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NK CELL EDUCATION AND ADHESION MOLECULES – IMPLICATIONS FOR IMMUNOTHERAPY

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NK cell Education and Adhesion Molecules – Implications for Immunotherapy

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"No one can do it alone. As you navigate through the rest of your life, be open to collaboration. Other people and other people's ideas are often better than your own. Find a group of people who challenge and inspire you, spend a lot of time with them, and it will change your life."

(…)

"… besides, it's much more fun to fail and succeed with other people."

Amy Poehler, Harvard Commencement speech 2011

ABSTRACT

NK cells have a key role in immune surveillance. They detect aberrant expression of stress-induced molecules and MHC class I molecules with an array of activating and inhibitory receptors. Inhibitory receptors are not only important during the effector phase to guarantee tolerance towards self cells, but also provide the means to learn what self is, during a process termed NK cell education. This mechanism ensures that only NK cells expressing inhibitory receptors for self MHC-I become functionally responsive to react to down-regulation of MHC-I in otherwise normal cells.

Recent evidence suggests that education is tunable and changes according to the net signaling input an individual NK cell receives. In **paper I**, we studied such retuning effects of NK cell education in different settings relevant to immunotherapy and asked whether they would impair anti-tumor responses. We used two mouse models of clinically relevant approaches to interfere with inhibitory receptor signaling, antibody mediated checkpoint blockade of inhibitory receptors, and allogeneic adoptive transfer. Despite adaptation of NK cell responsiveness resulting in tolerance to healthy MHC-I⁻ cells, we could still detect efficient anti-tumor responses.

To date, there is no known marker that distinguishes educated from hypo-responsive NK cells. In **paper II** we investigated the relationship between the adhesion molecule and activating receptor DNAM-1 and the education state of mouse NK cells. DNAM-1 expression appeared early in development prior to inhibitory receptors for MHC-I. We observed in several experimental situations that DNAM-1 levels are higher on NK cells that have Ly49 inhibitory receptors for self MHC-I, and thus are considered to be educated. Furthermore, we found an MHC-I independent, tight correlation between DNAM-1 expression and the inhibitory receptor NKG2A. Based on the data, we propose a model for how DNAM-1 levels may influence NK cell functions in different developmental and functional stages.

While NK cell education ensures strong tolerance to most normal cells, one exception is the interaction with DCs. Cross-talk between NK cells and DCs can lead to functional maturation of both cell types, but may also result in NK cell-mediated killing of the DC under certain conditions. In **paper III**, we analyzed the impact of an inflammatory environment on this interaction. We report that non-inflammatory DCs are relatively resistant to NK cell killing, while under inflammatory conditions, NK cells kill DCs via recognition of CD155 and ICAM-1 by DNAM-1 and LFA-1.

In **paper IV** we explored the possibility of NK cell activation by vaccination with alpha-galactosyl-ceramide (αGalCer) loaded exosomes directed to stimulate iNKT cells. We observed a preferential proliferation of educated NK cells in response to iNKT cell activation, as opposed to published results in virus models where preferential proliferation of uneducated cells is observed. This results in increased missing self killing of normal cells, and increased killing of both MHC-I⁺ and MHC-I⁻ tumor cells. Together, these findings contribute to illustrate the role of NK cells and NK cell education in immune surveillance against cancer cells. The thesis discusses these results in light of NK cells and their application in immunotherapy.

LIST OF SCIENTIFIC PAPERS

I.   **Arnika K. Wagner***, Stina L. Wickström*, Rossana Tallerico, Sadia Salam, Tadepally Lakshmikanth, Hanna Brauner, Petter Höglund, Ennio Carbone, Maria H. Johansson*, Klas Kärre*
Retuning of mouse NK cells after interference with MHC class I sensing adjusts self-tolerance but preserves anticancer response
Cancer Immunol Res. 2016 Feb;4(2):113-23.

II.   **Arnika K. Wagner**, Nadir Kadri, Johanna Snäll, Petter Brodin, Gunther Bernhardt, Petter Höglund, Klas Kärre* and Benedict J. Chambers*
Expression of DNAM-1 (CD226) is associated with education status of NK cells
Submitted

III.   Laura E. Smith, Marcin A. Olszewski, Anna-Maria Georgoudaki, **Arnika K. Wagner**, Thomas Hägglöf, Mikael C.I. Karlsson, Margarita Dominguez-Villar, Francisco Garcia-Cozar, Steffan Mueller, Inga Ravens, Günter Bernhardt and Benedict J. Chambers
Sensitivity of dendritic cells to NK-mediated lysis depends on the inflammatory environment and is modulated by CD54/CD226 driven interactions
Journal of Leukocyte Biology, 2016, volume 100

IV.   **Arnika K. Wagner***, Ulf Gehrmann*, Stefanie Hiltbrunner, Valentina Carannante, Nadir Kadri, Hanna Brauner, Klas Kärre* and Susanne Gabrielsson*
α-Galactosyl-Ceramide and exosome-induced iNKT cell activation mediate increased missing self recognition and preferential proliferation of educated NK cells
Manuscript

*   denotes equal contribution

PUBLICATIONS NOT INCLUDED IN THIS THESIS

Nadir Kadri, **Arnika K. Wagner**, Sridharan Ganesan, Klas Kärre, Stina L. Wickström, Maria H. Johansson, Petter Höglund
Dynamic Regulation of NK Cell Responsiveness
Curr Top Microbiol Immunol. 2016;395:95-114.

Joanna S. Kritikou, Carin I.M. Dahlberg, Marisa A.P. Baptista, **Arnika K. Wagner**, Pinaki P. Banerjee, Lavesh Amar Gwalani, Cecilia Poli, Sudeepta K. Panda, Klas Kärre, Susan M. Kaech, Fredrik Wermeling, John Andersson, Jordan S. Orange, Hanna Brauner, Lisa S. Westerberg
Interleukin-2 is sufficient to restore killing capacity of Wiskott-Aldrich syndrome protein-deficient NK cells in vivo
Under Revision

Thuy Thanh Luu, Sridharan Ganesan, **Arnika Kathleen Wagner**, Dhifaf Sarhan, Stephan Meinke, Natalio Garbi, Günter Hämmerling, Evren Alici, Klas Kärre, Benedict Chambers, Petter Höglund*, Nadir Kadri*
Continuous presence of dendritic cells is required to maintain responsiveness and homeostasis of NK cells under non-inflammatory conditions
Submitted

Rossana Tallerico, Laura Conti, Stefania Lanzardo, Rosa Sottile, Cinzia Garofalo, **Arnika K. Wagner**, Maria H. Johansson, Costanza Cristiani, Klas Karre, Ennio Carbone, and Federica Cavallo
NK cells control the breast cancer and related cancer stem cells hematological spreading
Submitted

Stefanie Hiltbrunner*, Pia Larssen*, Maria Eldh, Maria Jose Martinez Bravo, **Arnika K. Wagner**, Mikael Karlsson, and Susanne Gabrielsson
Exosomal cancer immunotherapy is independent of MHC molecules on exosomes
Oncotarget, in press

Hanna Brauner, Stina L. Wickström, **Arnika K. Wagner**, Marjet Elemans, Ramit Mehr, Maria H Johansson* and Klas Kärre*
MHC class I dependent shaping of the NK cell repertoire takes place already early during maturation in the bone marrow
Manuscript

*   denotes equal contribution

CONTENTS

LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen presenting cell
alphaGalCer	alpha-galactosylceramide
B6	C57BL/6 mouse strain
BM	Bone marrow
BMDC	Bone marrow-derived dendritic cell
BiKe	Bispecific killer cell engager
CAR	Chimeric antigen receptor
cDC	Conventional Dendritic cell
CD	Cluster of differentiation
CMP	Common myeloid progenitor
CMV	Cytomegalovirus
CLP	Common lymphoid progenitor
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cyotoxic T Lymphocyte-associated protein-4
DAP12	DNAX activating protein of 12 kDa
DC	Dendritic cell
DNAM-1	DNAX accessory molecule-1
FcR	Fc receptor
FasL	FAS ligand
Flt3L	Fms-like tyrosine kinase 3 ligand
GvL	Graft versus Leukemia effect
GvHD	Graft versus Host Disease
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage-colony-stimulating factor
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HCMV	Human Cytomegalovirus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif

ITSM	Immunoreceptor tyrosine-based switch motif
KIR	Killer cell immunoglobulin-like receptor
KLRG1	Killer cell lectin like receptor G1
LAK	Lymphokine activated killer
LN	Lymph node
LTi	Lymphoid tissue inducer
mAb	Monoclonal antibody
MCMV	Murine Cytomegalovirus
MDSC	Myeloid derived suppressor cell
MFI	Mean fluorescence intensity
MHC-I	Major histocompatibility complex class I
NCR	Natural cytotoxicity receptor
NK	Natural killer
NKC	NK gene complex
NKP	NK cell precursor
PAMP	Pathogen-associated molecular pattern
PD-1	Programmed cell death protein-1
pDC	Plasmacytoid dendritic cell
PRR	Pattern recognition receptor
R	Receptor
SH2	Src homology region 2
SHIP-1	SH2 domain-containing inositol-5 phosphatase 1
SHP-1/2	SH2 domain-containing phosphatase 1/2
TCR	T cell receptor
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIL	Tumor infiltrating lymphocyte
Tim-3	T cell immunoglobulin mucin-3
TLR	Toll like receptor
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
wt	wildtype
XLP	X-linked lymphoproliferative disease

1 INTRODUCTION

1.1 CANCER IMMUNOTHERAPY

Cancer immunotherapy strategies use the inherent ability of the immune system to recognize and kill tumor cells. This effect is mediated primarily by T cells, NK cells, NKT cells, B cells, dendritic cells (DC) and macrophages [1]. Therapy strategies that are based on the immune system include hematopoietic stem cell transplantation (HSCT) and adoptive transfer of specific immune cells, treatment with immune activating cytokines, vaccination, monoclonal antibodies (mAb) specific for antigens primarily expressed by tumor cells, blockade of natural inhibitory receptors, so called checkpoint blockade, and more recently, the use of engineered substances to specifically target cancer cells.

One of the first immune-based cellular therapies was the use of bone marrow (BM) stem cells in HSCT, which has provided long-term disease-free survival to many patients with hematologic cancers [2]. In HSCT, the patient's BM cells are replaced by donor hematopoietic stem cells (HSC) which give rise to a new immune system. Furthermore, cytotoxic lymphocytes within the graft can attack and eliminate cancer cells by a mechanism referred to as the graft-versus-leukemia (GvL) effect. This treatment however, can also cause attack of the patient's normal cells, a major drawback known as graft-versus-host disease (GvHD).

To refine cell-based procedures and reduce GvHD, one approach was to isolate blood lymphocytes, activate them *in vitro* with interleukin (IL)-2 to generate "lymphokine activated killer (LAK) cells". When transferred back to the patient in conjunction with IL-2 treatment, these LAK cells were capable of killing autologous tumor but not normal cells, which lead to complete tumor regression in some of the patients with metastatic cancers [3]. Much later it was appreciated that the majority of LAK cells were NK cells [4]. After these initial achievements with LAK cells, tumor-infiltrating lymphocytes (TILs), which are mainly T cells, were used with some promising results in the treatment of solid tumors [5] and likewise, syngeneic and allogeneic NK cells have been used for adoptive therapy of blood-borne cancers [6]. Isolation of certain subsets of immune cells has been further refined, and many different approaches have been or are tested in clinical trials.

Another approach to harness the immune system's anti-tumor effect was the administration IL-2, an immune-stimulatory cytokine, which activates NK cells and T cells and lead to regression of some solid tumors [7]. IL-2 also induces the generation of suppressive mechanisms, primarily by stimulating regulatory T cells (Tregs) which can counteract the beneficial effects. Recently, the use of IL-15 in the clinic has been approved [8], to circumvent the suppressive and other side effects of IL-2. Furthermore, engineered variants that combine IL-15 with its high-affinity receptor have been shown to activate specifically NK cells and cytotoxic T cells [9].

Antibodies that target molecules on cancer cells have been used for some years now. They work via several mechanisms, e.g. by blocking the function of the targeted molecule on the surface of the tumor cell, induction of apoptosis, or by labeling the targeted cell for attack by immune cells. The efficacy of many monoclonal abs used in therapy (anti-GD2 for neuroblastoma, cetuximab for metastatic colorectal cancer, trastuzumab for breast carcinoma, rituximab for several leukemias) have been shown to be at least partly dependent on NK cells [10, 11]. The Fc portion of many mAbs can be recognized and bound by Fc receptors (CD16) expressed on NK cells [12, 13]. Upon binding of the mAb, the Fc receptors transmit an activating signal that ultimately leads to antibody-dependent cell-mediated cytotoxity (ADCC) [11, 14]. Ideal targets for mAb therapy are neo-antigens, molecules that are expressed on tumor tissue (e.g. GD2 on neuroblastoma cells, HER2 on breast carcinoma and other types of cancer) with restricted expression on normal tissues. To include the benefit of T cells, and to increase specificity of antibody-therapy, bi- and tri-specific antibodies, that target molecules on cancer cells and on immune cells at the same time, have been engineered and successfully used in pre-clinical studies [15]. Furthermore, engineered chimeric antigen receptors (CARs) for NK or T cells or fusion proteins of pro-inflammatory cytokines with tumor-specific antigens are being introduced into clinical therapy [16].

A crucial part of the immune system is to distinguish between self and non-self, and to restrict activation of immune cells against healthy self cells. Inhibitory immune checkpoints are negative regulators expressed by antigen-presenting cells (APCs) that normally deliver inhibitory signals to limit effector T cell and NK cell responses and maintain self-tolerance during immune responses. In the tumor microenvironment, these inhibitory checkpoint ligands can suppress responses of cytotoxic lymphocytes. Blockade of these natural immune breaks with antibodies directed against cytotoxic T lymphocyte antigen-4 (CTLA-4), programed cell death-1 (PD-1) and its ligands, or inhibitory NK cell receptors, have been developed and approved for use in patients [17-19]. The emerging role of host immunity and the tumor-immunogenicity in the efficacy of cancer treatments has just started to become appreciated.

1.2 IMMUNE CELLS RELEVANT FOR IMMUNOTHERAPY

1.2.1 T cells

T cells play a central role in cellular immunity, and as such, in recognition of tumor cells. T cells have T cell receptors (TCRs) consisting of alpha and beta chains (αβ T cells), that are produced from re-arranged genes which creates a diverse T cell repertoire. A fraction of T cells have invariant gamma delta TCRs (γδ T cells) with limited diversity. αβ T cells recognize antigenic peptides in complex with major histocompatibility complex (MHC). CD4^{+} T helper cells bind to MHC-II on the surface of APCs, while CD8^{+} cytotoxic T lymphocytes recognize peptide presented by MHC-I on the surface of most cells of the body. The different subsets of T cells each have distinct functions; CD8^{+} cytotoxic T cells (CTLs) kill target cells, CD4^{+} helper T cells produce cytokines and provide stimulation to other lymphocytes and antigen-presenting cells (APCs), regulatory T cells (Tregs) can

suppress effector responses and mediate tolerance and γδ T cells are considered to be part of innate immunity with incompletely understood effects on healthy and diseased tissues. During an immune response, CD4$^+$ T helper cells can differentiate into several different functional classes. The most studied are Th1 cells, which secrete pro-inflammatory cytokines such as IFNγ, and orchestrate cell-mediated immunity, and Th2 cells, which secrete primarily IL-4, IL-5 and IL-13 and provide protection against many extra-cellular infections. Depending on the type of inflammation, Th1 or Th2 responses may be required for successful immune reactions [20]. In the context of a tumor, Th1 responses appear to be beneficial. Most immune-therapeutical approaches using T cells involve activation, modification or transfer of CD8$^+$ CTLs, but the effect of the therapy, including the pre-conditioning of the patients, may also affect CD4$^+$ cells and the balance of Th1 vs Th2 responses.

One of the first immunotherapies was infusion of autologous T cells with anti-tumor properties, which are isolated from resected tumor tissue and expanded *in vitro*. These tumor infiltrating T lymphocytes (TILs) are a heterogeneous population and only a fraction express a TCR specific for unique tumor-associated antigens. After infusion, TILs exert anti-tumor functions for a limited time, as they are unable to persist *in vivo*. Despite this apparent obstacle the response appears to be durable, and overall response rate for treatment of metastatic melanoma are 20-50% [5]. However, the efficacy of TILs seems to be restricted to melanoma, probably due to the high immunogenicity of this type of cancer. Furthermore, to obtain TILs, the tumor has to be surgically removed which limits the applicability of TILs to solid resectable cancers. Recently, infusion of autologous or heterologous unmodified selected T cells has been used for treatment of virally associated cancers and disorders [21].

Of late, new approaches to genetically modify T cells are being developed to redirect T cell specificity to tumor antigens. Two approaches have shown promising results in clinical studies and mouse models: (i) the use of bi-specific or tri-specific mAbs (BiKEs or TriKEs), and (ii) the introduction of a transgenic TCR or chimeric antigen receptor (CAR) [15, 16]. Since T cells do not express Fc receptors [15], they are unable to detect tumor cells that are bound by mAbs and do not perform ADCC as NK cells. BiKEs and TriKEs are fusion proteins composed of two or three different mAbs and are engineered to simultaneously bind to tumor cells and cytotoxic lymphocytes. By combining mAbs directed against tumor antigens with mAbs specific for CD3, T cells can be directed to kill tumor cells [15].

T cells can be transfected with a genetically modified TCR or a CAR with specificity against a tumor antigen [16]. These T cells can be isolated from the peripheral blood, transfected with a TCR specific for the desired antigen, expanded *in vitro* and then infused into the patient. Antigens that can be used for this type of therapy are molecules primarily expressed by tumor cells, so-called neo-antigens, viral antigens, or tissue-specific differentiation antigens, which are detected in many tumors but not in healthy tissues. An obstacle with using tumor-antigen-specific T cells is the appearance of MHC-I loss variants. To overcome this limitation, CAR T cells have been developed and are now being tested in clinical trials [16]. CARs are fusion proteins of the variable fragment of an antibody fused to intracellular signaling domains of

TCRs. The resulting modified T cells do not depend on proper antigen processing and presentation, and recognize the antigen in an MHC-I-unrestricted fashion. T cells with modified TCRs and CAR technology offers many advantages, but toxicity and the appearance of cytokine release syndrome warrant further studies. Malignancies of the B cell lineage, which displays restricted expression of CD19, have been successfully treated with CD19 CAR T cells [16].

Vaccination with antigenic peptides to treat cancers have been tested with limited success. Tumor-peptide, injected together with adjuvants or activated APCs, were thought to generate anti-tumor-specific immune responses. One such vaccination strategy lead to the approval of peptide-pulsed blood-derived monocytes for the treatment against advanced prostate cancer, however, treatment increased survival time of patients by only 4 months [22, 23]. Perhaps in the future, it will be possible to combine this approach with checkpoint blockade, thereby increasing the activation of effector cells.

T cells are activated via their TCR and co-stimulatory molecules. To control T cell responses and to prevent activation of potentially autoreactive T cells, APCs express molecules that inhibit T cell activation, so-called inhibitory immune checkpoint molecules. Among the most known are CTLA-4 and PD-1. CTLA-4 is the inhibitory counter-receptor to the co-activating receptor CD28. Both are expressed on T cells and recognize CD80 and CD86 on APCs. CD28 delivers a co-activation signal to T cells, while CTLA-4, which is upregulated on activated T cells, transmits an inhibitory signal to the T cell, thereby providing a mechanism to terminate the response. PD-1 is expressed by activated T cells, B cell precursors and has been found on NK cells. It binds to PD-L1 and PD-L2 which are expressed on many cell types, and prevents activation of the lymphocyte. This discovery has led to the development of checkpoint blockade antibodies, such as anti-CTLA-4, anti-PD-1 or anti-PD-L1 to restore T cell anti-tumor immunity [17, 18].

1.2.2 NKT cells

NKT cells are a distinct population of T cells with features and receptors of both the T cell and the NK cell lineage. They express an invariant TCR encoded by Vα14Jα18 in mice and Vα24Jα18 in humans, and several NK cell receptors, both activating and inhibitory. NKT cells recognize lipid antigens presented by the non-classical MHC class Ib molecule CD1d [24]. Upon recognition, they are rapidly stimulated and can in turn activate both innate and adaptive immune responses. NKT cells can be activated directly by tumor cells presenting lipid antigens, or indirectly by cross-presentation of antigens by DCs. NKT cells can kill tumor cells and rapidly produce high levels of cytokines, such as IL-4 and IFNγ, which influence the microenvironment and regulate responses of other immune cells [25]. With the discovery of α-Galactosyl-Ceramide (αGalCer) as an antigen that induces strong NKT cell activation, the critical role of NKT cells in activity against cancer cells could be demonstrated. Pre-clinical and clinical studies have demonstrated the potential for therapeutic use of NKT cell agonists, such as αGalCer [26]. In mouse models of melanoma, activation of

NKT cells and subsequent activation of NK cells lead to tumor protection. Production of IFNγ by both NKT and NK cells was required for the protective effect [26].

1.2.3 Dendritic Cells

Dendritic cells (DC) are antigen-presenting cells (APC) that belong to the myeloid lineage and originate from the BM. They populate the periphery as immature DCs where they take up substrates from the environment, e.g. dying or infected cells or proteins and scan the periphery for potential dangers via pathogen recognition receptors (PRRs) such as the Toll like receptors (TLRs) [20]. Cytokines important for DC differentiation and maturation are fms-like tyrosine kinase three ligand (Flt3L) and granulocyte-monocyte colony stimulating factor (GM-CSF). In the mouse, there are two major subsets, conventional DCs (cDC) and plasmacytoid DCs (pDC). cDCs (also known as myeloid DCs) are further subdivided into $CD8\alpha^+$ or $CD8\alpha^-$ populations, the former is involved in cross-priming CTLs via MHC-I, while the latter drive adaptive immune responses via MHC-II [27]. Immature DCs are particularly proficient in capturing and processing of antigen. Furthermore, immature DCs can induce tolerance through induction of regulatory T cells (Tregs) or deletion of potentially autoreactive T cells. DCs mature when they encounter microbial peptide antigens or sense conserved pathogen-associated molecular patterns (PAMPs). Upon maturation, DC up-regulate co-stimulatory molecules such as CD40, CD80, CD83 and CD86, and express high levels of MHC-I and MHC-II and express CCR7 which induces migration to the lymph node (LN) where antigen presentation to T cells occurs [28, 29]. Mature DCs become highly efficient in antigen presentation and secrete cytokines, such as IL-12, which drives Th1-type immunity and enhance NK cell and CTL responses [30-32]. Due to their role in both innate and antigen-specific immunity, and their potential to control both tolerance and immune responses, DCs are sentinels of the immune system [29]. With the capacity to present antigen and provide co-stimulation, DCs appear to be an optimal vaccine against cancer [33]. DCs can be generated *in vitro* from BM or circulating precursor cells and activated and matured with tumor-specific antigens. The antigens are obtained from the patient, or from other sources such as cancer cell lines. With the molecular identification of many tumor-antigens, these molecules can be generated and used to stimulate DC. When transferred to the patient, DCs may home to the LNs, recruit T cells and NK cells and activate tumor-specific immune responses [22, 23, 33-37]. Vaccine strategies using antigenic peptide alone work in a similar fashion; the tumor-antigen is combined with an adjuvant to activate autologous DCs, which in turn can process and present the peptide and thereby inducing anti-tumor responses [22, 23, 38].

1.2.4 Macrophages and other Myeloid Cells

Macrophages are leukocytes of the myeloid lineage that recognize diseased cells and pathogens by PRRs such as TLRs. Upon recognition, the target cell is eliminated via phagocytosis or by direct killing via the production of nitric oxide (NO) [20]. Furthermore, macrophages can act as APCs and thereby initiate and regulate adaptive immune responses. Macrophages also play a role in anti-inflammatory responses as in wound healing [20]. Based on this functional diversity, macrophages are classified as M1/kill-type which directly kill

target cells and stimulate Th1 responses, and M2/repair-type, which stimulate Th2 responses [39]. Macrophages that are found in high numbers in tumors usually belong to the M2-type where they participate in the generation of the immune suppressive tumor microenvironment, which is in part, responsible for the failure of cytotoxic lymphocytes to kill tumor cells [40]. On the other hand, M1 macrophages may directly recognize tumor antigens and facilitate tumor clearance [41]. Macrophages exhibit great plasticity and conversion from the immunosuppressive M2 to the M1 may prove beneficial for cancer therapy [42, 43]. Notably, while M2 macrophages can suppress anti-tumor responses by T cells and NK cells, they can be killed by NK cells via DNAM-1 and 2B4 [41]. Furthermore, IFNγ released by NK cells can induce a shift from M2 to M1 macrophages [41], thereby shaping the tumor microenvironment. Some macrophage developmental intermediates may be part of a heterogeneous group of cells sometimes referred to as myeloid-derived suppressor cells (MDSCs), to which also some developmental and functional subsets of granulocytes can contribute. MDSCs expand in pathological situations and possess strong immunosuppressive properties. They can interact with other cell types, thereby influencing immune-therapeutical approaches [44]. A high infiltration of many tumors has been associated with a poor prognosis. Further studies on MDSCs and their impact on tumor- and immune cells are needed.

1.3   NK CELLS AND OTHER INNATE LYMPHOID CELLS

1.3.1   Innate lymphoid cells

Until recently NK cells were thought to be the only innate lymphocytes, but in recent years it has become apparent that additional innate lymphoid cells (ILC) exist. ILCs differ from T and B cells in that they do not have receptor gene rearrangement and lack antigen-specific receptors. ILCs are found in tissues that have close contact to commensal microorganisms, such as the skin, the gut, the blood, mucosal sites of lung and urogenital tract. They rapidly produce effector cytokines upon stimulation and are thought to help fight infections at these barrier sites, but also to help the microbiota to return back to normal conditions after an inflammation [45, 46]. Furthermore, ILCs have a role in lymphoid tissue development, tissue repair and homeostasis and in regulating adaptive immune responses [47] ILCs include cytotoxic or killer ILC (conventional NK cells) and helper ILCs (ILC1, ILC2, ILC3) [48]. ILCs are a heterogenous group of cells, but based on dependency on transcription factors and similarities in effector cytokine secretion, they can be divided into 3 main groups, which resemble T helper subsets in terms of differentiation and key cytokine secretion [45, 46, 48-50]. Functional plasticity of and between the different ILCs are starting to emerge [45, 51].

Group 1 ILCs are characterized by the dependency on the transcription factor T-bet for lineage specification and the ability to produce IFNγ upon stimulation. They can be further subdivided into Eomes⁻ ILC1 and Eomes⁺ ILC1, the latter are known as conventional NK cells and will be called NK cells throughout this thesis. Eomes⁻ ILC1 have phenotypic resemblance to NK cells, but differ in expression of certain markers that can be used to distinguish the two. ILC1 express CD127 (IL-7Rα), NCRs (NKp46 in the mouse, NKp44 and

NKp30 in human), lack expression of MHC-I-specific inhibitory receptors and CD49b (DX5), express CXCR3 and CXCR6, and are positive for α1β1 integrin (CD49a) and TNF-related apoptosis-inducing ligand (TRAIL), which allows ILC1 to eliminate target cells without the use of granzymes and perforin and they secrete high levels of IFNγ and TNFα [45-47, 52]. ILC1 have been identified in liver, intestine, tonsils, and the peritoneal cavity and in limited numbers in the spleen [45, 47, 50].

The key signature transcription factors for ILC2 are GATA3 and RORα [45, 50]. ILC2s produce large amounts of Th2-type cytokines such as IL-5 and IL-13, but can also express amphiregulin, IL-4, IL-9 and IL-6 in response to IL-25, IL-33 or thymic stromal lymphopoietin (TSLP). ILC2 have been found in mesenteric fat-associated lymphoid clusters, mesenteric LN, lung, intestinal lamina propria, BM, liver and skin. ILC2 are critical for innate protection against helminths and nematode infections, by supporting epithelial barrier integrity and recruitment of eosinophils through secretion of IL-5 and IL-13. They can activate Th2 cell directly through MHC-II or indirectly through early type 2 cytokine secretion and recruitment of DCs, thereby initiating type 2 adaptive immune responses [45, 50].

Key transcription factors of all group 3 ILCs are TCF1 [53] and RORγt, which is expressed already by precursors committed to the ILC3 lineage both in human and in mouse [54-56]. These ILC3 committed precursors seem to leave the BM and develop into the different ILC3 subsets locally in other tissues [45]. Other transcription factors, e.g. T-bet, TOX or AHR and Notch signaling are required for the development of some ILC3 subsets [45, 50]. Some ILC3 subsets secrete type 17 cytokines, such as IL-17A or IL-22, and GM-CSF. IL-22 is required in certain intestinal infections in mice, extracellular bacteria and fungal infections at mucosal barriers. Lymphoid tissue inducer (LTi) cells constitute a subpopulation of ILC3 and have been identified in human and mouse. LTi cells are important for lymphoid organogenesis, as in absence of LTi cells (as in RORγt-deficiency) there is no development of peripheral LN and Peyer's patches. Furthermore, in adult mice, LTi cells produce IL-17A and IL-22 in the lamina propria of the intestine. Other ILC3 subsets can express classical NK cell markers such as NKG2D or NCRs (and CD56 in human), they secrete either IL-17A or IL-22 or both. Some NCR[+]ILC3 subsets produce IFNγ. ILC3s are found in tonsils, intestinal lamina propria, while LTi cells are mainly found in fetal liver and lymphoid organs [45, 46, 50].

1.3.2 NK cell biology

NK cells are innate lymphocytes that can kill virally infected or transformed cells by natural killing, i.e. without prior sensitization [57-60]. NK cells can kill tumor cells and they are important for the control of many virus and intracellular infections and thus have a critical role in immune surveillance [57-62]. They are rapidly recruited to sites of inflammation, where they participate in early defenses. While NK cells can kill target cells without prior sensitization, the do need priming by DCs via type I IFNs and IL-15 [63]. Upon activation, NK cells can kill target cells by releasing preformed granules that contain cytotoxic proteins such as perforin and granzyme or by transmitting death signals via Fas ligand (FasL) and

TRAIL to the target cell [64-68]. Furthermore, NK cell may recognize and kill antibody-covered cells with their Fc-receptor CD16, a process usually referred to as antibody-dependent cell-mediated cytotoxicity (ADCC) [12, 69]. Additionally, activated NK cells can rapidly secrete cytokines, such as IFNγ or TNFα, chemokines such as MIP-1α/β and growth factors such as GM-CSF and G-CSF [70, 71].

NK cells functions are controlled by integration of a multitude of signals from germline-encoded activating and inhibitory receptors, co-activation and adhesion molecules [72]. Some of the ligands for NK cell activating receptors are expressed at low levels on normal cells, and upregulated on infected or transformed cells. Most inhibitory receptors on NK cells bind to MHC-I. Normal cells are spared from NK cell attack when signals from activating receptors are balanced by inhibitory signals emanating from inhibitory receptors recognizing their cognate MHC-I ligand. Target cells that lack expression of MHC-I, are killed due to lack of inhibition, a concept termed recognition of "missing self" [73-75]. NK cells can also kill cells which express MHC-I if these cells express high levels of activating receptor ligands, a mechanism termed "induced self recognition" [76, 77] .

NK cells are found in many tissues, such as liver, peritoneal cavity, placenta, uterine mucosa, lungs, lymph nodes (LN), tonsils, spleen and blood [78]. They comprise 5-15% of lymphocytes in human peripheral blood and 3-5% of mouse spleen cells. NK cells are characterized as $CD3^-CD56^+NKp46^+$ cells in human and $CD3^-NK1.1^+$ or $CD3^-NKp46^+$ in mice, however, also other ILCs can express NKp46. About 90% of human NK cells in blood or spleen are $CD56^{dim}CD16^+$, which are potent killer cells. NK cells in LNs and tonsils on the other hand are $CD56^{bright}CD16^{dim/-}$ and are characterized by production of cytokines such as IFNγ, in response to type I IFNs, IL-12, IL-15 or IL-18 [78-80].

In addition to their function in control of tumors and protection against infections, NK cells have a role in maintenance of homeostasis in the lymphoid system and in reproduction [81, 82].

1.3.3 Missing self recognition

Soon after the initial description of NK cells, the relationship between NK cells and a phenomenon called "hybrid resistance" became acknowledged [83-85]. "Hybrid resistance" refers to a situation where BM grafts from either inbred parent are rejected in F1-generation offspring, while solid organ transplants are not [83]. This and other observations led to the hypothesis that NK cells search for self MHC-I on target cells and that expression of sufficient levels of self MHC-I would inhibit NK cell-mediated attack [73-75]. Further indications for such a recognition strategy were provided by studies showing that wildtype (wt) mouse NK cells attack cells from mice with mutations that result in low/absent MHC-I surface expression [86-89]. Similar results came from studies with human NK cells [90]. Furthermore, introduction of a transgene H2-D^d, enables NK cells to reject cells lacking this MHC-I, demonstrating that MHC-I genes themselves control how NK cells see missing self [91, 92]. The identification of inhibitory receptors specific for MHC-I in mice and humans has lent support to the missing self hypothesis [93-96].

1.3.4 NK cell development

NK cells arise from hematopoietic stem cells (HSC) that reside in the BM, where hematopoiesis, the generation of hematopoietic cells, takes place. Development of the different lineages occurs through several differentiation steps, guided by cytokines, transcription factors and interactions with BM stromal cells [20]. One of the first critical steps in hematopoiesis is fate decision between the myeloid and the lymphoid lineage to either common myeloid progenitor (CMP) or common lymphoid progenitor (CLP), which give rise to the myeloid lineages and lymphoid lineages respectively. T cells, B cells, NK cells and ILCs develop from CLPs, as well as some DC subsets [97, 98]. Early stages of NK development occur in the BM throughout life, and the presence of proper BM microenvironment is necessary for NK cell function, as agents that affect BM destroy support of NK maturation, e. g. [89]Sr [99] or estradiol [100, 101]. Some of the early papers describing NK cells show that BM is an important source of NK cells [84, 102] and NK cell progenitors could be transferred by BM graft [84, 103]. The CLP can give rise to progenitor cells that have been termed pre-pro NK cells by one group and pre-NK precursor (pre-NKP) by another and have lost the capacity to give rise to T, B, DCs [104, 105]. This progenitor cell lacks expression of most canonical NK cell markers, is characterized by a high expression of IL-7R, NKG2D and 2B4 and the transcription factor Id2, and lack of CD135 (Flt3) and CD122 (IL-2Rβ; component of the IL-15R), suggesting that commitment to the NK cell lineage does not require IL-15 [104, 105]. In the next step, the pre-pro NK/pre-NKP differentiates in NK cell precursors (NKP)s which start to express the IL-15R complex (IL-15Rα, IL-2Rβ, IL-2cγ chain) [106], by upregulating CD122, enabling this progenitor to respond to IL-15 [107]. Furthermore, NKPs express the transcription factors Ets-1, Id2 and GATA-3 [107]. NKPs progress into immature NK cells which express NK1.1 and NKG2D, but are not functionally mature [108, 109]. As they start to acquire expression of NK cell lineage markers such as DX5 and Ly49 receptors (Ly49r), they become immature NK cells, which are functional and egress from the BM into the periphery [108-110]. In the periphery, e.g. the spleen, immature NK cells develop via several intermediate stages, defined by expression of cell surface markers. NK1.1$^+$DX5$^+$ NK cells can be further subdivided using expression of CD27 and CD11b as markers, where immature NK cells (double negative DN) start to express CD27, then acquire CD11b (double positive , DP), and then down-regulate CD27 again [111, 112]. During the CD27^{high}CD11b^{low} stage, immature NK cells experience a proliferative burst and Ly49r expression commences [111]. The CD27^{high}CD11b^{high} subset is the most potent when it comes to effector functions such as cytokine production and cytotoxicity *in vitro* [111]. After down-regulation of CD27, expression of killer cell lectin-like receptor-1 (KLRG1) commences, resulting in CD11b^{high}KLRG1$^+$ terminally mature NK cells, with low proliferation and poor cytokine production capacity [113]. However, these terminally mature CD27^{low}KLRG1$^+$ NK cells have been shown to be the predominant effector cells that migrate to and perform cytotoxic functions against colon carcinoma cells and are necessary for the clearance of pulmonary metastases in mice [114, 115]

Different cytokines and contact with stromal BM cells appear to be necessary at different stages of NK cell development and maintenance [116-118]. Mice deficient for any component of the IL-15 signaling (IL-15 or IL-15R complex) are unable to generate NK cells, but the finding that NKPs lack CD122 suggest that IL-15 may be more important for proliferation and survival of NK cells than commitment to the NK cell lineage [105, 116]. It was recently demonstrated by fate mapping that all NK cells progress through an IL-7Rα$^+$ intermediate stage [119]. Signaling through the common IL-2 and IL-15 receptor pathway is pivotal for the maturation and the acquisition of effector functions of NK cells [120]

Similarly, different transcription factors regulate the progression through the distinct developmental stages. Functional development of NK cells is reliant of key transcription factors such as Ikaros (commitment to the lymphoid lineage) [121], PU.1 [122] and Id2 [123, 124], as well as the T-box transcription factor T-bet [125, 126] and eomesodermin (Eomes) [127]. GATA-3 is required for NK cell maturation, Ly49r expression and functional development [128]. T-bet is necessary for the differentiation into terminally mature CD27^{low}KLRG1$^+$ NK cells [111, 112, 125, 127, 129, 130].

1.3.5 Activating receptors

Each NK cell expresses several different activating receptors that can recognize ligands on target cells. Some of these ligands are stress-induced molecules that are up-regulated on the cell surface during transformation or infection, while others are constitutively expressed by healthy self cells. Some activating receptors specifically recognize pathogen-encoded ligands. Induction of NK cell cytotoxicity by resting NK cells requires engagement of several activating receptors, while NK cells activated in vitro with IL-2 do not need synergy of multiple receptors [131]. Activating receptors contain short cytoplasmic domains and require association with adaptor molecules to transduce a signal. Signaling results in cytoskeletal reorganization, secretion of cytokines and chemokines and release of preformed cytotoxic granules [72].

Signaling via Immunoreceptor tyrosine-based activation motifs

Most activating receptors either contain immunoreceptor tyrosine-based activation motifs (ITAM) within their cytoplasmic domain, or associate with adaptor molecules that harbor an ITAM. Upon recognition of its ligand, the ITAM becomes phosphorylated by Src family kinases [132]. In NK cells, three ITAM-containing adaptor molecules are expressed: FcRγ, CD3ζ and DAP12. Phosphorylated ITAMs serve as docking stations for the kinases Syk and Zap70, both of which are expressed in NK cells [133]. Syk and ZAP70 transmit downstream signaling involving activation of LAT, PI3K, PLCγ, PKC and VAV. These signaling molecules in turn trigger down-stream signaling events. [72, 132].

CD16

CD16 is a low-affinity receptor on NK cells which binds the Fc part of antibodies (IgG1 and IgG3 in human, IgG1 and IgG2 in mouse) [69] and induces ADCC [134]. Human CD16 can signal via FcRγ or CD3ζ, while mouse CD16 can only bind FcRγ [72]. CD16 is the only

activating receptor on resting NK cells that mediates killing of targets without engagement of another activating or co-activating receptor [131].

NK1.1

NK1.1 (NKR-P1C, KLR-B1C) is an activating receptor expressed on all NK cells in B6 mice, and is commonly used to identify NK cells in this strain [135-137]. It belongs to a family of C-type lectins that encompasses both activating and inhibitory members, and is encoded within the NK gene complex (NKC) [137-139]. NK1.1 associates with FcRγ for signal transduction [140]. To date, no ligand for the activating member NK1.1 has been identified, while the inhibitory members bind to Ocil/Clrb, another C-type lectin encoded in close proximity to the NKR-P1 genes [138, 141].

NKp46 and NCRs

The activating receptor NKp46 belongs to the natural cytotoxicity receptors (NCRs), a group of receptors primarily studied in human NK cell biology [142-144]. NKp46 is the only member of the NCRs that is also found in mouse, where it is expressed on almost all NK cells and commonly used for NK cell detection [145]. The NCRs NKp46, NKp44 and NKp30 trigger cytotoxicity against tumor targets [144]. The NCRs bind to intracellular proteins that may be expressed on the surface of stressed, proliferating or otherwise activated cells [144, 146-148], and recognition of viral hemagglutinins has been reported [147, 149, 150].

Activating Ly49rs, Ly49D and Ly49H

Ly49D is an activating member of the Ly49r family. It binds to H2-D^d and mediates rejection of D^d-positive BM or cell grafts [151-153]. The activation signal is transduced via DAP12 and crosslinking leads to cytotoxic granule release and cytokine production [154]. The affinity of Ly49D to D^d is lower than the affinity of the inhibitory Ly49A to the same ligand [155, 156]. NK cells expressing only Ly49D without inhibitory Ly49rs are disfavored and more prone to apoptosis in an H2-D^d mouse strain, probably to reduce the frequency of potentially autoreactive NK cells [157]. However, when NK cells are strongly stimulated by cytokines the inhibitory receptors are overridden and Ly49D$^+$ NK cells become activated even in the presence of the inhibitory receptor [158]. A physiological role for activating receptors that recognize MHC-I has been suggested in pregnancy [81, 159].

The activating Ly49H receptor, expressed in some mouse strains such as B6, directly binds m157 protein of murine cytomegalovirus (MCMV) and is responsible for the genetic resistance to the virus [160-165]. Ly49H associates with DAP12 for signaling [166, 167].

NKG2D

NKG2D is a heterodimer of the C-type lectin family. It is expressed by all NK cells and a subset of T cells. NKG2D associates and signals via DAP10 and DAP12 [168, 169]. NKG2D binds to structures that are rarely expressed by healthy cells, but induced upon cellular stress such as DNA damage, heat shock response or excessive proliferation and it thus recognizes cells that are infected or undergoing cellular transformation [170]. The ligands of NKG2D are

MICA/B and ULBP1-6 in human, and Rae1, Mult1 and H60 in the mouse, which share structural similarities with MHC-I molecules [170-173]. Most primary tumors express NKG2D ligands. Tumors can evade NK cell recognition by shedding their NKG2D ligands, a mechanism that not only reduces the level of ligands on the target cell, but also the soluble portions can desensitize NK cells and induce downregulation of the NKG2D receptor on NK cells [174-176]. However, an enhanced anti-tumor activity has been reported recently in response to shed NKG2D ligands [177, 178].

DNAM-1

DNAM-1 (DNAX accessory molecule-1; CD226) is a molecule that functions both as activating receptor and as adhesion molecule through interactions with CD155 and CD112 [179-181]. Expression of CD112 and CD155 is regulated by cellular stress[182, 183]. The DNAM-1 signal can be transduced via intracellular recruitment of the serine-threonine kinase PKC and the tyrosine kinase Fyn [184-187]. More recently, DNAM-1 mediated cytotoxicity was also found to be involved directly via Grb2 [188]. Both in mouse and man, the ligands for DNAM-1 are CD112/Nectin-2 and CD155/PVR which are members of the Nectin/Nectin-like family of adhesion molecules [180, 189, 190]. Nectins and nectin-like proteins mediate cell-cell adhesion, migration, cell polarization and formation of adherence junctions by binding to each other in *cis* or *trans* via homotypic and heterotypic interactions [191]. NK cells have three receptors that can recognize CD155, the co-activating receptors DNAM-1, the inhibitory receptor T cell immunoreceptor with Ig and ITIM domains (TIGIT) and CD96, for which both activating and inhibitory functions were reported [179]. DNAM-1/CD155 recognition is critical for NK-mediated tumor cell killing of several human cancers and DNAM-1-deficient mice have increased susceptibility for tumor cells or chemically induced tumor development [192-196].

1.3.6 Inhibitory receptors

Most NK cell inhibitory receptors recognize MHC-I molecules. Three families of MHC-I-specific inhibitory NK cell receptors exist, Ly49 receptors (Ly49r) in mouse, Killer-cell immunoglobulin-like receptors (KIR) in human and NKG2A in both species. The Ly49rs and KIRs are structurally different and belong to different receptor families, but they are expressed in a similar manner. Both gene families are polygenic and polymorphic, i.e. there are many genes with many allelic variants for the Ly49rs in mouse and the KIRs in humans [197-199]. Other species use either Ly49rs or KIRs, although independent evolution of KIR genes in different species has been suggested [198]. This is an example of convergent evolution, where different species have developed distinct recognition systems for MHC-I, thereby enabling NK cell recognition and inhibitory function [199, 200]. The diversity of genes and alleles has most likely arisen through gene duplication and gene conversion[198].

Signaling

Signaling by inhibitory receptors is mediated by an imunoreceptor tyrosine-based inhibitory motif (ITIM), a conserved sequence in the cytoplasmic domain of the receptors. Each Ly49r heterodimer contains two ITIMs (one in each molecule), which are phosphorylated at the

tyrosine upon receptor engagement. The phosphorylated ITIMs of Ly49rs recruit phosphatases, preferentially src homology region 2 (SH2) domain-containing phosphatase 1 (SHP-1) or SHP-2, but also SH2 domain-containing inositol-5 phosphatase 1 (SHIP-1) can bind via the SH2 domain [201]. SHP-1/2 function by dephosphorylating key signaling molecules needed for optimal transduction of activating signals, such as Vav-1, ZAP70, Syk, PLCγ1 and PLCγ2, LAT, SLP76 and the JAK kinases necessary for type I IFNs and cytokine-mediated activation [72]. SHIP-1 dephosphorylates signaling molecules necessary for sustained Ca^{2+} signaling [201]. Furthermore, engagement of inhibitory Ly49rs and NKG2A induces phosphorylation of Crk, a small adaptor protein, which leads to its dissociation from a multiprotein complex that connects Crk to the components of the cytoskeleton and prevents remodeling of the F-actin network [202, 203]. Remodeling of the cytoskeleton is important in granule polarization, NK cell-target cell conjugate formation and cytotoxicity [204].

NKG2A

The heterodimer CD94/NKG2A, which is encoded in the NKC, is expressed by both human and mouse NK cells and interacts with non-classical class Ib molecule Qa-1[b] in mouse or HLA-E in human. Both are dependent on β2m and present conserved leader peptides from the cleaved signal sequences of certain classical class Ia molecules [205-209]. This allows NK cells to probe the entire pathway involved in MHC-I synthesis, since either absence of the heavy chain, defects in the transport into the endoplasmatic reticulum and peptide loading, defects in transport to the cell surface or β2m light chain availability and expression impact on the expression of QDM-loaded Qa-1[b] [205, 208, 210]. NKG2A is expressed on some T cell subsets and about 50% of NK cells in a mono-allelic fashion [211-213]. During NK cell development, NKG2A is the earliest expressed MHC-I-specific inhibitory receptor and provides developing NK cells with the means to recognize MHC-I-deficient target cells [214].

KIRs

In humans, most MHC-I-specific inhibitory receptors belong to the KIR family. KIRs consist of a family of approximately 15 closely related Ig-like receptors expressed on human NK cells containing two (KIR2D) or three (KIR3D) Ig-like domains. Most KIRs have long (L) intracellular domains that contain ITIMs and are inhibitory, while the activating KIRs have a short (S) cytoplasmic region without signaling motifs [72, 215]. The ITIM of inhibitory KIRs, once phosphorylated, enables recruitment of SHP-1 and interference with activating signaling. Inhibitory KIRs recognize the HLA-I ligand groups HLA-C1, C2 and Bw4. Similar to the Ly49rs, some inhibitory and activating KIRs share the same ligand, but the affinity of the inhibitory version is superior. Each individual has different KIR profile (genes) and within an individual, not all NK cells have the same receptor expression profile, not all NK cells express even express a KIR that is specific for self-MHC-I [216]. KIR haplotype (group of 5-10 specific alleles in the KIR locus), and KIR expression is highly diverse among the population. Furthermore, the KIR locus is among the most polymorphic regions of the human

genome [72, 199]. The haplotypes are classified into two main categories, haplotype A, which consists of mostly inhibitory KIRs and is common in the Caucasian population, and haplotype B, which consist of inhibitory and activating KIRs and is common in Africa [217]. However, some KIR genes are shared by all haplotypes [72, 217].

Ly49rs

The Ly49 C-type lectin family of receptors (now also known as killer cell lectin-like receptors subfamily A, Klra) recognize MHC-I or related molecules [153, 155, 218]. Several closely related Ly49rs have been identified, all of which are encoded in the NKC on mouse chromosome 6 [93, 219, 220]. The Ly49rs and NKG2A are expressed on individual NK cells in a partly stochastic variegated fashion, i.e individual NK cells may express a random number of inhibitory receptors, so that each individual NK cell can end up with none, one, or any combination of inhibitory receptors. In a B6 mouse that is MHC-I$^+$, NK cells can express 0-6, but most cells express one or two inhibitory receptors [109, 157, 221-226]. Most Ly49rs have overlapping specificity to different MHC-I alleles [155, 222, 227]. It is noteworthy, that the Ly49rs and their ligands, MHC-I molecules, are located on different chromosomes, and thus inherited independently. As a result, NK cells can lack expression of inhibitory receptors that are specific for self-MHC-I [224]. A similar pattern of expression is seen for KIRs on human NK cells [216, 228]. A mechanism termed NK cell education ensures that only NK cells with inhibitory receptor for self-MHC-I can react to cells lacking MHC-I expression (but otherwise normal) (section 1.3.7).

During NK cell development, the Ly49rs are expressed in an orderly fashion, although distinct expression sequences have been determined by different experimental approaches. Surface detection after transfer of immature NK cells suggests expression in the order Ly49A, -G2, -C/I [110]. A study using *in vitro* BM stromal cell culture experiments corroborated this order [229]. However, when transcription was assessed by RT-PCR in NK cells generated *in vitro* from progenitor cells, inhibitory receptors were expressed in the order NKG2A and Ly49B, then Ly49G2, -C/I, and much later Ly49A, -D,-E, -F [109]. These results are not necessarily in conflict, they could rather suggest that transcription and surface expression are not linked. It has been shown by several studies that once expression of a certain inhibitory receptor has started, it remains stable over time [110, 218, 230, 231]. Ly49r$^+$ NK cells appear gradually during ontogeny and the appearance is concurrent with NK cell activity [110].

Most inhibitory Ly49rs are usually expressed in a mono-allelic fashion and transcriptional termination of the second allele is regulated by methylation [232-235]. Cessation of transcription affects each Ly49r gene independently and appears to a certain degree to be stochastic process [234, 236, 237]. An influence of MHC-I on mono-allelic expression could be demonstrated; in the presence of a cognate MHC-I molecule, bi-allelic expression of those Ly49rs that could bind has been reduced [236]. Expression of the activating receptors Ly49D and Ly49H on the other hand is bi-allelic [238]. Furthermore, Ly49D and Ly49H often appear together and are preferentially expressed on NKG2A- NK cells [239, 240].

MHC-I-dependent skewing of the Ly49r repertoire

Even though inhibitory receptors appear to be expressed in a seemingly random fashion, there is a striking influence of MHC-I expression on the distribution of Ly49rs. In the presence of MHC-I, NK cells with one or two inhibitory receptors are over-represented (as approximated by the product rule, which predicts frequencies by multiplying the frequency of NK cells expressing each receptor), while NK cells with many self-specific inhibitory receptors are scarce. This phenomenon is referred to as "skewing of the repertoire" and creates a diverse repertoire of NK cells with distinct receptors [157, 241, 242]. Using mouse models with different MHC-I alleles it has become evident that there is a reduction in total expression of each Ly49r in the presence of the cognate MHC-I molecule. This is apparent both in terms of frequency of Ly49r$^+$ NK cells (i.e. NK cells expressing the particular Ly49r), and the average expression level per cell (usually measured as mean fluorescence intensity [MFI]) [242-245]. It has to be noted that most of the reduction in MFI results from interactions of Ly49rs with their cognate MHC-I molecules in *cis*, i.e. on the same cell, which is described further below [246]. However, the frequency of NK cells expressing only one particular Ly49r recognizing host MHC-I, and none of the other inhibitory receptors, increases [157, 243, 247]. A skewed repertoire (an NK cell pool where many cells express only few self-specific inhibitory receptors) is potentially more useful in detecting loss or down-regulation of a certain MHC-I, which is a hallmark of both infection and tumor transformation. In humans, MHC-I-dependent shaping of the inhibitory receptor repertoire has been described for peripheral blood NK cells but not for cord blood-derived NK cells, while other studies report little or no influence by the presence of cognate MHC-I [228, 248].

Specificty of Ly49rs for different MHC-I alleles

Most Ly49rs recognize several MHC-I alleles with variable affinity [92, 155, 227, 249]. Depending on the method to determine recognition (e.g. MHC-I-tetramers for staining, inhibition of cytotoxicity, degree of receptor-downregulation, NK cell education), different studies suggest different receptor-ligand interactions. In B6 mice, the Ly49C and Ly49I receptors interact strongly with K^b, while Ly49A appears to interact weakly with D^b [155, 227, 249]. In mice transgenic for D^d, it is primarily the Ly49A$^+$ NK cells that can strongly interact with its ligand [157, 218, 249].

Mode of binding

NK cells and T cell both bind to MHC-I, yet both the result of this interaction, as well as the mode of binding, differ. The T cell receptor touches both germline-encoded components of the MHC-I as well as the peptide. Ly49rs, on the other hand require the presence of a bound peptide, but they do not directly bind to the peptide [250]. Some degree of peptide selectivity has been reported for Ly49C and Ly49I [155, 227], whereas Ly49A binds to the complex with any peptide tested [251, 252]. Ly49rs bind multiple H-2D and H-2K alleles underneath the peptide-bonding platform which is formed by β_2m and mainly non-polymorphic amino-acids of the heavy chain α3 domain [253]. Furthermore, different modes of binding have been reported for the distinct Ly49rs [254]. KIRs bind MHC-I molecules by engaging mostly

invariant (but polymorphic) amino-acid residues of the α1 and α2 helices with minimal interactions with the bound peptide [250]. Peptide-selectivity for KIR2D binding to HLA-C has been reported [72, 255]. In addition to binding to MHC-I in on other cells (in *trans*), some Ly49rs can also bind MHC-I molecules on the same cell (in *cis*) [256]. *Cis* binding reduces the availability of Ly49rs to binding in *trans*, thereby lowering the threshold for inhibition [256, 257]. *Trans* and *cis* binding is mediated by the same binding site [256]. *Cis* binding of KIRs has not been reported [258]

Non-MHC-I-specific inhibitory receptors

NK cells may express inhibitory receptors recognizing other ligands than MHC-I. Within the NKC on mouse chromosome 6 there are genes encoded belonging to three families of C-type lectin-like glycoproteins [94, 259]. Similar to the Ly49r and the NKG2 family, the NKR-P1 family contains activating and inhibitory members as well. NKR-P1A, -C, and –F are activating, while NKR-P1B and –D are inhibitory and recognize Ocil/Clr-b. Ocil/Clr-b is encoded in close proximity to the receptors within the NKC and is expressed on many hematopoietic cells. this receptor-ligand system can provide additional MHC-I-independent regulation of self-tolerance [259]. In humans, several additional inhibitory NK cell receptors exist, which have not been identified in the mouse.

The SLAM family member 2B4

Receptors of the SLAM family are expressed on hematopoietic cells and have important immune-modulatory functions. Most SLAM family members are self-ligands, except 2B4 which binds to CD48, a molecule expressed on all hematopoietic cells [260]. 2B4 has four immunoreceptor tyrosine-based switch motifs (ITSM) in its cytoplasmic domain, which become phosphorylated and recruit the small adaptor molecules SAP, EAT2 and ERT (ERT only in mice)[261]. These adaptor molecules mediate activation of NK cells via the Src family kinase Fyn and Vav-1, which results in cytokine secretion and cytotoxicity [262]. In the absence of SAP, as in immature NK cells or X-linked lymphoproliferative disease (XLP), the ITSM motifs bind to the phosphatases SHP-1, SHP-2 and SHIP1 instead, which leads to NK cell inhibition [261, 263-265]. In mice, 2B4 appears to mediate both inhibitory and activating signals, depending on the context and the model system [266, 267].

KLRG1

Killer cell lectin-like receptor 1 (KLRG1) is an adhesion molecule expressed by about 50% of naïve mouse and human NK cells and by activated and memory T cells [268, 269]. KLRG1 is a marker for mature NK cells and activated T cells, as expression on NK cells starts at the CD27$^-$CD11b$^+$ late maturation stage in NK cells and is induced on T cells after contact with virus-infected cells [130, 269-271]. KLRG1 binds to cadherins wich are expressed on epithelial and endothelial cells, as well as some hematopietic cells such as DCs [272-274]. KLRG1 contains an ITIM within its cytoplasmic domain which can recruit SHIP-1 and SHP-2, but inhibitory function under physiological conditions has only been demonstrated in human NK cells [271, 275, 276]. In mouse NK cells, KLRG1 form

multimers, while on human NK cells it exists predominantly as a homo-dimer, which appears to be crucial for inhibitory function [277]. An inhibitory function of KLRG1 for mouse NK cells could only be demonstrated with transfected KLRG1 at very high levels, but not under physiological conditions [277]. An association between KLRG1 expression and education had been suggested [157, 278].

1.3.7 NK cell education

NK cells need to determine whether a cell is harmful to the body or not, i.e. they need to distinguish healthy cells from cells that are infected or transformed. Reactivity of NK cells is controlled by the balance of signals received by activating and inhibitory receptors. NK cell inhibitory receptors (Ly49 receptors in mouse, KIRs in human, CD94/NKG2A in both species) and their ligands (different MHC-I alleles) are located on different chromosomes, and consequently inherited independently from each other [279]. Furthermore, expression of inhibitory receptors is seemingly random (although there are some patterns connected to host MHC-I), so that NK cells lacking a receptor that can bind to self MHC-I do exist [216, 224, 228]. This suggests that there must be other means than shaping the repertoire of inhibitory receptors to ensure self-tolerance. Both deletion and induction of anergy of potentially auto-reactive NK cell subsets have been discussed [86, 280-282]. To remain self-tolerant, NK cells need to undergo an *in vivo* education process, so that only those NK cells which have an inhibitory receptor specific for self MHC-I, acquire the capacity to kill cells based on missing self as the only or major phenotype alteration [216, 223, 224, 283, 284]. NK cells lacking expression of an inhibitory receptor specific for self-MHC-I remain in the population but display a reduced responsiveness MHC-I-deficient, but otherwise healthy cells [224, 228]. Education is dependent on the functional interaction of inhibitory receptors with a cognate MHC-I and the resulting phosphorylation of the Ly49r-ITIM, but not necessarily recruitment of SHP-1 [223, 285, 286].

Different models have been proposed for how NK cells adapt their responsiveness during development. The licensing model postulates that NK cells with self receptors acquire responsiveness due to MHC-I interactions with surrounding cells. Only NK cells that encounter and bind a cognate MHC-I molecule via its Ly49r will develop functionally [223]. In contrast, the disarming model assumes that initially responsive NK cells lacking self receptors acquire a hypo-responsive phenotype and become self-tolerant in situations where overstimulation by activating receptors in the absence of inhibitory signals are perceived [224]. The main argument for an active mechanism of tolerance in induction is that wt NK cells in chimeric or mosaic mice (mix of MHC-I$^+$ and MHC-I$^-$ cells) acquire a hypo-responsive phenotype, i.e., they are less responsive and do not attack surrounding MHC-I$^-$ cells [282, 287, 288]. In recent years, our group has proposed the "Rheostat model" for tuning of NK activation thresholds [283, 284], which postulates that NK cells are not just in an "on-" or "off-state" – but that there rather is a continuum of different degrees of responsiveness at the single cell level. Furthermore, the rheostat model suggests that the degree of responsiveness, at the single cell level, will depend qualitatively and quantitatively on inhibitory MHC-I input. According to the rheostat model, NK cell education is a continuous

process in which the responsiveness of an individual cell is set according to the amount of inhibitory input (interaction with MHC-I) they receive, and the combined strength of the resulting interactions, i.e. the number of inhibitory receptor-MHC-I interactions and the affinity of the individual inhibitory receptor for their cognate MHC-I ligands [283, 284]. Consequently, it should be possible to fine-tune responsiveness also in mature NK cells by changing the inhibitory input at the NK/receptor level, which we tested (paper I) [289].

As a result of this education process, only NK cells which express inhibitory receptors for self MHC-I, and consequently are inhibited from killing healthy self cells, have gained the functional capacity for effector functions when encountering a target cell which has too low MHC-I levels to prove that it is a healthy non-dangerous self cell. This inhibitory interaction during the effector phase, when the NK cell integrates activating and inhibitory signals and decides whether to kill or not to kill, is mediated by the same inhibitory receptor-MHC-I as the preceding education. Importantly, the threshold for education appears to be higher than that for inhibition so that weak interactions could lead to inhibition even if they do not impose education, most likely to establish a safety margin against auto-reactivity [249].

NK cells with multiple inhibitory receptors for self MHC-I respond better, and the different MHC-I molecules have distinct impact on responsiveness [92, 157, 284]. Similarly, also the MHC-I expression levels on encountering cells seems to play a role in education. This is exemplified by a study in mice where the impact of homo-versus hemizygosity of MHC-I expression was assessed [290], and could also be shown in humans [291, 292]. Any higher organism usually has multiple MHC-I alleles, each of which can bind to different inhibitory receptors with distinct affinity and avidity and thus educate different NK cells. NK cells can express individual Ly49rs in a seemingly random order, some express none, one, two or up to the number of MHC-I genes [293] (ranging from 0-6 in B6 mice).

Different MHC-I alleles have a different impact on education of an individual NK cell via a certain inhibitory receptor, i.e. there is a hierarchy of education impact [92, 283]. In the mouse the interaction of H2-D^d with the Ly49A results in a more responsive NK cell than interaction of H2-D^d with Ly49G$_2$ and interaction of H2-K^b with Ly49C has a stronger impact than H2-K^b on Ly49I-expressing NK cells [223, 284]. Similar hierarchy of educating impact of the different HLA-I alleles have been reported for human NK cells [228, 294]. Overall, when it comes to functional readouts of education, the most clear-cut results can be obtained by comparing Ly49A$^+$ with Ly49A$^-$ NK cells in a mouse that expresses a strong ligand for Ly49A, e.g. H2-D^d [157, 249, 284].

Recently, it has been proposed by several groups, that not only the inhibitory input an NK cells receives but also the activation signals are important for education and set the threshold for responsiveness [157, 289, 295-297]. As interactions via inhibitory receptors decreases the threshold, and thereby increases responsiveness to subsequent stimuli, the interactions via activating receptors may increase the threshold for responsiveness. This is to assure that every individual NK cell has the appropriate threshold to respond to changes in the levels of both MHC-I (and other inhibitory ligands) and ligands for the activating receptors.

Overstimulation via activating receptors thus leads to hypo-responsive NK cells [291, 296, 298-303].

In this thesis, education of NK cells via inhibitory interactions is studied and discussed. NK cell education by other inhibitory receptors such as 2B4, KLRG1 or inhibitory NKR-P1 receptors has been suggested [138, 267, 304]. The term "educated" in this thesis refers to an NK cell that has experienced MHC-I and can react to loss of the same, while an "uneducated" or "hypo-responsive" NK cell has not and thus does not kill a target solely on the basis of MHC-I down-regulation.

Uneducated, hypo-responsive NK cells are not useless. As they are not inhibited by MHC-I molecules, in case of a strong activation, their reduced responsiveness could be compensated by reduced inhibition during an effector phase. Indeed, during infection, it is mainly the uneducated NK cells that respond with proliferation and IFNγ production to MCMV [305], influenza [306], or Listeria monocytogenes[224]. Similarly, uneducated NK cells can respond quite well to stimulation by cytokines. Furthermore, NK cells do not need to have experienced education by MHC-I in order to kill MHC-I- targets. The balance of activating and inhibitory signals is what determines whether a target cell is killed or not. This quite counter-intuitive notion has been demonstrated by *in vitro* killing assays [88], and also *in vivo* (paper I) [289].

1.3.8 Adaptive features of NK cells

Interplay with other cells

An immune response in mammalians is mediated by two broad systems, innate immunity which provides a first-line defense against many pathogens, and adaptive immunity, which is antigen-specific and displays immunological memory [20]. NK cells were described to respond rapidly and non-specifically to infection, and therefore classified as being part of the innate immune system. However, NK cells can direct immune responses to a more pro-inflammatory, Th1 type response, they can help T cell responses by secretion of IFNγ and other pro-inflammatory mediators, and by early killing of target cells, thereby reducing antigenic load. The resulting cell debris may promote antigen presentation by APCs [307]. NK cells can also directly communicate with other immune cells, such as DCs, NKT cells and T cells and thereby shift adaptive immune responses [71, 308, 309]. NK cells kill recently activated CD8^{+} T cell in a perforin- and NKG2D-dependent manner, which may be important for termination of an immune response but can also lead to a reduction in T cell memory responses [310]. A vast body of literature describes cross-talk of NK cells with DCs [32, 35, 311-317]. The reciprocal activation of NK cells and DCs leads to polarization of the immune response, NK cells may release IFNγ, while DCs acquire the capacity to prime T cells towards Th1 responses. Furthermore, DCs provide tonic stimuli which are necessary for NK cell priming [63].

Memory NK cells

NK cells, with their limited repertoire of germ-line encoded receptors, are part of the innate immune system, and as such, have been thought to lack immunological memory (acquired immunity). However, recent studies suggest that NK cells can respond better to secondary stimulation, a feature that has been attributed to immunological memory. The first report illustrating NK cell memory functions, describes contact hypersensitivity responses against 2,4-dinitrofluorobenzene and oxazolone haptens in RAGcγ mice, which are devoid of T and B cells. These responses were acquired, antigen-specific, persisted for up to four weeks and depended on Ly49C/I^{+} NK cells, (educated in these mice) [318]. Later, it was shown that NK cells from liver, but not spleen, could develop enhanced responses to a secondary challenge, against antigens from several different viruses [319]. Furthermore, NK cells activated *in vitro* with IL-12, -18, and low dose IL-15 could be detected for up to 22 days after transfer to naïve mice. These transferred NK cells were phenotypically indistinguishable from naïve NK cells, but responded better to subsequent stimulation with cytokines and receptor-crosslinking [320]. Memory NK cells have been further characterized in a mouse model for MCMV infection. In B6 mice, it is NK cells with the activating Ly49H receptor that specifically recognize m157, a MCMV-encoded protein, and respond by proliferation and effector functions. After the infection is cleared, most NK cells disappear again during a so-called contraction phase. Some NK cells, however, persist for several months in lymphoid and non-lymphoid organs. Upon a second challenge with MCMV, these long-living, self-renewing NK cells respond with enhanced cytokine production and degranulation which results in protective immunity against higher doses of virus [321].

Similar to the expansion of the Ly49H^{+} NK cell subset during MCMV infection, HCMV seems to drive the expansion of certain NK cell subsets as well and selectively shapes the repertoire of NK cells. HCMV seropositive humans have an increase of NK cells expressing certain KIRs and NKG2C, which recognizes HLA-E [322]. These NKG2C^{+} NK cells displayed an increased responsiveness when challenged with HLA-E-expressing target cells or crosslinking of activating receptors. Furthermore, these in vivo expanded NKG2C^{+} NK cells had acquired the maturation marker CD57 and expressed KIRs specific for self-HLA-I [323-327]. Changes in signaling molecules have been detected which may facilitate stronger effector responses by these adaptive NK cells when encountering infected cells. Furthermore, adaptive NK cells have been shown to spare activated T cells thereby further influencing adaptive immune responses [133].

The generation of memory NK cells in MCMV mouse model is dependent on the pro-inflammatory cytokine IL-12 and the expression of Ly49H and DNAM-1 on the NK cell [321, 328, 329]. Furthermore, memory NK cells predominantly arise from KLRG1^{-} NK cells, even though memory NK cells generated in response to MCMV infection are KLRG1^{high} [330, 331]. Human memory-like NK cells generated *in vitro* with IL-12, -15 and -18 up-regulate CD25, the high affinity receptor for IL-2, which enables them to respond to picomolar concentrations of this cytokine with expansion and increased cytotoxicity [332]. All these reports indicate that NK cells, in certain situations and in response to certain stimuli,

can acquire the ability to respond more robustly against a secondary stimulus with the same antigen. The results from mouse studies using hapten-induced memory, and the human studies indicate that NK cell education may be helpful in the generation of memory cells.

1.3.9 NK cells in immunotherapy

As one of the first immune-based cellular therapies, allogeneic (from a donor to a patient) hematopoietic stem cell transplantation (HSCT) has provided disease-free survival to many patients with hematological malignancies. The usual conditioning with irradiation and chemotherapy already reduces the tumor burden substantially, while it allows stable engraftment of the new immune system. Mature lymphocytes in the graft, and cells arising from the new BM can eliminate remaining tumor cells, a process referred to as the graft versus leukemia (GvL) effect. A major drawback is graft versus host disease (GvHD), based on the recognition of the patient's healthy cells by mature T cells of the graft, which become activated by the recipients APCs presenting peptides of the patient to the donor T cells. However, a certain degree of GvHD appears to be beneficial in terms of long-term survival, most likely because the cytotoxic lymphocytes mediate both GvHD and GvL and a complete suppression of GvHD may also hamper anti-tumor responses.

The first clinical evidence that NK cell alloreactivity played a major role in tumor surveillance came in 2002 [333]. In a cohort of AML patients that received HSCT, the risk of relapse was substantially reduced if the patient received a KIR-HLA mismatched graft. For a long time, donors have been selected so that their MHC setup matches that of the patient. This can be achieved through sibling donors (25% chance for a complete match) or via BM donor registries (usually for a strong but not complete match). Due to lack of such optimal donors, different additional strategies have been launched, e.g. the use of haplo-identical (half of the MHC loci are matched) has been started. To avoid T cell-mediated GvHD, these haplo-identical grafts are extensively depleted of T cells. Optimal donors in such a situation are biological parents and most siblings, as their MHC setup display the condition of haplo-identical, i.e. that half of the MHC genes are identical to the patient's. Extensive T cell depletion before transplantation, and immune suppressive treatment after the procedure result in a situation where the patient is quite susceptible to infections. However, the mature donor NK cells in the graft have been shown be protective and to boost the anti-tumor response without causing GvHD [333]. In those donor-recipient-pairs where the NK cells display a KIR ligand mismatch in the donor vs recipient direction, donor-versus-recipient NK cell alloreactivity decreased leukemia relapse and protected from GvHD. The transferred NK cells attacked the patients remaining tumor cells (GvL) and the patients activated DCs, thereby preventing the induction of GvHD responses [333]. The reason for why donor NK cells killed the patient's tumor cells and DCs, but no other tissues of the patient, is probably the balance of activating and inhibitory signals provided by the cells. As all patient cells were haplo-identical, i.e. they lacked some of the MHC-I molecules that the donor's NK cells regard as self, there is low inhibition. Coupled with the high levels of activating ligands on tumor cells and on activated DCs, this would tip the balance towards NK cell activation and killing of such target cells.

These reports triggered the field of mismatched or haplo-identical transplantation and adoptive transfer of allogeneic mature haplo-identical NK cells. Many transplant centers around the world have started to study the outcome based on stratification of both HLA and KIR genotype in donors and recipients, albeit with varying results [334-343]. The conclusions that can be drawn from these studies are the following: (i) a KIR-HLA-I-ligand mismatch, where the donor has the HLA-ligand for the KIRs expressed by the NK cells, but the patient lacks these HLA-I genes, are preferable and lead to overall better outcome [333, 344-346], (ii) donor-recipient combinations where the donor had at least one group B haplotype (more activating KIR genes), had a better outcome [347, 348], and (iii) patients receiving NK cells with certain activating KIRs (e.g. KIR2DS1) where the donor was positive for the ligand (e.g. HLA-C2) had a reduced risk of relapse [291, 349]. Some, if not all of these observations may be explained by NK cell education and the search for missing self, as discussed in section 3.1 (Paper I).

As donor NK cell alloreactivity correlates with improved outcome in several studies, investigators started to use allogeneic mature NK cells for adoptive transfers to boost the NK alloreactivity effect after HSCT, or to reduce tumor burden before HSCT. Additionally, adoptive NK cell transfer has been used outside of HSCT in conjunction with IL-2 activation, and complete remission could be achieved in 5/19 AML patients [350]. This outcome could be increased to 50% complete remission by depleting the patient of Tregs, which appear to hamper NK cell expansion and activation [351]. With the clinical approval of IL-15, this treatment may be further enhanced by replacing IL-2 with IL-15, thereby limiting the activation of Tregs. Multiple groups are now investigating alternatives sources of NK cells, such as differentiation from progenitors, *ex vivo* expansion, and immortalized NK cell lines [352].

NK cell effector functions are negatively regulated by inhibitory receptors such as KIRs and NKG2A, as well as induced co-inhibitory receptors PD-1 and T cell immunoglobulin mucin-3 (Tim-3). Anti-KIR mAbs are used with the aim of blocking the inhibitory KIR-HLA-I signal and thereby enhancing NK cell function [353]. While pre-clinical studies in mice have given promising results, initial clinical studies have shown that this treatment did not demonstrate major responses when used as single agent [19, 354-358]. However, combining anti-KIR mAb with lenalidomide, which increases the expression of stress molecules such as ligands for NKG2D or DNAM-1 on tumor cells, has increased the response rate in a recent study [359].

Shortly after the discovery of NK cells, the ability to kill syngeneic and allogeneic tumor cells *in vivo* and thereby providing natural resistance against cancer was recognized [360]. Ever since, there have been numerous studies in mice on effector functions of NK cells and their role in host protection [72].

As for T cell immunotherapy discussed above, engineered BiKes and TriKes, and CARs for NK cells are being developed and tested in pre-clinical studies [352, 361, 362]. Targets for BiKes and TriKes include CD33$^+$ cells which is expressed on many hematopoietic

malignancies such as AML, or CD133, which is an antigen specifically expressed by colorectal cancer [361, 362]. NK cell CARs currently under investigation are anti-CD19 (B cell malignancies) and anti- EGFR targeted for breast cancer and brain metastasis [352]. The field of engineered receptors has just started, and it will be interesting to see what the future may hold.

2 AIMS

This thesis aims to study NK cell education, whether it can be modulated *in vivo* and how this impacts immunotherapy.

Specific Aims:

Paper I

To investigate if education of mature NK cells can be retuned by altered inhibitory input from normal cells in the environment and how this may affect tolerance to self and activity towards tumor cells.

Paper II

To characterize the expression of DNAM-1 on NK cells with respect to education imposed by MHC class I molecules.

Paper III

To assess the impact on inflammation on NK cell – DC cross-talk, and to study which molecules are involved.

Paper IV

To modulate NK cell missing self- responses by *in vivo* activation of iNKT cells and to compare soluble αGalCer versus αGalCer administered in the context of exosomes.

3 DISCUSSION

3.1 PRESERVED ANTI-CANCER RESPONSE DESPITE RETUNING OF NK CELL EDUCATION

The rheostat model for NK cell education postulates that responsiveness is set according the strength of the net signal resulting after balancing activating and inhibitory input, so that killing of normal healthy cells is avoided. As a consequence, the number of inhibitory MHC-I interactions and the affinity of the individual inhibitory receptor for their cognate MHC-I ligands will strongly influence the development of an activation threshold, i.e. more inhibitory input sensed by an individual NK cells will result in a stronger responsiveness [283, 284]. Furthermore, the model suggests that responsiveness is tuned continuously and it predicts that retuning of responsiveness should be possible. This was tested in studies performed in mouse, where mature NK cells where adoptively transferred from B6 mice (MHC-I$^+$) to β_2m$^{-/-}$ mice (MHC-I$^-$) and vice versa [363, 364]. NK cells transferred from B6 to β_2m$^{-/-}$ mice displayed reduced responsiveness when triggered via crosslinking of activating receptors *in vitro*, and against MHC-I-deficient target cells *in vivo*. The decrease in capacity to degranulate and to produce INFγ was specific to NK cells that expressed inhibitory receptors sensing the relevant MHC-I [364]. When mature hypo-responsive NK cells from β_2m$^{-/-}$ mice were adoptively transferred to an MHC-I-positive mouse strain, the ability to degranulate and produce IFNγ in response to *in vitro* receptor-crosslinking was increased [363]. These results advocate that mature NK cells can adapt their responsiveness according to the MHC-I environment, which is in support of the rheostat model of NK cell education. The results from these two mouse studies would predict that in a clinical setting, adoptively transferred NK cells from an HLA-mismatched donor to a patient where KIR ligands (HLA-I) are missing, would adapt and lose their capacity to kill the patient's tumor cells. However, in adoptive transfer or BM transplantation therapies, the KIR haplotype of the donor, and the relationship between the donor's inhibitory KIR expression and the patient's HLA genotype, do have an impact on clinical outcome in allogeneic settings. A longer progression-free survival post-transplantation is observed in leukemia patients that lack the HLA-I (KIR-ligand) for the donor's inhibitory KIRs [333, 343-346, 348, 365]. In the same studies it was shown that donor NK cells did not induce GvHD, in fact, the risk of GvHD after mismatched transplantations of HSC was reduced [333]. The donor's NK cells seem to kill the mismatched tumor cells but not the equally mismatched healthy tissues. This was explained by the finding that the transferred NK cells could kill the patient's DCs, thereby preventing activation of allogeneic T cells of the graft, which would attack the patient's tissues and cause GvHD. Furthermore, ongoing clinical trials use anti-KIR antibodies to block the inhibitory interaction of KIRs with MHC-I on tumor cells. The approach tested in these clinical trials, as well as results from pre-clinical studies, suggest increased killing of MHC-I-positive tumors by NK cells [19, 354-356, 366].

The strategies of these clinical studies are encouraging, but the sometimes limited effect of treatment could potentially be explained by retuning as demonstrated in mouse studies, where

transferred NK cells adapt and become tolerant to MHC-I-mismatched cells [364]. After transfer of mature NK cells from B6 to $\beta_2m^{-/-}$ mice, NK cell reactivity *in vivo* was tested only against spleen cells (MHC-I⁻) and the *in vivo* killing capacity of NK cells after transfer from $\beta_2m^{-/-}$ to MHC-I⁺ mouse strains was not tested at all [363, 364]. The results of both studies therefore only show that the activation threshold is altered after a change in MHC-I sensing. How this translates to killing of tumor cells, MHC-I⁺ or MHC-I⁻, was the starting point of paper I. We transferred mature splenic NK cells from B6 mice (MHC-I⁺) to $\beta_2m^{-/-}$ mice (MHC-I⁻), and tested the capacity to kill MHC-I⁻ target cells after 7 days. This time-point was chosen after initial kinetic experiments using spleen cells as targets. We found a variable induction of tolerance towards MHC-I⁻ spleen cells as early as day 4 (data not shown), which was very robust at day 7 (paper I, Fig. 3). NK cell responsiveness was down-tuned both when cell suspensions made from entire spleens were transferred, and when we isolated the NK cells prior to transfer (paper I, Fig. 3A). We then tested how well these NK cells could kill tumor cells, by using a pair of H-2^b (syngeneic to B6) T cell lymphoma lines, RMA (MHC-I⁺) and RMA-S (MHC-I⁻). RMA-S was generated by mutagenesis of the parental lymphoma line RBL-5 (later identified as a subline of the EL-4 cell line[367, 368]) followed by repeated selection for low binding of MHC-I antibody, while the RMA line underwent mutagenesis but not selection [73, 75]. The two cell lines have been cultured separately and could have acquired different mutations. We therefore tested a panel of ligands for NK cell activating receptors and detected no difference between RMA and RMA-S (Paper I, Suppl. Fig. 2). Notably, while B6 NK cells transferred to $\beta_2m^{-/-}$ mice became tolerant to MHC-I spleen cells, they were still able to recognize and efficiently kill MHC-I⁻ RMA-S tumor cells (paper I, Fig. 3D). This suggests that these NK cells were not simply exhausted by the multitude of interactions with MHC-I cells but actually tolerant. After all, educated NK cells fully capable of performing missing self-rejection, were placed into an environment full of cells representing the missing self phenotype, i.e. cells lacking MHC-I. We did not see any signs of auto-aggression or toxicity. This does not exclude the possibility that the transferred NK cells killed the first couple of MHCI⁻ cells they encountered. But ultimately, these NK cells adapted and became tolerant to healthy MHC-I⁻ cells while still being able to kill tumor targets with low MHC-I expression.

The previous studies showed that hypo-responsive NK cells, transferred from $\beta_2m^{-/-}$ mice to MHC-I⁺ recipients can respond better to stimulation *in vitro* [363, 364]. We wanted to test if this could translate to an *in vivo* situation, i.e. whether NK cells could up-tune their capacity to kill MHC-I⁻ target cells *in vivo*. As MHC-I⁺ recipients we used RAGcγ-double knockout mice. These mice lack T and B cells due to their defect TCR and BCR generation, and they lack NK cells since NK cell development requires functional IL-15 signaling and the common gamma chain is part of the IL-15R complex [116, 369]. We needed a mouse strain that would not reject the transferred NK cells because we wanted to avoid the use of a "mega-dose" of NK cells [363] to generate a more physiological and clinically relevant setting. NK cells transferred from an MHC-I⁻ donor to an MHC-I⁺ recipient did not kill spleen cells that lack MHC-I expression, rather, they remained tolerant (paper I, Fig. 4A+C). This may be in

apparent contrast with the *in vitro* data obtained by Elliot et al. and Joncker et al., as well as the predictions of the rheostat model [363, 364]. One possibility, however, was that the NK cells had been retuned but that this was not sufficient to kill cells with low MHC-I but otherwise healthy phenotype and hence not detectable using spleen cells as targets. We therefore wondered how these NK cells would perform when challenged with tumor targets. Again we used the lymphoma cell lines RMA and RMA-S, and observed that NK cells transferred from MHC-I⁻ mice could kill RMA-S better when exposed to MHC-I⁺ surrounding cells (paper I, Fig, 4B+D). $\beta_2 m^{-/-}$ NK cells were even better in RMA-S killing than B6 cells when transferred to RAGcγ mice and just as efficient as naïve B6 NK cells (paper I, Fig. 4B). These results show that mature NK cells can adapt their responsiveness to their surroundings, but also that self-tolerance is very robust. In both settings, NK cells tolerate the lack of MHC-I on healthy (spleen) cells, while they vigorously react to tumor cells that lack the same MHC-I molecules.

As mentioned before, blocking inhibitory receptors to decrease MHC-I-dependent inhibition of NK cells and thereby inducing killing of (MHC-I⁺) tumor cells, has given encouraging results both in a clinical trial for safety and in mouse studies [19, 354-356, 358, 366]. Importantly, killing of MHC-I⁻ normal cells has not been reported in these studies. We therefore wanted to test the effect of blocking Ly49C and Ly49I (self-specific inhibitory Ly49rs in B6 mice) on the killing of MHC-I⁻ target cells. Similar to the adoptively transferred NK cells, mature educated NK cells in B6 mice altered their behavior and became tolerant towards MHC-I⁻ spleen cells. Paper I is the first report of a change in NK cell behavior towards MHC-I⁻ cells after blockade of inhibitory NK cells receptors. The decrease in responsiveness seen *in vivo* was also observed after crosslinking activating receptors *in vitro*. Importantly, in the *in vitro* assay we could monitor the function of specific NK cell subsets by staining for functional markers and inhibitory receptors. To our surprise, only NK cells expressing Ly49I showed a significant decrease in IFNγ production and degranulation (paper I, Fig. 2A+C) even though the antibody can bind both Ly49I and Ly49C [358]. We speculate that either the binding efficiency of the antibody to the two receptors may differ, or that the two receptors respond differently to the blockade. A direct comparison on binding affinity has not been performed. However, it is known that Ly49I is more accessible to the antibody than Ly49C, which, in a B6 mouse (H2-K^b) is mostly bound in *cis* [370]. After establishing that after blockade of Ly49C/I by antibody binding, NK cells reset their responsiveness to MHC-I⁻ spleen cells, we wondered how these cells would perform when challenged with tumor targets. In this approach, we tested two MHC-I⁻ tumor cell lines, the previously described RMA-S and a recently generated MHC-I⁻ variant of C1498 [371]. Similar to the B6 NK cells that were adoptively transferred to MHC-I⁻ hosts, the blocked NK cells responded efficiently to MHC-I⁻ tumor cells (Paper I, Fig. 1D-F). Whether continuous blockade of inhibitory KIRs affects NK cell responsiveness has not been studied in humans. However, in a mouse model transgenic for a KIR and the cognate HLA-I molecule, *in vivo* blockade with anti-KIR mAb for up to 21 days did not affect *in vitro* responses to stimulation with αNK1.1 or tumor cells. *In vivo* rejection of healthy cells has however not been tested

[366]. As more clinical trials are undertaken with the anti-KIR mAb lirilumab, further studies may elucidate whether KIR blockade also leads to retuning in treated patients.

We suggested a model to explain our and other groups' findings (paper I, Fig. 6). We propose that the activation threshold is set according to the integration of activating and inhibitory input NK cells perceive. This is set at the single cell level, i.e. every NK cell senses a different extent of activation signals and inhibitory signals which are integrated into a net signaling that is perceived as normal and healthy/non-dangerous. Only target cells with ligands providing sufficient activating potential (signals from activating ligands exceeding the net activation signal that was perceived as normal/healthy) will make the NK cell react.
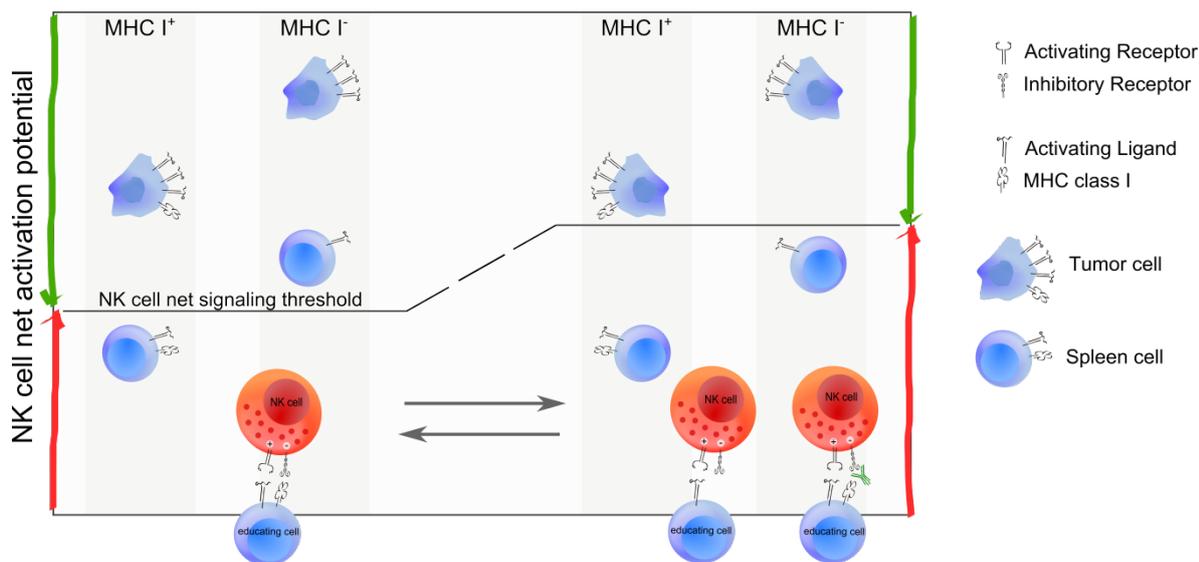

Figure 1: Model for retuning of NK cell responsiveness after a change in MHC-I-dependent inhibitory input. NK cells integrate inhibitory and activating signals they receive from surrounding cells. Based on this integrated signal, a net signaling threshold is set for each individual NK cell. This net signaling threshold needs to be overcome by the activation potential of target cells for killing to NK cell activation to occur. Adoptive transfer to an MHC-I-disparate environment or blockade of inhibitory receptors alters the inhibitory input. Activating and inhibitory signals are re-integrated which causes an adaptation of the net signaling threshold. Individual NK cells can still respond to cells where the activating ligands exceed inhibitory ligands, such as cancer cells. Signals provided by MHC-I-deficient tumor cells are above the net signaling threshold, while MHC-I-deficient healthy cells are not.

Reduction of inhibitory input, e.g. by blocking interactions of inhibitory Ly49rs or by adoptive transfer from an MHC-I$^+$ to an MHC-I$^-$ environment, increases the net signaling threshold, i.e. the same activation signals with less inhibition are now perceived as normal. This can explain the induction of tolerance towards MHC-I$^-$ spleen cells, as they provide exactly what is after retuning perceived as normal: less inhibition, but the same amount of activation. Tumor cells are still efficiently killed since they express more ligands for NK cell activating receptors (paper I, suppl. Fig. 2 and [171]) and their activation potential therefore overcomes the net signaling threshold of NK cells (paper I, Fig. 6). Additionally, tumor cells could potentially produce cytokines that activate NK cells. While RMA and RMA-S do not express IL-12 or IL-18 [372], they produce IL-2, a cytokine known to activate NK cells (Kritikou et al, manuscript under revision).

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Our model can also explain the increased ability of $\beta_2 m^{-/-}$ NK cells to kill MHC-I$^-$ tumor cells after transfer to MHC-I$^+$ mice, while at the same time tolerance towards MHC-I$^-$ spleen cells is observed. By being able to receive MHC-I-dependent inhibitory signals, the net signaling threshold is reduced, meaning that less net activation provided by ligands on target cells is required for an NK cell to kill the target (Fig. 1: Model for retuning of NK cell responsiveness after a change in MHC-I-dependent inhibitory input). Translated to the clinical setting with human NK cells, this would implicate that when NK cells are transferred from a donor that lacks the cognate HLA-I for some of the expressed KIRs to a patient where the particular HLA-I is present, these NK cells could gain responsiveness and start to kill the patient's tumor cells, while being tolerant to the healthy cells, that are equally mismatched.

Interactions of Ly49rs with cognate MHC-I give rise to strong inhibitory signals, but they are not the only source of inhibition and therefore non-MHC-I-dependent inhibitory interactions may also be involved in fine-tuning the net activation threshold [138, 267, 304]. However, since we interfered with sensing of MHC-I via Ly49rs, we can show that MHC-I-dependent inhibition is a major contributor in setting the net signaling threshold. Similarly, not only stress-induced activating ligands can be a source of activation, also MHC-I-specific activating interactions may contribute to the increase the net activation threshold. As described in section 1.3.7, recent studies report an impact of activating Ly49rs/KIRs on NK cell education. In clinical studies it was reported that patients receiving NK cell allografts from donors expressing activating KIRs (e.g. KIR2DS1) but lacking the ligand (e.g. HLA-C2), had less relapse than patients who received grafts where both KIR and KIR-ligand were expressed [291, 349, 373]. These studies suggest that NK cells with activating KIRs interacting with cognate ligand in the donor are hypo-responsive. The effect is even stronger, when two copies of the HLA-I gene are present, for example KIR2DS1$^+$ NK cells in donor's that are homozygous for HLA-C2 [349]. However, when the patients were homozygous for the ligand (HLA-C2/C2), the KIR2DS1-induced NK alloreactivity was gone and these patients had a higher risk of relapse [349]. This finding could be interpreted as a down-tuning in net activation threshold induced by increased activation input via recognition of C2 by KIR2DS1.

To date it is not known which cells provide the educating signal to NK cells. It could be a specific cell type, e.g. DCs or BM stromal cells or any hematopoietic cell expressing a certain molecule or secreting a necessary factor, or it could be any encounter with another cell, including fellow NK cells and even target cells, i.e. cells that are killed. It could also be so that every cell can provide the educating signal, but that NK cells are only responsive to that in a certain environment/niche, e.g., in a certain organ (lymphoid organs as the BM) or under specific conditions (cytokines, growth factors). A recent study performed in mice suggested that even MHC-I$^-$ tumors can alter the activation threshold and induce hypo-responsiveness in NK cells locally [372]. Initially it has been postulated that NK cells are educated in the BM [99, 100]. More recent data, including my own (paper I), have shown that education is not fixed, that mature NK cells can be re-educated and adjust their responsiveness according to the MHC-I input they receive throughout their life span and also in the periphery [289, 363,

364, 372, 374]. It has been proposed that interactions of Ly49rs with MHC-I in *cis*, i.e. on the same cell, influence NK cell education [375]. The data on adoptively transferred NK cells presented in paper I however, indicate that *trans* interactions are necessary to adapt to the new environment. If *cis* interactions were the sole cause for education, the transferred NK cells would not retune and change their net signaling threshold, as B6 NK cells transferred to $\beta_2 m^{-/-}$ mice could still sense MHC-I in *cis*, and $\beta_2 m^{-/-}$ NK cells transferred to MHC-I$^+$ mice would not sense MHC-I in *cis*. However, NK cells can acquire MHC-I from the surface of other cells, a process termed trogocytosis [376]. When we assessed the expression of K^b on transferred $\beta_2 m^{-/-}$ NK cells 7 days after transfer, we could see a small increase in K^b MFI when the recipients were MHC-I$^+$, but not MHC-I$^-$ (data not shown). The levels of K^b expression on transferred $\beta_2 m^{-/-}$ NK cells only reached about 1-2% of wt B6 levels, but we therefore cannot exclude trogocytosis and *cis* interactions entirely. However, transferred B6 NK cells adapted to the changed ability to interact with MHC-I in *trans* as well without a measurable reduction of MHC-I expression (data not shown), strongly suggesting that *trans* interactions are required for the observed retuning.

In the clinic, NK cells from haplo-identical (partly HLA-I mismatched) donors either as mature NK cells or as HSCs, are experimentally used to treat patients with several blood-borne cancers, such as acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), acute lymphoblastic leukemia (ALL), myelodysplastic syndrome (MDS). However, variable success rates have been reported. Some studies describe a beneficial effect when patients lack the HLA-I (KIR-ligand) for the donor's inhibitory KIRs [333, 340, 343, 346, 348, 365], while others see no such effects [377-379]. Differences in conditioning regimen, T cell depletion or immunosuppressive treatment may influence the net signaling threshold of educated donor cells which could provide an explanation for the differences in observed outcome. Furthermore, a difference in residual disease of different patients could have an impact on the killing capacity of transferred NK cells. The data from tumor outgrowth experiments, (paper I, table I) indicate that the increased net signaling threshold also affects tumor rejection. Treatment with anti-Ly49C/I antibody increased killing of the MHC-I$^+$ tumor cells (paper I, table I and [356, 358]). We interpret this as a result of reduced inhibition at the effector-target interaction phase where the tumor cells are not able to inhibit the NK cells from killing and fewer mice develop tumors. As described previously, killing of MHC-I$^-$ tumor cells was not affected (Paper I, Figs. 1, 3, 4). However, when we challenged the system with a higher dose of MHC-I$^-$ tumor cells, more mice in the group that received the anti-Ly49C/I treatment developed MHC-I$^-$ tumors (paper I, table 1). This suggests that missing self-rejection of tumor cells is also affected, as the net signaling threshold of blocked NK cells is increased. Our results show that the window where the treatment is beneficial might be narrow, which could explain the divergent outcome in clinical studies. To increase the positive effects of antibody blockade, we suggest discontinuous treatment so that NK cells will have the time to set their net activation threshold back to default and be able to respond efficiently to MHC-I$^-$ tumor cells. Furthermore, combining inhibitory receptor blockade with another treatment, such as immune-modulating cytokines or other mAbs specific for tumor

antigens, or adoptive transfer of donor NK cells might be successful. In fact, the study performed by Ardolino et. al. could recently show that the hypo-responsiveness induced by MHC-I⁻ tumors could be reversed by treatment with IL-2 or IL-12 and IL-18 [372]. Furthermore, in clinical trials for treatments of a broad range of solid and hematological tumors, the anti-KIR antibody lirilumab is tested in combination with several other anti-cancer drugs. These drugs include the newly approved anti-PD-1 mAb, anti-CTLA-4, an anti-SLAMF7 mab, the anti-CD20 mAb, and the demethylating agent 5-azacytidine (clinicaltrials.gov, as of 2016-05-05). Combining anti-KIR blockade with a mAb against the neo-antigen GD2, has already given promising results in the treatment of neuroblastoma. To improve therapy with adoptively transferred NK cells, we suggest similar strategies to enhance NK cell functions and to counter-act the adaptation processes. Pre-treatment with cytokines before transfer (e.g. IL-2 or IL-15), and *in vivo* activation with IL-15 are being used recently. It will be interesting to see the developments in the field in the coming years.

Taken together, our data in paper I demonstrate that retuning of MHC-I dependent education of mature NK cells can be achieved in both directions. We can furthermore show that despite induction of tolerance towards healthy MHC-I⁻ cells, tumor cells representing the same missing self phenotype are efficiently rejected. We suggest that this is because tumor cells, due to their higher expression of activating ligands, overcome the net activation threshold of NK cells even after retuning.

3.2 DNAM-1 IN EDUCATION OF NK CELLS

DNAM-1 is expressed on NK cells, T cells, DCs, monocytes and a subset of B cells [181]. It binds to CD155 and CD112, which are broadly expressed by hematopoietic, epithelial and endothelial cells [189, 190], upregulated on some tumor cells [380, 381] and modulated in response to infection [329, 382]. DNAM-1 contributes to various innate and adaptive immune responses, which include lymphocyte migration, activation, proliferation and differentiation of NK and T cells and polarization towards the Th1 type of CD4 T cells [181, 187, 194, 383, 384]. A physical and functional association with LFA-1 has been reported [185, 187, 385, 386], with a coordinated expression of DNAM-1 and LFA-1 in educated human NK cells [386]. Previous reports established that DNAM-1^{high} NK cells can produce more IFNγ, degranulate more upon stimulation and are superior in killing CD155-bearing tumor target cells [239, 386, 387]. Additionally, DNAM-1^{+} NK cells respond better to IL-15 stimulation and express genes involved in cell cycle and survival [387]. Most of these traits have been associated with educated NK cells [157, 223, 224]. Furthermore, in humans, where all NK cells are all positive for DNAM-1, educated NK cells have higher levels of DNAM-1 [216, 386]. Based on these observations, we sought to determine a possible role of DNAM-1 in NK cell education.

Based on the hypothesis that DNAM-1 is connected to NK cell education, we started with three predictions which we could test using different strains of mice and looking at different NK cell subsets based on inhibitory receptor expression. In contrast to human NK cells, NK

cells in laboratory mice have a bimodal expression of DNAM-1, resulting in DNAM-1$^+$ and DNAM-1$^-$ NK cells. For this reason, we assessed both the frequency of DNAM-1$^+$ NK cells, and the average expression level of DNAM-1 on NK cells that were positive for DNAM-1 (measured as DNAM-1 MFI). The two parameters correlated in all mouse strains with different combinations of MHC-I alleles (paper II, Suppl. Fig. 1).

The first prediction was that DNAM-1 should be higher in wt B6 mice and lower when mice lacked MHC-I surface expression. Indeed, in B6 wt mice, about 60% of NK cells express DNAM-1, while in β_2m$^{-/-}$, TAP-1$^{-/-}$ and K$^{b-/-}$D$^{b-/-}$ only 35-45% of NK cells are positive for DNAM-1 (paper II, Fig. 1 and data not shown). The second prediction was that expression of DNAM-1 should correlate to the educating impact of specific MHC-I alleles. Individual MHC-I alleles educate NK cells to different degrees, which results in different strength of responsiveness *in vitro* as well as of rejection capacity *in vivo* of cells that lack the particular MHC-I molecule [92, 284]. The educating impact correlates to differential binding strength and affinity between inhibitory receptors and the respective MHC-I ligand [249, 283, 284]. Indeed, the frequency of DNAM-1$^+$ NK cells, and the DNAM-1 MFI of DNAM-1$^+$ NK cells correlated positively with the educating impact of the particular MHC-I allele (paper II, Fig. 1) in a panel of mouse strains expressing one single MHC class I gene.

The third prediction was that DNAM-1 should be higher on NK cells that can bind to MHC-I versus NK cells that do not express an inhibitory receptor for self-MHC-I within the same mouse, and NK cells with several self-specific inhibitory receptors should have more DNAM-1 that NK cells with one receptor. We tested this by studying different NK cell subsets based on the expression of inhibitory Ly49rs and NKG2A in mice with different MHC-I alleles. In accordance with our prediction, in all mouse strains, NK cells expressing self-specific inhibitory receptors had higher levels of DNAM-1. The highest levels of DNAM-1 were observed on NK cells expressing NKG2A, as described previously [239]. For NKG2A$^-$ NK cells, the more Ly49rs were expressed, the more DNAM-1$^+$ NK cells were within that subset. In accordance to our prediction, NK cells with self-specific Ly49rs had higher levels of DNAM-1 in all mouse strains (paper II, Fig. 2 and Suppl. Fig. 2). NK cells with two self-MHC-I-specific Ly49rs had higher DNAM-1 levels than NK cells expressing either of the two Ly49rs alone, i.e. Ly49C$^+$Ly49I$^+$ NK cells had more DNAM-1$^+$ cells than Ly49C$^+$ or Ly49I$^+$ cells in a B6 mouse (paper II, Fig. 2A) and Ly49A$^+$Ly49G$_2$$^+$ cells than Ly49A$^+$ or Ly49G$_2$$^+$ NK cells in a D^d-single mouse (data not shown). This was true also when NK cells were divided based on the number of inhibitory Ly49rs (paper II, Fig. 3), most likely because with increasing number of receptors, the chance of expressing one that educates also increases.

Furthermore, we could show that DNAM-1$^+$ NK cells can detect the absence of MHC-I on tumor target cells and can perform missing self-reactions *in vitro* (paper II, Fig. 5). This could simply indicate that DNAM-1 is involved in tumor cell recognition and killing, however, we could show that the MHC-I$^+$ and MHC-I$^-$ tumor cells used have low and similar levels of CD155 and do not express CD112 (paper II, Suppl. Fig. 5). This is in line with recently

published results showing that even target cells devoid of any known DNAM-1 ligands are killed better by the DNAM-1^{high} human NK cells and indicates that DNAM-1 may serve as an intrinsic sensor for cytotoxic potential of NK cells [386].

We furthermore found that DNAM-1 expression is an early event during NK cell development, which precedes expression of NKG2A, the earliest NK cell inhibitory receptor expressed, and that the expression levels decrease upon NK cell development (paper II, Fig. 7). This is in line with a recent study showing that DNAM-1 expression decreases upon NK cell maturation [387]. However, DNAM-1 does not only appear early, the expression is also the same in MHC-I$^+$ and MHC-I$^-$ mice early in life (data not shown), and is then calibrated to the levels seen in adult mice after the onset of Ly49r expression, again suggesting a link between DNAM-1 and education. In addition, the expression levels are re-calibrated to the levels seen in the host after adoptive transfer of mature splenic NK cells. In our transfer experiments we could see down-regulation of DNAM-1 when mature educated NK (Ly49C$^+$ or Ly49I$^+$) cells were transferred to β_2m$^{-/-}$ recipients, but not when transferred to syngeneic MHC-I$^+$ mice. Uneducated NK cells from β_2m$^{-/-}$ mice, on the other hand, up-regulated DNAM-1 slightly when transferred to an environment expressing MHC-I (paper II, Fig. 8 and Suppl. Fig. 7). It has been shown that DNAM-1$^-$ NK cells develop from DNAM-1$^+$ NK cells when transferred into lymphopenic hosts, but not vice versa [387]. Furthermore, DNAM-1 is not essential for maturation [192, 239, 387]. Whether it is required in order to become educated has not been tested so far. NK cell education is tunable and can be changed even in mature NK cells [283, 289, 297, 363, 364]. To test the possibility that interactions via DNAM-1 are constantly needed to maintain the educated state, we blocked DNAM-1 for 2 days or 2 weeks, and then challenged NK cells with MHC-I$^-$ target spleen cells to assess killing due to missing self recognition. DNAM-1 blockade had no effect on missing self responses, even though complete blockade was achieved (paper II, Fig. 6 and Suppl. Fig. 6), showing that DNAM-1 is not needed to sustain education. Furthermore, this result shows that DNAM-1 is not involved in killing of MHC-I$^-$ spleen cells, at least not as a cell surface receptor recognizing cognate ligand. Whether DNAM-1 is necessary to reach the educated state could not be tested with this experiment. For this, the missing self rejection assay would need to be performed with DNAM-1$^{-/-}$ mice, which to my knowledge has not been done.

Three simplistic interpretations

There are at least three possible interpretations of our data offering simplistic models. Firstly, DNAM-1 expression on NK cells may be a prerequisite for education to occur *in vivo*, marking NK cells that are "educate-able". Secondly, DNAM-1 may be a major functional determinator of education, similar as proposed by Enqvist et. al.[386], i.e. induced by education and required to maintain that state. The third interpretation is that DNAM-1 does not induce or control education, but rather is a down-stream correlate of education. According to this interpretation, expression of DNAM-1 could simply be a passive marker for educated NK cells, but it could also endow NK cells with additional features that provide the educated

NK cells with increased functional capabilities, although these are not essential for the educated state (capacity to recognize and eliminate MHC-I-deficient cells) per se.

If DNAM-1 is indeed a pre-existing marker for "educate-able" NK cells, DNAM-1 should be present on NK cells before the education process imposed by MHC-I begins, i.e. before NK cells express inhibitory receptors specific for MHC-I. This is indeed what we observed; appearance of DNAM-1 expression is an early event during NK cell development, that precedes expression of the counter-receptor TIGIT as well as NKG2A, the earliest MHC-I-specific NK cell inhibitory receptor expressed. The surface levels then decrease upon NK cell maturation. While this is consistent with the idea that DNAM-1 may be required for NK cell education, the observation that the correlation between education and DNAM-1 expression is not absolute argues against this interpretation. Additionally, in humans all NK cells express DNAM-1, but not all NK cells are educated [216, 386].

The second possibility is that the DNAM-1 level is what makes NK cells more capable of detecting and reacting to lack of MHC-I on otherwise normal cells, i.e. act as "educated" by MHC-I. DNAM-1 might for example function in educated NK cells by inducing a tight attachment to a target cell and delivering an activating signal. This would be necessary for NK cells to scan a potential target cell for the presence of self-MHC-I. Our data on adoptively transferred mature NK cells lend some support to this interpretation, since DNAM-1 expression was indeed reversible according to the inhibitory input on individual cells, which could indicate that DNAM-1 is in fact what defines and maintains education. However, this model would predict induction of DNAM-1 expression as a result of education, i.e. after expression of Ly49rs during development in the bone marrow. The very early expression of DNAM-1 in developing NK cells that we observed thus argues against this interpretation. Furthermore our results with blockade of DNAM-1 *in vivo* argue against DNAM-1 as being the deterministic molecule for NK cell education, at least in a model where cognate interactions involving cell surface DNAM-1 would be required to allow NK cells attack of MHC-I-deficient target cells, or to maintain education. Finally, we note that if DNAM-1 were the functional determinator, then all educated NK cells should express DNAM-1. However, within the NK cell subsets carrying a self-specific Ly49r, there was always a population that was DNAM-1$^-$.

The third interpretation is that DNAM-1 is a down-stream consequence of education rather than a functional requirement. Again, the very early onset of DNAM-1 expression during NK cell development argues against this. The fact that DNAM-1 is expressed on NK cells of mice deficient for MHC-I$^{-/-}$, β_2m$^{-/-}$ or TAP-1$^{-/-}$, is also difficult to reconcile with this possibility, but it is intriguing that all NK cell subsets in these mice express lower levels of DNAM-1 and that the preferential expression on certain Ly49r-positive subsets is abolished. It could still be so that all NK cells express a certain baseline level of DNAM-1, whether they are educated via MHC-I or not, and the education process adds to that via interactions with MHC-I and signaling through Ly49rs. Alternatively, education by non-MHC-I-dependent mechanisms such as 2B4 [138, 267, 304] could account for the baseline levels of DNAM-1 in mice

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lacking surface expression of MHC-I or NK cells in wt mice that lack inhibitory Ly49rs for self-MHC-I.

An integrated model

Neither of the simplistic interpretations fits all the data, we therefore propose a model in which DNAM-1 is expressed on NK cells independently of education, and is gradually down-regulated during maturation, although this process is modulated by education. Our data on the early onset of DNAM-1 expression on NK cell precursors before any inhibitory receptors specific for MHC-I are expressed fits with this model, and so does the observation that DNAM-1 levels generally decrease during maturation (paper II, Fig. 7 and [387]). The notion that DNAM-1 is high on "young" NK cells is true during ontogeny (NK cells in young mice), and in developmentally immature NK cells (young NK cells in adult mice). During development and maturation, DNAM-1 levels decrease, and in some instances decrease so much that in mice individual NK cells become DNAM-1$^-$. Superimposed on this gradual decline are other events, such as education or infection, that maintain DNAM-1 at higher levels and "rescue" NK cells from losing DNAM-1 expression altogether. The correlation between DNAM-1 expression and NK cell education observed in mouse (paper II) and human [216, 386], is in accordance with this model. So is the observation that DNAM-1 levels increase in response to viral infection [329]. One may even speculate that the reason why all NK cells in humans are DNAM-1$^+$ is the many infections that humans are subjected to. To this end, it would be interesting to assess DNAM-1 expression in feral mice, which have been shown to have a more similar immune system to humans than laboratory mice [388]. The assumption that DNAM-1 levels are increased due to constant infections could explain the finding that in human NK cells, it is the more mature CD57$^+$ population that has the highest levels of DNAM-1 [386, 389], which is in contrast to the situation in the mouse (paper II, Fig. 7 and [387]).

Education may in some instances even increase DNAM-1 levels. We could observe an increase of DNAM-1 levels in two distinct experimental setups. By staining for maturation markers CD27 and CD11b, we could show that at the transition from the CD27$^-$CD11b$^-$ to the CD27$^+$CD11b$^-$ stage, expression of DNAM-1 increases. This could reflect a factual increase in DNAM-1 gene transcription, or it could be so that the CD27$^-$CD11b$^-$ NK cells transcribe the same amount of DNAM-1 but that it is down-modulated due to interactions with the ligands [381, 390, 391], e.g. during migration to the spleen. Additionally, transfer of mature NK cells from β_2m$^{-/-}$ mice to MHC-I$^+$ recipients lead to an increase of DNAM-1 levels on DNAM-1$^+$ NK cells.

We suggest that DNAM-1 is not a pre-requisite for MHC-I-dependent education to occur, nor is it what defines the educated, responsive state. Rather, DNAM-1 endows NK cells with functional advantages, during different stages of an NK cell's life. Early in development, it provides immature NK cells with a means to interact with surrounding cells to receive growth and survival and possibly education signals. In paper II, we discuss interactions with DCs, as DCs are important for providing tonic stimuli for NK cells under steady state conditions [63].

However, interactions with other cells in the BM may also be of importance, and DNAM-1 ligands are expressed by many cell types. Furthermore, NK cells may use adhesion via DNAM-1 while they egress from the BM. DNAM-1^{+} cells respond better to IL-15 signaling and express more genes connected to cellular survival [387], both traits may be of importance for developing NK cells.
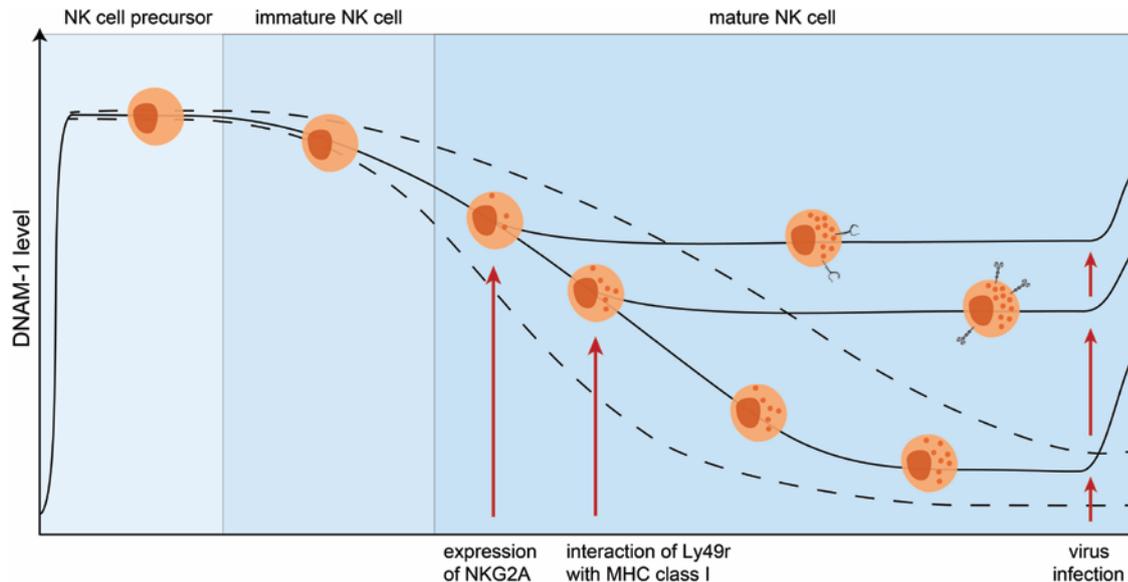

Figure 2: The gradual decline in DNAM-1 levels is rescued by expression of inhibitory NK cell receptors and interaction with MHC-I. Expression of DNAM-1 is initiated by NK cell precursors in the BM, before the onset of MHC-I-specific inhibitory NK cell receptors. During development, NK cells gradually decrease DNAM-1 levels, which may result in DNAM-1-negative NK cells. NK cells that start to express either NKG2A or inhibitory Ly49rs specific for self-MHC-I are rescued from maturation-induced loss of DNAM-1. Continuous interactions between inhibitory Ly49rs and MHC-I retain DNAM-1 expression and prevent complete loss of DNAM-1. DNAM-1 may be important for NK cell development and beneficial for educated NK cells during distinct maturation stages and in interactions with surrounding cells. Infection with a virus, e.g. MCMV induces expression of DNAM-1 in all NK cell subsets.

In the next step of an NK cells life, DNAM-1 may contribute to interactions with cells that provide educating signals. Many studies report the bi-directional crosstalk between NK cells and DCs, which result in enhanced effector functions of both cell types [35, 63, 311, 313-317]. We speculate that these continuous interactions may be facilitated by adhesion via DNAM-1 and its ligands, and furthermore may provide NK cells with a possibility to interact with MHC-I via their inhibitory receptors. As discussed in paper I, education is now considered to be a continuous process, and frequent interactions with MHC-I^{+} cells are necessary to maintain the educated state.

During effector responses of NK cells, DNAM-1 provides advantages as well. NK cells need to migrate to inflamed tissues and lymph nodes to interact with infected cells and DCs. A role for DNAM-1 and CD155 during monocyte transendothelial migration through endothelial junctions, specifically in the diapedesis step of extravasation has been shown [392]. DNAM-1 and CD155 may take part in the regulation of NK extravasation into the underlying tissues as well, similar as for monocytes. Healthy endothelial and epithelial cells express DNAM-1

ligands [393], which in this context might help the NK cell to migrate to inflamed tissues. Recirculation of leukocytes from blood to lymphoid organs or sites of inflammation is crucial for the induction of inflammatory responses. DNAM-1 may regulate the migration of NK cells, the interaction of NK cells with DCs, and the NK cell-mediated lysis of target cells, depending on the surface expression of MHC-I on the interacting cells. Additionally, DNAM-1 enables NK cells to efficiently dock to ligand-expressing target cells. Many tumor cells and MCMV-infected cells up-regulate DNAM-1 ligands, allowing DNAM-1^{+} NK cells to engage and possibly kill these cells [194, 329, 381, 383, 394-396]. Upregulation of CD155 is mediated by the DNA damage response and induced by therapeutic agents [182, 183, 397], and therefore is expressed by many tumors and acts as a danger signal alerting educated NK cells to cellular stress. In the absence of effective MHC-I inhibition, the engagement of DNAM-1 and other triggering receptors results in induction of cytotoxicity. It has recently been shown that education of NK cells alters the signaling of activating receptors after they have bound to their ligands on target cells [398]. Educated NK cells both in mouse and in human have increased inside-out signaling to LFA-1 upon cross-linking of activating receptor, which increases the frequency of conjugation and the strength of the NK cell adhesion to the target cell [398]. Furthermore, DNAM-1 crosslinking increases inside-out signaling to LFA-1 in T cells [185] and engagement of LFA-1 with ICAM-1 on target cells induces co-localization of DNAM-1 and LFA-1 at the immune synapse independent of expression of DNAM-1 ligands [386]. These data can explain the fact that co-engagement of DNAM-1 and LFA-1 by the ligands expressed on transfected insect cells triggered IFNγ production while expression of either ligand alone had no effects [399]. The synergistic effects of many activating receptors that are required for optimal NK cell activation [131] validate the idea that higher levels of DNAM-1, and other activating receptors and adhesion molecules, can provide educated NK cells with a means to overcome the inhibition mediated by the educating inhibitory receptors.

During infection with MCMV or influenza virus, NK cells without self-specific inhibitory Ly49rs dominate the response and are critical in protection against the respective virus [305, 306]. However, DNAM-1 is essential for optimal differentiation of memory NK cells during MCMV infection [329], thus having high DNAM-1 levels on educated NK cells may be an advantage for protection against secondary infections.

DNAM-1 and NKG2A

A tight correlation between DNAM-1 and NKG2A has been reported before [239], however, by staining for all relevant inhibitory receptors simultaneously, we could detect that almost all NK cells expressing only NKG2A (and no other inhibitory receptor) were positive for DNAM-1 in all MHC-I backgrounds. While this observation fits with our prediction, it was surprising that this correlation was seen in all strains of mice (paper II, Suppl. Fig. 2B). NKG2A is an inhibitory receptor which binds non-classical class Ib MHC-I molecules in complex with leader peptides of certain classical class Ia MHC-I molecules, Qa-1[b] in mouse and HLA-E in human [205, 206]. Qa-1[b] is predominantly loaded with a QDM-peptide (Qa-1

determinant modifier) from H2-D or H2-L alleles, and NK cells via NKG2A, can discriminate Qa-1^b complexes with substituted peptides [207]. Consequently, in a mouse that lacks H2-D alleles, as in K^b-single or K$^{b-/-}$D$^{b-/-}$ mice, Qa-1^b is not correctly loaded. Transfection of TAP-deficient RMA-S cells with a Qa-1^b-QDM construct protects these cells from NK cell attack [400], showing that recognition via NKG2A is sufficient to mediate NK cell inhibition. However, how this affects NK cell education has not been studied. Assuming that DNAM-1 is correlated to education, the observation that expression of NKG2A and DNAM-1 correlate even in the absence of QDM-loaded Qa-1^b indicates that either, the high expression of DNAM-1 on NKG2A$^+$ NK cells is independent of education, or that NK cells are educated via NKG2A even when Qa-1^b is loaded with other peptides than QDM. At present, we cannot distinguish between the alternatives and conclude that DNAM-1 expression is connected to NKG2A expression in a manner independent of MHC-I. This is emphasized by the observation that NKG2A is associated with DNAM-1 expression also in NK cells from β$_2$m$^{-/-}$ mice.

CD155 and Dap12

In mice that lack expression of CD155, the main ligand for DNAM-1 and TIGIT, DNAM-1 expression is slightly elevated. However, the pattern of DNAM-1 expression does not change, still the highest levels of DNAM-1 cells are found within the Ly49C$^+$ and/or Ly49I$^+$ subsets, when NKG2A-positive NK cells are excluded. The frequencies of DNAM-1$^+$ NK cells were only marginally elevated, showing a deviation from the correlation between frequency of DNAM-1$^+$ cells and MFI of DNAM-1$^+$ that we observed for all strains of mice that differed only in MHC-I expression (paper II, Suppl. Fig. 1 and manuscript on CD155$^{-/-}$ NK cells in preparation). Interaction between DNAM-1 and CD155 on other cells leads to down-regulation of DNAM-1 [381, 396] and this seems to be the reason for the increase in DNAM-1 levels in CD155$^{-/-}$ mice [401]. Interestingly, another exception from the observation (paper II, Suppl. Fig. 1) was seen in DAP12$^{-/-}$ mice, were the frequencies of DNAM-1$^+$ NK cells were elevated (paper II, Suppl. Fig. 3D), while the average expression level was not (data not shown). This supports our tentative conclusion that signaling by other activating receptors, such as Ly49D, may decrease the number of NK cells expressing DNAM-1 by counteracting the inhibitory signals during education. The DAP12$^{-/-}$ mice used in this study express a DAP12 with mutated ITAM which is signaling deficient, therefore Ly49D is expressed but the activating signals is not transmitted. In paper II we are quite cautious about this finding, as the DAP12$^{-/-}$ mice are on H-2^b background. There is only one study showing that Ly49D binds to an MHC-I molecule in this background, and that was done using non-glycosylated Ly49D [402]. Other than that there is no published evidence that D^b can serve as a ligand for Ly49D. However, this study and other unpublished results of our group argue for an interaction between Ly49D and D^b, albeit weaker than that of Ly49D with D^d. In paper II we propose that DNAM-1 levels are up-regulated by education via inhibitory Ly49rs. Further assuming that signals from activating receptors contribute to education by decreasing the net signaling threshold of NK cells, one can hypothesize that activating receptor signaling decreases DNAM-1 levels. The finding that NKG2D$^{-/-}$NKp46$^{-/-}$ double

knockout mice [403] and DAP12$^{-/-}$ mice (paper II, Suppl. Fig. 4D) have elevated frequencies of DNAM-1$^+$ NK cells lend some support to tuning of DNAM-1 levels by education.

The DNAM-1-TIGIT-CD96-CD155-CD112 network

Two additional adhesion molecules, CD96 and the inhibitory molecule TIGIT, are both expressed on NK and T cells and also bind to CD155 [404-407]. The receptors TIGIT and CD96 which bind to the same ligands as DNAM-1 but with distinct affinities could aid in fine-tuning NK cells responses reminiscent of the CD28-CTLA-4-CD80-CD86 network, which tightly regulates activation of T cells and thus ensures T cell activation only under appropriate circumstances [408]. TIGIT exerts inhibitory functions on NK cells by limiting granule polarization and thereby inhibiting the cytotoxicity against CD155-expressing target cells [405, 409, 410]. In addition, the phosphatases SHP-1, SHP-2 and SHIP-1 can be recruited by an ITIM in the cytoplasmic domain of TIGIT and contribute to inhibition of effector responses [409, 410]. For CD96 both activating and inhibitory functions have been described [410, 411], and in CD96$^{-/-}$ mice it has been shown that CD96 limits NK cell function competing with DNAM-1 for binding of CD155 [412]. When we analyzed CD96 and TIGIT on NK cells, we found that CD96 is expressed at similar levels on all NK cell subsets, and that TIGIT expression is inversely regulated to that of DNAM-1, with the highest levels on NK cells that express neither Ly49 inhibitory receptors nor NKG2A and similarly, higher levels in MHC-I-deficient mice than wt B6 mice (data not shown). The higher levels of DNAM-1 and lower levels of TIGIT on educated NK cells, combined with constant levels of CD96, may be a means to regulate effector functions of educated NK cells. The DNAM-1-TIGIT-CD96-CD155-CD112 network of positive and negative signaling receptors might regulate the threshold of NK cell activation and may thus be involved in maintenance of tolerance to self, while allowing for vigorous effector functions against infected or transformed cells [412]. It therefore does not seem astonishing that several groups could show that inhibitory interactions between TIGIT and CD155/CD112, very much like inhibitory Ly49 receptor interaction with MHC-I are dominant over the combined activating potential of the activating receptors including DNAM-1 [404, 405]. In cases of paired receptors sharing the same ligands, often the inhibitory receptor has a higher affinity for the shared ligand which implies that the activating receptor needs to out-compete the inhibitory receptor to signal. Blocking immune checkpoints with mAbs is proving successful as therapies against multiple types of cancers. Similarly, blocking the inhibitory interaction of CD96 with CD155 leads to increased anti-tumor responses of NK cells in a mouse model [412, 413]. Combination of anti-CD96 with anti-PD-1 has shown even greater responses in controlling lung metastasis [413]. Upregulation of DNAM-1 ligands by genotoxic drugs or irradiation therapy, combined with checkpoint blockade, e.g. anti-PD-1 or anti-KIR or possibly anti-CD96 may increase NK cell responses and reactivity against human tumors.

3.3   SURFACE RECEPTORS THAT CHANGE UPON RETUNING

In paper I, we employed several experimental models for retuning of NK cells. This allowed us to search for surface markers that would change during the re-tuning process, i.e. markers

that might be used to distinguish educated from hyporesponsive NK cells. Which changes occur during education are to date not known. How does education work at the molecular and mechanistic level? What are the molecular changes? What has been observed so far are very subtle changes, e.g. down-regulation of Ly49 per-cell-levels in the presence of the cognate MHC-I, small increases of those NK cell subsets that express only the particular Ly49r specific for self MHC-I, a general increase of cells expressing 1-2 and a decrease in cells expressing 3 or more inhibitory receptors. While these subtle differences may explain how the frequency of "potentially useful" NK cells increases, they do not explain what is so fundamentally different between an educated and a hypo-responsive NK cell. Several groups, including my own, have tried to determine changes in expression of genes on a global level, using microarrays to quantify mRNA. Most of these studies were inconclusive, which suggests that the difference may not be a matter of expression level of molecules, but rather their localization within the cell or within the cell membrane. Cell surfaces are compartmentalized into distinct regions, which differ in membrane composition. Some of these regions, so called membrane-rafts, attract specific proteins which results in signaling clusters. In fact, one group recently showed that the distribution of activating receptors is different, and that on non-MHC-I-educated NK cells, activating receptors are confined together with inhibitory receptors. In educated NK cells on the other hand, the activating receptors became dynamically compartmentalized in functional microdomains [414]. Furthermore, signaling via inhibitory KIRs leads to dissociation of the small adaptor protein Crk from cytoskeletal scaffold complexes, which enables subsequent activation events by activating receptors [202]. A possible conclusion from these and other studies is that education does not change the amount of certain receptors or adaptors, but rather the localization or availability or signaling complexes. Furthermore, education appears to induce changes that are close to the membrane, as changes in responsiveness induce alterations in the phosphorylation state of signaling molecules Akt and Erk [372].

There is no one single molecular marker that can be used to distinguish educated from non-educated NK cells. By stimulating NK cells *in vitro* with plate-bound mAb or tumor cells, and staining for inhibitory receptors in addition to the degranulation marker CD107 and intracellular cytokines, e.g. IFNγ, we can determine which cells are more responsive and can correlate this to the MHC-I alleles these NK cells have been educated against [415]. This knowledge can then be transferred to different situations, where we assess another marker, in my case DNAM-1 or KLRG1, together with inhibitory receptors in different mouse strains with known MHC-I expression. This approach allows the researcher to correlate markers with NK cell education. But it does not provide an answer as to the reason for the correlation, not to mention the possible mechanism. Finding such a molecular marker for educated NK cells would enable clinicians to sort and enrich these cells before using them in therapeutic approaches.

Of all surface markers we assessed, only expression of KLRG1 changed in all three settings in which we observed an adaptation of responsiveness. The surface expression of KLRG1 was reduced after blockade of inhibitory receptors (paper I, Fig. 2D-E) and after transfer of

B6 NK cells to $\beta_2 m^{-/-}$ mice, while it was increased after transfer of $\beta_2 m^{-/-}$ NK cells to an MHC-I$^+$ environment (paper I, Fig. 5C-D). This was detectable as an increase in NK cells that expressed KLRG1 (frequency of KLRG1$^+$ NK cells), and as an increase of average expression per cell (MFI). Furthermore, in the transfer settings, NKG2D surface levels were increased when NK cells gained responsiveness, and decreased when tolerance was induced (paper I, Fig. 5A-B). This could be seen only when MFI was analyzed, as still most NK cells were positive for NKG2D. Additionally, the surface expression of DNAM-1 behaved in a similar way after transfer in both directions (paper II, Fig. 8). The change in surface levels were marginal, but we could see an increase in average DNAM-1 levels per cells (MFI) when $\beta_2 m^{-/-}$ NK cells were placed into an MHC-I$^+$ environment and a decrease when the cognate MHC-I interaction was removed, which, similar to KLRG1, suggests that the cells expressing the respective marker adjust the levels of it.

KLRG1 and DNAM-1 are both adhesion molecules expressed by 50-60% of NK cells in wt B6 mice. While DNAM-1 is activating, KLRG1 has an ITIM in the cytoplasmic domain that can transmit an inhibitory signal upon engagement. However, due to the low affinity of KLRG1 to its ligands, it has been suggested that physiological levels of KLRG1 are not sufficient for inhibition [277]. The higher level observed on educated NK cells could therefore have a functional impact. DNAM-1 is expressed early during NK cell development, and the levels decrease during maturation ([387] and paper II). KLRG1 expression on the other hand, is initiated on terminally mature cells [130]. While both molecules are induced upon infection with MCMV [270, 271, 329], presence of DNAM-1 but absence of KLRG1 are beneficial for generation of MCMV-specific memory cells [329, 331]. Despite these opposing functions of the two markers, they are both preferentially expressed by educated NK cells (paper I, paper II and [157, 216, 278, 386]). One hypothesis is that it is primarily their function as adhesion molecules, and secondarily the activating/inhibitory function, that endows educated NK cells with a functional advantage. The ligands for both adhesion molecules are expressed on many cell types, including hematopoietic cells, which could provide educated NK cells with a means for interaction with surrounding cells.

3.4 NK CELL-MEDIATED KILLING OF DENDRITIC CELLS AND THE IMPACT OF PAIRED RECEPTORS

Education provides NK cells with a means to strongly react to dangerous and non-self cells, while at the same time robust tolerance to healthy self cells is achieved. One exception is the interaction with autologous DCs. NK cells can bi-directionally communicate with DCs, which can lead to an increase in the function of both cell types, but can also result in killing of the DC [32, 35, 63, 312-314, 316, 317, 416-418]. DCs produce cytokines such as IL-12 and IL-15 that are important for NK cell development and function [63, 417], and activated NK cells can secrete GM-CSF, TNFα and IFNγ cytokines which affect differentiation and maturation of DCs under inflammatory conditions [419]. Furthermore, NK cells can kill DCs in certain situations, which may help to modulate adaptive immune responses. It was proposed that during an infection, NK cells kill some DCs to select for those DC with the

propensity for potent stimulation of T cells and NK cells [311]. This model was later termed DC editing [418]. Most studies that have investigated the NK cell-mediated killing or activation of DCs involved DCs generated under inflammatory conditions [309, 314, 382, 420] or NK cells activated by MHC-I- deficient cells [421, 422]. It is still unclear which NK cell subsets are involved in the DC editing effect, although involvement of NKG2A$^+$KIR$^-$ or DNAM-1$^+$NK cells has been suggested [314, 423]. Both cell-to-cell interactions and various cytokines released by both cell types have been suggested to play a role during the early phases of innate immune responses [314].

In paper III we have investigated the effect of inflammatory conditions on the sensitivity of DCs to NK-mediated killing. We could show that DCs generated from BM (BMDC) in the presence of Flt3L or isolated from mouse spleen were relatively resistant to NK cell-mediated cytotoxicity. However, DCs became more susceptible in the presence of the inflammatory cytokine GM-CSF both *in vitro* and *in vivo* (paper III, Figs. 1+2). GM-CSF is produced by NK cells upon stimulation with IL-18, and this is partly responsible for the increase in sensitivity of DCs. Culture of DCs with GM-CSF, or treatment of mice with IL-18, resulted in an increase in the surface markers ICAM-1 (CD54), CD80, CD86, PD-L1 and the DNAM-1-ligands CD112 and CD155 (paper III, Fig. 3). Classical and non-classical MHC-I molecules and CD40 were also slightly up-regulated. In an attempt to find the receptor-ligand interactions that are responsible for the observed increase in susceptibility, we used knock-out mice for CD80, CD86, PD-1, CD112 or CD155 for generation of BMDCs, or blocked CD40, ICAM-1 or PD-L1 during the *in vitro* killing assay. A reduction in NK-mediated killing of the DCs was observed only in two situations; firstly, when interactions of ICAM-1 on DCs with LFA-1 on NK cells were blocked, and secondly, when DNAM-1 on NK cells was blocked from binding to its ligands on DCs (paper III, Fig. 5). Further we could show that it is a specialized subset of NK cells, expressing DNAM-1 but not NKG2A, which is superior in killing DCs. However, CD155$^{-/-}$ BMDCs were even more susceptible to killing by NK cells (paper III, Fig. 5).

These data suggest that under inflammatory conditions, such as in the presence of GM-CSF or IL-18, expression of CD155 and ICAM-1 are induced on DCs which makes them more vulnerable to NK cell attack. It has been previously hypothesized, that NK cell killing of DCs is a way of terminating an inflammatory response induced by DCs [311, 418, 421]. Along these lines, in has been shown that also IFNγ and TNFα produced by NK cells can cause up-regulation of ICAM-1 and induce conjugate formation and target cell cytolysis [424]. In support of this, GM-CSF has been used in combination therapy together with the anti-GD2 mAb (dinituximab) in a phase II clinical trial for neuroblastoma [425, 426].

Previous studies have mainly focused on inflammatory DCs [309, 314, 382, 420]. Our study shows that BMDCs generated under non-inflammatory conditions or isolated from mouse spleen, are relatively resistant to NK cell killing, and that the levels of ligands for both activating and inhibitory receptors are lower. Once DCs are matured, and NK cells activated, the balance of activating and inhibitory ligands is shifted and the expression of activating

receptors (such as DNAM-1) and inhibitory receptors (such as NKG2A) determines the outcome of that interaction. It is noteworthy, that the DNAM-1$^+$NKG2A$^-$ NK cell subset represent a small percentage of NK cells, probably because expression of NKG2A appears to maintain high levels of DNAM-1 (paper II). However, this subset is efficient in killing, as it is not inhibited by non-classical MHC-I, which is also higher on DCs in inflammation (paper III, Fig. 5).

Similar to DNAM-1$^+$ mouse NK cells, DNAM-1$^+$ human NK cells are involved in killing of human monocyte-derived DCs [314, 382, 420]. Furthermore, killing of pDCs has been shown to depend on expression of CD155, but not CD112 [314], which is similar to our results showing that CD112 does not play a role in NK cell-mediated killing of DCs. During HCMV infection, DNAM-1 ligands and HLA-E are reduced over time, making these cells more susceptible to DNAM-1$^-$NKG2A$^+$ NK cells [382] In contrast, a mouse study shows that both CD155 and CD112 are rapidly upregulated on infected DCs and macrophages upon infection with MCMV [329]. Additionally, it has been shown that anti-DNAM-1 could inhibit CD86 upregulation on DCs, and simultaneous inhibition of NKp30 and DNAM-1 completely abrogated maturation of human DCs [309]. It is therefore reasonable to assume that both in mouse and man, different subsets of NK cells play a role both in controlling DCs and in providing maturation stimuli at different time-points during an inflammation.

NK cells with inhibitory receptors specific for MHC-I can detect DCs which, during an immune response or under homeostatic conditions, do not reach sufficient levels of MHC-I expression. NK cells could favor DCs that have high levels of MHC-I but also co-stimulatory receptor ligands and are thus good for T and NK cell priming and eliminate those that fail to undergo maturation [311, 421, 423]. This of course requires that the NK cells have inhibitory receptors able to recognize autologous MHC-I, which we could show in paper II is correlated to expression of DNAM-1. Additionally, NK cells with Ly49r for self-MHC-I have higher levels other adhesion molecules, such as KLRG1 (paper I and [157]). The ligands, cadherins are expressed by DCs and may contribute to binding to NK cells and some T cell subsets that express KLRG1. Expression of MHC-I-specific inhibitory receptors and DNAM-1 enables NK cells to mediate the "editing program". We could show that DNAM-1$^+$NKG2A$^-$ NK cells are superior in killing DCs under inflammatory conditions (paper III, Fig. 5). However, when we tested killing of non-inflammatory DCs, we hardly observed any killing by this subset (unpublished results). DCs provide tonic stimuli needed to keep NK cells in a primed state [63] and Luu et al, manuscript submitted), possibly even providing NK cells with the opportunity to interact with MHC-I$^+$ cells to maintain education.

These data together with our findings that DCs were killed under inflammatory but not non-inflammatory conditions could be interpreted in such a way that in the absence of inflammation, there are interactions of the two cell types which do not result in killing of DC, but rather in a productive communication between DCs and NK cells which may result in enhancement of functions of both cell types such as education of NK cells. A recent report has shown that DNAM-1$^+$ NK cells respond better to IL-15 signaling and express more cell-

cycle and survival-related genes [387]. The lower IL-15 reactivity of DNAM-1^{-} NK cells might be a result of insufficient crosstalk between NK cells and DCs.

DCs generated from CD155^{-/-} BM were killed better by NK cells. This result was at first surprising, but can be explained by the complex nature of paired receptors that share the same ligands [427]. Besides DNAM-1, NK cells have two additional receptors that can recognize CD155, the inhibitory receptor TIGIT and CD96, for which both activating and inhibitory functions have been described [405, 410-412]. CD155 and CD112 are up-regulated on many tumor cells [189, 428], and it has been shown that the interaction of DNAM-1 with its ligands leads to destruction of the target cell, but on the other hand both molecules are expressed on healthy epithelial or endothelial cells and cells of the hematopoietic lineages. It would be detrimental should NK cell attack these normal healthy cells. It therefore does not seem astonishing that several groups could show that inhibitory interactions between TIGIT and CD155/CD112, very much like inhibitory Ly49 receptor interaction with MHC-I are dominant over the combined activating potential of the activating receptors including DNAM-1 and CD96 [404, 405, 410]. The DNAM-1-TIGIT-CD96-CD155-CD112 network of positive and negative signaling receptors might regulate the threshold of NK cell activation and may thus be involved in maintenance of tolerance to DCs under steady state conditions, while allowing for vigorous effector functions against DCs under inflammatory conditions [412]. This system may provide NK cells with a means of appropriate self-tolerance before the regulation by MHC-I-specific activating and inhibitory receptors during development. Our data on the early expression of DNAM-1, TIGIT (paper II, Fig. 7) and CD96 (manuscript in preparation) supports this hypothesis.

Fascinatingly, of the molecules that were increased on GM-CSF-treated DCs, only ICAM-1 and CD155 appeared to play a role in NK cell-mediated killing of inflammatory DCs. ICAM-1 is recognized by LFA-1, and this interaction is necessary for polarization of cytotoxic granules when NK cells interact with target cells [429]. LFA-1 and DNAM-1 are physically and functionally associated in lipid rafts [185-187] and engagement of LFA-1 and subsequent inside-out signaling recruits DNAM-1 to the immunological synapse and this is increased when both LFA-1 and DNAM-1 are engaged with ligands on the target cell [386]. Furthermore, educated NK cells are more prone to recruitment of DNAM-1 and activation-induced conformational changes of LFA-1 upon encounter with a target cell [386].

The bi-directional crosstalk between NK cells and DCs, which can result in increase of functions of both cell types, and in a selection for Th1-promoting DCs, should be taken into account for cell-based therapies. Moreover, studies in mice show that mature DCs could induce a Th1 polarization during the early phase of T cell priming which is defective in NK-deficient infection models [35, 430, 431].

Both NK cells and DCs are used in the clinic as cellular therapy for treatment of leukemia and solid tumors. It has been shown that in haplo-identical HSCT, donor NK cells kill the patient's DCs, thereby preventing the development of GvHD [333]. Additionally, DNAM-1^{+} NK cells can lyse activated T cells which express high levels of CD155 in response to

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proliferation, thereby limiting GvHD [183]. This effect could be even mediated by autologous DNAM-1$^+$NKG2A$^-$ or DNAM-1$^+$Ly49r$^-$ NK cells, as they are not inhibited by the expression of MHC-I on DCs or T cells. The interactions between the two cell types could also have an impact on transferred DCs generated *ex vivo*. Different clinical centers use different protocols to generate and activate the DCs. Our results in paper II demonstrate that the *in vitro* culture conditions have a profound impact on the outcome of the interaction.

Inflammatory conditions may influence NK cell immune surveillance. NK cells react upon infection with many pathogens, and kill cells with low or no MHC-I. It is however less clear how NK cells react to developing tumor cells. At the beginning of tumor development, NK cells most likely kill most cells with aberrant MHC-I expression. But once the number of MHC-I- cells overwhelms NK cells, they seem to become retuned and functionally impaired to kill MHC-I$^-$ tumor cells [372]. Acute inflammation as in MCMV infection, can revert anergy and break tolerance towards MHC-I$^-$ deficient cells [303]. The low levels of inflammation within many tumors may therefore diminish NK cell responses. On the other hand, it has been suggested that low grade inflammation is a driver for tumor development, but it has not been studied how this affects NK cells. Initiation of an inflammatory response may alert NK cells to the imminent danger of transformed cells. The differential expression of DNAM-1 and inhibitory receptors for MHC-I may ensure responsiveness under inflammatory conditions.

3.5   EXOSOMES AS ANTI-CANCER VACCINE

DCs regulate NK cell functions via cell-cell contacts and by release of cytokines and chemokines. In addition, DCs can secrete exosomes, 30-100 nm small vesicles consisting of a lipid bilayer that derive from multivesicular bodies (MVB) within the late endosomal compartment. In this thesis exosomes were used to study NK cell responses induced by stimulation of iNKT cells using αGalCer. Activation of iNKT cells by soluble αGalCer has been used successfully in mouse studies and clinical trials for cancer [25, 26]. However, iNKT cells become anergic after the first treatment with αGalCer, while αGalCer-loaded exosomes do not induce iNKT cell anergy [432]. Exosomes are shed from different cell types, such as DCs, B cells, mast cells, platelets, erythrocytes, and tumor cells during physiological and pathological conditions. The composition of lipids and membrane-proteins depends on the cell type which secretes the vesicle. Exosomes also contain mRNA and microRNA molecules and cytosolic proteins, which after fusion with another cell, can immediately regulate transcription of different genes.

 Exosomes usually contain a narrow range of different proteins and lipids, which suggests a non-random enrichment and loading into the exosome [433]. The surface proteins of exosomes can mediate co-activation, antigen-presentation, adhesion and fusion to other cells and are usually enriched in components necessary to present antigens, such as MHC-I, MHC-II and CD1. They were originally described as a mechanism for reticulocytes to release proteins that were no longer required. An immune modulating role has later been appreciated

with the discovery that APC-derived exosomes activate other cells and even can be taken up by other cells. On the other hand, exosomes released by tumor cells can suppress T cells and NK cells and promote a general immune-suppressive environment by enrichment of suppressive factors [433, 434].

Using DC-derived exosomes without additional antigen-loading in a mouse model, tumor growth could be suppressed and established tumors were eradicated [435]. After this first success, DC-derived exosomes were specifically loaded with tumor-specific antigens, e.g. MAGE, which is expressed on many melanoma cells and by other tumors. These exosomes elicited partial responses in phase I studies for melanoma and non-small cell lung cancer [436, 437]. Importantly, while no antigen-specific T cells could be detected, NK cell infiltration into the tumor and activation of NK cells with increased *in vitro* responses towards tumor targets could be observed [436, 437]. Assessment of surface molecules of DC-derived exosomes revealed the presence of IL-15Rα on exosomes from both human and mouse DCs, which stimulated NK cell proliferation [438]. Furthermore, ligands for the activating receptor NKG2D were detected on human DC-derived exosomes. Soluble ligands for NKG2D have been proposed to desensitize NKG2D on NK cells, but when presented in the context of an exosomal membrane, NK cells were activated via NKG2D [438].

In an experimental approach aiming to modulate exosome composition, mouse DCs have been cultured in the presence of peptide and αGalCer. Exosomes harvested from these cultures have the potential to stimulate antigen-specific T cell responses and activate iNKT cells via αGalCer presented on CD1d *in vivo*. Treatment of mice with exosomes loaded with both the peptide and αGalCer have been shown to elicit B and T cell responses superior to those after simultaneous treatment with exosomes loaded with either peptide or αGalCer, showing that the immunogenicity of DC-derived exosomes can be further enhanced when several cell types are targeted simultaneously [432].

In paper IV we addressed NK cell activation after treatment of mice with αGalCer, either soluble or exosome-bound. The exosomes we used were loaded with αGalCer to activate iNKT cells and the model peptide antigen OVA to provide stimulation for T cells. These exosomes were produced similarly to the ones used in the study by Gehrman et al. where synergistic induction of adaptive antitumor responses by co-delivery of peptide antigen and lipid antigen has been demonstrated [432]. The tumor used in that study, B16/OVA stably expresses the antigen and can thereby be recognized by OVA-specific T cells. However, since B16 naturally has only very low levels of MHC-I, it is also recognized by NK cells [439]. In paper IV, we therefore addressed NK cell anti-tumor functions and missing self recognition when activated in an iNKT cell-dependent manner. We could show that iNKT cells activated with αGalCer, either soluble or exosomal, mediate activation and proliferation of NK cells and induce up-regulation of KLRG1 (paper IV, Figs. 1 and 6). *Ex vivo* NK cell responses measured by degranulation and IFNγ production were augmented and this could be further increased by stimulation with YAC-1 tumor cells or crosslinking of the activating receptors NK1.1 or NKp46 (paper IV, Fig. 2 and data not shown). Interestingly, mainly the

KLRG1$^+$ NK cells responded with IFNγ production and degranulation. Previous studies on KLRG1 have demonstrated a rather weak inhibitory effect of KLRG1 at physiological level on mouse NK cells [277]. The observed drastic increase after iNKT cell activation may increase the inhibitory potential of this receptor and may thus allow KLRG1^{high} NK cells to functionally interact with cadherin-expressing cells, e.g. DCs without killing them. Furthermore, KLRG1 physically associates with the transferrin receptor in *cis*, sequestering KLRG1 from interacting with its ligands, which limits the inhibitory effect of KLRG1. Notably, transferrin receptor expression is induced on activated and proliferating cells, making KLRG1 available for binding to cadherins in *trans* [440].

Given the importance of educated NK cells in anti-tumor immunity, we determined whether proliferation and activation were different between NK cells that could recognize self-MHC-I and those that could not. Treatment with either soluble or exosomal αGalCer led to activation of all NK cell subsets (data not shown), but induced proliferation predominantly of educated NK cells (paper IV, Fig. 4). To confirm that it is specifically educated NK cells that preferentially proliferate, we used mouse strains expressing different MHC-I alleles. Indeed, the Ly49C$^+$Ly49I$^+$NKG2A$^+$ subset had a higher proliferation index in B6 mice, where Ly49C, Ly49I and NKG2A can bind to self MHC-I, while the Ly49A$^+$Ly49G$_2$$^+$ NK cell subset proliferated more in a mouse expressing D^d, which is recognized by Ly49A and Ly49G$_2$.

In a recent study it has been shown that during a response to MCMV infection, uneducated, hypo-responsive NK cells primarily proliferate and dominate the response to the infection [305]. However, in the MCMV model of infection, the virus is specifically recognized by the activating Ly49H receptor, which binds to the MCMV-encoded surface protein m157. About 50% of NK cells in B6 express Ly49H. Orr et al show that among the Ly49H$^+$ NK cells, the ones with self-specific Ly49C or Ly49I are inhibited due to recognition of self MHC-I and therefore respond less well. While the study clearly demonstrates that Ly49C/I NK cells respond better and dominate the response, it does not show that educated NK cells do not respond to viral infection or that they are useless. Activation via Ly49H is such a strong cue, which activates both educated and hypo-responsive NK cells. However, since educated NK cells are "inhibit-able" because they express Ly49rs specific for self-MHC-I, they respond less. The "gain in function" inflicted by education does not reach the unimposed activation of Ly49C/I NK cells. Experiments with Ly49H-deficient mice or Δm157-MCMV could provide information on whether educated or uneducated NK cells can do better during infection. However, another recent study analyzing influenza virus infection in mice confirms the dominant role of uneducated, hypo-functional NK cells in the response [306]. It is not known how NK cells interact with influenza-infected cells, but a specific recognition of influenza hemagglutinin by activating receptor NKp46 has been suggested [150]. In contrast, by using αGalCer-induced iNKT cell-dependent mechanism for NK cell activation, we could show that educated MHC-I-recognizing NK cells proliferate better. The difference may be explained by the different mode of stimulation, receptor-mediated versus cytokine-mediated, or it may be that the specific mixture of cytokines has different influence on educated versus

hypo-responsive NK cells. One hypothesis is that in our system, exosomes are taken up by host DCs, which in turn provide stimulation of NK cells, possible via surface-bound IL-15 [63, 417]. As educated NK cells can respond better to stimulation by IL-15 [157], these NK cell subsets would then proliferate preferentially in our system. Our findings support the notion that distinct NK cell subsets are useful in different physiological and pathological situations.

Previous studies have suggested that the functions of exosomes are mediated by uptake of injected DC-derived exosomes by host DCs [435]. In fact, in a recently accepted paper my collaborators can show that the whole proteins in exosomes are degraded and presented by host DCs on the host MHC-I (Hiltbrunner & Larssen, in press), which corroborates the idea that the effects are mediated by host DCs which have taken up the injected exosomes. This finding is promising for the clinical applicability of exosome immunotherapy, as it implies that exosomes do not necessarily need to be generated from autologous (the patient's) DCs.

To test whether the increased proliferation of educated NK cells would lead to any functional responses, we used *in vivo* methods to determine missing self responses and anti-tumor responses. The *in vivo* target cell elimination assay, which I have used throughout my studies, is the only way to measure NK cell responses that are entirely dependent on recognition of missing self. By simultaneously using two target cell populations that differ only in their MHC-I expression, the differential killing of the MHC-I$^-$ target cells, measured as preferential survival of the MHC-I$^+$ cells, can be determined. This assay allows assessment of a functional increase of specifically educated NK cells, as only NK cells educated on MHC-I will detect missing MHC-I on the MHC-I$^-$ target cells. Wt B6 mice treated with αGalCer, both soluble or exosomal, showed increased missing self responses (paper IV, Fig. 5-6). Importantly, the treatment did not induce killing of MHC-I$^-$ target cells in MHC-I$^{-/-}$ mice, despite comparable activation of NK cells in these mice (paper IV, Suppl. Fig. 1), which shows that tolerance is not broken and NK cells do not start to kill healthy autologous cells. Furthermore, this finding demonstrates that education is necessary for the αGalCer-enhanced missing self responses towards otherwise healthy cells.

When we tested the capacity to kill tumor cells, we used the same cells as in paper I, RMA and RMA-S. In this study however, the assay was somewhat modified. Spleen cells from NK1.1-depleted wt B6 mice were co-injected as reference cells together with the two tumor cells lines, as an increase in killing of both tumors was anticipated. Indeed, this reference population enabled us to detect an increase in killing of both tumors, MHC-I$^+$ and MHC-I$^-$. Interestingly, in this case, αGalCer-mediated iNKT cell activation lead to an increase of tumor killing in MHC-I$^{-/-}$ mice as well.

Previous studies suggested a major role of NKG2D ligands in exosome-mediated NK cell activation [438]. NKG2D ligands were absent from exosomes used in our study. Furthermore, we did not detect any changes in NKG2D levels on NK cells. However, non-significant down-regulation of NKp46 could be detected (data not shown), which may indicate that NK cells encounter NKp46 ligands and the receptor levels are lower due to

ligand-induced receptor down-regulation. Whether ligands for activating receptors on exosomes have activating or desensitizing effects, as suggested for soluble NKG2D ligands, is unknown. The study by Viaud et al. however could show NK cell activation after encounter with exosomal NKG2D ligands [438].

Exosomes, with the abundant expression of MHC-I, MHC–II, co-stimulatory molecules, and possible activating receptor ligands, can function as an antigen-presenting entity, and can be used as vehicles for antigen-based anti-tumor vaccines. Similar to DC-based vaccines, they induce activation and proliferation of immune cells. However, in the tumor microenvironment, DCs may be subjected to suppressive mechanisms and may down-regulate co-stimulatory molecules, which impairs antigen presentation functions. Exosomes should be unaffected by such mechanisms. Furthermore, the membrane lipids of exosomes can have adjuvant functions and can further boost an immune response. In fact, the first study using exosome therapy against tumors in a mouse model, demonstrates that not only can exosomes substitute for DCs, they also display superior immune stimulation which leads to eradication of established tumors [435].

In paper IV we show that iNKT cell activation can enhance NK cell-mediated missing self responses towards healthy cells with aberrant MHC-I expression and tumor cells, which should be taken into account when designing vaccines against cancer. By including the innate arm of the immune system, adaptive responses can be shaped and fine-tuned by production of cytokines and early target cell elimination, which in turn increases antigen presentation. By induction of CTL responses against MHC-I$^+$ tumors and NK cell responses against MHC-I$^-$ tumors, NKT cell-targeted therapy thus offers the potential for a successful therapy against cancer.

3.6 CONCLUDING REMARKS

Protection against infected and transformed cells is vital for any organism to survive. NK cells, with their inherent ability to detect loss or down-regulation of MHC-I as well as molecules induced by cellular stress, are key players in immune surveillance. With the discovery of missing self recognition 30 years ago, and the continuously growing knowledge about NK cell biology, it does not seem surprising that these cells are considered in many immune therapeutically approaches for treatment of cancer. In fact, the first cell-based adoptive immunotherapy approach, infusion of *ex vivo* activated LAK cells, turned out to be NK cells. This and other approaches have been and are refined ever since, fueled by the increasing appreciation of NK cell missing self responses against tumor cells.

This thesis aimed to explore how NK cell education may impact immunotherapy against cancer. The data and conclusions from the papers presented show that a balance between activation and inhibition is crucial for NK cells to perform anti-tumor responses while at the same time being tolerant to healthy and non-dangerous cells. Tolerance to healthy cells is robust, even in the absence of cognate MHC-I. This fine-tuned balance between being active against potentially dangerous cells, and being tolerant to cells that, despite low levels of self-ligands, do not represent a threat, is mediated by a multitude of inhibitory receptors, activating receptors and adhesion molecules. A tight correlation between some of these molecules appears to be beneficial.

The main conclusions from each paper of this thesis are as follows:

- NK cells adapt their responsiveness according to the integrated net signaling input from interactions with surrounding cells. This can occur in different forms of immunotherapy based on missing self-recognition. The adaptation ensures both tolerance to non-dangerous cells, while reactivity towards malignant cells is maintained (**paper I**).
- Expression of the adhesion molecule DNAM-1 is correlated to NK cell education. Furthermore, there is a tight association between DNAM-1 and NKG2A expression, independent of education. DNAM-1 is expressed at high levels early during development and then decrease gradually during maturation so that it is eventually lost in some NK cells. We propose a model in which NK cell education can prevent the loss of DNAM-1 and in some cases even increase the expression, which may be useful for educated NK cells to perform their functions (**paper II**).
- Although NK cell education appears to result in robust tolerance to normal host cells it is clear that NK cells can kill autologous DCs. In **paper III** we show that depending on the context - normal or inflammatory conditions - NK cells may kill autologous DCs. Production of GM-CSF by NK cells induces up-regulation of surface molecules on DCs, such as ICAM-1 and CD155, which makes them susceptible to NK cell attack.

- Activation of iNKT cells via (exosomal) αGalCer induces activation and strong proliferation of educated NK cells and potentiates killing of normal as well as tumor cells with the "missing self phenotype" (**paper IV**).

These conclusions allude to the potential that NK cells offer in therapy against cancer, and also point out the need for further study. They emphasize that combination of different approaches to increase NK cell responses to tumor cells is advisable. Furthermore, careful stratification of donor and recipient MHC-I genotype and as well as receptor phenotype of donor NK cells seems to be key for the success of immunotherapy.

What has been considered basic research just some few years ago, has a major impact on development of clinical therapies today. I truly believe that gathering knowledge about biology and function of the different cell types of our immune system will help to develop new and to improve existing therapies and may one day lead to cure for cancer.

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