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INTERLEUKIN 15 THROUGHOUT MURINE NATURAL KILLER CELL BIOLOGY

Thuy Thanh Luu

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INTERLEUKIN 15 THROUGHOUT NATURAL KILLER CELL BIOLOGY

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Thuy Thanh Luu

Principal Supervisor:
Professor Petter Höglund, M.D, Ph.D.
Karolinska Institutet
Department of Medicine, Huddinge
Center for Hematology and
Regenerative Medicine (HERM)

Co-supervisor(s):
Assistant Professor Nadir Kadri, Ph.D.
Karolinska Institutet
Science for Life Laboratory, Department of Medicine, Solna
Division of Infectious Diseases, Karolinska University Hospital

Assistant Professor Stephan Meinke, Ph.D.
Karolinska Institutet
Department of Medicine, Huddinge
Center for Hematology and
Regenerative Medicine (HERM)

Assistant Professor Evren Alici, Ph.D.
Karolinska Institutet
Department of Medicine, Huddinge
Center for Hematology and
Regenerative Medicine (HERM)

Opponent:
Professor André Veillette, Ph.D.
Université de Montréal, Canada
Department of Medicine

Examination Board:
Professor Ewa Sitnicka Quinn, Ph.D.
Lund University
Division of Molecular Hematology (DMH)

Associate Professor Jenny Mjösberg, Ph.D.
Karolinska Institutet
Department of Medicine, Huddinge
Center for Infectious Medicine (CIM)

Associate Professor Di Yu, Ph.D.
Uppsala University
Department of Immunology, Genetics and Pathology

To my dear parents

“Be still and know”

Thích Nhất Hạnh

ABSTRACT

Natural killer (NK) cells are innate immune cells that mount responses against virally infected, transformed and allogeneic transplanted cells. Equipped with cytolytic molecules and death receptors, NK cells mediate cytotoxicity against cells expressing low levels of MHC class I (MHC-I) or high levels of stress-induced molecules. NK cells are also immune-modulatory, releasing various cytokines and chemokines as well as kill immature immune cells. A tight regulation is available to endow NK cells with capacity to distinguish self and non-self, while making sure that they are well functional. Numerous extrinsic and intrinsic factors become parts of the regulatory network to control NK cell development, maturation and functional acquisition.

Interleukin 15 (IL-15) is an indispensable cytokine throughout NK cell biology. In this thesis, a few novel aspects of IL-15 in NK cell biology under non-inflammatory conditions were reported. In **paper I**, the importance of IL-15 expressed by dendritic cells (DCs) for NK cell homeostasis, maturation, and functions at steady-state were investigated. In **paper II**, the coordination of IL-15 with activating signaling DNAX-1-associated receptor (DNAM-1) in controlling the expression of linker for activation of T cells (LAT), an activating signaling molecule, was studied. In **paper III**, we studied the functional impact of brief contact with IL-15 ("priming") and its underlying molecular mechanism. Finally, **in paper IV**, the roles of forkhead box transcription factors of the O class (FOXO) transcription factors in NK cell development in relation to IL-15 receptor (IL-15R) expression and other transcription factors were explored **(Figure 1)**.

In summary, we have found that under non-inflammatory conditions, the presence of DCs is required to maintain NK cell homeostasis in regards to apoptosis and proliferation. NK cell maturation and receptor expression were also perturbed upon DC depletion. Importantly, DC derived IL-15 was required for "missing self" reactivity of NK cells in the absence of infection **(paper I)**. At steady-state, the brief contacts with IL-15 are functionally relevant as five-minute treatment with IL-15 augmented degranulation, cytokine production, and calcium flux triggered by activating receptor stimulation, as well as cytotoxicity against YAC1 cells **(paper III)**. Short-time IL-15 stimulation induced phosphorylation of activating signaling molecules, which is Janus Kinase 3 (JAK3) dependent. The functional impact of short time IL-15 stimulation remained up to three hours after IL-15 removal. In **paper II**, IL-15 stimulation was found to induce expression of LAT especially in DNAM-1^+ NK cells. The absence of DNAM-1 or its ligand, CD155, reduced LAT expression. The heightened level of LAT expression in DNAM-1^+ NK cells endows them with better responsiveness to NK1.1 stimulation, as measured by calcium flux, cytokine production and degranulation.

In the last paper, **paper IV**, mouse models with specific deletion of FOXO1 and FOXO3 in hematopoietic cells (Vav1^+iCre FOXO1,$3^{flox/flox}$) were employed to study the roles of these transcription factors in NK cell development. Upon depletion of FOXO1,3 in Vav1^+ cells, NK cell development was blocked at the transition from pre-NK cell progenitors (preNKP) (CD122^- or IL-15Rβ^-) to refined NK cell progenitors (rNKP) (CD122^+), resulting in very few committed NK cells in both bone marrow (BM) and spleen. The few NK cells developing in the absence of FOXO1,3 were less mature and display perturbed inhibitory and activating receptor expression. The transplantation experiment demonstrated that the effects of FOXO1,3 on NK cell development,

maturation and receptor expression were NK-cell-progenitor intrinsic. The experiment employing RNA sequencing (RNA-seq) and assay for transposase-accessible chromatin using sequencing (ATAC-seq) revealed a possible mechanism underlying the roles of FOXO1,3 in controlling NK cell development. The expression and/or DNA binding of ETS proto-oncogene 1 (ETS1), transcription factor T cell factor 7 (TCF7), and CD122 were affected in different stages of NK cell development in the absence of FOXO1,3. With the reduction in CD122 (IL-15Rβ), mature NK cells from both BM and spleen displayed an increase rate in undergoing apoptosis. Taken together, FOXO1,3 controlled IL-15R expression on NK progenitors and committed NK cells, which contributes to maintain NK cell population in mice.

In summary, we have found that, under non-inflammatory condition, IL-15 regulated NK cell homeostasis and functions, brief contacts with IL-15 were functionally relevant, and the cellular effect was coordinated with DNAM-1 signaling via controlling LAT expression. Lastly, FOXO1,3 were identified as novel transcription factors which control IL-15R expression and NK cell development.

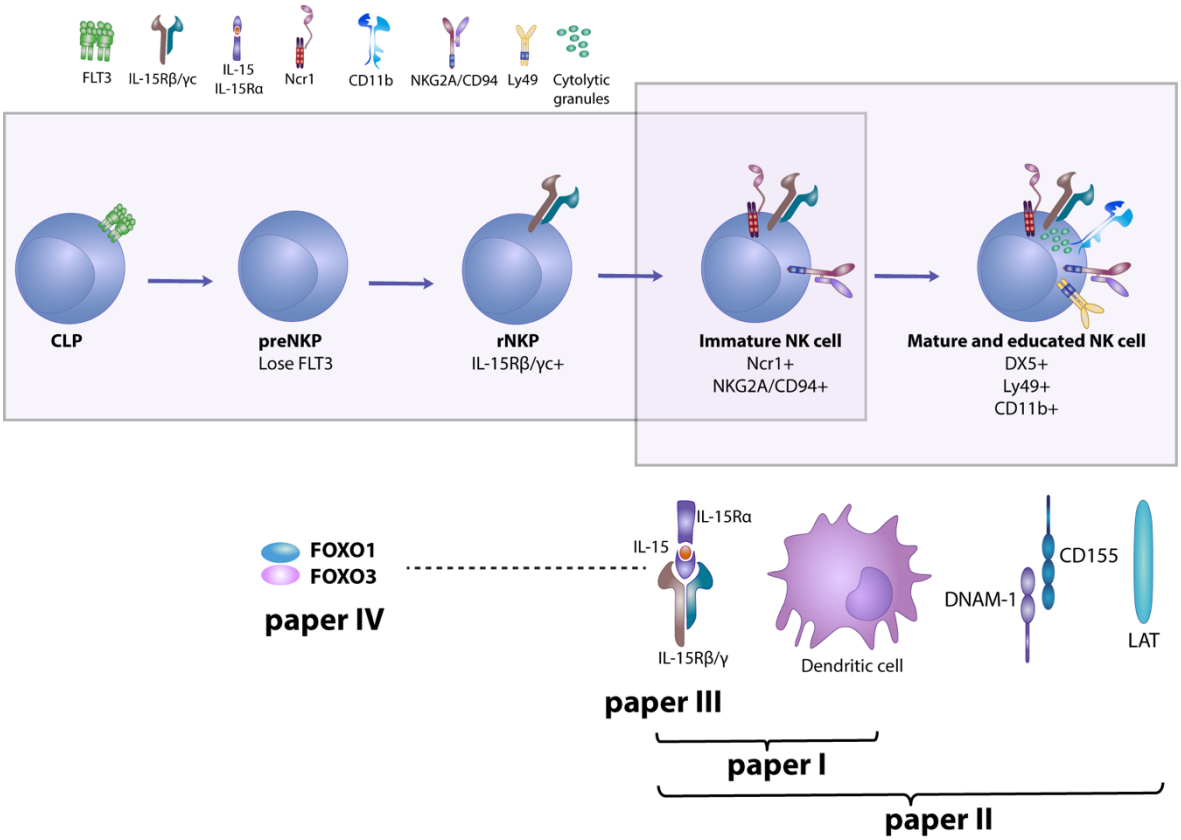

Figure 1. Summary of the work in this thesis. Murine NK cells develop from common lymphoid progenitors (CLP) which downregulate FLT3 to become preNKP. PreNKP acquire IL-15Rβ (CD122) to become rNKP, which subsequently express activating and inhibitory receptors to become immature NK cells. Immature NK cells express adhesion molecules, migrate to the periphery and acquire functional education. IL-15 plays a crucial role throughout NK cell biology including formation of NKP, expression of signaling molecules (LAT), survival, proliferation and maturation. FLT3: fms like tyrosine kinase 3, Ncr1: natural cell receptor 1.

LIST OF SCIENTIFIC PAPERS

I. ***Luu, T.T.***, Ganesan, S., Wagner, A.K., Sarhan, D., Meinke, S., Garbi, N., Hammerling, G., Alici, E., Karre, K., Chambers, B.J., Hoglund*, P. & Kadri*, N. (2016) Independent control of natural killer cell responsiveness and homeostasis at steady-state by CD11c+ dendritic cells. *Sci Rep*, **6**, 37996. (* shared last authors)

II. ***Luu, T.T***., Wagner, A.K., Schmied, L., Meinke, S., Freund, J.E., Kambayashi, T., Ravens, I., Achour, A., Bernhardt, G., Chambers, B., Hoglund, P. & Kadri, N. (2019) IL-15 and CD155 expression regulate LAT expression in murine DNAM1(+) NK cells, enhancing their effectors functions. *European journal of immunology*.

III. ***Luu, T.T***., Schmied, L., Nguyen, N.-A., Wiel, C., Mohammad, D., Meinke, S., Bergö, M., Alici, E., Kadri, N., Ganesan, S. & Höglund, P. (2020) Short-term IL-15 priming leaves a long-lasting signalling imprint in mouse NK cells independent of a metabolic switch. *Manuscript*.

IV. ***Luu, T.T***., Søndergaard, J.N.[#], Peña-Pérez, L.[#], Kharazi, S., Meinke, S., Schmied, L., Nicolai F., Heshmati, Y., Kierczak, M., Bouderlique, T., Wagner, A.K., Chambers, B.J., Achour, A., Kutter, C., Höglund, P., Månsson*, R. & Kadri*, N. (2020) Foxo1 and Foxo3 cooperatively control NK cell development. *Manuscript*. ([#] shared second authors, * shared last authors)

CONTENTS

LIST OF ABBREVIATIONS

γc	Gamma chain
AICD	Activation-Induced Cytidine Deaminase
AKT	Protein Kinase B
APOC3	Apolipoprotein C3
ATAC-seq	Assay for Transposase-Accessible Chromatin using sequencing
BCL6	B Cell Lymphoma 6
BM	Bone Marrow
CCR7	C-C Chemokine Receptor type 7
CD95L	CD95 Ligand
CDK	Cyclin Dependent Kinases
ChIP	Chromatin Immunoprecipitation
CLP	Common lymphoid progenitors
co-IP	co-Immunoprecipitation
CTLA4	Cytotoxic T-Lymphocyte-Associated protein 4
DAP12	DNAX Activating Protein of 12 kDa
DCs	Dendritic Cells
DNAM-1	DNAX-1-Associated Receptor
DT	Diptheria Toxin
DTR	Diptheria Toxin Receptor
EOMES	Eomesodermin
ETS1	ETS proto-oncogene 1
FASL	Fas Ligand
FLT3	Fms Like Tyrosine kinase 3
FOXO	Forkhead box transcription factors of the O class
Foxp3	Forkhead box P3
GPR17	G Protein-Coupled Receptor 17
GRB2	Growth factor Receptor-Bound protein 2
HLA-I	Human Leukocyte Antigen class I
HSCs	Hematopoietic Stem Cells
ID2	Inhibitor of DNA binding 2

IFN-α	Interferon α
IFN-γ	Interferon γ
iNK	Immature NK cells
IL-15	Interleukin-15
IL-15R	IL-15 Receptor
IL-7Rα	Interleukin-7 Receptor α chain
ILCs	Innate Lymphoid Cells
IRF	Interferon Regulatory Factor
IS	Immunological Synapse
ITAM	Immuno-receptor Tyrosine-based Activation Motif
ITIM	Immuno-receptor Tyrosine-based Inhibition Motif
JAK3	Janus Kinase 3
LAT	Linker for Activation of T cells
LCK	Lymphocyte-specific protein tyrosine kinase
LNs	Lymph nodes
LPS	Lipopolysaccharide
LTi	Lymphoid Tissue inducers
MaFIA	transgenic Macrophage Fas-Induced Apoptosis
MAPK	Mitogen-Activated Protein Kinase
MCP-1	Monocyte Chemoattractant Protein 1
MHC-I	MHC class I
MHC-II	MHC class II
mNK	Mature NK cells
mTOR	The mamalian target of rapamycin
NF-κB	Nuclear Factor κB
NFIL3	Nuclear Factor, Interleukin 3 Regulated
NK	Natural Killer
NKP	NK cell Progenitors
PI3K	Phosphoinositide 3-kinase
PKC-θ	Protein Kinase C θ
PLC-γ2	Phosphoinositide phospholipase C-γ2
preNKP	Pre-NK cell Progenitors

Rag1,2	Recombination Activating Gene 1,2
RNA-seq	RNA sequencing
rNKP	Refined NK cell Progenitors
RORα	RAR-related Orphan Receptor α
ROS	Reactive Oxygen Species
SAP	SLAM Associated Protein
SHC	Src Homology 2 domain-Containing
SHIP1	SH-2 containing Inositol 5' Polyphosphatase 1
SHP-1	Src homology 2 (SH2)-domain-containing phosphatase 1
SHP-2	Src homology 2 (SH2)-domain-containing phosphatase 2
SLAM	Signaling Lymphocytic Activating Molecule
SLP-76	SH2 domain-containing leukocyte phosphoprotein of 76kDa
SOD2	Superoxide dismutase 2
STAT5	Signal transducer and activator of transcription 5
T-BET	T-box protein expressed in T cells
TCF7	Transcription Factor T cell Factor 7
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor β
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor α
TOX	Thymocyte Selection Associated High Mobility Group Box
TRAPs	Transmembrane Adaptor Proteins
ZAP70	Zeta-chain-Associated Protein kinase 70
ZBTB46	Zinc Finger And BTB Domain Containing 46
SRC	Sarcoma kinase
mNK	Mature NK cells

1 INTRODUCTION

1.1 OVERVIEW OF NATURAL KILLER CELLS

Natural killer (NK) cells were first identified as a subset of large granular lymphocytes that exert non-specific cytotoxicity against various solid tumors and hematopoietic malignancies [1-3]. This lymphocyte subset was named NK cells owing to their capacity to exert "natural" cytotoxicity without a prior activation [4, 5]. NK cells are important not only for their cytotoxicity but also for being major players in the immune regulatory network, by producing a wide range of cytokines and chemokines, including IL-1β, chemokine (C-X-C motif) ligand 1 (CXCL8 or IL-8), tumor necrosis factor α (TNF-α), IL-10, IL-13, granulocyte macrophage colony stimulating factor (GM-CSF), interferon α (IFN-α), interferon γ (IFN-γ), transforming growth factor β (TGF-β), chemokine (C-C motif) ligand 3 and 4 (CCL3, CCL4) [6-10]. NK cell immune-modulatory effects were also attributed to their capacity to exert cytotoxicity towards other immune cells, including T, B, and dendritic cells (DCs) [11-14]. NK cells were recently showed to also regulate certain bacterial or fungal infections [7].

NK cells were originally distinguished from T cell cytotoxic lymphocytes due to their lack of receptor specificity based on genetic recombination (such as the B cell receptor and T cell receptor), their quick response without a need for sensitization, and their lack of a recall or memory response. This distinction has been challenged recently by numerous studies showing that NK cells indeed possess characteristics that were attributed to adaptive cells. Firstly, NK cells do need constant signals from accessory cells, namely macrophages, neutrophils, and DCs for their optimal functions. These cells provide both contact-dependent and independent signals to NK cells and reside in close areas with NK cells in the secondary lymphoid tissues. Secondly, some human NK cell inhibitory receptors can be highly polymorphic and display differential binding affinities to different human leukocyte antigen class I (HLA-I) groups and HLA-I ligands. The specificity is decided at the level of single amino acid substitution, creating numerous combinations of NK receptor-HLA-I pairs [15]. Even though the receptor pool is much more limited and not based on DNA recombination, such as in T and B cells, the degree of specificity is beyond what would be expected of an innate cell type. Lastly, upon certain viral infections, including cytomegalovirus (CMV), vaccinia virus, herpes simplex virus type 2, human immunodeficiency virus (HIV) and influenza, NK cells form a long-lived memory population which is capable of mounting a strong recall response [10, 16, 17].

In some reports, NK cells were classified to group 1 ILC together with ILC1. ILC, which include ILC1, ILC2, ILC3 and lymphoid tissue inducers (LTi), are the innate counterparts of T lymphocytes and are mostly tissue residents. They play a crucial role in maintaining tissue homeostasis, mainly in the intestine, adipose tissue and lung. NK cells are closely related to ILC1 in their capacity to produce IFN-γ. In mice, NK cells are circulating and dependent on eomesodermin (EOMES) while ILC1 are tissue residents and dependent on T-box protein expressed in T cells (T-BET) [18, 19]. NK cells are more cytotoxic and express higher levels of perforin than ILC1 [20]. ILC2 produce IL-4, IL-5, IL-13, and ligands for epithelial growth factor receptors, such as AREG, and are important for the control of large extracellular pathogens, i.e. helminths, and tissue repair [20]. ILC2 development is dependent on GATA3 and RAR-related orphan receptor alpha (RORα) [21]. ILC3 secrete IL-22 and IL-17 to control extracellular microbes and regulate tissue remodeling and their development depends on RORC [20]. LTi play a major role for lymphoid organ homeostasis by modulating the capacity of

mesenchymal stromal cells to release chemokines and adhesion molecules which are important for lymphoid organogensis [22].

1.2 NK CELL RECEPTORS

1.2.1 NK cell receptors and signaling

NK cells possess the potent cytotoxicity; and hence, it is crucial that a well-regulated mechanism would be developed to modulate their functions. The regulation is the integration of signals from two types of surface receptors, activating and inhibitory receptors [23]. These receptors are expressed in a stochastic and variegated pattern on NK cells, which results in the formation of subsets expressing different receptors and likely having distinct functions. Unlike T and B cells, NK cell receptors are germline-encoded, recognize a limited set of ligands and do not form based on the DNA rearrangement process. Likewise, NK cell self-tolerance is not dependent on the selective survivability of developing cells, but shaped via the counterbalancing between the reactive and inhibitory signals [24].

Activating receptors recognize ligands upregulated on transformed, stressed, or viral-infected cells [25, 26]. Some activating receptors also recognize constitutively expressed ligands on healthy cells, such as the mouse MHC-I molecule H2-D^d, which is recognized by an activating receptor – Ly49D. In mice, the most studied activating receptors are natural cytotoxicity receptors (NKp46), the Fcγ receptor IIIA (CD16), DNAM-1, NKG2D, C-type lectin-like Ly49 receptors (Ly49D, Ly49H), and signaling leukocyte activating molecule (SLAM) family receptors [27]. Activating receptors normally do not signal by themselves, but associate with adaptor molecules bearing immunoreceptor tyrosine-based activating motifs (ITAM), such as DNAX activating protein of 12 kDa (DAP12), FcεRIγ and CD3ζ [28]. Signaling through some receptors is ITAM-independent such as DNAM-1 and Ly49H [29, 30]. Following activation through ligand binding or receptor crosslinking by monoclonal antibodies, Sarcoma kinase (SRC) family members phosphorylate ITAMs, which then serve as the binding sites for Src2 homology 2 (SH2) of zeta-chain-associated protein kinase 70 (ZAP70) and SYK tyrosine kinases. Activated ZAP70/SYK phosphorylates LAT and SH2 domain-containing leukocyte phosphoprotein of 76kDa (SLP-76), which act as plasma membrane bound adaptor molecules. These adaptors recruit downstream signaling molecules and activate signaling cascades through Vav1, phosphoinositide phospholipase C-γ2 (PLC-γ2) or adhesion and degranulation- promoting adapter protein (ADAP), finally resulting in degranulation and transcriptional upregulation of immune regulators [31]. In detail, PLC-γ2 facilitates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to become inositol 1,4,5-trisphosphate (IP3) and diacyl glycerol (DAG). IP3 binds to calcium channels on the endoplasmic reticulum (ER) which facilitates the release of Ca^{2+} ions into the cytoplasm. The released Ca^{2+} acts on calcium-sensitive proteins like calmodulin to mediate actin cytoskeletal remodeling. The process is facilitated by Vav1, which activates the small GTPase, including Rho and Rac. DAG and Ca^{2+}-sensitive molecules activates mitogen-activated protein kinase (MAPK) pathways or protein kinase C θ (PKC-θ) that induce the transcription of effector molecules, including granzyme B, perforin, IFN-γ, TNF-α [32].

Inhibitory receptors recognize MHC-I molecules or non-classical MHC-I molecules (Qa-1(b), H_2M_3) expressed by healthy cells, counteracting activating signals and serving as “do not kill me” signals to prevent self-damage to endogenous tissue [33, 34]. In mice, most prevalent inhibitory receptors are

C-type lectin receptors, including Ly49 receptors (Ly49C, LY49I, Ly49A, Ly49G2, Ly49F) and CD94/NKG2A [27]. Transformed cells and virus infected cells with downregulated MHC-I expression or transplanted/grafted cells expressing mismatched MHC- I can induce a 'missing self' recognition by NK cells [35-37]. Binding of inhibitory receptors to MHC-I leads to activation of Src-family tyrosine kinases, such as lymphocyte-specific protein tyrosine kinase (LCK). Src kinases phosphorylate tyrosine-based inhibitory motifs (ITIMs) on the intracellular domains of inhibitory receptors. The phosphorylated sites will serve as docking motives for Src homology 2 (SH2)-domain-containing phosphatases (SHP-1 or SHP-2), which directly dephosphorylate Vav1 [38]. Besides signaling through phosphatases, stimulation through inhibitory receptors induces phosphorylation of CRK tyrosine adaptor, dissociating it from the complex with the scaffold protein Casitas B-lineage Lymphoma (c-CBL) and guanine exchange protein C3G [39, 40].

1.2.2 Mechanism of NK cell killing

Upon being activated and encounter with a target cell with an increased expression of activating ligands or a decrease in MHC-I expression, NK cells form an immunological synapse (IS) with target cells via which cytotoxic mediators are released. The cytotoxic mediators are packed in cytolytic granules which are polarized towards IS along the microtubules. Main cytotoxic mediators in NK cells are perforins, granzymes, granulysin, fas ligand (FASL), TNF-related apoptosis-inducing ligand (TRAIL) [41-43]. The mediators induce target cell death via two main molecular mechanisms.

The first mechanism involves perforins and granzymes. Granzymes are delivered to target cells via pores on their membrane formed by perforin molecules [43]. Target cell apoptosis is then induced via caspase-dependent or independent mechanisms which involve activation of a pro-apoptotic molecule, BH3 interacting-domain death agonist (BID), directly acting on the mitochondrial membrane and inducing cytochrome C release [44, 45]. Another killing mechanism involves death receptors including the TNF-related apoptosis-inducing ligand (TRAIL) receptor and FAS, being activated by cognate ligands on NK cells, namely TRAIL and fas ligand (FASL) [46]. The engagement of corresponding ligand-receptors induces target cell apoptosis via an extrinsic apoptotic pathway [47]. The extrinsic apoptotic pathway involves cytoplasmic death domain, capase-8, capase-10, and finally caspase-3, which mediates the release of cytochrome C from mitochondria and hence the formation of an apoptosome. Capase-3 induces DNA fragmentation [48] and the apoptosome enhances the cleavage of caspase-3 mediated by capase-8 and -10 [49], resulting in cell death. NK cells can be "serial killers" and induce cell death of many target cells during their life span [50]. Granzyme-mediated killings happen when NK cells encounter their first target cells, last in a short time-frame, and are critical for serial killing. Death-receptors- or caspase-mediated killings, on the other hand, take much longer than granzyme-mediated killings, and can happen only once and usually mark the end of NK cell "serial killing" [51]. NK cell population is apparently heterogenous with hypo-responsive subsets, intermediately functional subsets and highly potent subsets in regards to the number of kills [50]. Studies on NK cell developmental regulation, the detailed signaling proximally at the IS and downstream effector functions are providing insights into this heterogeneity.

1.2.3 DNAM-1

DNAM-1 (CD226) is a co-stimulatory receptor and an adhesion molecule in both mouse and human NK cells. DNAM-1 in mouse is expressed early on preNKP before the expression of CD122, NKp46,

and NKG2A [52]. The receptor recognizes CD155 or poliovirus receptor (PVR) and nectin-2 (CD122), which are upregulated upon transformation and viral infection [53, 54]. It is important for NK cell-dependent tumor control, IS formation, NK memory cell differentiation and contributes to pathologies of certain autoimmune diseases [55-59]. DNAM-1 signaling coordinates with Ly49H to control expansion and differentiation of memory NK cells in response to MCMV infection. DNAM-1 and its associated adhesion molecule, leukocyte function antigen-1 (LFA-1) were suggested to be markers of both human and murine NK cell education [52, 60]. DNAM-1 expression also endows NK cells with higher capacity to proliferate in response to IL-15 [61]. The permissiveness to IL-15 stimulation conferred by DNAM-1 expression suggested that these two signals might converge to control certain critical signaling molecules in NK cells. Therefore, in **paper II**, we studied the cooperation of DNAM-1 and IL-15 signaling in controlling LAT expression. Furthermore, we also examined the impact of the potential coordination in NK cell functions.

1.2.4 LAT

LAT (or LAT-1) together with non-T-cell activation linker (NTAL, also named LAT-2) belong to a group of transmembrane adaptor proteins (TRAPs). TRAPs act as membrane-associated scaffolding proteins that help to recruit signaling molecules to the region that is proximal to the plasma membrane, and hence allowing them to be phosphorylated by tyrosine kinases [62]. TRAPs themselves do not possess enzymatic activity but can integrate signals by bringing signaling molcules into the close proximity to each other. LAT is expressed in T cells, NK cells, mast cells, and platelets, while LAT-2 is critical for signal transduction in NK cells, B cells and mast cells [63, 64]. Structurally, LAT is mostly transmembrane and intracellular with only four extracellular amino acids. LAT is extremely acidic and contains nine conserved tyrosine residues [65].

In NK cells, LAT is particularly critical for signaling downstream of ITAM-associated receptors such as NKG2D, Ly49H and NKp46 in NK cells. LAT is activated upon its being phosphorylated by ZAP70/SYK, LCK or ITK [65]. Little is known, however, about the inactivation of LAT, which is probably mediated via dephosphorylation by CD148 [66]. Tyrosine residues on LAT, upon being phosphorylated recruit a range of signaling molecules that activate various pathways, including Growth-factor-Receptor-Bound protein 2 (GRB2) family, SLP-76, PLC-γ, and the p85 domain of PI3K. LAT molecules oligomerize together with LAT-binding proteins to form clusters that are proximal to the plasma membrane, and therefore serve crucial roles to transduce activation signals [67, 68]. NK cells from mice that are deficient for both LAT and LAT-2 exhibit defective responsiveness downstream of NK1.1. On the other hand, the absence of LAT solely compromises functions of IL-2 activated NK cells but does not affect resting cells [64, 69]. LAT expression was shown to be down-regulated in lymphokine-activated killer cells as compared to primary murine NK cells [64]. This infers a modulation of LAT expression by IL-2. Whether IL-15 controls LAT expression has not been studied. In **paper II**, we explored the regulation of LAT expression by DNAM-1/CD155 interaction and IL-15 stimulation.

1.3 NK CELL DEVELOPMENT

1.3.1 Overview of NK cell development

The original discovery of NK cells demonstrated the presence of NK cells in human peripheral blood [70, 71] and in rodent spleen [5, 72]. Recent discoveries have provided evidences that NK cells are present in numerous organs, including BM, spleen, lymph nodes (LNs), skin, gut, liver, tonsils, lung, and uterus [73]. Conventional NK (cNK) cells develop in the BM, subsequently migrate to secondary lymphoid tissues including LNs and spleen, and peripheral tissues. This thesis focuses on cNK cells and hereinafter, NK cells refers to cNK cells.

In the BM, NK cells develop from CLP, which also give rise to T cells, B cell, and ILC [10, 74]. CLP generation, expansion, or maintenance are dependent on FLT3L and IL-7 [75, 76]. The transition from CLP to NK cell progenitors (NKP) is marked by the downregulation of FLT3 expression, giving rise to preNKP, which do not express any NK cell markers, yet develop exclusively to NK cells as shown by *in vitro* and *in vivo* development experiments in the original study [74]. A recent study demonstrated the expression of DNAM-1 on the preNKP population, demonstrating that this population might already exhibit NK signatures [52]. Thereafter, the acquisition of CD122 (IL-2Rβ/IL-15Rβ) marks the formation of refined NKPs (rNKP) with responsiveness to IL-15 signals from surrounding cells [74, 77]. Subsequently, rNKP become immature NK cells (iNK) and then mature NK cells (mNK) upon the expression of various receptors and adhesion molecules as well as being stimulated by cytokines.

1.3.2 Transcription factors in NK cell development

NK cell lineage specification from BM progenitors depends on soluble and contact-dependent factors in the BM niche. The impact of exogenous factors on NK cell differentiation is generally mediated via activation of transcription factors, inducing expression of proteins crucial to NK cell homeostasis, migration, and functional maturation **(Figure 2).** Many of these proteins are downstream of IL-15 signaling and/or affect the expression of the IL-15R and IL-15-signaling molecules.

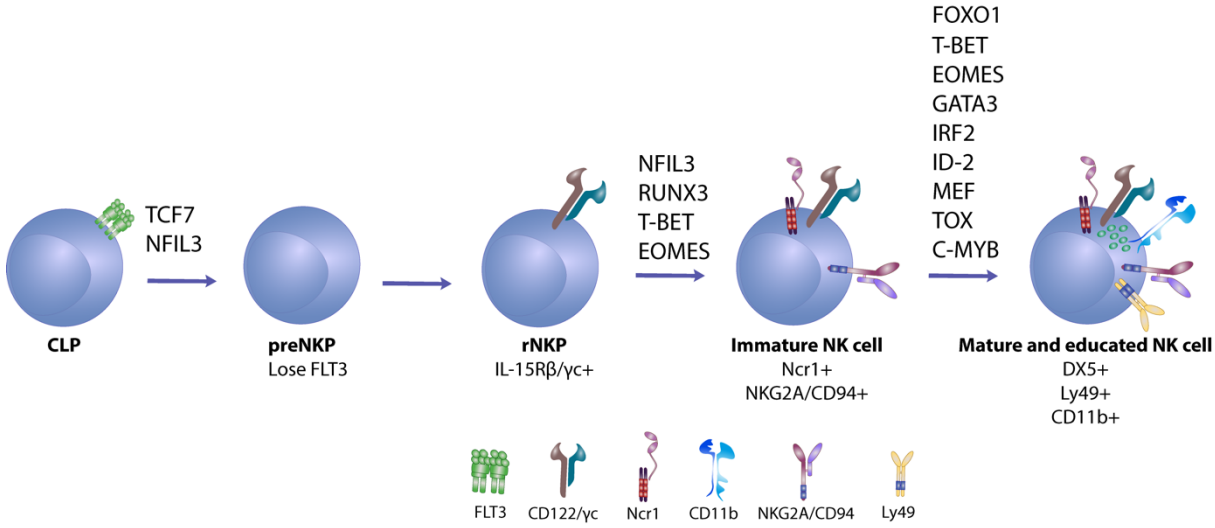

Figure 2. Transcription factors crucial for different stages of NK cell development and maturation

Murine NK cell development was so far known to be controlled by three transcription factors: T-Cell-specific transcription Factor 7 (TCF7), ETS proto-oncogene 1 (ETS1), and E4 Binding Protein 4

(E4BP4 or Nuclear Factor, Interleukin 3 Regulated-NFIL3) [78-80]. NFIL3 was shown to act downstream of IL-15R signaling through the PDK1-mTOR-NFIL3-CD122 circuit, and is hence critical for the expansion and survival of progenitors and committed NK cells [81]. ETS1 KO mice showed decreased preNKP and rNKP populations as compared to WT mice, which was suggested to be linked to the downregulation of inhibitor of DNA binding 2 (ID2) and T-BET [82, 83]. ETS1 KO DX5+ NK cells showed decreased CD122 expression and Chromatin Immunoprecipitation (ChIP) qPCR experiment demonstrated binding of ETS1 to CD122 promoter in mNK cells, suggesting ETS1-modulated IL-15 signaling [83]. TCF7 was shown to control T-cell specification genes including *Gata3, Bcl11b, IL2ra*, and *CD3e* [84], yet more explanation for the perturbed NK cell development in TCF7 KO mice was not given. In general, transcription factors that are crucial for NK cell development act in networks that appear to converge to modulate IL-15 signaling.

1.3.3 NK cell maturation

Following CD122 expression, NK cells acquire expression of surface receptors, adhesion molecules, and chemokine receptors, including NKG2D, NCR1 (NKp46), NKG2A, L-selectin (CD62L) and Leukosialin (CD43) [77, 85]. The acquisition of CD51 (Integrin αV), CD49b (DX5, Integrin VLA-2α), CD43, CD11b (Mac-1), killer cell lectin-like receptor subfamily G member 1 (KLRG1) define mature populations. The acquisition of inhibitory receptors that recognize MHC-I expressed by surrounding cells happens in parallel with the acquisition of adhesion molecules and the migration to the periphery. These receptors mark functionally competent subsets, being the "educated" or "licensed" subsets [77].

The most commonly used markers to define the maturation process of murine NK cells are CD27 and CD11b. The four subsets segregated by these two markers exhibit differences in functional responsiveness, localization, and proliferation [86, 87]. The most immature subset is negative for both markers, while the acquisition of CD27 and subsequently CD11b marks a more mature stage. The $CD27^{high}CD11b^{high}$ and $CD27^{low}CD11b^{high}$ subsets are comparably functionally competent, while the $CD27^{high}CD11b^{low}$ population possesses lower reactivity. $CD27^{low}CD11b^{high}$ subset appears to be the terminally mature cells as their proliferative capacity is declined as compared to the $CD27^{high}CD11b^{high}$ counterparts [87]. As the NK cells get mature, they migrate to the periphery, meaning that $CD27^{low}CD11b^{high}$ cells are found more in the periphery while $CD27^{low}CD11b^{low}$ and $CD27^{high}CD11b^{low}$ subsets are present more in the BM [87].

1.3.4 Innate lymphoid cell development

Like NK cells, ILC develop from CLP in the BM. ILC progenitors (ILCP) and NKP are overlapping populations with shared expression of some surface receptors and transcription factors. ILCP are characterized as $FLT3^{-}\alpha_4\beta_7^{+}ID2^{+}IL\text{-}7R\alpha^{+}$ [88]. PreNKP (defined as $Lin^{-}CD27^{+}2B4^{+}c\text{-}Kit^{+}IL7\text{-}R\alpha^{+}FLT3^{-}CD122^{-}$) and a significant fraction of this population (40 – 50 %) expresses ZBTB16 and $\alpha_4\beta_7$ [89]. Similarly, a variable CD122 expression, which is the marker for preNKP to rNKP transition, was shown in the ILCP population [89]. The fate decision of these ILCP/NKP population is then dependent on niche components. ILC1 and NK cells are dependent on IL-15 [90, 91] while IL-7 is crucial for ILC2 and ILC3 differentiation [92, 93]. After being induced by specific cytokines and cell-cell interaction, a set of specific transcription factors is expressed and cooperates to drive lineage specification.

ILCP and NKP formation are both dependent on ETS1, TCF7 and NFIL3. NFIL3 deficiency led to a severe reduction in all ILC subsets and NK cells [78, 88]. ILC differentiation from ILCP is dependent on GATA3 and while NKP are able to develop, despite subsequently into less mature cells in GATA3 KO mice [94, 95]. ILCP are also dependent on three other transcription factors namely ID2, Zinc Finger And BTB Domain Containing 16 (ZBTB16) and Thymocyte Selection Associated High Mobility Group Box (TOX). The involvement of ID2, ZBTB16, and TOX in NKP formation is still debated [89, 95-99].

1.4 NK CELL EDUCATION

NK cells acquire functional competency and self-tolerance through a process called "licensing" or "education", mostly being MHC-I mediated [100-102]. NK cells that are derived from an MHC-I deficient environment or do not express any inhibitory receptors are hyporesponsive [103, 104], ensuring self-tolerance in these particular situations. Educated NK cells are more responsive towards "missing-self" targets. NK cells belonging to all stages of maturation are capable of performing missing-self reactivity, hence the education and the maturation process likely progress in parallel [87]. The molecular mechanism of NK cell education is so far not well defined. Several models have been proposed to explain NK cell education, including "arming", "disarming", "rheostat", and "retuning" models [105].

In the arming model, NK cells are initially hyporesponsive until signals through MHC-I-specific inhibitory receptors actively endow them with functional competency [106]. Consistent with this model, deletion of critical signaling molecules downstream of inhibitory receptors, namely SHP-1, SH-2 containing Inositol 5' Polyphosphatase 1 (SHIP1), or Signaling Lymphocytic Activating Molecule (SLAM) Associated Protein (SAP), inhibited NK cell education in MHC-I sufficient environments and resulted in NK cells whose phenotypes and functions were similar to those arising from MHC-deficient environments [107-110]. On the other hand, in the disarming model, NK cells are 'disarmed' in MHC class I-deficient situations due to overactive activating signals, which in MHC-I-sufficient environments would have been counteracted [24]. Activating NK cell receptors and ligands on healthy cells that disarm NK cells in this model have not been identified [24]. In the "rheostat" model, our lab and others have demonstrated that education is not an on/off system but rather quantitative and tunable according to the level of MHC-I expression by surrounding cells [111-113]. The NK cell responsiveness is dependent on an interplay between the extent of MHC-I expression and the density and numbers of inhibitory receptors recognizing endogenous MHC-I [100, 111, 112]. NK cell education is a reversible, tunable process and requires continuous interactions between self-MHC-I and self-specific inhibitory receptors [111, 114]. Experiments employing blocking antibodies to interfere with inhibitory signals or cell transfer into an environment with different level of MHC-I expression confirmed that NK cell education indeed is tunable. The loss or reduction of MHC-I expression down-tuned NK cell responsiveness while the presence of novel MHC-I signals up-tuned the reactivity [115-118].

1.5 INTERLEUKIN 15 (IL-15) THROUGHOUT NK CELL BIOLOGY

1.5.1 Overview of IL-15

A wide range of cytokines have been reported to modulate NK-cell homeostasis and functions, including IL-2, IL-4, IL-12, IL-18, IL-27, IL-21, IL-9, IL-35, IFN-α, TGF-β and IL-15 [91, 119, 120]. Among cytokines that share the common gamma chain (γc) (IL-15, IL-2, IL-21, IL-7, IL-9, and IL-4), IL-15 demonstrates an absolute importance for orchestrating the entire life of an NK cell, including development, proliferation, survival, and activation **(Figure 1)** [91]. A wide range of cell types, including mononuclear cells, activated macrophages, and epithelial cells express IL-15; while IL-15Rα is expressed on stromal cells, DCs, macrophages, and hematopoietic precursors [121, 122]. IL-15 and IL-15Rα expression is induced upon infection. IL-15 forms a complex with the IL-15Rα chain in the endoplasmic reticulum and the complex is then shuttled to the cell membrane to be presented to neighboring cells expressing IL-15Rβ and the common γc (CD132), in a process called "trans-presentation" [123, 124]. Since IL-15 is a potent pro-inflammatory cytokine, its expression is tightly regulated at different levels, including transcription, translation and intracellular trafficking [125].

When the IL-15Rβγ complex on NK cells is activated upon being bound with IL-15/IL-15Rα on presenting cells, three different pathways can be initiated, including Janus Kinase- Signal Transducer and Activator of Transcription (JAK-STAT), or PI3K-AKT-mTOR (the Mammalian Target Of Rapamycin), or MAPK **(Figure 3)** [126]. The β chain of the IL15-Rβ/γc heterodimer induces JAK1/STAT3 activation while the γc component stimulates JAK3/STAT5 activation. Phosphorylated STAT3 and STAT5 form either homodimers or heterodimers and translocate into the nucleus to trigger transcription of target genes. Binding of IL-15-IL15-Rα to IL15-Rβ/γc also stimulates the PI3K-AKT pathway through Src Homology 2 domain-Containing (SHC)- GRB2 signaling, in which SHC serves as adaptor protein to activate GRB2 and hence recruits and triggers PI3K activation. In the third signaling pathway activated by IL-15, activated GRB2 binds guanine exchange factor Son of Sevenless (SOS), which in turn stimulates the RAS-RAF-MAPK pathway.

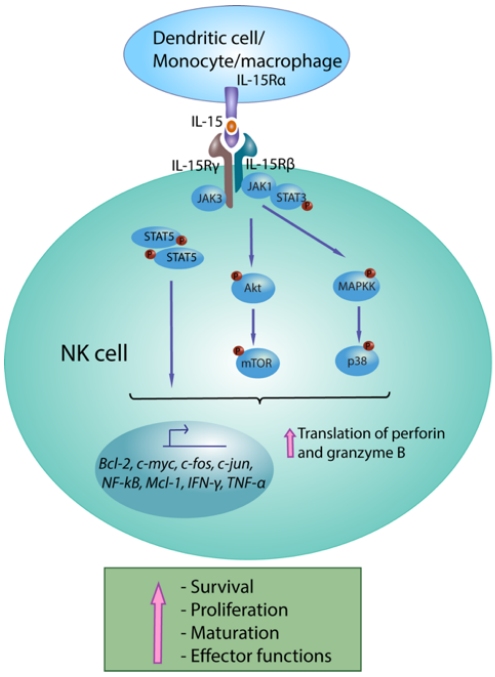

Figure 3. IL-15 signaling. IL-15 is mostly trans-presented to NK cells via IL-15Rα on DCs/monocyte/macrophages. IL-15/IL-15Rα interaction with IL-15Rβ/γc triggers three different signaling pathways, including JAK/STAT, AKT/mTOR and MAPK. The result is the increased transcription of genes involved in cell survival, proliferation, maturation and immune functions. IL-15 signaling also induces translation of perforin and granzyme B.

1.5.2 IL-15 in NK cell development

IL-15 is an indispensable cytokine for NK cell development. In mice deficient in expression of IL-15, IL-15Rα or IL-15Rβ, peripheral NK cells, together with γδ T cells, NKT cells and memory CD8 T cells, were almost absent [90, 127, 128]. Analysis of different knockout mice for cytokines sharing the γc as part of their receptors revealed that only IL-15-deficient mice showed the defective development of NK cells. On the other hand, mice deficient of IL-2, IL-7, IL-21, IL-9 and IL-4 possess relatively normal numbers of NK cells in the periphery [91, 127, 128]. The deficiency in either JAK3 or both STAT5a and STAT5b or mTOR in mice led to the block in NK cell development similarly to IL-15 or IL-15R KO mice [129-131]. Along the same line, mice overexpressing IL-15 have a higher proportion of NK cells, together with memory $CD8^+$ T cells, in the peripheral blood [132]. The difference between IL-15 and other cytokines sharing γc in their receptors suggests the uniqueness in the signaling pathways of IL-15.

IL-15 regulates NK cell development by supporting the survival and proliferation of progenitors as well as immature NK cells. Binding of IL-15Rα to IL-15Rβ/γc leads to increased survival of NK cells by stimulating expression of anti-apoptotic protein B-Cell Lymphoma 2 (BCL-2) and Myeloid Cell Leukemia 1 (MCL-1) downstream of JAK/STAT pathways and suppressing expression or activities of pro-apoptotic proteins FOXO, BIM and p53 Upregulated Modulator of Apoptosis (PUMA) through the PI3K-mTOR-AKT pathway. The proliferation effect is due to activation of the JAK/STAT and MAPK pathways, which increase expression of the proto-oncogenes *c-myc*, *c-jun*, and *c-fos* [133, 134].

1.5.3 IL-15 in NK cell maturation and functions

The link between IL-15 and NK-cell maturation has been noted in several studies. IL-15 stimulation can trigger NK cell maturation through the mTOR-AKT-FOXOs-T-BET signaling pathway. IL-15-induced PI3K phosphorylates mTOR, which in turn phosphorylates AKT on Ser 473, leading to phosphorylation of the FOXO transcription factors and inducing their nuclear export [129, 135]. FOXOs, in particular FOXO1, have recently been reported to negatively regulate NK cell maturation through inhibiting transcription of T-BET, an important transcription factor driving NK cell maturation [136]. Furthermore, NK cells from IL-15 or mTOR KO mice were shown to be less mature [91, 129]. Likewise, human NK cells displayed a more mature phenotype, $CD56^{low}CD16^+$, in response to the treatment with rhIL-15 plus IL-15Rα-Fc [137].

Relating to its role in supporting NK cell maturation, IL-15 also stimulates effector functions of NK cells. *In vitro* and *in vivo* experiments demonstrated that IL-15 trans-presentation by DCs primed NK cell functions upon the infection-like conditions induced by lipopolysaccharide (LPS) or TLR agonists [138, 139]. IL-15 modulates NK cell activation by increasing the translation of perforin and granzyme B, the important cytolytic molecules that mediate target cell killing by NK cells, without affecting the abundance of their mRNA [140]. It remains unknown whether, under steady-state conditions, IL-15 also supports NK cell functions or contributes to the education process. It is known that the education process is associated with shaping of the inhibitory receptor repertoire [141]. Using a transgenic mouse model with DCs as the only cell type expressing IL-15, it was reported that IL-15 from DCs was important for the up-regulation of several inhibitory and activating receptors on NK cells, including Ly49A/D, Ly49G2, Ly49I and Ly49D [142]. Indeed, many known downstream

molecules of the IL-15 receptor are essential for NK cell effector functions, including *c-jun*, *c-fos*, IFN-γ, and TNF-α [126]. Therefore, whether IL-15-IL-15Rα presentation, especially from DCs, contributes to NK-cell acquisition of the functional potency at the steady-state is a worth-to-pursue question and is the main research aim for **paper I**.

1.6 DENDRITIC CELLS AND NK CELLS

1.6.1 Overview of dendritic cells

DCs make up a critical subset of the innate immune system. They present foreign antigens to induce adaptive immune responses and self-antigens to induce tolerance. DCs are classified into conventional, plasmacytoid, and monocyte-derived DC based on their developmental paths, locations, receptor expression, and functions. Conventional DCs (cDCs) express high levels of CD11c and MHC-II. Zinc Finger And BTB Domain Containing 46 (ZBTB46), FLT3, c-KIT and GM-CSF regulate the development of cDCs [143]. cDCs populate both lymphoid and non-lymphoid tissues, and are superior compared to other DC subsets in sensing and capturing pathogens as well as self-antigens and present to T lymphocytes in the draining LNs [144]. Beside the roles in presentation and activation of T cells, cDCs also produce and wide range of cytokines and chemokines to modulate other immune cells including NK cells, e.g. IL-12, IL-18, IL-15, IL-1 and TNF-α [145]. As opposed to LNs, spleens contain only lymphoid tissue-resident cDCs [146]. Plasmacytoid DCs (pDCs) mainly reside in the primary and secondary lymphoid tissues and express TLR7 and TLR9 to sense viral infection. The development of pDCs is impaired in the absence of E2-2 transcription factor [147]. Monocyte-derived DCs, as their name indicates, are originally $CD14^{+}$ cells, which extravasate, upregulate MHCII, CD11c and costimulatory receptors to become DCs. Acting similarly to cDCs, monocyte-derived DCs become mature and migrate to draining LNs to present antigens to T lymphocytes [148]. In study I, we focused on the roles of cDCs (denoted hereinafter and in the paper as DCs) on NK cell homeostasis and functions in the mouse spleens.

1.6.2 Dendritic cells in NK cell homeostasis and functions

As opposed to T lymphocytes, NK cells were first identified to readily display effector functions without any prior priming from other immune cells. However, during the last decade, extensive studies have shown that myeloid cells, including neutrophils, macrophages and DCs, are required for NK cell activation [138, 149]. *In vitro* studies demonstrated that the cytotoxicity and IFN-γ production of NK cells were increased in the presence of DCs [139]. In this study, DCs were getting mature with TNF-α, LPS, or mycobacterium tuberculosis [139]. In addition, administration of FLT3L led to the expansion of DCs and hence also increased the mature NK cell pool by enhancing their survival and proliferation [150].

Recently, several mouse models in which DCs can be specifically depleted (CD11c.DTR, CD11c.DOG, and CD11c.DTRluci) have been created to facilitate the investigation of DC-NK interaction *in vivo* [138, 151-153]. Common to these models is the expression of human diphtheria toxin receptor (DTR) under the control of CD11c promotor; hence $CD11c^{high}MHCII^{high}$ cells can be depleted upon diphtheria toxin (DT) injection. Using these models, it was established that, under inflammatory conditions, DCs are crucial for the activation and homeostasis of NK cells [151].

Both soluble factors and contact-dependent molecules have been implicated in DC mediated modulation of NK cell activation *in vitro* and *in vivo*. From *in vitro* co-culture experiments and MCMV infection models, it has been shown that type I interferons and NKG2D interaction with its ligands are required for NK cell cytotoxicity, IL-12 and IL-18 for IFN-γ production, CXC3CL1/CXC3CR1 for IFN-γ production, and IL-15 for proliferation and survival of NK cells [149, 154-158]. In addition, *in vivo* DC-depletion experiments demonstrated that NK cell activation upon TLR ligand stimulation was enhanced by IL-15 *trans*-presentation from DCs [138].

Despite the well-characterized priming process happening upon the pathogen challenge, little is known about whether DC-NK interactions at steady-state play a role in functional acquisition or functional maintenance of NK cells. Therefore, in **paper I**, we studied the kinetic changes of NK cells upon DC depletion in mice, with regard to survival, proliferation, maturation, and functional responsiveness.

1.6.3 IL-15 priming

IL-15 priming refers to a process in which NK cells acquire a heightened activation state in the LNs owing to their contacts with cytokines or DCs, and thereafter are able to perform enhanced effector functions in peripheral tissues. Lucas and coworkers demonstrated that NK cells require DCs for their optimal effector functions upon infections [138]. Upon TLR stimulation, DCs rapidly upregulated IL-15 and IL-15Rα in a process that is dependent on type I IFNs. During a local infection, NK cells upregulate CD62 ligand and are then recruited to the draining LNs, where the priming takes place [138]. In LNs, recruited NK cells are present in the outer paracortex just beneath B cell follicles, where they make long and stable interactions with activated DCs [159]. In the absence of infection, more than 50 % of NK cells also form contacts with DCs in the LNs [160]. Even though 90 % of the contacts last less than 900 seconds, NK cell interaction with IL-15 at the steady-state might be functionally relevant. In **paper III**, we aimed at studying the functional outcomes of short time IL-15 stimulation, whether it is long lasting and its molecular mechanisms.

1.7 METABOLISM IN NK CELL FUNCTIONS

1.7.1 Metabolism in immune cell functions

Metabolic signaling has been shown to be important for differentiation and activation of various immune cell types [161]. Naïve T cells primarily rely on oxidative phosphorylation (OXPHOS) as their energy source. On the other hand, activated T cells switch to glycolysis for an increased anabolism for cell proliferation and a faster energy-supply, a process that is similar to the switch in cancer cell metabolism called the Warburg effect [162-164]. When activated T lymphocytes become the memory cells, they remain quiescent by changing back to more energy-producing metabolic pathways, including OXPHOS and fatty acid oxidation [165]. Therefore, in immune cells, metabolic changes are crucial to provide cellular energy in a right time frame and intermediates that are important for immune functions.

One of the responses of immune cells upon pathogen infection is the production of oxidative molecules including reactive oxygen species (ROS) and nitrogen reactive intermediates. ROS include free radicals, such as superoxide (O_2-) and hydroxyl radical (•OH), and non-free radical oxidative

molecules, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). The formation of superoxide is mediated by membrane-associated enzymes or cytoplasmic enzymes such as NAD(P)H oxidases and xanthine oxidases and redox-reactive compounds of electron transport chain in mitochondria, such as semi-ubiquinone. ROS are not only byproducts of cellular metabolism but also regulate multiple cellular signaling cascades via the activation of kinases, the inhibition of tyrosine phosphatases, and the induction of cytosolic Ca^{2+} by acting on calcium channels on plasma membrane or endoplasmic reticulum [166]. Treatment of the T cell membrane with H_2O_2 increased phosphorylation of proximal signaling molecules such as LCK, FYN, SYK, ZAP70, and CD3ς [167]. In addition, H_2O_2 also enhanced the capacity of T cells to produce IL-2 and IL-4 and to proliferate in response to CD3/CD28 stimulation by activating directly NF-κB [168].

1.7.2 IL-15, metabolism, and NK cell functions

IL-15 stimulation is known to induce metabolic changes in NK cells, in particular through mTOR activation [169]. IL-15 stimulation, for at least 16 hours, in mouse and human NK cells induces both glycolysis and OXPHOS [129, 169]. In addition, glucose-driven OXPHOS was shown to be critical for IFN-γ production triggered via activating receptor stimulation, which was bypassed by a treatment with a high-dose IL-15 [170]. Inhibiting mTORC1, a key metabolic regulator induced by IL-15, compromised NK cell cytokine production and cytotoxicity *in vitro* and *in vivo* [169, 171]. As opposed to T cells, studies on the effects of ROS on NK cell functions are scattered. Blazquez and co-workers showed in 1997 that antioxidant treatment reduced NF-κB activation and NK cell cytotoxicity [172]. In obesity, higher level of lipid accumulating in NK cells impairs glycolysis and OXPHOS, and hence their anti-tumor functions [173]. Whether metabolism and ROS are involved in short-time IL-15 stimulation has so far not been studied and hence has become one of the questions that we addressed in **paper III**.

1.8 FOXO TRANSCRIPTION FACTORS

1.8.1 Overview of the forkhead box transcription factors of the O class (FOXO) transcription factors

FOXO is a family of transcription factors that integrates external information, including growth factors and stress signals, to modulate various cellular processes. At the organism level, FOXO affects life span, metabolism, and fertility [174, 175]. At the cellular level, FOXO proteins modulate metabolism, stress response, cell fate decisions, and protein homeostasis [176]. FOXO controls cell homeostasis by regulating genes involved in cell cycle arrest and apoptosis, including cyclin dependent kinases (CDK), B cell lymphoma 6 (BCL6), FASL, TNF, etc. In a condition of starvation, FOXO controls gluconeogenesis, propensity for food intake and redox balance, by influencing expression of apolipoprotein C3 (APOC3), G Protein-Coupled Receptor 17 (GPR17), Superoxide dismutase 2 (SOD2), etc [177]. FOXO also regulates protein homeostasis by controlling expression of genes involved in mitophagy, proteasomal breakdown, and autophagy [178-180]. In addition, FOXO controls pluripotency via SOX2 and OCT4 [181]. Furthermore, emerging data show that FOXO proteins serve as critical transcription factors for various aspects of the immune system [182].

Due to the critical roles of FOXO in stress response and cell homeostasis, these proteins have been considered as tumor suppressors [183]. The activity of FOXO is controlled by the integration of

multiple signals. For example, independent phosphorylation of FOXO at three different sites by PI3K/mTORC2/AKT leads to their nuclear export and degradation [184, 185]. In addition, FOXO activity is also controlled acetylation, methylation, and ubiquitinylation [182].

1.8.2 FOXO in immune differentiation and immune responses

FOXOs are crucial for a wide range of immune processes. FOXO1 is critical for the survival and the homing of naïve T cells by controlling the expression of L-selectin, C-C chemokine receptor type 7 (CCR7) and IL-7Rα [186]. In addition, FOXO1 is involved in various stages of B cell differentiation, by regulating the expression of IL-7Rα, recombination activating gene 1,2 (Rag1,2), L-selectin, and activation-induced cytidine deaminase (AICD) [187-189]. FOXO1 and FOXO3a together are important for forkhead box P3 (Foxp3) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) expression and transforming growth factor β (TGF-β)-mediated downregulation of T-BET, which is crucial for regulatory T cell differentiation [190, 191]. Furthermore, FOXO3 controls interferon response factor 7 (IRF7) expression, which is critical for antiviral response of macrophages, or IL-6, monocyte chemoattractant protein 1 (MCP-1), and IFN-γ expression, which is important in DCs-mediated induction of T cell response [192, 193].

FOXO proteins regulate the survival of immune cells by being downstream of activating receptors and inflammatory cytokine receptors. Stimulations via NK-cell activating receptors, TCR or γc receptors induce PI3K-AKT pathway, which phosphorylates and retains FOXO in the cytoplasm **(Figure 4)**. In the absence of these signals, nucleus FOXO induces the upregulation of proapoptotic genes (Bim, CD95/CD95L, Puma), and cell cycle inhibitor genes ($p27^{Kip1}$) [194-197].

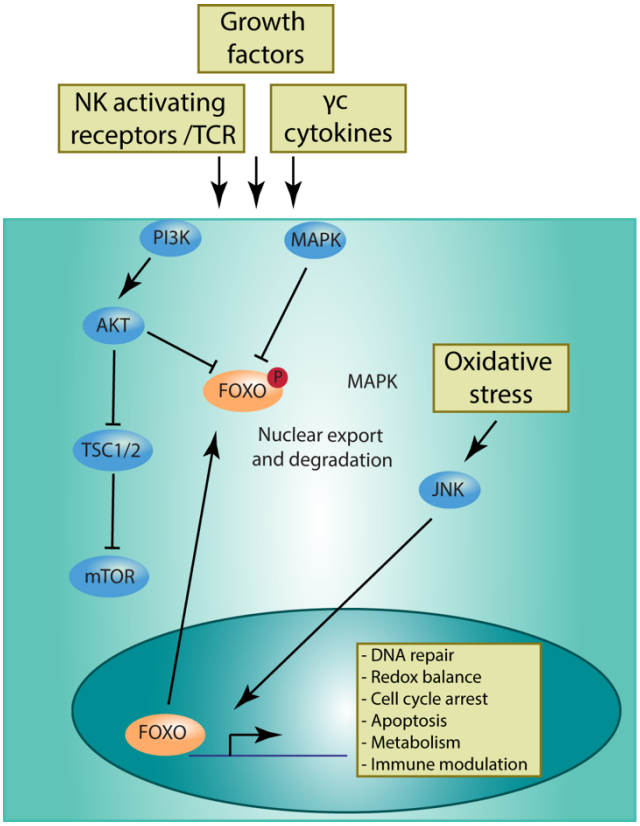

Figure 4. FOXO signaling in immune cells. Stimulations via NK-cell activating receptors, TCR or γc receptors induce the activation of AKT/mTOR or MAPK pathways which phosphorylate FOXO proteins. Phosphorylated FOXO proteins are exported from the nucleus, ubiquitinylated, and degraded. On the other hand, oxidative stress activates JNK which phosphorylates FOXO at a different site, and retain FOXOs in the nucleus to induce transcription of target genes. Target genes of FOXOs are involved in cell cycle arrest, DNA repair, ROS detoxification, metabolism, apoptosis, and immune functions.

1.8.3 **FOXO in NK cells**

Previous studies have reported conflicting findings on the roles of FOXO1 in NK cell development [136, 198]. Deng *et al.* demonstrated that FOXO1 deficiency specifically in NK cells ($Ncr1^{+}$iCre $FOXO1^{flox/flox}$) increased NK cell maturation and homing to LNs via the upregulation of T-BET and the downregulation of L-selectin. In this study, NK cell development was unperturbed with normal NK cell numbers in both BM and spleen [136]. Furthermore, FOXO1-deficient NK cells displayed an enhanced capacity to produce IFN-γ in response to IL-12 plus IL-18 stimulation, to kill YAC-1 cells *in vitro*, and to control B16F10 melanoma cells *in vivo* [136]. On the other hand, Wang *et al.* used a similar mouse model, yet with a different $Ncr1^{+}$iCre line, and showed that NK cell number in both BM and spleens were significantly reduced in the absence of FOXO1 due to an increase in ROS-mediated apoptosis. Wang and co-workers demonstrated that phosphorylated FOXO1 binds to Autophagy related protein 7 (ATG7) in the cytoplasm of immature NK cells, thereby inducing autophagy which protects NK cells from apoptosis-induced cell death [198]. As opposed to Deng *et al.*, Wang *et al.* reported a decreased function of FOXO1-deficient cells as they killed YAC-1 cells and controlled MCMV less efficiently as compared to littermate controls [198]. The contradicting data in the two studies were accounted to mouse housing conditions and the $Ncr1^{+}$Cre lines used [198].

In **paper IV**, we also have made $Ncr1^{+}$iCre $FOXO1^{flox/flox}$ to resolve the discrepancies between these two published works. Furthermore, we also made $Vav1^{+}$iCre $FOXO1,3^{flox/flox}$ to study the potential roles of FOXO1,3 in NK cell development as Vav1 is readily expressed in hematopoietic progenitors [199]. In this study, we also investigated the changes in NK cell maturation and receptor expression upon FOXO1,3 depletion and possible molecular mechanisms underlying any NK cell developmental effect.

2 RESULTS AND DISCUSSION

2.1 DENDRITIC CELLS THROUGHOUT MURINE NK CELL BIOLOGY

2.1.1 Dendritic cells are important for the homeostasis and functions of NK cells at steady-state

Making used of a conditional cell-depletion mouse model in which diphtheria toxin receptor (DTR) is expressed under the control of the CD11c promotor (CD11c.DOG), we explored the kinetic changes of NK cell homeostasis and functions in BM and spleens **(paper I)**. We have followed the changes in NK cell number and functions after up to 10 days of DC depletion. Even though DTR is expressed under the CD11c promotor control, we could confirm previous data that NK cells, with intermediate CD11c expression, were not affected directly by the DT treatment [151]. This key prerequisite for the study was confirmed by the fact that, in the spleen, DCs were readily depleted after 24 hours of DT injection while NK cell numbers remained unchanged. Using this model, we showed that depletion of DCs at the steady-state compromised NK cell homeostasis, perturbed the differentiation process with the accumulation of immature cells, and reduced the functional responsiveness of NK cells. The effects of DCs on NK cells were dependent on IL-15 expressed by DCs, as transferring back IL-15 KO DCs to DC-depleted mice did not restore missing-self killing capacity while the transfer of WT DCs did. We also showed the downregulation of genes involved in IL-15 pathway as well as the lower IL-15 responsiveness of NK cells obtained from DC-depleted mice **(Figure 5)**.

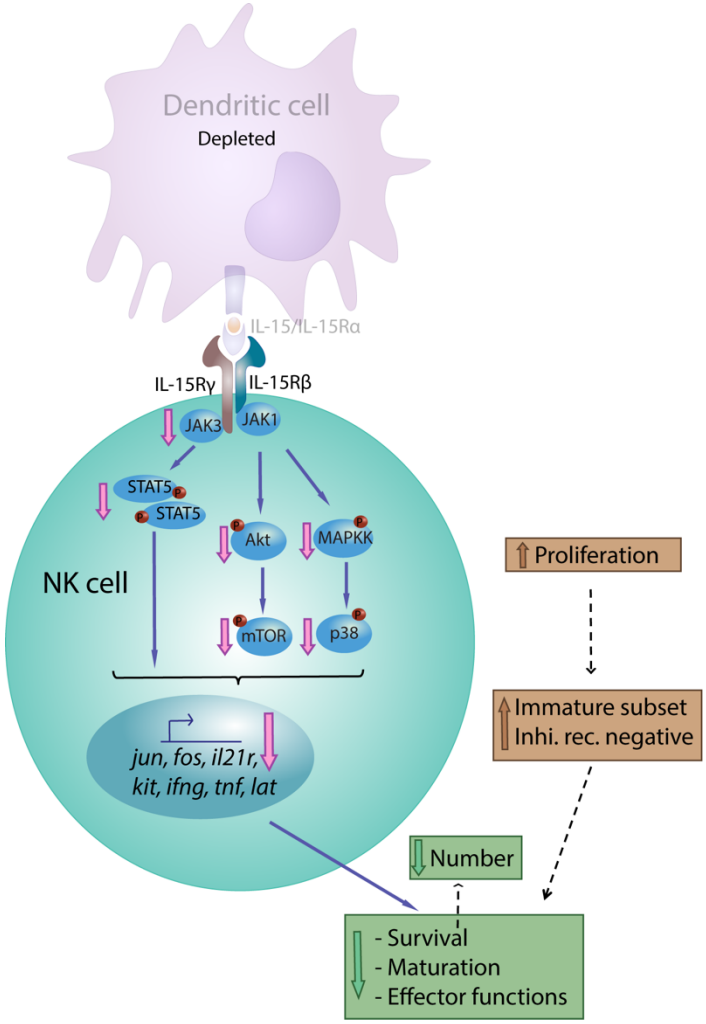

Figure 5. DC-depletion under steady-state condition affects NK cell homeostasis and functions. Early upon DC depletion (2-4 days-green boxes), NK cell numbers in both BM and spleen were reduced. IL-15 signaling was compromised in NK cells in the absence of DCs, as measured by the phosphorylation of STAT5, mTOR, AKT and p38. Expression of IL-15 target genes was down-regulated, including *jun, fos, ifng, tnf, lat, il21r, kit*. As a result, NK cell capacity to produce IFN-γ and kill "missing-self" targets was decreased. Late after DC depletion (6-10 days-brown boxes), NK cell homeostatic proliferation was induced, leading to the accumulation of immature subsets and inhibitory receptor negative subsets.

Using CD11b and CD27 as markers for NK cell maturation in mice, we found that there was an accumulation of the most immature subset, $CD27^{low}CD11b^{low}$ and a decrease in the mature subset, $CD27^{high}CD11b^{high}$. The accumulation of the immature subset starting to be apparent at day 4 might be accounted by their high proliferation rate as compared to other subsets. Therefore, the changes in the maturation status of splenic NK cells appeared to happen in parallel with the increased proliferation rate upon DC depletion. The work in this paper was performed from 2012 till 2016 when it was published and the knowledge on ILC was still limited. Therefore, little was explored and discussed in the paper regarding to the nature of the $CD27^{low}CD11b^{low}$ subset. In BM, this subset was shown to be largely ILC1 [89]. Therefore, it is possible that there was an expansion of ILC1 in the spleen upon DC depletion which was associated with a general reduction in Ly49 expression in the total $NK1.1^{+}CD3^{-}$ population. However, ILC1 linage specification and maintenance is also dependent on IL-15 [200]; and hence, the ILC1 population might be compromised similarly to NK cells. Future experiments to investigate the nature of the expanded $CD27^{low}CD11b^{low}$ population as well as the impact of DC depletion on ILC1 will shed light on the dynamic regulation of innate cell homeostasis by accessory cells.

In this study, we provided an evidence that, under non-inflammatory conditions, dendritic cells support NK homeostasis and functions via the effect of IL-15 signals. Upon 4 days of DC depletion, we detected that basal mTOR and AKT phosphorylation was reduced. Furthermore, gene set enrichment analysis of gene array data revealed that NK cells from DC-depleted mice down-regulated genes downstream of STAT5 pathway. *In vivo* functional assays demonstrated that IL-15 from DCs are crucial for missing-self reactivity against MHC-I deficient targets. In this experiment, transferring back of WT DCs restored NK cell functions from DC-depleted mice to the same level of NK cells from non-DC-depleted mice, while adding back IL-15 KO DC did not exert a similar effect. Nevertheless, add-back of IL-15 KO DCs still augmented the responsiveness of NK cells as compared to the non-add-back situation. Therefore, DCs might provide other factors that support NK cell functions at the steady-state, for example IL-12, IL-18, or MHC-I signal (discussed below in 2.1.2).

One observation consistent with the original study was that DC depletion resulted in the neutrophilia in the spleen of DC-depleted mice [151]. As a follow-up of paper I, we asked the question whether neutrophilia blurred the effect of DC depletion on NK cell development. In order to address this point, we injected A18 antibodies in an attempt to deplete neutrophils in the DC-depleted mice. We were able to restore the neutrophil number in the spleen back to a same number with non-DC-depleted mice and to partially reduce the neutrophil number in the BM. Upon 6 days of the antibody injection, NK cell population in the BM and spleen was reduced from mice with both DT and A18 injection to a higher extent as compared to DT injection alone. This suggests the coordinating roles of DC and neutrophils in NK cell development in the BM and homeostasis in the spleen. A thorough literature search did not give many evidences on the expression of IL-15 by neutrophils at the steady-state. A study detected IL-15 expression intracellularly and on the cell surface upon LPS treatment, but barely detected it in the absence of the stimulant [201]. Nevertheless, neutrophils possess the expression of all three components of the IL-15R complex, including IL-15Rα, IL-15Rβ and γc [202-204]. Therefore, we cannot exclude the possibility that neutrophils take up IL-15 from the BM niche and *trans*-present to NK cells and being the factor that blurred the effect of DC depletion in NK cell development in our model. Further experiments

to dissect carefully neutrophil as well as monocyte compartments in the BM will address the contributions of different hematopoietic cells to NK cell development.

Our system employs the cellular effect of DT, a potent toxin from *Corynebacterium diphetheriae,* which blocks cellular protein synthesis and induces cell death via apoptosis without inducing inflammation [205, 206]. Rodent cells also possess DTR, however, with three amino-acid different from human DTR, are much more resistant to DT. Transgenic mice with human DTR being expressed under specific promotors, in our study the $CD11c^{+}$ promotor, are useful for the selective depletion of $CD11c^{high}$ cDCs in a time-controlled manner. LC and pDC, on the other hand, are spared in this model [207]. However, as other DTR-DT models, CD11c.DOG mice were shown to develop antibody against DT and hence DC population was restored beyond 11 days of DT injection and kinetic study was not possible after this time point [151].

A limitation with our model was that CD11c is not only expressed on DCs but also on macrophages, NK cells, NKT cells, and some $CD11c^{+}$ T and B cells [208, 209]. Identifying unique markers for DCs or specific DC subsets has been a great challenge. Similar to CD11c, other DC markers, including FLT3, LYSM, CSF1R, CX3CR1, and CD11b, are also expressed by DC-related linages including monocytes and macrophages [209, 210]. In addition, the expression of the many markers is not stable and subjected to changes with the tissue niche components, including surrounding cell types and cytokine availability [209, 211]. At the same time with the starting of the work in paper I, a study showed a specific transcription factor, ZBTB46, identifies early committed cDC precursors and cDC, but not pDC, monocytes, or macrophages [143]. Even though ZBTB46 is dispensable for cDC development *in vivo*, its expression is crucial for the suppression of G-CSF and leukemia inhibitory factor receptors that usually happen during cDC development [143]. Making a new mouse model with expression of DTR under the control of ZBTB46 promotor will provide an opportunity to study more specifically the role of cDCs on NK cell development, homeostasis, and functions. In addition, further experiments to dissect the roles of a specific cDC subsets on NK cell development, homeostasis and functions can be done by using transgenic mice with expression of CD11c-driven Cre recombinase that targets the depletion of IRF8, BATF3, or components of the Notch signaling [143, 212-214].

2.1.2 Can dendritic cells be the main cell type providing IL-15 for NK cell development in the BM?

The formation and/or maintenance of splenic NK cells was shown to be dependent on IL-15 derived from hematopoietic cells but not stromal cells [150]. Regardless of this finding, the exact cell type residing in same BM niche with NK cells and supporting their development is still unknown. The hematopoietic candidates are monocytes, macrophages, DCs, and neutrophils, which express IL-15Rα. Indeed, IL-15 is required to be trans-presented to support NK cell development, as mice with only free IL-15 in the serum, but not IL-15α/IL-15 complex formed on the surface of BM DCs, possess significantly lower NK cell population size as compared to WT mice or parent mice with IL-15Rα expression [215]. Likewise, human NK cells arising from human hematopoietic stem cells in a transplantation mouse model of Rag2/γc KO, which do not have a lymphoid system [216], were significantly higher in the presence of exogenous rhIL-15/R but not soluble IL-15 [137]. Nevertheless, the exact cell type that provides IL-15 signals to support BM NK cell development is yet to be discovered.

So far, DCs have been shown to have numerous contacts with NK cells and reside in the same areas in the T cell zone in the LNs [160]. Yet, no study has been done to explore the localization and contacts between DCs and NK cells in the BM. Interestingly, a study using IL-15 reporter mice only demonstrated that in BM VCAM+ stromal cells are the main source of IL-15 [122]. Therefore, it is still unclear how much IL-15 is produced by BM DCs and whether DCs and NK cells are in frequent contacts in the BM like in the LNs.

2.1.3 Dendritic cells might provide MHC-I signal for NK cell education

The cell type that provides MHC-I signal essential for NK cell education is not yet known. Mature DCs were shown to form "regulatory synapses" with NK cells, which is a mixture of lytic and inhibitory synapse, with the actin reorganization in DCs and the accumulation of MHC-I molecules at the synapse [217]. However, little is known about NK-DC synapse under resting conditions. Resting NK cells do not kill immature DCs at low NK/DC ratio but induce their activation [12], suggesting that regulatory synapses might present between resting NK and immature DCs. We asked, in **paper I**, whether MHC-I expression on DCs was important for NK cell functions. Indeed, transferring back MHC-I KO DCs to DC-depleted mice did not restore the missing-self reactivity of NK cells as compared to WT DCs **(Figure 6)**. However, we could not exclude that transferred MHC-I KO DCs were killed by the host NK cells. We actually showed the presence of transferred MHC-I KO DCs in DC-depleted mice by CD45 markers, yet at a lower fraction as compared to transferred WT DCs. Therefore, we were still unsure of whether indeed MHC-I expressed by DCs is important or the number of transferred MHC-I KO DCs that have reached lymphoid organs was not sufficient. Further experiments to confirm this finding as well as studies on MHC-I KO mice with MHC-I expressed solely by DCs will answer this point.

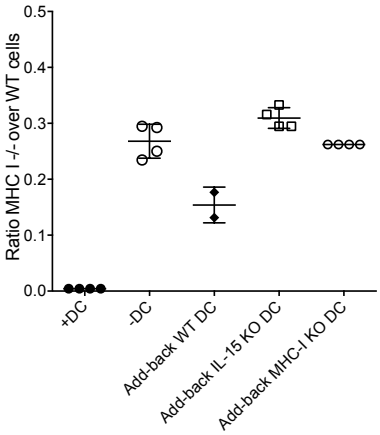

Figure 6. Transfer of WT DCs but not IL-15 KO or MHC-I KO DCs restored the *in vivo* missing-self reactivity of NK cells from DC-depleted mice. Design of the experiments was the same as Figure 4e from Paper I.

2.2 IL-15 THROUGHOUT MURINE NK CELL BIOLOGY

2.2.1 Functional effects of IL-15 short-time priming

NK cells bump into DCs with a high frequency and for very short durations in the LNs [160]. In **paper III**, we investigated whether the short-time IL-15 stimulation (five minutes) leaves any functional impact on NK cells, if it is long lasting, and what the molecular mechanism is. We have found that a five-minute stimulation augmented the calcium flux, IFN-γ production, degranulation of NK cells in response to activating receptor crosslinking, and their cytotoxicity towards YAC-1 cells. The priming could be explained by the crosstalk between IL-15 and activating receptors as

IL-15 stimulation alone induces phosphorylation of proximal signaling molecules downstream of activating receptor, including LCK and SLP-76.

The augmentation of calcium flux induced by five-minute treatment of IL-15 was long lasting after the removal of the cytokine, indicating a physiological relevance of this phenomenon. Based on these findings, we formulated a model for IL-15 priming of NK cell functions. In draining LNs, upon encountering with IL-15-transpresenting cells, DCs, even for a short period of time, the activation of activating receptor signaling molecules was increased above the basal level in unprimed cells. The small magnitude of activation, which is not sufficient to induce calcium flux, degranulation, or IFN-γ production, might be relevant. This helps to avoid potentially harmful production of inflammatory cytokines or cytotoxicity against LN endogenous cells, including DCs, endothelial cells, and stromal cells. Bearing a lower threshold for activation, primed NK cells migrate to the periphery and are ready to fight infections and control transformed cells. At the steady-state, there is a pool of NK cells in the peripheral tissues, being more functionally responsive due to previously encountering with DCs in the LNs. In fact, the brief contacts with IL-15 signal might help to avoid exhaustion induced by long-term IL-15 stimulations, which was observed for continuous IL-15 treatment in both human and mouse NK cells [218, 219]. In addition, overexposure to IL-15 signaling in a transgenic mouse model and uncontrolled autocrine/paracrine IL-15 expression led to a fetal leukemia of NK cells and T cells [132, 220]. This model points to a non-innate characteristic of NK cells, which is the requirement of accessory cells to maintain an optimal function state.

We have shown that IL-15/Rα complex also exerted a priming effect similarly to soluble IL-15. The first attention is on the efficacy of IL-15/Rα as compared to soluble IL-15. IL-15 acts mostly through trans-presentation due to its high affinity to IL-15Rα [124, 221]. The presence of IL-15Rα increased IL-15 efficiency to induce proliferation of $CD8^+$ and NK cell by more than 50 times [222]. It was intriguing that the same dose (10 μg/ml) of IL-15/Rα complex and IL-15 exerted comparable priming effects on calcium flux triggered via NK1.1 stimulation. However, our data on similar effects of IL-15 and IL-15/Rα complex might not be contradictory to previous finding. In fact, our study analyzed Ca^{2+} flux already after 20 minutes while Rubinstein *et al.* performed a much longer incubation of three to four days and investigated only the proliferation rate [222]. Indeed, the stimulation length of a cytokine might decide the cellular effects. This is in agreement with our findings that 20-minute IL-15 stimulation did not induce metabolic changes while 22-hour incubation did.

We have provided molecular explanations for short-time IL-15 priming, including crosstalk via JAK3 and ROS induction **(Figure 7)**. We made used of the selective JAK3 inhibitor (CP-690550), which has been proved *in vitro* and *in vivo* to severely block the development, proliferation, and activation of NK cell and CD8+ T cell [223]. JAK3 inhibition dampened the upregulation of STAT5 phosphorylation induced by five-minute IL-15 stimulation, proving the efficiency of the inhibitor on IL-15 signaling. In addition, the inhibition compromised IL-15 mediated induction of SLP-76 and LCK phosphorylation. Functionally, IL-15 mediated induction of IFN-γ and degranulation triggered via NK1.1 crosslinking was also dampened by the JAK3 inhibitor. Taken together, our data suggested that IL-15 might crosstalk with activating receptors proximally via JAK3 and LCK/SLP-76. JAK3 crosstalk between IL-15 signaling and activating receptor signaling

on NK cells was suggested in another study for the receptor NKG2D [224]. In this study, JAK3 was shown to associate with CD122 and γc in the NK cell line KY-1 or in LAK cells treated or untreated with pervanadate using co-immunoprecipitation (co-IP) experiments [224]. It would be the ultimate goal to prove physically that JAK3 interact with proximal signaling molecules downstream of NK1.1, i.e. FcRγ [225]. However, initial attempts to pull down FcRγ from primary unactivated murine NK cells were not successful due to its low expression level. Future co-IP experiments using NK cell lines expressing tagged JAK3 or FcRγ will possibly demonstrate the physical interaction between these two molecules upon a short-time IL-15 stimulation.

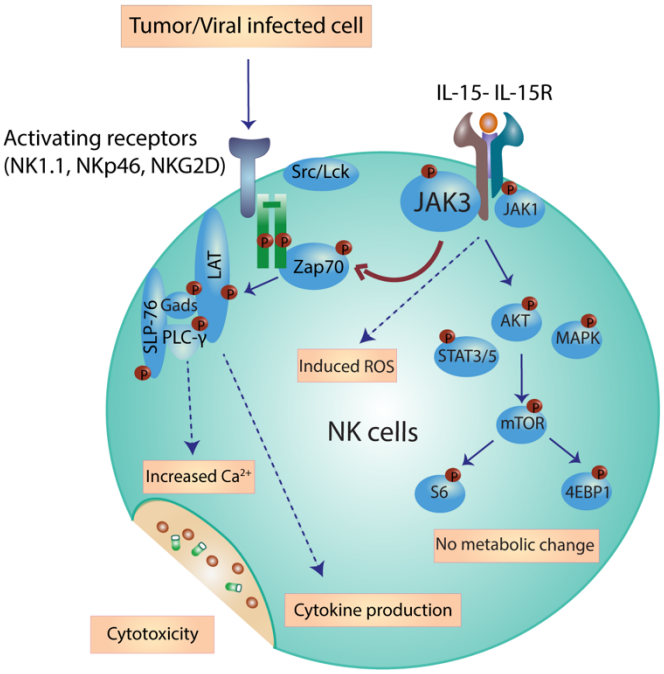

Figure 7. IL-15-short time priming effects on NK cell functions. Short-time IL-15 encounter (with soluble IL-15 or IL-15/Rα complex) induces JAK3 phosphorylation which cross-talks with proximal activating receptor signaling molecules including LCK and SLP-76. The crosstalk leads to the enhanced IFN-γ production, degranulation, and cytotoxicity of NK cells. Short-time IL-15 stimulation (20 minutes) does induce AKT/mTOR, JAK/STAT, MAPK pathways and ROS production, but not metabolism.

Further studies with more advanced techniques would shed light on the *in vivo* relevance of brief contacts between NK and DCs in the LNs. A photodegradable IL-15/IL-15R complex might be used to time the contacts more precisely, i.e. for 15 seconds, 30 seconds, 1 minute, 2 minutes and so on. Afterwards, target cells could be added directly to the NK cell culture and the calcium release might be monitored by microscopy to minimize the time that the cells are kept on ice. NK cells could also be left alone for certain periods of time between IL-15 being degraded and target cells being added, this is to study the long-lasting effect of short-time IL-15 contact. In a more advanced set-up, DCs could be transfected with photodegradable IL-15 and be used to replace IL-15/IL-15R complex. In this setting, DCs could also be activated with TLR agonist to study the kinetic of priming effects in the presence of infection. With the proposed settings, the functional relevance of short-time IL-15 priming could be explored in more detail.

2.2.2 IL-15 and metabolism in NK cell functions

Since long-term IL-15 stimulation was shown to induce metabolic changes in NK cells, we asked whether short-time IL-15 stimulation also resulted in a similar enhancement. mTOR mediates IL-15 induced metabolic changes in NK cells as mTOR inhibition compromised the effects [169]. In **paper III**, we found that mTOR and downstream molecules of mTOR, including S6 and 4EBP1, displayed an increased activation in response to 20-minute IL-15 stimulation. Yet to our surprise, we did not observe any changes in either glycolysis or OXPHOS upon a 20-minute stimulation

with IL-15. Therefore, we deduced that short time IL-15 stimulation augmented NK cell function independently of metabolic changes despite mTOR activation. MTOR induces metabolic changes via selectively modulating the translation of nuclear-encoded mitochondrial proteins as well as the upregulation of genes involved in nutrient uptake proteins, metabolic enzymes and mitochondrial structure proteins [226-228]. Like other immune cells, NK cells require an optimal level of cellular metabolism for functional responsiveness [170]. However, mTOR-mediated augmentation of both glycolysis and OXPHOS is dependent on protein translation, which is not likely to happen within five minutes. Therefore, it was reasonable to see a delay between mTOR activation and metabolic changes. Nevertheless, we do not exclude the involvement of metabolic changes in short time IL-15 priming. In the later stage when primed NK cells leave the sites of IL-15 contacts and migrate to the periphery, the activated mTOR might lead to metabolic changes. Analyzing primed cells upon a certain period of IL-15 starvation will address this question.

Despite the failure to detect any metabolic change upon short-time IL-15 stimulation, we have found a small increase in ROS production. ROS treatments themselves increased phosphorylation of proximal signaling molecules downstream of activating receptors, including LCK and SLP-76. The treatments with a ROS scavenger, N-acetylcysteine (NAC), reduced the augmented phosphorylation of LCK and SLP-76, which was induced by IL-15 stimulation. This indicates that ROS might contribute to the increased baseline in activation of signaling molecules upon IL-15 stimulation. However, we have also detected significant decreases in the phosphorylation of LCK and SLP-76 in untreated cells. Taken together, ROS might contribute to NK cell functional responsiveness by their effects in both resting and IL-15-treated cells.

2.2.3 DNAM-1, IL-15 in NK cell development and functions

In **paper II**, we showed that DNAM-1 and IL-15 signals coordinate to control LAT expression, which was crucial for NK cell functional responsiveness in response to NK1.1 stimulation **(Figure 8)**. DNAM-1^+ NK cells possess a higher level of LAT protein and the absence of DNAM-1 or its ligand, CD155, compromised LAT expression. LAT expression was also modulated by IL-15 stimulation and IL-15-induced upregulation of LAT was observed only for the DNAM-1^+ subset. The relevance of heightened LAT expression in DNAM$^+$ NK cells was shown for calcium flux, IFN-γ production and degranulation triggered via NK1.1 crosslinking. In agreement with our study, experiments on human NK cells demonstrated that higher responsiveness of DNAM-1^+ NK cells was also applied for target cells that do not express DNAM-1 ligand, including 721.221 and *Drosophila* S2 cells transfected with CD48 and ULBP1 [60]. The inside-out signal from DNAM-1 via LFA-1 conformation was suggested to be crucial for the immunological synapse formation towards the target cells and hence cytotoxic functions of NK cells [60]. Our study added another explanation, LAT-1 expression, for the heightened responsiveness of DNAM-1^+ NK cells.

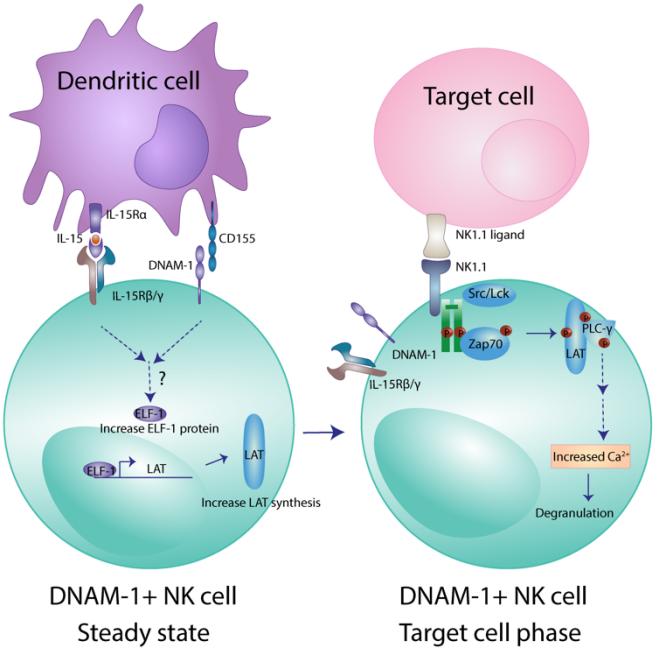

Figure 8. DNAM-1/CD155 and IL-15 cooperate to induce LAT expression. DNAM-1+ NK cells are more responsive to IL-15 stimulation with regards to LAT expression. The absence of DNAM-1 interaction with CD155 compromises LAT expression. The increased level of LAT expression in DNAM-1+ NK cells endowed them with higher capacity to response to activating receptor NK1.1, as measured by calcium flux, IFN-γ production, and degranulation.

In this study, we provided evidence showing that DC-derived IL-15 regulates LAT expression in NK cells via a transcriptional regulation. Upon 10 days of DC depletion, we found that ELF1, a member of ETS family of transcription factors, was significantly downregulated at the protein level in NK cells. In addition, adding back WT DC to DC-depleted mice restored the ELF-1 expression in NK cells to a similar level from non-DC depleted mice. The LAT promotor possesses two bindings sites for ETS transcription factor family and point mutations in these sites reduced LAT expression in Jurkat cells [229]. Antibody supershift experiments focusing on only four members of ETS family out of 16 members that are expressed in Jurkat cells, demonstrated that ELF1 and possibly also ETS1 bind to the LAT promotor [229]. It would be interesting to address why ELF-1 is particularly increased in DNAM-1$^+$ NK cells upon IL-15 stimulation. Is it a direct effect of DNAM-1 signaling? DNAM-1 stimulation experiments would provide an explanation.

We have showed that the expression of DNAM-1 endowed NK cells with heightened responsiveness towards NK1.1 triggering in regards to IFN-γ, degranulation, and calcium flux. DNAM-1 has been demonstrated to be a marker for NK cell education in both mice and human. The fractions of cells expressing DNAM-1 is higher in educated subsets as compared to uneducated subsets [52, 60]. An obvious question arising was whether the superior responsiveness of DNAM-1$^+$ over DNAM-1$^-$ NK cells was solely due to the education effect. In order to answer this question, we studied the reactivity of NK cells that are positive or negative for DNAM-1 from WT and MHC-I KO mice. In response to NK1.1 crosslinking, DNAM-1$^+$ NK cells from MHC-I KO showed a higher level of IFN-γ production and degranulation as compared to DNAM-1$^-$ counterparts. This demonstrated that the better responsiveness towards NK1.1 stimulation of DNAM-1$^+$ NK cells as compared to DNAM-1$^-$ cells was not accounted by the education effects.

In the same paper, **paper II**, we have demonstrated that the superior responsiveness of the DNAM-1$^+$ subset over DNAM-1$^-$ cells was owning to the direct interaction between DNAM-1 and its main ligand in mice, CD155. We showed that LAT expression is lowered in NK cells from CD155 and DNAM-1 KO mice as compared to those from WT mice. Nevertheless, our data was not in line with a study by Martinet and co-workers, in which interaction between CD155 and DNAM-1 was

claimed to not be important for the functional differences between DNAM-1$^+$ and DNAM-1$^-$ subsets [61]. However, in this paper, only cytokine production, including IFN-γ, IL-6 and MIP-1α in response to IL-12 plus IL-18 stimulation was evaluated. In addition, the absence of DNAM-1 expression in DNAM-1 KO mice did not render NK cells hyporesponsive, even though they displayed significantly lowered missing-self reactivity as compared to WT NK cells [52]. It is likely that DNAM-1$^+$ and DNAM-1$^-$ subsets possess a different potency towards different stimuli and that might depend on whether LAT is crucial in this certain stimulation. LAT and LAT2, were shown to play differential roles in IL-2 activated versus resting NK cells, in which the ZAP70-LAT pathway is more crucial in IL-2 activated NK cells while resting cells require SYK-LAT/LAT2 for an optimal responsiveness [230]. Further studies to explore the roles of LAT and LAT2, in DNAM-1$^+$ and DNAM-1$^-$ subsets will shed light on the developmental requirement of adaptor molecules.

With regard to the cell type providing CD155 signal to NK cells, CD11c$^+$MHC-II^{high} DCs from peripheral LNs express CD155 at the highest level as compared to other immune cell types analyzed from spleen, thymus, and LNs [231]. The frequent contacts between NK and DCs might be the results of their close proximity in the niche, or due to the DNAM-1-CD155 interaction, and hence allowing a higher responsiveness to IL-15 signals. Future experiments to study localization and movement of NK cells and DCs in the LNs in the presence or absence of DNAM-1-CD155 interaction will address this question.

2.2.4 IL-15 versus IL-2 in NK cell development and functions

The differences between IL-15 and IL-2 are interesting in relation to NK cell development and functions. Even though, IL-2 was not the focus of the thesis, I am captivated by the fact that IL-15 is indispensable for NK cell development while IL-2 is not. The question is intriguing while considering the fact that IL-15 and IL-2 stimulations lead to activation of exactly the same signaling pathways [232]. The same signaling is induced despite that IL-15 and IL-2 act on different α chains of the receptor complexes while sharing β and γc components. The differential roles of IL-15 and IL-2 in NK cell development could be attributed to cellular distribution of their cognate alpha-receptors and the modes of delivery. IL-2 seems to exert effects more efficiently in cis-presentation while IL-15 requires trans-presentation, due to differential affinity to their cognate receptor α chains (IL-2 and IL-2Rα: K_d = 10 nM, IL-15 and IL-15Rα: K_d = 50 pM) [124, 221]. In fact, NK cells and their progenitors do not highly express IL-2Rα at the steady-state. On the other hand, accessory cells, including DCs, macrophages, and stromal cells, are readily present in the bone marrow niche to trans-present IL-15 to NK cells through their IL-15Rα [233]. Of note, NK cell development *in vitro* can be induced by high amounts of IL-2 triggered via IL-2Rα lowly expressed in cis on NKP [234]. However, IL-2 is produced by activated cells, including T and NK cells, which are not readily present in the BM niche [235]. On mature NK cells, IL-2Rα is significantly upregulated upon activation and hence contributes to the functional priming and the survival support via the augmentation of the expression of perforin, granzyme B, and survival factors like BCL-2 or MCL-1. Hence, both IL-15 and IL-2 are crucial for homeostasis and functional responsiveness of mature NK cells [142, 236, 237], while only IL-15 is indispensable for NK cell development.

2.3 FOXO CONTROL NK CELL DEVELOPMENT AND MATURATION

The FOXO family of transcription factors plays such a diverse role in controlling immune cell homeostasis, differentiation, homing, memory formation, and effector functions [182]. FOXO1 KO mice are embryonically lethal due to the incomplete vascular development and FOXO3 KO female mice are infertile because of the abnormal ovarian follicle development [238]. Therefore, specific deletion of FOXOs is necessary to study their contributions to biology of certain immune cells. In **paper IV**, we made used of two mouse models that lack FOXO1,3 depleted in early hematopoietic cells or in committed NK cells. In the first model, $Ncr1^{+}$iCre FOXO1,$3^{flox/flox}$ (denoted as FOXO1,$3^{\Delta Ncr1}$), the Cre recombinase is expressed under the control of the Ncr1 promotor, the same mouse line that was used in Deng *et al.* [136], therefore, FOXO1,3 are depleted in committed NK cells that express Ncr1 (NKp46). Furthermore, in order to explore the roles of FOXO1,3 in early NK cell linage specification, we made mouse strains with the specific depletion of FOXO1, FOXO3, or both in $Vav1^{+}$ cells by crossing $Vav1^{+}$Cre with FOXO1,$3^{flox/flox}$ mice. The resulting strains are named in this thesis as FOXO$1^{\Delta Vav1}$, FOXO$3^{\Delta Vav1}$ and FOXO1,$3^{\Delta Vav1}$ [183]. The study with mice expressing lacZ reporter only in $Vav1^{+}$ cells demonstrated that Vav1 expression *in vivo* was restricted to hematopoietic and endothelial cells [199]. Due to the expression of Vav1 in the endothelial cells and the functions of FOXO3 for ovarian follicle development, the females which were used for breedings were $iCre^{-/-}$FOXO1,$3^{flox/flox}$, therefore preventing the deletion of FOXO3 in the ovary and infertility.

2.3.1 FOXO1,3 control NK cell development

Using the $Ncr1^{+}$iCre mouse model [239], we confirmed the findings by Deng *et al.* that deleting FOXO1 and FOXO3 does not compromise NK cell development, with normal NK cell compartments in both BM and spleen. On the other hand, FOXO1,3 depletion in early hematopoietic cells ($Vav1^{+}$) causes a block in NK cell development at the preNKP stage and a severe reduction in BM and splenic NK cells **(Figure 9)**. The few NK cells that develop in the absence of FOXO1,3 display an immature phenotype and perturbed expression of activating and inhibitory receptors. The effects of FOXO1,3 on the development and phenotype of NK cells are intrinsic as demonstrated by the transplantation experiments. Based on RNA seq and ATAC seq data, we suggest that FOXO1,3 coordinate to control expression and/or DNA binding of TCF7, ETS1 and CD122.

FOXO1,3 were showed to control the expression of proteins involved in cell homing, including L-selectin (CD62L) and sphingosine-1-phosphate receptor 1 (S1pr1) [136, 186, 240]. In line with these studies, we found that *S1pr*1 significantly down-regulated (8 folds) in preNKP of FOXO1,$3^{\Delta Vav1}$ mice as compared to controls. In addition, FOXO1,3 depletion compromised the survival of committed NK cells ($NK1.1^{+}CD3^{-}$) in both BM and spleen. This might be due to the defective autophagy and heightened ROS level as reported by Deng et al. [198]. Or the lower expression of CD122 might render NK cells less responsive to IL-15 in both BM and spleen. In fact, we believe that the dramatic effect on the NK cell population in the spleen of FOXO1,$3^{\Delta Vav1}$ mice could be the combined result of the lower egress from BM and the lower survival of NK cells in general.

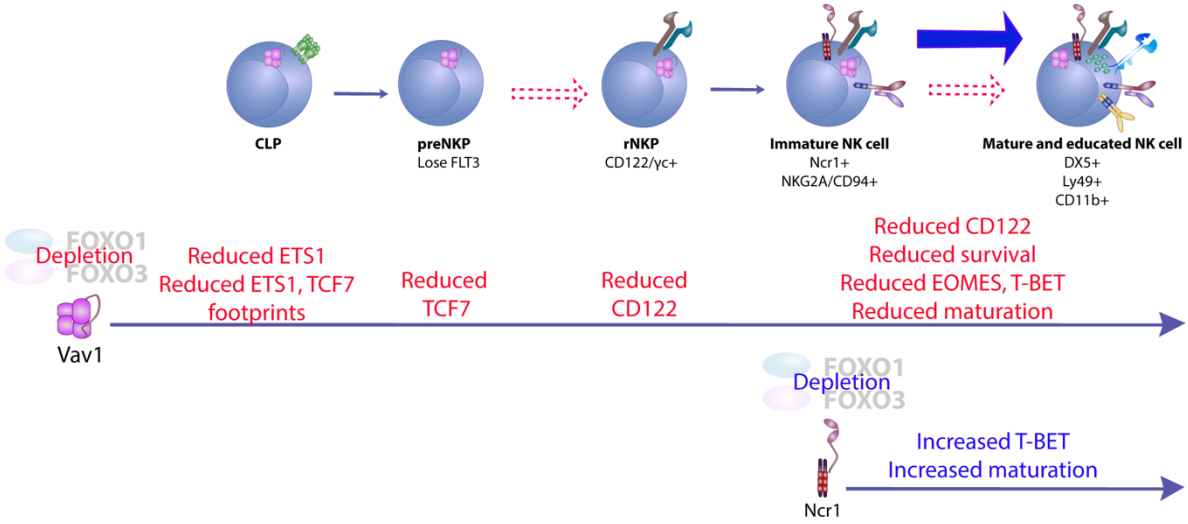

Figure 9. Deletion of FOXO1,3 in Vav1^{+} cells compromised NK cell development. Vav1 is expressed early in hematopoietic cells hence NK cell development from CLP can be studied in Vav1^{+}iCre FOXO1,3$^{flox/flox}$ mouse model. The results of FOXO1,3 depletion in Vav1^{+} cells are indicated in red arrows and letters. FOXO1,3 depletion led to a decreased transition from preNKP to rNKP. The reduction in expression and/or DNA binding of ETS1, TCF7 and CD122 might account for the developmental block. Furthermore, committed NK cells developing in the absence of FOXO1,3 show higher apoptosis and less mature phenotype. On the other hand, in our study and from [136], depletion of FOXO1,3 in Ncr1^{+} cells in the Ncr1^{+}iCre FOXO1,3$^{flox/flox}$ mouse model did not compromise NK cell development and numbers but resulted in an accelerated maturation process due to an increased T-BET expression. The results of FOXO1,3 depletion in Ncr1^{+} cells are indicated in blue arrow and letters.

FOXO1,3 regulate NK cell development in a hematopoietic intrinsic mechanism. In an attempt to address this question, we transplanted enriched BM cells from WT or FOXO1,3$^{\Delta Vav1}$ mice to irradiated recipient mice. Congenic markers were used to distinguish between donor and recipient cells (CD45.1 versus CD45.2). FOXO proteins were shown to support the long-term repopulating capacity of hematopoietic stem cells (HSCs) as their absence increased cell cycling and apoptosis rate of these cells [241]. Therefore, in the transplantation experiments, FOXO proteins were crucial for an efficient long-term reconstitution of donor BM cells [242]. We have encountered the apparent challenge which is the poor reconstitution of FOXO1,3$^{\Delta Vav1}$ BM cells despite being at a much higher number at the start as compared to WT cells (three million FOXO1,3$^{\Delta Vav1}$ cells as compared to 0.2 million WT cells). In order to overcome this problem, we injected a group of mice with three million CD45.2^{+} FOXO1,3$^{\Delta Vav1}$ cells together with 0.2 million CD45.1^{+} WT cells as support cells. Despite being surrounded by WT support cells, FOXO1,3$^{\Delta Vav1}$ BM progenitor cells still failed to develop to NK cells, suggesting that the requirement of having FOXO1,3 for NK cell development was intrinsic. Taken together, HSCs and NKP require FOXO1,3 expression by themselves in order to develop to NK cells.

2.3.2 FOXO1,3 control NK cell maturation

Using the Vav1^{+}iCre FOXO1,3$^{floxed/floxed}$ mouse model, we also studied the impact of FOXO1,3 deletion on NK cell maturation and receptor expression. Consistent with Deng *et al.*, NK cells from FOXO1$^{\Delta Vav1}$ mice were more mature as compared to WT controls with an increased CD27^{low}CD11b^{high} population. However, different from Deng *et al.*, NK cell maturation from

FOXO1,3$^{\Delta Vav1}$ was perturbed but not in the same pattern as FOXO1$^{\Delta Vav1}$. NK cells from FOXO1,3$^{\Delta Vav1}$ are less mature with an accumulation of the immature subsets, CD27^{low}CD11b^{low} and CD27^{high}CD11b^{low} and a smaller fraction of the mature subset, CD27^{low}CD11b^{high}. This finding points to the temporal effect of FOXO1,3 in modulation NK cell maturation, likely via T-BET expression. If FOXO3 is deleted early in Vav1^{+} hematopoietic cells, its role in positively controlling T-BET expression counteracts FOXO1-mediated-inhibition of T-BET expression. While, FOXO3 deletion in Ncr1^{+} committed NK cells fails to counteract the effect of FOXO1 deletion, as FOXO1,3$^{\Delta Ncr1}$ NK cells and FOXO1$^{\Delta Ncr1}$ are comparably mature [136]. FOXO have been known to decide cell fates by integrating external and internal signals. FOXO proteins are controlled by and modulate multiple cellular pathways. Therefore, our study suggests that the temporal difference in FOXO deletion might have resulted from the perturbation in different cellular pathways.

In an attempt to decipher the network of transcription factors involving FOXO1,3 that controls NK cell maturation, we examined the expression of EOMES and T-BET in BM immature NK cells from FOXO1,3$^{\Delta Vav1}$ and WT mice. EOMES expression in CD11b^{low} BM NK cells from FOXO1,3$^{\Delta Vav1}$ mice was significantly lowered as compared to those from WT controls, while T-BET expression showed a trend in reduction. EOMES has been shown to be important for NK cell maturation due to its controlling CD122 expression [243]. NK cells from Eomes$^{flox/flox}$Vav1^{+}iCre mice also displayed an immature phenotype with the accumulation of CD27^{high}CD11b^{low} subset, which is similar to EOMES negative NK cells from WT mice [243]. FOXO1 has been shown to bind indirectly to the promotor of *Eomes* [244] and FOXO3 binds to both the promotor and enhancer regions of *Eomes* [245]. Another explanation to account for the reduced maturation of NK cells in FOXO1,3$^{\Delta Vav1}$ is the lower responsiveness to IL-15 signal due to the lower CD122 expression. It was shown that the few NK cells that develop in spleens of Rag2 KO IL-15 KO nice were less mature with a lower CD43^{+}CD11b^{+} fraction [91]. Taken together, the deletion of FOXO1,3 in Vav1^{+} progenitor cells led to different changes in NK cell maturation as compared to their deletion in Ncr1^{+} committed NK cells.

2.3.3 FOXO1,3 in the network with other transcription factors that modulates IL-15 signaling

FOXO1 is located mostly in the nucleus of NKP while it resides preferentially in the cytosol of iNK and mNK [198]. This suggests potential transcriptional importance of FOXO1 in NKP, e.g. transcriptional control of IL7-Rα and ETS1. FOXO protiens control expression of numerous transcription factors and also interact with co-factors to induce or suppress gene expression [176, 246]. Motif analyses of target genes in both mice and *Caenorhabditis elegans* showed that many co-activators or co-suppressors might coordinate with FOXO proteins to control target gene expression. In mouse, the co-activators and co-suppressors belong to Forkhead, ETS, CTCF, bHLH, and CTF families [246]. In NK cells, it has been shown that FOXO1 is recruited to T-BET promoter via Sp1, to inhibit T-BET expression [136]. In our study, by RNA-seq and ATAC-seq, we uncovered a potential molecular mechanism underlying FOXO1,3-mediated NK cell development. RNA-seq was used to study the global transcriptional program while ATAC-seq is a useful tool to investigate the chromatin landscape.

ATAC-seq is a recently developed method to for epigenomic profiling that provides information regarding to DNA accessibility, regions of transcription factor bindings as well as nucleosome position [247, 248]. ATAC-seq is superior over older methods regarding to the low amount of starting material, the simple and short-time sample preparation, and the interrogation of different epigenetic information [248]. ATAC-seq makes use of hyperactive Tn5 transposase to simultaneously cut and ligate high-throughput sequencing adaptors to regions of good accessibility. Regions of transcription factor binding can be identified based on a sudden decrease of reads within a peak, called "footprint" [268]. ATAC-seq together with RNA-seq experiments demonstrated that ETS1 expression and footprints were reduced in FOXO1,3$^{\Delta Vav1}$ CLP as compared to littermate controls.

In addition to ETS1, RNA seq experiment on preNKP showed that expression of TCF7 was also down-regulated from FOXO1,3$^{\Delta Vav1}$ mice as compared to WT mice. TCF7 was shown to contribute to early NK cell development as TCF7 depletion compromised the formation of NKP in a similar way as ETS1 KO and FOXO1,3$^{\Delta Vav1}$ mice [78, 83, 249]. As opposed to the reduced expression in preNKP, TCF7 expression was significantly increased in CLP from FOXO1,3$^{\Delta Vav1}$ mice. Nevertheless, we found a reduction of 46 TCF7 footprints on DNA, which hints to its lower activity, in CLP from FOXO1,3$^{\Delta Vav1}$ mice. CLP and preNKP might possess different levels of co-activators or co-repressors, leading to differences in TCF7 expression changes upon FOXO1,3 depletion. Different from ETS1 and TCF7, NFIL3 did not exhibit changes in either expression or DNA binding capacity upon depletion of FOXO1,3 in both CLP and preNKP.

ETS1 has been shown to bind to the CD122 promotor and control its expression [83]. When CD122 is expressed at a very low level due to FOXO1,3 depletion, the rNKP population cannot be formed. FOXO1,3$^{\Delta Vav1}$, IL-15KO Rag2 KO and IL-15Rα KO mice [91] are similar with regards to NK cell progenitor development and maturation. Indeed, we found a similar reduction in NKP population from IL-15Rα KO mice (data not shown). We found a reduced EOMES expression in CD11b^{low} NK cells from BM in FOXO1,3$^{\Delta Vav1}$ mice, which might be the contributing factor for the lower CD122 expression as EOMES was shown to positively regulate CD22 expression [243]. As further confirmation of the reduction in IL-15 responsiveness, we showed that the fraction of splenic NK cells undergoing apoptosis was significantly increased in the absence of FOXO1,3. These findings suggest a network of transcription factors, including FOXO1,3, ETS1 and TCF7, modulates CD122 expression and hence NK cell development, survival and maturation.

The differences in NK cell development and maturation between depletion of FOX1,3 in early NKP (Vav1^{+}) and committed NK cells (Ncr1^{+}) are interesting. We found that NK cell development was compromised in FOXO1,3$^{\Delta Vav1}$ mice while largely spared in FOXO1,3$^{\Delta Ncr1}$ mice. We suggested that the developmental defect was due to the lower CD122 expression and hence the lower IL-15 responsiveness. FOXO1,3 might also control CD122 expression in committed NK cells. However, the phenotype might be minute due to the positive feedback loop of IL-15 signaling, that once CD122 is expressed, its expression is sustained. A positive feedback loop was found to mediate PDK-mTOR-EOMES-NFIL3-CD122 pathway, supporting the sustained expression of IL-15R and IL-15 signaling [81]. Besides, NK cell maturation from FOXO1,3$^{\Delta Vav1}$ and FOXO1,3$^{\Delta Ncr1}$ mice is also not similar, with an accelerated maturation in FOXO1,3$^{\Delta Ncr1}$ mice while a decreased maturation

in FOXO1,3$^{\Delta Vav1}$ mice. The difference can be accounted to the differences in T-BET and EOMES expression when FOXO1,3 are depleted in NKP or committed NK cells. When FOXO1,3 were depleted in committed NK cells, T-BET expression was increased and hence a higher proportion of mature NK cells was formed. However, when FOXO1,3 were depleted in NKP, T-BET and EOMES expression was significantly reduced. This difference might again be due to different networks of transcription factors present in NKP versus NK cells. So far, we have not found the possible underlying molecular mechanism accounting for the lower T-BET expression when FOXO1,3 were depleted in NKP. ChIP sequencing of FOXO binding regions will reveal any differences in the transcriptional network controlled by FOXO1 and FOXO3 in NKP and NK cells.

2.3.4 **FOXO1,3 control ILC development**

NK cells were recently placed together with ILC1 in group 1 ILC. Observing a severe defect in NK cell development on FOXO1,3$^{\Delta Vav1}$ mice, we asked whether the depletion of FOXO1,3 also affected ILC lineage specification. This is an apparent question since the preNKP and the rNKP populations indeed do share phenotypic characteristics with ILCP in the expression of GATA3, ZBTB16, α4β7 and CD122 expression [89]. In **study IV**, we showed that the ILC1 population was significantly reduced in BM while ILC3 was decreased in BM and thymus while increased in the spleen. On the other hand, the ILC2 population, especially ST2^{-} ILC2 displayed an expansion in all three organs. The reduction in ILC1 is in line with the defect in NK cell development as both ILC1 and NK cells rely on IL-15 for their development. The ILC3 population is dependent on the transcription factor RORC and we revealed that RORC is downregulated in both preNKP/ILCP and committed ILC3, as showed by RNA-seq and flow cytometry data. We also showed a reduction in *IL23r* expression in preNKP/ILCP population in FOXO1,3$^{\Delta Vav1}$ as compared to WT mice. The ILC2 and ILC3 require IL-7 for their development and the reduction in ILC3 might have led to an increase in ILC2 population. In addition, the expansion of ILC2 population might not be solely the indirect effect of the IL-7 niche. Indeed, FOXO expression or activity were linked with ZBTB16 and BCL11B [250-252], two indispensable transcription factors for ILC2 formation [97]. Taken together, in **paper IV**, we have shown that FOXO1,3 are crucial for NK cell and ILC development.

3 CONCLUSION AND OUTLOOK

IL-15 is a crucial factor orchestrating the entire life of an NK cell, including development, proliferation, survival, and activation **(Figure 1)** [91]. IL-15 exerts its effect mostly via *trans*-presentation mediated by the IL-15Rα expressed on accessory cells, including DCs. Upon brief contacts in the LNs, NK cells receive signals from DCs at the steady-state. IL-15 deficiency in mice results in a block in NK cell development. Likewise, mice with low expression of various transcription factors, or signaling molecules that act downstream of IL-15 signaling, including JAK3, STAT5, AKT and mTOR, also exhibited defective NK cell development. This thesis has addressed several aspects of IL-15 in murine NK cell biology, including i) the discovery of new transcription factors which regulate IL-15R expression, and hence, are crucial for NK cell development, ii) the exploration of functional effects of the short-time priming, iii) the study of the crosstalk between DNAM-1 and IL-15 signaling, and iv) the investigation of how homeostasis and functions of NK cells are controlled by DCs *in vivo*.

In **paper I**, we investigated changes in homeostasis and functions of NK cells upon conditional depletion of DCs. Making use of a mouse model in which DTR is expressed under the control of the CD11c promotor, we could specifically deplete DCs in the spleen under non-inflammatory conditions. In the absence of DCs, NK cells were less mature and expressed fewer inhibitory receptors and their *in vivo* missing-self reactivity was compromised. Gene array and flow cytometry analyses demonstrated a defect in IL-15 signaling in NK cells upon DC depletion. Our data demonstrated that, like T cells, NK cells require a tonic signal from accessory cells at the steady-state for their optimal homeostasis and functions.

Paper II explored the same system as in paper I and elaborated on the molecular consequences of DC interactions with NK cells. We demonstrated in this study that both DNAM-1 and IL-15 signals increased expression of the LAT protein, a key player in the transmission of activating signals in NK cells. Therefore, NK cells might receive various signals from DCs in the LNs, including among others IL-15, IL-12, IL-18, DNAM-1, MHC-I, to gain optimal functions and to be ready for fighting infections.

In **paper III**, we studied the outcomes of the brief stimulation of NK cells by IL-15, artificially mimicking what might take place when IL-5 is presented in short contacts with accessory cells *in vivo*. This work revealed that short-time contacts with IL-15 left remarkably robust signaling imprints and functional impacts on NK cells, including IFN-γ production, degranulation, and cytotoxicity. In addition, IL-15 stimulation increased phosphorylation of proximal signaling molecules downstream of activating receptors, providing a mechanistic link to the increased functional capacity. Furthermore, the short-time priming effects were long-lasting, indicating its possible physiological relevance. Albeit speculative, and open to further experimentation, it could be hypothesized from our data that the short-term interactions between DCs and NK cells that have been described *in vivo*, could, despite short contacts, result in an enhanced functional imprint that would be sufficiently long-lasting to allow migration to peripheral tissues and execution of effector functions against NK cell targets.

Paper IV takes a step back from the functionally oriented studies of the other papers and focuses on NK cell development in the bone marrow, specifically on the role of a set of transcription factors

for this process. FOXO1,3 depletion in hematopoietic progenitors resulted in a block in NK cell development with a significant decrease in rNKP and NK cell populations in both BM and spleen. In parallel with the compromised NK cell development, ILC development was also perturbed, with a tendency in an increase in ILC2 and a decrease in ILC1 and ILC3 populations. Mechanistically, RNA-seq and ATAC-seq data demonstrated a lower expression and DNA binding of ETS1. Furthermore, IL-15 responsiveness of NK cells was reduced, which could be explained by the lower level of IL-15Rβ expression upon FOXO1,3 depletion. Taken together, **paper IV**, demonstrated a crucial role of FOXO1,3 in NK cell development, owing to their direct or indirect regulation of IL-15R expression.

It is always difficult to predict the clinical importance of findings from the basic medical science, which does not directly aim to novel drugs or therapeutic implications. Nevertheless, aspects of the work in my thesis may be directly relevant for clinical medicine, or at least suggest new studies with a more clinical focus. Since FOXO transcription factors play a diverse role in controlling the immune system, therapeutic drugs targeting FOXOs are being developed against inflammatory diseases. Development of chemical inhibitors against transcription factors is still challenging due to the overlapping specificity within members of the same family [253]. Library screening has gained some advances in the search for inhibitors that inhibit FOXO phosphorylation [254-256]. Blocking FOXO, however, has to be taken cautiously as FOXO in mice were considered as tumor suppressors [183].

One challenge in NK cell therapy against leukemia has been to make NK cell proliferate, persist and maintain functional competence *in vivo* after adoptive transfer. Here, my findings on how NK cells respond to IL-15, in particular the mechanism regulating NK cell priming and functions after short-term IL-15 treatment, may suggest novel ways how to enhance these properties in patients. In addition, my studies on mechanisms resulting from NK cell/DC interactions *in vivo* may point to new ways in which these interactions could be enhanced. Thus, because IL-15 plays such an important role in supporting NK cell development and function, it has therapeutic potential to boost NK cell expansion and functions *in vivo*. Short half-life and poor bioavailability of IL-15 requires a development of modified IL-15 that is more stable, more readily accesses the tumor sites and has a higher affinity to its receptor.

While many novel insights have been provided by my work in this thesis, much remains to be discovered. Regarding the role of IL-15 in development, a key question is which cell type(s) among hematopoietic cells provide IL-15 for NK cell development in the BM? What do contacts between NK cells and IL-15 *trans*-presenting cells look like in the LNs, not only involving DCs but also macrophages, monocytes, endothelial cells etc.? Furthermore, does IL-15 coordinate with activating receptor signaling, e.g. DNAM-1, and MHC-I/inhibitory receptor signaling to endow NK cells with functional responsiveness against “missing self” targets? How does NK cell education link to NK cell development? Do the same cell types, or cellular interactions, control these two processes simultaneously or are they completely different processes? Which cell types provide MHC-I signal for NK cell education? What is the role of IL-15 in this process, and which signals downstream of MHC-I receptors regulate the acquisition of effector functions in NK cells? Finally, the exact role for FOXO1,3 in NK cell development needs to be determined, which might

give novel insights into the full maturation process of these cells and potentially lead to novel ways to boost NK cell development in stem cell transplantation.

Science has a start but no end. The more we discover, the more we realize that remains hidden. In fact, my notebook contains many more questions now compared to before my thesis work, which is a good sign that leaves plenty of interesting new projects for me to explore in the future.

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