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Natural Killer Cells in Cancer: Studies on Migration and Cytotoxicity

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NATURAL KILLER CELLS IN CANCER: STUDIES ON MIGRATION AND CYTOTOXICITY

Erik Wennerberg

Karolinska Institutet

Stockholm 2014

Foreword

The opportunity to write a thesis is a chance to finally summarize all of the work that you and your group have done over the past four years and to be proud of what you achieved together. The group started in the beginning of 2010 with only Andreas, Dhifaf and me and we were eager to get started with doing all the things that we heard Andreas had done back in the States. We had the aspiration that we would become a well-known NK cell group at KI and that our research would one day form the basis for a clinical trial testing NK cell-based therapy in cancer patients. Today, four years later, these aspirations are still very much alive and our contributions to the scientific community have finally started to bear fruit. And yes, the aspiration that NK cells can be used to treat cancer patients in Sweden is still there and within grasp. For helping me keep these dreams alive despite setbacks and disappointments, I can only thank my supervisor and friend Andreas Lundqvist. I know that the future is as bright and as confident as an NK cell expressing loads of NKG2D and CXCR3.

The product of mental labor - science - always stands far below its value, because the labor-time necessary to reproduce it has no relation at all to the labor-time required for its original production.

Karl Marx

Populärvetenskaplig sammanfattning

När en genetisk skada uppstår i en cell kan det leda till mutationer. Cellen försöker då att reparera skadan men om detta misslyckas försöker den ta död på sig själv genom så kallad programmerad celldöd. Om även detta misslyckas riskerar cellen att utsättas för ytterligare mutationer och utveckla egenskaper som bland annat gör att den börjar dela sig okontrollerat och bilda en tumör. Vårt immunförsvar består av flera olika celltyper som har till uppgift att bekämpa infektioner men som också hjälper kroppen att upptäcka och oskadliggöra cancerceller. I och med att cancerceller utvecklas från den egna kroppen så är det svårare för immunförsvaret att känna igen dem som något farligt i motsats till t.ex. bakterier och virus som kommer utifrån. Den immuncell som är mest effektiv på att känna igen och ta död på tumörceller är de så kallade natural killer (NK) cellerna. NK cellerna patrullerar våra kroppar under hela vår livstid och de interagerar ständigt med omgivande celler för att kontrollera om de har blivit infekterade med virus eller om de har kännetecken av tumörceller.

Immunterapi mot cancer kan utformas på många olika sätt och under senare år har potentialen av NK celler börjat utnyttjas för att behandla patienter med avancerad cancer. Så kallad adoptiv NK cellterapi går ut på att man isolerar NK celler från blodet på en cancerpatient eller en frisk donator. Dessa celler kan sedan odlas och manipuleras under varierande former i laboratoriet för att få NK cellerna att dela sig och för att få dem att bli bättre på att känna igen och döda olika typer av cancer. Denna process, vilken kallas expansionsfasen, tar ca två veckor och under tiden behandlas patienten med t.ex. cytostatika och stålningsterapi. Detta görs både för att bekämpa cancern men också för att ta bort patientens kvarvarande immunceller så att det finns plats i blodet när man sedan sprutar in de expanderade NK cellerna.

I den här avhandlingen har vi studerat hur man kan förbättra adoptiv NK cellterapi genom att angripa tre olika problem som i nuläget hindrar den kliniska effektiviteten av NK cellsterapi. 1) *Öka tumörcellernas känslighet för avdödning av NK celler*. Här har vi upptäckt att genom att behandla tumörer med låga doser av cytostatika så ökar deras känslighet för att bli dödade av NK celler. Vi har även studerat hur man kan förbättra NK cellers förmåga att döda tumörceller genom att manipulera dem i laboratoriet 2) *Öka migreringen av NK celler mot tumörer*. I detta projekt har vi i möss studerat hur NK celler som sprutas in i blodet kan förflytta sig mot tumörer och infiltrera dem. Här har vi även utvärderat vilken anti-tumör effekt som infiltrationen medför. 3) *Identifiera tumörer som är naturligt känsliga för NK cellterapi.* Vi har upptäckt att den mycket aggresiva tumörtypen anaplastisk sköldkörtelcancer både är naturligt känslig för avdödning av NK celler och utsöndrar signalsubstanser som får NK celler att migrera mot tumören. Dessa egenskaper gör att patienter med anaplastisk sköldkörtelcancer, vilka i nuläget inte har tillgång till någon botande behandling, i framtiden skulle hjälpas av NK cell-baserad immunterapi.

Abstract

The role of natural killer (NK) cells in cancer development has been studied extensively over the last four decades and the increasing knowledge on NK cell regulation has improved both safety and efficacy of treatment. Despite these recent advances the clinical success has to date been modest in treatment of solid tumors, owing both to suboptimal directed migration of NK cells and the tumor cell's resistance to NK cell-mediated lysis. This thesis focuses on strategies to overcome these critical issues thus improving the anti-tumor effect of adoptive NK cell therapy. In paper I, we have studied the sensitizing effect of doxorubicin on tumor cells to NK cell and T cell-mediated lysis. The potential clinical advantage of using doxorubicin as a preconditioning agent was highlighted in a xenograft mouse model, where mice receiving low-doses of doxorubicin prior to NK cell infusion had a stronger anti-tumor effect of a subsequent NK cell treatment compared to mice receiving only NK cell treatment. Further, we identified TRAIL-signaling as the main pathway responsible for the tumor sensitization due to decreased expression of the anti-apoptotic protein cFLIP. In paper II we have established that the cytotoxicity of NK cells can be augmented by co-culturing them with monocytes in presence of the biphosphonate zoledronic acid (ZA). We observed an upregulated expression of TRAIL on NK cells, through increased levels of monocyte-derived IFNγ in the culture. Thus, NK cells primed with ZA were able to lyse TRAIL-sensitive tumors both *in vitro* and *in vivo*. In paper III, we studied CXCL10-mediated migration of NK cells toward solid tumors. We found that *ex vivo* expansion of NK cells induced a 10-fold increase in CXCR3-receptor expression, which allowed them to migrate towards tumor cells in a CXCL10-dependent manner. In two separate xenograft models we could demonstrate the anti-tumor effect of CXCL10-induced migration of adoptively transferred CXCR3-positive NK cells by their selective targeting of CXCL10-producing tumors, which resulted in reduced tumor progression and prolonged survival. In paper IV, we identified anaplastic thyroid carcinoma (ATC) as a potential novel target for NK cell therapy. We found that ATC cells were sensitive to NKG2D-mediated lysis due to high expression of ULBP2 on tumor cells. In addition, tumor cells produced high levels of CXCL10 which attracted CXCR3-positive NK cells *in vitro*. In ATC tumor samples we found a suppressed NK cell population although enriched for CXCR3 expression suggesting that CXCL10 may have been involved in the chemoattraction of the NK cells.

In conclusion, we have studied some of the important aspects of how NK cells interact with tumor cells and suggested approaches that could improve the use of NK cells in cancer therapy. Moreover, we have identified the highly aggressive tumor ATC as being uniquely sensitive to NK cell lysis and have studied the prospects of developing NK cell therapies for ATC patients.

List of publications

I. **Wennerberg E**, Sarhan D, Carlsten M, Kaminskyy VO, D'Arcy P, Zhivotovsky B, Childs R, Lundqvist A. Doxorubicin sensitizes human tumor cells to NK cell- and T-cell-mediated killing by augmented TRAIL receptor signaling. *Int J Cancer*. 2013 Mar 18. doi: 10.1002/ijc.28163.

II. Sarhan D, D'Arcy P, **Wennerberg E**, Lidén M, Hu J, Winqvist O, Rolny C, Lundqvist A. Activated monocytes augment TRAIL-mediated cytotoxicity by human NK cells through release of IFN-γ. *Eur J Immunol*. 2013 Jan;43(1):249-57. doi: 10.1002/eji.201242735.

III. **Wennerberg E**, Kremer V, Childs R, Lundqvist A. *Ex vivo* expanded human NK cells migrate toward solid tumors through a CXCL10-dependent mechanism augmenting the antitumor effects of NK cell transfer in vivo. *Manuscript*

IV. **Wennerberg E**, Pfefferle A, Ekblad L, Kremer V, Kaminskyy V.O, Juhlin C.C, Höög A, Bodin I, Svjatoha V, Larsson C, Zedenius J, Wennerberg J, Lundqvist A. Human anaplastic thyroid carcinoma cells are sensitive to NK cell-mediated lysis via ULBP2 and chemoattract CXCR3-positive NK cells. *Manuscript*

List of associated publications

A. Sarhan D, **Wennerberg E,** D'Arcy P, Gurajada D, Linder S, Lundqvist A. A novel inhibitor of proteasome deubiquitinating activity renders tumor cells sensitive to TRAIL-mediated apoptosis by natural killer cells and T cells. *Cancer Immunol Immunother*. 2013 Aug;62(8):1359-68. doi: 10.1007/s00262-013-1439-1

B. Mao Y, Poschke I, **Wennerberg E**, Pico de Coaña Y, Egyhazi Brage S, Schultz I, Hansson J, Masucci G, Lundqvist A, Kiessling R. Melanoma-educated CD14+ cells acquire a myeloid-derived suppressor cell phenotype through COX-2-dependent mechanisms. *Cancer Res*. 2013 Jul 1;73(13):3877-87.

C. Tittarelli A, Mendoza-Naranjo A, Farías M, Guerrero I, Ihara F, **Wennerberg E**, Riquelme S, Gleisner A, Kalergis A, Lundqvist A, López MN, Chambers BJ, Salazar-Onfray F. Gap junction intercellular communications regulate NK cell activation and modulate NK cytotoxic capacity. *J Immunol*. 2014 Feb 1;192(3):1313-9

D. Okita R, Mougiakakos D, Ando T, Mao Y, Sarhan D, **Wennerberg E**, Seliger B, Lundqvist A, Mimura K, Kiessling R. HER2/HER3 signaling regulates NK cell-mediated cytotoxicity via MHC class I chain-related molecule A and B expression in human breast cancer cell lines. *J Immunol*. 2012 Mar 1;188(5):2136-45

E. Kiessling R, Okita R, Mougiakakos D, Mao Y, Sarhan D, **Wennerberg E**, Seliger B, Lundqvist A, Mimura K, Kono K. Opposing consequences of signaling through EGF family members: Escape from CTLs could be a bait for NK cells. *Oncoimmunology*. 2012 Oct 1;1(7):1200-1201

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List of abbreviations

ACT	Adoptive cell transfer
ADCC	Antibody-dependent cell-mediated cytotoxicity
AML	Acute myeloid leukemia
APC	Antigen presenting cell
ATC	Anaplastic thyroid carcinoma
BMT	Bone marrow transplantation
CAF	Cancer-associated fibroblast
CAR	Chimeric antigen receptor
CCL	Chemokine C-C motif ligand
CCR	Chemokine C-C motif receptor
CD	Cluster of differentiation
cFLIP	Cellular FLICE inhibitory protein
CMV	Cytomegalovirus
COX-2	Cyclooxygenase-2
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
CXCL	Chemokine C-X-C motif ligand
CXCR	Chemokine C-X-C motif receptor
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DcR	Decoy receptor
DISC	Death-inducing signaling complex
DNA	Deoxyribonucleic acid
DNAM-1	DNAX-accessory molecule 1
DR	Death receptor
EBV	Epstein-Barr virus
EC	Endothelial cell
ECM	Extracellular matrix
FADD	Fas-associated protein with death domain
FasL	Fas ligand
FcγR	Fc-gamma receptor
FDA	Food and drug administration
FGF	Fibroblast growth factor
FNA	Fine-needle aspirate
FTC	Follicular thyroid carcinoma
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMP	Good manufacturing practice
GVHD	Graft-versus-host disease
HLA	Human leukocyte antigen
HMGB1	High-mobility group box 1
HPV	Human papilloma virus
HSCT	Hematopoietic stem cell transplantation
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP-10	Interferon gamma-induced protein 10

ITAC	Interferon-inducible T cell alpha chemoattractant
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITAM	Immunoreceptor tyrosine-based activation motif
KIR	Killer cell immunoglobulin-like receptor
LPS	Lipopolysaccharide
MART-1	Melanoma-associated antigen recognized by T cells
MCA	Methylcholanthrene
MDA	Melanocyte differentiation antigens
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MIC	Major histocompatibility complex class I chain-related chain
MIG	Monokine induced by interferon gamma
NCR	Natural cytotoxicity receptor
NK	Natural killer
NKG2D	Natural killer group 2 member D
NSCLC	Non-small cell lung cancer
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
pDC	Plasmacytoid dendritic cell
PGE2	Prostaglandin E2
Poly I:C	Polyinosinic-polycytidylic acid
PPR	Pattern recognition receptor
PTC	Papillary thyroid carcinoma
RCC	Renal cell carcinoma
ROS	Reactive oxygen species
SCID	Severe combined immunodeficiency
SLO	Secondary lymphoid organ
T_{CM}	Central memory T cell
T_{EM}	Effector memory T cell
TGF	Transforming growth factor
T_H	Helper T cell
TIL	Tumor-infiltrating lymphocyte
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R	TRAIL-receptor
T_{REG}	Regulatory T cell
TSH	Thyroid-stimulating hormone
ULBP	UL 16-binding protein
VEGF	Vascular endothelial growth factor
ZA	Zoledronic acid

1. Introduction

Throughout history, all organisms on earth have developed defense mechanisms that allow them to cope with challenges in their surrounding environment. In higher species, these mechanisms are collectively known as the immune system. For organisms to be able to develop into more advanced creatures, a genome with a certain degree of instability is required. This allows for mutations to occur that can potentially lead to increased survival benefit which is the essence of evolution. However, the price of our ability to evolve is the risk of developing cancer resulting from mutations that cause irreparable genetic damage to the host cell resulting in additional mutations which ultimately initiates the growth of a tumor. The human immune system, which is extremely complex in its organization, has evolved not only to combat external threats such as viruses, bacteria, parasites or other pathogens but has also developed mechanisms that screen the body for cells that has been malignantly transformed and needs to be eliminated. The idea that the immune system is involved in the elimination of tumors dates back to the early 20th century when Paul Ehrlich hypothesized that, without an immune system, the body would be overrun by an "overwhelming frequency" of carcinomas [1]. However, it was not until decades later in 1957 that the concept of immune surveillance was first described by Sir F McFarlane Burnet and his seminal discovery paved the way for future research in the field of tumor immunology [2-4]. The natural killer (NK) cell was described in 1975 and when its functional regulation was established in the early 1980s by researchers at Karolinska Institutet, it sparked a boom in NK cell research which later showed that NK cells play a major role in the natural immune response against malignant cells due to their simple, yet elegant, function of recognizing cells unable to show that they belong to the host [5-8].

In this thesis I will discern how NK cells can be used in treatment of cancer. The prospects, as well as the hurdles, will be discussed and I will focus on the central questions that determine whether a cancer is or can become susceptible to NK cell therapy.

I will start to give a general introduction to the human immune system and proceed with a more detailed explanation of the subjects that are essential to understand and interpret my findings.

1.1 Overview of the immune system

The human immune system is composed of two arms that complement each other in the defense against different pathogens; the innate and adaptive immune system. The innate immune system consists of phagocytes such as macrophages and neutrophils as well as dendritic cells (DCs) and NK cells. The innate effector cells are equipped with germline encoded receptors that are known as pattern recognition receptors (PPRs). With the use of PPRs, phagocytes can recognize pathogen-associated molecular patterns (PAMPs), which are structures specifically expressed by for instance bacteria (lipopolysaccharides (LPS)) or viruses (single stranded DNA). Upon ligation of the PPRs, the phagocytes are activated and triggered to either engulf the bacteria or infected cells or alternatively release cytotoxic

granules against their targets. Meanwhile, a subset of phagocytic cells known as antigen presenting cells (APC) of which DCs are the most potent, display fragments of the engulfed pathogens (antigens) on their cell surface and travel to the secondary lymphoid organs (SLOs)[1]. Here, they interact with adaptive immune cells (B and T cells) that can recognize and respond to an almost unlimited amount of different pathogens. This potential is achieved by random genetic rearrangement of the cells' recognition receptors creating a vast pool of cells in the SLOs that all have different specificities and are ready to be primed by any divisible pathogen. When the APCs come in contact with a lymphocyte that expresses a receptor that can recognize the presented antigen, the lymphocyte starts to clonally expand ultimately generating an army of clonally selected lymphocytes that are ready to engage the invading pathogens with high specificity. In contrast to innate immune cells, B and T cells can generate memory cells that can quickly and with a stronger magnitude respond to future exposures of the same antigen.

Human T cells are comprised of two main subsets; the $CD8^+$ T cells, also known as cytotoxic T lymphocytes (CTLs) bind to MHC class I molecules on APCs and are the main effectors cells that, after a clonal expansion, are deployed to specifically target and eliminate the pathogen-infected or cancerous cells. The $CD4^+$ T cells also known as the helper T cells (T_H), bind to MHC class II molecules on APCs and are, like the name suggests, important regulators of immune responses by activating other immune cells such as B cells, phagocytic cells as well as CTLs. The cellular immune response is also complemented by the humoral component of the immune system which is comprised of soluble macromolecules including antimicrobial peptides, complement proteins and most importantly antibodies (immunoglobulins). When B cells are primed they differentiate into plasma cells that are potent producers of antibodies. Antibodies that bind molecules on the microbe surface have several mechanisms of action; firstly, by blocking the binding of the microbe with host cells they neutralize their interaction. Secondly, they facilitate the engulfment of the microbe by acting as a flag for phagocytes to recognize and take up the microbe. A process termed opsonization. Thirdly, bound antibodies can be recognized by cytotoxic lymphocytes that express receptors for the Fc-portion of antibodies and subsequently induce antibody-dependent cell-mediated cytotoxicity (ADCC) directed against the opsonized target cells. Depending on the nature of the invading microbe, the immune responses can be skewed in different ways to most effectively clear the infection. In the case of microbes that infect and replicate in host cells, such as viruses or intracellular bacteria, a T_H1 response is elicited. In contrast, a T_H2 response is generated in response to extracellular bacteria or parasites [9].

[1] Lymph nodes and spleen

1.1.1 Cytokine signaling

Immune cells communicate with each other and with other cells in the body both through direct cell-cell contact but also through soluble proteins called cytokines. Through this communication they can orchestrate the actions of an immune response by regulating a variety of cellular responses including differentiation, proliferation and activation. Importantly, cytokine signaling is also used to maintain homeostasis of the immune system when an infection is cleared. The type I interferons (IFNs) are important regulators during viral infections. They are released from infected cells and modulate surrounding cells to prevent them from taking up the virus while also stimulating antigen presentation by APCs and increased activation of NK cells [10]. Type II interferon or interferon-gamma (IFNγ) is produced by immune cells (primarily T cells and NK cells) and trigger activation of several immune cells including increased microbicidal function of macrophages, isotype switching of B cells and T_H1 polarization of T cells. To increase antigen presentation and priming of T cells a wide variety of cells respond to IFNγ by upregulating major histocompatibility (MHC) class I as well as MHC class II receptors on the cell surface [11]. I will discuss the clinical implications of this phenomenon later in this thesis. Among the interleukins (ILs), IL-2 is one of the major players in regulating the actions of immune cells during an immune response. IL-2 is produced by T cells and by other cells such as NK cells and DCs, although in smaller amounts, and stimulates survival, proliferation and activation of T cells, NK cells and other lymphocytes [12]. After antigen exposure, IL-2 production is greatly increased and promotes both T cell expansion as well as memory generation [13]. Lately, IL-15 has been shown to be an important cytokine to promote NK cells survival and proliferation [14-16].

1.1.2 Lymphocyte migration and chemokines

When a lymphocyte commits to move in a particular direction, the morphology of the actin cytoskeleton is polarized to elongate the cell, forming a wide leading edge (pseudopod) in the direction of locomotion and a tail-like structure (uropod) in the trailing end [17]. In the case of chemokine-induced migration, the chemokine receptors accumulate in the leading pseudopod to allow for increased perception of the chemokine gradient [18]. A lymphocyte that travels through a vessel can responds to immobilized chemokines on the walls of endothelial cells (ECs) by tethering to the vessel wall followed by rolling, increased adhesion to the ECs and ultimately transmigration through the vessel wall [19]. In contrast, when soluble blood-borne chemokines bind to chemokine receptors on circulating lymphocytes, they inhibit their adhesion to ECs thus promoting continued circulation.

There are currently fifty chemokines and twenty chemokine receptors described in human and through their interplay the migratory patterns of immune cells are orchestrated. By directing migration of leukocytes and other cells to sites of inflammation, such as an infected wound or an emerging tumor, chemokines control the recruitment and retention of particular subsets of cells. Although chemokine signaling is generally promiscuous in its receptor-ligand interactions, where many of the genes are clustered in the same chromosomal loci, the so called “homeostatic” chemokines (e.g. CXCL14, CCL19 and CCL21) are constitutively

expressed and direct the homing of $CCR7^+$ naïve T cells and DCs to SLOs for priming [20]. After priming, some T cells lose expression of CCR7 to release them from recirculation in SLOs and acquire expression of inflammatory chemokine receptors allowing further migration to sites of inflammation. These T cells are termed effector memory T cells (T_{EM}). The central memory T cells (T_{CM}) retain their CCR7-expression and co-express CD62L allowing them to home to lymph nodes. Upon a second stimulation by DCs they can rapidly differentiate to effector T cells [21, 22]. Upon initiation of a humoral immune response, $CD4^+$ T cells acquire expression of CXCR5 allowing them to respond to CXCL13 which is secreted from follicles and promotes interaction with B cells.

Pro-inflammatory chemokine signaling has traits of both pleiotropism and redundancy meaning that chemokines can bind several receptors triggering different functions and that chemokine receptors can be stimulated by several different chemokines triggering the same response [23, 24]. Pro-inflammatory chemokines can have antagonistic functions shaping the immune response between T_H1 and T_H2 responses [25]. The chemokines CXCL9[2], CXL10[3] and CXCL11[4] are agonists to cells expressing the CXCR3 receptor including activated T_H1 cells and CTLs as well as innate lymphocytes such as NK cells [26]. However, these chemokines are natural antagonists to the CCR3 receptor which is expressed on the basophils and eosinophils [27, 28], cells that are part of a T_H2 immune response. The CXCR3-ligands are inducible by IFNγ, a T_H1 cytokine which is produced in areas of inflammation as well as in the tumor microenvironment. CXCL10 together with other inflammatory chemokines including CX3CL1[5] has been identified as major predictive factors for infiltration of CTLs in colorectal cancer as well as other cancers [29]. Galon and colleagues has pioneered the concept of immunoscoring which is a novel system for staging of tumors where the localization and density of infiltrating lymphocytes forms the basis for the prediction of tumor progression and clinical outcome [30]. The strength of the prediction by immunoscoring is another indication of how important the immune system is in the control of tumor growth and dissemination.

1.2 NK cells

NK cells are large granular lymphocytes that constitute an important part of the innate immune system. They were identified by Dr. Rolf Kiessling and colleagues in 1975 by analysis of their functional ability to kill tumor cells without prior sensitization [5, 31]. A decade later, Kärre and colleagues formulated the "missing self hypothesis" based on the finding that NK cells target cells with low or absent expression of MHC class I molecules [8].

[2] Monokine induced by interferon gamma (MIG)
[3] Interferon gamma-induced protein 10 (IP-10)
[4] Interferon-inducible T cell alpha chemoattractant (I-TAC)
[5] Fractalkine

In this thesis I have exclusively studied human NK cells and will only sparingly discuss findings from NK cell mouse models for the sake of clarity. NK cells originate from lymphoid progenitors in the bone marrow, where they acquire expression of NK cell specific receptors. The first to appear are the natural cytotoxicity receptor NKp46 and CD161c as well as the chemokine receptor CXCR4 of which the latter is needed to retain the developing NK cells in the bone marrow that contain high levels of the CXCR4 ligand CXCL12 [32, 33]. An important developmental step, which the NK cells share with other lymphoid cells, is the acquisition of the IL-2 receptor γ-chain since cells depend on stimulation by both IL-2 and IL-15 for their differentiation, proliferation and survival. NK cells can be subdivided into two phenotypically and functionally distinct subsets based on their expression of CD56 on the cell surface. The $CD56^{bright}$ NK cells comprise around 10% of circulating NK cells and have an immunoregulatory role by secretion of proinflammatory cytokines although they have poor cytotoxic capacity. The $CD56^{dim}$ NK cell subset, which is believed to be in a more mature state than the $CD56^{bright}$ NK cells, are highly granular and have potent cytolytic capacity [34]

Aside from their direct cytotoxicity toward virally infected cells or tumor targets, NK cells have a role in directing the immune response by interacting with other immune cells. They do this either through secretion of pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and IFNγ which has diverse and potent anti-viral effects but also by direct cell-cell contact with DCs [35-38]. Although NK cells are part of the innate immune system, they have recently been found to possess traits that, under certain experimental conditions, resemble adaptive immune cells. Sun and colleagues demonstrated that NK cells expressing the virus-specific Ly49H receptor, responded to a murine cytomegalovirus (mCMV) infection with a potent proliferation phase followed by the generation of $Ly49H^{+}$ cells that resided in the lymphoid organs for several months. When the "memory-like" NK cells were adoptively transferred to naïve syngeneic mice that were challenged with murine CMV, they underwent a secondary expansion phase conferring protection against the virus [39]. In humans, the existence of memory NK cells has not been established although it has been demonstrated in viral infections including hanta-virus, chikungunya virus and human cytomegalovirus (hCMV), that a certain subset of terminally differentiated $NKG2C^{+}$ NK cells can undergo re-expansion when transferred to a second seropositive host [40-42].

1.2.1 Regulation of NK cell cytotoxicity

NK cells express a range of germline encoded receptors with the capacity to induce cytotoxic functions of the NK cells when ligated with their target molecules. These target molecules are normal self proteins that are generally expressed in low levels in healthy cells but can be upregulated upon cellular stress such as infection of transformation thus gaining the potential to activate NK cells. The actions of NK cells are however tightly regulated by inhibitory receptors which bind to MHC class I molecules which are expressed on all nucleated cells [43]. It is the net sum of activating and inhibitory signals that NK cells receive from its interactions with target cells that determine whether it is activated to kill the target cell or not [44-47] (figure 1). This control mechanism safeguards normal cells from triggering activation of NK cells and being eliminated.

1.2.1.2 Inhibitory receptors

In humans, the killer-cell immunoglobulin-like receptors (KIRs) are the main inhibitory receptors. They recognize and bind to the classical MHC class I molecules human leukocyte antigen (HLA)-A, -B and -C while the CD94/NKG2A dimer binds to the non-classical MHC class I molecule HLA-E [48-50]. The MHC class I molecules are expressed on all healthy nucleated cells but may be downregulated or lost after viral infection or malignant transformation of cells or as a result of immune evasion by an evolving tumor [51-53]. The KIRs that have long cytoplasmic tails deliver their inhibitory signal via immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that can shut down NK cell activation through dephosphorylation of activating adaptor molecules. In contrast, there are KIRs with short cytoplasmic domains that can mediate activation instead of inhibition [54]. The finding that every individual person differ in their repertoire of KIR genes and that individual NK cells express different KIR gene products inspired researchers to investigate how the KIR repertoire is established and how tolerance is achieved. Indeed, when an NK cell expressing a specific KIR, interacts with a target cell that lacks a specific HLA-allele corresponding to the KIR, the NK cell is activated and the target cell is lysed in accordance with the missing self hypothesis. However, in MHC class I-deficient mouse models, NK cells were hyporesponsive [55]. Moreover, in humans where NK cells without any expression of inhibitory receptors targeting MHC class I were identified, these cells were also hyporesponsive indicating that the missing self hypothesis needed further fine-tuning [56, 57]. A hypothesis was put forward to explain these phenomena and how NK cells maintain their tolerance to self. The term "licensing" was born which proposed that NK cells need to interact with self MHC class I molecules to become fully mature and acquire their license to kill [58].

1.2.1.3 Activating receptors

There is a multitude of membrane bound activating receptors that can act in synergy to deliver intracellular activation signals to NK cells [59, 60]. The activating receptors employ different intracellular signaling pathways as opposed to inhibitory receptors which all recruit Src homology 2 domain-containing phosphatase 1 (SHP-1) to dephosphorylate cytoplasmic ITIMs thus delivering their inhibitory signal [61]. The natural cytotoxicity receptors (NCRs) NKp30 and NKp46 are expressed on resting NK cells while NKp44 is expressed only on activated NK cells [62-65]. These receptors as well as the Fcγ-receptor CD16 signal via the immunoreceptor tyrosine-based activation motif (ITAM) that associates with the tyrosine kinases Syk and ζ-associated protein of 70 kDa (ZAP-70) [66]. Efforts to characterize the NCR ligands are currently underway and so far the only well-defined ligand is the NKp30 ligand B7-H6 which has been found to be expressed on tumor cells as well as proinflammatory monocytes and neutrophils [67, 68].

Other important activating NK cell receptors are natural killer group 2 member D (NKG2D), DNAX-accessory molecule-1 (DNAM-1) and the CD2 family members which, upon IL-2 activation of NK cells, can act in synergy to induce lysis of target cells [69]. The ligands for NKG2D are the major histocompatibility complex class I chain-related chain (MIC) A and B and the UL16 binding proteins (ULBP) 1-6 [70-74]. It has been demonstrated that NKG2D-

ligands are upregulated upon cellular stress and that several NKG2D-ligands are overexpressed in human cancers [75-77]. Moreover, mice bearing tumors with ectopic expression of NKG2D-ligands overcome MHC-class I-induced inhibition of NK cells, thus promoting rejection of the tumors [78]. The binding of integrin-receptor lymphocyte function-associated antigen-1(LFA-1) to the intercellular adhesion molecule (ICAM) -1 or -2 on target cells triggers an adhesion and early activation of NK cells which is important for subsequent polarization and degranulation of the NK cell against its target [79].

1.2.1.4 Regulation of NK cell killing

A feature that NK cells share with CTLs is the capacity to exocytose granules containing perforin and granzyme as well as other lytic proteins [80]. These proteins work in concert to permeabilize and induce apoptosis in the target cells [81, 82]. The importance of the perforin/granzyme mechanism for NK cell and T cell cytotoxicity against tumors has been highlighted in several murine models [83, 84]. NK cells as well as T cells are able to employ an entirely different mode of cytotoxicity by engagement of so called death ligands including TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL). These ligands bind to death receptors (DRs) on target cells and initiate apoptosis through activation of the caspase-8 intracellular pathway [85-87] (figure 1). TRAIL is expressed on activated NK cells and can be upregulated by stimulation with IL-2 or IL-15 [88]. The role of NK cell-mediated elimination of tumors via TRAIL has been demonstrated in murine tumor models, where treatment with neutralizing antibodies against TRAIL promoted progression of subcutaneously inoculated TRAIL-sensitive tumors [89]. The activating receptors for TRAIL are the TRAIL-R1[6] and TRAIL-R2[7] which upon ligation with TRAIL trimerizes the ligand leading to recruitment of Fas-associated protein with death domain (FADD) and assembly of the death-inducing signaling complex (DISC) which subsequently autocatalytically cleaves caspase-8 [90-92]. From here, the propagation of the apoptotic signal can either go through the extrinsic pathway via direct cleavage of the effector caspase-3, alternatively, Bid is cleaved and its truncated bi-product (tBID) can translocate to the mitochondria triggering the intrinsic pathway of apoptosis ultimately cleaving caspase-9 which in turn cleaves caspase-3 resulting in cell death [93]. In addition to TRAIL-R1 and -R2, TRAIL can bind to two additional receptors that either lack or have non-signaling, truncated intracellular domains. Decoy receptor (DcR) 1 and 2 bind to TRAIL with high affinity but are unable to propagate an apoptotic signal [94, 95]. Targeting of tumor cells by TRAIL-induced killing is a promising concept due to the constitutive expression of TRAIL-R1 and -R2 in many tumor tissues and the fact that DcR1 and DcR2 are more frequently expressed in healthy tissue compared to transformed tissue indicating a low risk of toxicity [96]. FasL-mediated lysis of virus-infected or cancerous target cells is employed by both NK cells and T cells by binding to the single receptor FAS[8]

[6] DR4
[7] DR5
[8] CD95

triggering intracellular caspase-8 activation in a similar fashion as TRAIL-induced signaling [97, 98]. Moreover, NK cells and CTLs have the ability to secrete soluble FasL to induce caspase-dependent apoptosis in target cells. FAS is also expressed on activated lymphocytes enabling homeostatic regulation of immune responses by FasL-mediated elimination of lymphocytes [99]. Moreover, tumor cells can take advantage of this sensitivity by shedding FasL from the cell surface thus counteracting the anti-tumor response by killing the attacking lymphocytes [100, 101]. An example of the bridging between the innate and adaptive immune system is the killing of target cells that have been opsonized by antigen-specific antibodies by cells of the innate immune system such as monocytes, neutrophils and NK cells [102]. NK cells express only the high affinity Fcγ-receptor CD16 and not the lower affinity Fcγ-receptors CD64 and CD32 nor the inhibitory Fcγ-receptor CD32b, and they have been shown to be one of the main cell types that contribute to the ADCC effect in cancer patients treated with monoclonal antibodies such as trastuzumab [103, 104].

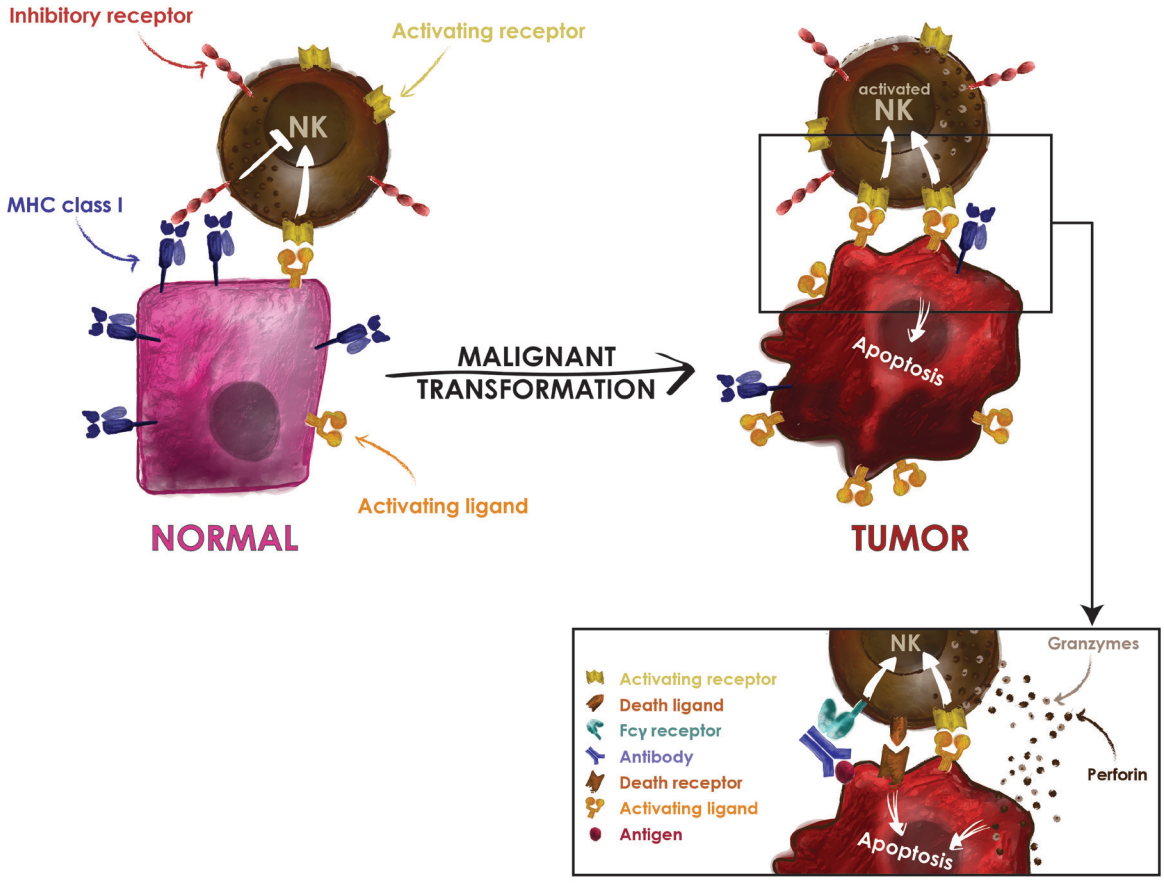

Figure 1. NK cell recognition of tumor cells. The cytolytic machinery of NK cells is controlled by a balance of activating and inhibitory signals received from interactions with their target cells. When NK cells interact with normal cells, which express both inhibitory receptors as well as low levels of activating receptors, the net sum of signals received by the NK cells triggers inhibition of the NK cells and the normal cell is spared. During malignant transformation, tumor cells often lose some or all of their MHC class I expression (missing self) while stress-induced activating ligands are upregulated. As a consequence, NK cells interacting with tumor cells receive enough activating signals to overcome the weak inhibitory signal by MHC class I leading to NK cell activation and lysis of the target cell by degranulation of perforin and granzymes or death receptor ligation. Opsonization of target cells with tumor antigen-specific

antibodies may also contribute to the activation of NK cells through interaction with Fcγ-receptors in a process called antibody-dependent cell-mediated cytotoxicity (ADCC).

1.2.4 Regulation of NK cell migration

In order for NK cells to efficiently take part in immunosurveillance they need to be motile and in constant surveillance of the peripheral organs [105]. NK cells express a wide range of receptors that can induce chemotaxis to several different tissues. During pregnancy, trafficking of NK cells to the uterus is induced through recruitment via CCR2 and CCR5 [106]. Under steady-state conditions $CCR7^{+}CD62L^{+}CD56^{+}$ NK cells can be found in peripheral lymph nodes [107] and under inflammatory conditions NK cells can be recruited by DCs via CXCR3-induced migration to spleen and lymph nodes where they secrete high levels of IFNγ and engage in reciprocal interactions with DCs [108, 109]. This recruitment leads to maturation of both NK cells and DCs thus aiding in priming of T cells and T_H1-polarization [110-113]. Similarly, NK cells, which are among the first immune cells to appear at inflamed tissues, have the ability to secrete the CCL3, -4 and -5 enabling them to recruit DCs and other immune cells expressing CCR1 and CCR5 to the site of inflammation [114].

NK cells are known to home to and infiltrate various different cancers where they in several cases have been shown to influence the patient's prognosis [115-117]. In viral infections as well as in tumor sites, high titers of both type I and type II IFNs induce secretion of the chemokines CXCL9, CXCL10 and CXCL11 [26]. These ligands are potent chemokines attracting CXCR3-positive NK cells towards solid tumors, which has been exemplified by Wendel et al in elegant mouse models [118]. CCR5 has also been implicated in homing of NK cells towards tumors where plasmacytoid dendritic cells (pDCs) have been shown to be the main source of chemokine secretion [119].

1.3 Cancer

Cancer is initiated with a single cell acquiring damage to its DNA. It happens due to a variety of both endogenous and exogenous factors. In metabolically active cells, reactive oxygen or nitrogen species are constantly formed which continually cause single strand breaks in our DNA. Also, there are countless environmental factors, such as exposure to chemicals (food) or UV-radiation, which can directly or indirectly compromise the integrity of our genome and cause DNA damage. This type of damage occurs frequently in our cells every day and in the majority of cases the damage is either repaired by the actions of enzymes that are solely responsible to maintain the genetic structure of the cells [120, 121]. Alternatively, the cell undergoes programmed cell death which is known as apoptosis [122, 123]. DNA damage can cause mutations affecting the very control mechanisms that regulate DNA repair or induction of apoptosis, for example in the so-called oncogenes or tumor-suppressor genes. These genes code for proteins that are directly involved in maintaining the defense mechanisms that prevent uncontrollable cell growth and when they are mutated the cell can start to acquire traits that send it on a slippery slope to becoming a cancerous cell. These traits have been categorized as the "hallmarks of cancer" including the ability to; *sustain proliferative*

signaling, evade growth suppressors, resist cell death, induce angiogenesis, enable replicative immortality and activate invasion and metastasis [124]. In recent years, as the knowledge about tumors cells and their interactions with neighboring cells has increased dramatically, the complexity of the tumor microenvironment has become more apparent. This has led to the addition of emerging hallmarks of cancer including *deregulation of cellular energetics* and importantly the ability of cancer cells to *avoid immune destruction* [125].

On average, a cancer cell acquires around ten mutations that can generate tumor-specific antigenic peptides. Moreover, as the tumor develops and undergoes dedifferentiation tumor-associated antigens can emerge. Both of these antigens can be presented by APCs to trigger an adaptive immune response against the tumor [126]. Despite this, tumors very rarely regress due to spontaneously induced immune responses [127, 128]. In the following chapters I will discuss why this is the case and how we can help the immune system to fight cancer.

3.3.1 Anaplastic thyroid cancer

The thyroid gland is an endocrine organ located behind the laryngeal prominence (”Adams apple”) where it lies protected behind a cartilage shield. The healthy thyroid is histologically composed of follicles where the thyroid hormones are stored in the form of thyroglobulin. Upon stimulation of thyroid-stimulating hormone (TSH) secreted from the anterior pituitary gland in the brain, the thyroid hormones are released into the blood stream to exert their actions which include regulation of heart rate, digestion, metabolic rate, etc. In addition to the hormones produced by follicular cells, parafollicular cells[9] produce and secrete calcitonin which is an important regulator of calcium uptake and bone metabolism.

There are several different subtypes of tumors that originate in the thyroid and they can have very different progression patterns as well as prognosis. The well-differentiated tumors include papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) which have a relatively low proliferation rate and rarely metastasize unless in advanced disease stages. PTC and FTC patients often respond to treatment with radioactive iodine and the prognosis is generally good. Poorly differentiated thyroid carcinoma (PDTC) represents the middle stage between the well-differentiated thyroid carcinomas and the undifferentiated anaplastic thyroid carcinoma (ATC) which is the most aggressive and lethal of the thyroid cancers. ATC can arise *de novo* although in most cases it develops due to a dedifferentiation of an existing PTC or FTC [129, 130] (figure 2).

[9] C cells

The frequency of ATC varies in different parts of the world, from 1.7 % in USA to 7.9 % in the Netherlands [131, 132]. ATC is a disease of the elderly (mean age of diagnosis is 55-65 years) and it is more common in women than in men. Due to the aggressive nature of ATC, the prognosis for patients is very poor. A meta-analysis of clinical reports from 1949-2007 including 1771 ATC patients revealed a median survival of only five months [133]. Upon diagnosis, ATC commonly presents with tracheal invasion and in more than 50% of all patients, distant metastasis to the lungs, bones and brain [134]. There are several traits of ATC that contribute to its rapid progression and lethality. Somatic mutations in several genes regulating angiogenesis, growth rate and cellular adhesion are commonly found in late stage ATC including p53, PI3KCA and β-catenin [133]. As of today, there are no curative treatments available for ATC.

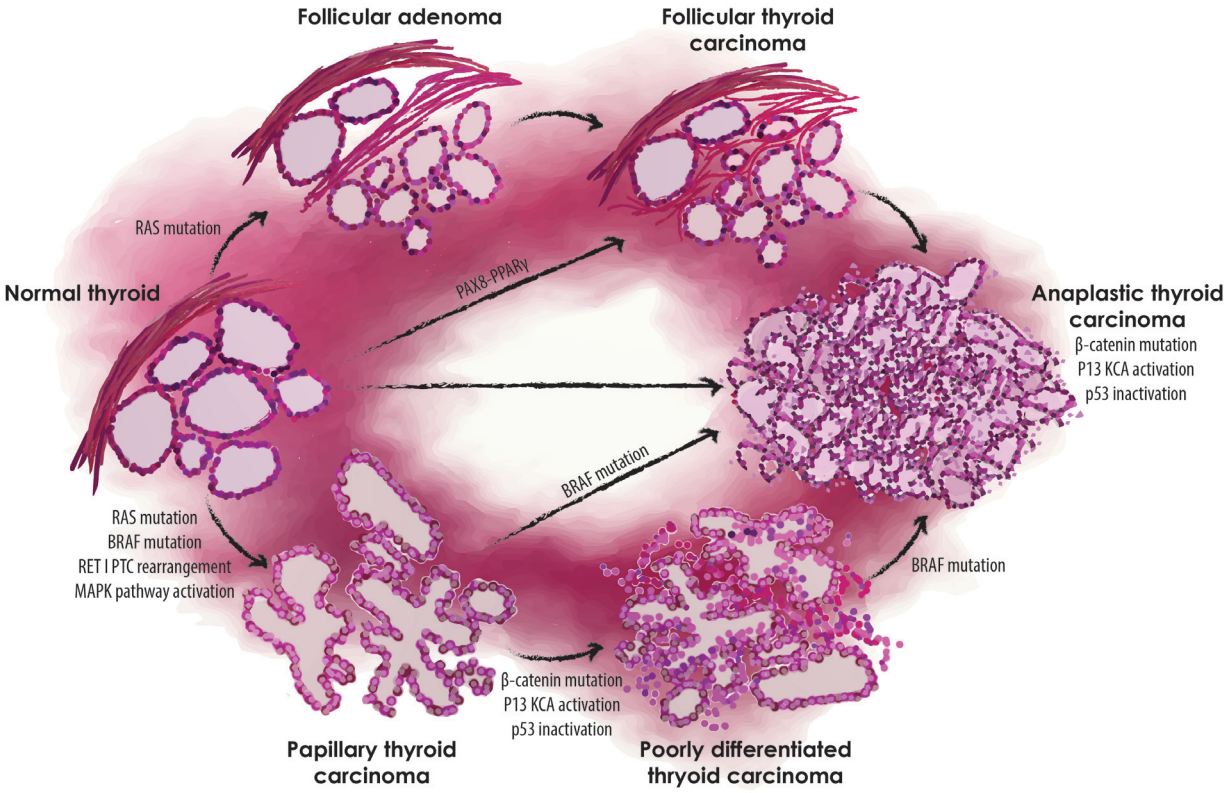

Figure 2. Progression of thyroid malignancy. Thyroid follicular cells can undergo genetic changes that transform them into different tumor types. Follicular adenoma is characterized by follicular cell dedifferentiation and capsule formation. Upon invasion or penetration of the capsule the tumor is considered a follicular thyroid carcinoma. The papillary thyroid carcinomas are characterized by morphological changes in the follicular cells with an optically clear appearance or so called "orphan Annie nuclei" as well as the formation of tree-like papillae. When the cancerous cells accumulate mutations in p53, PI3KCA and β-catenin, they can undergo extensive dedifferentiation leading to development of poorly differentiated anaplastic thyroid carcinoma. Here, the cellular morphology is heterogeneous within the tumor including squamoid, spindle cell and giant multinucleated cells and the growth pattern is invasive extending to vessels and extrathyroidal structures. In many of the undifferentiated thyroid carcinomas, there are areas of well-differentiated thyroid tumor indicating that they have de-differentiated from a pre-existing well-differentiated cancer.

1.3.1 The tumor microenvironment

As tumor cells proliferate and start to form a tumor mass, the environment around it will be sculpted to aid the tumor as it grows. Tumor cells communicate through paracrine signaling with surrounding cells, creating a dynamic equilibrium that favors tumor progression. Mesenchymal stromal cells such as fibroblasts and pericytes are commonly transformed by tumors to promote tumor growth and metastasis via secretion of growth factors like fibroblast growth factor (FGF) or stromal-derived factor (SDF)-1α. [135-138]. The emergence or recruitment of cancer-associated fibroblasts (CAFs) in the tumor microenvironment is an important step in tumor development as they promote both tumor angiogenesis through production of vascular endothelial growth factor (VEGF) as well as tumor growth and metastasis through protease-mediated degradation of the extracellular matrix (ECM) [139, 140]. Due to the chaotic structure of the neovasculature in rapidly growing tumors the oxygen levels are highly unstable and hypoxic regions commonly develop influencing several aspects of tumor growth including altered metabolic conditions as well as the differentiation and activity of CAFs [141, 142]. Hypoxia affects many immune cells including NK cells which under hypoxic conditions lose the ability to upregulate activating receptors in response to cytokine stimulation [143]. NK cells are highly influenced by cells and cytokines produced in the tumor microenvironment. In non-small cell lung cancer (NSCLC) the phenotype of intratumoral NK cells was modified showing reduced expression of NKp30, DNAM-1, NKG2D and CD16 compared to NK cells in the patient's blood [144]. Tumors and tumor infiltrating myeloid and granulocytic cells have been shown to produce large amounts of reactive oxygen species (ROS) which is an essential part of these cells natural effector mechanism against pathogens. In physiological concentrations, ROS is beneficial for NK and T cells function but at high concentrations ROS can induce loss of function and even apoptosis of NK cells and other lymphocyte subsets due to induction of oxidative stress [145-150]. Taken together, the tumor microenvironment can with time develop into a very hostile site for lymphocytes to operate in. However, it was recently reported that tumor cells and tumor-associated stromal cells were able to activate infiltrating NK cells in the microenvironment via trans-presentation of IL-15. The activated NK cells released large amounts of cytotoxic granules which resulted in eradication of large solid tumors. [151].

1.3.2 Immune surveillance of tumors

The role of the immune system in surveillance of emerging tumors is underpinned by the finding that patients undergoing organ transplantation who are immunosuppressed for long periods of time have a greatly increased risk of developing tumors [152-155]. The risk is higher for developing tumors driven by oncogenic viruses such as human papilloma virus (HPV), hepatitis B and C virus (HBV/HCV) and Epstein-Barr virus (EBV) but is also seen for tumors that are not linked to viral oncogenesis [155]. Engel and colleagues established mouse models where methylcholanthrene (MCA)-induced tumors develop faster in immunocompetent mice compared with immunodeficient nude or severe combined immunodeficiency (SCID) mice [156, 157] thus arguing for immunosurveillance of chemically-induced tumors. The impact of NK cells in protection against tumors was

highlighted in a mouse model where NK cells in BALB/c mice were selectively depleted using the anti-asialo-GM antibody. Mice with functional NK cells had improved protection from MCA-induced fibrosarcoma compared with NK cell-depleted mice [158].

1.3.3 Tumor immunoediting

As developing tumors are infiltrated and attacked by immune cells, the composition of the tumor is edited over time and is classically divided into three phases; elimination, equilibrium and escape (figure 3). In the elimination phase the tumor is at its most immunogenic. Innate immune cells recognize and target tumor cells expressing ligands for TRAIL, NKG2D, FasL etc. allowing for both direct and perforin-dependent cytolytic activity. Tumor cells present tumor antigens on MHC class I molecules that, together with a rich cytokine environment (high in type I and type II IFNs), supports recruitment of additional lymphocyte populations and aides in DC cross-presentation of tumor antigens to CD8+ T cells [159]. Macrophages of the M1 phenotype are important in the early stages of the elimination phase to secrete pro-inflammatory cytokines such as TNFα, IL-12 and IL-1 that further boosts the activation of infiltrating immune cells and killing of the tumor. During the elimination phase, many tumor cells die by apoptosis but also from necrosis which in turn further stimulates the immune response by release of danger-associated molecular patterns (DAMPs) including high-mobility group protein B1 (HMGB1) [160].

When the anti-tumor immune response enters the equilibrium phase, there has been a selection of less immunogenic tumor cells that are not accessible or recognizable to the host's immune system. Here, both adaptive and innate immune cells keeps the tumor growth in check and prevents both outgrowth and metastasis of tumor cells while at the same time sculpting the tumor to become more and more immune-resistant. The equilibrium phase can last the entire lifespan of the host. An example of a long equilibrium phase was the case of a woman with polycystic disease who received a kidney transplant from a donor that had been diagnosed, treated and "cured" of melanoma 16 years prior to the transplantation. When the kidney was transferred to a new host, the melanoma tumor cells in the kidney that had been under control of the donors immune system, now reemerged and subsequently killed the recipient [161].

The constant immunological pressure on a tumor creates a darwinistic selection of the least immunogenic tumor cells. In the event that tumor cells alter their phenotype to the point that they can avoid recognition by the immune cells, the tumor can escape the immune control, progress and disseminate. Tumor cells can escape immune cell attack by different mechanisms. Firstly, they avoid recognition by CTLs either by shedding tumor antigens or alternatively by interfering with processing of antigens or downregulation of MHC class I on the cell surface [53, 162]. Moreover, tumor cells may downregulate or shed ligands for activating NK cell receptors, thus avoiding recognition by NK cells and inducing inhibition or even lysis of the NK cells or other lymphocytes [163-167]. In patients with colorectal cancer, NK cells has been shown to have lowered expression of both NKG2D as well as NKp44, CCR7 and CXCR1 due to high serum levels of soluble MICA and MICB molecules [168].

Secondly, tumor cells can develop a resistance to lysis by immune cells either by upregulating anti-apoptotic proteins such as Bcl-2 and cellular FLICE inhibitory protein (cFLIP) [169-171]. Thirdly, tumors can create a surrounding milieu that is immunosuppressive and which converts and skews the resident immune cell population to a more immunoregulatory phenotype [172]. Myeloid-derived suppressor cells (MDSCs) are frequently found in the tumor microenvironment and they are often induced by tumor-derived factors including prostaglandin-E2 (PGE2) and granulocyte-macrophage colony-stimulating factor (GM-CSF) [173-177]. The activation of MDSCs is often triggered by cytokines such as IFNγ and transforming growth factor beta (TGFβ), secreted from infiltrating T cells or tumor stroma [178, 179]. Several studies have shown that MDSCs are able to suppress both cytokine secretion and cytotoxicity of NK cells through production of PGE2 and ROS as well as through cell-cell interactions [174, 180-182]. However, in mice bearing B16 melanoma tumors, treatment with polyinosinic-polycytidylic acid (poly I:C) induced production of IFNα by MDSCs which mediated activation of NK cells and subsequent growth retardation of the B16 tumors[183].

Regulatory T cells (T_{REG}) are defined as $CD4^{+}CD25^{+}CD127^{low/neg}$ and express the forkhead box P3 (FoxP3) transcription factor [184]. They are important regulators of peripheral tolerance that can either delete or induce anergy in autoreactive effector T cells [185]. However, T_{REG} are often induced or recruited to the tumor microenvironment where they can hamper the anti-tumor immune response [186, 187]. The effect of T_{REG} on NK cells has been studied in mouse models where depletion of T_{REG} augmented NK cell clearance of tumors while adoptive transfer of T_{REG} inhibited NK cell activity and favored tumor progression [188, 189]. T_{REG} can either inhibit NK cells activity by secretion of soluble TGFβ or ligation with membrane bound TGFβ [190], although a major mechanism of T_{REG}-mediated suppression is to act as a cytokine sink[10] for IL-2 produced by $CD4^{+}$ T helper cells thus depriving NK cells of one of their main activating cytokines [191].

[10] The cytokine sink effect is a term to describe how a cell type by depleting one or several cytokines in their environment will deleteriously effect other cell types that rely on stimulation by that particular cytokine.

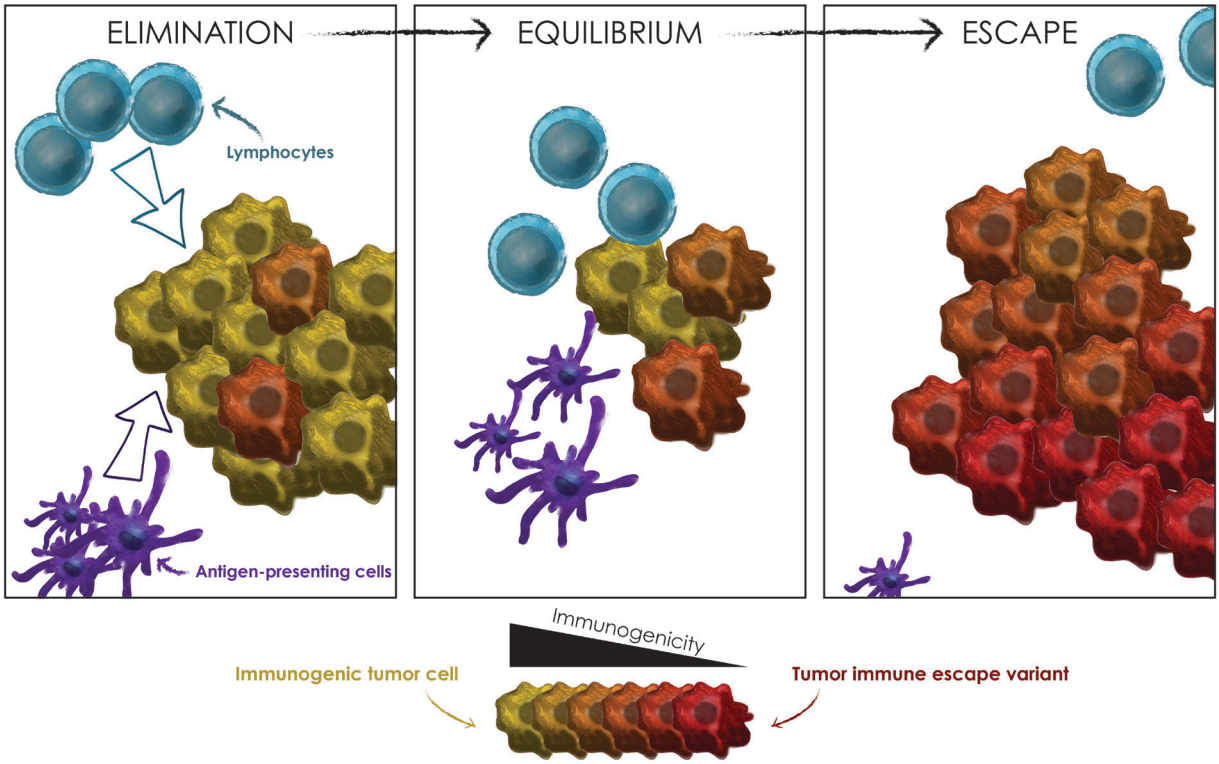

Figure 3. Tumor immunoediting. When a tumor is subjected to an immune response, it can undergo three stages of immunoediting. During the elimination phase, the immunogenic tumor cells which express strong tumor-antigens on their cell surface and high levels of stress-induced activating ligands, stimulate the priming of tumor-specific CTLs and are recognizable by innate immune cells. During the equilibrium phase, the most immunogenic tumor cells have been eliminated and the tumor is under control by the immune system. If the least immunogenic tumor cells are able to avoid immune recognition and start proliferating, the immune escape phase is initiated. Here, the immune system no longer has the strength nor the specificity to target the expanding tumor, leading to uncontrolled growth and dissemination of the tumor

1.3.4 Immune therapy of cancer

The idea of utilizing the potential strength and specificity of the immune system to treat cancer has been around for many decades although it has been met with a great deal of skepticism over the years. It is not until in recent years, with the evidence of curative treatments of cancer patients using adoptively transferred tumor-specific T cells and the large scale randomized clinical trials with monoclonal antibody treatments that the field of cancer immunotherapy is really starting to gain traction.

1.3.4.1 Cancer vaccines

While prophylactic cancer vaccines targeting oncogenic viruses has reduced the incidence of cervical and other cancers dramatically worldwide, the struggle to decipher the clue to efficient therapeutic cancer vaccination continues [192]. The idea of cancer vaccination is to induce both a therapeutically effective immune response that can combat the existing cancer and to establish protective immunity to prevent tumor relapse [193]. Several approaches has been tested to achieve these goals including vaccination with autologous or allogeneic tumor,

either as a lysate or as whole tumor cells, peptides mimicking tumor specific epitopes, DNA encoding tumor antigen or DCs pulsed with either of the above [194-197]. Specific humoral and cellular responses have been detected in animal models as well as in patients but the clinical success have so far been poor [198]. Cancer vaccination relies on powerful adjuvants to aid the host's resident APCs to take up and present the often weak tumor antigens that are injected [199]. In DC vaccination strategies, DCs are both loaded with antigen and matured under controlled conditions *ex vivo* ensuring potent interaction with the naïve immune system once injected to the patient [200]. In 2010, Provenge was approved by the food and drug administration (FDA). It is a DC vaccine targeting the prostate-specific antigen prostatic acid phosphatase (PAP) fused with GM-CSF. Phase III clinical trials have shown the 3-year survival improved by 40 % [201].

1.3.4.2 Checkpoint blockade

T cells require two signals from APCs to become fully activated. The first is the T cell receptor interaction with the antigen presented on MHC molecules. The second signal is delivered by the co-stimulatory molecules CD80[11] and CD86[12] expressed on DCs when they interact with CD28 on the T cells [202-204]. Whereas the CD28 receptor triggers activation of the T cell, the cytotoxic T lymphocyte antigen 4 (CTLA-4) receptor, which is upregulated after priming of the T cell, is able to transmit an inhibitory signal that prevents over-activation of the T cell [205]. The development of monoclonal antibodies targeting CTLA-4 (ipilimumab) has resulted in many clinical trials where its efficacy has been greatest in patients with metastatic melanoma [206, 207]. Moreover, CTLA-4 is constitutively expressed on T_{REG} and part of the clinical effect of anti-CTLA-4 treatment in cancer patients is through *in vivo* abrogation of T_{REG} function [208]. Programmed cell death protein 1 (PD-1) is another regulatory receptor expressed on T cells after antigen priming, although it is also expressed on NK cells, B cells and on myeloid cell subsets [209]. Upon ligation with its ligands PD-L1 and PD-L2 on target cells in the tumor microenvironment, the PD-1 receptor conveys an inhibitory signal to the T cells thus acting as a regulator of peripheral tolerance [210]. Antibody blockade of PD-1 (nivolumab) and PD-L1 has been tested in clinical trials where it has been shown to be safe and has generated objective responses in patients with NSCLC, renal cell carcinoma (RCC) and melanoma [211, 212]. Since anti-CTLA-4 and anti-PD-1 respectively controls the immune checkpoints of both central and peripheral tolerance, the prospect of combining the two treatments could potentially act in synergy to augment both priming and activation of T cells against their tumor targets.

[11] B7-1
[12] B7-2

1.3.4.3 T cell therapy

The idea of adoptive cell transfer is to extract lymphocytes from a cancer patient, manipulate and expand the cells *ex vivo* and finally reinfuse them to the patient. The T cells are isolated either from peripheral blood or from infiltrating lymphocytes in the patient's tumor. When isolating tumor-infiltrating lymphocytes (TILs) from a naturally immunogenic tumor such as melanoma, the chances of finding T cells with specificity against a tumor-antigen is high. By removing the tumor-reactive T cells from the suppressive tumor microenvironment and culturing them in stimulatory cytokines such as IL-2, it alleviates the inhibition from immunosuppressive cytokines, immunoregulatory cells and PD-1 and CTLA-4 blockade, allowing the T cells to proliferate to large numbers. To further augment the specificity of the T cell product, some protocols employ purification and cloning of antigen-specific TILs before starting the *ex vivo* expansion [213]. Melanoma tumor cells normally expresses high levels of melanocyte differentiation antigens (MDAs) for which specific T cells can be isolated and expanded for adoptive TIL therapy. Targeting of a tissue-specific differentiation antigen often generates off-target toxicity to normal cells expressing that same antigen [214]. Melanoma patients treated with TILs against MDAs have suffered from adverse events such as vitiligo, partial blindness and deafness [215-217]. Because of the inherent genetic instability of melanoma and other tumors, there have been several antigens described that are truly tumor-specific in the sense that they are products of mutated genes [218]. These tumor-antigens as well as oncogenic viral antigens often drive the cancer progression and are easily targeted by T cell since they have not been tolerized to the antigens in the thymus [219].

The strength of the anti-tumor effect of adoptively transferred tumor-specific T cell can vary between each patient and the purely cytolytic effect is mediated via both release of toxic granules but also by death ligand-induced apoptosis. However, the T cells that home to the tumor site (including both $CD8^+$ and $CD4^+$ T cells) are also potent producers of both chemokines and cytokines such as TNFα and IFNγ. Tissue resident DCs respond to the cytokine stimulation by upregulating their expression of MHC class I and MHC class II which together with $CD4^+$ T cell cross-talk leads to increased recruitment and activation of NK cells and other innate immune cells [220]. There are several barriers that limit the large-scale use of adoptive cell therapy. Firstly, to achieve long-term persistence and boost the anti-tumor effect of the infused lymphocytes, the patients are required to undergo heavy preconditioning prior to the adoptive transfer. To make space for the infused cells and avoid the cytokine sink effect as well as to eliminate immunosuppressive lymphocyte subsets, patients receive lympho-depleting chemotherapy, which can be supplemented with directed or total body irradiation [221]. This regimen together with high dose adjuvant IL-2 treatment, to maintain proliferation of lymphocytes *in vivo*, reduces the inclusion criteria to patients with good performance status [222, 223]. Secondly, isolation of strong tumor-antigen reactive T cells from a biopsy is not possible for all patients, nor is the ability to generate the large numbers of T cells that are required for lymphocyte infusion. Thirdly, isolation and expansion of the lymphocyte product *ex vivo* is an extremely laborious and expensive process that requires specialized good manufacturing practice (GMP) facilities. However, when adoptive treatment of TILs is successful, the clinical responses can be dramatic. In three sequential clinical trials where a

total of 93 patients with metastatic melanoma were treated with autologous TILs and IL-2, the objective response rate ranged between 49% to 72% and complete regression of the tumor was achieved in 22% of the patients [224]. In recent years, the development of chimeric antigen receptors (CARs), with increasingly stronger intracellular signaling capacity, has sparked the interest in using genetically modified T cells to treat cancers that are otherwise difficult to target with conventional T cells [225]. Retargeting T cell lysis against a particular cancer-related molecule (including antigens other than proteins) in a non-MHC-restricted manner has led to efficient targeting of leukemias by CD19- and CD20-CARs and NY-ESO-1-CARs in multiple myeloma (MM) and several other cancer types [226-228].

1.3.4.4 NK cell therapy

The NK cell is a promising cell type to utilize for adoptive cell therapy due to several different reasons. They do not require priming or prior sensitization to interact with and kill tumor cells, they can be isolated directly from peripheral blood from both patients and healthy donors and they can be expanded and genetically modified *ex vivo* (figure 4).

1.3.4.4.1 NK cell therapy against hematological tumors

The first truly potent clinical effect of NK cells against cancer was demonstrated when Ruggeri and colleagues transplanted patients suffering from acute myelogenous leukemia (AML) with T cell depleted KIR-ligand mismatched bone marrow transplantation (BMT) [229]. The patients receiving grafts with KIR-ligand incompatibility had 60% probability of event-free survival after 5 years compared to 5 % in the patients receiving KIR-ligand compatible grafts. Moreover, the incidence of graft-versus-host disease (GVHD) was also lower in patients receiving KIR-mismatched BMT. The authors hypothesized that the contribution of the alloreactive NK cells in the graft was three-fold. Firstly, the NK cells target the residual leukemic blasts, thus preventing disease relapse. Secondly, NK cell-mediated elimination of recipient APCs prevents their presentation of host-antigen to donor derived T cells thus reducing the risk of GVHD. Thirdly, by targeting the residual recipient T cells, the NK cells promote the engraftment of the BMT [230, 231]. There are numerous recent and ongoing clinical trials in patients with different hematological tumors that assess the importance of donor selection based on KIR/HLA phenotype of NK cells both in the setting of NK cell adoptive therapy or as an adjunct to hematopoietic stem cell transplantation (HSCT) [232, 233]. Miller et al. showed in 2005 that adoptive transfer of haploidentical NK cells was safe and mediated anti-tumor effect in lymphodepleted AML patients receiving daily low-dose injections of IL-2 [233]. Persistence of NK cells was higher in patients receiving preconditioning and correlated with *in vivo* expansion and clearance of leukemia.

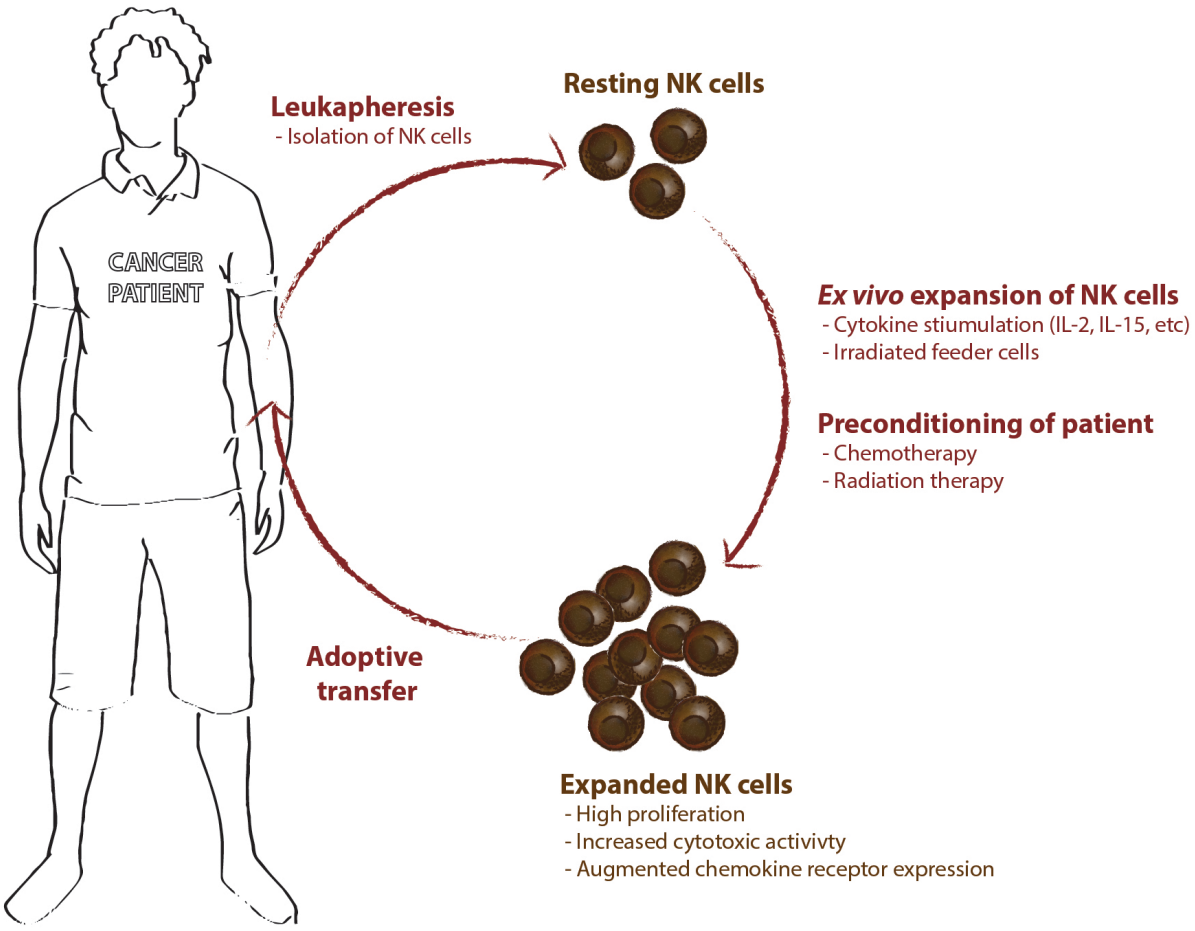

Figure 4. Isolation and expansion of NK cells for adoptive transfer. Peripheral blood mononuclear cells (PBMCs) are cells purified from peripheral blood of a cancer patient and the NK cells are isolated using CD56-specific magnetic bead selection. In order to obtain high numbers of cytotoxic NK cells, the isolated NK cells are cultured in the presence of high doses of NK cell-stimulatory cytokines such as IL-2 and an irradiated feeder cell population. The expansion phase can take up to several weeks and meanwhile the patient can undergo preconditioning to make space for the infused cellular product as well as to sensitize the patients tumor to NK cell lysis. The expanded NK cells have a high proliferation rate (after two weeks of expansion the NK cells can expand up to 1000-fold), high expression of activating receptor resulting in high cytotoxicity and elevated expression of chemokine receptors allowing them to home toward chemokines secreted from tumors. The expanded NK cell product is infused to the patient intravenously in bolus doses of up to 1x 10^8 NK cells/kg of recipient body weight.

1.3.4.4.2 NK cell targeting of solid tumors

Although the anti-tumor effect of NK cells *in vivo* has been demonstrated in several hematological malignancies, the use of NK cells in treatment of solid tumors has yet to show clinical benefit [234-236]. There are several explanations for the difficulty of targeting solid tumors. Firstly, cells that form solid tumors have the advantage of creating a tumor microenvironment that is suppressive towards NK cells, where the actions of T_{REG} and MDSCs play a major role. Therefore, adoptively transferred NK cells must have properties allowing them to withstand 1) inhibition by suppressive cytokines and cells, 2) a hostile and often hypoxic microenvironment and 3) direct cytotoxic counteractive measures employed by the tumor cells and associated cells [237]. Secondly, tumor cells that undergo immune editing often downmodulate their expression of ligands that are targetable by NK cell receptors [238, 239]. Thus, it is difficult for NK cells to recognize and destroy immune escape variants of cancer cells. This highlights the need for pre-sensitization of established tumors to NK cell lysis, as well as identification of predictive markers for NK cell therapy (discussed in paper I and IV). Also, NK cells may need to be pre-treated or *ex vivo* expanded in different fashions depending on the nature of the tumor that they are intended to target (discussed in paper II). Thirdly, only a fraction of all adoptively transferred NK cells actually reach the tumor site [240]. Expression of the appropriate chemokine receptors on the infused NK cells as well as induction of chemokine secretion from tumors or tumor-associated cells is essential for efficient recruitment of NK cells to the tumor site where they can elicit their tumoricidal effect (discussed in paper III).

1.3.4.4.3 Cytokine-induced activation of NK cells

NK cells depend on cytokine stimulation for their function and survival. IL-2 has for many years been used to stimulate NK cells *ex vivo* and has been given to patients with cancer for several decades showing clinical responses both as a single therapy, in combination with cancer vaccines or in adoptive cell transfer [241, 242]. However, administration of high dose IL-2 is associated with severe toxicity such as capillary leak syndrome and other symptoms [243]. So far, adjuvant IL-2 treatment has generated *in vivo* proliferation and long-term persistence of adoptively transferred NK cells although the anti-tumor effect has been modest [236]. Therefore, additional NK-stimulating cytokines has been evaluated both for *ex vivo* manipulation and for *in vivo* use including those naturally derived from DCs. Depending on the subset of DC and what PAMPs they have engaged in the periphery, DCs can induce different types of activation signals for NK cells by either secreting or presenting cytokines to NK cells in SLOs [244]. IL-12 and IL-18 is secreted to activate and induce secretion of cytokines by NK cells, IL-15 is trans-presented by DCs to induce proliferation and provide survival signals to NK cells while type I IFNs can induce NK cell cytotoxicity [245-248]. Presumably, culturing of NK cells in a combination of cytokines prior to infusion would be a good strategy to allow for persistence and cytotoxicity *in vivo*. In a recent study, Cerwenka and colleagues found that in mice bearing solid tumors, administration of NK cells pretreated with a cocktail of IL-12, IL-15 and IL-18, in combination with radiotherapy, was superior in delaying tumor growth compared to IL-15-treated NK cells plus radiotherapy or radiotherapy

alone [249]. The IL-12/15/18-primed NK cells displayed properties that resemble "memory-like" NK cells including high proliferative capacity and *in vivo* persistence. Moreover, the traits were retained over several generations of NK cells [250]. The importance in IL-15 stimulation of NK cells has also been demonstrated by Szczepanski and colleagues who has shown that the cytotoxic potential of suppressed NK cells from AML patients could be restored *ex vivo* by exposure to IL-15 [251].

1.3.4.4.3 *Ex vivo* expansion and genetic modification of NK cells

There have been different opinions on the pros and cons and the potential benefit of *ex vivo* expansion of NK cells prior to infusion [252]. Preparation of the cellular product from peripheral blood mononuclear cells (PBMCs) is done by different variations of magnetic bead selection under GMP-conditions where the CliniMACS system (Miltenyi) is currently the only system approved by the FDA to be used in clinical trials. In an autologous setting, contamination of T cells is generally acceptable while in an allogeneic setting, a depletion of T cells using anti-CD3 magnetic beads is required to prevent induction of GVHD. After CD3 depletion the product still contains large amounts of monocytes and B cells and a second step of $CD56^+$ cell magnetic bead selection can be employed, usually yielding a more than 99% purity of NK cells [253, 254]. By expanding the NK cell population using feeder cell populations, the fold expansion of NK cells can be increased compared to using only cytokines. A larger absolute number of NK cells in the infused cell product could increase the chances of targeting cancer cells, although the risk of encountering replicative senescence is a factor to consider, as it has been shown with TILs that a shorter activation regimen *ex vivo* allows for higher *in vivo* proliferation [255].

When choosing the method for *ex vivo* activation it is also important to consider the selective expansion of certain NK cell subsets and the effect of the particular protocol on phenotype and function of the NK cells. Although cytokine stimulation with IL-2 has shown nearly 200-fold expansion rates of NK cells, culturing of NK cells with the MHC-class I deficient leukemic cell line K562 expressing 41BB-ligand and IL-15 provided stimulation and trans-presentation of IL-15 that induced proliferation of NK cells up to 1000-fold expansion, with increased cytotoxicity and IFNγ secretion [256, 257]. Similar proliferative capacity was observed when NK cells were cultured with irradiated EBV-transformed B cell lines which also augmented NK cell killing of tumors with marked upregulation of TRAIL, FasL and NKG2D [258]. Although the field of genetic modification of NK cells is struggling with low transductions rates, NK cells have been successful transduced with a CD19-specific CAR with a CD3 ζ-chain which improved the NK cells killing capacity against CD19-positive leukemia cells [256]. The NK cell line NK92 has also been successfully transduced with a HER2/NEU-CAR inducing cytotoxicity against a HER2/NEU positive cell line that was completely resistant to untransduced NK92 cells [259].

1.3.4.4.4 Reducing suppression of NK cells

T_{REG} have a strong inhibitory effect on NK cells and in combination with IL-2 treatment of patients undergoing NK cell transfer, T_{REG} are generated and preferentially activated thus

contributing both to immune suppression of NK cells and to a cytokine sink effect [260, 261]. A recent study showed that, in a BALB/c mouse model, administration of IL-2 diphteria toxin prior to adoptive transfer of syngeneic IL-15-treated NK cells, selectively depleted T_{REG} and resulted in NK cell mediated boosting of adaptive immune responses, reduced tumor clearance and increased survival [262]. As described earlier, MDSCs are potent inhibitors of NK cell function. Efforts to limit the conversion of myeloid cells into MDSCs and to inhibit their actions have recently shown beneficial effects in mouse models of cancer. By administration of nitro aspirin and the phosphodiesterase 5 inhibitor Sildenafil in mice, inducible nitric oxide synthase (iNOS) and arginase I (Arg1) production in MDSCs is reduced respectively, resulting in MDSCs displaying a less suppressive phenotype and increased tumor infiltration and efficacy of adoptively transferred T cells in tumor bearing mice [263-265]. However, none of the treatments resulted in decreased tumor progression. By treating mice as well as humans with either cyclooxygenase 2 (COX-2) inhibitors, reduced levels and reduced suppressive capacity of MDSCs has been observed [174, 266].

1.3.4.4.5 Improving homing of NK cells towards tumors

Studies on the recruitment of lymphocytes to tumor sites have mostly been performed in the context of CTL [267]. There has been evidence of tumor infiltration of adoptively transferred melanoma-associated antigen recognized by T cells (MART-1)-specific T cells in melanoma patients where up to 38% of infiltrating T cells were MART-1 specific but also in MART-1 expressing non-malignant tissues leading to vitiligo [268]. Although there little known about the rate of tumor infiltration and quantification of absolute numbers of adoptively transferred cells, one study showed that only 0.016 % of the total number of infused cells in a patient with melanoma was localized per gram of tumor [240]. This finding together with observations of tumor progression despite high levels of circulating tumor-antigen specific T cells after adoptive transfer highlight the importance of efficient lymphocyte homing to achieve a clinical response [269]. In mouse models it has been shown that transduction of tumors with the chemokine CCL21 resulted in increased cytotoxic activity of adoptively transferred T cells and prolonged survival in the mice although increased T cell infiltration could not be detected [270]. In other studies, introduction of chemokine genes such as CCL3 and XCL1 induced migration of T cells to the tumor site but failed to induce tumor regression [271]. In another model, tumors adenovirally transduced with CX3CL1 had increased accumulation of both T cells and NK cells which contributed to suppression of tumor growth. Expression of CX3CL1 as well as CXCL10 in tumor tissues has been shown to be one of the main predictive factors for infiltration of CTLs in colorectal cancer, although the correlation did not apply to tumor infiltration of NK cells [29]. However, CX3CL1 receptor CX3CR1[13] has been shown to be a major governing chemokine receptor for successful homing of NK cells to the central nervous system during experimental autoimmune encephalomyelitis and

[13] Fractalkine receptor

induction of CX3CL1 in murine lymphoma cell lines induced NK cell recruitment and inhibition of tumor development which was abrogated in $RAG^{-/-}$ mice[14] suggesting that NK cells play a major role in the anti-tumor effect [272].

Expression of the relevant chemokine receptors is essential for the induction of homing of lymphocytes towards tumors. Genetic manipulation of the infused cell product as a strategy to augment the chemokine receptor expression and induce tumor-directed migration has been shown to be beneficial in mouse models. Peng and colleagues introduced the CXCR2 gene in pMEL-1 T cells recognizing the gp100 peptide on melanoma cells. The melanoma cells spontaneously produce the CXCL1 and CXCL8 chemokines that ligate and attract CXCR2 positive lymphocytes. Adoptively transferred CXCR2-transduced T cells showed increased localization to the tumors resulting in reduced tumor progression and prolonged survival [273]. The importance of CXCR3-induced migration of NK cells towards tumors was demonstrated by Wendel et al. They showed in a mouse model that CXCR3-positive NK cells could home to and reduce the progression of tumors that ectopically express CXCR3-ligands or alternatively are treated with IFNγ to overexpress the ligands. In $CXCR3^{-/-}$ mice the accumulation of NK cells in the tumors was impaired [118].

1.3.4.4.6 Combination therapies

Chemotherapy agents are designed to kill human tumor cells while causing minimal toxicity to normal cells. However, the secondary effects that they may have in an immune competent host are rarely assessed before they are put to clinical use [274]. Although there is a risk that chemotherapy would induce a selection of tumor cells that are resistant to cellular stress and would therefore become resistant to both continued chemotherapy and immune cell attack, several studies has shown that many different chemotherapy agents can boost the anti-tumor immune response by several different mechanisms [275]. Induction of immunogenic cell death can be achieved by several pharmacological compounds. Anthracyclines and platinum-based drugs have been shown to induce relocation of calreticulin to the cell surface, thus promoting phagocytosis and antigen presentation of tumor cell peptides. A second effect is the increased release of danger signals such as HMGB1 which binds the PPR toll-like receptor 4 (TLR-4) on DCs thus facilitating cross-presentation of the processed peptides [276, 277].

Enhancing the sensitivity of tumor cells to killing by lymphocytes by chemotherapy treatment has been demonstrated in several studies. By upregulating the tumor-antigen carcino-embryogenic antigen (CEA) on colon and breast cancer cells, treatment of tumor cells with 5-fluoracil enhanced their sensitivity to killing by HLA-A2 restricted T cells [278]. Upregulation of mannose-6-phophate receptors on tumor cells induced by the chemotherapy agents paclitaxel, cisplatin and doxorubicin enhanced the tumor cell's permeability to

[14] Deficient in B and T cells

granzyme B making them sensitive to killing by tumor-specific T cells. This approach also induced a bystander effect when adjacent tumor cells that did not express tumor antigen were targeted by the cytotoxic effect of granzyme B released from the T cells [279]. The ligands for NKG2D are inducible by cellular stress and it has been shown that treatment of cell lines with proteasome inhibitors or histone deacetylase (HDAC) inhibitors upregulated expression of ULBP2, MICA and MICB on tumor cells [280]. In some studies, cell lines treated with HDAC inhibitors displayed increased sensitivity to lysis by NK cells [281, 282] which could be synergistically enhanced in combination with ionizing radiation [283]. Elevated sensitivity to NK cell lysis via upregulation of TRAIL-R2 has also been reported in cell lines and in mice treated with the proteasome inhibitor bortezomib [284, 285]. There have also been reports of radiotherapy regimens that augment immune responses [277, 286]. A study showed that *in situ* tumor destruction by radiofrequency ablation induced an immune response in a B16-OVA melanoma model which was greatly enhanced in combination with anti-CTLA-4 antibody treatment [287]. Chemotherapeutic agents often have immunosuppressive side effects where some of the more well known, including methotrexate and imatinib, have deleterious effect on peripheral T cells [288, 289] Although cyclophosphamide and fludarabine (Cy/Flu) are commonly used to lymphodeplete patients with leukemia or as a preconditioning to adoptive cell transfer, residual T cells after Cy/Flu treatment have a mature phenotype, are predominantly T_H1 polarized and are more responsive to mitogen stimulation [290]. Moreover, Cy/Flu treatment as well as 5-fluoracil can aid anti-tumor immune responses by selectively depleting suppressive immune subsets [291-293]. Myeloid cells are particularly sensitive to chemotherapy agents although there are differences among drugs. At therapeutic concentrations, doxorubicin, cisplatin and vinblastine can easily trigger DC apoptosis while the toxicity of etoposide and 5-fluorouracil is relatively low [294]. Several targeted therapies are also found to work in concert with the immune system. By inhibiting VEGF, which has been shown to inhibit differentiation and maturation of DCs, the anti-VEGF monoclonal antibody bevacizumab or tyrosine kinase inhibitors sorafenib or sunitinib can restore function of tumor resident DCs while simultaneously inhibiting tumor vascularization [295]. In cancer patients receiving monoclonal antibody treatment such as cetuximab, which targets the epidermal growth factor, the ADCC effect by NK cells has been shown to contribute to the anti-tumor effect [296]. There has also been a correlation found between NK cell function and the response of trastuzumab in patients with metastatic breast cancer [103].

Antibody-mediated blocking of NK cell inhibitory receptors has been tested in a mouse leukemia model where an antibody targeting the murine equivalent of KIR, the Ly49-receptor, demonstrated anti-tumor effect with low toxicity [297]. Importantly, subsequent studies showed that long-term exposure to Ly49-targeting antibodies did not induce autoimmunity or NK cell anergy suggesting that NK cell licensing was not disrupted by the antibody treatment [298]. Moreover, combination of the immunostimulatory drugs lenalidomide with KIR-blocking antibodies has shown synergistic effect in a MM mouse model [299, 300]. A human antibody targeting KIR2DL1/2/3 augments NK cell lysis of MM and AML blasts *in vitro* and was shown to be tolerable in a phase I trial [301]. In clinical trials and in pre-clinical studies checkpoint inhibitors have been combined with each other as well as with other targeted therapies. Wolchok et al showed that concomitant treatment of

patients with advanced melanoma with ipilimumab and nivolumab resulted in that 53% of patients had objective tumor responses and 80% of the responders had tumor regression [302]. Ipilimumab has also been combined with autologous tumor lysate vaccination plus GM-CSF in patients with advanced ovarian carcinoma which generated clinical responses. Here, the extent of tumor necrosis correlated with an increased CTL/T_{REG} ratio [303] indicating that CTLA-4-meditaed T_{REG}-depletion by ipilimumab may have contributed to the specific CTL-response. Although few, there have been reports of patients experiencing tumor regression of distant metastasis after directed radiotherapy to their primary tumors [304-306]. This phenomenon has been termed "abscopal effect" and provides striking evidence for how localized radiotherapy can induce immunogenicity of a tumor that is strong enough to elicit a systemic anti-tumor immune response [307]. It is known that when a tumor is subjected to ionizing radiation, several events occur that may promote both infiltration and tumor-recognition by lymphocytes, including chemokine release, presentation of tumor-antigen and upregulation of death receptors and adhesion molecules [308-311].

Considering the complexity and dynamic nature of the interactions between a tumor and the immune system, it is best to take a multimodal approach when designing immune therapeutic strategies against cancer. With increasing knowledge of how our most commonly used chemotherapeutic drugs and radiation regimens affect the phenotype of tumor cells and the micromileu of the tumor surroundings, their clinical use can be fine-tuned to work in synergy with the anti-tumor immune response. Furthermore, with the recent development of new immunomodulatory agents and targeted therapies against cancer we now have a better capability to tailor immune therapies to each individual cancer patient. For NK cell-based therapy, few cancer types have been tested in clinical trials and the use of combination treatments have not yet been evaluated extensively. Therefore, there are still many hurdles that need to be bridged for NK cells to reach their full therapeutic potential. In this thesis I have focused on three of them; low sensitivity of tumors to NK cell lysis, poor migration of NK cells toward solid tumors and lack of tumor targets that are prone to killing by NK cells.

2. Aims of the thesis

The general aims of this thesis is to increase the understanding of how NK cells interact with tumor cells with the overall purpose to bridge the hurdles that are currently facing NK cell-based therapies. The thesis is divided into three parts and their respective aims are:

1. **Enhancing NK cell cytotoxicity towards tumor cells:** exploring ways to enhance the anti-tumor effect of adoptively transferred NK cells, either by sensitizing tumor cells to NK cell mediated lysis or by boosting the cytotoxic potential of *ex vivo* expanded NK cells. (Paper I and II)

2. **Augmenting NK cell migration toward solid tumors:** studying how chemokine-induced migration of expanded NK cells affects their homing toward solid tumors and how it impacts the anti-tumor effect. (Paper III)

3. **Finding novel tumor targets for NK cell therapy**: assessing the interaction between NK cells and anaplastic thyroid carcinoma cells in terms of mechanism of NK cell-mediated lysis as well as chemoattraction and regulation of NK cell activity. (Paper IV).

3. Results and Discussion

3.1 Enhancing NK cell cytotoxicity against tumor cells

In the past few decades, breakthroughs in technical development of cell culture systems, selection of donors and subsets of NK cells for infusion as well as improved GMP facilities, have increased the ability to treat cancer patients in a personalized setting with large amounts of *ex vivo* expanded NK cells. However, the inherent regulation of NK cell cytotoxicity is still a factor that needs to be considered when NK cells are infused and expected to find and kill a tumor. The inhibition of NK cells by MHC class I molecules as well as the downregulation or shedding of recognition receptors on tumor cells reduces the chances of generating an anti-tumor effect by infused NK cells, even if the NK cells are able to reach and interact with the patient's tumor. Two strategies to overcome the decreased targeting are to either sensitize tumor cells to NK cell lysis or to manipulate NK cells *ex vivo* to improve their targeted cytotoxicity.

3.1.1 Paper I

The aim of this paper was to study chemotherapy-induced sensitization of tumor cells to lysis by activated NK cells and T cells. The proteasome inhibitor bortezomib has previously been shown to render tumor cells sensitive to NK cell-mediated lysis [284]. However, due to the interference with antigen processing following bortezomib treatment, tumor cells were rendered almost completely resistant to lysis by tumor-specific T cells [312]. An appealing prospect in the field of adoptive cell transfer is to combine both NK cell- and T cell-infusions to increase the chances of targeting tumor immune escape variants. Therefore, we aimed to screen several different chemotherapy agents for their potential to induce sensitivity to both NK cell and T cell mediated lysis. We found that the chemotherapy agent doxorubicin[15], which had previously been described to induce sensitivity to lysis by recombinant TRAIL, could sensitize tumor cells to lysis by both *ex vivo* expanded NK cells and T cells expressing high levels of surface bound TRAIL (figure 5). Through blocking studies we could confirm that when TRAIL was blocked in co-cultures where NK cells or T cells were incubated with doxorubicin-treated tumor cells, the level of tumor cell lysis was significantly reduced. As previously described, we found that expression of the anti-apoptotic protein cFLIP in tumor cells was almost completely abolished already after a few hours incubation with subapoptotic levels of doxorubicin [313]. cFLIP inhibits the cleavage of pro-caspase-8 into active caspase-8 which subsequently cleaves effector caspases and ultimately induces apoptosis in the tumor cell. Alternatively, active caspase-8 can cleave Bid which propagates the apoptotic signal via

[15] Doxorubicin is an anthracycline antibiotic that is routinely used to treat a variety of different cancer types. By intercalation of DNA, doxorubicin inhibits the enzyme topoisomerase II which results in inhibition of replication in the affected cell.

the mitochondrial pathway [314]. We observed that when tumor cells were exposed to low levels of doxorubicin or NK cells, we measured only minimal activation of caspase-8 and cleavage of Bid. However, if we added NK cells to a tumor cell culture that had been pretreated with doxorubicin, there was a marked increase in both activation of caspase-8 and Bid-cleavage, indicating a synergistic effect.

Next, we assessed whether doxorubicin could sensitize tumors to adoptively transferred NK cells in a xenogeneic mouse model where immunodeficient mice bearing subcutaneous tumor received infusions of human expanded NK cells and/or tumor-specific T cells. Mice received intravenous injections of low levels of doxorubicin 24 h prior to the lymphocyte infusions. We observed that, while the doxorubicin treatment alone did not affect the tumor progression, it reduced the tumor progression in the mice that received NK cells, T cells or a combination of NK and T cells. Interestingly, in doxorubicin treated mice, the combination of NK and T cells generated the greatest anti-tumor effect when compared with infusions of NK cells alone or T cells alone. From our findings, we can conclude that doxorubicin has the potential to sensitize tumors to lysis by NK cells or T cells through increasing TRAIL-mediated signaling. Thus, doxorubicin could potentially be used as an adjuvant therapy for NK cell or T cell-based adoptive transfer therapies. There have been other immune-related effects of doxorubicin that could synergize with the induction of NK cell sensitivity. By triggering immunogenic cell death, the chances of priming of tumor-specific T cell responses increases which could be further augmented by the tumor cells doxorubicin-induced sensitivity to TRAIL-mediated T cell lysis [276]. Recently, it has been shown that doxorubicin selectively depletes MDSCs and reduces MDSC suppressive function in mice thus augmenting degranulation of NK cells and CTLs in doxorubicin-treated mice. In humans, isolated MDSCs were sensitive to doxorubicin toxicity *in vitro* [315]. This reduction of immune suppression would further aid in the NK and T cell-mediated lysis of doxorubicin-treated tumors.

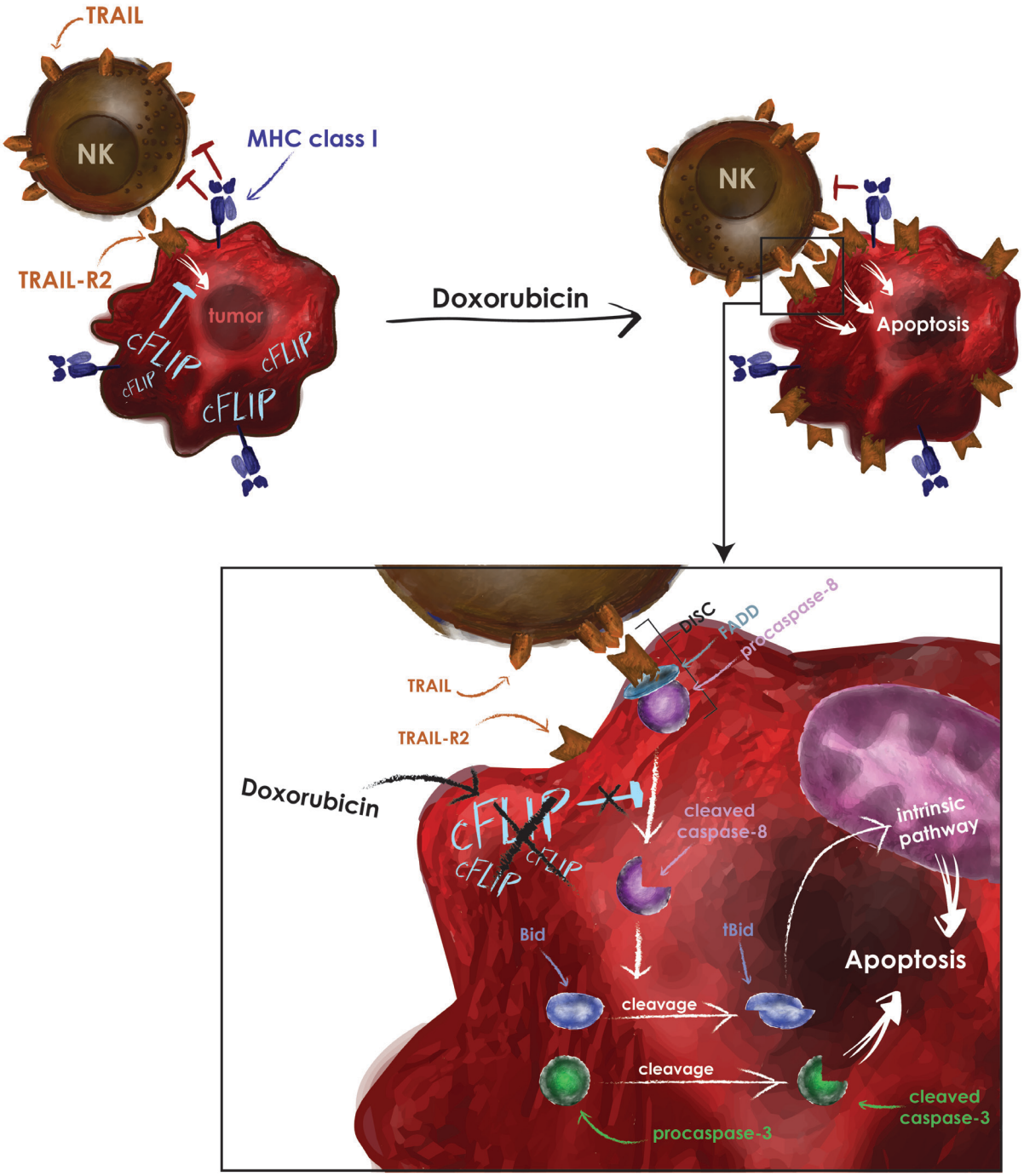

Figure 5. Doxorubicin sensitizes tumor cells to NK cell lysis via increased TRAIL-signaling. Doxorubicin has a dual effect in sensitizing tumor cells to lysis by expanded NK cells expressing high levels of TRAIL. When added to tumor cells at sub-apoptotic concentrations, doxorubicin induces cell surface expression of TRAIL-R2 which promotes binding of membrane-bound TRAIL. The ligation results in recruitment of pro-caspase-8 to the DISC complex which is cleaved into active caspase-8 resulting in initiation of an intracellular apoptotic cascade. The second effect of doxorubicin is the induced downregulation of the anti-apoptotic protein cFLIP in tumor cells. cFLIP inhibits the cleavage of pro-caspase-8, which in untreated cells abrogates the apoptotic cascade. Therefore, in doxorubicin-treated cells, both the TRAIL/TRAIL-R2 interaction as well as the intracellular TRAIL-signal is amplified leading to an increased sensitivity of the tumor cells to NK cell-mediated lysis. Signaling via TRAIL is described in detail on page 21.

3.1.2 Paper II

In this paper we aimed to study the immunostimulatory effect of zoledronic acid (ZA) on human NK cells. ZA is used to treat cancer patients suffering from bone metastasis by preventing bone resorption thus reducing the risk of skeletal complications. Here we show that monocytes stimulated with ZA are stimulated to produce IFNγ. Selective expansion of γδ T cells[16] has been observed in PBMC cultures treated with ZA and IL-2 and ZA have also been shown to sensitize colon cancer cells to lysis by γδ T cells [316, 317]. ZA-treated DCs can in turn activate NK cells via production of IL-18 and IL-1β and NK cells can be further co-stimulated by ZA-activated γδ T cells [318]. We found that monocyte-derived IFNγ was responsible for the induction of TRAIL expression on NK cells (figure 6). When NK cells were co-cultured with monocyte-depleted PBMCs in presence of ZA, induction of TRAIL expression on NK cells was abolished. Indeed, co-culture of NK cells and purified monocytes provided an additional boost of TRAIL-expression on NK cells and also increased secretion of soluble TRAIL and FasL. NK cells primed with ZA in a monocyte co-culture also acquired an increased cytotoxic effect against TRAIL-sensitive tumor cell lines *in vitro*. The clinical significance of ZA as an immune stimulatory drug was demonstrated in an immunodeficient SCID/Beige mouse model, where infusion of ZA-primed NK cells reduced tumor progression and prolonged survival of tumor-bearing mice when compared with mice receiving unprimed NK cell infusions. In the clinic, a combined treatment with ZA and IL-2 in patients with metastatic prostate cancer has shown to be safe where treatable side effects have been attributable to the IL-2 treatment. Patients maintained high levels of soluble TRAIL produced largely by activated γδ T cells whose numbers correlated with clinical outcome [319]. Our study shows that ZA could be used both in combination with autologous monocyte cultures in the priming of NK cells prior to infusion in patients carrying TRAIL-R -positive tumors. Alternatively, patients could be pretreated with chemotherapy inducing sensitivity to TRAIL-mediated lysis, including doxorubicin or the novel proteasome-inhibitor bAP-15 [320-322].

[16] The γδ T cell is a relatively uncharacterised cell type which has properties resembling both innate and adaptive immune cells. It has a non MHC-restricted T cell receptor and they are most frequent in epithelial tissues such as in the skin and in the intestine.

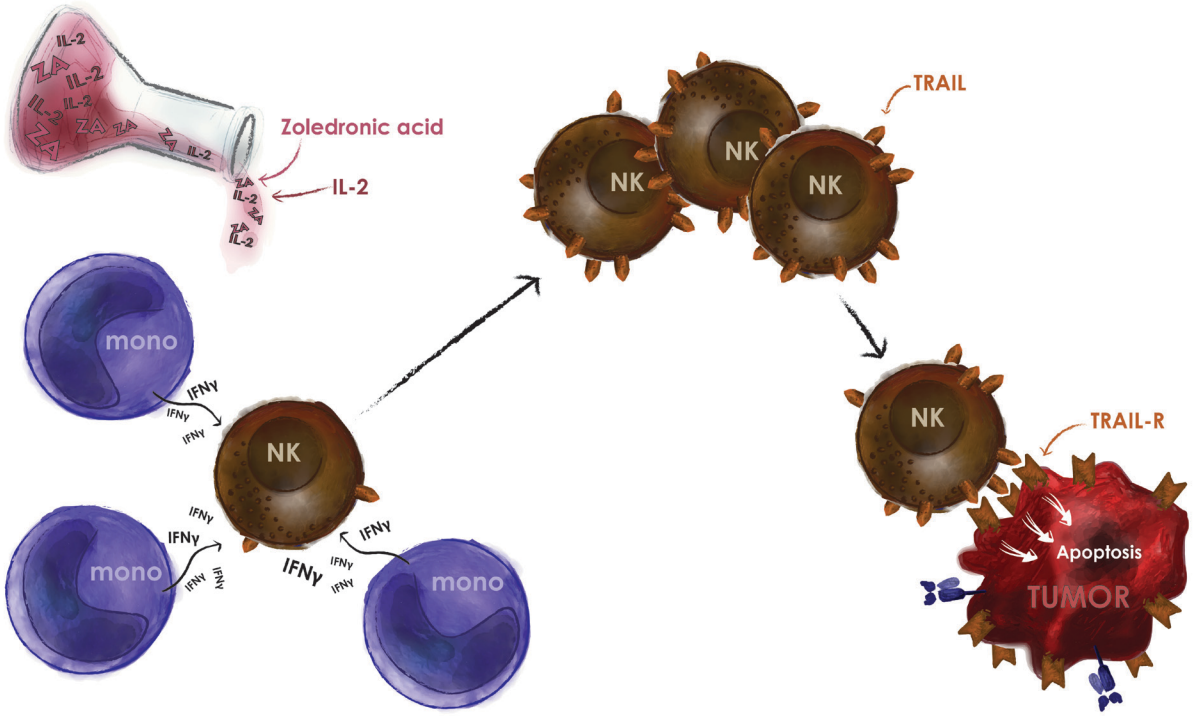

Figure 6. ZA upregulates TRAIL on NK cells. In a co-culture of NK cells and autologous monocytes, addition of ZA in combination with IL-2 induces secretion of IFNγ from the monocytes which in turn triggers upregulation of TRAIL on NK cells. ZA-primed NK cells display enhanced cytotoxicity against TRAIL-sensitive tumor targets *in vitro* and *in vivo*.

3.2 Augmenting NK cell migration toward solid tumors

NK cell therapy has been most effective against hematological malignancies but has yet to generate strong anti-tumor effects in patients with solid tumors. Although studies have shown that NK cells are better at lysing tumor target cells of hematopoietic origin compared to tumor cells derived from other tissues [323], several investigators have shown that expression of both ULBPs and NCRs is low on leukemic blasts while loss of HLA class I expression is less frequent in AML than in melanoma and other solid tumors [324, 325]. Insufficient homing of NK cells has been proposed as one of the main contributing factors to the discrepancy in clinical effect between hematological and solid tumors [233, 236, 326]. In line with this hypothesis, it has been shown in several cancer types that intratumoral infiltration of NK cells correlates with good prognosis [115-117, 327, 328]. It has been demonstrated that in melanoma patients receiving indium-labeled lymphocytes without pre-conditioning, only a fraction of a percent of the infused lymphocytes actually end up at the tumor site [240]. There have been several attempts made at augmenting the homing capacity of lymphocytes by inducing chemokine receptor expression, either by stimulation or by viral transduction [273, 329]. Other studies have showed that induced secretion of chemokines from tumor cells or tumor-associated cells results in increased directed lymphocyte homing and infiltration [118].

3.2.1 Paper III

In this paper we aimed to demonstrate the clinical significance of CXCL10-induced migration by adoptively transferred *ex vivo* expanded NK cells. There are several conflicting studies on how the expression of CXCR3 is modulated on NK cells upon activation [330, 331]. By using an NK cell expansion protocol involving high dose IL-2 and a feeder cell population of irradiated EBV-transformed B cells, we achieved a more than 10-fold increase in surface expression of CXCR3. In transwell migration assays we showed that expanded NK cells migrated better towards both recombinant CXCL10 and IFNγ-stimulated tumor cells (figure 7). To confirm that an augmented CXCL10-directed homing of NK cells would have an effect on tumor growth, we established a xenogeneic mouse model. In the first step we inoculated mice with tumors that were negative for CXCL10-production subcutaneously in the left flank and with tumors that were lentivirally transduced to overexpress CXCL10 in the right flank. When tumors became palpable the mice were treated with fluorescently labeled expanded human NK cells and migration of the NK cells was monitored using *in vivo* imaging. This model demonstrated that NK cells preferentially migrated towards the CXCL10-positive tumors. In a second xenograft model, we implanted CXCL10-negative tumors in one group of mice and CXCL10-positive tumors in another group. Both groups were injected with fluorescently labeled NK cells. By measuring the fluorescence intensity of excised tumors we found that NK cell infiltration was higher in the CXCL10-positive tumors than in CXCL10-negative tumors. The impact of CXCR3-ligand expression in tumors on the migration and infiltration of lymphocytes has been exemplified both in patients and in mouse models [29, 118]. However, we show for the first time that CXCL10-induced migration of adoptively transferred human NK cells has a significant impact on the anti-tumor effect of the infused NK cells.

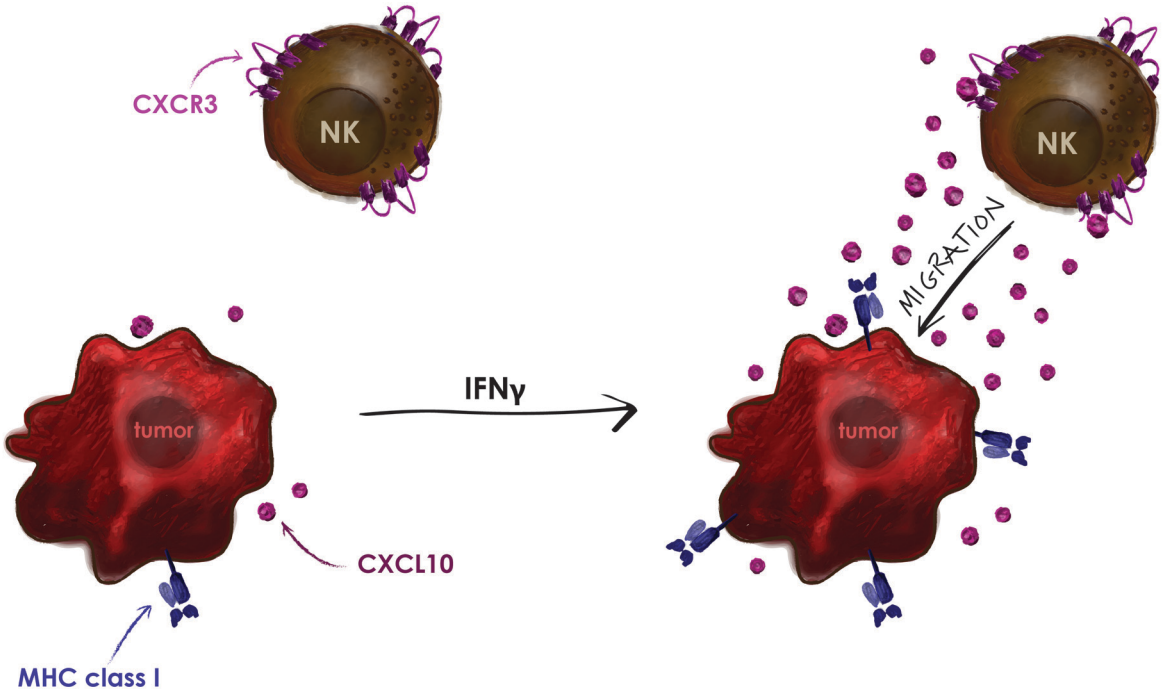

Figure 7. IFNγ-induced secretion of CXCL10 chemoattracts CXCR3-positive NK cells. Tumors can be stimulated to secrete the NK cell-attracting chemokine CXCL10 by stimulation with the pro-inflammatory cytokine IFNγ. Although the IFNγ-stimulation leads to upregulation of MHC class I on tumor cells thus conveying an increased resistance to NK cell lysis, the augmented secretion of CXCL10 leads to increased capacity of tumor cells to chemoattract NK cells expressing the chemokine receptor CXCR3.

3.3 Finding novel tumor targets for NK cell therapy

NK cell therapy against solid tumors has been tested only in a few trials and in a very limited range of malignancies. Although clinical responses of NK cell treatment has been observed in RCC, malignant glioma and metastatic breast cancer, durable tumor regression has so far been limited [332-334]. For T cell therapy, melanoma has been identified as the single most immunogenic tumor type, although for NK cell treatment that has not been the case [335]. The identification of tumor types that display the characteristics needed, at least in theory, to be targetable by activated NK cells is one important aspect to consider on the path to successful treatment of cancer patients with NK cell therapy.

3.3.1 Paper IV

The aim of this paper was to study the interactions between NK cells and ATC cells in terms of sensitivity to lysis by expanded NK cells as well as proneness to chemoattract NK cells (figure 8). The idea of the project was formulated when we were screening cell lines for their response to doxorubicin in terms of sensitivity to NK cell lysis. In all of the ATC cell lines that we tested we observed sensitivity to lysis by NK cells at a level that we had not seen with

any other cell lines irrespective of histiotype. We continued by obtaining a larger panel of ATC cell lines, of which half were in low-passage, and proceeded with elucidating the mechanisms behind the unprecedented sensitivity. We established that the NKG2D-pathway was involved in NK cell-mediated killing of ATC cell lines and further analysis revealed that ULBP2 was highly expressed on ATC cells and that the level of expression correlated with sensitivity to NK cell-mediated lysis. Blocking of NKG2D on NK cells or ULBP2 on tumor cells in a co-culture abrogated the NK cell-mediated lysis of ATC cells.

Analysis of fine-needle aspirates (FNA) and PBMCs from untreated ATC patients revealed a suppressed phenotype of tumor-infiltrating NK cells compared to NK cells from peripheral blood. The total frequency of NK cells among lymphocytes and the level of $CD56^{dim}$ NK cells in the NK cell population were lower in FNA than in PBMCs. Several investigators have reported a skewed $CD56^{dim}/CD56^{bright}$ ratio in chronic inflammatory conditions such as tuberculosis and rheumatoid arthritis [336, 337]. In cancer, which in many cases also can be considered as a site of chronic inflammation, it has been reported that circulating $CD56^{dim}$ NK cells are prone to undergo apoptosis [338]. It has also been proposed that $CD56^{dim}$ NK cells are more susceptible to ROS produced in the tumor microenvironment due to them having lower anti-oxidative capacity compared to $CD56^{bright}$ NK cells [150]. Although our analysis of CXCR3-expression in ATC patients only involves three samples we found that in two out of three patients, the $CD56^{dim}$ NK cells had a lower expression of CXCR3 compared to $CD56^{bright}$ NK cells. This could provide an alternative explanation to the skewed $CD56^{dim}/CD56^{bright}$ in ATC tumors due to preferential migration of $CD56^{bright}$ NK cells to the tumor site. In ATC patient samples we also observed that NKG2D surface expression was lower in FNA compared with PBMCs although CD69 expression was elevated in FNA. Expression of CD69 on NK cells is a sign of activation but can also be a general inhibitor of immune responses against tumors. NK cell cytotoxicity was augmented in $CD69^{-/-}$ mice as a consequence of altered cytokine signaling, including reduced lymphocyte secretion of TGFβ and increased release of MCP-1 and IL-1β [339].

We wanted to investigate if the poor condition of the NK cells was a result of immune suppressive factors secreted from the tumor, so therefore we cultured NK cells in the presence of supernatant from ATC cell lines. We found that tumor-derived supernatants from COX-2-positive cell lines where more suppressive of NKG2D-expression on NK cells than supernatant from COX-2-negative cell lines. Furthermore, when we neutralized PGE2 in the co-culture, both NKG2D-expression and cytotoxic activity of NK cells was restored to normal. The importance of NKG2D-mediated surveillance and elimination of tumors has been demonstrated in an NKG2D-deficient mouse model [340]. In *in vitro* culture, we observed that ATC cell lines were prone to secrete high levels of CXCL10 after stimulation with minute doses of IFNγ. Consequently, CXCR3-positive NK cells were able to actively migrate toward supernatant from IFNγ-treated ATC tumors in a transwell migration assay. In line with this finding, we found a dramatically increased percentage of CXCR3-positive NK cells in FNA compared to peripheral blood NK cells. Taken together, our findings indicate that ATC is a potential target for adoptive NK cells therapy due to sensitivity to NKG2D-mediated lysis by NK cells and proneness to secrete CXCL10.

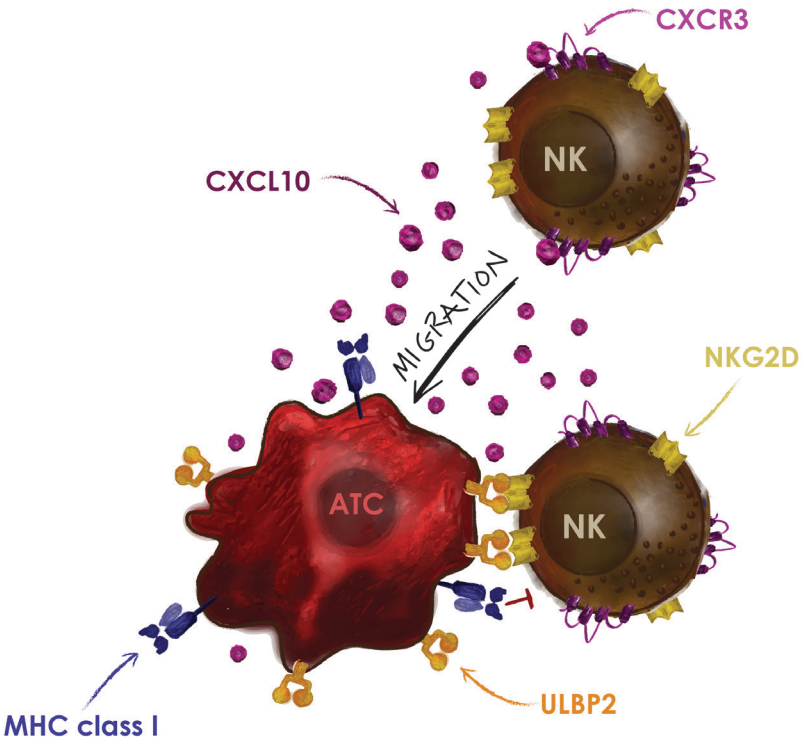

Figure 8. ATC cells are sensitive to NK cell lysis and chemoattract CXCR3-positive NK cells. The highly aggressive tumor type ATC express high levels of the NK cell activating ligand ULBP2 rendering them sensitive to NKG2D-mediated lysis by NK cells without prior sensitization. Moreover, ATC cells are prone to secrete high levels of the NK cell-attracting chemokine CXCL10. Taken together, these characteristics of ATC cells make ATC a promising target for NK cell-based therapies.

Concluding remarks

Successful NK cell-based immunotherapy relies on a variety of factors that are needed to ensure that NK cells reach their target and are able to eliminate it without being suppressed in the process. Therefore, continued studies on the intrinsic mechanisms of NK cell biology and regulation are essential in order to develop safe and efficient therapies. In a pre-clinical setting, screening of pharmaceutical capable of augmenting NK cell cytotoxicity and improving the chemotactic capacity of NK cells are important in order to find more effective ways of priming NK cells before infusion. In addition, by testing how chemotherapy agents and small molecules can be used to sensitize tumors to NK cell lysis and act in synergy with NK cells, will allow for the development of new combination strategies.

NK cell therapy is a *bona fide* individualized treatment form where the patient and the particularities of that patient's tumor need to be in focus. This requires a careful analysis of the parameters that will influence the chances of success, including donor selection and priming of the cellular product, choice of preconditioning and evaluation of predictive markers for each individual patient.

With ongoing clinical testing of immunomodulatory therapeutics and different combination strategies, which are nowadays carefully immune monitored, our knowledge of how NK cells interact with tumors and other components of the immune system will continue to increase. This knowledge, in combination with technical advances in the typing and selection of suitable NK cell donors and processing of cellular products, our ability to design effective NK cell therapies will likely increase in the future.

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