Year : 2014

The role of the transcription factor Tcf-1 for the development and the function of NK cells

Gehrig Jasmine

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Ludwig Center for Cancer Research

The role of the transcription factor Tcf-1 for the development and the function of NK cells

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

Jasmine Gehrig

Master de l'Université de Zürich

Jury

Prof. Luc Tappy, Président
Prof. Werner Held, Directeur de thèse
Prof. Sanjiv Luther, expert
Prof. Daniela Finke, expert
Prof. Fabienne Tacchini-Cottier, PhD program representative

Lausanne, May 2014

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Ludwig Center for Cancer Research

The role of the transcription factor Tcf-1 for the development and the function of NK cells

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

Jasmine Gehrig

Master de l'Université de Zürich

Jury

Prof. Luc Tappy, Président
Prof. Werner Held, Directeur de thèse
Prof. Sanjiv Luther, expert
Prof. Daniela Finke, expert
Prof. Fabienne Tacchini-Cottier, PhD program representative

Lausanne, May 2014

UNIL | Université de Lausanne
Faculté de biologie et de médecine

Ecole Doctorale

Doctorat ès sciences de la vie

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Président	Monsieur	Prof. Luc **Tappy**
Directeur de thèse	Monsieur	Prof. Werner **Held**
Experts	Madame	Prof. Fabienne **Tacchini-Cottier**
	Madame	Prof. Daniela **Finke**
	Monsieur	Prof. Sanjiv **Luther**

le Conseil de Faculté autorise l'impression de la thèse de

Madame Jasmine Gehrig

Master of Science de l' Université de Zürich

intitulée

The role of the transcription factor Tcf-1 for the development and the function of NK cells

Lausanne, le 23 mai 2014

pour La Doyenne
de la Faculté de Biologie et de Médecine

Prof. Luc Tappy

Summary

Natural Killer (NK) cells are innate immune cells that can eliminate malignant and foreign cells and that play an important role for the early control of viral and fungal infections. Further, they are important regulators of the adaptive and innate immune responses. During their development in the bone marrow (BM) NK cells undergo several maturation steps that directly establish an effector program. The transcriptional network that controls NK cell development and maturation is still incompletely understood. Based on earlier findings that NK cell numbers are reduced in the absence of the transcription factor T cell factor-1 (Tcf-1), my thesis has addressed the precise role of this transcription factor for NK cell development, maturation and function and whether Tcf-1 acts as a nuclear effector of the canonical Wnt signaling pathway to mediate its effects. It is shown that Tcf-1 is selectively required for the emergence of mature BM NK cells. Surprisingly, the emergence of BM NK cells depends on the repressor function of Tcf-1 and is independent of the Wnt pathway. In BM and peripheral NK cells Tcf-1 is found to suppress Granzyme B (GzmB) expression, a key cytotoxic effector molecule required to kill target cells. We provide evidence that GzmB over-expression in the absence of Tcf-1 results in accelerated spontaneous death of bone marrow NK cells and of cytokine stimulated peripheral NK cells. Moreover, Tcf-1 deficient NK cells show reduced target cell killing, which is due to enhanced GzmB-dependent NK cell death induced by the recognition of tumour target cells. Collectively, these data provide significant new insights into the transcriptional regulation of NK cell development and function and suggest a novel mechanism that protects NK cells from the deleterious effects of highly cytotoxic effector molecules.

Resumé

Les cellules NK (de l'anglais Natural Killer) font partie du système immunitaire inné et sont capables d'éliminer à elles seules les cellules cancéreuses ou infectées. Ces cellules participent dans la régulation et la coordination des réponses innée et adaptative. Lors de leur développement dans la moelle osseuse, les cellules NK vont acquérir leurs fonctions effectrices, un processus contrôlé par des facteurs de transcription mais encore peu connu. Des précédentes travaux ont montré qu'une diminution du nombre de cellules NK corrélait avec l'absence du facteur de transcription Tcf-1 (T cell factor-1), suggérant un rôle important de Tcf-1 dans le développement de cellules NK. Cette thèse a pour but de mieux comprendre le rôle du facteur de transcription Tcf-1 lors du développement et la maturation des cellules NK, ainsi que son interaction avec la voie de signalisation Wnt. Nous avons montré que Tcf-1 est essentiel pour la transition des cellules immatures NK (iNK) à des cellules matures NK (mNK) dans la moelle osseuse, et cela de manière indépendamment de la voie de signalisation Wnt. De manière intéressante, nous avons observé qu'en absence du facteur de transcription Tcf-1, les cellules NK augmentaient l'expression de la protéine Granzyme B (GzmB), une protéine essentielle pour l'élimination des cellules cancéreuses ou infectées. Ceci a pour conséquence, une augmentation de la mort des cellules mNK dans la moelle osseuse ainsi qu'une diminution de leur fonction «tueuses». Ces résultats montrent pour la première fois, le rôle répresseur du facteur de transcription Tcf-1 dans l'expression de la protéine GzmB. L'ensemble de ces résultats apporte de nouveaux éléments concernant le rôle de Tcf-1 dans la régulation du développement et de la fonction des cellules NK et suggèrent un nouveau mécanisme cellulaire de protection contre les effets délétères d'une dérégulation de l'expression des molécules cytotoxique.

Acknowledgements

First of all, I want to thank Prof. Werner Held for giving me the opportunity to do work on the fascinating subject of NK cell development and function in the absence of Tcf-1. Throughout my doctoral work, Werner guided me whenever I needed advice on a strategic decision. Werner has taught me to work independently and to think critically, especially about any possible control before starting an experiment. In the individual meetings we had, I always learned many new things and he taught me to look at a problem from many different angles.

I further wish to express my gratitude to the members of my thesis committee, Prof. Daniela Finke, Prof. Sanjiv Luther and Prof. Fabienne Tacchini-Cottier for their advice and critical reading and commenting on the manuscript.

A very special thank you goes to all the present and past members of the Held lab: Camille Grandclément, Beena Jeevan-Raj, Irene Pizzitola, Georgi Angelov, Stéphanie Bessoles, Mika Kijma, Caroline Boudousquié, Noémie Gardiol, Elisenda Alari Pahissa, Mélanie Charmony, Maxime Danilo, Laurène Pousse and Leo Scarpellino for their help, the fruitful discussions and the cheerful atmosphere.
Stéphanie, thanks for your humor and for taking me under your "NK cell wing". I almost adapted to the southern French accent while sharing the office with you and the Ludwig gang. Mika, thanks for the wonderful self-made sushi and your capturing explanations about Japanese culture and way of life. Camille, thanks for all the shared scientific and non scientific thoughts, for helping me with the B-ALL model and for taking care of my mice, while I was writing this manuscript. Thanks for showing me the French Jura, especially the "course de chiens de traîneau et le ski de fond". Beena, thanks for your help, especially for performing the Chip-seq analysis to further unravel the Tcf-1 GzmB relationship. I enjoyed your introduction to indian dance and to taste your delicious baked creations. Thanks for carefully reading this manuscript. Irène, thanks for your cheerfulness, criticism and scientific spirit and for always sharing some ideas on our projects and life over a cup of coffee. Georgi, thanks your for your scientific input and for fixing everything that broke down in the lab. Mélanie, thanks for correcting the French abstract.

To the staff from the Ludwig Institute I owe my gratitude for all the help I received over the years. I enjoyed the openness and interactions between the people from all over the world. I felt at home thanks to the nice friendships I could experience. In particular, I would like to thank, Rachel Perret for being my desk neighbor and for patiently answering my questions concerning FACS analysis and statistics. Thanks for the good moments and for always giving me a helping hand. I will never forget what a real Kiwi is. Jan Dudda, thanks for sharing your scientific knowledge and for taking the Ludwig gang on unforgetable ski trips. Thanks, for your great friendship and for introducing me in the art of beekeeping and for the tasty honey. Julie Leingardner, thanks for always being ready to share a protocol or a reagent and the good discussions we had over “French-English” lunches. A special thanks to the Ludwig-Biochemistry swim club: Jan, Jean-Paul, Katrin and Marie for the sporty spirit and the good times we shared in the pool. Thanks to Anne Wilson, who always was ready to answer my questions concerning the best FACS staining or antibody and for correcting the English of my thesis. Danny Labes, thanks for your great FACS assistance, training and tips whenever I needed them and for the good chats and for making me laugh while cells were sorted. Danijel, thanks for always being there to motivate me, whenever I stuck with a long experiment in the middle of the night. Manuele, thanks for your support, for believing in me, the wonderful moments and for sharing your passion for science. I wish you all the luck on this earth that you soon might open your own lab. Stephanie, thanks for your technical advise and for the fun.

My deepest gratitude I owe to my fiancé Lukas. Thanks for your love, encouragement and optimism. Thanks for your understanding and for cheering me up whenever I felt desperate. Thanks for critical reading the manuscript and your support. Thanks for the wonderful braids and for the best time ever.

Finally, I would like to thank my family for the help and support. Thanks to my parents for teaching me the basic wisdom of life and for all their love. Huge thanks to my sister and my two wonderful nieces for always cheering me up.

Table of Contents

Abbreviations

Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
Ag	Antigen
APC	Antigen presenting cell
APC	Adenomatous polyposis coli
ATF2	Activating transcription factor 2
BM	Bone marrow
BCR	B cell receptor
β-TRCP	β-transducin-repeat-containing protein
BM	Bone marrow
CBFb	Core-binding factor b
CD	Cluster of differentiation
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
DC	Dentridic cell
FDG	Fluorescein di-β-galactopyranoside
DN	Double negative
DP	Double positive
DPP-I	Dipeptidyl peptidase I
Dsh	Dishevelled
Eomes	Eomesodermin
E4bp4	E4-binding protein 4
ETP	Early thymic precursor
Fz	Frizzled receptor
GMP	Granulocyte/macrophage precursor
Gro	Groucho
GSK-3β	Glycogen-synthase kinase 3β
GvT	Graft versus tumor

GVHD	Graft versus host disease
GzmB	Granzmye B
HLA	Human leukocyte antigen
HMG	High mobility group
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplants
Id2	Inhibitor of DNA-binding 2
IFNγ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
IL-2Rγ	IL-2 receptor common γ chain
ILC	Innate lymphoid cells
Irf	Interferon regulatory factor
ITIM	Intracytoplasmic immunoreceptor tyrosine-based inhibition motifs
INF	Interferon
iNK	Immature natural killer
ISP	Immature single positive
ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
KIR	Killer Ig-like receptor
LAMP-1	Lysosome-associated membrane protein 1
LCMV	Lymphocytic choriomeningitis virus
Lef-1	Lymphoid enhancer factor-1
LMPP	Lymphoid-primed multipotent progenitor
LSK	$Lineage^{-}$ $c\text{-}Kit^{+}$ $IL\text{-}7Ra^{+}$
LTi	Lymphoid Tissue inducer
MCA	Methylcholanthrene -induced sarcomas
MCMV	The murine cytomegalovirus
MHC	Major histocompatibilty complex
MHV	Murine hepatitis virus
MEP	Megakaryocyte/erythrocyte precursors
MFI	Mean fluorescent intensity
MMP	Matrix metalloproteinase
mNK	Mature natural killer

NK	Natural killer
NKp	Natural killer precursor
NKT	Natrual killer T cell
PRR	Pattern-recognition receptor
Runx	Runt-related transcription factor
PAMP	Pathogen-associated molecular patterns
Prf1	Perforin
Poly IC	Polyinosinic:polycytidylic acid
RAG	Recombination-activating gene
SCID	Severe combined immunodeficiency
SLAM	Signaling lymphocyte activation molecule
SLT	Secondary lymphoid tissues
Socs	Suppressor of cytokine signaling
S1P	Sphingosine 1-phosphate
S1P5	Drivespingosine-1-phosphate receptor 5
Spi-6	Serine protease inhibitor 6
T-bet	T-box transcription factor TBX21
TCR	T cell receptor
Tcf-1	T cell factor-1
Th1	T helper 1
TNF	Tumor-necrosis factor
Tg	Transgene
TLR	Toll-like receptors
TNF	Tumor-necrosis factor
Treg	Regulatory T cells
Wnt	Wingless/Integration-1
Wt	Wild-type

1 Introduction

1.1 Cells and organs of the immune system

The lymphoid organs such as the thymus, spleen and lymph nodes are central components of the mammalian immune system. The thymus and the bone marrow (BM) are the primary (or central) lymphoid organs. They provide an appropriate microenvironment for the development and maturation of lymphocytes. All blood cells including all immune cells arise from haematopoietic stem cells (HSC). This process, called haematopoiesis, takes place within the bone marrow throughout adult life. HCS have the capacity for self-renewal and are maintained at a stable level. Early in haematopoiesis, pluripotent stem cells can differentiate either into common lymphoid progenitor (CLP) cells or common myeloid progenitor (CMP) cells. These cells have lost the capacity of self-renewal and are committed to a particular cell lineage. CLP cells give rise to B, and natural killer (NK) cells. CMP cells generate progenitors of red blood cells (erythrocytes), many of the various white blood cells (neutrophils, eosinophils, basophils, monocytes, mast cells), and platelet-generating cells called megakaryocytes.

The cells of the adaptive immune system (B and T lymphocytes) undergo further differentiation steps within primary lymphoid organs (T cells in the thymus and B cells in the bone marrow) to acquire their specificity and to generate antigen (Ag)-receptor diversity. This is achieved through the clonal expression of B cell receptors (BCRs) and T cell receptors (TCRs) with distinct antigen specificities, the diversity of which results from random somatic DNA rearrangements. The recombination events yield a high number of antigen receptors, some of which are potentially self-reactive. A central selection process eliminates potentially harmful T and B cells that possess self-reactive receptors.

The secondary lymphoid tissues (SLT) including lymph nodes (LNs), the spleen, Peyer`s patches and the gut- and bronchus-associated lymphoid tissues play an important role during the development of an adaptive immune response. They are highly organized structures, which trap and concentrate the Ag and allow the interaction of Ag-bearing antigen-presenting cells (APCs) with the rare Ag specific T and B cells.

1.2 Innate and adaptive immunity

The mammalian immune system consists of the Ag-non-specific innate immunity and the Ag-specific adaptive immunity. These two arms interact with each other. In contrast to innate immunity, which develops within hours after encountering a pathogen, the adaptive immunity develops over the course of days. Additionally, the adaptive immunity exhibits memory that allows a faster and stronger response upon re-exposure to the Ag.

The first defense against pathogens is our body surface, the skin and the epithelial barriers of gastrointestinal, respiratory and urogenital tracts. These barriers are populated by sensors of the innate immune system, such as dendritic cells (DCs) and macrophages. As a component of the innate immune system, they screen their environment for pathogens using germ line encoded receptors called pattern-recognition receptors (PRRs). PRR recognize a limited but highly conserved set of molecular structures, the pathogen-associated molecular patterns (PAMPs), which are absent in the host (Imler and Hoffmann, 2001). If a pathogen is recognized by components of the innate immune system, a cascade of events takes place within a few hours and this results in an inflammatory response. This leads to the recruitment of more cells of the innate immune system to this site of infection to eliminate the pathogens.

The most prominent PRRs expressed by macrophages and DCs, are the scavenger receptors and the Toll-like receptors (TLRs). The TLRs are capable of recognizing organisms ranging from bacteria to fungi, protozoa and viruses, and they play a major role in innate immunity (Uematsu and Akira, 2008). To date, 12 TLRs are known in mice and 11 in humans. Stimulation of TLRs results in an increased phagocytic activity, a release of inflammatory mediators and the secretion of cytokines. The increased phagocytic activity of the APCs leads to a higher chance of capturing Ags for presentation by major histocompatibility complex (MHC) molecules (Reis e Sousa, 2006). The Ags taken up are cleaved into peptides in the endocytic compartments and loaded onto MHC-II molecules (MHC-II) , which present the Ag on the cell surface to CD4 T cells. In contrast MHC class I (MHC-I) presents antigens derived from cytosolic or nuclear proteins. Exogenous antigenic peptides can also be presented by MHC-I molecule using a process called cross-presentation. Cross-presentation is essential for inducing cytotoxic T-lymphocyte (CTL) responses

against most tumors and against viruses that do not readily infect antigen-presenting cells and it is important for induction and maintenance of tolerance directed to self antigens (Banchereau and Steinman, 1998; Bevan, 2006; Neefjes and Sadaka, 2012). DCs are the only APCs able to activate naïve T lymphocytes and are the most powerful APCs in higher vertebrates. Following maturation induced by inflammatory mediators and microbial products and the uptake of Ags, DCs migrate to the T-cell area of the draining lymph nodes. During their journey they down-regulate their capacity to take up Ags and up-regulate Ag-MHC-II complexes, co-stimulatory molecules (e.g. cluster of differentiation (CD)40, CD80 and CD86) and the chemokine receptor CCR7. The CCR7 is the main lymph-node homing receptor and enables the DCs to migrate to lymph nodes through peripheral lymphatic vessels (Forster et al., 1999). There they initiate the activation and expansion of T cells recognizing their cognate Ag.

The central event in the generation of both humoral and cell-mediated immune responses is the activation and clonal expansion of rare antigen-specific T and B cells. Mature T cells are constantly circulating through the blood, lymph and SLTs such as LNs and the spleen. SLs have evolved in order to trap and concentrate Ag and to allow the interaction of Ag-laden APCs with rare Ag-specific T and B cells. The highly organized SLTs contain defined compartments consisting of T cell areas and B cell areas.

Circulating T lymphocytes enter the LN via the T cell area where they interact with Ag-laden DCs. T lymphocytes specific for the foreign Ag are thus activated and expand clonally. Upon differentiation, they either leave the LNs in order to migrate through the body in search of their cognate Ag displayed by resident APCs, infected or malignant cells. Alternatively they home to the B cell areas to assist in Ag-specific antibody production. T lymphocytes only recognize processed peptide antigens presented on MHC molecules. The cytotoxic $CD8^+$ T cells react to peptides bound on MHC-I that are expressed by virtually all cells of the body. The $CD4^+$T cells recognize MHC-II bound peptides. MHC-II molecules are only expressed by professional APCs. A contact between an APC providing a suitable MHC/peptide complex and an additional co-stimulatory signal is required for the activation of a $CD4^+$ T cell, a process called priming. The $CD4^+$ T cells provide help during the process of $CD8^+$ T cell and B cell priming. Dependent on the cytokine environment the $CD4^+$ T cells can differentiate into various subset types with functions that depend on the array of

cytokines they produce. For example, regulatory T cells that maintain peripheral tolerance or Th17 cells that promote inflammation (Welsh and Selin, 2002).
Activated $CD8^+$ T cells are able to directly destroy target cells that present their cognate Ag on MHC-I molecules. They induce apoptosis of the target cell either via the release of the cytotoxic molecules Perforin (Prf1) and Granzymes (Gzm) or trough Fas-Fas-ligand interactions. Most of the clonally expanded $CD8^+$ T cells die once the pathogen is cleared, leaving behind a population of long-lived memory $CD8^+$ T cells.

1.3 NK cells in health and disease

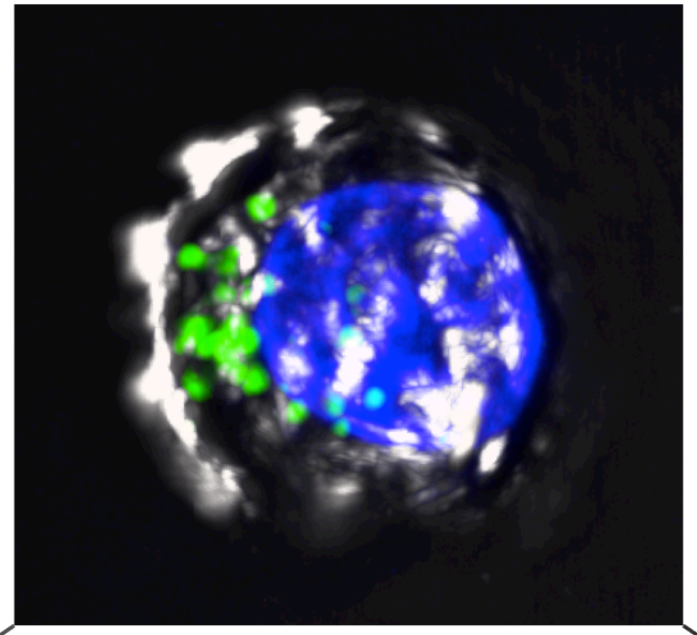
The NK cells belong to the innate branch of the immune system. They were named based on their ability to eliminate tumor cells, without the need for prior sensitization. NK cells circulate in the blood and they can be found in the bone marrow, spleen, liver, lung and lymph nodes. They are rapidly attracted to the sites of inflammation. NK cells are able to directly kill infected or aberrant cells and to provide activating signals to DCs and macrophages. Therefore they are important to maintain a first line of defense while the adaptive immune response is building up (Hepworth and Sonnenberg, 2014; Moretta et al., 2005; Vivier et al., 2011) (Fig. 1). They play a role in the early control of viral infections in humans and mice. The control of several virus infections in mice including influenza virus, murine hepatitis virus (MHV), ectromelia poxvirus and murine cytomegalovirus (MCMV), depends on NK cells. Mice show increased susceptibility or resistance to MCMV after NK cell depletion or NK cell and adoptive transfer, respectively (Bukowski et al., 1983; Lee et al., 2007). In line with those findings human patients lacking NK cells are susceptible to herpesvirus infections that are otherwise harmless (Biron et al., 1989; Orange, 2006).
Recently, a critical role for NK immunity to systemic fungal infections has been reported. NK cell deficiency causes high susceptibility to *C. albicans* infections, and adoptive transfer of NK cells restores the ability of mice to control the infection (Bar et al., 2014). The role of NK cells in the control of bacterial infections is less well defined yet.
NK cells can also play a role in tumor control. Mouse NK cells are involved in the *in vivo* rejection of transplanted tumors, in a manner dependent upon the presence or absence of NK cell receptor ligands expressed by the tumor (Diefenbach et al., 2001;

Marcus et al., 2014). Furthermore it has been shown that NK cells are able to eliminate methylcholanthrene (MCA)-induced sarcomas (Smyth et al., 2001; Yokoyama, 2005). NK cell infiltrates in human tumor biopsies, such as in colorectal carcinoma and gastric carcinoma, are associated with favorable prognoses (Coca et al., 1997; Ishigami et al., 2000). As a consequence, several recent therapeutic approaches aim at boosting the tumoricidal activity of NK cells (Ames and Murphy, 2014).

NK cells also have the ability to reject bone marrow allografts in lethally irradiated mice. Hematopoietic stem cell transplants (HSCT) using a MHC-I mismatch between recipient and donor can be rejected by NK cells. Conversely, in the context of hematological malignancies NK cells can improve the therapeutic success of partial human leucocyte antigen (HLA) mismatched hematopoietic stem cell transplantation by mediating a graft versus tumor (GvT) effect. A GvT occurs when cytotoxic immune cells like NK cells or T cells of the graft recognize malignant cells of the host as “foreign” and mount an immune response against them (Ruggeri et al., 2002). While NK cells can mediate GvT, they do not mediate graft versus host disease (GVHD), which is in contrast to T cells. GVHD occurs when grafted immune cells attack normal host tissues such as the skin, liver, and lungs.

Activated NK cells are an early source of interferon-γ (INFγ), which can directly affect the survival of pathogens. Furthermore they can secrete the tumor-necrosis factor (TNF) that together with INFγ can prevent the growth and spread of tumors.

NK cells also play an important role as regulators of adaptive immune responses. They can kill activated T cells, APC and endothelial cells and thereby dampen potentially harmful immune responses. Conversely NK cells can enhance adaptive immune responses by mediating DC maturation and by promoting T helper 1 (Th1) cell priming in lymph nodes via the secretion of INFγ (Laouar et al., 2005; Martin-Fontecha et al., 2004) (Fig.1).



Functions of activated NK cells

Cytotoxicity	DC maturation	T cell priming	Tolerance	Cytokine and chemokine production
Kill transformed, virus infected and stressed cells	Mediate DC editing and maturation by killing immature myeloid-DCs and selecting for the `most fit` DC to cause T cell priming	INFγ produced by NK cells influences Th1 priming. Influence DC-induced polarisation of naïve T cells	NK cell can secrete the regulatory cytokines TGFβ and IL10	Activated NK are an early source of INFγ what directly affects the survival of pathogens Are a source for chemokines that might atrract other immune cells to the sites of infalmmation
Mediate killing of activated T cells, APCs and endothelial cells	Can enhance INFα production by plasmacytoid DCs	Enhance $CD8^+$ T cell responses	NK cells can directly eliminate regulatory T cells	Can secrete tumor-necrosis factor (TNF) that together with INFγ can enhance the growth and spread of tumors

Figure 1. NK cells play an important role in innate and adaptive immunity
The table shows the most important functions of activated NK cells in innate and adaptive immunity.
Picture was taken by confocal microscopy and shows an activated NK cell at day 5 of IL-2 stimulation. The green granules within the cytoplasm depict Granzyme B (GzmB). The nucleus was stained with DAPI and is shown in blue. The white reflections depict parts of the the cytoplasm.

Regulatory T cells are important for the maintenance of peripheral tolerance. Their presence and/or activity is reduced in autoimmune conditions and increased in tumors. Regulatory T cells can inhibit NK cell activity by restricting the availability of IL-2, which is released by activated $CD4^+$ T cells. Conversely, NK cells can eliminate regulatory T cells. It has been shown in several autoimmune diseases, like multiple sclerosis, that NK cells can attack host tissues (Kaur et al., 2013). In contrast, certain NK cell subsets may prevent autoimmunity, as they can secrete the regulatory cytokines TGFβ and IL10 (Fig. 1) (Kerdiles et al., 2013; Poggi and Zocchi, 2014).
Alltogether, besides direct protective effects against infection, NK cells are modulating many aspects of adaptive immunity (Fig. 1).

1.4 NK cell development

During embryogenesis NK cells develop mainly in the fetal liver, whereas in adult mice the primary site for NK cell development is the BM. Nevertheless, a small number of NK cells continue to develop in the liver and the thymus (Constantinides et al., 2014; Daussy et al., 2014). NK cells develop from the common lymphoid progenitor (CLP) that can be characterized as lineage-negative (Lin-) (negative for mature cell lineage markers) and by the expression of the interleukin (IL)-7Rα (CD127) and the growth factor receptor c-Kit (lineage$^-$ IL-7Rα^+ c-Kit$^+$). CLPs are oligopotent and can also give rise to B cells and to a small extend to T cells, but they have lost the myeloid lineage potential. Carotta *et al*. recently identified the earliest committed NK cell progenitor with the help of an inhibitor of DNA binding-2 (Id2)-GFP reporter mouse. They found a small population of GFP^+ cells in the Fms-related tyrosine kinase 3 ligand $(Flt\text{-}3L)^+$ CLP fraction (Lin$^-$ Id2$^+$ stem cell antigen-1 $(Sca1)^+$ CD127$^+$ c-Kit^{low} Flt-3L$^+$ CD122$^-$). These GFP^+ cells efficiently differentiated into NK cells *in vitro* while having lost T and B cell potential (Carotta et al., 2011). At the same time Fathman *et al*. identified an NK cell progenitor within the CLP population using the surface markers CD244 (2B4) and CD27 (Lin$^-$CD27$^+$CD244$^+$CD127$^+$). *In vivo* and *in vitro* assays revealed that those cells were committed to the NK cell lineage (Fathman et al., 2011).
Subsequently, NK cell committed progenitors up-regulate the IL-15 receptor complex, composed of IL-15 receptor α (IL-15Rα), IL-2Rβ (CD122), and IL-2 receptor common γ (IL-2Rγc) chains. At this stage they are named natural killer progenitor (NKP) and they have lost the ability to differentiate into a B or T cell (FIg. 2). The NKP cells

subsequently progress to an immature NK cell (iNK) stage defined by the acquisition of NK1.1 as well as NKG2D receptors (Rosmaraki et al., 2001). The iNK cell stage correlates with the expression of receptors for MHC class I-molecules including CD94 in combination with NKG2A/C/E, and Ly49 receptors, a family of c-type lectin-like receptors.

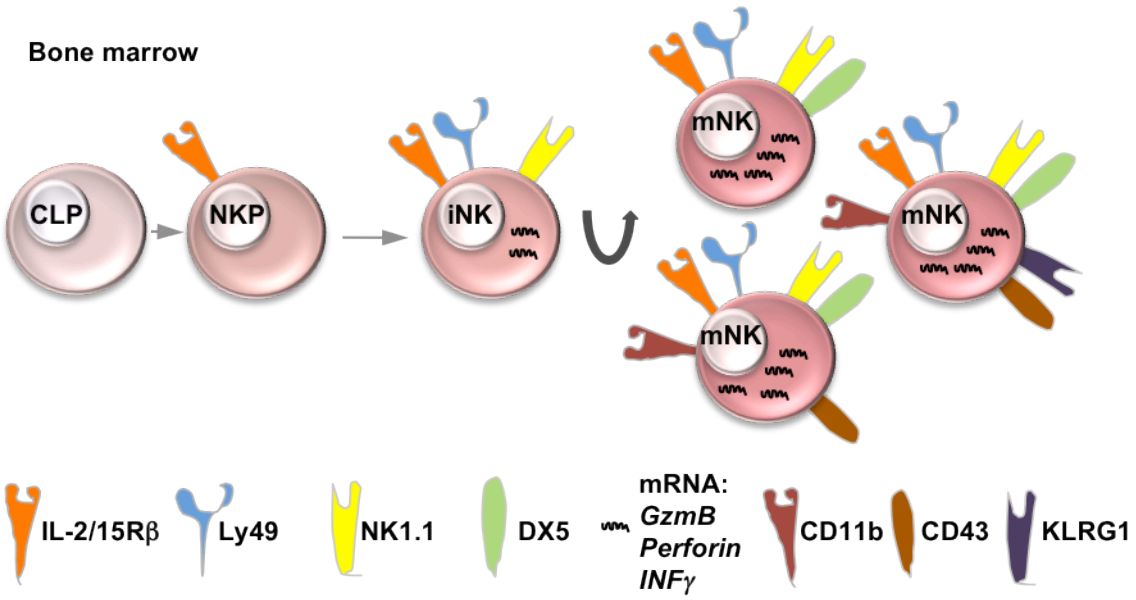


Figure 2. NK cell development in the bone marrow.
NK cells arise from a common lymphoid progenitor (CLP). The first progenitors that are exclusively committed to the NK cell lineage are called NK cell precursors (NKP) and they can be identified by the expression of the IL-2/15Rβ receptor. Immature NK cells (iNK) cells can be characterized by the expression of NK1.1 and the lack of DX5. The later marks the mature (mNK) stage. During the iNK to mNK transition NK cell sequentially up-regulate the expression of activating and inhibitory receptors including the Ly49 family of receptors. The mNK stage correlates with extensive expansion and the up-regulation of maturation markers like CD11b, CD43 and KLRG1. During their maturation process NK cells are programmed for their effector potential. At the iNK stage they start to accumulate an mRNA pool for GzmB, Perforin and INFγ but the expression of these effector molecules is prevented until priming. The model shows the essential markers that we use in the result section to analyze BM development.

In the next developmental step, BM NK cells up-regulate the integrin DX5/CD49b and are considered mature (mNK) cells. The hallmark of this phase is the extensive expansion and the acquisition of effector functions such as cytotoxic activity and INFγ production. NK cells then sequentially up-regulate the maturation markers CD11b followed by CD43 and S1PR5, a receptor for sphingosine-1 phosphate that induces their exit from the BM to the periphery (Jenne et al., 2009). During this process CD27, a marker for immature NK cells, is down regulated. Few mNKs in the BM express KLRG1, which correlates with end stage maturation. KLRG1 and CD27 are expressed in a mutually exclusive fashion on NK cells (Huntington et al., 2007). In

the periphery KLRG1 is up-regulated upon homeostatic proliferation or infection and marks cellular senescence (Fig. 2) (Huntington et al., 2007; Robbins et al., 2004).

1.5 NK cell function

NK cells use germline-encoded receptors to distinguish altered or non-self cells from normal host cells. NK cells employ two different categories of receptors: one that delivers inhibitory signals and another that delivers activation signals. NK cells constantly scan the cells of the body and integrate the activating and inhibitory signals that they receive (Barton et al., 1998). Healthy cells express MHC-I molecules that are recognized by specific inhibitory Ly49 (mouse), CD94/NKG2A (mouse or human) or KIR family receptors (human). Moreover, they constitutively express low amounts of self-ligands that are recognized by activating receptors on NK cells. Contact with a healthy cell that expresses MHC class I molecules signals balance and results in self-tolerant NK cells.

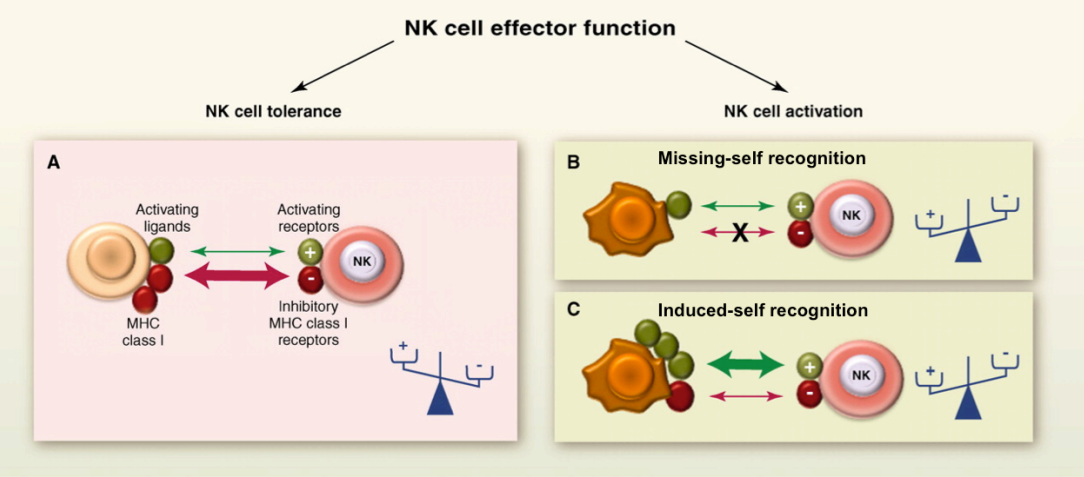


Figure 3. The regulation of NK cell function (adapted from (Vivier et al., 2011)).
NK cells employ two different categories of receptors: one that delivers inhibitory signals and another that delivers activation signals. NK cells constantly scan the cells of the body and integrate the activation and inhibitory signals that they receive. (A) The contact with a healthy cell that expresses self-MHC-I molecules and low amounts of activating ligands leads to an excess of inhibitory signals (bold red arrow) and a self-tolerant NK cell. A cell that is perturbed by viral infection or cellular transformation can either down-regulate MHC-I molecules (B) what is called missing self or up-regulate stress induced activating ligands (induced self) (C). Both situations result in an excess of activating over inhibitory signals what triggers the activation of the NK cell and the subsequent killing of the target cell. +, activating receptors; –, inhibitory receptors. The tilt of the balance indicates if the integration of the inhibitory and activating signals that NK cell receives upon target cell recognition leads to inhibition (tilt to the right) or activation (tilt to the left) of the NK cell.

A cell that is perturbed by viral infection or cellular transformation can either down-regulate MHC-class I molecules and/or up-regulate stress-induced self molecules such as NKG2D ligands. This results in excessive NK cell activation and leads to the killing of the target cell (Karre et al., 1986; Vivier et al., 2004; Vivier et al., 2011).
The lack of MHC class I on a target cell leads to an NK cell activation due to the loss of inhibitory signals. This phenomenon is named missing self-recognition. Conversely, induced self-recognition defines NK cell activation due to strong activating signals triggered by stress induced self-ligands. In this situation the strong activating signals override the inhibitory signals delivered by MHC class I molecules (Fig. 3) (Long et al., 2013; Poggi and Zocchi, 2014).
Mouse NK cells express a variety of MHC class I–specific lectin-like Ly49 inhibitory receptors on their cell surface that function by signaling through intra-cytoplasmic immune-receptor tyrosine-based inhibition motifs (ITIMs) (Karlhofer et al., 1992; Vivier et al., 2004). Ly49 receptors are aquired during BM development in a stochastic, variegated and overlapping manner, and are involved in a process that has been referred to as 'NK cell education'. Mature NK cells that express at least one inhibitory NK cell receptor that is engaged by self-MHC-I molecules show full functional competence (Held, 2013; Held et al., 2011; Orr and Lanier, 2010; Raulet and Vance, 2006). NK cells that do not recognize MHC-I are hypo-responsive. However the Ly49 family of genes also encodes activating receptors like Ly49H, which recognizes the mouse cytomegalovirus (MCMV) protein m157 (Sun et al., 2009). Indeed MCMV clearance is dependent on Ly49H NK cells (Biron et al., 1999).
Other NK cell activating receptors like NKG2D detect self-molecules that are over-expressed upon initiation of cellular distress (Raulet and Guerra, 2009; Smith et al., 2002). Most NK cells express CD16 (FcγRIIIa), an activating low-affinity receptor for the Fc domain of IgG-isotype antibodies. Through CD16, NK cells recognize and kill antibody-coated cells, a process called antibody-dependent cell-mediated cytotoxicity (ADCC).
NK cells start to express high levels of Perforin, GzmB and IFNγ mRNAs during their development in the bone marrow (Fehniger et al., 2007; Stetson et al., 2003). The trigger for the acquisition of this effector program during bone marrow maturation is currently not known, eventhough IL-17 may play a role (Bar et al., 2014). Although naïve NK cells constitutively express high levels of Perforin, GzmB and IFNγ mRNAs the translation into protein does not occur (Fehniger et al., 2007; Stetson et al., 2003).

The translation of preformed Perforin and GzmB mRNA into protein is dependent on NK cell priming by cytokines such as IL-15 (Lucas et al., 2007). Indeed, NK cells in a naïve mouse have poor cytotoxic activity. Early in an immune response, infected or activated pDCs can activate NK cells in a type I IFN-α/β–dependent manner (Miyagi et al., 2007; Schleicher et al., 2007). Activated dendritic cells and macrophages produce the cytokines IL-15 and IL-18 (Degli-Esposti and Smyth, 2005; Newman and Riley, 2007; Walzer et al., 2005). Dendritic cell–mediated IL-15 trans-presentation results in the translation of pre-existing Perforin and GzmB mRNA pools in the NK cell (Chaix et al., 2008; Fehniger et al., 2007; Gordon et al., 2012; Lucas et al., 2007; Stetson et al., 2003).

NK cell killing of target cells is initiated by the pore-forming protein Perforin and executed by the Granzyme family of serine proteases (Held, 2013; Masson and Tschopp, 1987). Mice have 10 different Granzyme genes (A-G, K, M and N) that are localized in clusters on three different chromosomes.

After expression, the mRNA is translated to a pro-GzmB, an inactive precursor protein that carries an N-terminal signal peptide to ensure the packaging into secretory granules. The cleaving of the dipeptide Gly-Glu at the N-terminus of pro-GzmB by the cysteine protease cathepsin C generates the enzymatically active form of GzmB. The principal cytoplasmic inhibitor of GzmB is the serine protease inhibitor 6 (Spi-6) (Ida et al., 2003; Laforge et al., 2006; Phillips et al., 2004; Zhang et al., 2006). Spi-6 prevents the processing of the N-terminal pro-peptide by the protease cathepsin and thereby protects the NK cell from GzmB mediated self inflected apoptosis (Fig. 4). (Caputo et al., 1993; Pham and Ley, 1999; Sun et al., 1997; Zhang et al., 2006).

Granzymes and Perforin are stored within secretory lysosomes were they are kept inactive by low pH and calcium concentrations. Upon target cell recognition lytic granules are polarized towards the immunological synapse, where they fuse with the plasma membrane. Their content is relased into the synaptic cleft between effector and target cell. Subsequently Granzmye B (GzmB) enters the target cells with the help of Perforin, and rapidly induces apoptosis via both, caspase-dependent and caspase-independent mechanisms. Perforin can either directly form pores in the phospholipid bilayer of target cells to facilitate entry of granzymes or alternatively disrupt the membranes of target-cell endosomes that contain Granzymes (Long et al., 2013; Voskoboinik et al., 2006; Wang et al., 1996) (Fig. 4).

The second way by which killer cells induce apoptosis in target cells is via the expression of death ligands like TNF (tumour-necrosis factor), Fas-L and TRAIL (TNF-related apoptosis inducing ligand) that are recognized by death receptors on the target cell. This interaction activates caspase-mediated death pathways in the target cell (Lodolce et al., 1998; Suzuki et al., 1997).

In addition to killing, NK cells produce inflammatory cytokines like INFγ, TNFα and GM-CSF or suppressive cytokines as IL–10 and TGFβ. Further they secrete chemokines, recruiting different effector cells (such as ATAC/lymphotactin, Mig, MIP-1α and RANTES) (Fehniger et al., 2007; Lucas et al., 2007; McCartney et al., 2009; Russell and Ley, 2002).

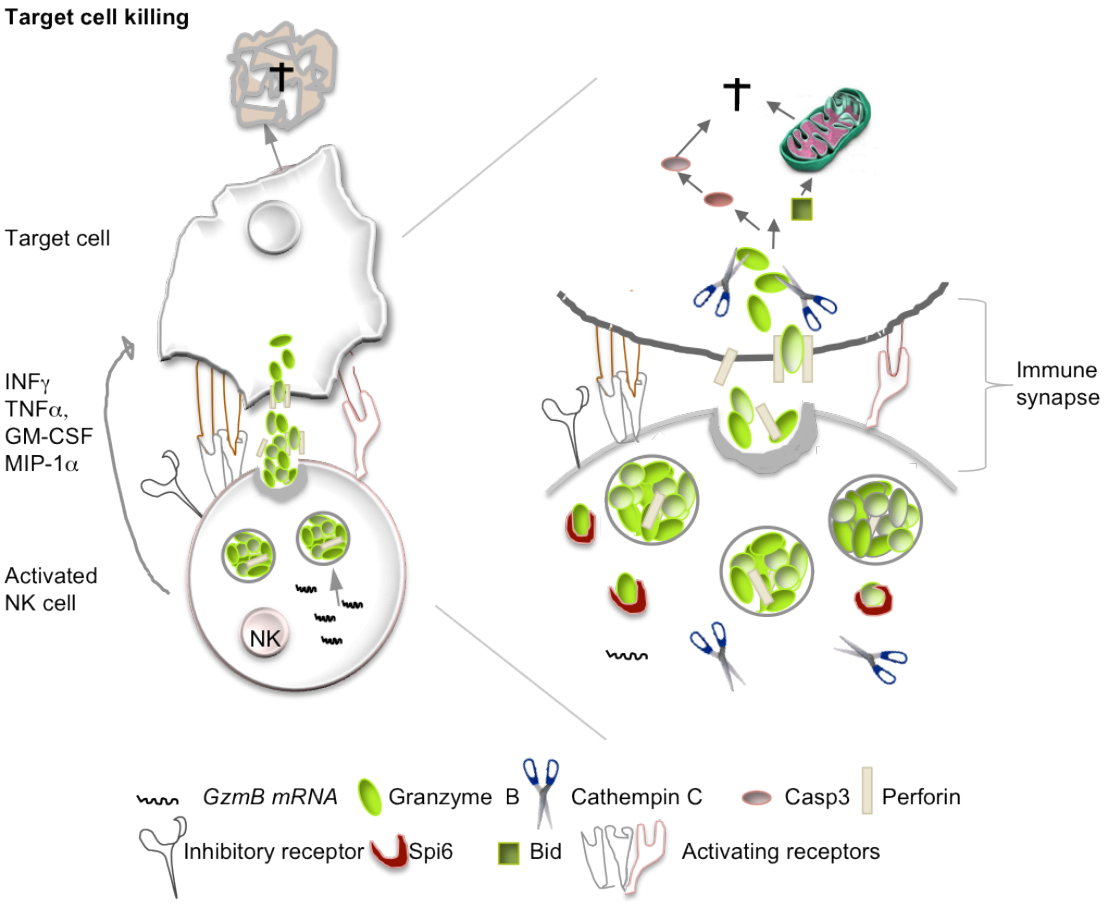


Figure 4. Target cell killing by NK cells.
Following priming by cytokines, NK cells translate pre-formed mRNA for GzmB, INFγ and Perforin (small arrow within the NK cell). Subsequently, target cell recognition and activation triggers the polarization of lytic granules filled with GzmB and Perforin, towards the immunological synapse. There they fuse with the plasma membrane and release their contents into the synaptic cleft between the effector and the target cell. GzmB enters the target cells with the help of Perforin, and rapidly induces apoptosis via caspase-dependent and caspase-independent mechanisms. Pro-GzmB is an inactive precursor protein. Removal of a dipeptide at the N-terminus of pro-GzmB by the cysteine protease Cathepsin C generates the enzymatically active form of GzmB. The principal cytoplasmic inhibitor of GzmB is the serine protease inhibitor 6 (Spi-6). Spi-6 prevents the processing of Pro-GzmB by the protease Cathepsin C. This mechanism protects the NK cell from GzmB mediated self inflected apoptosis. A process that we will discuss in detail in this thesis.

1.6 Cytokines in NK cell development and function

The NK cell committed progenitors up-regulate the IL-15 receptor complex, consisting of the IL-15 receptor α (IL-15Rα), IL-2Rβ (CD122), and the IL-2 receptor common γ chains (IL-2Rγc). This receptor complex is making the progenitor cells responsive to IL-15, a cytokine necessary for NK development, survival and proliferation. Mice that lack IL-15 or its high affinity receptor (IL-15Rα) display dramatic defects in NK cell development and lack peripheral NK cells. Similar defects have been observed for mice lacking the activator for the transcription of STAT5 a downstream signaling component of the IL-15 receptor (Eckelhart et al., 2011; Kennedy et al., 2000; Suzuki et al., 1997). During homeostasis IL-15 plays a major role to ensure the survival and proliferation of peripheral NK cells, via the maintenance of the antiapoptotic factors Bcl-2 and Bcl-X_L (Ranson et al., 2003). Developing NK cells express the cytokine receptor Fms-like tyrosine kinase-3 (FLT3). NK cell development and NK cell effector functions are impaired in mice that lack the *Flt3* ligand (Cheng et al., 2009; McKenna et al., 2000). Recently it has been shown that IL-17Rα is expressed in NKPs and iNK cells, but not in the mNK cells in the BM. Mice that lack the IL17Rα have normal NK cell counts in the BM and spleen. Upon priming IL17Rα deficient NK cells up-regulate CD69 but they show a defect in their ability to produce IFNγ. Furthermore their cytotoxic activity towards YAC-1 target cells is strongly impaired. NK cells that develop in the absence of IL-17 are unable to clear the mouse cytomegalovirus (MCMV) (Bar et al., 2014). Thus IL-17Rα signalling in NK cell precursors is a crucial trigger for the aquisition of their effector potential.

1.7 Transacting factors in NK cell development and function

The maturation of NK cells is governed by different transcription factors. The interplay between these transacting factors is still poorly understood and additional factors are likely involved. In the following part some of the most important transcription factors involved in NK cell development and function are discussed (Fig. 5 and 6).

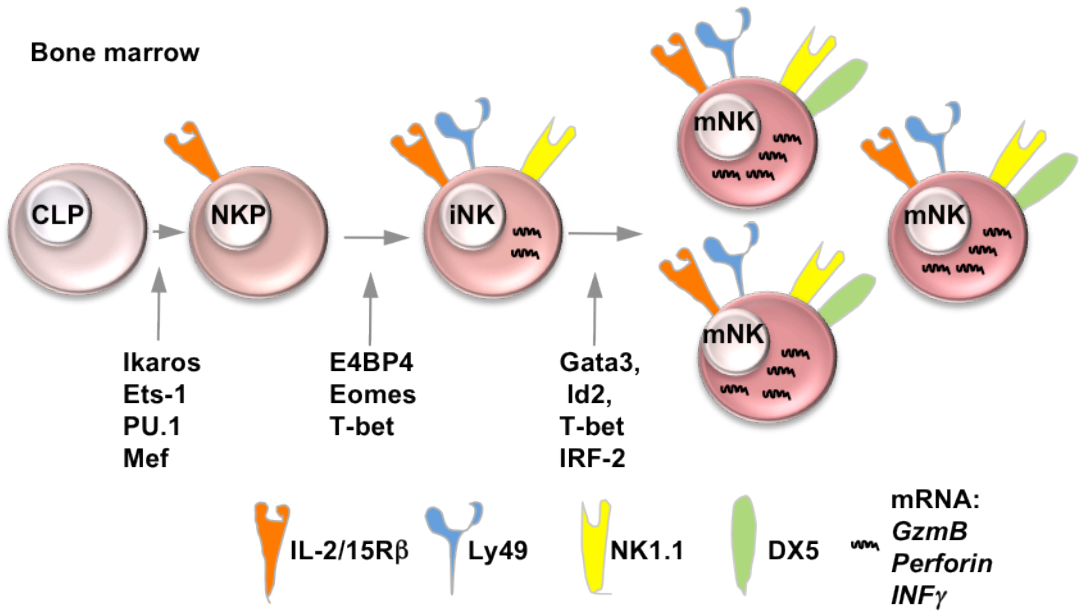


Figure 5. Transcription factors in NK cell development.
Arrows indicate the stages where the transcription factors play a crucial role. CLP, common lymphoid precursors, NKP, NK cell precursors; iNK, immature NK cell; mNK, mature NK cells (compare Fig. 2).

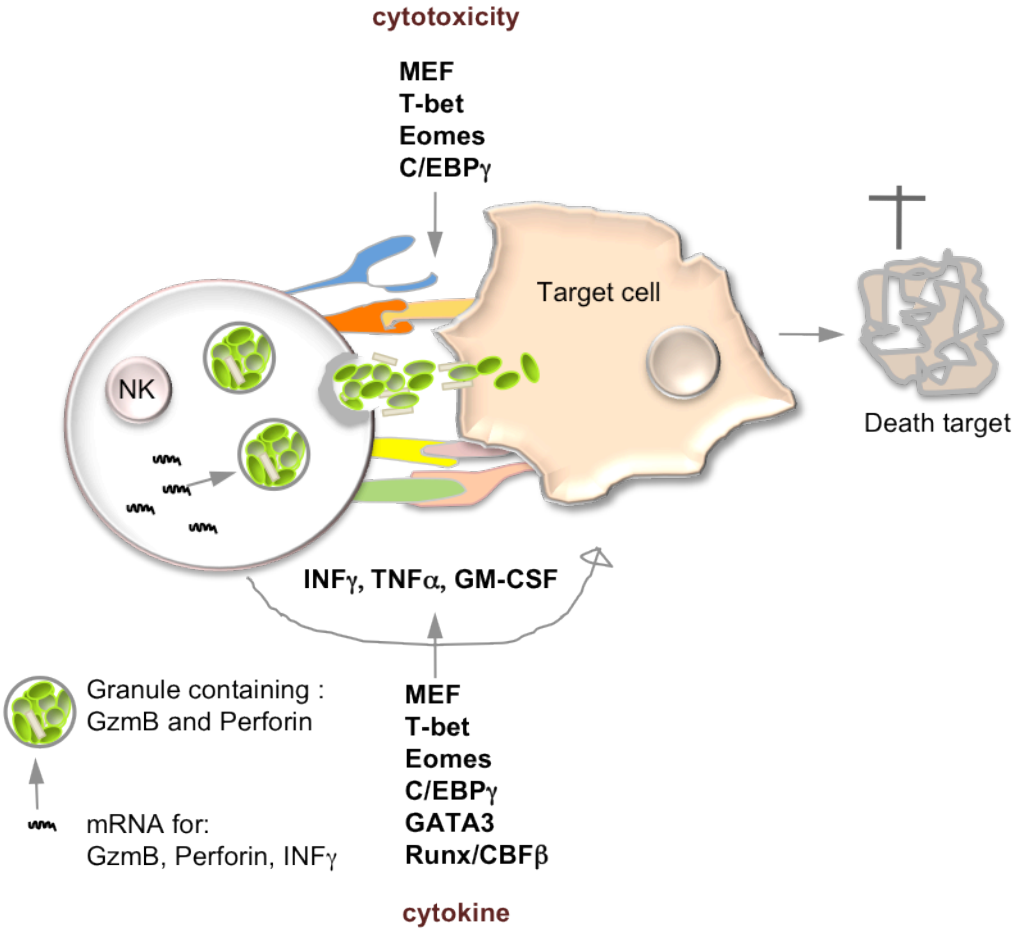


Figure 6. Transcription factors in NK cell function.
Indicated are the transcription factors that play an essential role for cytokine production or cytotoxicity during the process of target cell killing (compare Fig. 4).

1.7.1 Ikaros

The zinc finger transcription factor Ikaros is crucial for the differentiation of HSC into lymphoid progenitor cells and for their maintenance (Arranz et al., 2012; Yoshida et al., 2006). Ikaros-deficient mice do not express the cytokine receptor Flt3 and express reduced levels of the stem cell growth factor receptor c-kit. Ikaros-deficient precursors fail to express CD122, which is crucial for IL-15 responses. Consequently, Ikaros deficient mice do not possess fetal T, B and NK cells and are deficient for adult T cells and NK cells (Wang et al., 1996).

1.7.2 Ets-family transcription factors: Ets-1, PU.1 and Mef

The winged helix-turn-helix transcription factors Ets-1, PU.1 and the myocyte enhancer factor (Mef) play an important role for NK cell development during the NKP to iNK transition and beyond. Mature NK cells are strongly reduced and not functional in mice deficient for any of those transcription factors (Barton et al., 1998; Colucci et al., 2001). NK cell development in Ets-1 deficient mice is blocked at the iNK cell stage or earlier as BM mNK cells are absent. Ets-1 deficient NK cells are not able to kill target cells like RMAS or YAC (Barton et al., 1998). Mef-deficient NK cells have a defect in INFγ production. Furthermore, they express reduced levels of Perforin. Mef can bind to the Perforin locus and induce its expression. As a consequence the NK cells that lack Mef show strongly reduced effector functions. PU.1 deficient mice kill YAC-1 targets but are defective in their ability to proliferate. PU.1 deficient NK cells do not expand after IL-2 stimulation due to a cell intrinsic cell cycle defect (Barton et al., 1998; Colucci et al., 2001).

1.7.3 Id proteins and repression of E-box proteins

Id2 belongs to the inhibitor of DNA-binding (Id) protein family which contains a helix-loop-helix (HLH) dimerization domain but lacks a basic DNA-binding domain. Id proteins can bind to E proteins that contain a basic DNA-binding region adjacent to a HLH dimerization domain and thereby prevent E proteins from binding to DNA and activating target genes (Kee, 2009). Regulation of the E and Id protein ratio allows the control of differentiation programs. In the absence of Id2, peripheral NK cells are strongly reduced and they have decreased effector function capability (Yokota et al., 1999). The function of Id proteins in NK cell differentiation is to repress E proteins at the NKP and mNK stage. Over-expression of E proteins inhibits NK cell development

whereas the elimination of E proteins in Id2 deficient mice restored NK cell development. The role of Id proteins in NK cell precursors might be to suppress the expression of other lineage programs and thereby promote NK cell development (Ji et al., 2008; Quong et al., 2002).

1.7.4 E4bp4

The transcription factor E4-binding protein 4 (E4bp4), encoded by the *Nfil3* gene, is highly expressed during the progression from the NKP to iNK and iNK to mNK stages (Gascoyne et al., 2009; Male et al., 2011). E4bp4-deficient mice have a severe NK cell deficiency whereas T and B cell numbers are normal. The NK cell development in E4bp4-deficient mice is blocked at the NKP to iNK transition with the consequence that the number of iNK and mature NK cells are drastically reduced (Gascoyne et al., 2009; Kamizono et al., 2009). The NK cell differentiation defect is cell intrinsic, as differentiation was not rescued when E4bp4-deficient bone marrow was transplanted into irradiated wild-type recipients (Gascoyne et al., 2009; Male et al., 2011). NK cells that lack E4bp4 express lower levels of the activation receptors 2B4, Ly49D, and Nkp46 and the integrin CD11b. Moreover, they have a defect in their ability to produce INFγ and to kill target cells.

E4bp4 induces the expression of inhibitor of DNA-binding 2 (Id2), which is essential for the subsequent iNK-mNK transition (see above). Reconstitution of E4bp4-deficient mice with ectopically expressed Id2 restores the development of NK cells (Boos et al., 2007; Gascoyne et al., 2009; Yokota et al., 1999). Despite the near absence of NK cells, these mice do not manifest increased development of spontaneous cancers or other immune system disorders (Gascoyne et al., 2009).

1.7.5 T-bet and Eomes

T-bet deficiency leads to a reduction in the numbers of mNK cells. It has been shown that T-bet controls the repression of the immaturity markers CD27 and c-kit and the induction of CD43 and KLRG1 by NK cells. The lack of T-bet leads to a strong increase of iNK cell number in the bone marrow. Peripheral NK cell numbers are strongly reduced with the exception of lymph nodes where the frequency of NK cells is elevated (Townsend et al., 2004). The altered distribution of NK cells relies on the fact that the absence of T-bet leads to a reduced expression of S1P5. S1P5 belongs to a receptor family that controls lymphocyte trafficking and governs the egress of NK

cells from BM and lymph nodes. S1P5 is a direct target of T-bet (Jenne et al., 2009). NK cells display increased basal rates of proliferation and apoptosis in the absence of T-bet. This might be an additional cause for the reduced numbers of NK cells found in the periphery. T-bet represses CD27 and c-kit and induces CD43 and KLRG1. Moreover, adult T-bet deficient mice lack a population of liver NK cells that expresses the death receptor TRAIL (DX5-TRAIL^{+}) (Gordon et al., 2012).

In contrast to T-bet deficient mice, where DX5^{+} mNK cells can develop, Eomes deficient mice lack mature NK cells that are defined by high expression of DX5. Further analysis revealed that bone marrow NK cell development is blocked at the iNK stage.

In the absence of both, T-bet and Eomes, mice were nearly devoid of mature NK cells. Both lineages depend on IL-15 and it has been shown that T-bet and Eomes cooperate to induce high expression of CD122 (Gordon et al., 2012). Eomes and T-bet double-deficient mice have a normal NKP population, however, they are not able to acquire the NK cell lineage markers NK1.1 and NKp46 and to differentiate into mature NK cells. Experiments with bone marrow chimeras have confirmed that the observed effects for T-bet and Eomes are cell intrinsic.

A recent study suggests the existence of two alternative pathways of NK cell development occurring in the BM and in the liver. An Eomes-GFP reporter has shown that Eomes-GFP^{-} NK cells preferentially home to the liver, whereas Eomes-GFP^{+} NK cells mainly home to the spleen. Further experiments revealed that NK cell precursors in the bone marrow express low levels of T-bet, which allows their development into mature Eomes^{+}Trail^{-} NK cells. Reciprocally, the liver environment permits high expression of T-bet early during NK cell development, which results in Eomes repression and instructs the development of Eomes^{-} Trail^{+} DX5^{-} Itga1^{+} NK cells. These NK cells mainly produce the cytokines IL-2 and TNF and may have specific immunoregulatory functions that are crucial for the local protection of the liver. The mutually exclusive expression of T-bet and Eomes in NK cell precursors is thought to drive NK cell lineages with complementary functions (Daussy et al., 2014).

In the absence of T-bet, susceptibility to metastatic cancer, including melanoma is increased. Most likely this is due to the combined effects of reduced cytotoxicity, homing ability and IFNγ production (Werneck et al., 2008). However T-bet-deficient NK cells still are able to express some INFγ and Perforin and they are only mildly defective in killing target cells (Aberle et al.).

1.7.6 GATA-3

The zinc-finger transcription factor GATA-3 binds the consensus (A/T)GATA(A/G) motif and can activate or repress transcription. Peripheral NK cell counts in the spleen and the blood of GATA-3 deficient mice are normal but hepatic and thymic $CD127^+$ NK cells do not develop (Samson et al., 2003). GATA-3 plays a crucial role during the final maturation of mNK cells. In the absence of GATA-3, mNK cells expand normally but they show reduced expression of the maturation markers CD11b, CD43 and KLRG1. GATA-3 deficient NK cells show normal target cell killing. However they have a reduced ability to produce INFγ (Samson et al., 2003).

1.7.7 Runx proteins

The transcription factor Runt-related transcription factor 3 (Runx3) and its binding partner Core-binding factor subunit β (CBFβ) are expressed during the NK cell development from the NKP stage onwards. CBF can both, activate and repress target genes by forming complexes with other transcription factors and by recruiting histone acetyltransferases and histone deacetylases. Mice deficient for Runx3 are embryonically lethal. *Ohno et. al.* produced transgenic (Tg) mice that express the Runt domain under the control of the CD2 promoter. In contrast to the full-length Runx the truncated form Runt contains the domain for both DNA binding and hetero-dimerization with CBFβ, but lacks the trans-activating domain. This dominant negative form binds with a higher affinity to CBFβ and the DNA target sequence than to full-length Runx proteins. Mature NK cells in transgenic mice showed reduced expression of the maturation markers CD11b and CD43 and of the Ly49 receptors. IFNγ production was greatly enhanced in these mice. They show normal cytotoxicity against YAC-1 target cells. Knock-in mice that express Runx3 together with a fluorescent protein showed expression from the NKP stage onwards (Levanon et al., 2014; Ohno et al., 2008).

1.7.8 IRF-2

The interferon regulatory factor (IRF) plays an important role for the survival of mNK cells in the bone marrow. In IRF-2 deficient mice $DX5^+$ NK cells are generated, but they fail to acquire CD11b and to expand due to accelerated apoptosis. As a consequence the peripheral NK cell numbers are strongly reduced. However these

NK cells do not show a defect in cytotoxicity and they express normal levels of the Ly49 receptor (Lohoff et al., 2000; Taki et al., 2005).

1.8 Tcf/Lef transcription factor family

Our laboratory has discovered that peripheral NK cell numbers are reduced in the absence of T cell factor-1 (Tcf-1) (Held et al., 1999). This suggests a role for this trans-acting factor in NK cell development. In vertebrates the Tcf/Lef family comprises 4 members: Lef-1, Tcf-1 (*Tcf7*), Tcf-3 and Tcf-4 (Arce et al., 2006; Archbold et al., 2012). Tcf-1 was discovered as a T-cell-specific transcription factor that binds to the CD3ϵ enhancer (Oosterwegel et al., 1991b). Lef-1 was cloned from early B cells by a subtraction strategy and was shown to bind and activate the T-cell receptor α enhancer (Travis et al., 1991; Waterman et al., 1991). Tcf-1 and Lef-1 contain a HMG (high mobility group) box (van de Wetering et al., 1991), which binds a specific DNA sequence in the minor groove of the double helix. Like other HMG box-containing transcription factors Tcf-1 and Lef-1 enforce a bend between 90° and 127° in the DNA helix facilitating the formation of large nucleoprotein complexes (Giese et al., 1995; Love et al., 1995). Tcf-1/Lef-1 possess no intrinsic ability to modulate transcription but are usually associated with a transcriptional repressor (Groucho (Gro) in Drosophila, Groucho-related gene in mouse or transducin-like enhancer protein in humans) that is bound to a central domain of Tcf-1/Lef-1 and inhibits the expression of target genes (Cavallo et al., 1998). A long isoform of Tcf-1 (termed p45), which is transcribed from a distal promoter, contains a β-catenin interaction domain at its N-terminus. Association with β-catenin leads to replacement of Gro and the transcriptional activation of target genes. The short isoform of Tcf-1 (termed p33) is transcribed from a proximal promoter, lacks the β-catenin binding domain and may have dominant-negative effects (Fig. 7). In postnatal mice Tcf-1 is mainly expressed by lymphoid cells and regulates the survival, proliferation and differentiation of these cells (Ioannidis et al., 2001; Reya et al., 2003; Verbeek et al., 1995). Tcf-1 is highly expressed in T cells, innate lymphoid cells and NK cells whereas Lef-1 is expressed by T cells and by pre-B cells.

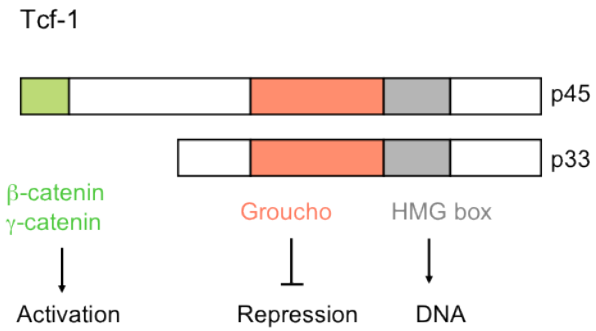


Figure 7. Different isoforms of Tcf-1
Shown are the Tcf-1 isoforms named p33 and p45. They derive from alternative promoter usage and alternative splicing. Both isoforms contain a HMG (high mobility group) box (grey), which binds DNA via a specific consensus sequence and a central Groucho binding domain (red). The Tcf-1 isoforms possess no intrinsic ability to modulate gene transcription but they are usually associated with Groucho that represses target genes. The long p45 isoform of Tcf-1 contains a β/γ-catenin interaction domain at the N-terminus (green). Binding of β/γ-catenin to the N-terminus of p45 leads to the replacement of Groucho and the activation of Wnt target genes.

1.9 The canonical Wnt pathway

Tcf/Lef are nuclear effectors of the canonical Wnt signaling pathway. Wnt signaling is initiated by secreted lipid-modified glycoproteins called Wingless/Integration-1 (Wnt) (Willert et al., 2003) that bind to a cell surface receptor complex composed of the frizzled receptor (Fz) together with the lipoprotein receptor-related proteins LRP6 and LRP5. In the absence of a Wnt ligand β-catenin is sequestered in a multi-protein complex and phosphorylated by the glycogen-synthase kinase 3 β (GSK3β). Phosphorylated β-catenin is subsequently ubiquitinylated by the β-transducin-repeat-containing protein (β-TRCP) and targeted for proteosomal degradation. In contrast, the binding of a Wnt ligand to the Fz/LRP receptor leads to the disruption of the β-catenin degradation complex (Mao et al., 2001a; Mao et al., 2001b; Tolwinski et al., 2003). Consequently β-catenin is stabilized and can translocate to the nucleus, where it binds to the N-terminus of the Tcf/Lef family members (Fig. 8). This association induces the transient transcription of Tcf/Lef target genes such as c-myc, cyclin D1 and Axin2 (conductin) (Kikuchi et al., 2006).

The canonical Wnt signaling pathway play essential roles during embryonic development especially for the body axis formation and for the development of the mesoderm that gives rise to the hematopoietic system. Furthermore, this pathway is critically involved in the maintenance of self-renewing tissues including the skin, the

gut, hair follicles and hematopoietic stem cells. Aberrant or constitutive Wnt signaling is the cause of various cancers like colon cancer (see below) (Reya and Clevers, 2005).

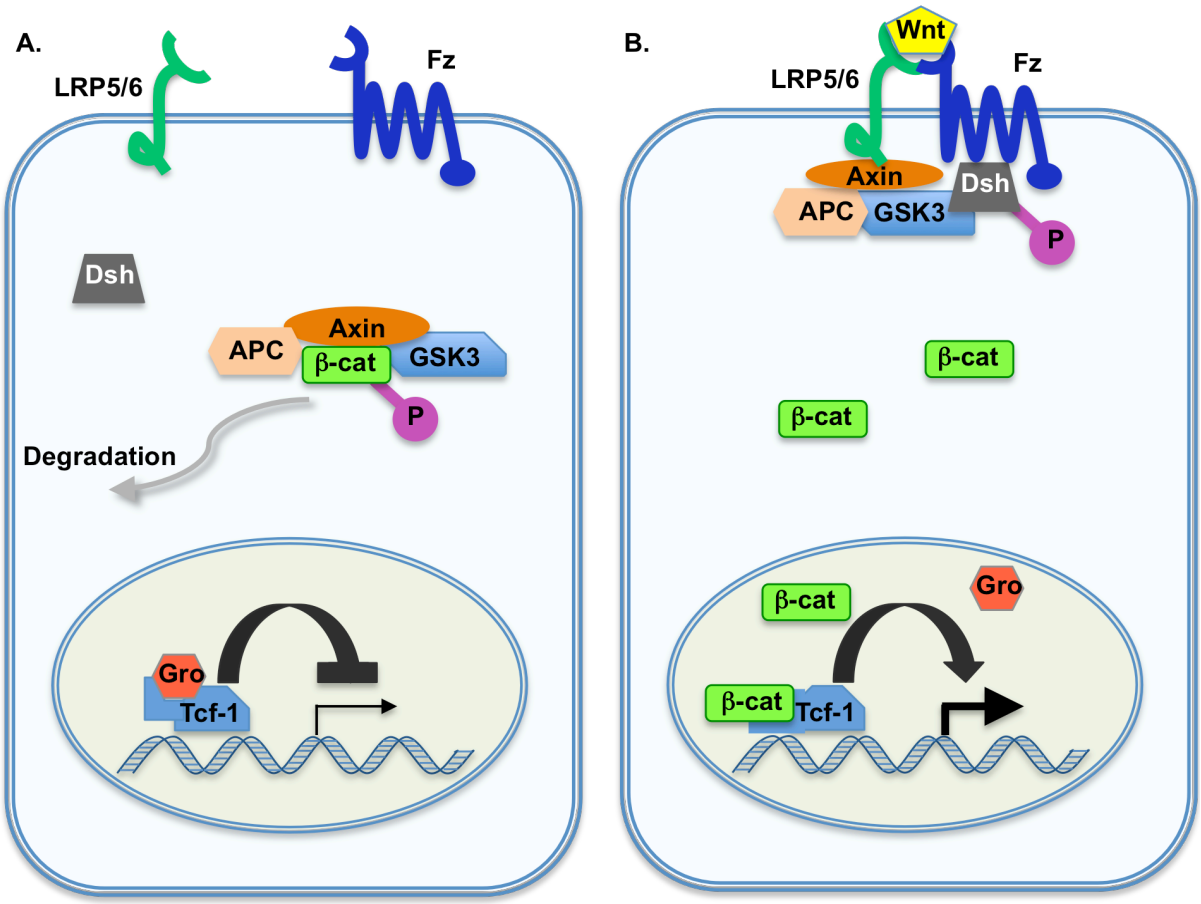


Figure 8. Canonical Wnt pathway.
(A) When Wnt is not present, β-catenin is phoyphorylated by a complex formed by adenomatous polyposis coli (APC), glycogen-synthase 3β (GSK3β), Axin and other proteins. Subsequently β-catenin is tagged with ubiquitin for proteasomal degradation. The nuclear effector of canonical Wnt signaling Tcf-1 is associated with Groucho (Gro) what leads to target cell repression. (B) Wnt binding to its receptors low-density-lipoprotein-receptor related protein 5/6 (LRP5/6)/Frizzled results in the inactivation of GSK3 by Dishevelled (Dvl). Stabilized β-catenin migrates to the nucleus where it binds to the N-terminus of Tcf-1 in order to activate target genes.

1.10 The Wnt pathway and cancer

Constitutive activation of the Wnt signaling pathway due to mutations in the multi-domain protein APC or β-catenin is a hallmark of colon cancer. In the absence of Wnt signaling, APC together with GSK-3β target β-catenin for degradation by the proteasome. However in APC or β-catenin mutant colon cells, degradation is disrupted and levels of free β-catenin rise dramatically. Free β-catenin can

translocate to the nucleus, where it engages DNA-bound Tcf/Lef transcription factors by binding to the N-terminus. This leads to the replacement of the repressor Groucho (Gro) and the constitutive activation of genes involved in proliferation and/or in antagonizing apoptosis (Korinek et al., 1997; Morin et al., 1997; Rubinfeld et al., 1997) (Figure 8, above).

Furthermore, the removal of *Tcf7* leads to the development of adenomas in the gut and mammary glands of mice. Moreover *Tcf7* deficient mice develop thymomas that are characterized by high *Lef-1* mRNA expression. In colon cells the isoform of Tcf-1 that is lacking the β-catenin interaction domain is expressed at much higher levels than the full-length form of Tcf-1. It has been suggested that the loss of the short Tcf-1 isoform is the cause for the development of adenomas in the gut and mammary glands of *Tcf7* defidient mice (Roose et al., 1999). Thus Tcf-1 might also function as a tumor suppressor.

1.11 Role of Tcf-1 and the Wnt pathway for lymphocyte development and function

A Tcf-1 deficiency results in partial blocks at multiple stages of T cell development. These T cell development stages can be followed by the sequential expression of cell surface markers: the double negative (DN)1 ($CD4^-CD8^-CD44^+CD25^-$) to the DN2 ($CD4^-CD8^-CD44^+CD25^+$) transition, the DN3 ($CD4^-CD8^-CD44^-CD25^+$) to DN4 ($CD4^-CD8^-CD44^-CD25^-$) transition as well as the immature single positive (ISP) to the double positive $CD4^+CD8^+$ (DP) transition (Goux et al., 2005; Ioannidis et al., 2001; Jeannet et al., 2008b; Schilham et al., 1998; Verbeek et al., 1995). The developmental blocks caused by Tcf-1 deficiency coincide with stages of extensive cell proliferation (DN2 and DN4). Thymocytes lacking Tcf-1 normally produce and express a functional pre-T cell receptor (TCR). Subsequently *Tcf7* deficient DN3 cells proliferate efficiently, however icTCRβ$^+$ DN4 cells do not accumulate due to rapid cell death (Goux et al., 2005). DP cells of Tcf-1 deficient mice undergo accelerated cell death due to reduced levels of the anti apoptotic molecule Bcl-xL. Even though the survival of DP thymocytes was rescued in Bcl-2−Tg *Tcf7*$^{-/-}$ mice, the thymus cellularity did not improve (Ioannidis et al., 2001). The ®-catenin/Tcf-1 pathway enhances DP thymocyte survival in a RORγt-dependent manner (Wang et al., 2011).

The ®-catenin/Tcf-1 acts genetically upstream of the transcription factor RORγt that up-regulates Bcl-x_L to increase the lifespan of DP cells (Sun et al., 2000; Xie et al., 2006). Taken together, Tcf-1 is required for the survival of proliferation of thymocytes at the preTCR stage and for the survival of differentiating DP thymocytes. The mechanims through which Tcf-1 regulates these processes might be different. A genetic complementation approach showed that the N-terminal β-catenin-binding domain of Tcf-1 (amino acids 1-117) was required to rescue thymocyte development in *Tcf7* deficient mice (Ioannidis et al., 2001). Importantly, T cell development is normal in the combined absence of β- and γ-catenin (Cobas et al., 2004; Jeannet et al., 2008b; Koch et al., 2008). That suggests the existence of either an additional Wnt independent role for Tcf-1 or the presence of an additional transmitter of canonical Wnt signals. Indeed it has been suggested that the transcription factor activating transcription factor 2 (ATF2) can mediate Wnt signaling in leukemia and in lymphoma cells by associating with the N-terminus of Tcf-1 and Lef-1 (Grumolato et al., 2013).
The expression of Tcf-1 is strongly induced upon activation of Notch signaling in early thymic progenitors (ETP) and Tcf-1 subsequently drives the T cell specification (Germar et al., 2011; Weber et al., 2011).

1.11.1 Role of Tcf-1 in innate lymphoid cell development

Tcf-1 is essential for the development and/or the differentiation of several innate lymphocyte subsets including NK cells, ILC2 and NKp46$^+$ ILC3 (Held et al., 2003; Mielke et al., 2013; Yang et al., 2013), innate lymphocytes that lack Ag-receptors but functionally match subsets of T helper cells (Spits and Cupedo, 2012). As mentionned above Tcf-1 deficiency results in a reduced number of peripheral NK cells (Held et al., 2003; Held et al., 1999). NK cells, LTis and ILC share a common progenitor (IL-7Rα$^+$α4β7^{high}) but the subsequent lineage specific developmental commitment is distinct. Tcf-1 expression in ILC2 and NKp46$^+$ ILC3 seems to be Notch dependent (Lee et al., 2012; Ribeiro et al., 2010). There is no evidence for a role of Notch in NK cell development (Ribeiro et al., 2010). Thus the exact role of Tcf-1 for NK cell development remains to be defined.

1.11.2 Role of Tcf-1 in T cell function

CD8$^+$ T cells lacking Tcf-1 mount a normal primary response to infection with lymphocytic choriomeningitis virus (LCMV). However, CD8$^+$ LCMV immune T cells

from Tcf-1$^{-/-}$ mice fail to expand upon secondary infection. Thus Tcf-1 is essential for the generation of functional CD8 T cell memory. A corresponding defect is observed in the absence of β- and γ-catenin, suggesting that memory formation depends on Tcf-1 function in the context of the Wnt pathway (Jeannet et al., 2010). Furthermore it has been shown that Tcf-1 and β-catenin positively regulate GATA-3 expression in TCR-activated CD4$^+$ T cells. This led to an increased IL-4 production by these activated T cells resulting in the subsequent polarization into T helper type 2 (Th2) cells (Yu et al., 2009). A further a role for Wnt/β-catenin signaling in mature T cells is demonstrated by experiments using CD4$^+$CD25$^+$ regulatory T (Treg) cells that have been transduced with stable β-catenin. These cells survived longer *in vitro* and outcompeted control Treg cells *in vivo* (Ding et al., 2008). Endothelial cell derived Wnt proteins have been shown to mediate the up-regulation of matrix metalloproteinase 2 (MMP2) and MMP9 in effector T cells and increase T cell transmigration. This process could be inhibited *in vitro* and *in vivo* by a soluble form of the Wnt receptor Fz5 (sFz5-Fc) (Wu et al., 2007). Further Tcf-1 is required to epigenetically maintain the IL-17 gene locus in a repressed state. In the absence of Tcf-1 the chromatin of the IL-17 locus is less dense, resulting in a high potential for differentiation of T cells into Th-17 cells (Ma et al., 2011). Taken together these studies show that Tcf-1 plays an essential role to modulate T cell function.

1.12 Aim

The transcriptional network that controls NK cell development and function is incompletely understood. The aim of my thesis was to define whether Tcf-1 plays a role in NK cell development, homeostasis and/or function and to determine whether Tcf-1 acts in the context of the canonical Wnt signaling pathway to mediate some or all of its functions. Taken together the aim of my thesis was to obtain novel insights into the control of the development and the function of NK cells.

2 Results

2.1 Canonical Wnt signaling and Tcf-1 expression during NK cell development

The first aim was to test if and when the T cell factor-1 (Tcf-1, encoded by the *Tcf7* gene) is expressed during natural killer (NK) cell development and if we can detect Wingless/Integration-1 (Wnt) signal transduction. By intracellular FACS we could not detect Tcf-1 expression in the NK cell precursor (NKp) cells. However Tcf-1 was abundantly expressed in immature NK (iNK) cells and slightly less in mature NK (mNK) cells compared to *Tcf7*$^{-/-}$ controls (Fig. 9A). The lymphoid enhancer factor-1 (Lef-1) was absent in all three developmental stages (Fig. 9B). Furthermore, we detected Tcf-1 in a subset of splenic NK cells from naïve mice whereas Tcf-1 levels were reduced in NK cells from Polyinosinic:polycytidylic acid (Poly IC) primed mice (Fig. 9C). Lef-1 was absent in naïve and primed NK cells (Fig. 9D). Therefore we conclude that during bone marrow development Tcf-1 levels peak at the iNK stage and are maintained in mNK cells. Upon activation by Poly IC the Tcf-1 levels decrease.

To determine whether NK cells receive canonical Wnt signals during development, we measured the expression of conductin/axin2. Conductin is induced in response to Wnt signals (Jho et al., 2002; Lustig et al., 2002). Moreover, conductin is a direct Tcf-1 target in CD8^{+} T cells (Zhou et al., 2010). We used mice in which the endogenous open reading frame of one conductin allele was replaced with a bacterial β-galactosidase reporter gene (termed conductin^{LacZ}). B-galactosidase activity was used to measure Wnt signal transduction at the single cell level *ex vivo*. While we detected significant β-galactosidase activity in T cells but not in B cells (Fig. 10A), we failed to detect conductin expression in NKp cells. We detected strong reporter activity in the iNK population and maintenance of the signal in a subpopulation of mNK cells (Fig. 10B). Similar to bone marrow (BM) mNK cells, we detected significant β-galactosidase activity in a subset of splenic NK cells (Fig 10A and B). Importantly the conductin signal was entirely dependent on Tcf-1 both in splenic as well as in BM mNK cells (Fig. 10B). Concordant with Tcf-1 expression, Wnt signaling

is induced during the NKp to iNK transition and is maintained in a subpopulation of mNK cells.

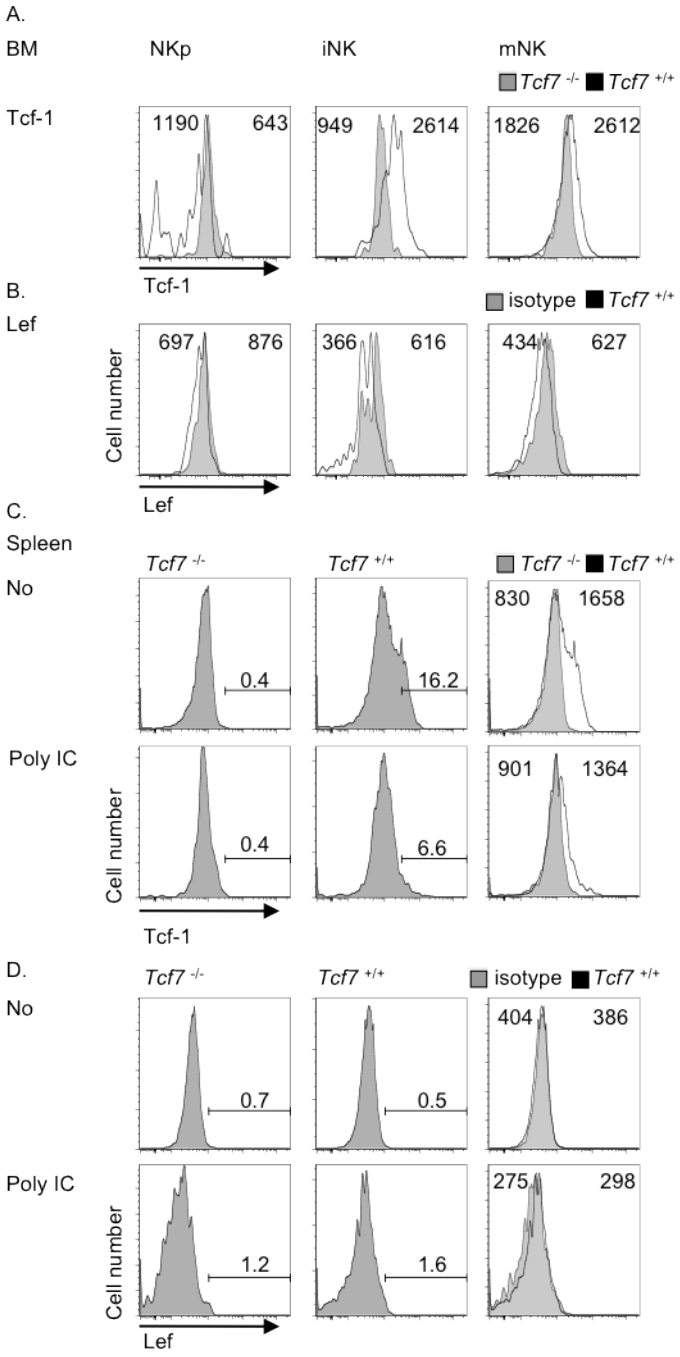


Figure 9. Tcf-1 is expressed in developing and mature NK cells.
In A and B it was gated on BM NKp ($CD3^-IL\text{-}2/15R\beta^+NK1.1^-DX5^-$), iNK ($CD3^-IL\text{-}2/15R\beta^+NK1.1^+DX5^-$) and mNK ($CD3^-IL\text{-}2/15R\beta^+NK1.1^+DX5^+$) cells and in C and D on splenic NK cells ($CD3^-NK1.1^+$). Histograms show Tcf-1 (A and C) or Lef-1 expression (B and D) in $Tcf7^{+/+}$ NK cells (black line) as compared to controls (filled grey) (i.e. $Tcf7^{-/-}$ NK cells in A and C and isotype controls in B and D. Histograms in C and D depict Tcf-1 (C) or Lef-1 (D) expression in either naïve (upper row) or Poly IC primed (lower row) splenic $Tcf7^{+/+}$ NK cells ($CD3^-NK1.1^+$) as compared to controls (filled grey) (i.e. $Tcf7^{-/-}$ NK cells in C and an isotype control D.

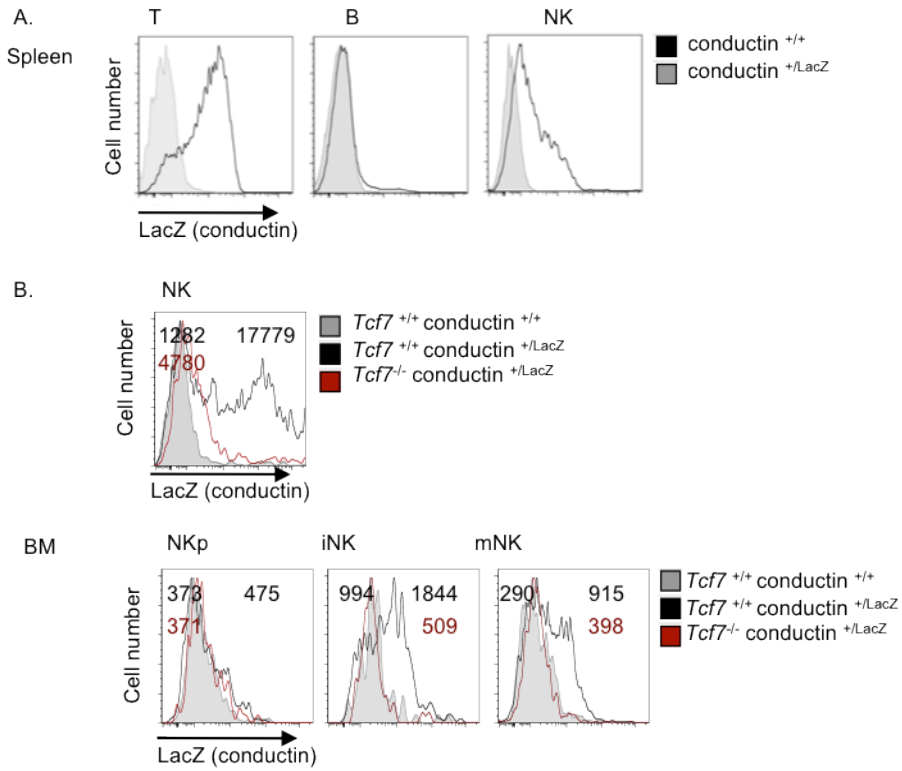


Figure 10. Wnt signal transduction in NK cells
A. Histograms show β-galactosidase (conductin^{LacZ}) activity in gated T, B and NK cells in the spleen. Overlays represent background staining using conductin$^{+/+}$ cells (grey shad) as compared to the signal obtained using cells form conductin$^{+/LacZ}$ cells (black line) B. Histograms show conductin^{LacZ} expression in splenic NK CD3$^-$ NK1.1$^+$ cells and in bone marrow NKp (IL-2/15Rβ$^+$CD3$^-$DX5$^+$NK1.1$^-$), iNK (IL-2/15Rβ$^+$CD3$^-$DX5$^+$NK1.1$^-$) and mNK (IL-2/15Rβ$^+$CD3$^-$DX5$^+$NK1.1$^+$) cells. Overlays represent background staining using *Tcf7*$^{+/+}$ conductin$^{+/+}$ cells (grey shad) as compared to *Tcf7*$^{+/+}$ conductin$^{+/LacZ}$ cells (black line) and *Tcf7*$^{-/-}$ conductin$^{+/LacZ}$ cells (red line). Numbers indicate the mean fluorescence intensity (MFI) of LacZ staining of the indicated populations of cells. Shown is one representative experiment out of at least 3.

2.2 An essential role of Tcf-1 for NK cell development

Based on the finding that Tcf-1 is strongly expressed during the iNK to mNK transition we next addressed whether NK cell development is affected by the loss of Tcf-1. Detailed analyses of hematopoiesis and lymphopoiesis showed that *Tcf7*$^{-/-}$ mice harbor a normal LSK (lineage$^-$ Sca-1$^+$ c-Kit$^+$) compartment, which includes hematopoietic stem cells (HSC). In addition, the number of CLPs (Common Lymphoid Progenitors, lineage$^-$ c-Kit$^+$ IL-7Rα$^+$) is not altered (not shown). The number of NKp and iNK cells was not significantly different as compared to control mice. In contrast, mNK cell numbers were considerably reduced (7 fold) in *Tcf7*$^{-/-}$ as compared to control mice (Fig. 11A and D) as reported earlier (Held et al., 1999;

Ioannidis et al., 2003). Splenic and liver NK cells were decreased 3 fold (Fig. 11 B, C and E). Taken together, we found that Tcf-1 deficiency partially blocks NK cell development at the mNK stage.

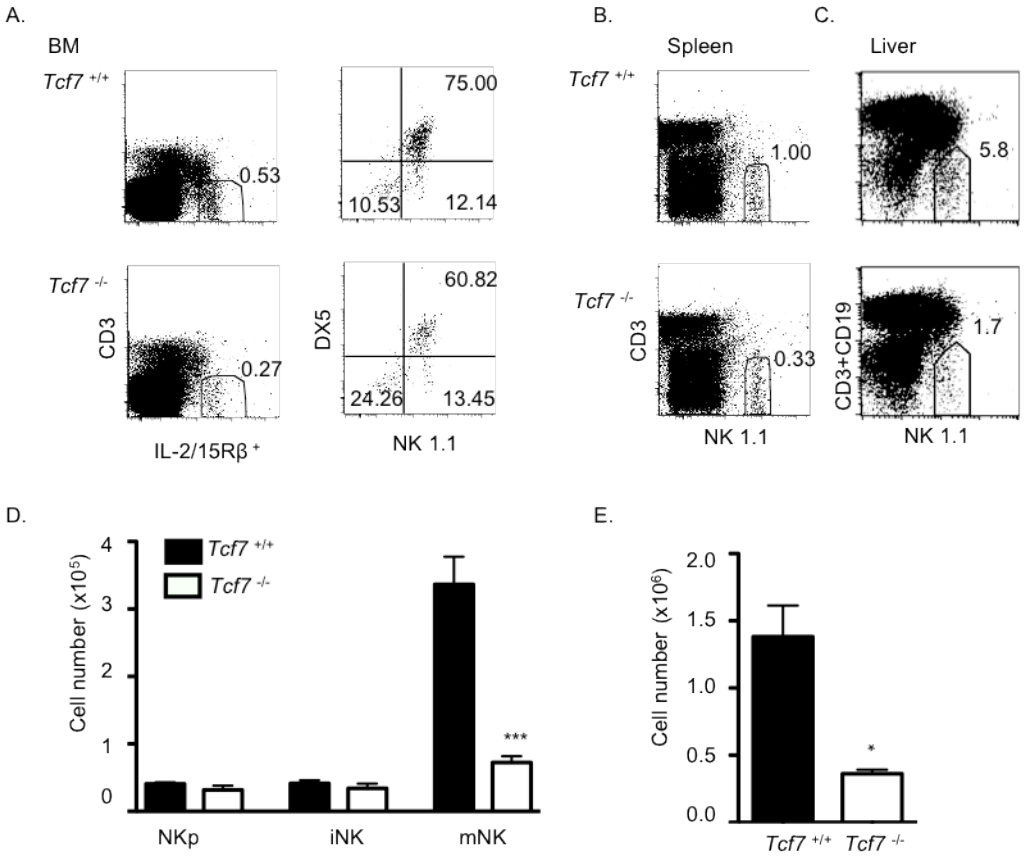


Figure 11. NK cell development in the absence of *Tcf7*.
Density plots of *Tcf7*$^{+/+}$ and *Tcf7*$^{-/-}$ BM (A), spleen (B) and liver (C) cells stained for the indicated markers. Numbers indicate the percentage of gated cells (for the left panel of A, the panels B and C the numbers are the percentage of total cells; for the right panel of A numbers are the percentage of gated IL-2/15Rβ $^{+}$CD3^{-} cells). D and E. Numbers of cells of the indicated population in the BM (D) and spleen (E) of *Tcf7*$^{+/+}$ and *Tcf7*$^{-/-}$ mice. D. Bar graphs represent the mean number of NKp, iNK and mNK cells in one hind leg ± SEM of n=10 mice. The statistical significance of differences was calculated using two-tailed t-test and (*), (**) and (***) show significant differences (p<0.05), (p<0.01) and (p<0.001) respectively, compared to *Tcf7*$^{+/+}$ mice. When nothing is marked, the values are not significantly different (p>0.05).

2.3 A cell intrinsic role for Tcf-1 in NK cell development

To address the basis for the impaired NK cell development in the absence of Tcf-1, we next determined whether NK cell development depends on Tcf-1 expression by NK cells or by the surrounding cells. Lethally irradiated wild type (wt) recipient mice (CD45.1^{+}) were reconstituted with a mixture of equal proportions of *Tcf7*$^{-/-}$ (CD45.2^{+})

and wild type (CD45.1$^+$) BM cells. Mixtures of *Tcf7*$^{+/+}$ (CD45.2$^+$) and wild type (CD45.1$^+$) BM cells served as controls. After stable engraftment, chimeric mice were analyzed for the contribution of CD45.2$^+$ cells to the NK cell compartment. As expected, a sizeable CD45.2$^+$ NK cell population was detected in the spleen and BM of control chimeras receiving *Tcf7*$^{+/+}$ BM (Fig. 12A, B and C). In contrast, very few CD45.2$^+$ splenic or BM NK cells were derived from Tcf-1-deficient BM precursors (Fig. 12A and B). Compared to controls, absolute numbers of *Tcf7*$^{-/-}$ NK cells per spleen and BM were reduced approximately 10 and 15 fold, respectively (Fig. 12C).

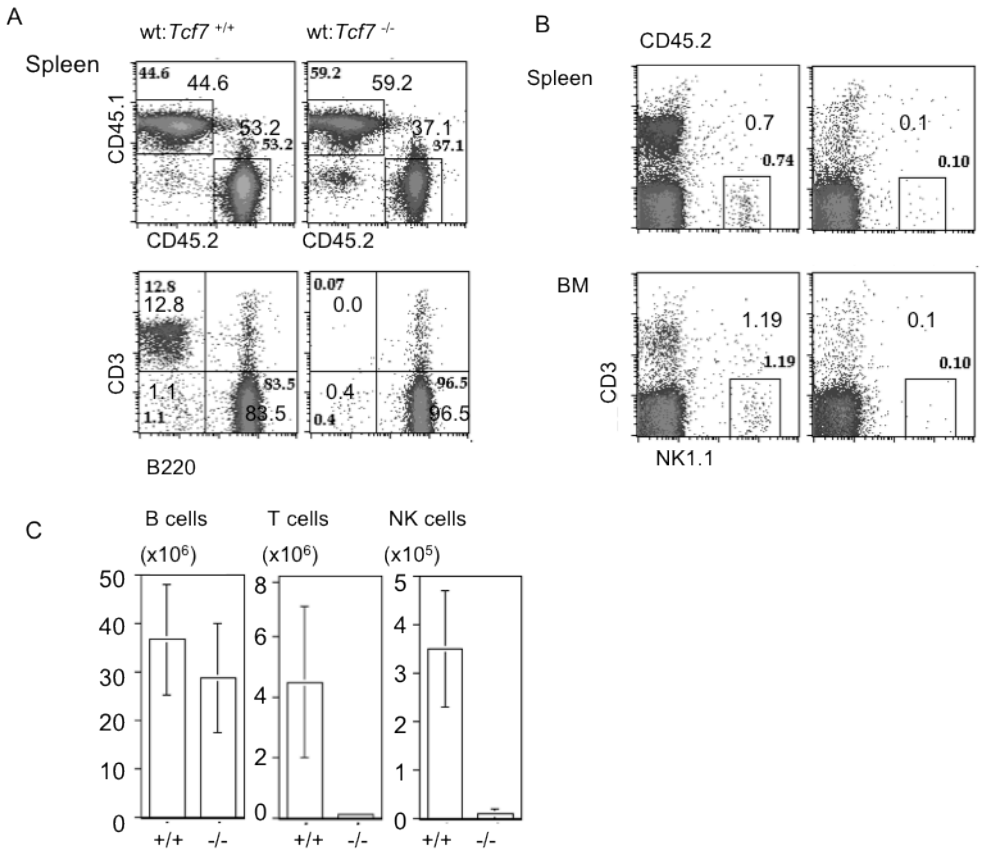


Figure 12. Cell intrinsic role of Tcf-1 for NK cell development.
Spleens from mixed BM chimeras were analyzed 2-3 months after reconstitution with a 1:1 mixture of competitor wt (CD45.1) and tester *Tcf7*$^{+/+}$ (CD45.2) or wt (CD45.1) and *Tcf7*$^{-/-}$ (CD45.2) BM cells. Density plots show spleen cells stained with mAbs to CD45.1 and CD45.2. Gated CD45.1$^-$CD45.2$^+$ populations were analyzed for CD3 versus B220 expression (A) or CD3 versus NK1.1 expression in spleen or BM (B). Numbers indicate the percentage of cells in the respective region. (C) The bar graph shows the mean absolute number (±SD) of CD45.2$^+$ cells per spleen in mixed chimeras of the indicated type. Data are derived from 8 – 10 chimeras.

Contribution:
Experiments shown in Figure 12. were performed and analyzed by Werner Held.

T cell development from Tcf-1-deficient BM precursors was similarly impaired, in agreement with a previous report (Weber et al., 2011). In contrast, the development of other hematopoietic lineages such as B cells, dendritic cells ($CD11c^+$ MHC class II^+) (Ferrero et al., 2002), Macrophages ($CD11b^+$), Granulocytes ($Gr\text{-}1^+$) and nucleated erythroid cells ($Ter119^+$) were not dependent on Tcf-1 (data not shown). These data show that NK cell development depends on Tcf-1 expression by NK cells and not by the surrounding cells. Indeed, Tcf-1-deficient hosts readily supported NK cell development from Tcf-1 sufficient BM progenitors (W. Held, unpublished data). Overall these data show that Tcf-1 ensures NK cell development in a cell autonomous fashion.

2.4 Normal NK cell development in the combined absence of β- and γ-catenin

Although Tcf-1 binds DNA, it lacks intrinsic transcriptional activity. Tcf-1 represses target genes via its association with Groucho co-repressors. Target gene activation in response to Wnt signals depends on the binding of the co-activators β-catenin and γ-catenin to the N-terminus of Tcf-1 (Grigoryan et al., 2008; Jeannet et al., 2008a). We analyzed NK cell development in mice lacking both β– and γ-catenin. Mice that have a null mutation of β-catenin or γ-catenin are embryonic lethal (Huelsken et al., 2000; Ruiz et al., 1996). Therefore we crossed mice harboring floxed alleles of β catenin (Huelsken et al., 2001) and γ-catenin with mice expressing Cre recombinase under the control of the hematopoietic-specific *Vav* regulatory elements (Katzav et al., 1989; Ogilvy et al., 1999; Stadtfeld and Graf, 2005). We found that BM NK cell numbers and their developmental progression are normal in the combined absence of β-catenin and γ–catenin (Fig. 13A). Splenic NK cells were also normal (Fig. 13A). BM and splenic NK cells showed normal expression of maturation markers and of common inhibitory and activating receptors (data not shown). Moreover, the expansion of NK cells in response to IL-2 was normal in the combined absence of β-catenin and γ-catenin (Fig. 13B). Overall these data show that β-catenin and γ–catenin are dispensable for NK cell development.

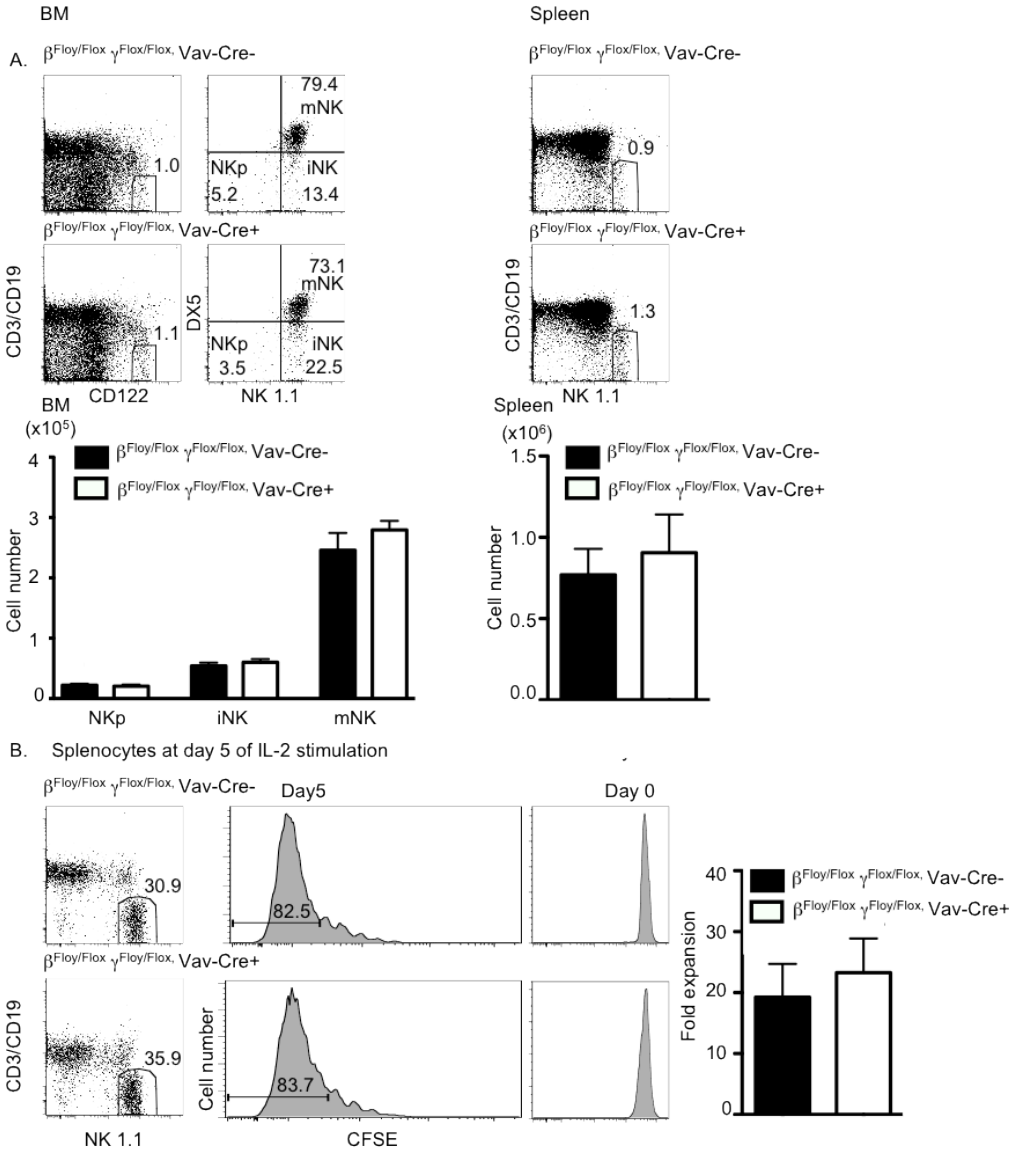


Figure 13. NK cell development is normal in the absence of β and γ catenin.
(A) Density plots of $\beta^{+/+}$ and $\gamma^{-/-}$ BM (left) and spleen cells (right) stained for the indicated markers. Numbers indicate the percentage of gated cells (for BM left panel and the spleen panel, numbers are the percentage of total cells, whereas for the BM right panel numbers are the percentage of gated IL-2/15Rβ $^{+}$CD3^{-} cells). Bar graphs represent the mean number of NKp, iNK and mNK cells in one hind leg (left) and splenic NK cells (right) ± SEM of n=4 mice. (B) Nylon wool non-adherent splenocytes were labeled with CFSE and stimulated with IL2 for 5 days. Numbers indicate the percentage of cells in the indicated gates. These data are representative of 4 experiments. Bar Graphs show the fold expansion of NK cells after 5 days of stimulation ± SEM. The statistical significance of differences was calculated using two-tailed t-test and (*), (**) and (***) show significant differences (p<0.05), (p<0.01) and (p<0.001) respectively, compared to *Tcf7*$^{+/+}$ mice. When nothing is marked, the values are not significantly different (p>0.05).

2.5 The repressor domain in Tcf-1 mediates NK cell development

While Tcf-1 was crucial for NK cell development, the two known Wnt signaling transmitters β-catenin and γ-catenin were dispensable. Thus we next tested whether the N-terminal catenin-binding domain of Tcf-1 was needed for NK cell development. To study the role of different domains of Tcf-1 for NK cell development, our laboratory has used a genetic complementation approach. A Tcf-1 transgene (Tg) containing the catenin-binding domain plus the repressor domain (p45) rescued T cell development in *Tcf7*$^{-/-}$ mice while a Tg containing only the repressor domain (p33) failed to do so (Ioannidis et al., 2001). We used the same approach to determine the mechanism of Tcf-1 function in NK cell development. Intriguingly, BM NK cell development is fully restored in p33 and in p45 Tg *Tcf7*$^{-/-}$ mice (Fig. 14). In addition, the splenic NK cell compartment was restored to normal in the p33 and the p45 Tg *Tcf7*$^{-/-}$ mice (Fig. 15). Data shown in Fig. 14 and 15, will be discussed in more detail later on. These data show that the short Tcf-1 isoform (p33), that contains the DNA- and Groucho-binding domains but lacks the β-catenin interaction site (Oosterwegel et al., 1991a; Van de Wetering et al., 1996), is sufficient to restore NK cell development.

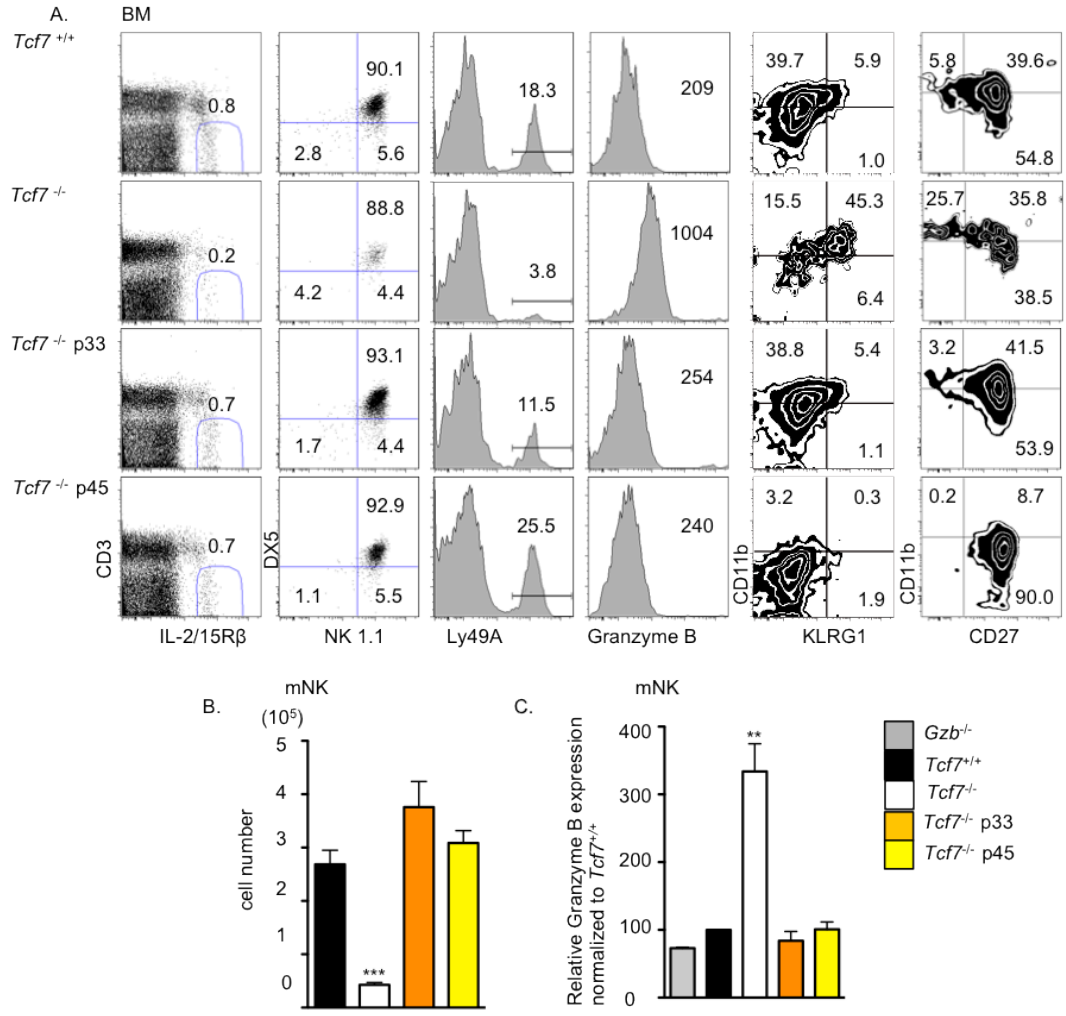


Figure 14. The repressor domain of Tcf-1 mediates BM NK cell development.
(A) Shown are dot plots of *Tcf7*$^{+/+}$, *Tcf7*$^{-/-}$, p33 *Tcf7*$^{-/-}$, p45 *Tcf7*$^{-/-}$ BM cells stained for the indicated markers. Numbers indicate the percentage of gated cells (for A left panel, numbers are the percentage of total cells whereas for the right panel of A numbers are the percentage of gated IL-2/15Rβ $^{+}$CD3^{-} cells). Histograms and density plots show Ly49A, GzmB, KLRG1, CD11b and CD27 expression in gated mNK (IL-2/15Rβ $^{+}$CD3^{-}NK1.1^{+}DX5^{+}) cells. Numbers indicate the percentage of gated cells and the mean fluorescence intensity (MFI) of GzmB staining. Shown is one representative experiment out of four independent experiments. Bar graphs show the mean number of NK cells in one hind leg ± SEM of n=4 mice (B). (C) The graph depicts the relative mean fluorescence intensity (MFI) ±SEM of Granzyme B expression in *GzmB*$^{-/-}$, *Tcf7*$^{-/-}$, p33 *Tcf7*$^{-/-}$, p45 *Tcf7*$^{-/-}$ mNK cells normalized to *Tcf7*$^{+/+}$ controls that were set to 100%. (n=4). Based on the two-tailed t-test (*), (**) and (***) depict significant differences (p<0.05), (p<0.01) and (p<0.001) respectively, compared to *Tcf7*$^{+/+}$ controls. When nothing is marked, the values are not significantly different (p>0.05).

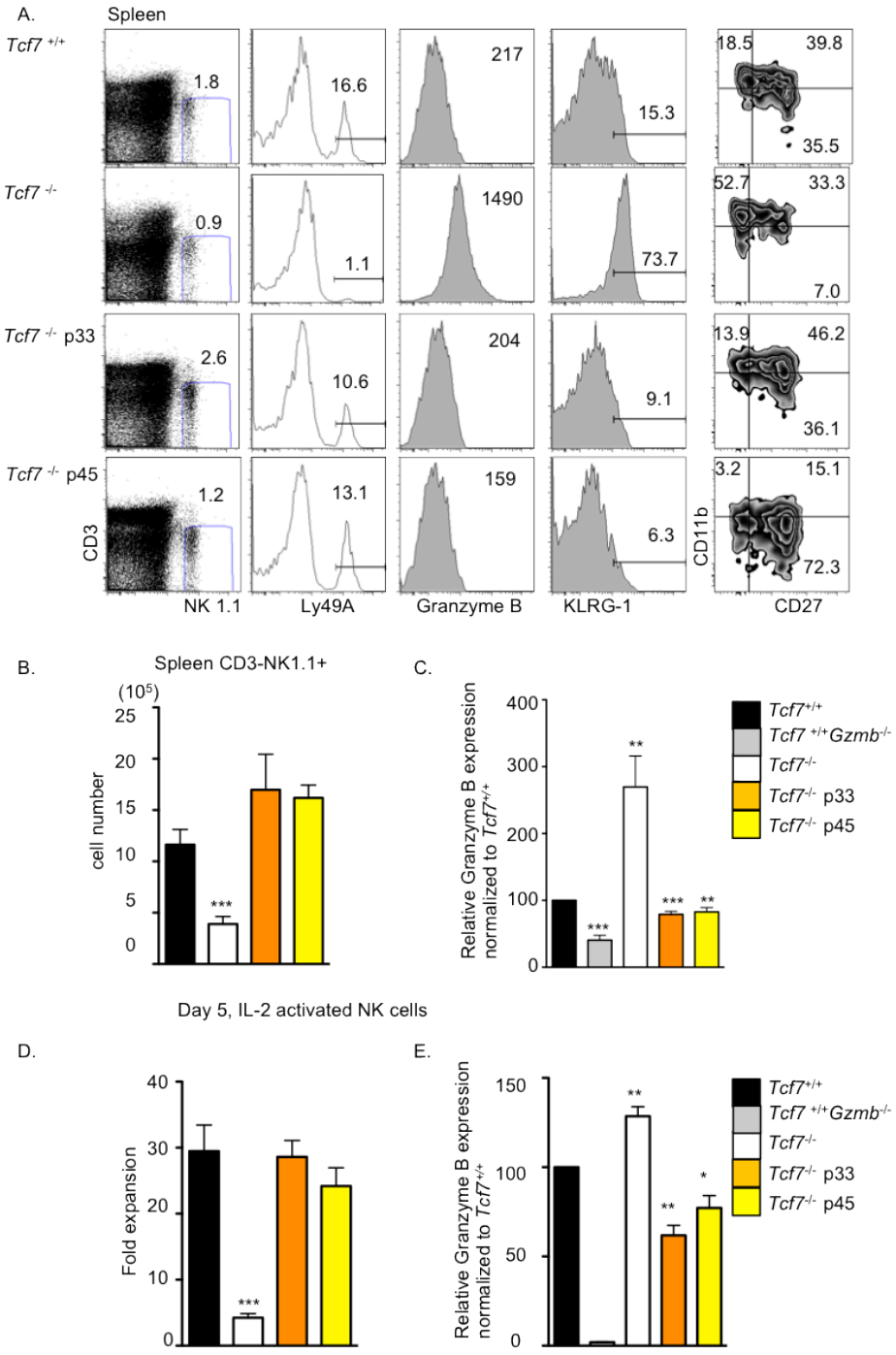


Figure 15. The repressor domain of Tcf-1 restores the splenic NK cell compartment
Shown are dot plots of *Tcf7*$^{+/+}$, *Tcf7*$^{-/-}$, p33 *Tcf7*$^{-/-}$, p45 *Tcf7*$^{-/-}$ spleen cells stained for CD3 versus NK1.1. Numbers indicate the percentage of gated cells. Histograms and density plots show Ly49A, GzmB, KLRG1, CD11b and CD27 expression in gated NK (CD3^{-}NK1.1$^{+)}$ cells. Numbers indicate the percentage of gated cells and the mean fluorescence intensity (MFI) of GzmB staining. Shown is one representative experiment of at least four independent experiments. (B) Bar graph represent the mean number of NK cells in the spleen ± SEM of n=4 mice. (C) The graph depicts the MFI ±SEM of GzmB expression in *GzmB*$^{-/-}$, *Tcf7*$^{-/-}$, p33 *Tcf7*$^{-/-}$, p45 *Tcf7*$^{-/-}$ NK cells normalized to *Tcf7*$^{+/+}$ controls that were set to 100%. (n=4). (D) The bar graph represents the fold expansion of *Tcf7*$^{+/+}$, *Tcf7*$^{-/-}$, p33 *Tcf7*$^{-/-}$, p45 *Tcf7*$^{-/-}$ NK cells between day 0 and day 5 of IL-2 stimulation (n=5). (E) The graph depicts the MFI ±SEM of GzmB expression in *GzmB*$^{-/-}$, *Tcf7*$^{-/-}$, p33 *Tcf7*$^{-/-}$, p45 *Tcf7*$^{-/-}$ NK cells at day 5 of IL-2 stimulation normalized to *Tcf7*$^{+/+}$ controls, that were set to 100% (n=5). Based on the two-tailed t-test (*), (**) and (***) depict significant differences (p<0.05), (p<0.01) and (p<0.001) respectively, compared to *Tcf7*$^{+/+}$ controls. If nothing is marked, the values are not significantly different (p>0.05).

2.6 Basis for impaired NK cell development in the absence of Tcf-1

During their development in the BM, NK cells up-regulate the IL-15 receptor complex followed by inhibitory and activating surface receptors and finally maturation markers. The next aim was to define the basis for impaired NK cell development in the absence of Tcf-1. We first tested whether *Tcf7* deficiency altered the expression of activating receptors, which control NK cell function upon interaction with ligands on target cells. We could not detect any significant changes in the expression levels of NKG2D, NKp46, NK1.1 or CD16 in BM or spleen NK cells in the absence of Tcf-1 (Fig. 16A and B and data not shown). We also could not detect any significant changes in the expression levels of the signaling lymphocyte activation molecule (SLAM) family receptors (SLAM, 2B4, CD84, Ly-9, Ly-108 and CD2-like receptor activating cytotoxic cells (CRACC)) in mNK or splenic NK cells in the absence of Tcf-1 (Fig. 16A and B).

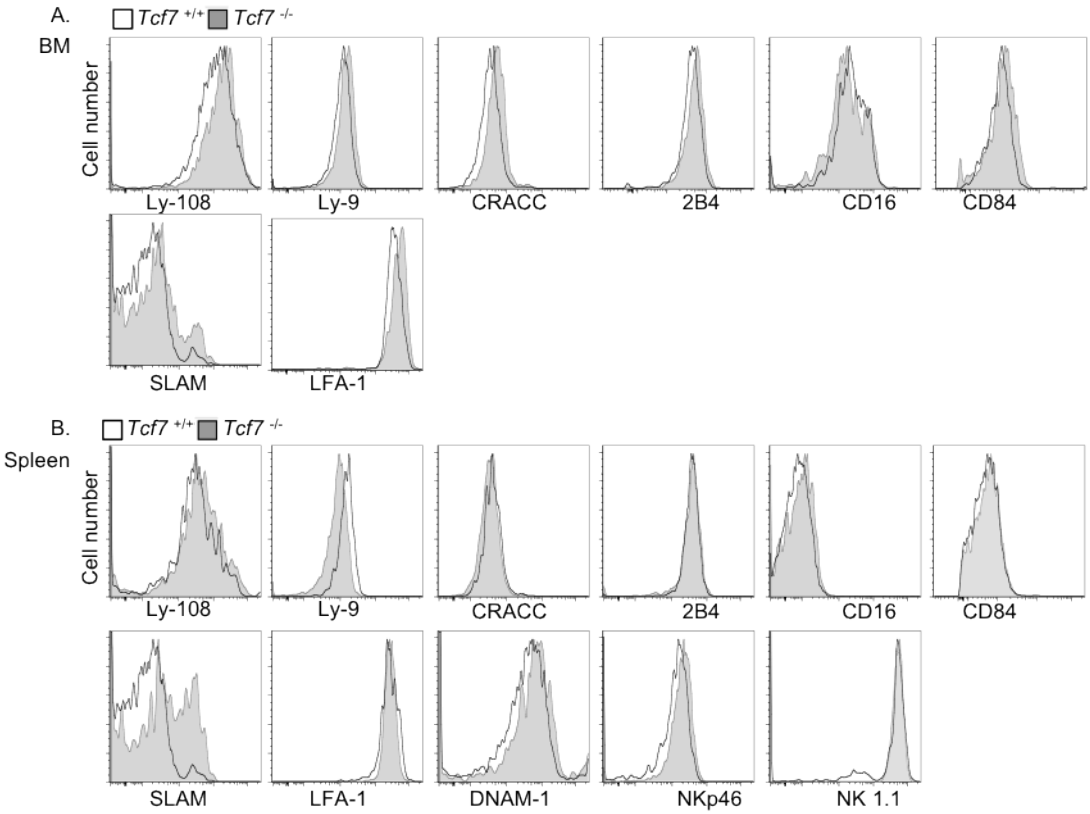


Figure 16. Activating receptors are normally expressed in the absence of Tcf-1.
Histograms in show overlays of *Tcf7*$^{+/+}$ (black line) and *Tcf7*$^{-/-}$ (filled grey) mNK ($CD3^-$IL-$2/15R\beta^+NK1.1^+DX5^+$) cells (A.) or splenic NK cells ($CD3^-NK1.1^+$) (B). Shown is the expression of different activating receptors including various SLAM family members.

NK cells also acquire receptors for major histocompatibility complex (MHC) class I-molecules including CD94 /NKG2A, and Ly49 receptors. The Ly49 receptor family includes both inhibitory (Ly49A, Ly49C/I, Ly49F, Ly49G2, Ly49I) and activating receptors (Ly49D and Ly49H). The expression of Ly49D is significantly decreased wherease the expression of Ly49H, which recognizes the mouse cytomegalovirus (MCMV) protein m157, is normal in the absence of *Tcf7* (Table 1). Our group has previously shown that the *Ly-49a* gene is a direct Tcf-1 target and that the Ly49A receptor is essentially absent in the absence of *Tcf7* (Held et al., 1999). Expression of the Ly49A receptor in *Tcf7*$^{-/-}$ NK cells is fully and partially restored by the p45 and the p33 Tgs, respectively (Ioannidis et al., 2003). The expression of the inhibitory Ly49 receptors (Ly49C/I, Ly49G2 and Ly49I) was not affected by the loss of *Tcf7,* whereas expression of the Ly49F, NKG2/ CD94 were decreased (Table 1). Thus, expression of several inhibitory receptors was decreased in the absence of Tcf-1.

Table 1. Expression of Ly49 and CD94/NKG2 receptors.

		NK1.1$^+$ CD3$^-$			
	n	B6	Tcf7-/-	p45 Tcf7-/-	p33 Tcf7-/-
Ly49A	11	19.1±2.8	1.3±0.4	20.9±5.2	3.5±0.6
Ly49C/I	10	50.3±7.3	67.8±8.3	36.5±6.9	58.0±5.9
Ly49F	3-4	7.5±0.9	4.1±1.2	12.7±1.5	8.1±3.2
Ly49G2	10	56.8±3.8	64.2±8.1	56.3±8.7	60.0±6.5
Ly49I	3	54.5±4.5	59.4±8.5	39.3±3.6	57.9±5.2
NKG2	2-3	50.2±2.4	15.9±2.7	61.9±4.6	17.8±7.8
CD94	2-3	49.6±3.3	16.6±5.7	62.0±3.7	17.8±8.2
Ly49D	10	55.9±4.9	20.2±4.6	29.1±6.1	29.7±8.7
Ly49H	3	57.2±7.5	54.6±3.7	58.5±7.1	57.2±7.5

Shown is the Mean % ± SD of different inhibitory and activating Ly49 family receptors and CD94/NKG2 expressed by splenic NK cells of the indicated genotypes. Red numbers indicate $p>0.05$ *vs* B6. Black numbers indicate no significant difference from B6 controls.
Contribution:
This table was adapted and completed based on data provided by Werner Held.

We next tested whether the loss of Tcf-1 had an impact on NK cell maturation. The iNK-mNK cell transition is associated with up-regulation of CD11b followed by CD43 (Kim et al., 2002; Rosmaraki et al., 2001). We found that *Tcf7*$^{-/-}$ mice have an increased proportion (2 fold) of CD11b^{hi} and CD11b^{hi}CD43^{hi} BM mNK cells compared to wt (Fig. 14A). Likewise, the proportion of CD11b^{hi}CD43^{hi} NK cells was increased in the liver and the spleen of Tcf-1-deficient mice (1.4 fold) (Fig. 15A and data not shown). NK cells can be divided into CD11b$^-$ CD27$^+$ (immature), CD11b$^+$CD27$^+$ (mature1; M1), and CD11b$^+$ CD27$^-$ (mature2; M2) subsets. The CD11b$^+$CD27$^+$

subset is the most potent in acquiring effector functions whereas the M2 population mostly co-expresses KLRG1 that marks terminal maturation (Hayakawa and Smyth, 2006). The M2 CD11b^{+} CD27^{-} subset was strongly increased in the BM and the spleen of mice that lack Tcf-1 compared to wild type controls. Furthermore in the absence of Tcf-1 the bulk of mNK (50-60%) and splenic NK cells (75%) expressed KLRG1 compared to (5-8%) and (30%) of *Tcf7*$^{+/+}$ NK cells (Fig. 14A). These data show that Tcf-1 deficient NK cells have a more mature phenotype. The expression of maturation markers in p33 Tg *Tcf7*$^{-/-}$ mice was comparable to wild type controls. However p45 Tg *Tcf7*$^{-/-}$ NK cells showed a very immature phenotype. Around 90% of the BM and 72% of splenic NK cells of p45 Tg *Tcf7*$^{-/-}$ mice were immature (CD11b^{-}CD27^{+}) compared to wt controls BM (54.8%) and spleen (35.5%), respectively. The mature M1 (CD11b^{+}CD27^{+}) NK cell fraction was strongly reduced in the BM and the spleen and the M2 (CD11b^{+}CD27^{-}) population was completely absent (Fig. 15A). Furthermore, the NK cells of p45 Tg *Tcf7*$^{-/-}$ mice did not up-regulate KLRG1. We conclude that enforced Tcf-1 p45 signaling prevents maturation whereas the absence of Tcf-1 accelerates maturation.

2.7 Tcf-1 ensures the survival of BM and cytokine stimulated NK cells

The analysis of NK cell surface markers in the absence of *Tcf7* did not directly reveal why NK cell development was blocked at the mNK stage. The emergence of mNK cells coincides with rapid cycling of precursor cells (Kim et al., 2002). Thus the reasons for the reduced number of *Tcf7*$^{-/-}$ mNK cells could be a defect in cycling and/or survival. To examine cell proliferation we measured the DNA content of the cells using Hoechst staining. We found that Tcf-1-deficient BM mNK cells cycled (S/G2/M) at least as efficiently as control mNK cells (Fig. 17A). There was also no difference in the proportion of cycling NKp and iNK cells (Fig. 17A). Thus, even though Tcf-1-deficient BM NK cells cycle normally, their number fails to increase, suggesting that *Tcf7*$^{-/-}$ NK cells have a survival defect.

Freshly isolated *Tcf7* deficient BM mNK cells did not show increased Annexin V staining, which detects apoptotic cells (data not shown). However, following *in vitro* culture (4h) in the absence of exogenous growth factors a significantly increased

fraction of *Tcf7*$^{-/-}$ BM NK cells became Annexin V^{+} (44.0±1.4% n=5) as compared to wild type BM NK cells (27.5±1.9% n=5) (Fig. 17B). This observation was specific to BM NK cells since splenic NK cells did not show an increase in Annexin V^{+} cells following *in vitro* culture (4h) (not shown). Overall this suggests that Tcf-1 ensures the survival of BM NK cells.

We further tested whether cytokine stimulation of splenic NK cells resulted in a corresponding impairment of expansion. In contrast to wild type NK cells, Tcf-1-deficient NK cells failed to efficiently accumulate in number in response to either IL2 or IL-15 stimulation (Fig. 17C). In response to IL-2 (or IL-15) *Tcf7*$^{-/-}$ NK cells showed normal cell cycle progression based on the combined analysis of BrdU incorporation and 7-AAD staining (Fig. 17E). In addition, as shown by the efficient dilution of CFSE, *Tcf7*$^{-/-}$ NK cells underwent as many cell divisions as wild type control NK cells (Fig. 17F). At day 5 of cytokine stimulation, significantly more *Tcf7*$^{-/-}$ NK cells were Annexin V^{+} (19±2.0%) as compared to wild type (8±1.0%) (Fig. 17G). Corresponding data were obtained at day 3 and based on TUNEL assays (*Tcf7*$^{+/+}$ 5.3±0.3% Tunnel^{+}, *Tcf7*$^{-/-}$ 9.9±0.8% Tunnel $^{+}$) (Fig.17F). When cell death (Annexin V) and cell division (CFSE) were simultaneously monitored we noted increased rates of cell death at each division both at day 3 and day 5 of cytokine stimulation, suggesting that at each division a certain fraction of proliferating cells undergoes apoptosis (not shown). Similar to BM NK cell development we found that the presence of the p33 Tg was sufficient to restore NK cell accumulation upon cytokine stimulation (Fig. 15D). Taken together, these data show that the poor accumulation of BM and cytokine stimulated splenic NK cells is caused by increased rates of cell death. Furthermore, we found that the suppressor activity of Tcf-1 prevent the apoptosis of cycling NK cells.

Next we determined whether the death of cytokine activated NK cells idepended on Tcf-1 expression by NK cells or by the surrounding cells. Splenic *Tcf7*$^{-/-}$ (CD45.2) and *Tcf7* $^{+/+}$ (CD45.1) were mixed at a ratio of 1:1 and cultured for 5 days *in vitro* in the presence of IL-2. While Tcf-1 sufficient NK cells expanded normally, Tcf-1-deficient NK cells failed to efficiently accumulate (Fig. 18). Taken together these data show that Tcf-1 ensures the survival of proliferating BM mNK cells and cytokine stimulated peripheral NK cells in a cell autonomous fashion.

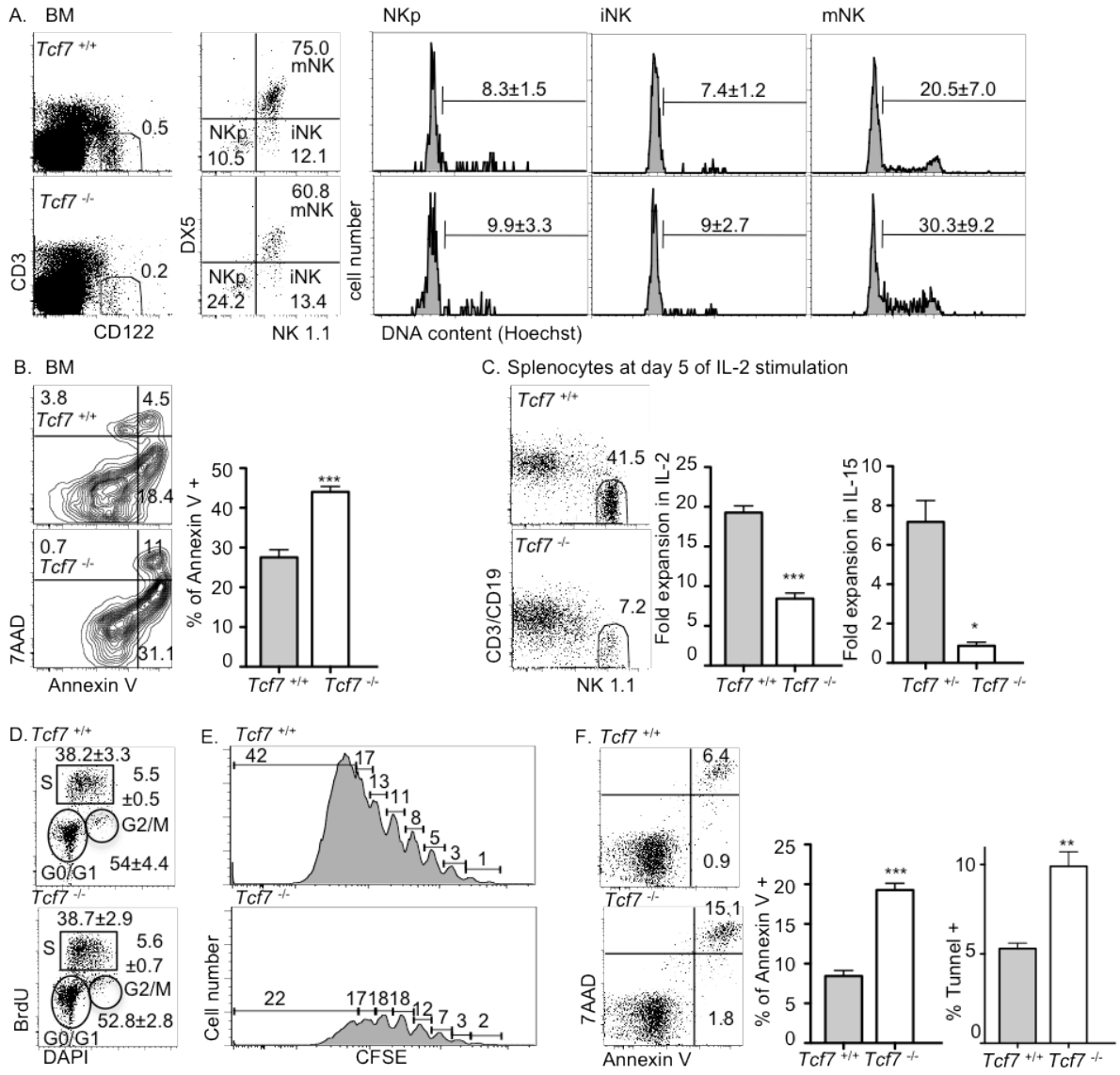


Figure 17. Tcf-1 is required for NK cell expansion.
A. Hoechst (DNA) staining of the indicated NK cell populations in BM of *Tcf7*$^{+/+}$ or *Tcf7*$^{-/-}$ mice. Hoechst positive cells are in S, G2, M phases of the cell cycle. Numbers show the mean percentage ± SD of Hoechst positive cells in the different populations. These data are representative of 3 experiments. B. Annexin V (marker of apoptosis) staining of the indicated BM NK cell populations in *Tcf7*$^{+/+}$ and *Tcf7*$^{-/-}$ mice cultured for 4 hours at 37°. Bar graphs depict the mean percentage (±SEM) of AnnexinV and 7AAD positive cells (n=5). The statistical significance of differences was calculated using two-tailed t-test and * shows statistical significance ($p<0.05$) compared to *Tcf7*$^{+/+}$ mice. C**.** Nylon wool non-adherent splenocytes from *Tcf7*$^{+/+}$ or *Tcf7*$^{-/-}$ mice were stimulated with IL-2 or IL-15. Shown is one representative dot plot of at least 10 experiments for IL-2 stimulation. Bar graphs show the fold expansion of NK cells between day 0 and day 5 of stimulation ± SEM. These data are representative of at least 10 experiments for IL-2 stimulation and for 3 experiments for IL-15 stimulation. The statistical significance of differences was calculated using two-tailed t-test and * shows statistical significance ($p<0.05$) compared to *Tcf7*$^{+/+}$ (C2) or *Tcf7*$^{+/-}$ (C3) mice. D. Scatter plots depict the cell cycle distribution of gated NK cells at day 5 after IL-2 stimulation following treatment with 1 BrdU for 45 min. Bivariate analysis of the DNA content (DAPI staining) and the incorporation of BrdU was performed. These data are representative of at least 3 experiments. E. Nylon wool non-adherent splenocytes were labeled with CFSE and stimulated with IL2 for 5 days. Numbers indicate the percentage of cells in the indicated gates. These data are representative of 9 experiments. F. Nylon wool non-adherent splenocytes were stimulated with IL2 for 5 days. The numbers indicate the percentage of cells in the indicated gates. These data are representative of at least 7 experiments. Bar Graphs: percentage of AnnexinV (left panel) or Tunnel (right panel) postive cells after 5 days of stimulation ± SEM (n=4 for Annexin V and n=3 for Tunnel assays).

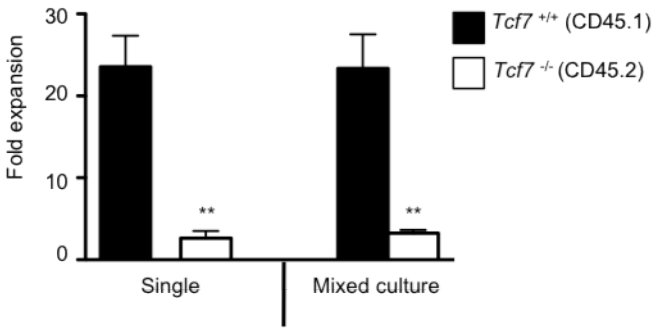
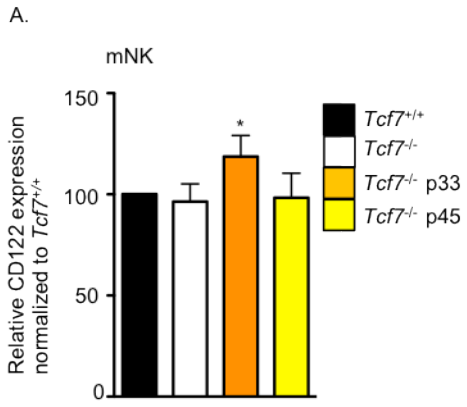


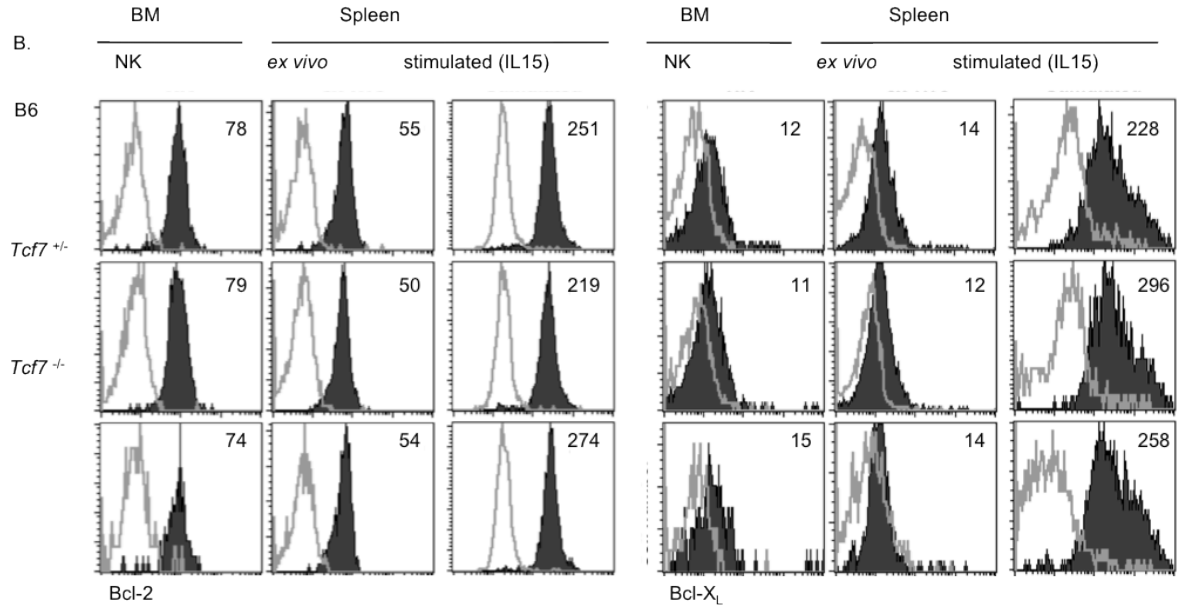
Figure 18. Cell intrinsic role of Tcf-1 for NK cell expansion.
Bar graph depicts fold expansion of *Tcf7* $^{+/+}$ (CD45.1+) and *Tcf7* $^{-/-}$ (CD45.2+) NK cells were cultured separately (left) or as a 1:1 mixture (right) for 5 days with IL-2. NK cell numbers were determined by gating on CD45.1^{+}CD3^{-}NK1.1^{+} for *Tcf7* $^{+/+}$ and on CD45.2^{+}CD3^{-}NK1.1^{+} for *Tcf7* $^{-/-}$ cells. Bar graphs represent the mean number of indicated NK cells ± SEM of 3 independent experiments. (**) depicts significant differences ($p<0.01$) on the student`s t-test.

NK cell development and survival is critically dependent on cytokines. IL-15 deficiency blocks NK cell development at the iNK stage (Vosshenrich et al., 2005). This cytokine is also required for NK cell homeostasis and may be necessary for NK cell expansion (Koka et al., 2003). The IL-15 receptor complex is composed of the IL-15 receptor α (IL-15Rα), IL-2Rβ (CD122), and the IL-2 receptor common γ chains (IL-2Rγc). Compared to their wild type counterparts, *Tcf7*$^{-/-}$ BM mNK and splenic NK cells expressed equivalent amounts of IL-2/15Rβ (Fig. 19A). IL-15R signaling mediates the survival of NK cells by promoting the expression of high levels of anti-apoptotic Bcl-2 and Bcl-X_L. Moreover it has been shown that Bcl-2 family members regulate cytokine deprivation dependent apoptosis (Carson et al., 1997; Cooper et al., 2002; Ranson et al., 2003). Intracellular staining showed that the expression of Bcl-2 and Bcl-X_L in freshly isolated or cytokine stimulated NK cells was unperturbed in the absence of Tcf-1 (Fig. 19). Taken together we conclude that the survival defect of Tcf-1 deficient NK cells is not caused by the reduced expression of the IL-15 receptor complex or by a decrease of the anti-apoptotic molecules Bcl-2 and Bcl-X_L.

Figure 19. The IL-15 receptor and the anti-apoptotic molecules Bcl-2 and Bcl-X are normally expressed in the absence of Tcf-1.
A. The graph depicts the MFI ±SEM of IL-2/15Rβ expression in *Tcf7*$^{-/-}$, p33 *Tcf7*$^{-/-}$, p45 *Tcf7*$^{-/-}$ NK cells normalized to *Tcf7*$^{+/+}$ controls that were set to 100%. (n=5). B. Expression of Bcl-2 and Bcl-X_L in freshly isolated BM and splenic NK cells or IL-2 day 7 stimulated NK cells. The open histogram represents the isotype controls. Numbers indicate the MFI of staining. These data are representative of 3 experiments for freshly isolated cells and from a single experiment for stimulated NK cells.

Contribution:
Experiment for figure 19B was done by Grégoire Jeannet.





2.8 Tcf-1-deficient BM NK cells over-express Granzymes

To elucidate the mechanisms underlying apoptosis of NK cells, we established the global gene expression profiles of FACS-purified BM mNK cells ($CD3^-CD19^-CD122^+NK1.1^+DX5^+$) isolated from *Tcf7*$^{+/+}$ and *Tcf7*$^{-/-}$ mice using Affymetrix arrays. Based on three independent data sets, 364 of 28'000 genes were differentially expressed (False discovery rate FDR (adjusted P value)< 10% and fold change (FC) > 1.5). A total of 205 genes were up-regulated and 159 genes were down regulated in *Tcf7*$^{+/+}$ compared to *Tcf7*$^{-/-}$ BM NK mNK cells (Table 2).

Table 2. Gene expression profile of mNK cells derived either form *Tcf7*$^{-/-}$ or *Tcf7*$^{+/+}$ mice.

Cut-off	UP	DOWN
FDR < 5%	15	5
FDR < 10% and FC > 1.5	205	159

Based on three independent data sets, 20 FDR < 5% and 364 (False discovery rate FDR (adjusted P value)< 10% and fold change (FC) > 1.5) of 28'000 genes were differentially expressed). A total of 205 genes were significantly up-regulated (green) and 159 genes were down regulated (red) in *Tcf7*$^{+/+}$ compared to *Tcf7*$^{-/-}$ BM NK mNK cells

As expected, *Tcf7* transcripts were down regulated in NK cells, yet still detectable, which is in agreement with data obtained by qRT-PCR. However, in *Tcf7*$^{-/-}$ NK cells, Tcf-1 protein was not detected by Western blot (Held et al., 1999). *Tcf7*$^{-/-}$ NK cells showed higher mRNA levels of genes encoding Minichromosome maintenance factors (Mcm) (Mcm10, Mcm5, Mcm3), cyclin (cyclinF, cyclinA2, cdk14, cdk inhibitor Cdnk2c (p18, inhibits Cdk4, repressed by c-Myc)), E2Fs (E2F8, E2F2) in agreement with efficient cycling of *Tcf7*$^{-/-}$ mNK cells (Table 3, The Affimetrix microarray has been done by the Center for Integrative Genomics at the University of Lausanne). The transcript levels of the pro-apoptotic Bcl-2 family members Bim, Puma, Noxa or Bax in *Tcf7*$^{-/-}$ mNK cells were comparable to wt controls. Amongst the most up-regulated transcripts upon loss of *Tcf7* was the gene encoding for *GzmB* (2.5-fold as determined by microarray, $p<0.5$ and 8.7-fold as determined by qRT-PCR analysis (Table 2 and Fig. 20D). Genes coding for other Granzyme family proteins, including Granzyme C (2.5-fold, $p<0.05$) and Granzyme K (2-fold, $p<0.1$) were also up-regulated (Table 3). GzmB is a serine protease and the main component of exocytic granules used by cytotoxic T cells (CTL) and natural killer (NK) cells to destroy cells infected with intracellular pathogens or tumor cells (Russell and Ley, 2002). Recently several groups have reported GzmB-leakage-induced cell death in NK cells and CTLs where GzmB escapes into the cytoplasm, and thereby provokes apoptosis of the GzmB producing cell itself (Ida et al., 2003; Zhang et al., 2006).

Table 3. Gene expression profile of mNK cells derived either form *Tcf7*$^{-/-}$ or *Tcf7*$^{+/+}$ mice.

Gene accession nr.	Gene	Gene description	adj. P.Val	*Tcf7*$^{-/-}$ mean	*Tcf7*$^{+/+}$ mean	Fold_ Change
NM_009640	*Angpt1*	angiopoietin 1	0.020	5.1	7.0	-3.7
NM_016970	***Klrg1***	**killer cell lectin-like receptor subfamily G**	**0.030**	**9.7**	**7.6**	**4.1**
NM_013473	*Anxa8*	annexin A8	0.036	7.3	4.3	7.8
NM_027127	*Gpx8*	glutathione peroxidase 8 (putative)	0.039	6.5	4.7	3.6
NM_139198	*Plac8*	placenta-specific 8	0.039	9.3	8.1	2.3
NM_009848	*Entpd1*	ectonucleoside triphosphate diphosphohydrolase 1	0.039	9.7	7.4	4.8
NM_013542	***GzmB***	**granzyme B**	**0.039**	**11.4**	**10.1**	**2.5**
NM_009987	*Cx3cr1*	chemokine (C-X3-C) receptor 1	0.039	8.0	6.6	2.8
NM_010371	***GzmC***	**granzyme C**	**0.039**	**6.5**	**5.1**	**2.5**
NM_024477	*Ttc28*	tetratricopeptide repeat domain 28	0.039	7.0	8.2	-2.4
NM_011311	*S100a4*	S100 calcium binding protein A4	0.039	11.2	10.2	2.0
NM_030712	*Cxcr6*	chemokine (C-X-C motif) receptor 6	0.039	10.3	8.6	3.3
NM_177660	*Zbtb10*	zinc finger and BTB domain containing 10	0.039	6.3	7.2	-1.9
NM_009858	*Cd8b1*	CD8 antigen, beta chain 1	0.040	9.3	5.6	13.1
NM_001163608	*Plxdc1*	plexin domain containing 1	0.044	5.4	4.5	1.8
NM_011920	*Abcg2*	ATP-binding cassette, sub-family G	0.048	6.4	7.8	-2.5
NM_008479	*Lag3*	lymphocyte-activation gene 3	0.050	6.6	5.6	2.1
NM_010740	*Cd93*	CD93 antigen	0.050	6.7	5.7	1.9
NM_011496	*Aurkb*	aurora kinase B	0.050	9.4	8.5	1.9
NM_009331	***Tcf7***	**transcription factor 7, T-cell specific**	**0.050**	**9.8**	**10.8**	**-2.0**
NM_027290	***Mcm10***	**minichromosome maintenance deficient 10**	**0.059**	**7.6**	**6.9**	**1.7**
NM_026410	***Cdca5***	**cell division cycle associated 5**	**0.059**	**8.4**	**7.5**	**1.8**
NM_001013368	***E2f8***	**E2F transcription factor 8**	**0.059**	**8.4**	**7.6**	**1.8**
NM_007634	***Ccnf***	**cyclin F**	**0.059**	**7.6**	**6.8**	**1.8**
NM_008196	***GzmK***	**granzyme K**	**0.059**	**9.3**	**8.3**	**2.0**
NM_177733	***E2f2***	**E2F transcription factor 2**	**0.059**	**7.6**	**6.8**	**1.8**
NM_007671	***Cdkn2c***	**cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)**	**0.059**	**9.0**	**8.2**	**1.7**
NM_011074	***Cdk14***	**cyclin-dependent kinase 14**	**0.059**	**7.2**	**6.5**	**1.6**
NM_009828	***Ccna2***	**cyclin A2**	**0.073**	**10.8**	**10.0**	**1.7**
NM_008566	***Mcm5***	**minichromosome maintenance deficient 5**	**0.073**	**8.8**	**8.2**	**1.4**
NM_008563	***Mcm3***	**minichromosome maintenance deficient 3**	**0.077**	**9.0**	**8.3**	**1.5**
NM_011452	***Serpinb9b***	**serine (or cysteine) peptidase inhibitor, clade B, member 9b**	**0.497**	**7.9**	**7.7**	**1.2**
NM_009256	***Serpinb9 (Spi-6)***	**serine (or cysteine) peptidase inhibitor, clade B, member 9**	**0.808**	**9.5**	**9.4**	**1.1**
NM_009982	***Ctsc***	**cathepsin C**	**0.812**	**9.3**	**9.2**	**1.1**
NM_011453	***Serpinb9c***	**serine (or cysteine) peptidase inhibitor, clade B, member 9c**	**0.831**	**3.1**	**3.2**	**-1.0**
NM_011456	***Serpinb9e***	**serine (or cysteine) peptidase inhibitor, clade B, member 9e**	**0.844**	**2.8**	**2.9**	**-1.1**
NM_183197	***Serpinb9f***	**serine (or cysteine) peptidase inhibitor, clade B, member 9f**	**0.901**	**3.3**	**3.2**	**1.0**
NM_011455	***Serpinb9g***	**serine (or cysteine) peptidase inhibitor, clade B, member 9g**	**0.963**	**2.8**	**2.8**	**1.0**

cDNA from FACS sorted BM mNK cells was analyzed on an Affymetrix microarray. Table 2 shows the gene accession number, symbol and description, the adjusted p-value, the mean expression of three independent data sets for *Tcf7*$^{+/+}$ and *Tcf7*$^{-/-}$ and the fold change. Adjusted p-values have been computed for each comparison by the Benjamini-Hochberg method, controlling for false discovery rate (FDR). Expression values are measured using RMA algorithm from the BioConductor affy package. The expression values are on the log 2 scale and range from 0 to 16. First section: annotated genes with an FDR < 5%. Second section: genes with an FDR < 10% relevant for this thesis. Red: genes that are increased; green: genes that are decreased in *Tcf7*$^{-/-}$. The last panel shows genes of interest that do not show a significant change in expression. Genes that are mentioned in this thesis are highlighted in bold.

Mature NK cells from naïve mice transcribe *GzmB* and *Prf1* mRNA constitutively. However, such NK cells are minimally cytotoxic because of a block in *Prf1* and *GzmB* mRNA translation that is only released by NK cell priming (Fehniger et al., 2007). In agreement with these data, splenic NK cells express little GzmB but they abundantly express GzmA as reported previously (Fehniger et al., 2007) (Fig. 20A). We also could not detect GzmB protein in NKP, iNK and mNK cells from wt mice. In contrast, *Tcf7* deficient iNK and mNK cells abundantly expressed GzmB protein as determined by intracellular FACS staining (Fig. 20B).

We decided to analyze the subcellular localization of GzmB in activated NK cells. We detected a granular GzmB localization in the cytoplasm of wild type NK cells, which co-localized with lysosome-associated membrane protein 1 (LAMP-1) (Fig. 21A). In sharp contrast, the activated *Tcf7*$^{-/-}$ NK cells showed a diffuse cytoplasmic distribution of GzmB and LAMP-1, which partly co-localized (Fig. 21A). Based on these observations we hypothesized that GzmB mediated, self-inflected damage may explain the increased cell death of proliferating Tcf-1 deficient NK cells.

A major determinant of GzmB-leakage-induced cell death is an excess of cytosolic GzmB over the serine protease inhibitor 6 (Spi-6), its principal cytosolic inhibitor (Ida et al., 2003; Laforge et al., 2006; Phillips et al., 2004; Zhang et al., 2006). Spi-6 prevents the processing of the N-terminal GzmB pro-peptide by the protease cathepsin C (or DPPI), which is required to unlock the proteolytic activity of GzmB (Caputo et al., 1993; Pham and Ley, 1999; Sun et al., 1997; Zhang et al., 2006). Based on the Affymetrix arrays Cathepsin C (Ctsc) and Spi-6 (serpinb9) or homologues (serpinb9b to serpinb9g) transcript levels were not different between wild type and *Tcf7* deficient mNK cells (Table 3). In the absence of Tcf-1, Spi-6 levels in naïve splenic NK cells and in IL-2 activated NK cells were comparable to wild type controls (Fig. 21B). Thus in the absence of *Tcf7,* activated NK cells show increased levels of cytosolic GzmB, whereas Spi-6 levels remain unchanged. Spi-6 might therefore not be able to prevent GzmB induced self-inflected apoptosis.

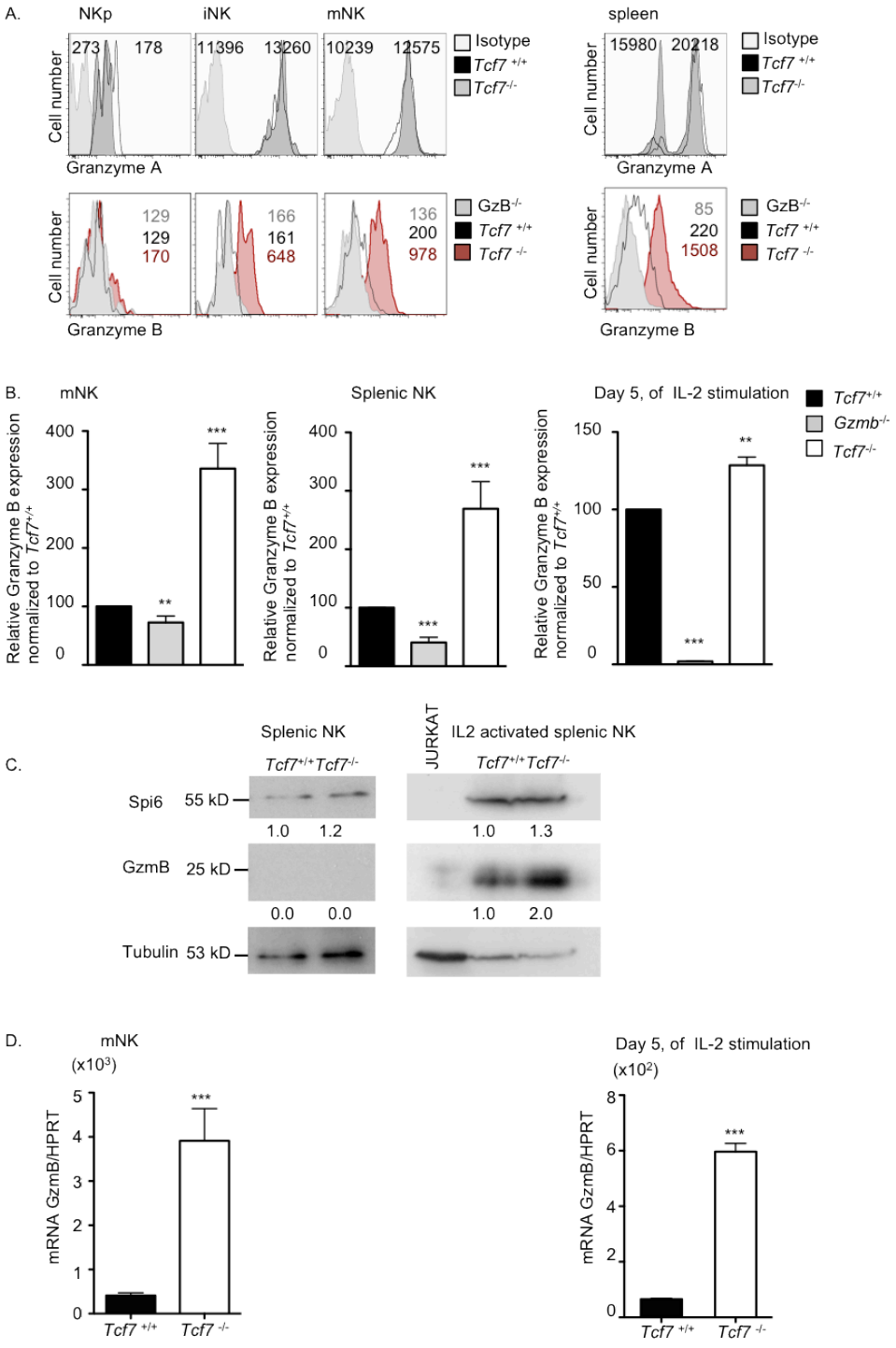


Figure 20. GzmB is over-expressed in the absence of *Tcf7*.
(A) Histograms show overlays of Granzyme A (upper panel) and Granzyme B (lower panel) expression in $Tcf7^{+/+}$ (black line), $Tcf7^{-/-}$ (filled grey for Granzmye A or filled red for Granzmye B) and $GzB^{-/-}$ or an isotype control (filled light grey) BM NKp, iNK and mNK (left) and splenic NK (right) cells like indicated. Following gating strategy was used for NKp ($CD3^{-}$IL-2/15R$\beta^{+}$$NK1.1^{-}$$DX5^{-}$), iNK ($CD3^{-}$IL-2/15R$\beta^{+}$$NK1.1^{+}$$DX5^{-}$), mNK ($CD3^{-}$IL-2/15R$\beta^{+}$$NK1.1^{+}$$DX5^{+}$) and splenic NK ($CD3^{-}$$NK1.1^{+}$) cells respectively. (B) The bar graphs depict the MFI ±SEM of Granzyme B expression in $Tcf7^{-/-}$ and $GzmB^{-/-}$ NK cells in the spleen (mid panel) and in the BM (left panel) and at day 5 of IL-2 expansion (right panel), normalized to the $Tcf7^{+/+}$ controls that were set to 100%. Shown are n=4 (IL-2 expansion) and n=12 (splenic and mNK) independent experiments. (C) Western Blot showing the expression of Spi6 and GzmB in naïve splenic $Tcf7^{+/+}$ and $Tcf7^{-/-}$ NK cells (left panel) and in NK cells at day 5 of IL-2 stimulation (right panel). JURKAT cell lysates were used as negative controls. Numbers indicate the relative density of $Tcf7^{-/-}$ adjusted to the Tubulin (53kD) loading control and to $Tcf7^{+/+}$. The 50kD band represents Spi6

and the 22kD band shows GzmB. (D) Quantitative RT-PCR analysis of the *GzmB* gene expression *Tcf7*$^{+/+}$ *and Tcf7*$^{-/-}$ mNK cells (left) and in IL-2 expanded NK cells (right) (n=3). Based on the two-tailed t-test (*), (**) and (***) depict significant differences (p<0.05), (p<0.01) and (p<0.001) respectively, compared to *Tcf7*$^{+/+}$ controls.

Contribution:
RT-PCRs shown in (Fig. 20C) were performed and analyzed by Beena-Jeevan-Raj (IL-2 activated NK cells) and by Georgi Angelov (mNK).

It has previously been reported, using Spi-6 deficient cytotoxic T cells, that an excess of GzmB disrupts lytic granules (Zhang et al., 2006). To see whether the absence of Tcf-1 or an excess of GzmB influenced the granule stability in activated NK cells, we analyzed *Tcf7*x*GzmB* double deficient NK cells. The granular morphology of LAMP-1^{+} exocytic lysosomes was restored in *Tcf7*-deficient NK cells lacking GzmB (Fig. 21). We estimated the average number of GzmB and LAMP-1 positive granules per NK cell cross-section. A cytokine activated NK cell showed on average 5 GzmB positive granules per cross-cell section that co-stained for LAMP-1 (Fig. 22A). In contrast, NK cells lacking Tcf-1 had only 0.9 clear GzmB positive granules per section but showed abundant diffuse GzmB staining that partly overlapped with similarly diffuse LAMP-1 staining (Fig. 22A and B). In the combined absence of GzmB and Tcf-1, LAMP-1^{+} lysosomes were back to normal in terms of morphology and number (Fig. 22 A and B). Taken together, we find that GzmB is abundantly present in the cytoplasm of *Tcf7* deficient NK cells. Moreover, the integrity of LAMP-1^{+} granules is disrupted, and this was restored to normal by the absence of *GzmB*. This suggests that over-expression of GzmB due to the absence of *Tcf7* leads to abundant GzmB in the cytoplasm, which may induce self-inflected NK cell death.

Based on the findings in IL-2 activated NK cells, we explored the localization of GzmB in mNK cells. Translation of *GzmB* mRNA is prevented in wild type NK cells, and GzmB was only occasionally detected by confocal microscopy. The absence of Tcf-1 leads to enhanced GzmB mRNA expression and translation in mNK cells (Fig. 20 and 21). Similar to activated NK cells, BM NK cells lacking Tcf-1 showed diffuse cytoplasmic/nuclear localization for GzmB. Only rare Lamp-1 positive granules could be detected and they did not co-stain for GzmB (Fig. 23A and B). This suggested that *Tcf7*-deficient mNK cells cannot compartmentalize GzmB in exocytic granules and that this leads to a cytpolsamic/nuclear accumulation of GzmB. Taken together, in the absence of Tcf-1 the *GzmB* gene is over-expressed. Furthermore, GzmB mRNA is translated into GzmB protein that accumulates in the cytoplasm and nucleus of mNK cells where it may induce apoptosis of the NK cell.

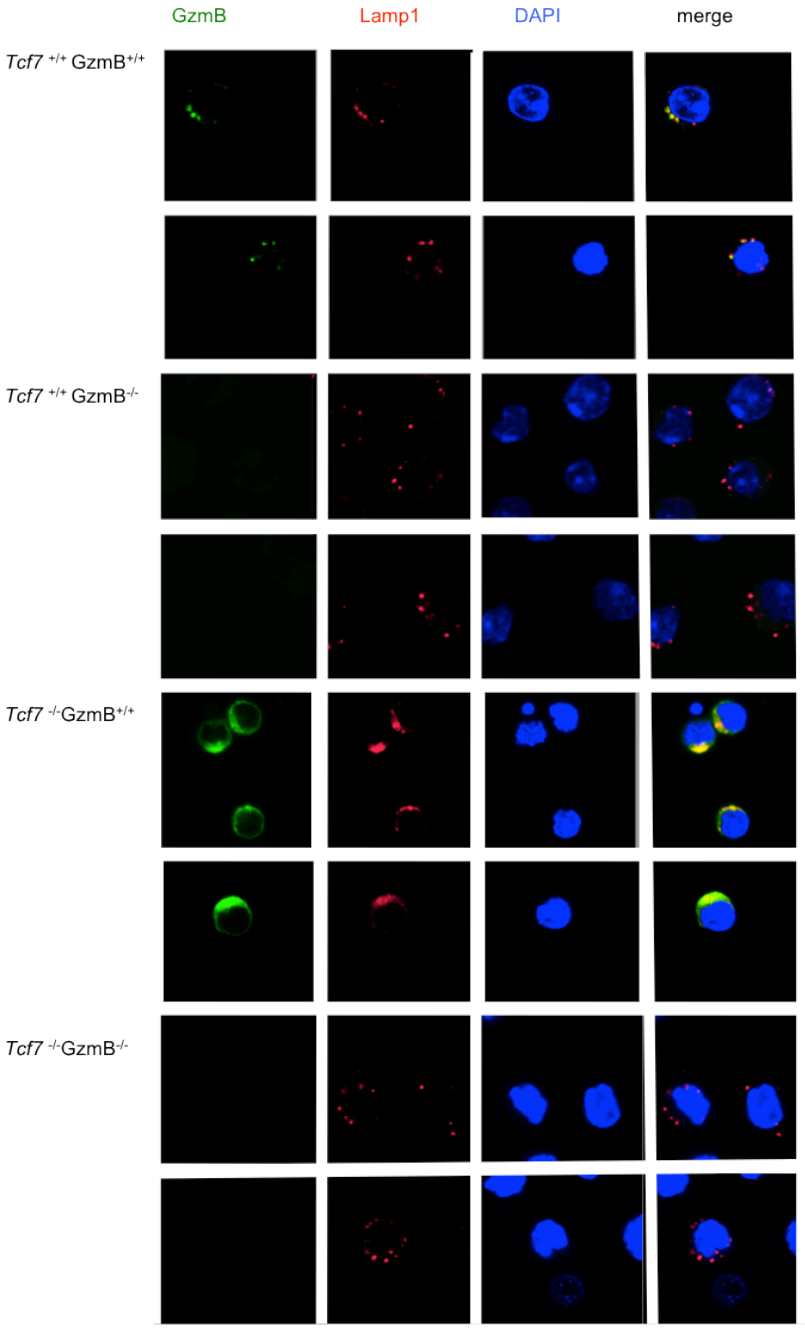


Figure 21. GzmB leaks into the cytoplasm of IL-2 activated splenic *Tcf7*$^{-/-}$ NK cells.
Purified *Tcf7*$^{+/+}$*Gzmb*$^{+/+}$, *Tcf7*$^{+/+}$*Gzmb*$^{-/-}$, *Tcf7* $^{-/-}$*Gzmb*$^{+/+}$ and *Tcf7*$^{-/-}$*Gzmb*$^{-/-}$ NK cells were expanded for 5 days in IL-2 and FACS sorted for (CD3^{-}NK1.1^{+}). Subsequently NK cells were stained for Granzyme B (green), Lamp-1 (red) and DAPI (nuclear dye) (blue). Shown are representative NK cell cross sections out of at least three independent experiments with GzmB and Lamp-1 visualized by Laser-scanning Confocal Microscopy. Images are as follows: from left to right, Granzyme B, Lamp1, DAPI and their overlay. Isotype matched mAbs were used as negative controls for the Lamp1 staining and *GzmB*$^{-/-}$ NK cells were used as a negative control for the GzmB staining.

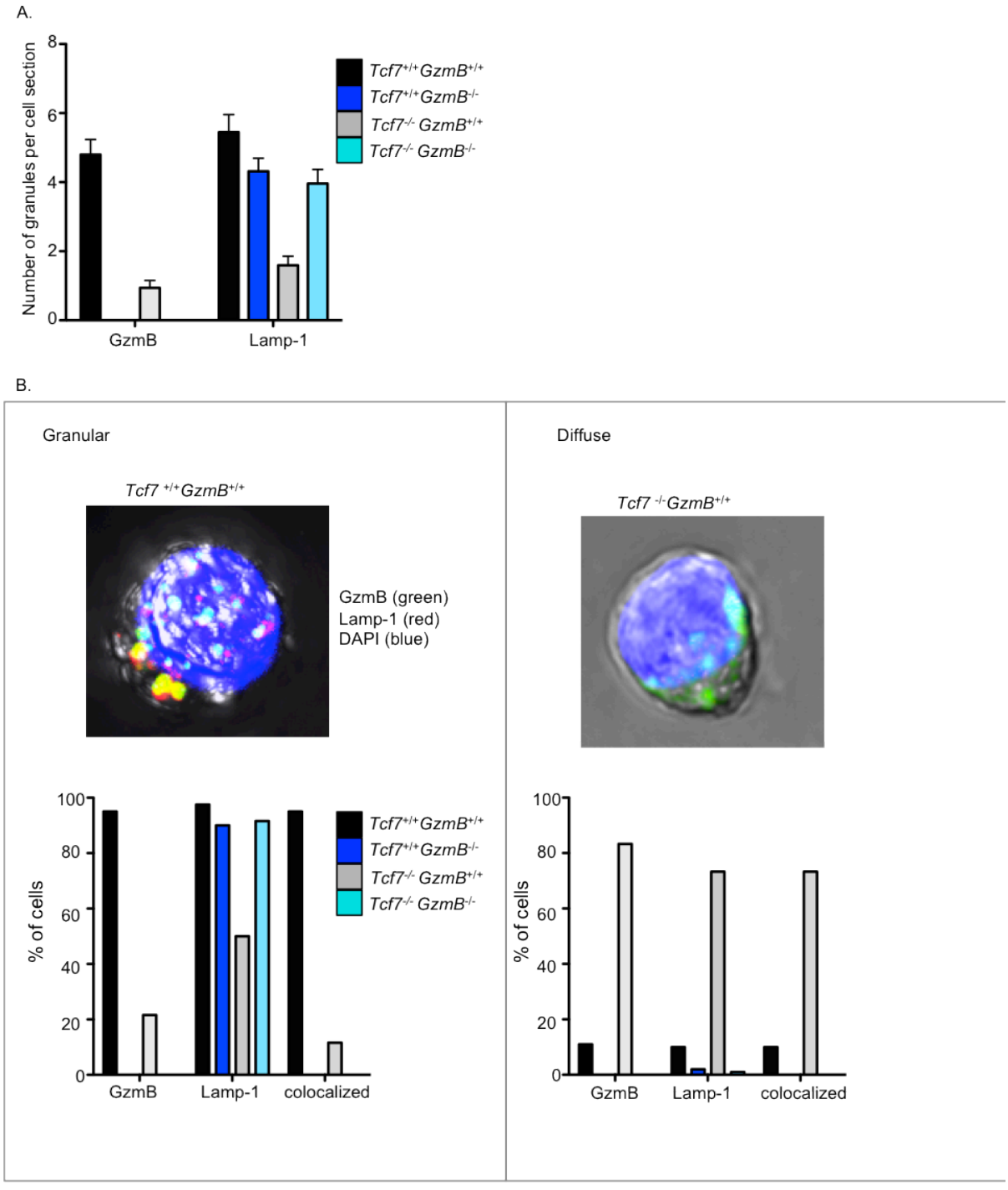


Figure 22. GzmB leaks into the cytoplasm of IL-2 activated splenic *Tcf7*$^{-/-}$ NK cells.
(A) Bar graphs depict the number of granules per cell section for GzmB (left) and Lamp-1 (right) of (n=60) cells of the indicated genotypes. (B) A minimum of 80 cells per genotype (*Tcf7* $^{+/+}$*Gzmb*$^{+/+}$, *Tcf7* $^{+/+}$*Gzmb*$^{-/-}$, *Tcf7*$^{-/-}$*Gzmb*$^{+/+}$ and *Tcf7*$^{-/-}$*Gzmb*$^{-/-}$) where analyzed in detail for the expression of GzmB (green) and LAMP-1 (red) and their co-localization (yellow), which was either defined as granular (left) or diffuse (right) as exemplified in (B). Bar graphs in (B) (left side) show the percentage of cells that either depicts GzmB or Lamp-1 granules and the percentage of cells in which Lamp-1 and GzmB co-localize. Bar graphs in the right panel show the percentage of cells with diffuse GzmB or Lamp-1 expression and the percentage of cells were GzmB and Lamp-1 co-localize.

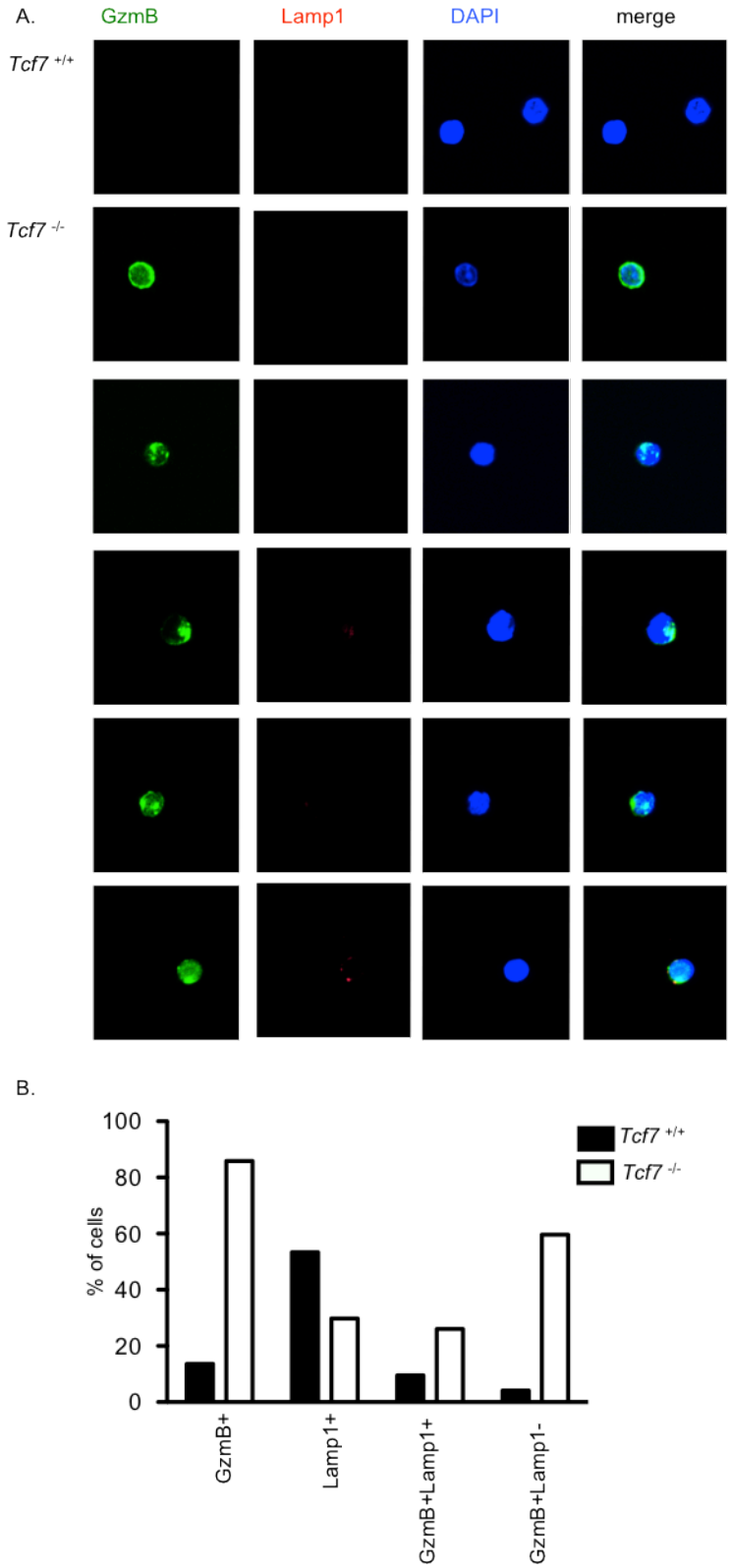


Figure 23. *Tcf7* deficient BM mNK cells express high levels of Granzyme B.
(A) FACS sorted *Tcf7*$^{+/+}$ and *Tcf7*$^{-/-}$ mNK cells stained for GzmB (green) and Lamp1 (red) and nucleus (blue). Shown are cross sections visualized by confocal microscopy. Images from left to right: GzmB, Lamp-1, nucleus and overlay. Shown are representative samples out of at least three independent experiments with n=6 mice, each. (B) Bar graphs depict the percentage of cells (n=70) that express GzmB, Lamp-1 or that show co-localized GzmB and Lamp-1 expression.

While GzmB protein is not expressed during BM development and in unprimed peripheral NK cells of wild type mice, we detected abundant GzmB protein in iNK, mNK and naïve peripheral NK cells in the absence of Tcf-1. The short Tcf-1 isoform (p33) was sufficient to suppress GzmB protein expression in *Tcf7*$^{-/-}$ iNK, mNK and peripheral NK cells. GzmB was also expressed at lower levels in cytokine-activated p33 Tg *Tcf7*$^{-/-}$ NK cells as compared to controls (Fig. 15E). These data show that p33 Tcf-1 is sufficient to prevent GzmB expression, which correlates with a rescue of BM NK cell development.

2.9 Absence of GzmB does not rescue development of Tcf-1-deficient NK cells

To verify the importance of GzmB repression for NK cell development, we used mice deficient in GzmB. In the mouse a large number of Granzyme genes (including (5′->3′) GzmB, C, F, G, L, N, D, and E) are present in the *GzmB* gene cluster. Beside *GzmB*, *GzmC* transcripts were also up regulated in Tcf-1 deficient mNK cells. *GzmB*$^{-/-}$*PGK-neo* mice are deficient in GzmB and express reduced levels of GzmC and F due to the integration of the *PGK-neo* cassette (Pham et al., 1996; Revell et al., 2005). We generated *GzmB*$^{-/-}$ *Tcf7*$^{-/-}$ mice. The mNK cell pool was similarly reduced (6.5 fold) in *GzmB*$^{-/-}$ *Tcf7*$^{-/-}$ mice as in *Tcf7* deficient mice (Fig. 24A and B). In addition, peripheral NK cell numbers were also reduced (Fig. 25A and B). Thus, impaired mNK development in *Tcf7* deficient mice was not rescued by the deletion of *GzmB*. Tcf-1 seems to control additional factors that are required for mNK cell survival.

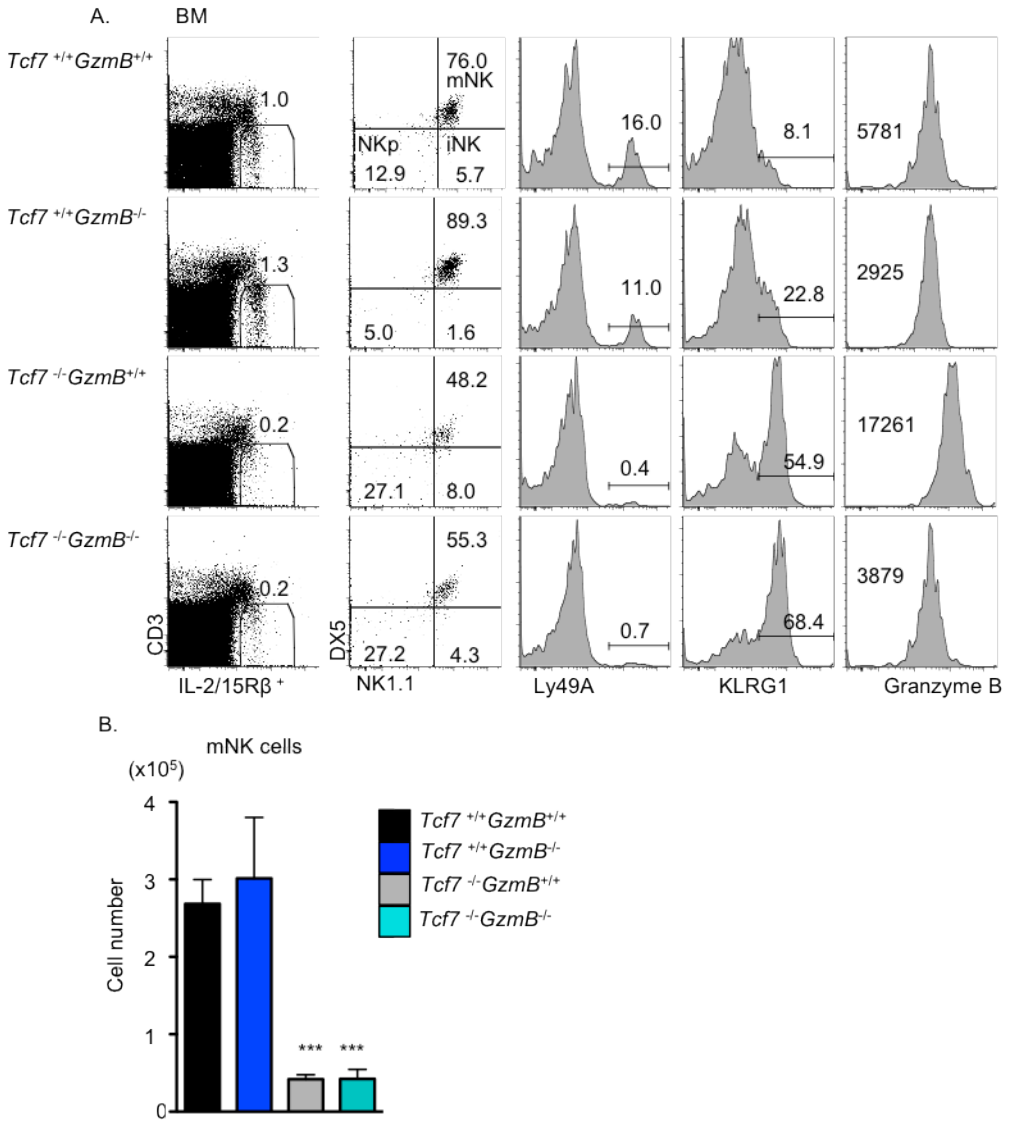


Figure 24. The absence of GzmB is not sufficient to restore BM NK cell development in the absence of *Tcf7*.
A. Dot plots of *Tcf7* $^{+/+}$*Gzmb*$^{+/+}$, *Tcf7* $^{+/+}$*Gzmb*$^{-/-}$, *Tcf7* $^{-/-}$*Gzmb*$^{+/+}$ and *Tcf7* $^{-/-}$*Gzmb*$^{-/-}$ BM cells stained for the indicated markers. Numbers indicate the percentage of gated cells (for A left panel, numbers are the percentage of total cells whereas for the right panel of A numbers are the percentage of gated IL-2/15Rβ $^{+}$CD3^{-} cells). Histograms show Ly49A, KLRG1 and GzmB expression in gated mNK cells. Numbers indicate the percentage of gated cells and the MFI of GzmB staining. (B) Bar graphs represent the mean number of cells in one hind leg ± SEM of n=4 mice. Based on the two-tailed t-test (*), (**) and (***) depict significant differences (p<0.05), (p<0.01) and (p<0.001) respectively, compared to *Tcf7* $^{+/+}$*Gzmb*$^{+/+}$ controls. When nothing is marked, the values are not significantly different (p>0.05).

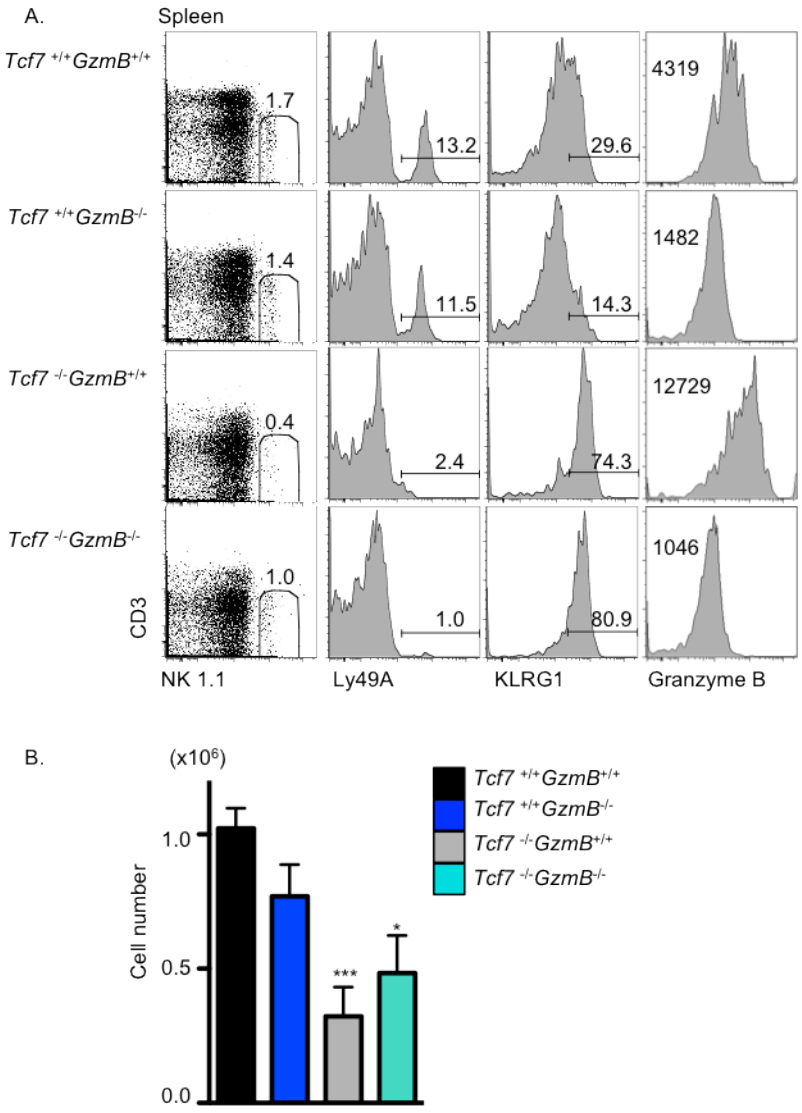


Figure 25. The absence of GzmB is not sufficient to restore splenic NK cell numbers in the absence of *Tcf7*.
A. Dor plots of *Tcf7* $^{+/+}$*Gzmb*$^{+/+}$, *Tcf7* $^{+/+}$*Gzmb*$^{-/-}$, *Tcf7* $^{-/-}$*Gzmb*$^{+/+}$ and *Tcf7* $^{-/-}$*Gzmb*$^{-/-}$ spleen cells stained for NK1.1 versus CD3. Histograms show Ly49A, KLRG1 and GzmB expression in gated NK (CD3^{-} NK1.1^{+}) cells. Numbers indicate the percentage of gated cells and the MFI of GzmB staining. (B) Bar graphs represent the mean number of cells ± SEM of n=4 mice. Based on the two-tailed t-test (*), (**) and (***) depict significant differences (p<0.05), (p<0.01) and (p<0.001) respectively, compared to *Tcf7* $^{+/+}$*Gzmb*$^{+/+}$ controls. When nothing is marked, the values are not significantly different (p>0.05).

2.10 Absence of GzmB restores cytokine driven expansion of Tcf-1-deficient NK cells

In contrast to wild type NK cells, Tcf-1-deficient NK cells failed to efficiently accumulate in number in response to cytokine stimulation (Fig. 26). Intriguingly, in the absence of GzmB, the expansion of *Tcf7* deficient NK cells was restored (Fig. 26). Compared to the wild type NK cells (9±2%), a significantly increased fraction of the *Tcf7*$^{-/-}$ NK cells was Annexin V^{+} (20±2%). The absence of GzmB in *Tcf7* deficient mice led to a strong reduction of the apoptotic cell fraction (6±1% Annexin V^{+}) (Fig. 26B,E).

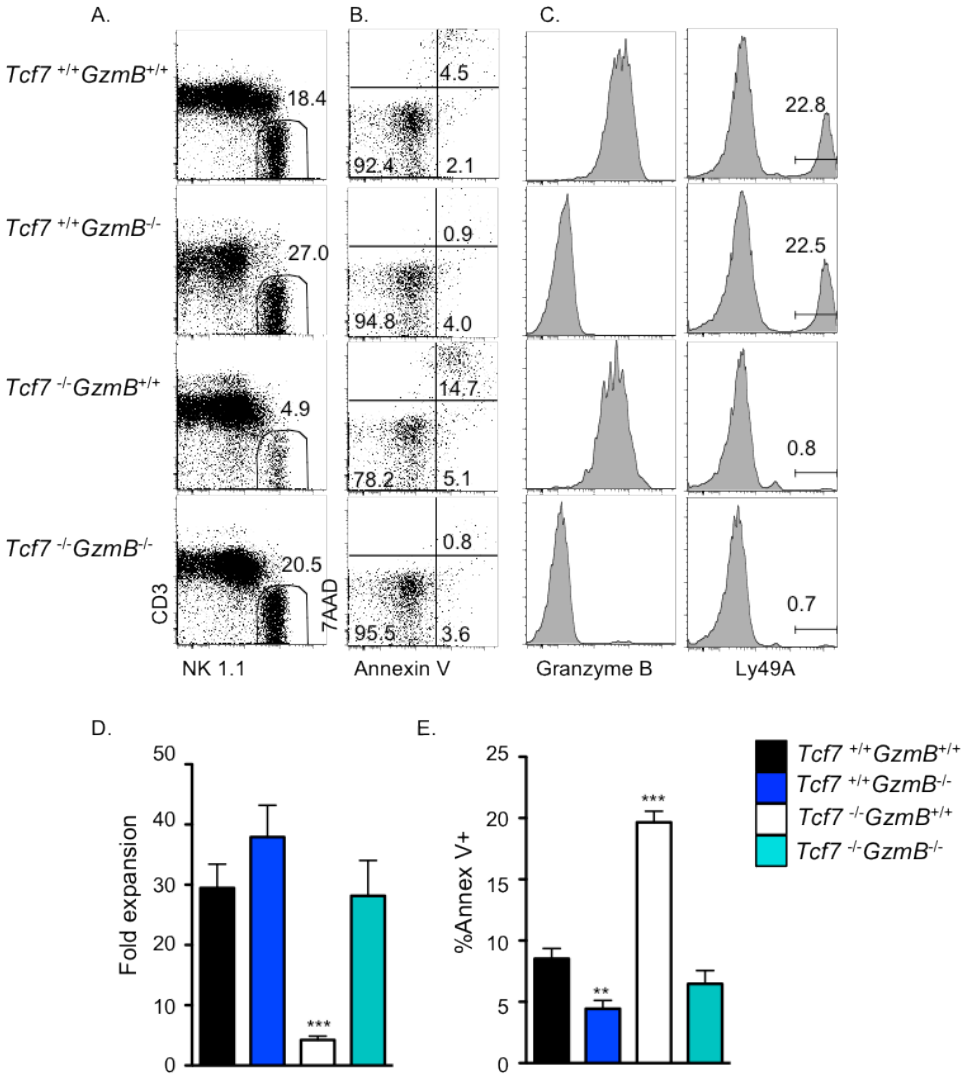


Figure 26. The expansion of *Tcf7* deficient NK cells is restored by the absence of GzmB. *Tcf7* $^{+/+}$*Gzmb*$^{+/+}$, *Tcf7* $^{+/+}$*Gzmb*$^{-/-}$, *Tcf7* $^{-/-}$*Gzmb*$^{+/+}$ and *Tcf7* $^{-/-}$*Gzmb*$^{-/-}$ splenocytes at day 5 of IL-2 stimulation. (A) Numbers in dot plots, indicate the percentage of gated NK1.1^{+}CD3^{-} cells. (B) Scatter plots depict the expression of Annexin V versus 7AAD in the NK1.1^{+}CD3^{-} cells. Numbers indicate the

percentage of cells in the respective quadrant. Shown is one representative sample of at least three independent experiments. Histograms in (C) show GzmB and Ly49A expression in gated $NK1.1^+CD3^-$ cells. (D and E) Bar graphs show fold expansion (D) and the percentage of Annexin V positive $NK1.1^+CD3^-$ cells (E) ± SEM of n=4 (D) and n=3 (E) experiments. The statistical significance of differences was calculated using two-tailed t-test and (*), (**) and (***) show significant differences (p<0.05), (p<0.01) and (p<0.001) respectively, compared to *Tcf7* $^{+/+}$*Gzmb*$^{+/+}$ mice. When nothing is marked, the values are not significantly different (p>0.05).

These data show that the expansion of Tcf-1 deficient NK cells can be rescued by the absence of GzmB. This suggests that the absence of *Tcf7* and as a consequence the up-regulation of GzmB induces apoptosis of the NK cells.

2.11 NK cell function in the absence of Tcf-1

We could not detect significant changes in the expression of activating NK cell receptors while certain inhibitory receptors were significantly reduced in the absence of Tcf-1. We next tested the ability of poly IC primed NK cells from *Tcf7*$^{-/-}$ mice to mount an effector response. As compared to wild type NK cells, stimulation with various tumor cells (RMA/S, RMA-H60, YAC-1) or monoclonal antibodies (mAbs) to activating NK cell receptors (NKp46, NK1.1 or NKG2D) induced a comparable production of the cytokine IFN-γ or of the chemokine MIP1α and a normal release of secretory granules (LAMP-1 release) by *Tcf7*$^{-/-}$ NK cells (Figs. 27 and 28). The only exception was a reduced IFNγ production in response to cross-linking with anti-NK1.1 antibody (Fig. 28).

We further tested the ability of *Tcf7*$^{-/-}$ NK cells to kill susceptible target cells *in vitro.* As expected, the Poly IC primed splenocytes from normal mice efficiently killed RMA/S tumor target cells while syngeneic MHC-I^+ RMA cells were not killed (Figs. 28A, B and C). In contrast, *Tcf7*$^{-/-}$ mice had a 5-10 fold reduced ability to kill RMA/S cells (Fig. 29B). *Tcf7*$^{-/-}$ mice also showed significantly reduced antibody-dependent cell-mediated cytotoxicity (ADCC) towards Thy1 coated RMA cells. In addition, they showed impaired killing of RMA-m157 (Ly49H) and of YAC-1 (in part NKG2D) target cells (Fig. 29C). As an exception, *Tcf7*$^{-/-}$ NK cells killed RMA-H60 cells as efficiently as wild type controls, suggesting that high levels of NK cell activation through NKG2D can restore normal target cell killing by *Tcf7*$^{-/-}$ NK cells (Fig. 29C).

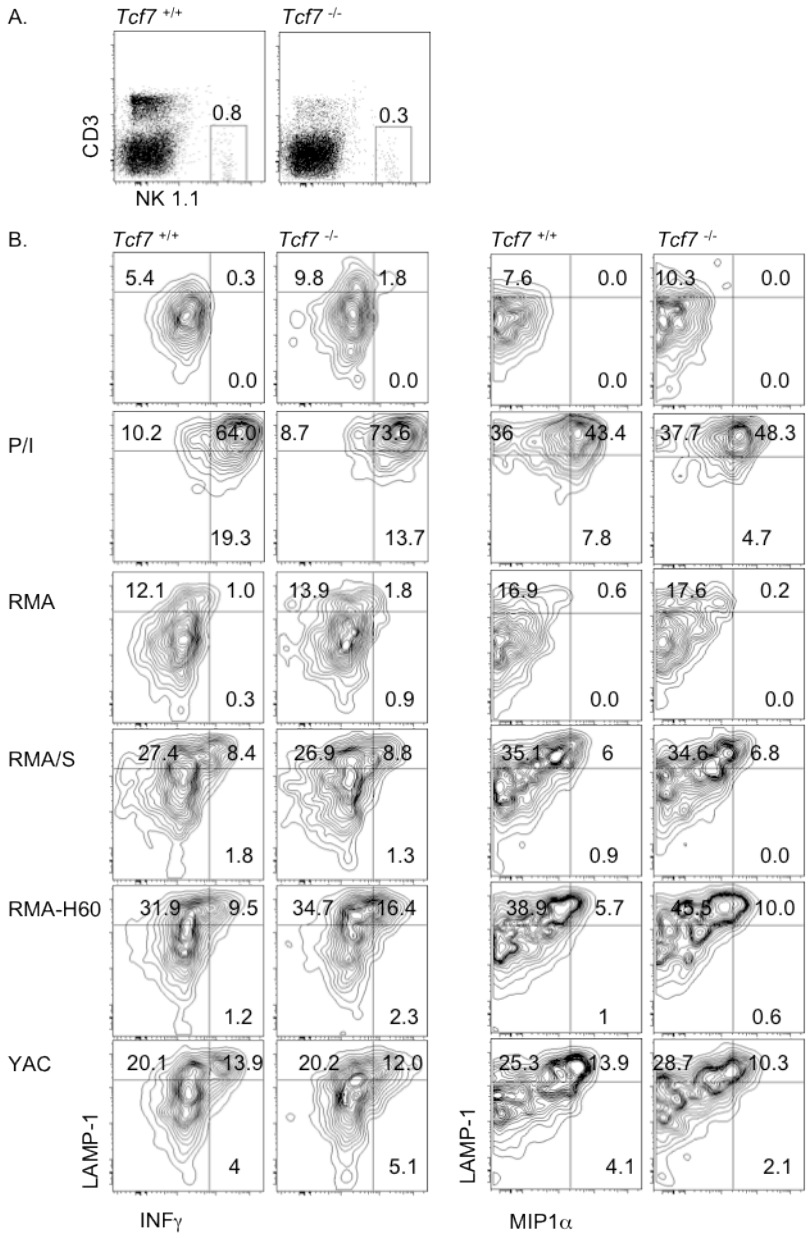


Figure 27. *Tcf7* deficient NK cells mount produce cytokine and chemokine upon target cell recognition.
(A) Dot plots show splenocytes of poly IC primed mice gated for $CD3^-$ versus $NK1.1^+$. (B) Density plots show representative examples of LAMP-1 release versus INFγ (left panel) or versus MIP1a (right panel) by poly IC primed *$Tcf7^{+/+}$ and $Tcf7^{-/-}$* NK cells in response to the MHC-I expressing RMA cells (negative control) or of different tumor target cell lines that are either MHC-I low (RMAS) (missing-self) or over-express activating NKG2D ligands (RMA-H60, YAC-1). As a positive control cells were stimulated with Phorbol 12-myristate 13-acetate (PMA) and Ionomycin (I) indicated as (P/I). Numbers represent the percentage of positive cells within the respective gates. Shown is one representative experiment out of at least 4.

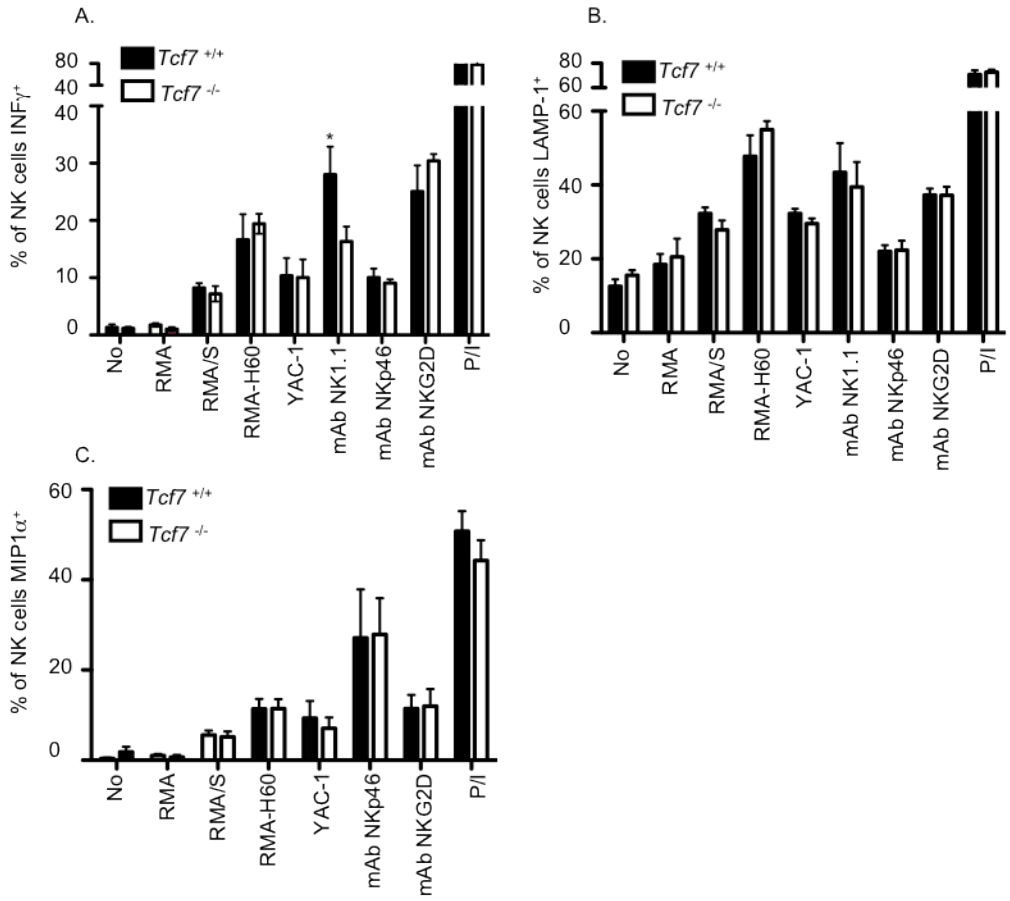


Figure 28. *Tcf7* deficient NK cells mount a normal cytokine and chemokine response upon target cell recognition or mAb stimulation.
A. Total spleen cells from Poly IC primed *Tcf7* $^{+/+}$ and *Tcf7* $^{-/-}$ mice were exposed to MHC-I expressing RMA cells (negative control) or to different tumor target cell lines that are either MHC-I low (RMAS) (missing-self) or over-express activating NKG2D ligands (RMA-H60, YAC-1) (induced-self) respectively. Further some NK cells were stimulated via activating receptors with immobilized mAb (NK1.1, NKp46, NKG2D). As a positive control PMA and Ionomycin (P/I) was added. After 5 hours NK cells ($CD3^-CD19^-DX5^+$) were analyzed for intracellular (ic) IFNγ (A.) or MIP-1a production (C.). The capacity of NK cells to de-granulate upon target cell recognition was measured by LAMP-1 expression (B.). Bar graphs depict the mean percentage (±SD) of (A.) IFNγ-positive (C.) MIP1α positive or (C) LAMP-1 positive cells among gated NK cells (n=10). Statistical significance of differences between *Tcf7* $^{+/+}$ controls and *Tcf7* $^{-/-}$ NK cells were determined with the two-tailed Student's t test: * $p < 0.05$; When nothing is marked, the values are not significantly different ($p>0.05$).

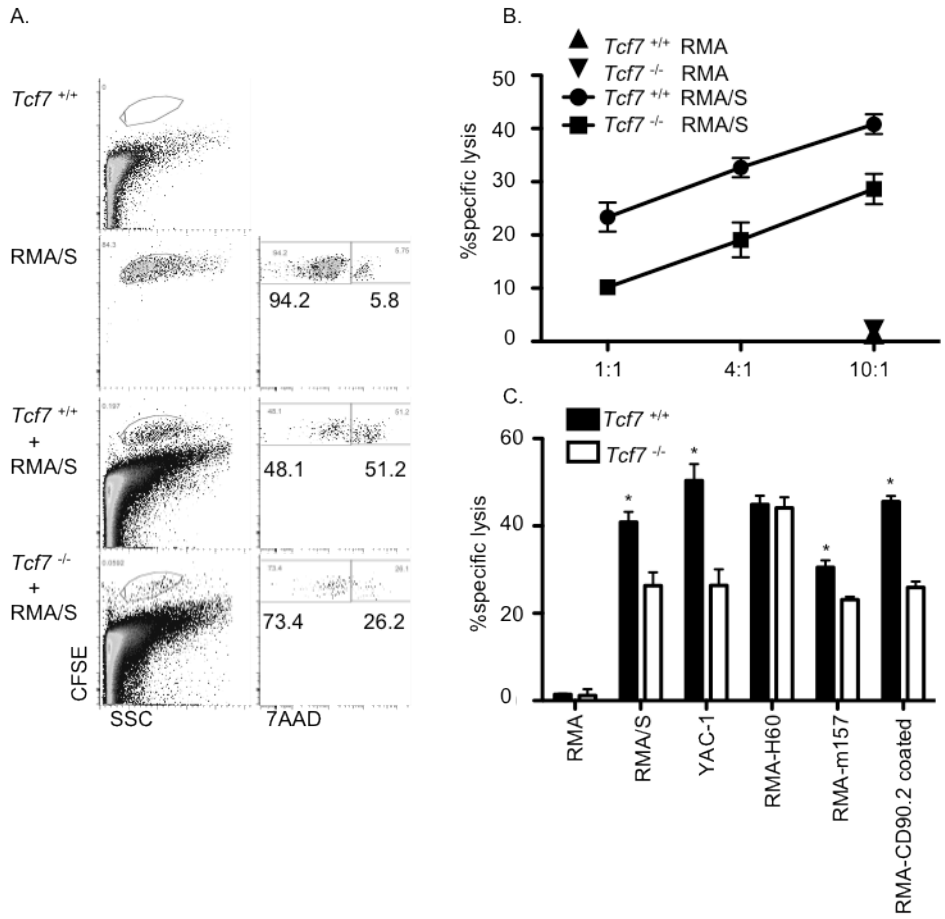


Figure 29. NK cells from *Tcf7* deficient mice show reduced target cell killing.
(A) The lytic activity of Poly IC-activated NK cells, from *Tcf7* $^{+/+}$ and *Tcf7* $^{-/-}$ mice was tested against different tumor cell lines. Target cells were labeled with CFSE and cell death within the CFSE positive population was measured by 7AAD incorporation. B. Poly IC primed *Tcf7* $^{+/+}$ and *Tcf7* $^{-/-}$ splenocytes were exposed to RMA (H-2b) or with RMA/S (MHC-I$^{low)}$ tumor cells at different effector:target ratios. Five hours later the percentage of dead target cells was determined by FACS Graph in B shows the mean percentage of specific lysis (spontanious death is subtracted) of at least 10 independent experiments. C. Bar graphs represent the percentage of specific lysis using the indicated target cells (RMA (H-2b), RMAS MHC-I^{low}, RMA-H60 or RMA-m157). Antibody dependent cellular cytotoxicity (ADCC) was tested using anti-Thy1 (CD90.2) coated RMA cells. Bar graphs show the mean % of specific lysis ± SD of n=6 independent experiments. Based on the two-tailed t-test (*), (**) and (***) depict significant differences (p<0.05), (p<0.01) and (p<0.001) respectively, compared to *Tcf7*$^{+/+}$ controls. When nothing is marked, the values are not significantly different (p>0.05).

Finally we tested the functionality of NK cells in *Tcf7*$^{-/-}$ mice using *in vivo* lysis of β2m-deficient (MHC-I^{low}) splenocytes. In contrast to wild type and *Tcf7*$^{+/-}$ mice, which readily rejected β2m-deficient splenocytes, *Tcf7*$^{-/-}$ mice completely failed to reject β2m-deficient splenocytes (Fig. 30A). We verified whether deficient rejection was due to impaired NK cell priming. In response to Poly IC, the *Tcf7*$^{-/-}$ NK cells efficiently up-regulated CD69 (Fig. 30B) (Lucas et al., 2007), and expressed intracellular GzmB and Perforin (Fig. 30C) (Fehniger et al., 2007), thus indicating that *Tcf7*$^{-/-}$ NK cells

were efficiently primed. Collectively these data suggest that *Tcf7*$^{-/-}$ NK cells can efficiently respond to cellular targets by releasing cytokines and chemokines. Cell-mediated lysis *in vitro* and particularly *in vivo* is impaired, despite proper priming and the expression of Perforin and Granzmye B protein.

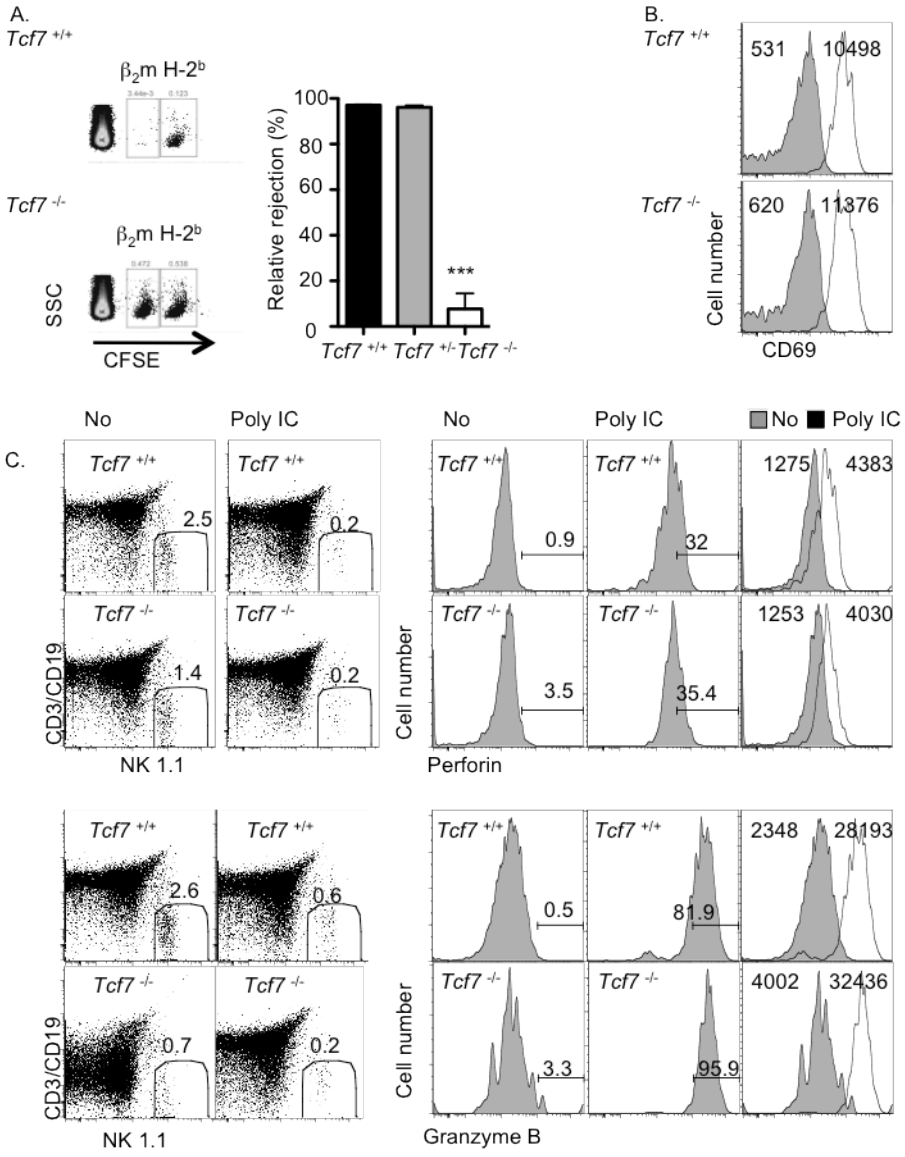


Figure 30. *Tcf7* deficient mice fail to reject β2m-deficient cells *in vivo*.
A. The functionality of NK cells in *Tcf7*$^{-/-}$ mice was tested using *in vivo* lysis of β2m-deficient (MHC-I^{low}) splenocytes. Poly IC primed wilde type and deficient mice were injected with a 1:1 mixture of β_2m (CFSE low) and H-2^b (CFSE high) splenocytes. Bar graphs show the percentage of relative rejection calculated as follows {100-[(% final β_2m cells / % final H-2^b cells)/(% initial of β_2m cells/ % initial H-2^b cells) x 100])}. The statistical significance of differences between groups of data was calculated using a one-way analysis of variance (one-way ANOVA) along with the Dunnet's post-test to adjust for multiple comparisons, using a 95% confidence interval. (***) depicts statistical significance ($p<0.001$) compared to *Tcf7*$^{+/+}$ (n=5). B and C. Histograms show the expression of the activation marker CD69 (indicates that NK cell are primed) or Perforin (C upper panel) or GzmB (C lower panel) in gated NK (CD3$^-$CD19$^-$ NK1.1$^+$) in Poly IC primed (black line) or unprimed (filled grey) *Tcf7*$^{+/+}$ *and Tcf7*$^{-/-}$ mice. Shown is one representative example out of at least 3 independent experiments.

To test the ability of Tcf-1 deficient NK cells to control tumor growth *in vivo*, we established β2m-deficient (MHC-I^{low}) B-ALL (B cell acute lymphoid leukemia) cell lines, which are rejected in a NK cell dependent fashion. To this end, β2m$^{-/-}$ BM cells were infected with pMSCV–*BCR-ABL1 (p185)* -IRES-eGFP retrovirus. Following 7 days in culture, 90% of the BM cells were transformed (GFP^{+}) and positive for markers characteristic for B-ALL (B220^{+}, CD43^{+}, BP1^{+}). Tail vein injections of 2 x 10^{6} infected bone-marrow cells lead to the rapid onset of B-ALL disease in NK cell depleted wild type hosts whereas control mice were resistant to the disease (Fig. 31B).

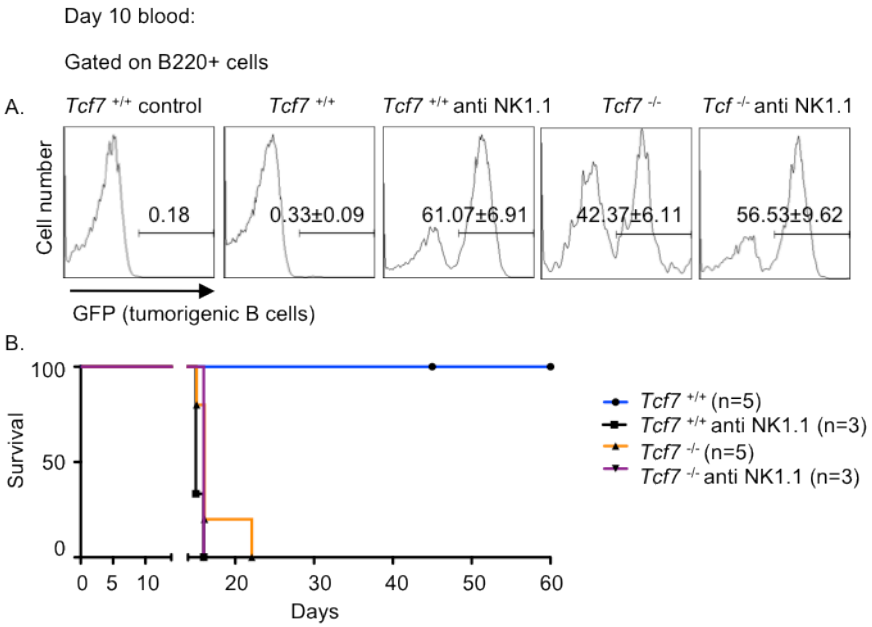


Figure 31. Tcf-1 deficient NK cells are not able to control tumor growth *in vivo*.
β2m$^{-/-}$ BM cells were infected with pMSCV–*bcr/abl p185*-IRES-eGFP retrovirus. Subsequently 2 x 10^{6} infected bone marrow cells (90% (B220+, CD43+, BP1+ GFP+)) were injected into the tail vein of non-irradiated *Tcf7*$^{+/+}$ and *Tcf7*$^{-/-}$ mice that had been depleted of NK cells (with anti NK1.1) or not. (A) Histograms show GFP expression in blood B cells (B220+) of *Tcf7*$^{+/+}$, *Tcf7*$^{+/+}$ + anti NK1.1, *Tcf7*$^{-/-}$ and *Tcf7*$^{-/-}$ + anti NK1.1 mice at day 10 after tumor cell injection. Numbers indicate the percentage of gated cells ±SD of two independent experiments. (n=5 for NK cell sufficient and 3 for the NK cell deficient controls). (B) Graph shows the survival curves of the indicated types of mice that have been injected with β2m$^{-/-}$ BALL cells.
Contribution:
Camille Grandclement established the B-ALL model and provided B_ALL cells for the injections.

These data show that growth of β2m$^{-/-}$ B-ALL is controlled by NK cells *in vivo*. We next addressed whether Tcf-1 deficient mice controlled tumor growth *in vivo***.** At day 10 after injection *Tcf7*$^{+/+}$ mice had no GFP^{+} B220^{+} B-ALL cells (0.33±0.09%) in the blood whereas GFP^{+} B220^{+} B-ALL cells (42.37±6.11%) were very abundant in mice that lack *Tcf7* (Fig. 31A). There was no significant difference when *Tcf7*$^{-/-}$ mice were

NK cell depleted (56.53±9.62%), indicating that NK cells in mutant mice are entirely unable to control B-ALL (Fig. 31A). In contrast to wild type mice, which remained healthy, all *Tcf7*$^{-/-}$ mice had to be sacrificed around day 17-22 because they showed severe disease symptoms (Fig. 31B). Symptoms included weight loss, weakness, hunched posture and/or paralysis of the posterior legs, due to secondary tumors in the spinal cord. Further analysis of sacrificed animals revealed a moderate splenomegaly, enlarged LN and a dramatic expansion of cells with a pre-B cell phenotype, B220^{+} CD43^{-} BP-1^{+} (not shown). Taken together these data suggest that the NK cells in *Tcf7* deficient mice are not able to control tumor growth *in vivo*.

2.12 Granzyme B mediates target cell induced death of *Tcf7*$^{-/-}$ NK cells

Tcf-1-deficient NK cells are unable to efficiently lyse tumor target cells *in vitro* and *in vivo* (Figs. 29 and 31). Based on the over-expression of GzmB, we hypothesized that NK cells undergo self- inflected damage upon activation by target cells. Therefore we assessed NK cell death in response to target cells. Splenocytes from Poly IC primed *Tcf7*$^{+/+}$ and *Tcf7*$^{-/-}$ mice were co-cultured with RMA/S (MHC-I^{-}) tumor target cells. As compared to wild type NK cells (8.4±1.9% 7AAD^{+} n=5) a significantly increased fraction of *Tcf7*$^{-/-}$ NK cells underwent cell death (21.1±1.8% 7AAD^{+} n=5) (Fig. 32) in response to susceptible RMA/S target cells. In the absence of target cells or the presence of NK cell resistant RMA cells we did not detect an increased fraction of 7AAD+ Tcf-1 deficient NK cells (Figs. 32A and B). Thus *Tcf7* deficient NK cells undergo increased cell death only upon target cell recognition.

Next we determined whether the increased NK cell death is mediated by GzmB. The addition of a GzmB inhibitor (z-AAD-CMK) prevented the increased cell death of Tcf-1 deficient NK cells in response to RMA/S targets whereas a control inhibitor (z-FA-CMK) did not rescue NK cells (Figs. 31A and B). Furthermore, blockade of cathepsin C (dipeptidyl peptidase I, DPP-I) whose activity is required for GzmB activation (Meade et al., 2006) also reduced the death of *Tcf7*$^{-/-}$ NK cells (Figs. 32 A and B). On the other hand z-VAD, which blocks the activity of several major caspases, did not prevent the death of Tcf-1 deficient NK cells (Figs. 32A and B). Thus Poly IC activated Tcf-1 deficient NK cells undergo accelerated cell death in response to

susceptible target cells and this can be prevented by the inhibition of GzmB or cathepsin C.

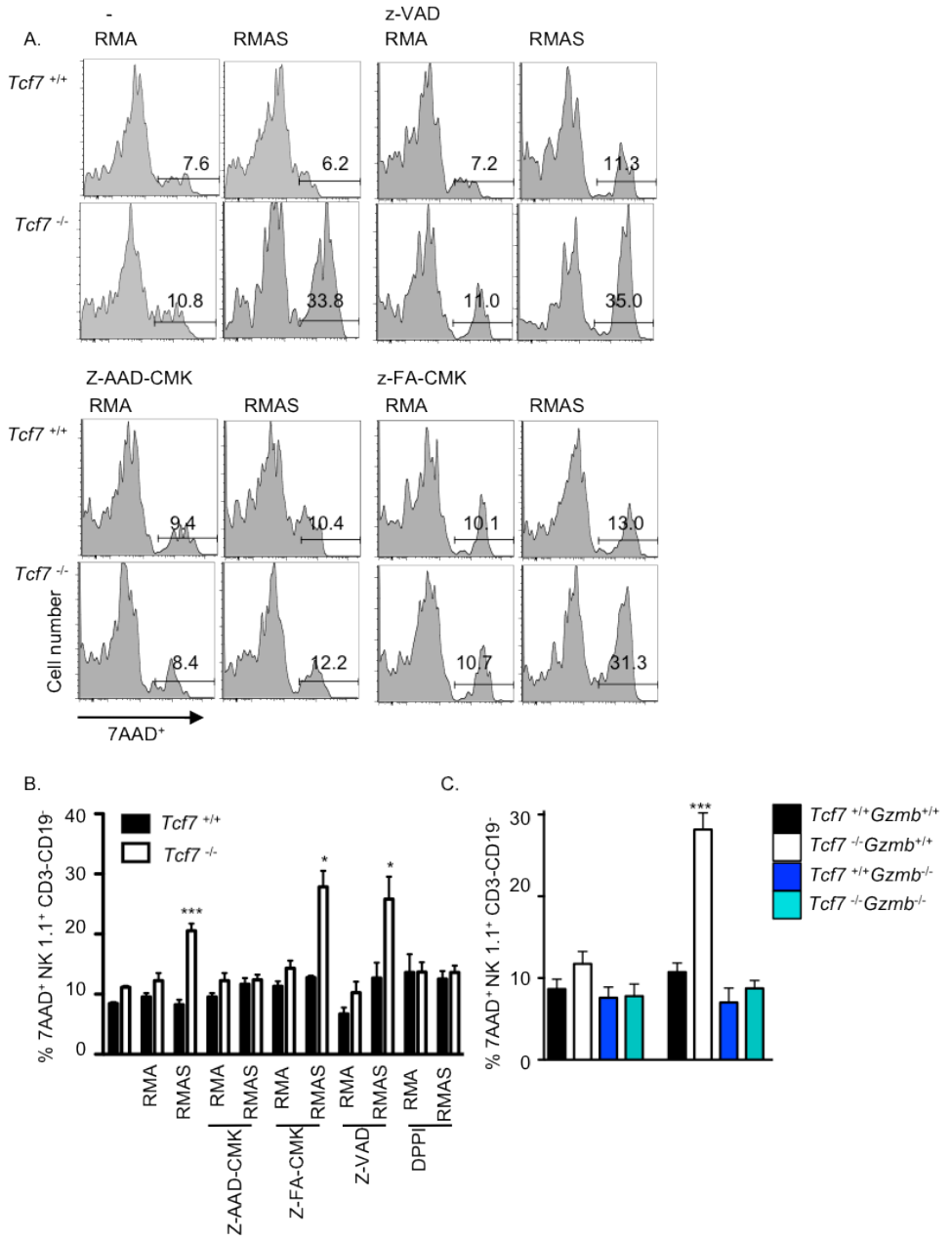


Figure 32. Accelerated cell death of *Tcf7* deficient NK cells upon activation by target cells depends on GzmB.
A. Histograms depict splenic NK cells ($CD3^-CD19^-NK1.1^+$) from Poly IC primed *Tcf7* $^{+/+}$ and *Tcf7* $^{-/-}$ mice that have been exposed to susceptible RMA/S or resistant RMA tumor cells. Numbers show the percentage of dead (7AAD+) NK cells. The assays was performed n the presence of the GzmB inhibitor (z-AAD-CMK) (100uM), its negative control peptide (z-FA-CMK (100uM)), the caspase inhibitor (z-VAD (50uM) or the cathepsin C inhibitor (DPPI (15-25 uM)). These data are a representative of at least 3 independent experiments n=3-9. (B) Bar graphs depict the mean percentage (±SEM) of 7AAD-positive *Tcf7*$^{+/+}$ and *Tcf7*$^{-/-}$ NK cells. (C) Histogram show the mean percentage (±SEM) of dead (7AAD+) *Tcf7* $^{+/+}$*Gzmb*$^{+/+}$, *Tcf7* $^{+/+}$*Gzmb*$^{-/-}$, *Tcf7* $^{-/-}$*Gzmb*$^{+/+}$ and *Tcf7* $^{-/-}$ *Gzmb*$^{-/-}$ NK cells that have been primed exposed to susceptible RMA/S or resistant RMA tumor cells (*), (**) and (***) depict significant differences ($p<0.05$), ($p<0.01$) and ($p<0.001$), respectively based on the student`s t-test. When nothing is marked, the values are not significantly different ($p>0.05$).

Finally we used *Tcf7*x*GzmB* double ko mice to verify whether NK cells undergo cell death in response to susceptible tumor target cells. Indeed the absence of GzmB rescued *Tcf7* deficient NK cells from target induced cell death (Fig. 32C). We conclude that Tcf-1 is essential for the killing of tumor target cells by protecting NK cells from GzmB mediated self-inflected injury.

Data contribution: All experiments and analyses have been performed by Jasmine Gehrig if not stated otherwise.

3 Discussion

3.1 An essential role of Tcf-1 for NK cell development

In the first part of this thesis we defined the T cell factor-1 (Tcf-1) dependent stages of NK cell development. We found that Tcf-1 is highly expressed at the immature NK (iNK) and mature NK (mNK) cell stages. Next, we addressed the question of whether NK cell development is associated with canonical Wingless/Integration-1 (Wnt) signaling. Tcf-1 binds to DNA but lacks intrinsic transcriptional activity. Target gene activation in response to Wnt signals depends on the co-activators ®-catenin and γ-catenin, which bind to the N-terminus of Tcf-1. In contrast, target gene repression is mediated via the association with the Groucho (Gro) co-repressors that bind to a more central domain of Tcf-1 (Grigoryan et al., 2008; Jeannet et al., 2008a). We found that Wnt signaling (assessed by conductin reporter activity) is strongly induced during the NKp to iNK transition and is maintained in a subset of mNK cells. Importantly, this signal was entirely dependent on Tcf-1 in both splenic, as well as BM mNK cells. Thus the up-regulation of Tcf-1 at the iNK stage correlates with Tcf-1 mediated Wnt signaling.

In order to define if Wnt signaling drives NK cell development, we used a genetic complementation approach. Surprisingly we found that the short Tcf-1 isoform (p33), which only contains the DNA- and Gro-binding domains (Oosterwegel et al., 1991a; Van de Wetering et al., 1996), is sufficient to restore NK cell development in Tcf-1-deficient mice (Fig. 14 and 15). NK cell development is also restored by a p45 transgene (Tg), which includes the N-terminal β-catenin-bining domain of Tcf-1. In agreement with these findings, we found that β- and γ-catenin are dispensable for NK cell development (Fig. 13). Thus, NK cell development is dependent on binding of Gro co-repressors to Tcf-1 at the DNA, whereas canonical Wnt signaling mediated by the N-terminal domain of Tcf-1 is dispensable.

Our laboratory has previously shown that T cell development depends on the N-terminus of Tcf-1, i.e. the p45 Tcf-1 isoform. Importantly, in this thesis we show that the regulation of the NK cell development differs from T cell development. NK cell development is restored by p33 and does not rely on signals mediated through the N-terminal domain of Tcf-1. Thus, we found an important molecular difference between

T and NK cell development. The two subsets depend on different Tcf-1 domains that mediate different functions. T cell development depends on target cell activation via the N terminal domain, whereas the repressor function of Tcf-1 is crucial for NK cell development. Here we demonstrate for the first time that the repressor function of Tcf-1 specifically drives an immunological process.

Recently it has been shown that Tcf-1 is essential for the development and/or differentiation of innate lymphocyte subsets including group 2 innate lymphoid cells (ILC2) and NKp46+ ILC3 cells (Mielke et al., 2013; Yang et al., 2013). However, the precise role of Tcf-1 in this process still remains to be elucidated. As NK cells and the ILC lineages are closely related, it would be interesting to test if the repressor p33 isoform of Tcf-1 is sufficient to restore ILC development as well.

Even though NK cell development is restored by both, Tcf-1 p33 and p45, there are differences between the two: Enforced p45 expression in *Tcf7* deficient mice reduces the maturation of developing NK cells. Such mNK cells do not up-regulate KLRG-1, CD11b and CD43, but maintain high levels of CD27, a hallmark of immature NK cells. Conversely, in the absence of *Tcf7*, NK cells have a more mature phenotype. These NK cells express KLRG-1, CD11b and CD43, while CD27 is reduced compared to wild type. The maturation status of *Tcf7* deficient NK cells that express the p33 Tg was comparable to that of wild type (Fig. 14 and 15). Peripheral NK cells can be divided into $CD11b^{-}$ $CD27^{+}$ (immature), $CD11b^{+}CD27^{+}$ (mature1; M1), and $CD11b^{+}CD27^{-}$ (mature2; M2) cells, with the immature $CD11b^{-}CD27^{+}$ NK cell population having a very low cytotoxic potential (Huntington et al., 2007). In p45 Tg *Tcf7* deficient mice, almost all NK cells show an immature $CD11b^{-}CD27^{+}$ phenotype. We propose a model in which distinct stages of NK cell maturation depend on different functions of Tcf-1. While enforced canonical Wnt signaling (p45 function) keeps NK cells at a very immature stage, the p33 isoform allows normal maturation, whereas total loss of Tcf-1 results in accelated maturation (Fig. 33).

In contrast to several other transcription factors that are required for NK cell development and have been shown to promote NK cell maturation, e.g. the E4-binding protein 4 (E4bp4), the interferon regulatory factor (IRF), GATA-3 and T-bet (Gascoyne et al., 2009; Gordon et al., 2012; Lohoff et al., 2000; Male et al., 2011; Samson et al., 2003; Taki et al., 2005), Tcf-1 dampens NK cell differentiation and thus plays a unique role for NK cell maturation (Fig. 33). Those findings suggest that

NK cell maturation is governed by the interplay of transcription factors that drive or inhibit NK cell differentiation, respectively.

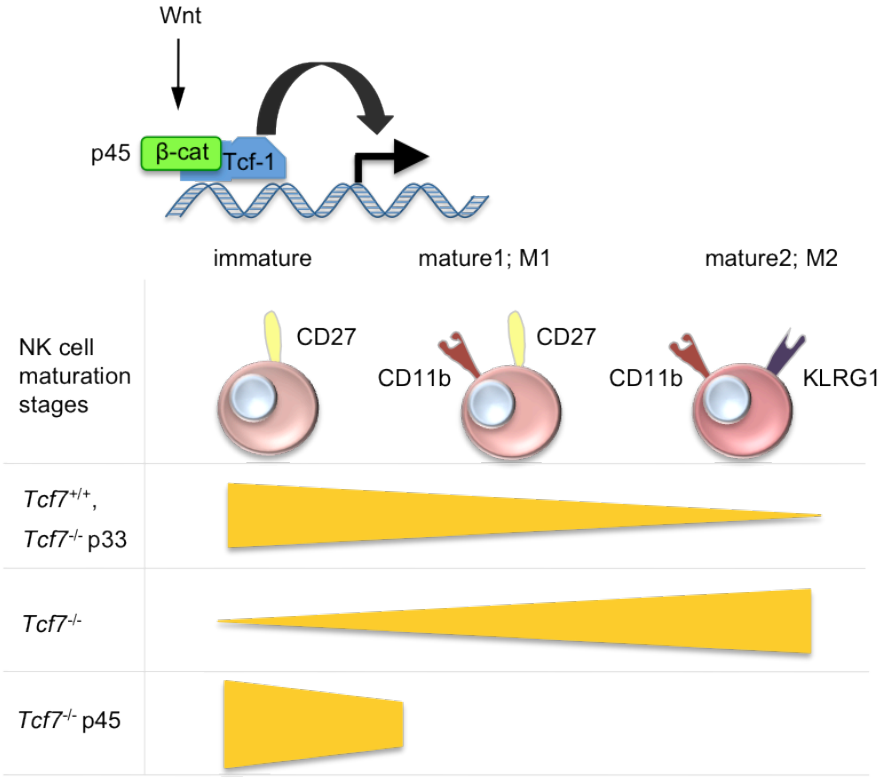


Figure 33. Enforced canonical Wnt signaling (p45 function) prevents maturation of NK cells. NK cells can be divided into CD11b$^-$ CD27$^+$ (immature), CD11b$^+$CD27$^+$ (mature1; M1), and CD11b$^+$ CD27$^-$ (mature2; M2) subsets. The CD11b$^+$CD27$^+$ subset is the most potent in acquiring effector functions whereas the M2 population mostly co-expresses KLRG1 that marks terminal maturation. In *Tcf7*$^{+/+}$, and *Tcf7*$^{-/-}$p33 mice the M2 subset represents the smallest population. In the absence of Tcf-1 maturation is accelerated and most NK cells show a terminal mature M2 CD11b$^+$ CD27$^-$ KLRG$^+$ phenotype. Conversely, in p45 Tg *Tcf7* deficient mice, almost all NK cells show an immature CD11b$^-$CD27$^+$ phenotype, suggesting that enforced canonical Wnt signaling (p45 function) prevents maturation. The yellow bar represents the relative number of cells in the different maturation stages for the indicated genotype.

3.2 Basis for impaired NK cell development

The emergence of mNK cells coincides with the rapid cycling of BM NK cells (Kim et al., 2002). We found that Tcf-1 deficient NK cells in the bone marrow cycle as efficiently as in control mNK cells, but undergo accelerated apoptosis (Fig. 17).To identify candidates that could mediate apoptosis of Tcf-1 deficient mNK cells, we established the global gene expression profiles of BM mNK cells isolated from *Tcf7*$^{+/+}$ and *Tcf7*$^{-/-}$ mice using Affymetrix arrays. Amongst the most up regulated transcripts in the absence of Tcf-1, was the gene encoding *Granzyme B* (*GzmB)* (Fig. 34). Genes coding for other Granzyme family proteins, including *GzmC* and *GzmK* were also up-

regulated (Table 1 and 2). The Affimetrix analysis did not show any differences for Perforin transcript levels.

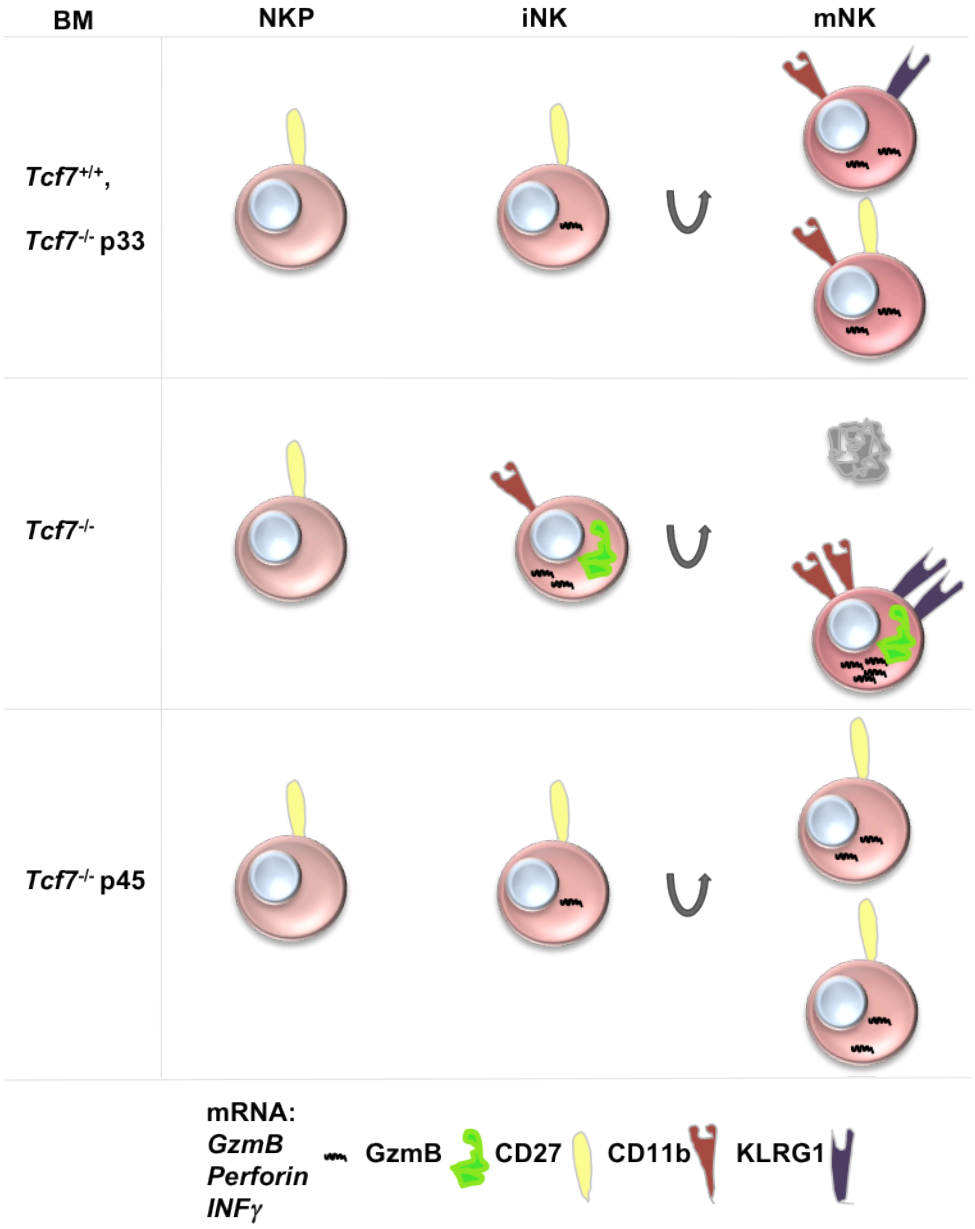


Figure 34. The repressor function of the short p33 Tcf-1 isoform is sufficient to control GzmB expression and to ensure survival of proliferating NK cells.
Shown is the sequential maturation of NK cells in the BM of *Tcf7*$^{+/+}$ and *Tcf7*$^{-/-}$p33, *Tcf7*$^{-/-}$ or *Tcf7*$^{-/-}$p45 mice. The model proposes that the the suppressor activity of Tcf-1 (p33) is vital to ensure the survival of expanding mNK cells and for the suppression of GzmB (top panel). In the absence of *Tcf7* NK cell maturation is accelerated, GzmB is up-regulated and translated and the expanding mNK cells undergo increased cell death (middle panel). Enfored signalling mediated by the N-terminal domain of Tcf-1 (present in p45) prevents maturation (bottom panel).

GzmB is a serine protease that together with Perforin is a main component of exocytic granules in cytotoxic T cells (CTL) and NK cells. Such granules are a hallmark of cell types that are able to kill cells infected with intracellular pathogens or

tumor cells (Russell and Ley, 2002). Upon target cell recognition, lytic granules are polarized towards the immunological synapse where they fuse with the plasma membrane. Their content is relased into the synaptic cleft between effector and target cell. Subsequently GzmB enters the target cells through pores in the plasma membrane formed by Perforin (Long et al., 2013; Voskoboinik et al., 2006; Wang et al., 1996). Once present in a target cell, GzmB can activate multiple caspases, including caspases 1, 2, 3, 6, 7, 9, and 10 (Lieberman, 2003), but apoptosis induced through the activation of caspase 3 seems to be the main pathway in GzmB mediated cytotoxicity (Ewen et al., 2012). GzmB can directly activate some caspase substrates as well, including PARP and NuMA (Beresford et al., 1999). Additionally, GzmB has been shown to promote permeability of the mitochondrial outer membrane and to activate t-BID as well as to cleave ICAD independently of caspases (Barry et al., 2000; Thomas et al., 2001).

In addition to killing other cells, GzmB-leakage into the cytoplasm can induce cell death in NK cells and CTLs (Ashton-Rickardt, 2013). It has previously been shown that primed NK cells undergo increased cell death when they are stimulated with antibodies against certain activating receptors (Ida and Anderson, 1998; Ida et al., 1997). CD2-induced activation of IL-2 primed NK cells leads to GzmB mediated self-inflected damage (Ida et al., 2003). In addition, T cell receptor-induced cell death (TCR-ICD) of Th2 cells is mediated by GzmB-induced self-inflicted damage, independently of caspase activation (Devadas et al., 2006). Moreover, it has been shown that iNKT cells undergo increased GzmB mediated self-inflicted damage in response to Ag stimulation (Ansari et al., 2010). Finally *c-Myc* and *N-Myc* deficient HSCs over-express GzmB and undergo increased cell death (Laurenti et al., 2008).

Mature and BM NK cells in naïve mice express GzmB and Perforin mRNA constitutively. Importantly, resting or BM NK cells are minimally cytotoxic, because Perforin and GzmB mRNAs are not translated. In agreement with these data we could not detect any GzmB and Perforin protein in developing NK cells from wild type mice. The mechanisms that prevent the translation are currently not well understood. This translational block is overcome upon NK cell priming by cytokines like IL-15, IL-2 and IL-18 that are produced early in an immune response by infected or activated dendritic cells (DCs) and macrophages (Fehniger et al., 2007; Stetson et al., 2003; Walzer et al., 2005). In contrast, in the absence of *Tcf7*, GzmB is detected in BM (from the iNK stage onwards) and spleen NK cells even in the absence of priming,

suggesting that Tcf-1 is involved in the regulatory mechanism responsive to NK cell priming. The presence of the short p33 domain of Tcf-1 engaging Gro co-repressors was sufficient to regain wild type like GzmB control (Fig. 34).

Upon cytokine stimulation NK cells increase in size and develop a high number of GzmB containing cytotoxic granules in their cytoplasm. Activated NK cells that lack Tcf-1 express much higher levels of GzmB mRNA and slightly more GzmB protein than their wild type counterparts. We show that cytokine stimulation of Tcf-1 deficient splenic NK cells results in the impairment of expansion. Similar to bone marrow mNK cells, cytokine stimulated NK cells are cycling normally but undergo accelerated cell death (Fig. 17). The presence of the short p33 domain of Tcf-1 was sufficient to restore NK cell expansion in the absence of *Tcf7*. Furthermore, enforced p33 expression significantly decreased GzmB levels compared to wild type controls.

GzmB is produced as a pro-peptide and needs to be cleaved by the protease cathepsin C (or DPPI) to gain proteolytic activity. In cytokine activated and primed wild-type NK cells, GzmB is stored in exocytic Lamp-1 positive granules present in the cytoplasm. Granzymes are packaged into the cytotoxic granules via the mannose 6-phosphate receptor (MPR) pathway (Lopez et al., 2012). In the granules, Granzymes and Perforin are kept inactive by low pH and calcium concentrations. In fact, activated NK cells and CD8 T cells contain small amounts of GzmB in their cytoplasm (Ashton-Rickardt, 2013). This suggests that some GzmB might leak from the granules or that some newly synthesized GzmB does not properly traffick to the granules but may be secreted via the constitutive secretory pathway as described previously (Isaaz et al., 1995). However, the serine protease inhibitor 6 (Spi-6) that is present in the cytoplasm of activated cytotoxic cells captures free GzmB and prevents its processing and activation as a protective mechanism (Fig. 35). Free GzmB in the cytoplasm of Spi-6 deficient cytotoxic T cells disrupts lytic granules, this results in GzmB-mediated apoptosis (Ansari et al., 2010; Zhang et al., 2006). Therefore, the major determinant of GzmB-leakage-induced cell death seems to be an excess of free active GzmB over its principal cytoplasmic inhibitor Spi-6 (Ida et al., 2003; Laforge et al., 2006; Phillips et al., 2004; Zhang et al., 2006). Here, we show that Spi-6 was expressed at normal levels in naïve and activated Tcf-1 deficient NK cells, whereas GzmB levels were significantly elevated (Fig. 20).

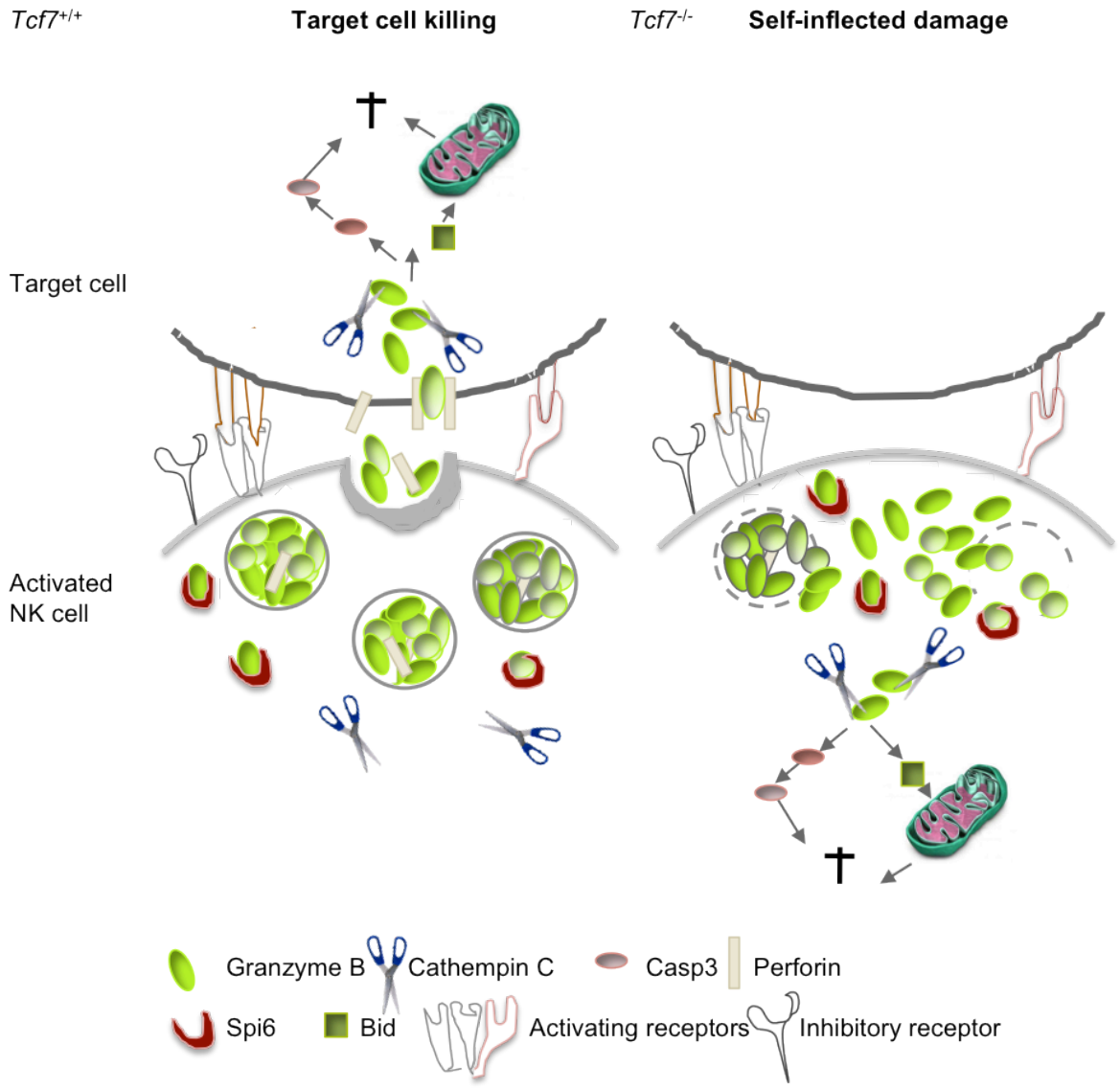


Figure 35. Tcf-1 deficient NK cells undergo self-inflected damage upon target cell recognition. Following priming by cytokines NK cells translate pre-formed mRNAs for GzmB, INFγ and Perforin. Subsequently in a wild type situation ($Tcf7^{+/+}$) target cell recognition and activation triggers polarization of lytic granules filled with GzmB and Perforin towards the immunological synapse, where they fuse with the plasma membrane and release their contents into the synaptic cleft between effector and target cell (left panel). GzmB enters the target cells with the help of Perforin, and rapidly induces apoptosis via both, caspase-dependent and caspase-independent mechanisms. GzmB is expressed as an inactive precursor protein that carries an N-terminal signal peptide that directs packaging of the protein into secretory granules. Removal of the dipeptide Gly-Glu at the N-terminus of pro-GzmB by the cysteine protease cathepsin C generates the enzymatically active form of GzmB. The principal cytoplasmic inhibitor of GzmB is the serine protease inhibitor 6 (Spi-6). Spi-6 prevents the processing of an N-terminal pro-peptide by the protease cathepsin. NK cells that lack *Tcf7* (right panel) over-express GzmB while Spi-6 levels remain unchanged. That leads to free pro-GzmB in the cytoplasm that can be cleaved by Cathepsin C. Active GzmB induces apoptosis and death of the host cell (right panel). Therefore NK cells show impaired in target cell killing in the absence of *Tcf7*.

In contrast to the discrete Lamp1 positive granules observed in cytokine activated wild type NK cells, cytokine activated Tcf-1 deficient NK cells show a diffuse distribution of GzmB and Lamp-1 in the cytoplasm (Fig. 21 and 22). To exclude that Tcf-1 deficiency plays a direct role for granule stability in activated NK cells, we

analyzed *Tcf7*x*GzmB* double deficient NK cells and found granular LAMP-1 staining to be restored. Therefore, we conclude that it is the excess of GzmB that destroys the integrity of lytic granules and not the absence of Tcf-1 per se or different downstream targets that lead to an accumulation of free GzmB in the cytoplasm.

To determine whether GzmB mediates accelerated NK cell death, we generated mice deficient for both, *GzmB* and *Tcf7*. *GzmB* deficiency restored the expansion of peripheral NK cells lacking *Tcf7.* Thus the observed self-inflicted cell death is indeed caused by the elevated GzmB levels (Fig. 35). However, the absence of GzmB was not sufficient to restore NK cell development in *Tcf7*-deficient mice. Besides GzmB, GzmC was also up regulated in *Tcf7* deficient mNK cells. *GzmB* deficient mice also express reduced levels of GzmC and F, which are just downstream in the *GzmB* gene cluster (Pham et al., 1996; Revell et al., 2005). It is thus not likely that GzmC or F play a significant role at this stage of NK cell development.
Based on these findings we conclude that Tcf-1 regulates an additional factor that is required for the survival of mNK cells. A third Granzyme that is increased in the absence of *Tcf7* is GzmK, which is located in a distinct cluster together with *GzmA*. GzmK can also induce rapid caspase-independent cell death in target cells (Zhong et al., 2012). So far it is not known whether GzmK is constitutively expressed in NK cells like GzmA, or whether it is translated and/or transcribed upon priming like GzmB. A single study with human NK cells showed that GzmK is mostly expressed in the $CD56^{high}$ NK cell population. Conversely, GzmB is primarily expressed in the $CD56^{low}$ NK cell subset, while GzmA is constitutively expressed in both subsets. These findings suggest that similar to GzmB, GzmK expression is not constitutive, but depends on specific triggers (Bratke et al., 2005). Although it is not known whether GzmK can mediate self-inflected damage, it is tempting to speculate it might act in a similar manner as GzmB when present in the cytoplasm. Therefore, further experiments are needed to test if GzmK mediates accelerated cell death in Tcf-1 deficient mNK cells lacking GzmB. Employing a GzmK specific inhibitor or *Tcf7*, *GzmB* and *GzmK* deficient mice would be a way to test if GzmK mediates self-inflected damage of *Tcf7* and *GzmB* deficient mNK cells.

Taken together, the data suggest that under steady state conditions GzmB expression is suppressed by Tcf-1. Two reasons could account for the enhanced

presence of GzmB protein in the absence of Tcf-1. First, the control mechanisms that block translation are over-come by the elevated GzmB mRNA levels. Second, the mediators that control GzmB translation are directly or indirectly affected by the loss of Tcf-1. Control of GzmB translation is not well understood and further experiments are needed to address these questions.Accordingly, it remains to be determined in further experiments if GzmB is a direct target gene of Tcf-1 regulation. To test whether Tcf-1 binds to the GzmB locus we are currently performing Tcf-1 chromatin immunoprecipitation followed by parallel sequencing (ChIP-seq) analysis of NK cells. The aim is to use the ChIP-seq data to identify Tcf-1 binding sites and to relate this information to the presence of activating (histone H3 trimethylated at lysine 4, H3K4me3) and repressive (H3K27me3) histone marks. Our hypothesis is that in wild type NK cells, Tcf-1 is bound to the *GzmB* locus and that this is associated with repressive histone marks. Consequently, in Tcf-1 deficient NK cells we would expect the active histone marks to be more abundant. If we identify a Tcf-1 binding sequence within the *GzmB* locus, we would need to perform reporter assays to confirm that Tcf-1 binding to this site results in repression or activation of *GzmB* transcription.

If direct binding of Tcf-1 to the GzmB promoter region cannot be shown, the observed regulatory effects of Tcf-1 on GzmB expression must involve further, yet unidentified molecular components. The iNK to mNK transition is impaired in the absence of the two T-box transcription factors T-bet and Eomes (Gordon et al., 2012). This process appears similar to our observations on Tcf-1 deficiency. Eomes is a direct target of Tcf-1 in CD8 T cells (Zhou et al., 2010). Eomes and T-bet coordinately regulate the expression of key effector molecules in cytotoxic lymphocytes such as Perforin, Granzymes and INFγ (Cichocki et al., 2013; Pearce et al., 2003; Szabo et al., 2000). Therefore, T-bet and Eomes may be downstream targets of Tcf-1 in NK cell development. While Eomes levels in BM NK cells that lack Tcf-1 did not significantly change, T-bet levels were slightly but significantly increased (around 30%). T-bet expression levels were reduced to normal in the p33 and the p45 Tg Tcf7$^{-/-}$ NK cells, respectively (data not shown). Thus, in addition to a possible direct effect, Tcf-1 might also control *GzmB* expression indirectly through the repression of T-bet. Our Chip-seq analysis may shed light on this question as well.

3.3 NK cell function in the absence of Tcf-1

We tested whether *Tcf7* deficient NK cells are able to mediate normal effector functions. Upon priming of mice with poly IC, wild type and *Tcf7* deficient NK cells comparably induced CD69 and expressed GzmB and Perforin. In response to target cells*, Tcf7* deficient NK cells normally released cytokines, chemokines and granule contents (Lamp-1). However, their ability to kill target cells *in vitro* and particularly *in vivo* was impaired. Rather than killing target cells, NK cells themselves underwent increased cell death upon the recognition of susceptible target cells. Using inhibitors, we found that the increased cell death upon target cell recognition was prevented by blocking the activity of GzmB or Cathepsin C. Moreover, the increased cell death upon target cell exposure was prevented when Tcf-1 defcient NK cells also lacked GzmB (Fig. 32).

Based on the data in this report our working model is that under steady state conditions, Tcf-1 suppresses GzmB levels. In an inflammatory context, NK cells are primed and this leads to a down-regulation of Tcf-1 and NK cells gain their full cytotoxic potential through the expression of GzmB. Indeed, we found that Tcf-1 is strongly decreased yet still expressed at low levels in NK cells of Poly IC primed mice (Fig. 9). Low levels of Tcf-1 may restrict GzmB production and thus prevent GzmB mediated self-inflected damage. If an inflammation is reaching an extent that is dangerous for the organism, Tcf-1 expression may shut off, leading to more free GzmB in the cytoplasm. This may induce apoptosis of highly activated NK cells that potentially harm the host itself (Fig. 35 and 36).

It remains to be explored in future experiments if the enforced expression of the Tcf-1 Tgs prevents maturation (N-terminus, p45) and maintains GzmB repression (p33) during viral infections such as the mouse cytomegalovirus (MCMV). Furthermore, it has to be determined if such Tg expression leads to decreased cytotoxicity and/or self inflected death. Since it has been suggested that immature NK cells have a very low cytotoxic potential (Huntington et al., 2007), it remains to be tested if the p45 Tg NK cells show a decreased functional competence to respond to tumor target cells (Fig. 34). This would allow to better describe the relationship between maturation markers and cytotoxic potential.

GzmB mediated self-inflected damage may be a naturally occurring mechanism to remove terminally differentiated effector cells. This mechanism may dampen immune responses and prevent damage to host tissue by highly activated NK cells. Tcf-1 would represent a major regulator of this process. Controlling Tcf-1 expression is thus expected to be key in the maturation and survival of NK cells during an immune response. It will be important to elucidate the specific signals which regulate Tcf-1 *in vivo* during the immune response. A Tcf-1 GFP reporter mouse would therefore represent a useful tool.

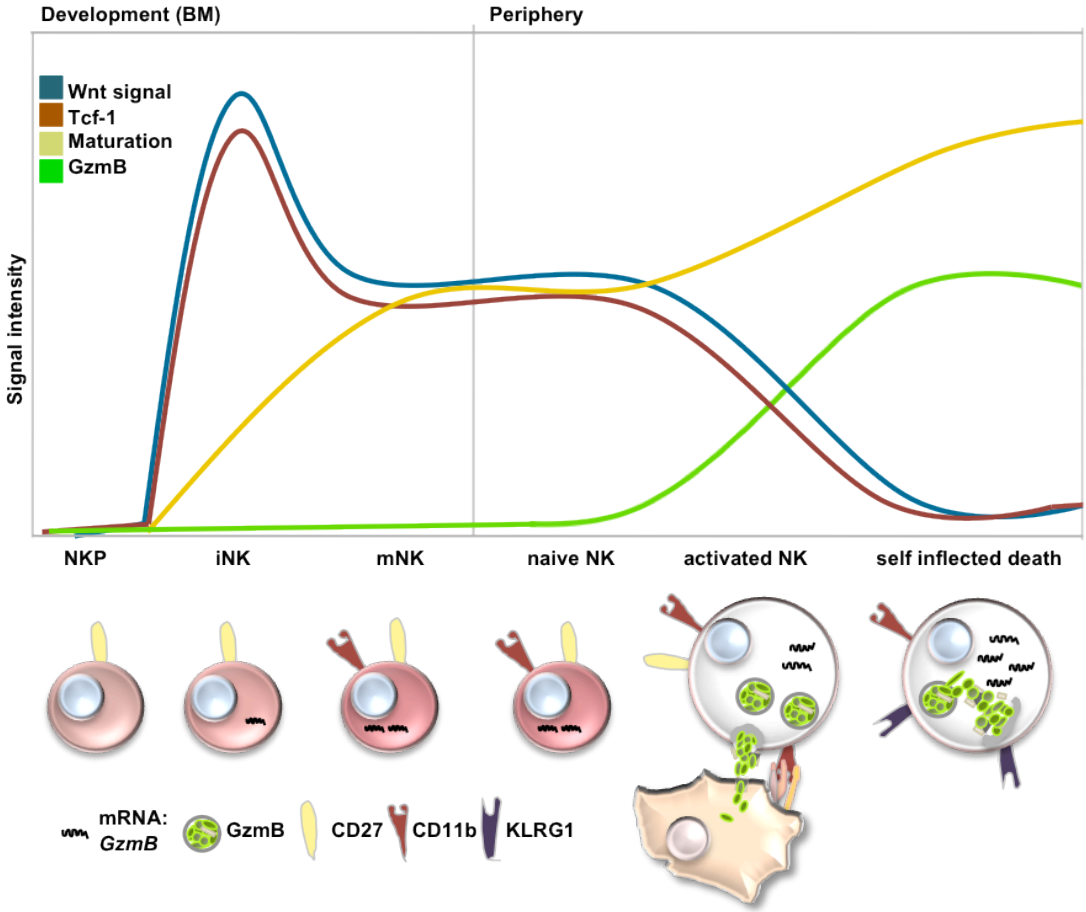


Figure 36. Schematic representation of the model that we developed based on the results shown and discussed in this thesis.
The Wnt signal mediating N-terminal domain of Tcf-1 is crucial to prevent the maturation of iNK cells and a subset of mNK cells, whereas the suppressor (p33) activity of Tcf-1 is vital to ensure the survival of expanding mNK cells and for the suppression of GzmB. In steady state conditions Tcf-1 suppresses GzmB, whereas in an inflammatory context, Tcf-1 levels may decrease. This leads to GzmB expression and up-regulation of the maturation markers KLRG1 and CD11b and the downregulation of CD27 (indicated in violet, purple and yellow, respectively) and subsequent target cell killing upon recognition. During this process some Tcf-1 is still present to prevent GzmB mediated self-inflected damage. In a persistent inflammatory context Tcf-1 levels further decline what leads to increased GzmB expression and self-inflected death. Curves indicate Wnt signaling (blue), Tcf-1 expression (red), maturation (yellow) and GzmB expression (green).

NK cells that have been attracted to the sites of inflammation could represent a highly desirable vehicle for viruses to ascertain rapid spread throughout the host. Viruses that do not infect endothelial cells are unable to enter or leave the bloodstream to infect peripheral tissues. Previously, it has been shown that *GzmA* and *GzmB* double deficient mice have higher virus titers upon infection with the cytopathic pox-viruses. The authors suggest that this observation is linked to the enhanced survival of CTLs and NK cells due to their inability to commit GzmB-mediated suicide. Therefore, the virus might use these cells as Trojan horses to extravagate and infiltrate other organs (Balkow et al., 2001). This suggests that GzmB mediated self-inflected death might also represent an important mechanism to prevent viral spread by activated and highly mobile cytotoxic cells.

Based on the findings shown in my thesis we propose a model where Tcf-1 plays a major role for the suppression of the highly cytotoxic effector molecule GzmB in NK cells, thereby preventing self-inflected damage in these cells. Concordantly, the Wnt signal mediating N-terminal domain of Tcf-1 is crucial to prevent the maturation of iNK cells and of a subset of mNK cells. Our data suggest that upon inflammation, Tcf-1 levels may decrease, that in turn would lead to GzmB expression and to terminal maturation. During the course of an immune response, Tcf-1 levels might further decline, triggering GzmB mediated self-inflected damage of highly activated NK cells (Fig. 36). This mechanism might be important to remove terminally differentiated effector NK cells and thus to dampen immune responses in order to prevent collateral damage of the host tissue. Blocking Tcf-1 expression in highly activated NK cells may therefore be a way to induce self-inflected cell death. In the context of autoimmune inflammation this mechanism could be exploited to eliminate effector cells that attack host tissue. The identification of the factors that modulate Tcf-1 expression during inflammation may therefore allow the identification of potential therapeutic targets.

4 Methods

Mice

C57BL/6 (B6) mice were purchased in Harlan OLAC (The Netherlands). CD45.1 congenic B6 were bred in the specific pathogen free animal facility of our institut. *Tcf7*$^{-/-}$ (Verbeek et al., 1995), *Tcf7*-p33 and *Tcf7*-p45 (Ioannidis et al., 2001), conductin^{lacZ} (Lustig et al., 2002), β-catenin$^{lox/lox}$ (Huelsken et al., 2001), γ-catenin$^{lox/lox}$ (a kind gift from Prof. Jörg Huelsken), Vav-Cre transgenic (Almarza et al., 2004; de Boer et al., 2003), *GzmB*$^{-/-}$ (*GzmB*$^{-/-}$*/ΔPGK-neo*) (Pham et al., 1996; Revell et al., 2005) (a kind gift form Prof. Christoph Borner) mice have been described. B-catenin $^{Flox/Flox}$ γ-catenin $^{Flox/Flox}$, *Vav-Cre*$^{+}$ mice have been crossed by Camille Grandclément in our lab and the *GzmB*$^{-/-}$ (*GzmB*$^{-/-}$*/ΔPGK-neo*) *Tcf7*$^{-/-}$ have been bred by myself. For BM chimeras, 2:1 or 3:1 mixtures of experimental (CD45.2^{+}) and wild type (CD45.1^{+}) BM cells were used (3-6x10^{6} cells total). Chimeras were tail bled at the indicated time points and lymphoid tissues were analyzed 2-12 months after reconstitution.

Flow cytometry

Spleen and bone marrow cells were treated with ACK (NH_4Cl 0.15M, $KHCO_3$ 1 mM, EDTA 0.1 mM, pH 7.3) to remove erythrocytes. Subsequently cells were incubated with Aquadead (Invitrogen) (to exclude dead cells) and anti-CD16/32 (2.4G2) hybridoma supernatant to block Fc receptors before staining with fluorescent mAbs to NK1.1 (PK136), CD49b (DX5), CD122 (TMB1), KLRG1 (2F1), CD27 (LG.7F9), CD11b (M1/70), CD69 (VEA), CD84 (mCD84.7), CRACC/SLAMF7 (235614), Ly-108 (13G3-19D), Ly-9 (Ly9ab3, BioLegend), 2B4 (244F4), CD16 (93, BioLegend), (LFA-1 (FD44.8), Ly-49A (JR9/YE1/48.10.6/A1), Ly-49H (3D10), Ly-49D (4E5), Ly-49G (4D11), Ly-49C/I (5E6), Ly49F (HBF), Ly49I (YLI90), NKp46 (29A1.4), CD3ε (17A2), CD19 (6D5), BP-1 (6C3), B220 (RA3-6B2), CD45.1 (A20), CD45.2 (104.2), CD43 (S/7), CD8a (53-6.7), CD25 (GCG1), GR-1 (Ly6G), Sca1 (D7), Ter119 (Ter119), CD4 (RM4-5), F4/80 (F4/80), CD90.2 (BOH12), TCRβ (H57), TCRγδ (GL3) and CD117 (ACK2) (if not indicated otherwise from BD Biosciences). A cocktail of FITC-conjugated anti-TCRβ, TCRγδ, CD3ε, CD4, CD8, CD11b, B220, NK1.1, Ter119 and GR-1 mAbs was used to gate lineage-negative (lin^{-}) cells. Intracellular staining for

GzmB (16G6, invitrogen), Perforin (OMAK-D, e-Bioscience), GzmA (3G8.5, Santa Cruz) IFN-γ (XMG1.2, e-Bioscience), T-bet (eBio4B10, e-Bioscience), Eomes (Dan11mag, e-Bioscience), MIP-1 alpha (39624, e-Bioscience), Tcf-1 (C63D9, Cell Signal) and Lef (C12A5, Cell Signal) was performed using the BD Cytofix/Cytoperm Fixation/Permeabilzation Kit (BD Biosciences) for IFN-γ, MIP-1α, Bcl-2 (PharMingen) and Bcl-XL (7B2.5, SouthernBiotech) or the Transcription factor staining buffer set" (eBioscience) for GzmB, GzmA, T-bet, Eomes, Tcf-1 and Lef according to the manufacturer's protocol. Data were acquired on an LSRII flow cytometer (BD Biosciences) or a FACSCanto flow cytometer (Becton Dickinson, San Jose, CA) and analyzed with FlowJo (Tree Star, Ashland, OR). Alternatively cells were subjected to sorting on a FACSAria machine (Becton Dickinson, San Jose, CA).

LacZ staining

For measuring LacZ activity, cells were counted and resuspended at $20x10^6$ cells per mL in HBSS (Gibco) containing 2% FCS, 10mM Hepes (Gibco) and 1% Pen-Strep (Gibco)). $2x10^6$ cells were incubated for 10 minutes at 37°C and loaded with pre-warmed Fluorescein Di-β-D-Galactopyranoside 2mM (FDG, Invitrogen) in H_2O for 1 minute. Cells were then quenched with complete HBSS and left for 90min on ice before washing. The cells were surface stained for flow cytometry as described previously. The vital dye 7-AAD (Biolegend) was added 10 min prior to the FACS analysis. Samples were run on LSRII or FACScanto flow cytometers (Becton Dickinson, San Jose, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR).

NK cell expansion

Splenic NK cells were purified by non-adherence to nylon wool followed by depeleting non-NK cells using the NK Cell Isolation Kit II and a MACS separator (Miltenyi Biotec). Purified NK cells were cultured for 5 days in DMEM with 10% FCS, Gentamicin (10mg/ml), 2-Mercaptoethanol (50mM), MEM NEAA (0.1mM), L-Glutamine (4 mM) and 500mg/ml recombinant human IL-2 (a gift from N. Rufer, University of Lausanne). After 5 d, cells were harvested and stained for surface antibodies and for Annexin V (PE Annexin V Apoptosis Detection Kit I, BD Pharmigen) according to the manufacturer's protocol. To identify DNA strand breaks we performed a TUNEL staining (In Situ Cell Death Detection Kit, Roche) that labels

free 3'-OH termini with modified nucleotides in an enzymatic reaction. Data were acquired on an LSRII flow cytometer (BD Biosciences).

***In vitro* NK cell activation to measure cytokine and chemokine release**

Spleen cells (2×10^6) from polyinosinic-polycytidylic acid (Poly IC) primed mice were stimulated with plastic immobilized mAbs to NK1.1 (PK136, BioXCell) (5mg/ml), NKG2D (191004, R&D Systems) (5 mg g/ml), NKp46 (29A1.4 eBiosciences) (2.5 mg/ml), Ly49D (4E5, BD Pharmingen) (2.5 mg/ml) or by target cells RMA (H-2^b), RMA/S (MHC-I^{low}), RMA-H60, RMA-m157 or YAC-1 cells (2×10^6) or by the addition of PMA (50 ng/ml) and Ionomycin (1 mg/ml). GolgiPlug, GolgiStop and LAMP mAb (1D4B, eBiosciences) were added after 1 h of culture and the cells were harvested 4 h later. Cells were incubated with Aquadead (Invitrogen) (to exclude dead cells) and anti-CD16/32 (2.4G2) hybridoma supernatant before staining with fluorescent mAbs to NK1.1 (PK136), NKp46 (29A1.4), CD3ε (17A2), CD19 (6D5) (all from BD Biosciences). Intracellular staining for IFNγ (XMG1.2, e-Bioscience) and MIP-1 alpha (39624, e-Bioscience) was performed using the BD Cytofix/Cytoperm Fixation/Permeabilzation Kit (BD Biosciences). Cells were analyzed on a FACSCanto flow cytometer (BD Biosciences) and analyzed with FlowJo (Tree Star).

FLOKA ASSAY

Mice were intravenously injected with 100ug polyinosinic-polycytidylic acid (Poly IC) (Labforce, tlrl-pic) to prime the NK cells. 24 hours later the mice were sacrificed and the spleens were harvested. The NK cell numbers (effectors) were determined by FACS analysis. Target cells RMA (H-2b), RMAS MHC-I^{low}, RMA-H60 or RMA-m157 were resuspended at a concentration of $1x10^7$ cells/ml in PBS containing 0.3 µM CFSE (5- and 6-carboxyfluorescein diacetate succinimidyl ester, Molecular Probes) and incubated for 5 min at 37°C in the dark. CFSE labelled cells were washed three times in DMEM containing 10% FCS. Subsequently the effector cells were added and the cells were cultured at 37° in a FACS tube with 500 ul of medium (DMEM and 10% FCS) at an effector: target ratio of 1:1, 3:1 or 10:1 (10 000 target cells were used and the effectors were adjusted accordingly). As a control targets and effectors were cultured separately. Four hours later 10 ul of 7AAD (PD Pharmigen) was added. The percentage of death cells ($7AAD^+$) cells within the CFSE positive target cell population was determined by FACS analysis like described earlier. The

spontaneous cell death within the target cells that were cultured without effectors was used to calculate the percentage of cells that underwent cell death due to killing. Inhibitors used: Caspase inhibitor Z-VAD-FMK (Promega), GzmB inhibitor Z-AAD-CMK (Enzo), Cathepsin C inhibitor, Gly-Phe-DMK (MP BIOMEDICALS)

In vivo target cell rejection

Mice were injected intra peritoneal with 100 µg Poly IC (Sigma-Aldrich). 24 h later a 1:1 mixture of 1 × 10^7 H-2^b (labeled 2.0 µM CFSE [Invitrogen]) and 1 × 10^7 β_2m-knockout (MHC-I^{low}) splenocytes (labeled with 0.2 µM CFSE) were injected intraveniously. Spleens of recipient mice were analyzed 24 h later for the presence of transferred cells. The percentage of specific rejection was calculated as follows: 100 − [(percentage of H-2^b cells ($CFSE^{hi}$) final/percentage of β_2m-knockout cells ($CFSE^{low}$) final)/(percentage of H-2^b cells ($CFSE^{hi}$) initial/percentage of β_2m-knockout cells ($CFSE^{low}$) initial)] × 100.

Confocal microscopy

BM mNK cells or splenic NK cells at day 5 of IL-2 stimulation were FACS sorted to get rid of non-NK cells. 5x10^4 NK cell blasts or 1x10^5 mNK cells/ 100uL were put on Poly-L-Lysin (0.1%) coated Super-Frost slides (VWR International). Subsequently cell were fixed for 10 min in 3.7% PFA at room temperature (RT). After three washing steps with PBS cells were permeabilized for 10 min at RT with 0.2% Triton in PBS. After washing cells were blocked for 1 hour with PBS+15% Goat serum (SIGMA) and then 1 hour in 15% Rat serum (SIGMA). Following an over night incubation at 4C° with the primary antibody (GzmB (BAF1865) (R&D); Goat anti-Mouse; biotynilated; and Anti mLAMP1 (1D4B) (BioLegend) Rat anti-Mouse) cells were washed rigorously with BPS and stained for 1 hour at RT with the secondary antibodies in blocking solution (Alexa-Fluor-488 -Strepatvidin) (invitrogen) and Alexa-Fluor- 555 Goat anti-Rat (Life technologies)). After being washed cells were mounted in ProLongGold antifade reagent with DAPI (invitrogen) and covered with a glass. Samples were examined with a LSM 510 confocal microscope (Zeiss) and images were recorded in multitracking mode.

B-ALL induction

For B-ALL like disease induction BM cells from β2m deficient mice were infected with the MCMV-bcr-ablp185/IRES-gfp virus. Before the viral supernatant was collected after transient transfection of 293T cells. The β2m deficient BM cells were cultured with the viral supernatant for 1 hour in the presence of 10ng/mL IL7, 10% WEHI supernatant (as a souce of IL3) and 5µg/mL of Polybrene. The infected BM cells were subsequently cultured for 7 days at 37°C, 5% CO2 in RPMI medium containing 5% FCS. After 7 days of culture, more than 90% of bone-marrow cells were B220+, $CD43^+$, $BP1^+$ and GFP^+. For tail vein injections, 2 x 10^6 infected bone marrow cells were re-suspended in 200µL of PBS and injected via the tail vein in B6 or *Tcf7*$^{-/-}$ recipient mice. In some cases, NK cells were depleted with the NK-depleting antibody PK136 (BioXcell, 200µg/mouse, IV) 2 days before the injection of B-ALL cells and the depletion was then performed once in a week. Mice were checked daily for the onset of the disease. Blood samples were analyzed 10 days after the injection of B-ALL cells to follow the disease. Sick mice were killed and analyzed for spleen weights, NK cell counts and the presence of leukemic cells.

Western Blot

Western blots on total cell lysates from FACS sorted mNK, splenic NK or IL-2 stimulated NK cells (100 000-500 000 cells per lane) were performed as described (Ioannidis et al., 2001). Blots were incubated overnight at 4°C with primaryantibodies: Rb polyclonal Ab anti-GzmB (abcam), PI-9 (PI9-17) (Santa Cruz Biotechnology) or anti-Tubulin (B-5-1-2, Sigma). Western blots were revealed with HRP- conjugated anti-mouse and anti-rabbit Abs (Sigma) followed by enhanced chemiluminescence detection (Pierce, Rockford, IL).

Microarray

Global gene expression profiles of FACS-purified BM mNK cells ($CD3^-CD19^-$ $CD122^+NK1.1^+DX5^+$) isolated from *Tcf7*$^{+/+}$ and *Tcf7*$^{-/-}$ mice were established using Affymetrix arrays. A minimum of 3x6 *Tcf7*$^{+/+}$ and *Tcf7*$^{-/-}$ mice were sorted. RNA was extracted using Tryzol Reagent (Invitrogen). Gene expression profiling was performed according to the manufacturer' 's protocol (Affymetrix). Labeled cRNA (from total RNA) was generated and applied to Affymetrix muU74Av2 microarray. All experiments were performed twice using RNA samples from two independently

sorted cell subsets. Results were analyzed using Microarray Analysis Software v4 and v5 (Affymetrix).

Quantitative PCR

Total mRNA was isolated from sorted cells using RNA easy kit (Qiagen), SV Total RNA Isolation System (Promega) or High Pure RNA Tissue Kit (Roche). First strand DNA was synthesized with oligo-dT (Amersham Biosciences) and AMV reverse transcriptase (Roche Diagnostics). RNA was digested with RNAse One (Promega) and DNA purified with Mini Elute PCR purification Kit (Qiagen). Quantitative PCR reactions were performed with the LightCycler FastStart DNA Master SYBR Green I kit and run on the LightCycler II (Roche Diagnostics). Amplification plots were analyzed using the second-derivative method with LC data analysis 3.5 software (Roche), and the relative quantification was determined using the LightCycler relative quantification software 1.0 (Roche).

Statistical Analysis

Statistical significance was determined using an unpaired two-tailed Student *t* test for unequal variances. *P* values are indicated as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and ns, statistically not significant. The statistical significance of differences between groups of data was calculated using a one-way analysis of variance (one-way ANOVA) along with the Dunnet's post-test to adjust for multiple comparisons, using a 95% confidence interval.

5 References

Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997). beta-catenin is a target for the ubiquitin-proteasome pathway. Embo J *16*, 3797-3804.

Almarza, E., Segovia, J.C., Guenechea, G., Gomez, S.G., Ramirez, A., and Bueren, J.A. (2004). Regulatory elements of the vav gene drive transgene expression in hematopoietic stem cells from adult mice. Exp Hematol *32*, 360-364.

Ames, E., and Murphy, W.J. (2014). Advantages and clinical applications of natural killer cells in cancer immunotherapy. Cancer Immunol Immunother *63*, 21-28.

Ansari, A.W., Temblay, J.N., Alyahya, S.H., and Ashton-Rickardt, P.G. (2010). Serine protease inhibitor 6 protects iNKT cells from self-inflicted damage. J Immunol *185*, 877-883.

Arce, L., Yokoyama, N.N., and Waterman, M.L. (2006). Diversity of LEF/TCF action in development and disease. Oncogene *25*, 7492-7504.

Archbold, H.C., Yang, Y.X., Chen, L., and Cadigan, K.M. (2012). How do they do Wnt they do?: regulation of transcription by the Wnt/beta-catenin pathway. Acta Physiol (Oxf) *204*, 74-109.

Arranz, L., Herrera-Merchan, A., Ligos, J.M., de Molina, A., Dominguez, O., and Gonzalez, S. (2012). Bmi1 is critical to prevent Ikaros-mediated lymphoid priming in hematopoietic stem cells. Cell Cycle *11*, 65-78.

Ashton-Rickardt, P.G. (2013). An emerging role for Serine Protease Inhibitors in T lymphocyte immunity and beyond. Immunol Lett *152*, 65-76.

Balkow, S., Kersten, A., Tran, T.T., Stehle, T., Grosse, P., Museteanu, C., Utermohlen, O., Pircher, H., von Weizsacker, F., Wallich, R*., et al.* (2001). Concerted action of the FasL/Fas and perforin/granzyme A and B pathways is mandatory for the development of early viral hepatitis but not for recovery from viral infection. J Virol *75*, 8781-8791.

Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245-252.

Bar, E., Whitney, P.G., Moor, K., Reis, E.S.C., and Leibundgut-Landmann, S. (2014). IL-17 Regulates Systemic Fungal Immunity by Controlling the Functional Competence of NK Cells. Immunity *40*, 117-127.

Barry, M., Heibein, J.A., Pinkoski, M.J., Lee, S.F., Moyer, R.W., Green, D.R., and Bleackley, R.C. (2000). Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. Mol Cell Biol *20*, 3781-3794.

Barton, K., Muthusamy, N., Fischer, C., Ting, C.N., Walunas, T.L., Lanier, L.L., and Leiden, J.M. (1998). The Ets-1 transcription factor is required for the development of natural killer cells in mice. Immunity *9*, 555-563.

Beresford, P.J., Xia, Z., Greenberg, A.H., and Lieberman, J. (1999). Granzyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation. Immunity *10*, 585-594.

Bevan, M.J. (2006). Cross-priming. Nat Immunol *7*, 363-365.

Biron, C.A., Byron, K.S., and Sullivan, J.L. (1989). Severe herpesvirus infections in an adolescent without natural killer cells. N Engl J Med *320*, 1731-1735.

Biron, C.A., Nguyen, K.B., Pien, G.C., Cousens, L.P., and Salazar-Mather, T.P. (1999). Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu Rev Immunol *17*, 189-220.

Boos, M.D., Yokota, Y., Eberl, G., and Kee, B.L. (2007). Mature natural killer cell and lymphoid tissue-inducing cell development requires Id2-mediated suppression of E protein activity. J Exp Med *204*, 1119-1130.

Bratke, K., Kuepper, M., Bade, B., Virchow, J.C., Jr., and Luttmann, W. (2005). Differential expression of human granzymes A, B, and K in natural killer cells and during CD8+ T cell differentiation in peripheral blood. Eur J Immunol *35*, 2608-2616.

Bukowski, J.F., Woda, B.A., Habu, S., Okumura, K., and Welsh, R.M. (1983). Natural killer cell depletion enhances virus synthesis and virus-induced hepatitis in vivo. J Immunol *131*, 1531-1538.

Caputo, A., Garner, R.S., Winkler, U., Hudig, D., and Bleackley, R.C. (1993). Activation of recombinant murine cytotoxic cell proteinase-1 requires deletion of an amino-terminal dipeptide. J Biol Chem *268*, 17672-17675.

Carotta, S., Pang, S.H., Nutt, S.L., and Belz, G.T. (2011). Identification of the earliest NK-cell precursor in the mouse BM. Blood *117*, 5449-5452.

Carson, W.E., Fehniger, T.A., Haldar, S., Eckhert, K., Lindemann, M.J., Lai, C.F., Croce, C.M., Baumann, H., and Caligiuri, M.A. (1997). A potential role for interleukin-15 in the regulation of human natural killer cell survival. J Clin Invest *99*, 937-943.

Cavallo, R.A., Cox, R.T., Moline, M.M., Roose, J., Polevoy, G.A., Clevers, H., Peifer, M., and Bejsovec, A. (1998). Drosophila Tcf and Groucho interact to repress Wingless signalling activity. Nature *395*, 604-608.

Chaix, J., Tessmer, M.S., Hoebe, K., Fuseri, N., Ryffel, B., Dalod, M., Alexopoulou, L., Beutler, B., Brossay, L., Vivier, E.*, et al.* (2008). Cutting edge: Priming of NK cells by IL-18. J Immunol *181*, 1627-1631.

Cheng, M., Charoudeh, H.N., Brodin, P., Tang, Y., Lakshmikanth, T., Hoglund, P., Jacobsen, S.E., and Sitnicka, E. (2009). Distinct and overlapping patterns of cytokine regulation of thymic and bone marrow-derived NK cell development. J Immunol *182*, 1460-1468.

Cichocki, F., Miller, J.S., Anderson, S.K., and Bryceson, Y.T. (2013). Epigenetic regulation of NK cell differentiation and effector functions. Front Immunol *4*, 55.

Cobas, M., Wilson, A., Ernst, B., Mancini, S.J., MacDonald, H.R., Kemler, R., and Radtke, F. (2004). Beta-catenin is dispensable for hematopoiesis and lymphopoiesis. J Exp Med *199*, 221-229.

Coca, S., Perez-Piqueras, J., Martinez, D., Colmenarejo, A., Saez, M.A., Vallejo, C., Martos, J.A., and Moreno, M. (1997). The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. Cancer *79*, 2320-2328.

Colucci, F., Samson, S.I., DeKoter, R.P., Lantz, O., Singh, H., and Di Santo, J.P. (2001). Differential requirement for the transcription factor PU.1 in the generation of natural killer cells versus B and T cells. Blood *97*, 2625-2632.

Constantinides, M.G., McDonald, B.D., Verhoef, P.A., and Bendelac, A. (2014). A committed precursor to innate lymphoid cells. Nature.

Cooper, M.A., Bush, J.E., Fehniger, T.A., VanDeusen, J.B., Waite, R.E., Liu, Y., Aguila, H.L., and Caligiuri, M.A. (2002). In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells. Blood *100*, 3633-3638.

Daussy, C., Faure, F., Mayol, K., Viel, S., Gasteiger, G., Charrier, E., Bienvenu, J., Henry, T., Debien, E., Hasan, U.A.*, et al.* (2014). T-bet and Eomes instruct the development of two distinct natural killer cell lineages in the liver and in the bone marrow. J Exp Med.

de Boer, J., Williams, A., Skavdis, G., Harker, N., Coles, M., Tolaini, M., Norton, T., Williams, K., Roderick, K., Potocnik, A.J.*, et al.* (2003). Transgenic mice with hematopoietic and lymphoid specific expression of Cre. Eur J Immunol *33*, 314-325.

Degli-Esposti, M.A., and Smyth, M.J. (2005). Close encounters of different kinds: dendritic cells and NK cells take centre stage. Nat Rev Immunol *5*, 112-124.

Devadas, S., Das, J., Liu, C., Zhang, L., Roberts, A.I., Pan, Z., Moore, P.A., Das, G., and Shi, Y. (2006). Granzyme B is critical for T cell receptor-induced cell death of type 2 helper T cells. Immunity *25*, 237-247.

Diefenbach, A., Jensen, E.R., Jamieson, A.M., and Raulet, D.H. (2001). Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. Nature *413*, 165-171.

Ding, Y., Shen, S., Lino, A.C., Curotto de Lafaille, M.A., and Lafaille, J.J. (2008). Beta-catenin stabilization extends regulatory T cell survival and induces anergy in nonregulatory T cells. Nat Med *14*, 162-169.

Eckelhart, E., Warsch, W., Zebedin, E., Simma, O., Stoiber, D., Kolbe, T., Rulicke, T., Mueller, M., Casanova, E., and Sexl, V. (2011). A novel Ncr1-Cre mouse reveals the essential role of STAT5 for NK-cell survival and development. Blood *117*, 1565-1573.

Ewen, C.L., Kane, K.P., and Bleackley, R.C. (2012). A quarter century of granzymes. Cell Death Differ *19*, 28-35.

Fathman, J.W., Bhattacharya, D., Inlay, M.A., Seita, J., Karsunky, H., and Weissman, I.L. (2011). Identification of the earliest natural killer cell committed progenitor in murine bone marrow. Blood.

Fehniger, T.A., Cai, S.F., Cao, X., Bredemeyer, A.J., Presti, R.M., French, A.R., and Ley, T.J. (2007). Acquisition of murine NK cell cytotoxicity requires the translation of a pre-existing pool of granzyme B and perforin mRNAs. Immunity *26*, 798-811.

Ferrero, I., Held, W., Wilson, A., Tacchini-Cottier, F., Radtke, F., and MacDonald, H.R. (2002). Mouse CD11c(+) B220(+) Gr1(+) plasmacytoid dendritic cells develop independently of the T-cell lineage. Blood *100*, 2852-2857.

Forster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Muller, I., Wolf, E., and Lipp, M. (1999). CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell *99*, 23-33.

Gascoyne, D.M., Long, E., Veiga-Fernandes, H., de Boer, J., Williams, O., Seddon, B., Coles, M., Kioussis, D., and Brady, H.J. (2009). The basic leucine zipper transcription factor E4BP4 is essential for natural killer cell development. Nat Immunol *10*, 1118-1124.

Germar, K., Dose, M., Konstantinou, T., Zhang, J., Wang, H., Lobry, C., Arnett, K.L., Blacklow, S.C., Aifantis, I., Aster, J.C.*, et al.* (2011). T-cell factor 1 is a gatekeeper for T-cell specification in response to Notch signaling. Proc Natl Acad Sci U S A *108*, 20060-20065.

Giese, K., Kingsley, C., Kirshner, J.R., and Grosschedl, R. (1995). Assembly and function of a TCR alpha enhancer complex is dependent on LEF-1-induced DNA bending and multiple protein-protein interactions. Genes Dev *9*, 995-1008.

Gordon, S.M., Chaix, J., Rupp, L.J., Wu, J., Madera, S., Sun, J.C., Lindsten, T., and Reiner, S.L. (2012). The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation. Immunity *36*, 55-67.

Goux, D., Coudert, J.D., Maurice, D., Scarpellino, L., Jeannet, G., Piccolo, S., Weston, K., Huelsken, J., and Held, W. (2005). Cooperating pre-T-cell receptor and TCF-1-dependent signals ensure thymocyte survival. Blood *106*, 1726-1733.

Grigoryan, T., Wend, P., Klaus, A., and Birchmeier, W. (2008). Deciphering the function of canonical Wnt signals in development and disease: conditional loss- and gain-of-function mutations of beta-catenin in mice. Genes Dev *22*, 2308-2341.

Grumolato, L., Liu, G., Haremaki, T., Mungamuri, S.K., Mong, P., Akiri, G., Lopez-Bergami, P., Arita, A., Anouar, Y., Mlodzik, M.*, et al.* (2013). beta-Catenin-independent activation of TCF1/LEF1 in human hematopoietic tumor cells through interaction with ATF2 transcription factors. PLoS Genet *9*, e1003603.

Hayakawa, Y., and Smyth, M.J. (2006). CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity. J Immunol *176*, 1517-1524.

Held, W. (2013). Inhibitory receptors and their mode of action: key insights from NK cells. J Immunol *191*, 3489-3490.

Held, W., Clevers, H., and Grosschedl, R. (2003). Redundant functions of TCF-1 and LEF-1 during T and NK cell development, but unique role of TCF-1 for Ly49 NK cell receptor acquisition. Eur J Immunol *33*, 1393-1398.

Held, W., Kijima, M., Angelov, G., and Bessoles, S. (2011). The function of natural killer cells: education, reminders and some good memories. Current opinion in immunology *23*, 228-233.

Held, W., Kunz, B., Lowin-Kropf, B., van de Wetering, M., and Clevers, H. (1999). Clonal acquisition of the Ly49A NK cell receptor is dependent on the trans-acting factor TCF-1. Immunity *11*, 433-442.

Hepworth, M.R., and Sonnenberg, G.F. (2014). Regulation of the adaptive immune system by innate lymphoid cells. Current opinion in immunology *27C*, 75-82.

Huelsken, J., Vogel, R., Brinkmann, V., Erdmann, B., Birchmeier, C., and Birchmeier, W. (2000). Requirement for beta-catenin in anterior-posterior axis formation in mice. J Cell Biol *148*, 567-578.

Huelsken, J., Vogel, R., Erdmann, B., Cotsarelis, G., and Birchmeier, W. (2001). beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. Cell *105*, 533-545.

Huntington, N.D., Tabarias, H., Fairfax, K., Brady, J., Hayakawa, Y., Degli-Esposti, M.A., Smyth, M.J., Tarlinton, D.M., and Nutt, S.L. (2007). NK cell maturation and peripheral homeostasis is associated with KLRG1 up-regulation. J Immunol *178*, 4764-4770.

Ida, H., and Anderson, P. (1998). Activation-induced NK cell death triggered by CD2 stimulation. Eur J Immunol *28*, 1292-1300.

Ida, H., Nakashima, T., Kedersha, N.L., Yamasaki, S., Huang, M., Izumi, Y., Miyashita, T., Origuchi, T., Kawakami, A., Migita, K*., et al.* (2003). Granzyme B leakage-induced cell death: a new type of activation-induced natural killer cell death. Eur J Immunol *33*, 3284-3292.

Ida, H., Robertson, M.J., Voss, S., Ritz, J., and Anderson, P. (1997). CD94 ligation induces apoptosis in a subset of IL-2-stimulated NK cells. J Immunol *159*, 2154-2160.

Imler, J.L., and Hoffmann, J.A. (2001). Toll receptors in innate immunity. Trends Cell Biol *11*, 304-311.

Ioannidis, V., Beermann, F., Clevers, H., and Held, W. (2001). The beta-catenin--TCF-1 pathway ensures CD4(+)CD8(+) thymocyte survival. Nat Immunol *2*, 691-697.

Ioannidis, V., Kunz, B., Tanamachi, D.M., Scarpellino, L., and Held, W. (2003). Initiation and limitation of Ly-49A NK cell receptor acquisition by T cell factor-1. J Immunol *171*, 769-775.

Isaaz, S., Baetz, K., Olsen, K., Podack, E., and Griffiths, G.M. (1995). Serial killing by cytotoxic T lymphocytes: T cell receptor triggers degranulation, re-filling of the lytic granules and secretion of lytic proteins via a non-granule pathway. Eur J Immunol *25*, 1071-1079.

Ishigami, S., Natsugoe, S., Tokuda, K., Nakajo, A., Che, X., Iwashige, H., Aridome, K., Hokita, S., and Aikou, T. (2000). Prognostic value of intratumoral natural killer cells in gastric carcinoma. Cancer *88*, 577-583.

Jeannet, G., Boudousquie, C., Gardiol, N., Kang, J., Huelsken, J., and Held, W. (2010). Essential role of the Wnt pathway effector Tcf-1 for the establishment of functional CD8 T cell memory. Proc Natl Acad Sci U S A *107*, 9777-9782.

Jeannet, G., Scheller, M., Scarpellino, L., Duboux, S., Gardiol, N., Back, J., Kuttler, F., Malanchi, I., Birchmeier, W., Leutz, A.*, et al.* (2008a). Long-term, multilineage hematopoiesis occurs in the combined absence of b-catenin and g-catenin. Blood *111*, 142-149.

Jeannet, G., Scheller, M., Scarpellino, L., Duboux, S., Gardiol, N., Back, J., Kuttler, F., Malanchi, I., Birchmeier, W., Leutz, A.*, et al.* (2008b). Long-term, multilineage hematopoiesis occurs in the combined absence of beta-catenin and gamma-catenin. Blood *111*, 142-149.

Jenne, C.N., Enders, A., Rivera, R., Watson, S.R., Bankovich, A.J., Pereira, J.P., Xu, Y., Roots, C.M., Beilke, J.N., Banerjee, A.*, et al.* (2009). T-bet-dependent S1P5 expression in NK cells promotes egress from lymph nodes and bone marrow. J Exp Med *206*, 2469-2481.

Jho, E.H., Zhang, T., Domon, C., Joo, C.K., Freund, J.N., and Costantini, F. (2002). Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Mol Cell Biol *22*, 1172-1183.

Ji, M., Li, H., Suh, H.C., Klarmann, K.D., Yokota, Y., and Keller, J.R. (2008). Id2 intrinsically regulates lymphoid and erythroid development via interaction with different target proteins. Blood *112*, 1068-1077.

Kamizono, S., Duncan, G.S., Seidel, M.G., Morimoto, A., Hamada, K., Grosveld, G., Akashi, K., Lind, E.F., Haight, J.P., Ohashi, P.S.*, et al.* (2009). Nfil3/E4bp4 is required for the development and maturation of NK cells in vivo. J Exp Med *206*, 2977-2986.

Karlhofer, F.M., Ribaudo, R.K., and Yokoyama, W.M. (1992). MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells. Nature *358*, 66-70.

Karre, K., Ljunggren, H.G., Piontek, G., and Kiessling, R. (1986). Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature *319*, 675-678.

Katzav, S., Martin-Zanca, D., and Barbacid, M. (1989). vav, a novel human oncogene derived from a locus ubiquitously expressed in hematopoietic cells. Embo J *8*, 2283-2290.

Kaur, G., Trowsdale, J., and Fugger, L. (2013). Natural killer cells and their receptors in multiple sclerosis. Brain *136*, 2657-2676.

Kee, B.L. (2009). E and ID proteins branch out. Nat Rev Immunol *9*, 175-184.

Kennedy, M.K., Glaccum, M., Brown, S.N., Butz, E.A., Viney, J.L., Embers, M., Matsuki, N., Charrier, K., Sedger, L., Willis, C.R.*, et al.* (2000). Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. J Exp Med *191*, 771-780.

Kerdiles, Y., Ugolini, S., and Vivier, E. (2013). T cell regulation of natural killer cells. J Exp Med *210*, 1065-1068.

Kikuchi, A., Kishida, S., and Yamamoto, H. (2006). Regulation of Wnt signaling by protein-protein interaction and post-translational modifications. Exp Mol Med *38*, 1-10.

Kim, S., Iizuka, K., Kang, H.S., Dokun, A., French, A.R., Greco, S., and Yokoyama, W.M. (2002). In vivo developmental stages in murine natural killer cell maturation. Nat Immunol *3*, 523-528.

Koch, U., Wilson, A., Cobas, M., Kemler, R., Macdonald, H.R., and Radtke, F. (2008). Simultaneous loss of beta- and gamma-catenin does not perturb hematopoiesis or lymphopoiesis. Blood *111*, 160-164.

Koka, R., Burkett, P.R., Chien, M., Chai, S., Chan, F., Lodolce, J.P., Boone, D.L., and Ma, A. (2003). Interleukin (IL)-15R[alpha]-deficient natural killer cells survive in normal but not IL-15R[alpha]-deficient mice. J Exp Med *197*, 977-984.

Korinek, V., Barker, N., Morin, P.J., van Wichen, D., de Weger, R., Kinzler, K.W., Vogelstein, B., and Clevers, H. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. Science *275*, 1784-1787.

Laforge, M., Bidere, N., Carmona, S., Devocelle, A., Charpentier, B., and Senik, A. (2006). Apoptotic death concurrent with CD3 stimulation in primary human CD8+ T lymphocytes: a role for endogenous granzyme B. J Immunol *176*, 3966-3977.

Laouar, Y., Sutterwala, F.S., Gorelik, L., and Flavell, R.A. (2005). Transforming growth factor-beta controls T helper type 1 cell development through regulation of natural killer cell interferon-gamma. Nat Immunol *6*, 600-607.

Laurenti, E., Varnum-Finney, B., Wilson, A., Ferrero, I., Blanco-Bose, W.E., Ehninger, A., Knoepfler, P.S., Cheng, P.F., MacDonald, H.R., Eisenman, R.N.*, et al.* (2008). Hematopoietic stem cell function and survival depend on c-Myc and N-Myc activity. Cell Stem Cell *3*, 611-624.

Lee, J.S., Cella, M., McDonald, K.G., Garlanda, C., Kennedy, G.D., Nukaya, M., Mantovani, A., Kopan, R., Bradfield, C.A., Newberry, R.D.*, et al.* (2012). AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch. Nat Immunol *13*, 144-151.

Lee, S.H., Miyagi, T., and Biron, C.A. (2007). Keeping NK cells in highly regulated antiviral warfare. Trends Immunol *28*, 252-259.

Levanon, D., Negreanu, V., Lotem, J., Bone, K.R., Brenner, O., Leshkowitz, D., and Groner, Y. (2014). Runx3 Regulates Interleukin-15 Dependent Natural Killer Cell Activation. Mol Cell Biol.

Lieberman, J. (2003). The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. Nat Rev Immunol *3*, 361-370.

Lodolce, J.P., Boone, D.L., Chai, S., Swain, R.E., Dassopoulos, T., Trettin, S., and Ma, A. (1998). IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. Immunity *9*, 669-676.

Lohoff, M., Duncan, G.S., Ferrick, D., Mittrucker, H.W., Bischof, S., Prechtl, S., Rollinghoff, M., Schmitt, E., Pahl, A., and Mak, T.W. (2000). Deficiency in the transcription factor interferon regulatory factor (IRF)-2 leads to severely compromised development of natural killer and T helper type 1 cells. J Exp Med *192*, 325-336.

Long, E.O., Kim, H.S., Liu, D., Peterson, M.E., and Rajagopalan, S. (2013). Controlling natural killer cell responses: integration of signals for activation and inhibition. Annu Rev Immunol *31*, 227-258.

Lopez, J.A., Brennan, A.J., Whisstock, J.C., Voskoboinik, I., and Trapani, J.A. (2012). Protecting a serial killer: pathways for perforin trafficking and self-defence ensure sequential target cell death. Trends Immunol *33*, 406-412.

Love, J.J., Li, X., Case, D.A., Giese, K., Grosschedl, R., and Wright, P.E. (1995). Structural basis for DNA bending by the architectural transcription factor LEF-1. Nature *376*, 791-795.

Lucas, M., Schachterle, W., Oberle, K., Aichele, P., and Diefenbach, A. (2007). Dendritic cells prime natural killer cells by trans-presenting interleukin 15. Immunity *26*, 503-517.

Lustig, B., Jerchow, B., Sachs, M., Weiler, S., Pietsch, T., Karsten, U., van de Wetering, M., Clevers, H., Schlag, P.M., Birchmeier, W.*, et al.* (2002). Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. Mol Cell Biol *22*, 1184-1193.

Ma, J., Wang, R., Fang, X., Ding, Y., and Sun, Z. (2011). Critical role of TCF-1 in repression of the IL-17 gene. PLoS One *6*, e24768.

Male, V., Nisoli, I., Gascoyne, D.M., and Brady, H.J. (2011). E4BP4: an unexpected player in the immune response. Trends Immunol.

Mao, B., Wu, W., Li, Y., Hoppe, D., Stannek, P., Glinka, A., and Niehrs, C. (2001a). LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. Nature *411*, 321-325.

Mao, J., Wang, J., Liu, B., Pan, W., Farr, G.H., 3rd, Flynn, C., Yuan, H., Takada, S., Kimelman, D., Li, L.*, et al.* (2001b). Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway. Mol Cell *7*, 801-809.

Marcus, A., Gowen, B.G., Thompson, T.W., Iannello, A., Ardolino, M., Deng, W., Wang, L., Shifrin, N., and Raulet, D.H. (2014). Recognition of tumors by the innate immune system and natural killer cells. Advances in immunology *122*, 91-128.

Martin-Fontecha, A., Thomsen, L.L., Brett, S., Gerard, C., Lipp, M., Lanzavecchia, A., and Sallusto, F. (2004). Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. Nat Immunol *5*, 1260-1265.

Masson, D., and Tschopp, J. (1987). A family of serine esterases in lytic granules of cytolytic T lymphocytes. Cell *49*, 679-685.

McCartney, S., Vermi, W., Gilfillan, S., Cella, M., Murphy, T.L., Schreiber, R.D., Murphy, K.M., and Colonna, M. (2009). Distinct and complementary functions of MDA5 and TLR3 in poly(I:C)-mediated activation of mouse NK cells. J Exp Med *206*, 2967-2976.

McKenna, H.J., Stocking, K.L., Miller, R.E., Brasel, K., De Smedt, T., Maraskovsky, E., Maliszewski, C.R., Lynch, D.H., Smith, J., Pulendran, B.*, et al.* (2000). Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. Blood *95*, 3489-3497.

Meade, J.L., de Wynter, E.A., Brett, P., Sharif, S.M., Woods, C.G., Markham, A.F., and Cook, G.P. (2006). A family with Papillon-Lefevre syndrome reveals a requirement for cathepsin C in granzyme B activation and NK cell cytolytic activity. Blood *107*, 3665-3668.

Mielke, L.A., Groom, J.R., Rankin, L.C., Seillet, C., Masson, F., Putoczki, T., and Belz, G.T. (2013). TCF-1 controls ILC2 and NKp46+RORgammat+ innate lymphocyte differentiation and protection in intestinal inflammation. J Immunol *191*, 4383-4391.

Miyagi, T., Gil, M.P., Wang, X., Louten, J., Chu, W.M., and Biron, C.A. (2007). High basal STAT4 balanced by STAT1 induction to control type 1 interferon effects in natural killer cells. J Exp Med *204*, 2383-2396.

Moretta, A., Marcenaro, E., Sivori, S., Della Chiesa, M., Vitale, M., and Moretta, L. (2005). Early liaisons between cells of the innate immune system in inflamed peripheral tissues. Trends Immunol *26*, 668-675.

Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K.W. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science *275*, 1787-1790.

Neefjes, J., and Sadaka, C. (2012). Into the intracellular logistics of cross-presentation. Front Immunol *3*, 31.

Newman, K.C., and Riley, E.M. (2007). Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. Nat Rev Immunol *7*, 279-291.

Ogilvy, S., Metcalf, D., Gibson, L., Bath, M.L., Harris, A.W., and Adams, J.M. (1999). Promoter elements of vav drive transgene expression in vivo throughout the hematopoietic compartment. Blood *94*, 1855-1863.

Ohno, S., Sato, T., Kohu, K., Takeda, K., Okumura, K., Satake, M., and Habu, S. (2008). Runx proteins are involved in regulation of CD122, Ly49 family and IFN-gamma expression during NK cell differentiation. Int Immunol *20*, 71-79.

Oosterwegel, M., van de Wetering, M., Dooijes, D., Klomp, L., Winoto, A., Georgopoulos, K., Meijlink, F., and Clevers, H. (1991a). Cloning of murine TCF-1, a T cell-specific

transcription factor interacting with functional motifs in the CD3-epsilon and T cell receptor alpha enhancers. J Exp Med *173*, 1133-1142.

Oosterwegel, M.A., van de Wetering, M.L., Holstege, F.C., Prosser, H.M., Owen, M.J., and Clevers, H.C. (1991b). TCF-1, a T cell-specific transcription factor of the HMG box family, interacts with sequence motifs in the TCR beta and TCR delta enhancers. Int Immunol *3*, 1189-1192.

Orange, J.S. (2006). Human natural killer cell deficiencies. Curr Opin Allergy Clin Immunol *6*, 399-409.

Orr, M.T., and Lanier, L.L. (2010). Natural killer cell education and tolerance. Cell *142*, 847-856.

Pearce, E.L., Mullen, A.C., Martins, G.A., Krawczyk, C.M., Hutchins, A.S., Zediak, V.P., Banica, M., DiCioccio, C.B., Gross, D.A., Mao, C.A.*, et al.* (2003). Control of effector CD8+ T cell function by the transcription factor Eomesodermin. Science *302*, 1041-1043.

Pham, C.T., and Ley, T.J. (1999). Dipeptidyl peptidase I is required for the processing and activation of granzymes A and B in vivo. Proc Natl Acad Sci U S A *96*, 8627-8632.

Pham, C.T., MacIvor, D.M., Hug, B.A., Heusel, J.W., and Ley, T.J. (1996). Long-range disruption of gene expression by a selectable marker cassette. Proc Natl Acad Sci U S A *93*, 13090-13095.

Phillips, T., Opferman, J.T., Shah, R., Liu, N., Froelich, C.J., and Ashton-Rickardt, P.G. (2004). A role for the granzyme B inhibitor serine protease inhibitor 6 in CD8+ memory cell homeostasis. J Immunol *173*, 3801-3809.

Poggi, A., and Zocchi, M.R. (2014). NK Cell Autoreactivity and Autoimmune Diseases. Front Immunol *5*, 27.

Quong, M.W., Romanow, W.J., and Murre, C. (2002). E protein function in lymphocyte development. Annu Rev Immunol *20*, 301-322.

Ranson, T., Vosshenrich, C.A., Corcuff, E., Richard, O., Muller, W., and Di Santo, J.P. (2003). IL-15 is an essential mediator of peripheral NK-cell homeostasis. Blood *101*, 4887-4893.

Raulet, D.H., and Guerra, N. (2009). Oncogenic stress sensed by the immune system: role of natural killer cell receptors. Nat Rev Immunol *9*, 568-580.

Raulet, D.H., and Vance, R.E. (2006). Self-tolerance of natural killer cells. Nat Rev Immunol *6*, 520-531.

Reis e Sousa, C. (2006). Dendritic cells in a mature age. Nat Rev Immunol *6*, 476-483.

Revell, P.A., Grossman, W.J., Thomas, D.A., Cao, X., Behl, R., Ratner, J.A., Lu, Z.H., and Ley, T.J. (2005). Granzyme B and the downstream granzymes C and/or F are important for cytotoxic lymphocyte functions. J Immunol *174*, 2124-2131.

Reya, T., and Clevers, H. (2005). Wnt signalling in stem cells and cancer. Nature *434*, 843-850.

Reya, T., Duncan, A.W., Ailles, L., Domen, J., Scherer, D.C., Willert, K., Hintz, L., Nusse, R., and Weissman, I.L. (2003). A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature *423*, 409-414.

Ribeiro, V.S., Hasan, M., Wilson, A., Boucontet, L., Pereira, P., Lesjean-Pottier, S., Satoh-Takayama, N., Di Santo, J.P., and Vosshenrich, C.A. (2010). Cutting edge: Thymic NK cells develop independently from T cell precursors. J Immunol *185*, 4993-4997.

Robbins, S.H., Tessmer, M.S., Mikayama, T., and Brossay, L. (2004). Expansion and contraction of the NK cell compartment in response to murine cytomegalovirus infection. J Immunol *173*, 259-266.

Roose, J., Huls, G., van Beest, M., Moerer, P., van der Horn, K., Goldschmeding, R., Logtenberg, T., and Clevers, H. (1999). Synergy between tumor suppressor APC and the beta-catenin-Tcf4 target Tcf1. Science *285*, 1923-1926.

Rosmaraki, E.E., Douagi, I., Roth, C., Colucci, F., Cumano, A., and Di Santo, J.P. (2001). Identification of committed NK cell progenitors in adult murine bone marrow. Eur J Immunol *31*, 1900-1909.

Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E., and Polakis, P. (1997). Stabilization of beta-catenin by genetic defects in melanoma cell lines. Science *275*, 1790-1792.

Ruggeri, L., Capanni, M., Urbani, E., Perruccio, K., Shlomchik, W.D., Tosti, A., Posati, S., Rogaia, D., Frassoni, F., Aversa, F.*, et al.* (2002). Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science *295*, 2097-2100.

Ruiz, P., Brinkmann, V., Ledermann, B., Behrend, M., Grund, C., Thalhammer, C., Vogel, F., Birchmeier, C., Gunthert, U., Franke, W.W.*, et al.* (1996). Targeted mutation of plakoglobin in mice reveals essential functions of desmosomes in the embryonic heart. J Cell Biol *135*, 215-225.

Russell, J.H., and Ley, T.J. (2002). Lymphocyte-mediated cytotoxicity. Annu Rev Immunol *20*, 323-370.

Samson, S.I., Richard, O., Tavian, M., Ranson, T., Vosshenrich, C.A., Colucci, F., Buer, J., Grosveld, F., Godin, I., and Di Santo, J.P. (2003). GATA-3 promotes maturation, IFN-gamma production, and liver-specific homing of NK cells. Immunity *19*, 701-711.

Schilham, M.W., Wilson, A., Moerer, P., Benaissa-Trouw, B.J., Cumano, A., and Clevers, H.C. (1998). Critical involvement of Tcf-1 in expansion of thymocytes. J Immunol *161*, 3984-3991.

Schleicher, U., Liese, J., Knippertz, I., Kurzmann, C., Hesse, A., Heit, A., Fischer, J.A., Weiss, S., Kalinke, U., Kunz, S.*, et al.* (2007). NK cell activation in visceral leishmaniasis requires TLR9, myeloid DCs, and IL-12, but is independent of plasmacytoid DCs. J Exp Med *204*, 893-906.

Smith, H.R., Heusel, J.W., Mehta, I.K., Kim, S., Dorner, B.G., Naidenko, O.V., Iizuka, K., Furukawa, H., Beckman, D.L., Pingel, J.T.*, et al.* (2002). Recognition of a virus-encoded ligand by a natural killer cell activation receptor. Proc Natl Acad Sci U S A *99*, 8826-8831.

Smyth, M.J., Crowe, N.Y., and Godfrey, D.I. (2001). NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. Int Immunol *13*, 459-463.

Spits, H., and Cupedo, T. (2012). Innate lymphoid cells: emerging insights in development, lineage relationships, and function. Annu Rev Immunol *30*, 647-675.

Stadtfeld, M., and Graf, T. (2005). Assessing the role of hematopoietic plasticity for endothelial and hepatocyte development by non-invasive lineage tracing. Development *132*, 203-213.

Stetson, D.B., Mohrs, M., Reinhardt, R.L., Baron, J.L., Wang, Z.E., Gapin, L., Kronenberg, M., and Locksley, R.M. (2003). Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. J Exp Med *198*, 1069-1076.

Sun, J., Ooms, L., Bird, C.H., Sutton, V.R., Trapani, J.A., and Bird, P.I. (1997). A new family of 10 murine ovalbumin serpins includes two homologs of proteinase inhibitor 8 and two homologs of the granzyme B inhibitor (proteinase inhibitor 9). J Biol Chem *272*, 15434-15441.

Sun, J.C., Beilke, J.N., and Lanier, L.L. (2009). Adaptive immune features of natural killer cells. Nature *457*, 557-561.

Sun, Z., Unutmaz, D., Zou, Y.R., Sunshine, M.J., Pierani, A., Brenner-Morton, S., Mebius, R.E., and Littman, D.R. (2000). Requirement for RORgamma in thymocyte survival and lymphoid organ development. Science *288*, 2369-2373.

Suzuki, H., Duncan, G.S., Takimoto, H., and Mak, T.W. (1997). Abnormal development of intestinal intraepithelial lymphocytes and peripheral natural killer cells in mice lacking the IL-2 receptor beta chain. J Exp Med *185*, 499-505.

Szabo, S.J., Kim, S.T., Costa, G.L., Zhang, X., Fathman, C.G., and Glimcher, L.H. (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell *100*, 655-669.

Taki, S., Nakajima, S., Ichikawa, E., Saito, T., and Hida, S. (2005). IFN regulatory factor-2 deficiency revealed a novel checkpoint critical for the generation of peripheral NK cells. J Immunol *174*, 6005-6012.

Thomas, D.A., Scorrano, L., Putcha, G.V., Korsmeyer, S.J., and Ley, T.J. (2001). Granzyme B can cause mitochondrial depolarization and cell death in the absence of BID, BAX, and BAK. Proc Natl Acad Sci U S A *98*, 14985-14990.

Tolwinski, N.S., Wehrli, M., Rives, A., Erdeniz, N., DiNardo, S., and Wieschaus, E. (2003). Wg/Wnt signal can be transmitted through arrow/LRP5,6 and Axin independently of Zw3/Gsk3beta activity. Dev Cell *4*, 407-418.

Townsend, M.J., Weinmann, A.S., Matsuda, J.L., Salomon, R., Farnham, P.J., Biron, C.A., Gapin, L., and Glimcher, L.H. (2004). T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. Immunity *20*, 477-494.

Travis, A., Amsterdam, A., Belanger, C., and Grosschedl, R. (1991). LEF-1, a gene encoding a lymphoid-specific protein with an HMG domain, regulates T-cell receptor alpha enhancer function [corrected]. Genes Dev *5*, 880-894.

Uematsu, S., and Akira, S. (2008). Toll-Like receptors (TLRs) and their ligands. Handb Exp Pharmacol, 1-20.

Van de Wetering, M., Castrop, J., Korinek, V., and Clevers, H. (1996). Extensive alternative splicing and dual promoter usage generate Tcf-1 protein isoforms with differential transcription control properties. Mol Cell Biol *16*, 745-752.

van de Wetering, M., Oosterwegel, M., Dooijes, D., and Clevers, H. (1991). Identification and cloning of TCF-1, a T lymphocyte-specific transcription factor containing a sequence-specific HMG box. EMBO J *10*, 123-132.

Verbeek, S., Izon, D., Hofhuis, F., Robanus-Maandag, E., te Riele, H., van de Wetering, M., Oosterwegel, M., Wilson, A., MacDonald, H.R., and Clevers, H. (1995). An HMG-box-containing T-cell factor required for thymocyte differentiation. Nature *374*, 70-74.

Vivier, E., Nunes, J.A., and Vely, F. (2004). Natural killer cell signaling pathways. Science *306*, 1517-1519.

Vivier, E., Raulet, D.H., Moretta, A., Caligiuri, M.A., Zitvogel, L., Lanier, L.L., Yokoyama, W.M., and Ugolini, S. (2011). Innate or adaptive immunity? The example of natural killer cells. Science *331*, 44-49.

Voskoboinik, I., Smyth, M.J., and Trapani, J.A. (2006). Perforin-mediated target-cell death and immune homeostasis. Nat Rev Immunol *6*, 940-952.

Vosshenrich, C.A., Ranson, T., Samson, S.I., Corcuff, E., Colucci, F., Rosmaraki, E.E., and Di Santo, J.P. (2005). Roles for common cytokine receptor gamma-chain-dependent cytokines in the generation, differentiation, and maturation of NK cell precursors and peripheral NK cells in vivo. J Immunol *174*, 1213-1221.

Walzer, T., Dalod, M., Robbins, S.H., Zitvogel, L., and Vivier, E. (2005). Natural-killer cells and dendritic cells: "l'union fait la force". Blood *106*, 2252-2258.

Wang, J.H., Nichogiannopoulou, A., Wu, L., Sun, L., Sharpe, A.H., Bigby, M., and Georgopoulos, K. (1996). Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. Immunity *5*, 537-549.

Wang, R., Xie, H., Huang, Z., Ma, J., Fang, X., Ding, Y., and Sun, Z. (2011). T cell factor 1 regulates thymocyte survival via a RORgammat-dependent pathway. J Immunol *187*, 5964-5973.

Waterman, M.L., Fischer, W.H., and Jones, K.A. (1991). A thymus-specific member of the HMG protein family regulates the human T cell receptor C alpha enhancer. Genes Dev *5*, 656-669.

Weber, B.N., Chi, A.W., Chavez, A., Yashiro-Ohtani, Y., Yang, Q., Shestova, O., and Bhandoola, A. (2011). A critical role for TCF-1 in T-lineage specification and differentiation. Nature *476*, 63-68.

Welsh, R.M., and Selin, L.K. (2002). No one is naive: the significance of heterologous T-cell immunity. Nat Rev Immunol *2*, 417-426.

Werneck, M.B., Lugo-Villarino, G., Hwang, E.S., Cantor, H., and Glimcher, L.H. (2008). T-bet plays a key role in NK-mediated control of melanoma metastatic disease. J Immunol *180*, 8004-8010.

Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates, J.R., 3rd, and Nusse, R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature *423*, 448-452.

Wu, B., Crampton, S.P., and Hughes, C.C. (2007). Wnt signaling induces matrix metalloproteinase expression and regulates T cell transmigration. Immunity *26*, 227-239.

Xie, H., Huang, Z., Wang, R., and Sun, Z. (2006). Regulation of thymocyte survival by transcriptional coactivators. Crit Rev Immunol *26*, 475-486.

Yang, Q., Monticelli, L.A., Saenz, S.A., Chi, A.W., Sonnenberg, G.F., Tang, J., De Obaldia, M.E., Bailis, W., Bryson, J.L., Toscano, K.*, et al.* (2013). T cell factor 1 is required for group 2 innate lymphoid cell generation. Immunity *38*, 694-704.

Yokota, Y., Mansouri, A., Mori, S., Sugawara, S., Adachi, S., Nishikawa, S., and Gruss, P. (1999). Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. Nature *397*, 702-706.

Yokoyama, W.M. (2005). Natural killer cell immune responses. Immunol Res *32*, 317-325.

Yoshida, T., Ng, S.Y., Zuniga-Pflucker, J.C., and Georgopoulos, K. (2006). Early hematopoietic lineage restrictions directed by Ikaros. Nat Immunol *7*, 382-391.

Yu, Q., Sharma, A., Oh, S.Y., Moon, H.G., Hossain, M.Z., Salay, T.M., Leeds, K.E., Du, H., Wu, B., Waterman, M.L.*, et al.* (2009). T cell factor 1 initiates the T helper type 2 fate by inducing the transcription factor GATA-3 and repressing interferon-gamma. Nat Immunol *10*, 992-999.

Zhang, M., Park, S.M., Wang, Y., Shah, R., Liu, N., Murmann, A.E., Wang, C.R., Peter, M.E., and Ashton-Rickardt, P.G. (2006). Serine protease inhibitor 6 protects cytotoxic T cells from self-inflicted injury by ensuring the integrity of cytotoxic granules. Immunity *24*, 451-461.

Zhong, C., Li, C., Wang, X., Toyoda, T., Gao, G., and Fan, Z. (2012). Granzyme K inhibits replication of influenza virus through cleaving the nuclear transport complex importin alpha1/beta dimer of infected host cells. Cell Death Differ *19*, 882-890.

Zhou, X., Yu, S., Zhao, D.M., Harty, J.T., Badovinac, V.P., and Xue, H.H. (2010). Differentiation and persistence of memory CD8(+) T cells depend on T cell factor 1. Immunity *33*, 229-240.