-X Enzyme and RT Enzyme mix. The 96-well plate also contained three negative controls in duplicate prepared with qRT-PCR mastermix without a template. The prepared 96-well plate was placed into the qRT-PCR instrument with following settings: reverse transcriptase reaction (42°C, 5min) (95°C, 10s); 40-cycle qPCR, denaturation (95°C, 5s) and annealing (60°C, 30s); dissociation curve.

2.4 Flow Cytometry

To assess expression of NK lineage markers, such as CD34 and CD56, we perform flow cytometry analysis at specific time-points. Cells were raised with TrypLE Express and resuspended in PBS mixed with 2% FBS and 1% Pen-Strep. Samples were stained with PE-Cy7-CD34 and APC-CD56 (Fluorochrome-Antibody) (BioLegend). After diluting in PBS 2% FBS and adding mouse serum (1/100), samples were incubated at 4°C for 30min. Washing step with 3mL of PBS 2% FBS 1% Pen-Strep was performed, followed by 350g 5min centrifugation. Fluorescence Minus One (FMO) control, single color and unstained samples were used as controls. Finally, 1µg/mL DAPI (BioLegend) was added, and samples were analyzed in BD LSRFortessa flow cytometer (BD Biosciences). Data analysis was performed using FlowJo Software (version 10.6.1, FlowJo, LLC).

Table 3 - List of antibodies, fluorochromes and laser colors used for flow cytometry.

Marker (antibody)	Fluorochrome	Laser
CD34	PE-Cy7	Yellow-Green
CD56	APC	Red
Viability dye	DAPI	Violet

2.5 Live Cell Imaging

Emerging tdTomato positive (tdT+) cells were analyzed 3-12 days after transduction by fluorescence microscopy. The entire well of a 6-well plate was acquire in an automated Zeiss Celldiscoverer 7 at 37°C 5% CO_2 to maintain cell viability. Bright field was used as focus reference. Images were processed using Adobe Illustrator software and tdT+ colonies were counted manually. One colony was defined as 5 or more cells.

2.6 Single-cell RNA sequencing (scRNA-seq)

2.6.1 Sample Preparation

Unstransduced MEFs and MEFs transduced with Ets1, Nfil3, T-bet and Eomes were dissociated using TrypLE Express, resuspended in PBS 2% FBS and FACS sorted at day 3 (tdT+), day 6 (tdT+), and day 12 (tdT+). Purified MEFs (5000 cells), tdT+ day 3 (5000 cells), tdT+ day 6 (5000 cells), tdT+ day 12 (5000 cells). Sorted tdT+ cells from NCR1-tdTomato mice bone marrow (5000 cells) and spleen (5000 cells) were used as control. Bone marrow cells were harvest from tibia and femur bones by flush. Spleen cells were homogenized with a syringe pestle. Red blood cells were lysed using BD Pharm Lyse (BD Biosciences) for 8 min at room temperature. For barcoded TFs scRNA-seq experiment, HEFs transduced with three different pools of candidate TFs (Pool A, Pool B and Pool C) or with the 4 TFs (Ets1, Nfil3, T-bet and Eomes) were dissociated using TrypLE Express, resuspended in PBS 2% FBS and FACS sorted at day 6 and day 12 (DAPI negative cells – live cells). Live cells Pool A day 6 (5000 cells), live cells Pool A day 12 (5000 cells), live cells Pool B day 6 (5000 cells), live cells Pool B day 12 (5000 cells), live cells Pool C day 6 (5000 cells), live cells Pool C day 12 (5000 cells). PBMCs $CD56^+$ (5000 cells) from two donors were used as control. Cells were collected in PBS 2% FBS and filtered through a 70µm cell strainer (BD Biosciences). All cells were sorted using BD FACS ARIA III and loaded on a 10× Chromium (10× Genomics) according to manufacturer's protocol. Single-cell RNA-seq libraries were prepared using Chromium Single Cell 3' v3.1 Reagent Kit (10×

Genomics) according to manufacturer's protocol. Single cells were isolated into droplets together with gel beads coated with unique primers bearing 10× unique molecular identifiers (UMI) and poly(dT) sequences. Reverse transcription reactions were performed to generate barcoded full-length cDNA. Indexed sequencing libraries were constructed using the from the Chromium Single cell 3' v3.1 Reagent Kit (10× Genomics). Library quantification and quality assessment was determined using Qubit and Agilant TapeStation. Indexed libraries were sequenced on an Illumina NovaSeq 6000 S2 100 FlowCell v1. Coverage of approximately 100,000 reads per single cell was obtained.

2.6.2 Pre-processing of scRNA-seq data

Paired-end sequencing reads of single cell RNA-seq were processed using the 10x Genomics software Cell Ranger v6.1.0 (https://support.10xgenomics.com/single-cell-gene-expression/software). Firstly, cellranger mkfastq was used to convert binary base call files to FASTQ files and to decode the multiplexed samples simultaneously. Next, cellranger count was applied to FASTQ files and alignment to either mouse (mm10) or human (hg38) genome assemblies.

2.6.3 TF barcode detection

To find TF barcodes associated with cells, we performed a search on non-mapped reads using the Cell Ranger generated bam files. We searched for a constant flanking region of 6 bp masking our variable 8bp barcode sequence. Additionally, we designed a computational tool that take the barcode sequence for a particular TF and build a dictionary of similar sequences within the user-specified mismatch range. Then sequence is matched to a particular TF not only if it has an exact match but also if it matches to the sequence from corresponding dictionary.

2.6.4 scRNA-seq data analysis

The sparse expression matrix generated by cellranger analysis pipeline was used as input to Scater library, and cells and genes passing quality control thresholds were included according to the following criteria: 1) total number of UMIs detected per sample greater than 3 lower median absolute deviations (MADs); 2) number of genes detected in each single

cell greater than 3 lower MADs; 3) percentage of counts in mitochondrial genes less than 7.5%. The resulting expression matrix was filtered by Scatter analysis pipeline and used as input to the Seurat library v4. To account for technical variation, we performed batch integration. Firstly, we normalized each batch separately using "LogNormalize" with the scale factor of 10,000 and identified 5,000 variable features. Next, we performed batch integration by finding corresponding anchors between the batch using 30 dimensions. We then computed 50 principal components and tested their significances by JackStraw. We selected the first 30 principal components for subsequent tSNE, UMAP and clustering analyses.

2.6.5 NK classification

In order to classify our mouse induced NKs (iNKs) we used a scPred library (69) and natural NK cells. To train classifier using scPred method (implemented as R library), the default parameters for getFeatureSpace and trainModel, was used as defined in tool vignette. For classification of mouse iNKs, the scPredict function was used with default parameters, except for a threshold = 0.99.

2.7 Statistical Analysis

Comparisons among groups were performed by Kruskal-Wallis test with GraphPad Prism 5 software. A *p value* ≤ 0.05 was considered statistically significant.

Chapter 3

3 Results

3.1 Candidate TFs – Ets1, Nfil3, T-bet, Eomes – induce NK specific reporter in MEFs

To determine minimal and optimal combination of TFs capable of inducing NK cell fate, we tested 19 candidate TFs identified through global gene expression analysis and literature mining (Supplementary Table 1). To evaluate candidate TF ability for NK reprogramming, our group (in collaboration with Ewa Sitnicka, Lund University) developed MEFs genetically modified with NCR1-tdT NK-specific reporter. NCR1 expression is specific for NK cells comparing with other mouse tissues and it is expressed across NK lineage, including progenitor stages. NCR1-tdT reporter MEFs were transduced with candidate TFs individually cloned in a doxycycline inducible vector (FUW-TetO). After 12 days of Dox inducible cultures, the emergence of tdT+ in iNK was scored by immunofluorescence (Figure 7A, B and C). NK cell identity is observed by the expression of tdT fluorescence protein. The initial 19 candidates were tested all together and divided in 5 different pools. The results show that at day 12 of reprogramming, the number of tdT+ colonies was higher in a pool containing 5 TFs (Ets1, Nfil3, Id2, T-bet, and Eomes) in comparison with other pooled TFs (Figure 7D). Fluorescence Microscopy pictures of MEFs co-transduced with M2rTA and Ets1, Nfil3, Id2, T-bet, and Eomes show an increasing number of tdT+ colonies over time (day 3, 6, 9, and 12) (Figure 7E). The next step was to take one of the TFs from Ets1, Nfil3, Id2, T-bet, and Eomes combination to assess if we could increase number of tdT+ colonies. The data show that without Id2, the combinatorial activity of the other 4 TFs results in higher number of tdT+ colonies (Figure 7F, top panel). With the same approach, was found that removing one TF from Ets1, Nfil3, T-bet, and Eomes (TENE) combination is not beneficial for tdT emergence (Figure 7F, bottom panel). To confirm the involvement Ets1, Nfil3, T-bet, and Eomes in NK cells, we analyzed the combined expression of the 4 TFs through several mouse tissues and cell types. Ets1, Nfil3, T-bet, and Eomes are enriched in NK cells (Figure 7G).

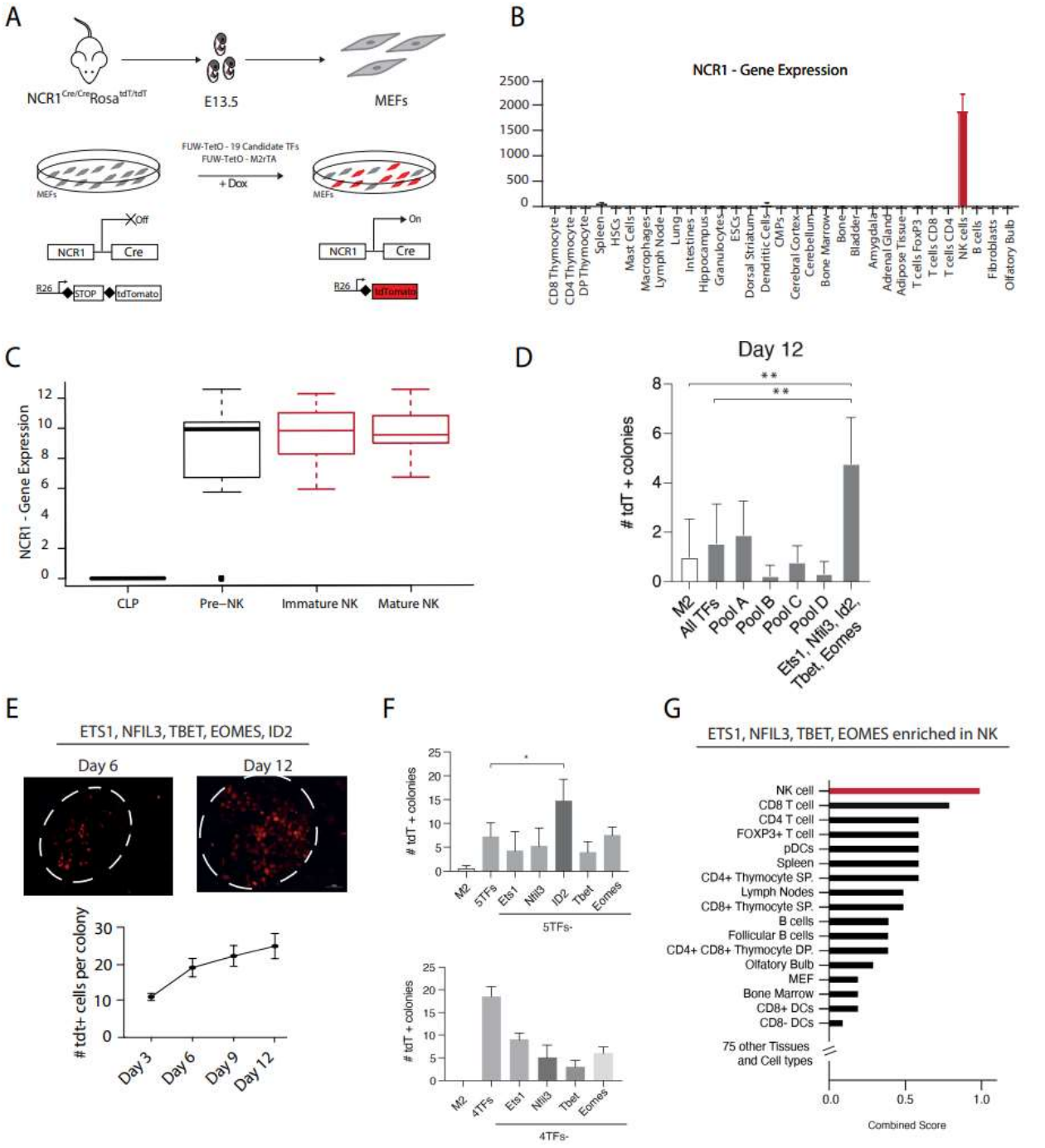


Figure 7 Combined expression of Ets1, Nfil3, T-bet and Eomes induce transdifferentiation to NK cell fate. (A) Experimental strategy to screen NK-inducing transcription factors (TFs). NCR1-cre/R26.Stop.f(/f).tdTomato double transgenic mouse embryonic Fibroblasts (MEFs) were cotransduced with lentiviral particles encoding 19 candidate TFs and M2rTA and cultured in presence with Dox. (B) NCR1 expression across several mouse tissues and cell types (Tabula Muris). (C) NCR1 expression in Common Lymphoid Progenitor (CLP), Pre-NK, immature NK and Mature NK. (D) Quantification of tdTomato+ colonies at day 12 of reprogramming with different pools of candidate TFs. (E) Fluorescence Microscopy pictures of MEFs co-transduced with M2rTA (M2) and ETS1, NFIL3, T-BET, EOMES and ID2, 6 and 12 days after adding Dox. Number of tdTomato cells per colony between day 3 and day 12. Mean ± SEM of thirteen colonies is shown. (F) Quantification of tdTomato+ colonies per TF combination at day 12. MEFs were transduced with control M2rTA, co-transduced with ETS1, NFIL3, ID2, T-BET and EOMES or additional combinations where one transcription factor was individually removed from the 5 TF (top panel) or 4 TF (bottom panel) pool (Mean ± SD; n = 2; $p \leq 0.05$). (G) ETS1, NFIL3, T-BET and EOMES expression in several mouse tissues and cell types (GeneAtlas MOE430).

3.2 TENE combination induces global gene expression changes in MEFs

To characterize transcriptional profile of mouse iNK, single-cell mRNA-seq (10x genomics) was performed at multiple time-points (day 3, 6, and 12) after transduction with TENE combination. NCR1 positive cells from bone marrow and spleen were sorted as comparison with reprogrammed cells (Figure 8A and B). The transcriptional profile of lymphoid cells was also assessed, and cells were clustered in different cell types: ILCs, NK, NKP, and NKT (Figure 8C, right panel). Cellular groups were defined based on specific markers (Supplementary figure 1). Overall, the data show that TENE combination induces global gene expression changes in MEFs.

3.2.1 TENE combination drives reprogrammed cells from fibroblast state towards NK identity

Ectopic expression of Nfil3, Ets1, T-bet and Eomes results in alteration of normal genetic program in MEFs. By analyzing the expression program of fibroblast-associated genes, such as Col1a2, Lox, and Fbln2, we notice a downregulation on the expression of these genes (Figure 8D). These results suggest that fibroblast identity is being shut down opening space for other new genetic landscapes. Fibroblast gene turn-off enforced by TENE expression enable chances of NK reprogramming. Downregulation also appears to be stronger over time, as expression of fibroblast-associated genes decreases from day 3 to day 12 of reprogramming, suggesting that reprogrammed cells are gradually losing fibroblast identity.

TENE combination also promotes the expression of NK-related genes. TENE combination is responsible for increasing not only Ets1, Nfil3, T-bet, and Eomes endogenous expression, but also inducing the expression of all candidate TF at later time-points of reprogramming (Figure 8E). The expression of all TFs increases over time, as we see lower expression on reprogrammed cells at day 3 but notice a slight increase on day 6 and day 12. By inducing the expression of several TFs involved in NK development, TENE TFs can be seen as key regulators of NK transcriptional core (Figure 8E, top panel). Furthermore, Ets1, Nfil3, T-bet, and Eomes endogenous expression evidently increases in later time-points of reprogramming (day 12) (Figure 8E, bottom panel). Enforced expression of TENE combination promotes endogenous expression of TENE TFs, suggesting that these 4 TFs are responsible for altering the genetic program of reprogrammed cells.

After enforced expression of Ets1, Nfil3, T-bet, and Eomes, downregulation of fibroblasts-associated genes and higher expression of NK identity related genes, support

the idea that the combinatorial action of TENE TFs change global gene expression of MEFs and contribute to impose NK cell fate.

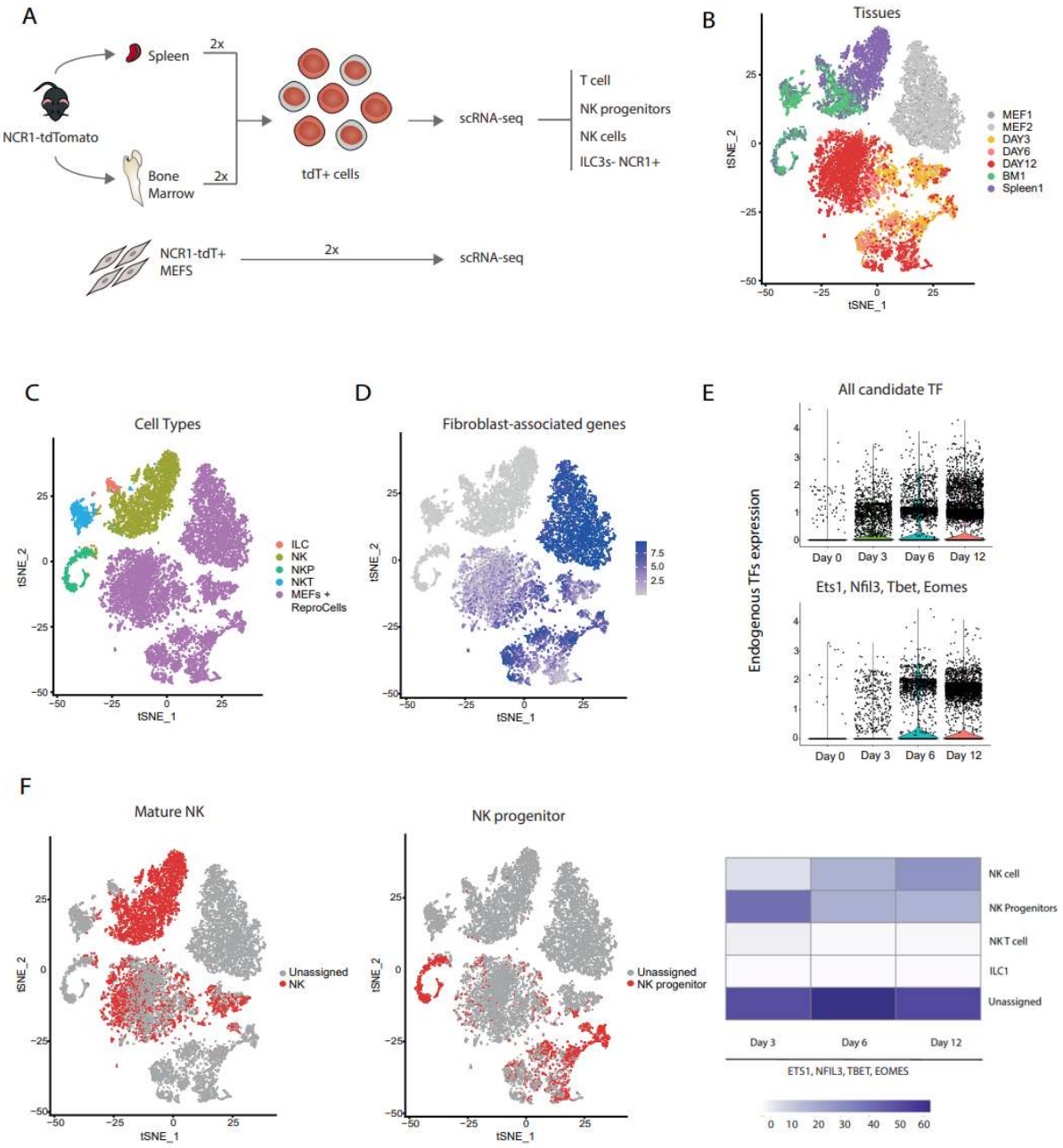


Figure 8 - Enforced expression of Ets1, Nfil3, T-bet and Eomes induce transcriptional NK profile. (A) Isolation of NCR1-tdTomato positive bone marrow and spleen cells and MEFs of 2 different mice for single-cell mRNA-seq analysis. (B) TSNE visualization of Mouse embryonic ¬fibroblasts (MEFs), NCR1-tdTomato positive MEFs at day 3, 6 and 12, bone marrow and spleen cells. (C) TSNE visualization of reprogrammed MEFs at day 12, NK, NKP, NKT and ILC. (D) Expression of ¬fibroblast-associated genes (Col1a2, Lox, Fbln2). (E) Plot quantification of endogenous expression of all candidate transcription factors (TFs) and 4 identified TFs (Ets1, Nfil3, T-bet, Eomes). (F) TSNE visualization of mature NK cell signature (left panel) and NK progenitor signature (center panel). Heat-map of Ets1, Nfil3, T-bet and Eomes expression in NK, NKP, NKT and ILC at day 3, 6 and 12 of reprogramming.

3.2.2 TENE combination induces NK mature and progenitor state

TENE combination induces NK state by controlling the expression of NK lineage specifying factors, inducing a global gene expression change in reprogrammed cells. In addition, our data suggests that these 4 TFs induce NKP signature at early time-points of reprogramming (day 3 and 6) (Figure 8F, center panel) similar to what we see with some of the cells assigned to bone marrow cluster. By analyzing the cluster in light green (Figure 8C) and BM cluster in green (Figure 8B) we identify an overlapping explained by the fact that NKP are mainly identified in BM, where NK development takes place. On the other hand, at day 12 after transduction is possible to identify a mature NK signature (Figure 8F, left panel). Clusters of mature NK overlap with spleen, where NK cells locate after maturation (Figure 8C). The affiliation with NKT cells and ILC1 was almost null (Figure 8F, right panel).

TENE combination is inducing progenitor signature at early times of reprogramming with shift towards mature NK signature at day 12, suggesting that NK cell reprogramming might be happening through an intermediate progenitor state.

3.3 Defined minimal TF network is conserved in humans

Ets1, Nfil3, T-bet, and Eomes expression is enriched in human NK cells (Figure 9A). To assess if the transcriptional network defined in the mouse system is conserved in humans, human embryonic fibroblasts (HEFs) were transduced with TENE combination present in two different lentiviral systems, Fuw-TetO and SFFV. Polycistronic and bicistronic constructs were also tested to further investigate reprogramming efficiency. Mature NK (CD56) and NK progenitor (CD34) markers were assessed. CD34 and CD56 expression was evaluated by flow cytometry at day 6 and day 12 after transduction (Figure 9B).

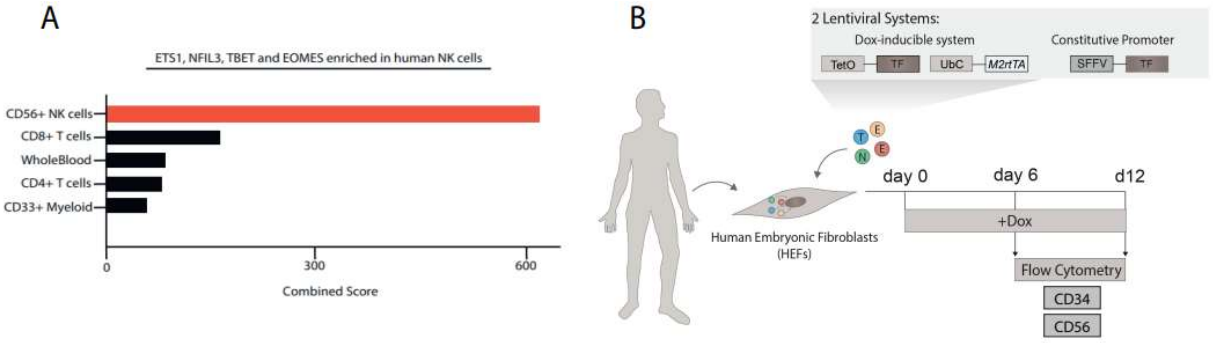
3.3.1 Constitutive expression of TENE induces CD34 expression in HEFs

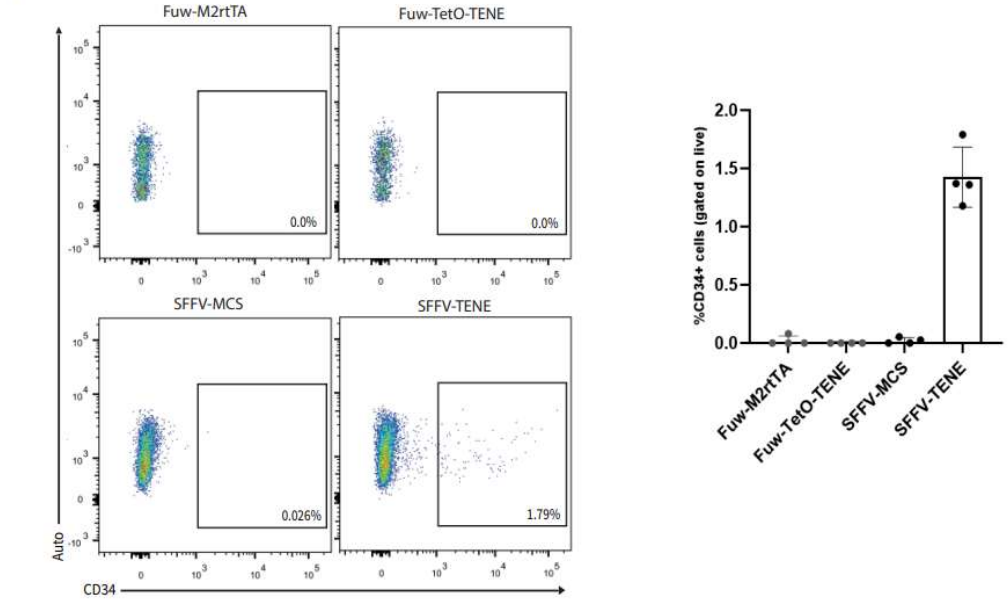
HEFs were transduced with lentiviral particles harboring the 4 TFs identified in the mouse system – Ets1, Nfil3, T-bet, and Eomes. To test reprogramming of human fibroblasts towards NK identity, Fuw-TetO and SFFV vectors were used. TENE TFs were individually cloned both in Fuw-TetO and SFFV (Fuw-TetO-TENE and SFFV-TENE conditions, respectively). Polycistronic constructs were also prepared in Fuw-TetO to assure the distribution of 4 TFs in the reprogrammed cells. Two different TF positions were tested:

Fuw-TetO-p(ENET) (Ets1, Nfil3, Eomes, T-bet) and Fuw-TetO-p(NEET) (Nfil3, Ets1, Eomes, T-bet). For SFFV, instead of polycistronics, bicistronic constructs were pooled together. Two different TF positions were also tested: SFFV-b(EN)+b(ET) (Ets1, Nfil3 + Eomes, T-bet) and SFFV-b(NE)+b(ET) (Nfil3, Ets1 + Eomes, T-bet). CD34 expression was assessed by flow cytometry at day 12 of reprogramming.

CD34 expression was not detected in Fuw-TetO-TENE (Figure 9C, top plots) or polycistronic conditions (Supplementary Figure 2), indicating that although the dox-inducible system was an efficient lentiviral vector in mouse fibroblasts, it was ineffective to enforce TF expression in HEFs. Alternatively, Rosa et al. has recently demonstrated that SFFV promoter can be used to reprogram human fibroblasts into DCs with increased efficiencies comparing to FUW-TetO system (68). Therefore, we tested SFFV promoter to enforce TENE expression in HEFs. CD34 expression was detectable in SFFV-TENE, as we observe approximately 1,5% of live cells expressing CD34 (Figure 9C, bottom plots). After a significant increase on CD34 expression compared with control sample with empty vector (SFFV-MCS) and Fuw-TetO vector, SFFV was used in the following experiments. Furthermore, this data suggest that TENE combination is conserved in human fibroblasts, as we see NK progenitor markers expression as observed in MEFs. We consider this to be the first indication that TENE is inducing direct alterations in the genetic program of HEFs, reprogramming these cells towards NK identity. As for bicistronic conditions, CD34 expression of SFFV-b(EN)+b(ET) is similar to SFFV-TENE (Figure 9D). However, SFFV-b(NE)+b(ET) marker expression is significantly lower than the other two conditions, with only 0,5% of average expression between samples. These results indicate that higher expression of Ets1 might be needed compared to Nfil3 to start genetic regulation of CD34 marker, as we see higher presence of CD34 when Ets1 appears in the first position of bicistronic order. Further investigation is needed to confirm Ets1 and other TENE TFs involvement in CD34 expression and NK development.

Overall, Fuw-TetO system is ineffective to stimulate CD34 expression in HEFs and TENE combination enforced expression with SFFV successfully induce NK progenitor marker expression. Bicistronic conditions in SFFV do not add a significant increase in reprogramming efficiency.


A
ETS1, NFIL3, TBET and EOMES enriched in human NK cells
CD56+ NK cells
CD8+ T cells
WholeBlood
CD4+ T cells
CD33+ Myeloid
0
300
600
Combined Score
B
2 Lentiviral Systems:
Dox-inducible system
Constitutive Promoter
TetO
TF
UbC
M2rtTA
SFFV
TF
Human Embryonic Fibroblasts (HEFs)
day 0
day 6
d12
+Dox
Flow Cytometry
CD34
CD56


C
Fuw-M2rtTA
Fuw-TetO-TENE
SFFV-MCS
SFFV-TENE
0.0%
0.0%
0.026%
1.79%
Auto
CD34
%CD34+ cells (gated on live)
2.0
1.5
1.0
0.5
0.0
Fuw-M2rtTA
Fuw-TetO-TENE
SFFV-MCS
SFFV-TENE

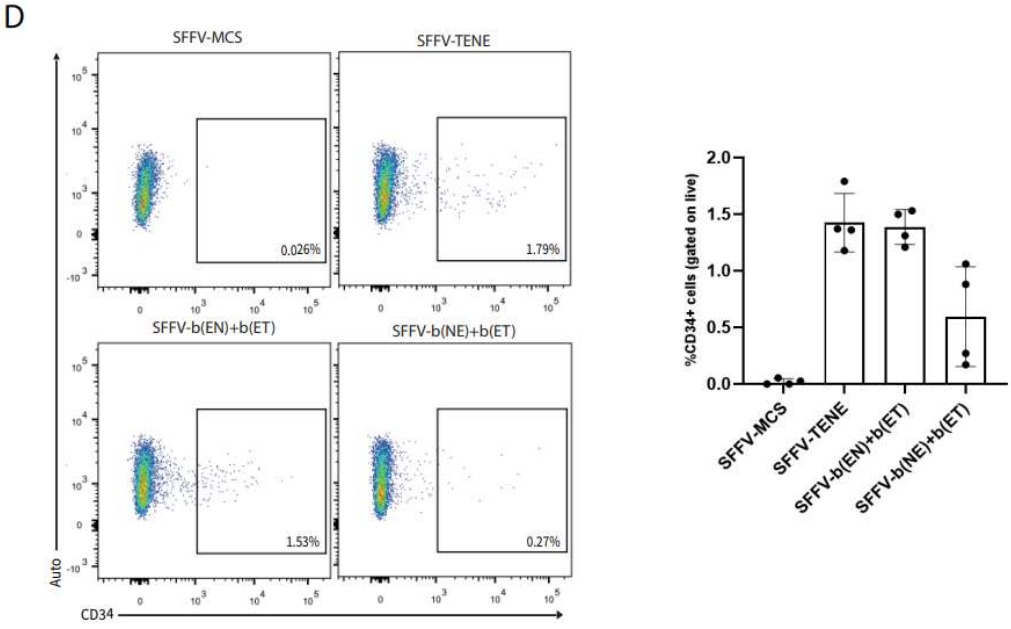

D
SFFV-MCS
SFFV-TENE
SFFV-b(EN)+b(ET)
SFFV-b(NE)+b(ET)
0.026%
1.79%
1.53%
0.27%
Auto
CD34
%CD34+ cells (gated on live)
2.0
1.5
1.0
0.5
0.0
SFFV-MCS
SFFV-TENE
SFFV-b(EN)+b(ET)
SFFV-b(NE)+b(ET)

Figure 9 - Enforced expression of Ets1, Nfil3, T-bet and Eomes induce CD34 expression in HEFs. (A) Ets1, Nfil3, T-bet, and Eomes expression in human tissues (GeneAtlas Human, BioGPS). (B) Enforced expression of TENE combination in HEFs using a dox-inducible vector (Fuw-TetO) and constitutive promoter (SFFV). Assess CD34 and CD56 expression at day 6 and day 12 after transduction by flow cytometry. (C) CD34 expression gated on live cells in HEFs transduced with Ets1, Nfil3, T-bet, and Eomes, individually clone in Fuw-TetO and SFFV, and controls Fuw-M2rtTA and SFFV-MCS. Percentage of CD34 positive cells gated on live cells. (D). CD34 expression gated on live cells in HEFs transduced with control SFFV-MCS, SFFV-TENE and bicistronic constructs in SFFV according to TF position: SFFV-b(EN)+b(ET) (Ets1 and Nfil3 + Eomes and T-bet) and SFFV-b(NE)+b(ET) (Nfil3 and Ets1 + Eomes and T-bet). Percentage of CD34 positive cells gated on live cells.

3.3.2 TENE expression increases over time

SFFV-TENE induces CD34 expression at day 12 after transduction. To understand if marker expression can be detected earlier, marker expression was assessed 6 days after transduction with Ets1, Nfil3, T-bet, and Eomes. In fact, CD34 expression was observed at day 6 of reprogramming. Approximately, 0.75% of live cells were positive for marker expression compared with the SFFV-MCS control. This expression increased over time to 2.0% of $CD34^{+}$ cells at day 12 (Figure 10). These data suggest that TENE enforced expression is resulting in a gradual improvement in the expression of the progenitor marker. It is possible that CD34 positive cells are expanding in culture promoting higher detection of NKP marker. Alternatively, NK reprogramming might be asynchronous in human fibroblasts and therefore resulting higher number of reprogrammed cells in later timepoints. To understand if TENE is driving CD34 positive cells to a mature stage or if these cells are locked in a progenitor stage, we need to further investigate.

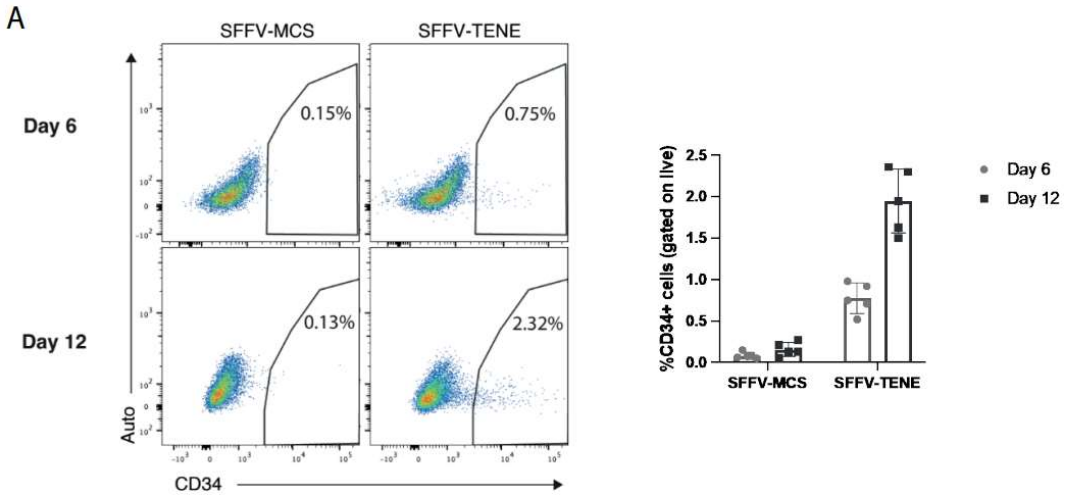


Figure 10 - TENE-induced CD34 expression in HEFs higher at later time point. (A) CD34 expression gated on live cells in HEFs transduced with SFFV-MCS control and SFFV-TENE at day 6 and day 12 (left). Percentage of CD34 positive cells gated on live cells (right).

3.3.3 TENE expression induces mature NK marker in HEFs

In order to understand if TENE combination is capable of inducing a mature state in human fibroblasts, CD56 expression was assessed 6 days after transduction. CD56 is expressed by mature NK cells (1). We could detect a significant increase in the number of CD56 positive cells in SFFV-TENE condition compared to the SFFV-MCS control (Figure 11A). Around 1,50% of live cells express CD56 in SFFV-TENE, which lead us to believe that TENE combination is promoting transcriptional changes in HEFs towards NK fate. Moreover, CD34 presence was assessed and once again marker expression was detected. However, $CD34^{+}CD56^{+}$ double positive cells were not present as we could expect. While CD34 is a progenitor marker that is not expressed in mature NK cells, CD56 expression is a strong indication of NK mature state. Therefore, combined presence of the two markers would be difficult. These data corroborate mouse results (Figure 5D) where two different signatures, NKP and mature NK, were detected at different points of reprogramming. Collectively, results suggest that TENE TFs combinatorial activity that induce NK cell identity is conserved in human system. Even though, we detect low percentage of positive cells, this can be seen as good evidence that NK reprogramming is being induced in HEFs. Strategies to improve reprogramming efficiency need additional research.

3.3.4 Cytokine cocktail improves CD34 expression in HEFs

To test whether cytokines would improve reprogramming efficiency, DMEM complete media was supplemented with SCF, IL-3, Flt3L, IL-7, IL-15. This cytokine cocktail is used to induce NK cell state from iPSCs (48). CD34 and CD56 expression was assessed by flow cytometry 12 days after transduction with TENE combination. Cytokine treatment induced an increase in CD34 expression (2,50% of CD34 positive cells) when compared with non-cytokine condition (±1,2%). CD34 expression is enhanced by cytokine cocktail, that combined with enforced expression of Ets1, Nfil3, T-bet, and Eomes induce NK progenitor state (Figure 11). CD56 expression was not enhanced by cytokine activity (Supplementary figure 3), suggesting that combinatorial action of these cytokines is not sufficient to push NK cell maturation. Even though specific role of cytokines in NK development remains unclear, research shows that SCF, IL-3, Flt3L, IL-7 and IL-15 synergized activity is specifically associated with lymphocytes development and proliferation (46,70–72). This is corroborated by the results where we observe increased CD34 expression in cytokine enriched samples. Conversely, IL-15 is also associated with later NK maturation (71), but in this case even with IL-15 presence the cytokine cocktail is insufficient to help NK final

development by promoting CD56 expression. One possible explanation is that by promoting expansion of CD34 positive cells, cytokine-induced processes are removing the space for later maturation steps.

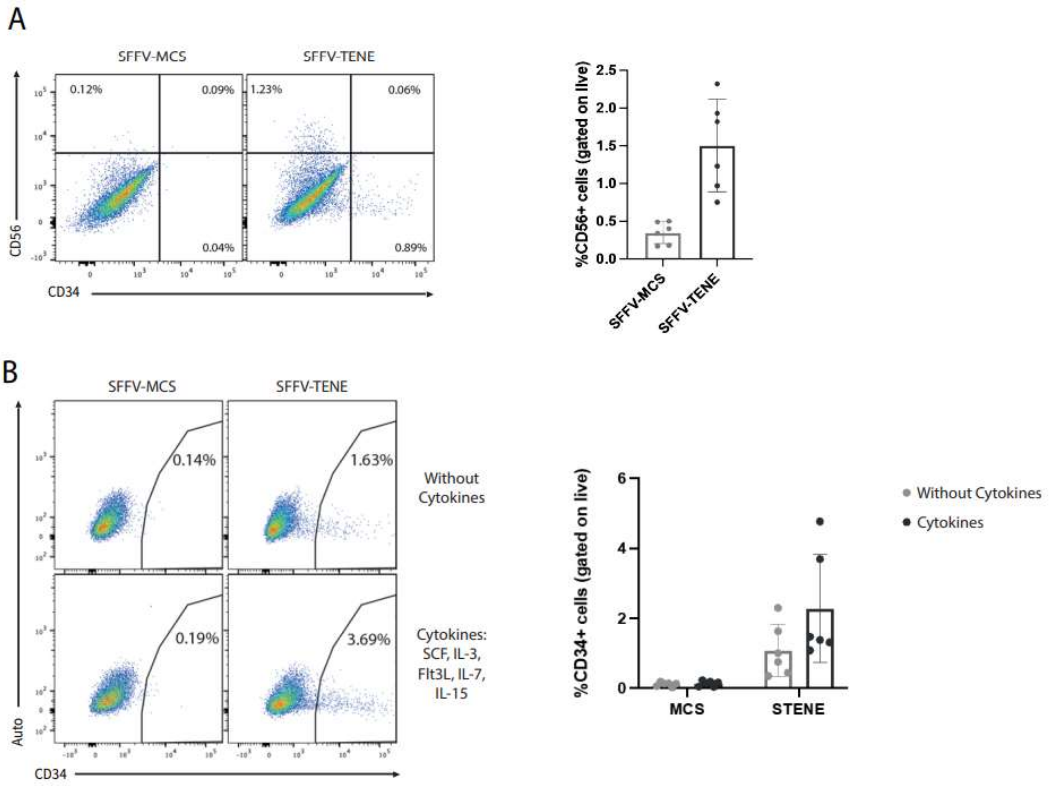
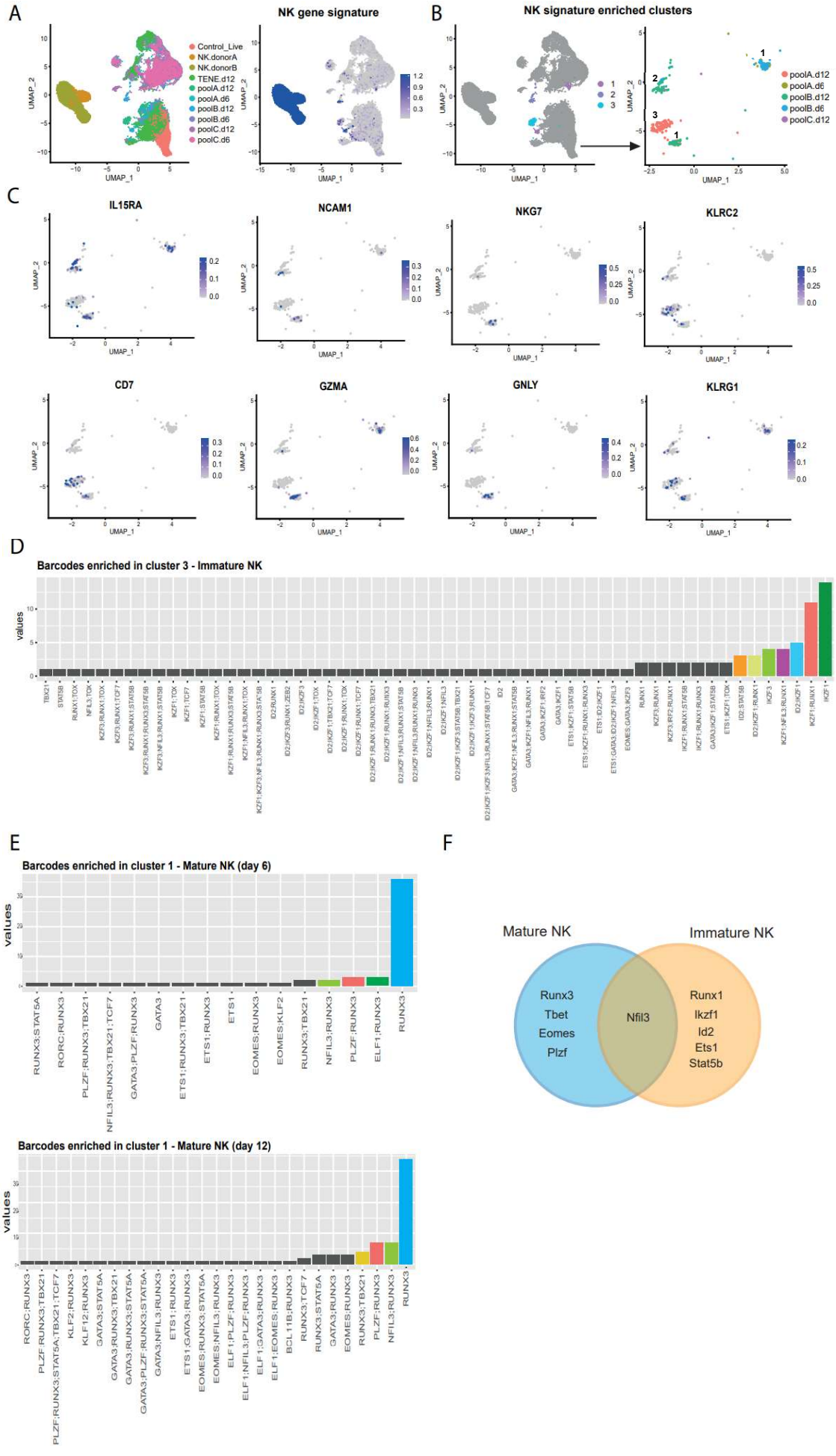


Figure 11 - Enforced expression of Ets1, Nfil3, T-bet and Eomes induce CD56 and CD34 expression in HEFs. Cytokine cocktail increase CD34 expression. (A) CD34 and CD56 expression gated on live cells in HEFs transduced with SFFV-MCS control and SFFV-TENE (left). Percentage of CD56 positive cells gated on live cells (right). (B) CD34 expression gated on live cells in HEFs transduced with SFFV-MCS control and SFFV-TENE treated with SCF, IL-3, Flt3L, IL-7, and IL-15 or without cytokines (right). Percentage of CD34 positive cells gated on live cells (left).

3.4 Barcoding strategy at single-cell level suggest TFs involvement in different NK developmental stages

To better understand NK transcriptional network, we transduced HEFs with Pooled TFs (Pool A, B, and C) specifically barcoded (Supplementary Table 2). Through single-cell mRNA-seq is possible to associate a specific cluster with different TFs expression by recognizing what barcodes are enriched in those clusters (73). Two time-points of reprogramming were assessed for each condition, day 6 and day 12 (Figure 12A, left panel). TENE-transduced HEFs were also profiled by scRNA-seq to test whether TENE was sufficient to induce NK transcriptional program in human fibroblasts. $CD56^{+}$ NKs isolated

from PBMCs of two donors were used as control to define NK gene signature (Figure 12A, right panel). NK gene signature was defined by the expression of specific genes: IL15RA, KLRG1, KLRC2, KLRC3, NCAM1, NKG7, GNLY, GZMA, GZMB, CD7, CCL5, FGFBP2, SERPINB9. Three NK signature enriched clusters were determined (Figure 12B, left panel). Next, we defined which pool of TFs was enriched in each NK signature enriched cluster (Figure 12B, right panel). While cluster 3 is formed by cells from pool A at day 12, cluster 1 is mostly composed by pool B at day 6 and 12. Interestingly, although the two timepoints of pool B are classified as cluster 1, they are segregated by timepoint in the UMAP. These results indicate that despite pool B day 6 and day 12 share NK-enriched genes, the transcriptional profile is still different. Note that Pool C, at day 6 is not associated with NK signature. The assignment with NK signature is confirmed by analyzing which NK genes are expressed for each cluster (Figure 12C). In fact, the results suggest that cluster 3 could be associated to immature NK cell signature, as see higher expression on CD7. CD7 is expressed by precursors of NK cells, suggesting that these could be progenitor or immature cell. On the other hand, data analysis indicates that cluster 1 could be associated to mature NK cell signature, as we see higher expression on GZMA (encode: Granzyme A), GNLY (encode: Granulysin), NKG7, and NCAM1 (encode: CD56) genes. Cells from cluster 1 present enrichment of cytotoxic genes and maturation marker for NK cells, leading to believe that these cells are close to mature NK profile. Correlating the data of Pooled TFs with defined clusters, we found that some TFs are enriched. For instance, in cluster 3 Ikzf1 and Runx1 are present in higher percentage (Figure 12D). Runx1 is mainly related with NK proliferation, while Ikzf1 deficient mice usually present impaired development of lymphocytes (74,75). These TFs are involved in normal NK development and, here, seem to contribute for immature signature maintenance. On the other hand, Runx3 is quite enriched in cluster 1 cells suggesting that this factor could be associated with mature NK cells (Figure 12E). Both at day 6 and day 12 of reprogramming we see Runx3 enriched, where we also saw increased expression of granzyme A, CD56, and granulysin. These results suggests that Runx3 is possibly implicated in late maturation steps contributing for NK functionality. As mentioned, other TFs are involved in NK development and some of them detected in our clusters (Figure 12F). T-bet and Eomes were present in cluster 1, confirming their involvement in NK maturation stage and corroborating the importance on our TENE combination. Plzf is also associated with these cluster. Additionally, Id2 and Ets1 are associated with NK cell commitment and take part in progenitor cluster (cluster 3). Stat5b is also implicated here. Finally, Nfil3 appears to be expressed in both clusters what goes in line with Nfil3 role in several developmental stages.


A
NK gene signature
Control_Live
NK.donorA
NK.donorB
TENE.d12
poolA.d12
poolA.d6
poolB.d12
poolB.d6
poolC.d12
poolC.d6
UMAP_1
UMAP_2
B
NK signature enriched clusters
poolA.d12
poolA.d6
poolB.d12
poolB.d6
poolC.d12
C
IL15RA
NCAM1
NKG7
KLRC2
CD7
GZMA
GNLY
KLRG1
D
Barcodes enriched in cluster 3 - Immature NK
values
E
Barcodes enriched in cluster 1 - Mature NK (day 6)
RUNX3;STAT5A
RORC;RUNX3
PLZF;RUNX3;TBX21
NFIL3;RUNX3;TBX21;TCF7
GATA3;PLZF;RUNX3
GATA3
ETS1;RUNX3;TBX21
ETS1;RUNX3
ETS1
EOMES;RUNX3
EOMES;KLF2
RUNX3;TBX21
NFIL3;RUNX3
PLZF;RUNX3
ELF1;RUNX3
RUNX3
Barcodes enriched in cluster 1 - Mature NK (day 12)
RORC;RUNX3
PLZF;RUNX3;TBX21
PLZF;RUNX3;STAT5A;TBX21;TCF7
KLF2;RUNX3
KLF12;RUNX3
GATA3;STAT5A
GATA3;RUNX3;TBX21
GATA3;RUNX3;STAT5A
GATA3;PLZF;RUNX3;STAT5A
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EOMES;RUNX3;STAT5A
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ELF1;PLZF;RUNX3
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ELF1;EOMES;RUNX3
BCL11B;RUNX3
RUNX3;TCF7
RUNX3;STAT5A
GATA3;RUNX3
EOMES;RUNX3
RUNX3;TBX21
PLZF;RUNX3
NFIL3;RUNX3
RUNX3
F
Mature NK
Immature NK
Runx3
Tbet
Eomes
Plzf
Nfil3
Runx1
Ikzf1
Id2
Ets1
Stat5b

Figure 12 - Single-cell mRNA-seq analysis of reprogrammed cells transduced with barcoded TFs. (A) UMAP visualization of all clusters (left panel) including PBMCs $CD56^{+}$ NK cells from 2 donors (NK donors), day 12 transduced HEFs with TENE combination, day 6 and day 12 transduced with Pool A, B, and C TFs. UMAP visualization of combined expression of NK signature genes (IL15RA, KLRG1, KLRC2, KLRC3, NCAM1, NKG7, GNLY, GZMA, GZMB, CD7, CCL5, FGFBP2, SERPINB9). (B) UMAP visualization of NK signature enriched clusters (1, 2, and 3). Simplified UMAP visualization of NK signature enriched clusters associated with TFs Pools and timepoint. (C) UMAP visualization of NK signature enriched genes. (D) Graph most enriched barcodes in cluster 3 (immature NK). (E) Graphic visualization of barcodes enriched in cluster 1 (mNK) at day 6 and day 12. (F) Venn diagram model of enriched TFs related with mature NK and immature NK signatures.

Chapter 4

4 Discussion

NK cells are important immune regulators that control cancer development and viral infections. Due to their cytotoxic abilities and low toxic effect against the host, NK cells have been explored to investigate several cancer immunotherapy strategies. Unfortunately, current sources of NKs still have limitations that challenge the use of these cells in immunotherapy. Cell fate reprogramming studies show that is possible to generate several types of cells from a completely different somatic cell by regulating specific transcription factors. Here, we present a novel approach to generate NK cells by direct reprogramming, which provide a better understanding on lymphoid transcriptional network and contributes to discover a new platform to generate patient-specific NK cells.

To define TF combinations to induce NK cell identity, we identified a pool of 19 candidate TFs through global gene expression and literature mining. From the initial list of candidate TFs, 4 TFs – Ets1, Nfil3, T-bet, and Eomes - were found to activate the expression of NCR1 NK-specific reporter in mouse fibroblasts. NCR1 is specifically expressed in mouse NK lineage, both in mature NK and NK progenitors. Even though several TFs are associated with NK lineage, their role in specific development stages is not entirely understood. Ets1 and Nfil3 are commonly associated with the regulation of different genes contributing to open chromatin landscapes for the binding of specific factors. For instance, it is believed that the two TFs modulate the expression of Id2 important on NK commitment. Higher number of tdT+ colonies when removing Id2, could be justified by the overexpression of Nfil3 and Ets1, which activate Id2 expression (17,22,23). Together with Nfil3 and Ets1, T-bet and Eomes also help NCR1 activation in MEFs corroborating their involvement in NK development. Our results also showed that all 4 TFs are needed for efficient NCR1 expression. Based on expression analysis across several mouse tissues, TENE TFs are associated with NKs. However, the question on how these TFs interact to regulate the genetic program that induce NK cell fate is still not answered.

Through single-cell mRNA-sequencing analysis, we found that combinatorial activity of TENE TFs induced a global gene expression change imposing NK cell identity on MEFs. Downregulation of fibroblast-associated genes is the first indication that TENE is altering the normal epigenetic landscapes of fibroblasts. Releasing the selective pressure of defining epigenetic regulators that induce specific cell fate is an important step to reprogram another cell type. For instance, reducing the expression of fibroblast specific genes by enforced expression of Myocd, Mef2C, and Gata-6 facilitates direct reprogramming of smooth muscle cells (76). Even though inducing lower expression of fibroblast genes is not

enough to change cell identity, it facilitates the induction of a new cell gene expression program.

Ectopic expression of TENE TFs modulates expression of NK transcriptional network in reprogrammed cells. By analyzing the levels of candidate TFs in TENE-transduced cells, we detect a slight increase compared to untransduced mouse fibroblasts. Both bone marrow and spleen NK cells express candidate TFs, confirming their association with NK development. Endogenous expression of Ets1, Nfil3, T-bet, and Eomes is also enhanced in reprogrammed cells, as we see similar expression levels of these 4 TFs on bona fide NKs and reprogrammed cells at day 12. Inducing their endogenous expression, indicates that NK genetic program is being initiated in mouse fibroblasts. The ability to upregulate specific factors is an important step to convert a new cellular type. Overall, TENE TFs impose NK cell identity by upregulating NK-related TFs.

Ets1, Nfil3, T-bet, and Eomes are also enriched in human NK cells. Even tough, it is believed that NK development in mouse is similar to human system, the conservation of lineage-specifying TFs is still to be elucidated. We overexpressed TENE combination in human fibroblasts and assessed NK cell identity through the analysis of specific markers. CD34 expression was induced by TENE combination in HEFs. It is known that CD34 expression is related with NK precursor cells, as we see marker expression in several NK developmental stages in human (77). Because HSC and CLPs also express this marker, it is possible that we are inducing an heterogenous population that contains cells from different steps of lymphoid differentiation. However, reprogramming mediated by TENE in mouse fibroblasts resulted in the induction of NK lineage signature, suggesting that the same is happening in human. Moreover, CD56 expression was present after ectopic expression of TENE TFs. NK cells usually express this marker in late steps of NK development (77). The data suggest that TENE combination not only induces precursor markers, but also promotes the NK mature signature. The results in human corroborate what was found in mouse, where at day 12 NK mature state was assigned to reprogrammed cells. Consistently, $CD34^{+}CD56^{+}$ double positive cells are not identified by exogenous expression of TENE TFs. As CD34 expression is lost after NK maturation and CD56 is present from this step forward, presence of both markers is less likely. In mouse, we also see NK mature signature and NKP signature at different reprogramming time-points, suggesting that TENE combination is inducing several development stages. According to the present data, it is possible that NK reprogramming is occurring through an intermediate progenitor state arguing that progenitor cells are converted in mature NKs in the last days of reprogramming. Alternatively, TENE TFs can induce two separate populations, $CD34^{+}$ (precursors) and $CD56^{+}$ (mature). If that is the case, it could be that association of three or

less TFs inside of the initial TENE combination induce separate NK lineage stages. Taken together, these results indicate that the minimal TF combination identified in the mouse is conserved in the human.

Although TENE combination imposes NK cell program in fibroblasts, markers expression is still low. Reprogramming efficiency is determined by several factors that need to be optimized, including the delivery system of the TFs. Our results show that two lentiviral vectors promote different responses in mouse and human fibroblasts. Although dox-inducible vector can be used to induce global gene expression changes in mouse fibroblasts, NK cell identity could only be replicated in human using a constitutive vector. Interestingly, the reprogramming of DCs was initially done with Fuw-TetO system but the substitution of the promotor for SFFV resulted in improved reprogramming efficiency (68). We hypothesize that Fuw-TetO system is not so efficient to reprogram human fibroblasts due to doxycycline-associated cytotoxicity. Otherwise, SFFV can induce higher levels of TF expression than Fuw-TetO or Fuw-TetO system can be involved in gene silencing. Additionally, culture conditions of reprogrammed cells can also be optimized. It has been demonstrated that specific cytokines can induce lineage conversion. For instance, synergize effect between instructor TFs and inflammatory cytokines was observed in DCs reprogramming (68). In other matter, cytokines can also be used to facilitate differentiation of immune cells. iPSCs-derived NK cells are differentiated with a cytokine cocktail containing IL-15, IL-7, IL-3, SCF, and Flt3L (48). Using the same combination, we assess reprogramming efficiency in TENE-transduced fibroblasts. In fact, CD34 expression was increased in HEFs cultured with DMEM supplemented with cytokines, suggesting that iPSCs cytokine cocktail can facilitate the induction of NK cell identity by direct reprogramming. Conversely, we did not observe an increase in CD56 expression. It is possible that cytokines are actively inducing CD34 positive cells expansion, sustaining a higher percentage of this population and ceasing NK acquisition of mature markers. Even though the involvement of cytokines in NK development is not entirely understood, we know that these chemotactic molecules present an important role not only modulating immune responses but also supporting immune cells differentiation. Several studies point that synergized activity of Flt3L, IL-3, and IL-7 help proliferation of hematopoietic progenitor cells (HPCs) and their differentiation into CLPs (71,78). Other study suggest that Flt3L induce the generation of a $CD34^{+}CD122^{+}$ NK cell intermediate that is responsive to IL-15 and originate NKP (70). Finally, combination of SCF with IL-15 also facilitates earlier cell-cycle entry what stimulates NK cell proliferation (72). Taken together, the data is in agreement with these reports, as cytokine cocktail stimulates CD34 positive cells expansion and potentially help reprogramming and development of NK cells. On the other hand, IL-15 is

specifically crucial for NK cell commitment and defines late steps of NK maturation (71), but in this case, in combination with the other cytokines IL-15 activity possibly enhances cell expansion, rather than contributing for the development of mature NKs. It would be interesting to induce mature NK signature using IL-15 combined with TENE TFs, isolated from the other cytokines. In alternative, the addition of all cytokines at early time-points of reprogramming replaced with culture media containing only IL-15 in the last days of reprogramming could also facilitate the understanding of IL-15 activity in NK cell fate. Overall, the optimization of culture conditions is important for efficient NK reprogramming being that the environment were cells are differentiated affects desired outcome.

Ensuring that all candidate TFs are correctly delivery to the cells can also secure better reprogramming efficiency. For that reason, we tested polycistronic and bicistronic constructs harboring Ets1, Nfil3, T-bet, and Eomes. However, these constructs did not increase NK reprogramming efficiency. Usually, direct cell reprogramming is enhanced by promoting better TF distribution provided by polycistronics (67,68). The low efficiency of our constructs could be explained by the TF order utilized in polycistronic conception. Even though Nfil3 and Ets1 are responsible for promoting main epigenetic changes facilitating DNA binding for other TFs, could be that Eomes and T-bet help stimulate marker expression by activating specific pathways. In addition, the combined length of the 4 TFs could also challenge the success of DNA integration. Together our 4 TFs have ~7000 pair bases (pb) what represents a long DNA product. As reference, Pu.1, Irf8, Batf3 construct used to reprogram human fibroblasts in antigen presenting DCs only harbors ~2400pb (67).

To further improve NK reprogramming efficiency and better understand the TF codes set in motion during NK lineage commitment, we applied a barcoding strategy at single-cell level. This approach provided information on TFs that are associated with specific NK cell signatures. NK signatures were defined based on expression of specific NK-related genes associated with mature or immature stage. In the cluster associated with immature NK signature characterized by higher CD7 expression, Runx1 and Ikzf1 were the most enriched TFs. However, Ikzf1 is not related with late NK maturation, this TF is crucial for normal CLPs development, directly affecting the development of NK lineage stages (75). In addition, Runx1 is an important regulator of NK proliferation by controlling the cell-cycle program (74). Other TFs are also identified in some of the cells of this cluster, namely, Id2, Ets1 and Stat5b. Specifically, this corroborates that Id2 and Ets1 are important regulators of NK cell commitment in early progenitors (20,24). Furthermore, is possible to detect a cluster with mature signature highlighted by the expression of specific maturation genes, such as GZMA, GNLY, and NCAM1. Runx3 is clearly enriched in the cells present in this cluster, raising questions about the importance of this TF in NK maturation. In fact, Runx3 loss is

associated with impaired IL-15 mediated program (79). IL-15-mediated response is an important maturation step acquired by NK cells during their development, so that NK cells can be activated. Therefore, Runx3 is a key TF for NK cell functionality. All-inclusive, we detect a pattern on the regulatory activity of Runx family transcription factors, as we see Runx1 implicated in immature signature, while Runx3 is enriched in cells associated to mature state. Interestingly, activity of T cell is also regulated by these TFs. For instance, Runx1 participate in T cell receptor acquisition and help define T cell fate. Later, Runx1 is downregulated and Runx3 overexpression induce T cell cytotoxicity (80). The balance between Runx TFs drives T cell development from a progenitor stage to a maturation stage. Developmental implication of Runx family in NK cells is still to be clarified, however, could be that a similar mechanism can regulate NK development. Nevertheless, a low number of cells was associated with NK cell signature in our analysis, thus more studies are needed to validate these results.

Overall, our work shows that a minimal combination of 4 transcription factors – Ets1, Nfil3, T-bet, Eomes – is sufficient and required to induce NK-specific reporter in MEFs. TENE combination promotes global gene expression changes that push mouse fibroblasts towards NK cell identity. We also provide evidence that TENE combinatorial activity induces NKP and mature NK signatures in mouse fibroblasts and that the lineage-specifying mechanisms are conserved in human. In addition, it was demonstrated that the combination with cytokines enhances the expression of NKP marker. Finally, we provide proof-of-concept that barcoding strategies at single-cell level can be employed to identify specific TFs that are associated with different NK cell signatures. This project contributes to understand transcriptional regulation of NK lineage and anticipate the first proofs on generation of NK cells applying direct cell reprogramming in cancer immunotherapy.

References:

1. Renoux VM, Zriwil A, Peitzsch C, Michaëlsson J, Friberg D, Soneji S, et al. Identification of a Human Natural Killer Cell Lineage-Restricted Progenitor in Fetal and Adult Tissues. Immunity. 2015 Aug;43(2):394–407.

2. Grégoire C, Chasson L, Luci C, Tomasello E, Geissmann F, Vivier E, et al. The trafficking of natural killer cells. Immunol Rev. 2007 Dec;220(1):169–82.

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

4. Paul S, Lal G. The Molecular Mechanism of Natural Killer Cells Function and Its Importance in Cancer Immunotherapy. Front Immunol. 2017 Sep 13;8:1124.

5. Vitale M, Chiesa MD, Carlomagno S, Pende D, Aricò M, Moretta L, et al. NK-dependent DC maturation is mediated by TNFα and IFNγ released upon engagement of the NKp30 triggering receptor. Blood. 2005 Jul 15;106(2):566–71.

6. Schuster IS, Coudert JD, Andoniou CE, Degli-Esposti MA. "Natural Regulators": NK Cells as Modulators of T Cell Immunity. Front Immunol [Internet]. 2016 Jun 14 [cited 2022 Jul 18];7. Available from: http://journal.frontiersin.org/Article/10.3389/fimmu.2016.00235/abstract

7. 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 Feb;172(5):1022-1037.e14.

8. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol. 2001 Nov;22(11):633–40.

9. Vosshenrich CAJ, García-Ojeda ME, Samson-Villéger SI, Pasqualetto V, Enault L, Goff ORL, et al. A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. Nat Immunol. 2006 Nov 1;7(11):1217–24.

10. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate Lymphoid Cells: 10 Years On. Cell. 2018 Aug;174(5):1054–66.

11. Stehle C, Rückert T, Fiancette R, Gajdasik DW, Willis C, Ulbricht C, et al. T-bet and RORα control lymph node formation by regulating embryonic innate lymphoid cell differentiation. Nat Immunol. 2021 Oct;22(10):1231–44.

12. Yang Q, Bhandoola A. The development of adult innate lymphoid cells. Curr Opin Immunol. 2016 Apr;39:114–20.

13. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate Lymphoid Cells: 10 Years On. Cell. 2018 Aug;174(5):1054–66.

14. Constantinides MG, McDonald BD, Verhoef PA, Bendelac A. A committed precursor to innate lymphoid cells. Nature. 2014 Apr 17;508(7496):397–401.

15. Walker JA, Clark PA, Crisp A, Barlow JL, Szeto A, Ferreira ACF, et al. Polychromic Reporter Mice Reveal Unappreciated Innate Lymphoid Cell Progenitor Heterogeneity and Elusive ILC3 Progenitors in Bone Marrow. Immunity. 2019 Jul;51(1):104-118.e7.

16. Lim AI, Li Y, Lopez-Lastra S, Stadhouders R, Paul F, Casrouge A, et al. Systemic Human ILC Precursors Provide a Substrate for Tissue ILC Differentiation. Cell. 2017 Mar;168(6):1086-1100.e10.

17. Male V, Nisoli I, Kostrzewski T, Allan DSJ, Carlyle JR, Lord GM, et al. The transcription factor E4bp4/Nfil3 controls commitment to the NK lineage and directly regulates Eomes and Id2 expression. J Exp Med. 2014 Apr 7;211(4):635–42.

18. Seillet C, Huntington ND, Gangatirkar P, Axelsson E, Minnich M, Brady HJM, et al. Differential Requirement for Nfil3 during NK Cell Development. J Immunol. 2014 Mar 15;192(6):2667–76.

19. Lacorazza HD, Miyazaki Y, Di Cristofano A, Deblasio A, Hedvat C, Zhang J, et al. The ETS Protein MEF Plays a Critical Role in Perforin Gene Expression and the Development of Natural Killer and NK-T Cells. Immunity. 2002 Oct;17(4):437–49.

20. 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.

21. 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.

22. Kamizono S, Duncan GS, Seidel MG, Morimoto A, Hamada K, Grosveld G, et al. Nfil3/E4bp4 is required for the development and maturation of NK cells in vivo. J Exp Med. 2009 Dec 21;206(13):2977–86.

23. Gascoyne DM, Long E, Veiga-Fernandes H, de Boer J, Williams O, Seddon B, et al. The basic leucine zipper transcription factor E4BP4 is essential for natural killer cell development. Nat Immunol. 2009 Oct;10(10):1118–24.

24. Sun XH. Id Proteins Idl and Id2 Selectively Inhibit DNA Binding by One Class of Helix-Loop-Helix Proteins. MOL CELL BIOL. :9.

25. Yokota Y, Mansouri A, Mori S, Sugawara S, Adachi S, Nishikawa SI, et al. Development of peripheral lymphoid organs and natural killer cells depends on the helix–loop–helix inhibitor Id2. Nature. 1999 Feb;397(6721):702–6.

26. Das R, Sant'Angelo DB, Nichols KE. Transcriptional control of invariant NKT cell development: Regulation of NKT cell differentiation. Immunol Rev. 2010 Nov;238(1):195–215.

27. Geiger TL, Sun JC. Development and maturation of natural killer cells. Curr Opin Immunol. 2016 Apr;39:82–9.

28. Abel AM, Yang C, Thakar MS, Malarkannan S. Natural Killer Cells: Development, Maturation, and Clinical Utilization. Front Immunol. 2018 Aug 13;9:1869.

29. Zhang S, Li T, Zhang B, Nong L, Aozasa K. Transcription factors engaged in development of NK cells are commonly expressed in nasal NK/T-cell lymphomas. Hum Pathol. 2011 Sep;42(9):1319–28.

30. Read KA, Jones DM, Freud AG, Oestreich KJ. Established and emergent roles for Ikaros transcription factors in lymphoid cell development and function. Immunol Rev. 2021 Mar;300(1):82–99.

31. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, et al. Innate or Adaptive Immunity? The Example of Natural Killer Cells. Science. 2011 Jan 7;331(6013):44–9.

32. Spranger S, Gajewski TF. Mechanisms of Tumor Cell–Intrinsic Immune Evasion. Annu Rev Cancer Biol. 2018 Mar 4;2(1):213–28.

33. Galluzzi L, Chan TA, Kroemer G, Wolchok JD, López-Soto A. The hallmarks of successful anticancer immunotherapy. Sci Transl Med. 2018 Sep 19;10(459):eaat7807.

34. Esfahani K, Roudaia L, Buhlaiga N, Del Rincon SV, Papneja N, Miller WH. A Review of Cancer Immunotherapy: From the Past, to the Present, to the Future. Curr Oncol. 2020 Apr 1;27(12):87–97.

35. Andtbacka RHI, Collichio F, Harrington KJ, Middleton MR, Downey G, Öhrling K, et al. Final analyses of OPTiM: a randomized phase III trial of talimogene laherparepvec versus granulocyte-macrophage colony-stimulating factor in unresectable stage III–IV melanoma. J Immunother Cancer. 2019 Dec;7(1):145.

36. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the Pd-1 Immunoinhibitory Receptor by a Novel B7 Family Member Leads to Negative Regulation of Lymphocyte Activation. J Exp Med. 2000 Oct 2;192(7):1027–34.

37. Robert C, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, et al. Ipilimumab plus Dacarbazine for Previously Untreated Metastatic Melanoma. N Engl J Med. 2011 Jun 30;364(26):2517–26.

38. Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, et al. Phase I Study of Single-Agent Anti–Programmed Death-1 (MDX-1106) in Refractory Solid Tumors: Safety, Clinical Activity, Pharmacodynamics, and Immunologic Correlates. J Clin Oncol. 2010 Jul 1;28(19):3167–75.

39. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, Activity, and Immune Correlates of Anti–PD-1 Antibody in Cancer. N Engl J Med. 2012 Jun 28;366(26):2443–54.

40. Schmid P, Rugo HS, Adams S, Schneeweiss A, Barrios CH, Iwata H, et al. Atezolizumab plus nab-paclitaxel as first-line treatment for unresectable, locally advanced or metastatic triple-negative breast cancer (IMpassion130): updated efficacy results from a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol. 2020 Jan;21(1):44–59.

41. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, et al. Sipuleucel-T Immunotherapy for Castration-Resistant Prostate Cancer. N Engl J Med. 2010 Jul 29;363(5):411–22.

42. Kranz LM, Diken M, Haas H, Kreiter S, Loquai C, Reuter KC, et al. Systemic RNA delivery to dendritic cells exploits antiviral defence for cancer immunotherapy. Nature. 2016 Jun;534(7607):396–401.

43. Sabado RL, Balan S, Bhardwaj N. Dendritic cell-based immunotherapy. Cell Res. 2017 Jan;27(1):74–95.

44. Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, et al. Cancer Regression and Autoimmunity in Patients after Clonal Repopulation with Antitumor Lymphocytes. Sci New Ser. 2002;298(5594):850–4.

45. Garfall AL, Maus MV, Hwang WT, Lacey SF, Mahnke YD, Melenhorst JJ, et al. Chimeric Antigen Receptor T Cells against CD19 for Multiple Myeloma. N Engl J Med. 2015 Sep 10;373(11):1040–7.

46. Wang QJ, Yu Z, Hanada K ichi, Patel K, Kleiner D, Restifo NP, et al. Preclinical Evaluation of Chimeric Antigen Receptors Targeting CD70-Expressing Cancers. Clin Cancer Res. 2017 May 1;23(9):2267–76.

47. Singh AK, McGuirk JP. CAR T cells: continuation in a revolution of immunotherapy. Lancet Oncol. 2020 Mar;21(3):e168–78.

48. Li Y, Hermanson DL, Moriarity BS, Kaufman DS. Human iPSC-Derived Natural Killer Cells Engineered with Chimeric Antigen Receptors Enhance Anti-tumor Activity. Cell Stem Cell. 2018 Aug;23(2):181-192.e5.

49. Shimasaki N, Jain A, Campana D. NK cells for cancer immunotherapy. Nat Rev Drug Discov. 2020 Mar;19(3):200–18.

50. Passweg JR, Tichelli A, Meyer-Monard S, Heim D, Stern M, Kühne T, et al. Purified donor NK-lymphocyte infusion to consolidate engraftment after haploidentical stem cell transplantation. Leukemia. 2004 Nov;18(11):1835–8.

51. Bachanova V, Cooley S, Defor TE, Verneris MR, Zhang B, McKenna DH, et al. Clearance of acute myeloid leukemia by haploidentical natural killer cells is improved using IL-2 diphtheria toxin fusion protein. Blood. 2014 Jun 19;123(25):3855–63.

52. Peighambarzadeh F, Najafalizadeh A, Esmaeil N, Rezaei A, Ashrafi F, Hakemi MG. Optimization of In Vitro Expansion and Activation of Human Natural Killer Cells against Breast Cancer Cell Line. 2020;12(1):7.

53. Suen WCW, Lee WYW, Leung KT, Pan XH, Li G. Natural Killer Cell-Based Cancer Immunotherapy: A Review on 10 Years Completed Clinical Trials. Cancer Invest. 2018 Sep 14;36(8):431–57.

54. Knorr DA, Ni Z, Hermanson D, Hexum MK, Bendzick L, Cooper LJN, et al. Clinical-Scale Derivation of Natural Killer Cells From Human Pluripotent Stem Cells for Cancer Therapy. Stem Cells Transl Med. 2013 Apr 1;2(4):274–83.

55. Tarn YK, Martinson JA, Doligosa K, Klingernann HG. Ex vivo expansion of the highly cytotoxic human natural killer cell line NK-92 under current good manufacturing practice conditions for clinical adoptive cellular immunotherapy. Cytotherapy. 2003;5(3):259–72.

56. Gurdon JB. The Developmental Capacity of Nuclei taken from Intestinal Epithelium Cells of Feeding Tadpoles. :20.

57. Schnleke AE, Campbell KHS. Viable offspring derived from fetal and adult mammalian cells. 1997;5.

58. Hochedlinger K, Jaenisch R. Monoclonal mice generated by nuclear transfer from mature B and T donor cells. Nature. 2002 Feb;415(6875):1035–8.

59. Harris H, Miller OJ, Klein G, Worst P, Tachibana T. Suppression of Malignancy by Cell Fusion. Nature. 1969 Jul;223(5204):363–8.

60. Pires CF, Rosa FF, Kurochkin I, Pereira CF. Understanding and Modulating Immunity With Cell Reprogramming. Front Immunol. 2019 Dec 11;10:2809.

61. Robert L. Davis, Harold Weintraub, Andrew B. Lassar,. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Available from: https://doi.org/10.1016/0092-8674(87)90585-X.

62. Takahashi K, Yamanaka S. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell. 2006 Aug;126(4):663–76.

63. Xu J, Du Y, Deng H. Direct Lineage Reprogramming: Strategies, Mechanisms, and Applications. Cell Stem Cell. 2015 Feb;16(2):119–34.

64. Pereira CF, Lemischka IR, Moore K. Reprogramming cell fates: insights from combinatorial approaches: Reprogramming factors to instruct cell fate. Ann N Y Acad Sci. 2012 Aug;1266(1):7–17.

65. Pereira CF, Chang B, Qiu J, Niu X, Papatsenko D, Hendry CE, et al. Induction of a Hemogenic Program in Mouse Fibroblasts. Cell Stem Cell. 2013 Aug;13(2):205–18.

66. Xie H, Ye M, Feng R, Graf T. Stepwise Reprogramming of B Cells into Macrophages. :14.

67. Rosa FF, Pires CF, Kurochkin I, Ferreira AG, Gomes AM, Palma LG, et al. Direct reprogramming of fibroblasts into antigen-presenting dendritic cells. Sci Immunol. 2018 Dec 14;3(30):eaau4292.

68. Rosa FF, Pires CF, Kurochkin I, Halitzki E, Zahan T, Arh N, et al. Single-cell transcriptional profiling informs efficient reprogramming of human somatic cells to cross-presenting dendritic cells. Sci Immunol. 2022 Mar 4;7(69):eabg5539.

69. Alquicira-Hernandez J, Sathe A, Ji HP, Nguyen Q, Powell JE. scPred: accurate supervised method for cell-type classification from single-cell RNA-seq data. Genome Biol. 2019 Dec;20(1):264.

70. Yu H, Fehniger TA, Fuchshuber P, Thiel KS, Vivier E, Carson WE, et al. Flt3 Ligand Promotes the Generation of a Distinct CD34⁺ Human Natural Killer Cell Progenitor That Responds to Interleukin-15. :11.

71. Wang X, Zhao XY. Transcription Factors Associated With IL-15 Cytokine Signaling During NK Cell Development. Front Immunol. 2021 Mar 18;12:610789.

72. Benson DM Jr. tem cell factor and interleukin-2/15 combine to enhance MAPK-mediated proliferation of human natural killer cells.

73. Biddy BA, Kong W, Kamimoto K, Guo C, Waye SE, Sun T, et al. Single-cell mapping of lineage and identity in direct reprogramming. Nature. 2018 Dec;564(7735):219–24.

74. Moritz Rapp.

75. Boast B, Miosge LA, Kuehn HS, Cho V, Athanasopoulos V, McNamara HA, et al. A Point Mutation in IKAROS ZF1 Causes a B Cell Deficiency in Mice. J Immunol. 2021 Apr 1;206(7):1505–14.

76. Hirai H, Yang B, Garcia-Barrio MT, Rom O, Ma PX, Zhang J, et al. Direct reprogramming of fibroblasts into Smooth Muscle-Like Cells with Defined Transcription Factors. 2019;16.

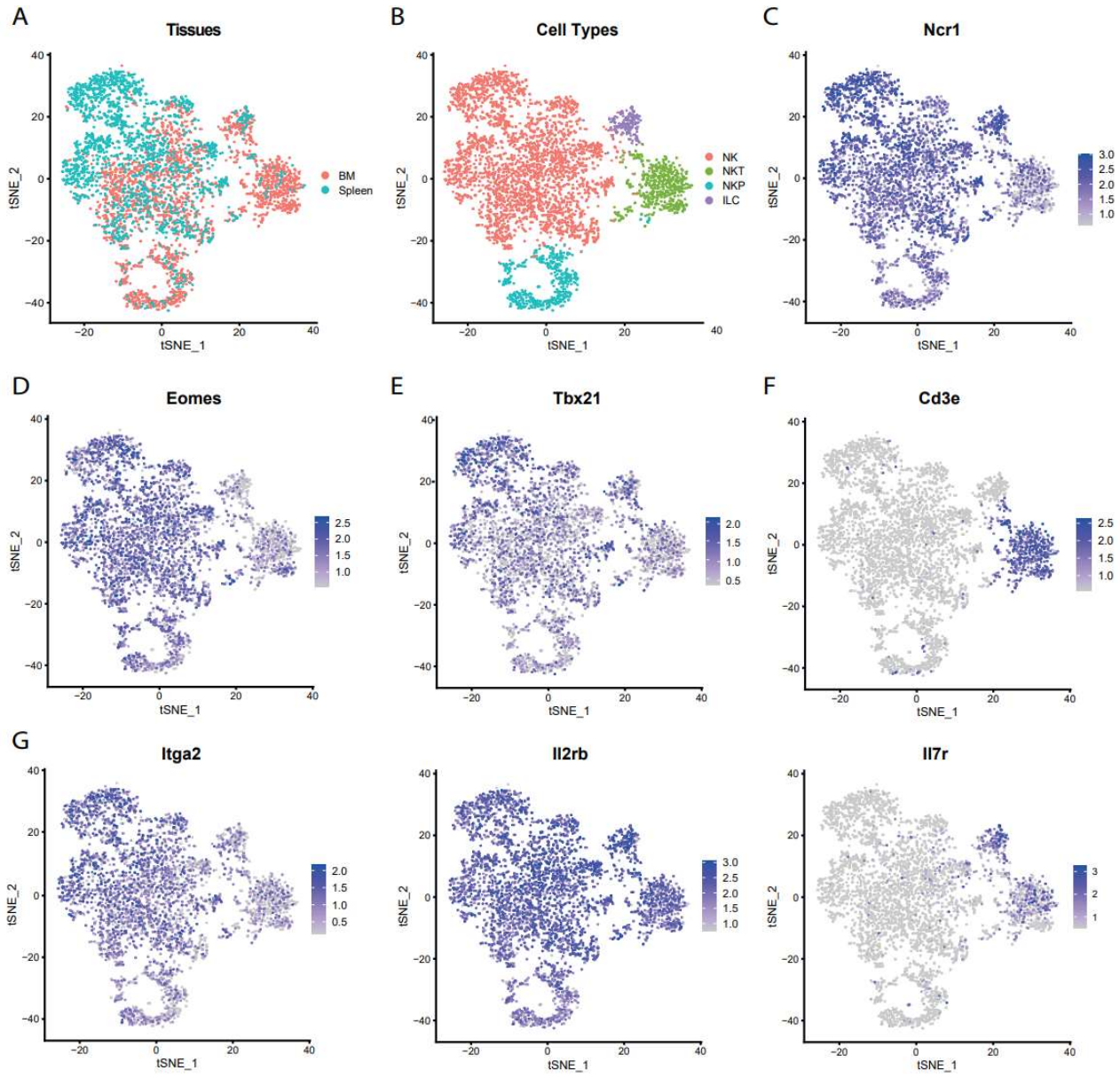
77. Bi J, Wang X. Molecular Regulation of NK Cell Maturation. Front Immunol. 2020 Aug 11;11:1945.

78. Wu Y, Tian Z, Wei H. Developmental and Functional Control of Natural Killer Cells by Cytokines. Front Immunol. 2017 Aug 4;8:930.

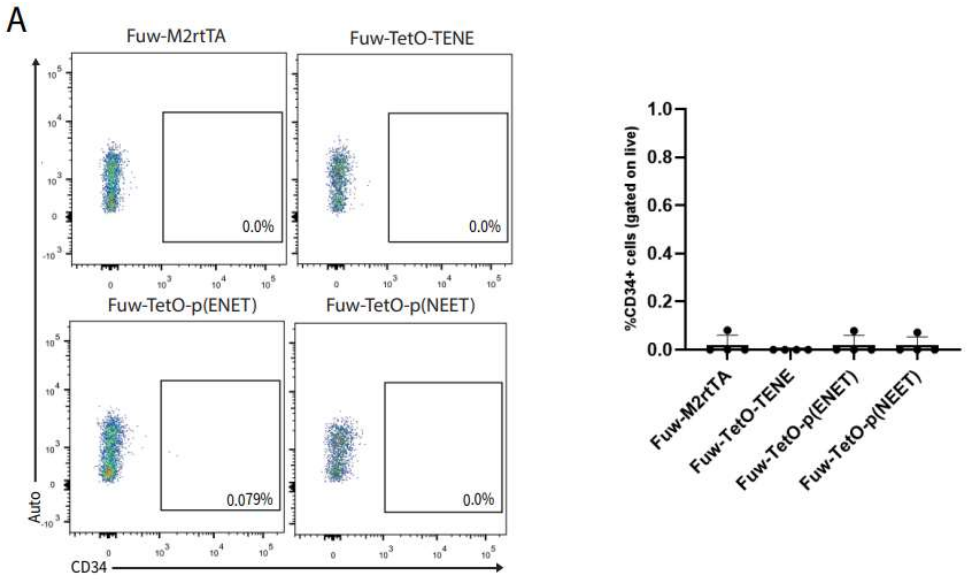
79. Lai CB, Mager DL. Role of Runt-related Transcription Factor 3 (RUNX3) in Transcription Regulation of Natural Cytotoxicity Receptor 1 (NCR1/NKp46), an Activating Natural Killer (NK) Cell Receptor. J Biol Chem. 2012 Mar;287(10):7324–34.

80. Wong WF, Kohu K, Chiba T, Sato T, Satake M. Interplay of transcription factors in T-cell differentiation and function: the role of Runx: Runx transcription factor and T lymphocytes. Immunology. 2011 Feb;132(2):157–64.

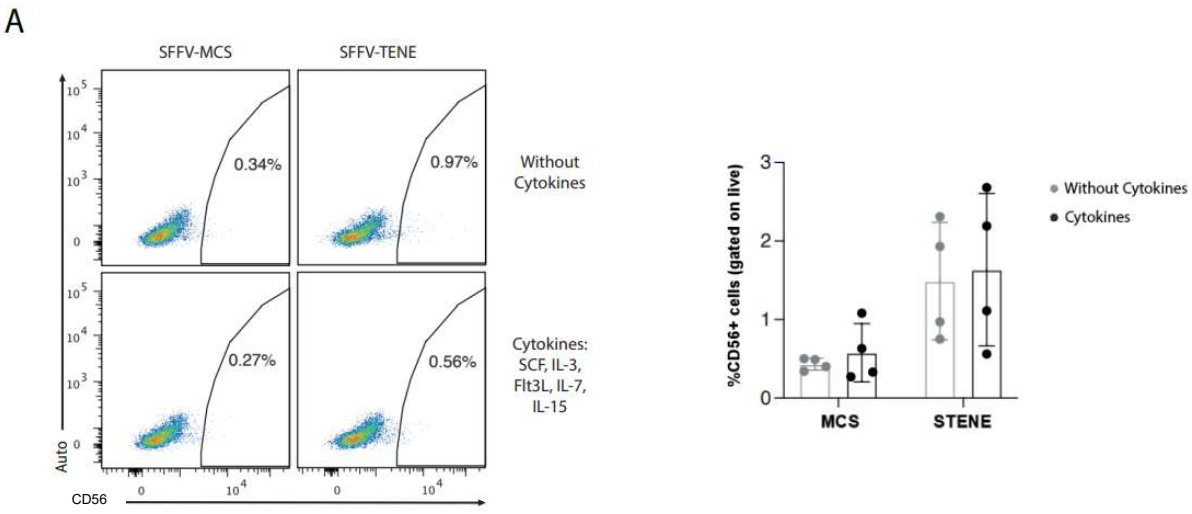
Supplementary information



Supplementary Figure 1 – Single-cell mRNA-seq analysis of different cell types using specific markers. (A) TSNE visualization of mouse NCR1-tdTomato positive bone marrow and spleen cells. (B) TSNE visualization of NK, NK progenitor (NKP), NKT and innate lymphoid cells (ILCs). (C) Expression of NCR1 marker confirming NK cell identity. (D) Expression of TF Eomes mostly upregulated in NK cells. (E) Expression of Tbx21, encoding T-bet, expressed associated to ILCs and NKs. (F) Expression of CD3e specific for NKT cells. (G) Expression of Itga2 (left panel), Il2rb (center panel), and Il7r (right panel) for NKP assignment. NKP cells are $CD7^{+}Il7r^{-}Il2rb^{low}Itga2^{low}$.



Supplementary Figure 2 – Fuw-tetO system inefficient to induce CD34 expression in HEFs. (A) CD34 expression gated on live cells in HEFs transduced with Fuw-TetO conditions and control Fuw-M2rtTA (left). Percentage of CD34 positive cells gated on live cells (right). Cells transduced with individually cloned TFs (Fuw-TetO-TENE), two polycistronic according to TF position: Fuw-TetO-p(ENET) (Ets1, Nfil3, Eomes, and T-bet) and Fuw-TetO-p(NEET) (Nfil3, Ets1, Eomes, and T-bet).



Supplementary Figure 3 – Cytokine cocktail does not promote CD56 expression in HEFs transduced with TENE combination. (A) CD56 expression gated on live cells in HEFs transduced with SFFV-MCS control and SFFV-TENE treated with SCF, IL-3, Flt3L, IL-7, and IL-15 or without cytokines (right). Percentage of CD56 positive cells gated on live cells (left).

Supplementary Table 1 – Candidate TFs tested in mouse.

Candidate TFs		
Eomes	Gata3	Elf1
T-bet	Ikzf3	Elf4
Id2	Tox	Gata2
Ets1	Zbtb16	Ikzf1
Runx3	Stat5	Zeb2
Klf12	Zfp105	Irf2
Nfil3		

Supplementary Table 2 – Pool A, B, and C of candidate TFs barcoded.

Pool A	Pool B	Pool C
Ets1	Ets1	Ets1
T-bet	T-bet	T-bet
Tcf7	Tcf7	Tcf7
Nfil3	Nfil3	Nfil3
Eomes	Eomes	Eomes
Id2	Gata3	Prdm1
Ikzf1	Stat4	Myb
Ikzf3	Bcl11b	Lef1
Runx1	Klf2	Satb1
Runx2	Bach2	Tcf12
Runx3	Rorα	Nr4a3
Zfp105	Rorc	Mllt3
Gata3	Nfatc3	Zfpm1
Stat5b	Klf12	Gfi1
Elf4	Plzf	Fos
Cbfβ	Znf683	Maf
Irf2	Elf1	Nfatc1
Gata2	Stat5a	Foxo1
Tox	Ikzf2	Irf4
Zeb2	Runx3	Hoxb5