Cancer immunotherapies

Cancer immunotherapies: challenges and opportunities for NK cells in the tumor microenvironment

Femke Ehlers

Cover design: Femke Ehlers & Gildeprint

Print: Gildeprint

ISBN: 978-94-6419-701-3

The research presented in this thesis was performed within GROW, School for Oncology and Reproduction at Maastricht University. Illustrative figures in this thesis were created using biorender.com and SMART - Servier Medical ART.

Cancer immunotherapies: challenges and opportunities for NK cells in the tumor microenvironment

DISSERTATION

to obtain the degree of Doctor at Maastricht University,
on the authority of the Rector Magnificus, Prof. dr. Pamela Habibović,
in accordance with the decision of the Board of Deans,
to be defended in public

on Thursday, February 16th 2023 at 16:00 hours

by

Femke Auguste Irene Ehlers

Supervisors:

Dr. Lotte Wieten

Prof. dr. Gerard M. J. Bos

Assessment Committee:

Prof. dr. Axel zur Hausen (chair)
Prof. dr. Patrick Derksen (University Medical Center Utrecht)
Dr. Eva M. König (St. Anna Children's Cancer Research Institute, Vienna)
Dr. Sander Rensen
Prof. dr. Dirk De Ruysscher

Table of contents

Chapter 1	General introduction	7
Chapter 2	Tuning Natural Killer Cell Anti-Multiple Myeloma Reactivity by Targeting Inhibitory Signaling via KIR and NKG2A	31
Chapter 3	NKG2A expression is not per se detrimental for the anti-multiple myeloma activity of activated natural killer cells in an in vitro system mimicking the tumor microenvironment	47
Chapter 4	ADCC-Inducing Antibody Trastuzumab and Selection of KIR-HLA Ligand Mismatched Donors Enhance the NK Cell Anti-Breast Cancer Response	75
Chapter 5	Profiling NK cells within the tumor microenvironment of HER2- and HER2+ human breast cancer	107
Chapter 6	Activated natural killer cells withstand the relatively low glucose concentrations found in the bone marrow of multiple myeloma patients	141
Chapter 7	Exploring the potential of combining IL-2 activated NK cells with an anti-PDL1 monoclonal antibody to target multiple myeloma-associated macrophages	161
Chapter 8	Polymorphic differences within HLA-C alleles contribute to alternatively spliced transcripts lacking exon 5	189
Chapter 9	General discussion	209
Appendices	Impact paragraph	236
	Summary/Samenvatting/Zusammenfassung	239
	Acknowledgements/Dankwoord/Danksagung	250
	Curriculum Vitae & List of publications	255
	Abbreviations	258

1

General introduction

The immune system as cancer treatment

The immune system is our defense mechanism that protects us against infectious agents such as viruses, bacteria and fungi and also plays a role in many other diseases including cancer. It comprises a highly complex network containing various immune cell types that cooperate to fight the diseased cells and afterwards restore tissue homeostasis[1]. Because the immune system is equipped with powerful tools to destroy cancer cells, harnessing the immune system's power against cancer are attractive novel therapy approaches, termed cancer immunotherapies[2]. Immunotherapies can modulate immune responses specifically against tumor cells, which is an advantage over conventional cancer treatments (e.g., chemotherapy or radiotherapy) which do not only target cancer cells, but can produce massive damage to normal tissues, too. Different approaches of immunotherapies have been used to activate immune cells or to exploit the anti-tumor functions of immune cells. Such approaches include adoptive transfer of immune cells, donor bone marrow stem cell transplantation, monoclonal antibodies, cytokine therapy, cancer vaccines, immune checkpoint inhibitors and T cells engineered with chimeric antigen receptors (CAR-T cells)[3]. Immunotherapies have the potential to induce long-lasting remissions, but the success is mostly limited to a fraction of patients, while other patients experience recurrence or do not respond to the treatment[4,5]. Why this happens is not understood in detail, but tumors can for instance be equipped with strategies to escape immune cells, resulting in immune cell exclusion from tumors or dysfunctional immune cells inside tumors due to an immunosuppressive environment[5,6]. Therefore, much research is devoted to restoring and enhancing the functions of the immune cells to more effectively fight cancer cells. In our research group, we focus on natural killer (NK) cells for immunotherapies as NK cells can mediate potent and quick anti-tumor responses and are safe to administer to patients. To develop effective NK cell-based immunotherapies, we traditionally focus on two cancer types as models namely multiple myeloma, a form of blood cancer, and breast cancer as a solid cancer, since improved therapy approaches are desired for both tumor types.

Multiple Myeloma

Multiple myeloma (MM) is an incurable B cell malignancy, characterized by aberrant plasma cells in the bone marrow that usually develop from one clone and produce monoclonal, dysfunctional antibodies named paraproteins[7]. The disease develops from an asymptomatic phase called monoclonal gammopathy of undetermined significance (MGUS), i.e. monoclonal antibodies present in the blood but no further disease stigmata, which might progress to smoldering MM and eventually to symptomatic MM[8]. MM represents 13% of hematological malignancies and primarily occurs in the elderly population[9]. Therapy options have greatly advanced in the past two decades from standard chemotherapy and stem cell transplants to broader treatment options including immunomodulatory drugs, proteasome inhibitors, and monoclonal antibodies (e.g., anti-CD38 antibody Daratumumab, anti-SLAMF7 antibody Elotuzumab), which have substantially improved survival[10]. Despite these

improvements, the large majority of patients will develop therapy resistance and will relapse eventually[10]. Because MM progression is strongly associated with dynamic changes in the surrounding bone marrow environment, comprehensive understanding of alterations in the MM environment could help to overcome therapy resistance and further improve treatment strategies[8].

Breast cancer

Breast cancer is worldwide the most commonly diagnosed cancer in women and remains a leading cause of cancer death[11]. Despite improved clinical management and survival rates, around 30% of breast cancer patients develop metastases and succumb to the disease[11,12]. Occurrence of metastases many years after the initial diagnosis is especially prominent in estrogen-positive breast cancer, where the 20-year recurrence rate is between 10 - 40%[13]. Breast cancer is a highly heterogeneous disease and are traditionally classified into molecular subtypes based on the presence or absence of the hormone receptors for estrogen and progesterone, the human epithelial growth factor receptor (HER2) or the absence of all three of these receptors (triple-negative breast cancer). Standard therapy approaches for all subsets comprise surgery, radiation or systemic treatments such as chemotherapy. For the 15-20% of patients with $HER2^+$ breast cancer, who used to have worse prognosis than patients with other subtypes, the approval of the HER2-targeting antibody Trastuzumab significantly improved the clinical outcomes for a subgroup of patients, but the clinical efficacy remains heterogeneous as 25-50% of $HER2^+$ breast cancer patients do not respond or eventually relapse[14].

Natural killers and more - Functions of NK cells

NK cells are involved in the first line of defense against tumors and microbial infections[15]. When they were first described in 1975, NK cells received their name due to their ability to kill tumor cells 'naturally', meaning without previous antigen stimulation and in a short period of time[16,17]. Later, it became clear that NK cells have many other functions as well, which include protection against certain viruses, parasites and intracellular bacteria as well as tissue-specific functions e.g., in the uterus[15,18]. NK cells account for 5-15% of the mononuclear cells in peripheral blood and they can be identified as $CD3^-$ $CD56^+$ cells. Based on their CD56 expression, NK cells can be further divided into $CD56^{dim}$ and $CD56^{bright}$ subsets. $CD56^{dim}$ NK cells make up the majority of NK cells in the peripheral blood (around 90%) and are generally considered the more cytotoxic subset, whereas $CD56^{bright}$ NK cells are more abundantly present in tissues and are generally less cytotoxic but strong cytokine producers[19]. However, more recent studies demonstrated that NK cell subsets in blood and tissues are much more diverse in phenotype and function and that the functional status of NK cells cannot solely be based on their phenotype[20]. The NK cell functions are described below in more detail with a focus on their anti-tumor functions.

NK cell-mediated cytotoxicity

NK cells constantly patrol the body and monitor whether the cells they encounter are healthy or diseased. When encountering a diseased cell, NK cells can readily kill the diseased target cell because NK cells are always in an activated state and loaded with cytotoxic granules[21]. Upon binding to target cells, an immunological synapse is formed and the NK cell's cytoskeleton is rearranged to establish close contact between NK cell and target cell and to facilitate cytotoxic responses against target cells[21]. The cytotoxic response of NK cells can be mediated via two different pathways, granule-mediated and death receptor-mediated killing of target cells. During granule-mediated cytotoxicity, the granule-containing lysosomes are guided towards the immunological synapse, where lysosomes fuse with the NK cell membrane and release the cytotoxic granules into synapse between NK- and target cell[22]. The granules contain perforins, which form holes in the target cell to facilitate granzyme entry and granzyme-induced apoptosis of the target cell[22]. The secretion of cytotoxic granules is termed degranulation, which can be measured by CD107a in experimental assays. CD107a, also named lysosomal-associated membrane protein-1 (LAMP-1), is a protein that is part of the lysosomal membrane and that gets integrated in the cell surface when lysosomes fuse with the cell membrane to release their content. An alternative way of inducing target cell death is death receptor-mediated cytotoxicity, which is induced when the ligands Fas ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL), expressed by NK cells, engage with the corresponding death receptors Fas or TRAIL-R1/R2 on the target cell. Subsequent conformational changes of the death receptor cause apoptosis of the target cell[23].
Of these two cytotoxic mechanisms by NK cells, the granule-mediated killing is a very rapid process that can occur within the first hour, whereas death-receptor mediated cell death requires more time[24]. After killing their first target cell, NK cells can usually continue to kill other target cells, a process that is termed 'serial killing'. How many target cells are killed can differ between NK cells with some NK cells killing many targets, while others might kill none[25].

Cytokine production by NK cells

When activated, NK cells are capable of producing large amounts of inflammatory cytokines such as interferon- γ (IFN-γ), tumor necrosis factor- α (TNF-α), granulocyte-macrophage colony stimulating factor (GM-CSF) and various chemokines[26,27]. By secreting cytokines, especially IFN-γ, NK cells can directly affect tumor cells for example by changing expression of cell surface molecules on tumor cells such as upregulation of death receptors and by stimulation of antigen presentation[28]. Through cytokine secretion, NK cells additionally promote macrophage and dendritic cell activation via IFN-γ and TNF-α[29]. Moreover, NK cells can stimulate adaptive immune responses such as priming of $CD4^+$ T helper cells type 1 by secreting IFN-γ[30]. Thereby, NK cells can contribute to anti-tumor immune responses in an indirect manner[30,31]. Depending on the context, NK cells can also adopt functions that support the tumor, for instance by secreting pro-angiogenic factors like vascular endothelial growth factor (VEGF) in response to hypoxia[32]. As this example

illustrates, the factors secreted by NK cells depend on the tissue NK cells reside in and the environmental cues they receive.

Antibody-dependent cellular cytotoxicity

In addition to their natural cytotoxic function, NK cells can kill their target cells with the support of antibodies; this mechanism is termed antibody-dependent cellular cytotoxicity (ADCC). NK cells recognize the Fc portion of human immunoglobulin G (IgG) antibodies through the receptor CD16, also named Fc gamma receptor IIIA (FcγRIIIA)[33]. When the antibodies bound to both their target and to NK cells, it creates a bridge between target cell and NK cell and can lead to NK cell-mediated target cell killing and secretion of large amounts of cytokines (**Figure 1**). ADCC is a key mechanism for NK cells to recognize tumor cells and significantly contributes to the clinical efficacy of some tumor-targeting monoclonal antibodies such as Daratumumab (anti-CD38) for multiple myeloma, Rituximab (anti-CD20) for B cell malignancies, or Trastuzumab (anti-HER2) for breast cancer[34].

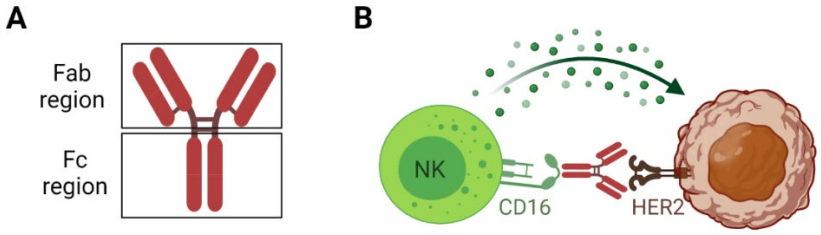


Figure 1. Antibody-dependent cellular cytotoxicity (ADCC). A) Structure of an IgG antibody. **B)** An antibody, in this example the anti-HER2 antibody Trastuzumab, bind its antigens (HER2) on the target cell via the Fab region. The NK cell recognizes the antibody through the CD16 receptor binding to the antibody's Fc region, whereby the antibody bridges the NK cell and target cell and signals the NK cell to kill the target cell. The figure was created using BioRender.com.

NK cell activation and recognition of target cells

NK cells can recognize diseased cells and distinguish them from healthy cells by using an extensive array of receptors. These receptors can be grouped into activating- and inhibiting receptors, which recognize molecules on the potential target cells that then provide activating or inhibiting signals to the NK cell[19]. Whether or not an NK cell starts to attack the target kill is determined by the sum of all activating and inhibitory signals that an NK cell receives: When the net balance of all integrated signals is shifted towards more activating signals, the NK cell gets activated and vice versa when inhibitory signals are dominant, NK cells are inhibited[35]. **Figure 2** shows four scenarios of NK cells encountering potential targets cells and the corresponding outcomes:

1.) Virtually all healthy nucleated cells express inhibitory molecules that deactivate the killing process in NK cells and usually do not express activating ligands. Thereby, healthy cells are protected from NK cell attacks. Human Leukocyte Antigen (HLA) class I molecules are the most important group of inhibitory ligands for NK cells (**Figure 2A**).
2.) In the absence of inhibitory ligands, healthy cells are also spared from NK cell attacks when activating ligands are absent as well. Red blood cells are an example of healthy cells that do not express the inhibitory ligands HLA class I (**Figure 2B**).
3.) Diseased cells might lose the expression of inhibitory ligands, for instance transformed tumor cells or virally-infected cells often lose HLA class I expression to escape from $CD8^+$ T cells. For NK cells, the loss of inhibitory ligands results in less inhibition and thus in a lower activation threshold. Consequently, NK cell-mediated killing is initiated if activating signals are present on the target cell. This scenario is also called missing-self hypothesis (**Figure 2C**).
4.) Alternatively, diseased cells can be killed by NK cells when they express high levels of activating ligands for NK cells, such as cellular stress- and virus-associated molecules. This way, NK cells can attack target cells also in the presence of inhibitory ligands, if the activating signals outweigh the inhibitory ones and thus shift the balance towards NK cell activation. This scenario is called induced-self hypothesis (**Figure 2D**).

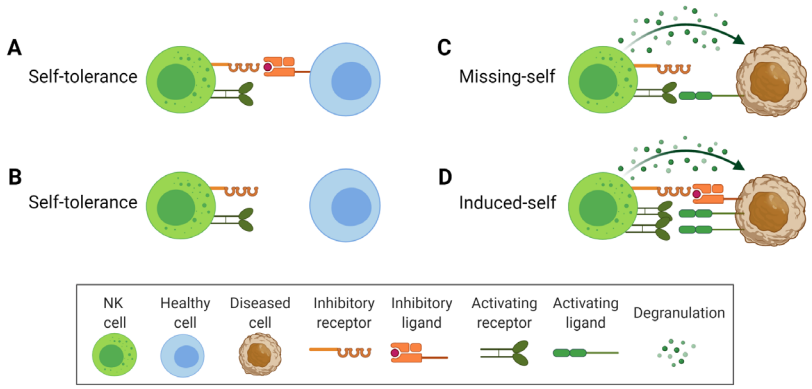


Figure 2. NK cell recognition of a potential target cell. A-B) Two scenarios of NK cell tolerance towards healthy cells: A) A healthy cell inhibits the NK cell through expression of inhibitory ligands and is tolerated by NK cells. **B)** A healthy cell that lacks both inhibitory ligands (no inhibition) and activating ligands (no activation) is spared from NK cell attacks due to the absence of activating signals. **C-D)** Two scenarios where NK cells respond to target cells, indicated by the release of cytotoxic granules: **C)** The 'missing-self' hypothesis describes a target cell that lacks expression of inhibitory ligands combined with expression of activating ligands and the target cell is thus attacked by the NK cell. **D)** The 'induced-self' hypothesis describes a target cell that expresses inhibitory ligands combined with sufficient activation ligands, which outweigh the inhibitory signals and therefore tip the balance towards NK cell activation and killing. The figure is adapted from [35] and was created using BioRender.com.

NK cell activation through cytokine stimulation

In addition to stimulation through activating receptors, NK cells are also responsive to a broad range of soluble factors such as cytokines, which can influence NK cell survival, migration, activation and effector functions[36]. Among these cytokines are IL-2, mainly produced by activated $CD4^+$ T cells, as well as IL-12, IL-15, and IL-18, which can be produced by myeloid cells such as activated dendritic cells and macrophages[36]. These cytokines can exert multiple activating functions on NK cells. While IL-2 is well-known for stimulating NK cell proliferation, IL-15 can stimulate NK cell survival, and all named cytokines can potently activate effector functions of NK cells[37]. Of note, NK cells also respond to cytokines that have suppressive effects on NK cell functions such as TGF-β or IL-10, which are important mediators of immunosuppression as further introduced below[36].

Activating receptors

The main activating receptors comprise the natural cytotoxicity receptors (NCRs, such as NKp30, NKp44, NKp46), NKG2D and DNAM1, which can typically bind to molecules that are upregulated on diseased cells in response to cellular stress, viral infections or transformation to tumor cells[38]. The NKG2D receptor engages with the stress-induced MICA/MICB molecules and UL16-binding proteins (ULBP1-6), which are expressed in response to malignant transformation, infection or DNA damage[19]. DNAM1 can bind to the poliovirus receptor CD155 and to the nectin adhesion molecule CD112, which can both be expressed on tumor cells[19]. For NCRs, the ligands are still insufficiently characterized. Known ligands include viral proteins such as hemagglutinins that engage with NKp44 and NKp46[39,40]. Moreover, ligands for NKp30 are the HLA-B associated transcript 3 (BAT3) protein and B7H6, a molecule which is often expressed on tumor cells[41]. Recently, HLA-DP molecules, belonging to the HLA class II family, have been identified as ligand for NKp44[42].

CD16 (FcγRIIIA) is another important activating receptor on NK cells through which NK cells can bind IgG antibodies and mediate ADCC, as explained above. CD16 is abundantly expressed on the majority of $CD56^{dim}$ NK cells and therefore on the majority of NK cells in the blood, whereas CD16 is absent or expressed at low levels on $CD56^{bright}$ NK cells[43]. Engagement of the CD16 receptor can induce very strong NK cell activation and is considered one of the most potent activating receptors on NK cells because antibody stimulation through CD16 receptors alone is sufficient to activate NK cells[44].

Inhibitory receptors

Among the pool of inhibitory receptors, NK cells are predominantly inhibited by Killer Immunoglobulin-like receptors (KIR) and NKG2A, which interact with the inhibitory HLA class I molecules HLA-ABC and HLA-E, respectively. Almost all healthy nucleated cells express the inhibitory ligands HLA class I. Due to the importance of HLA and its receptors KIR and NKG2A for NK cell function, they will be further introduced in a separate paragraph below. In addition to HLA class I-dependent inhibition, NK cells express several other inhibitory receptors that mediate NK cell tolerance in an HLA-

independent manner. Among these receptors are Programmed Death-1 (PD-1), T cell immunoglobulin and ITIM domain (TIGIT), and T cell immunoglobulin and mucin domain containing molecule 3 (TIM-3). As the names suggest, these receptors are also found on T cells and have relatively recently been described as inhibitory checkpoints for NK cells[45]. The immune checkpoint receptor PD-1 bind to its ligand PD-L1 and is one of the first receptors for which immune checkpoint inhibitors were used in the clinic[6]. TIGIT competes with the activating receptor DNAM1 as both receptors share the ligands CD112 and CD155, while TIM-3 receptors bind to soluble ligands (Galectin-9 and HMGB1) as well as cell surface ligands (Ceacam-1 and Phosphatidyl serine)[46]. The engagement of each of the three named receptors has been shown to restrict NK cells within tumors[45].

The NK cell receptors KIR and NKG2A

The KIR family is encoded by 14 highly polymorphic genes and consists of both inhibiting and activating receptors[47]. The best described KIRs are the inhibitory receptors KIR2DL1, KIR2DL2/3 and KIR3DL1, which engage with HLA-C and with HLA alleles containing Bw4 motives (Table 1). Identifying the ligands for activating KIRs has been more challenging, in part due to the sequence homology with inhibiting KIRs[48]. Some of the ligands identified for activating KIRs serve as ligands for inhibitory KIRs as well, but the inhibitory KIRs have generally a higher binding affinity to these shared ligands, resulting more often in NK cell inhibition[49]. Unless activating KIRs as specifically mentioned, this thesis focuses on inhibitory KIRs.

Not only KIR, but also HLA class I molecules are polymorphic. HLA class I are encoded by one of the most polymorphic genes in humans, which enables HLA molecules to bind and present a large variety of peptides to $CD8^+$ T cells for immunosurveillance[50]. Expression levels of HLA class I are tightly regulated at the transcriptional level by several mechanisms including HLA polymorphisms, epigenetic regulators, and tissue-specific transcription factors[51]. Further modulation of HLA class I expression levels can occur post-transcriptionally through proinflammatory cytokines, microRNAs or alternative splicing[52]. Notably, for NK cells, only some HLA-A and less than half of HLA-B alleles, but all HLA-C alleles serve as ligands for at least one KIR, which could suggest that HLA-C has a more dominant role in regulating NK cell activity and which may be related to the role of KIRs in pregnancy where HLA-C and HLA-E are expressed, while the other HLA molecules are virtually absent[53].

Table 1. Inhibitory receptors KIR and NKG2A and their corresponding ligands.

Inhibitory NK cell receptor	Ligands
KIR2DL1	HLA-C group 2 alleles (C2)
KIR2DL2, KIR2DL3	HLA-C group 1 alleles (C1)
KIR3DL1	HLA alleles with a Bw4 motif: HLA-B alleles, HLA-A*23, HLA-A*24, HLA-A*32
NKG2A	HLA-E

The inhibitory NKG2A receptor recognizes the non-classical HLA-E molecule, which is expressed at low levels on all nucleated cells (Table 1)[54]. NKG2A belongs to the receptor family NKG2 that forms a heterodimer with CD94 on the cell surface and comprises the inhibitory receptors NKG2A and NKG2B as well as the activating receptors NKG2C/D/E/H[55]. Of these, NKG2A and NKG2C are most extensively described to bind HLA-E. Similar to KIRs, the inhibitory receptor NKG2A has a stronger binding affinity to HLA-E than the activating receptor NKG2C, presumably to prevent overactivity[56]. But unlike KIR and HLA, NKG2A and HLA-E are not very polymorphic. Until now, few allelic variants are described for NKG2A and two main protein variants for HLA-E are known, which can result in different expression levels of HLA-E, but both variants can be bound by NKG2A[54,57].
A more extensive description of the KIR and NKG2A receptors can be found in **chapter 2**.

NK cell education: Acquiring "license to kill" to prevent autoreactivity

Because KIR and HLA genes are located on different chromosomes, different KIR and HLA combinations can be expressed in one individual[58]. Subsequently, NK cells can express KIRs hat recognize its corresponding HLA ligand, but also KIRs that recognize an HLA ligand, which is not expressed in the same individual. This second situation could potentially become dangerous because the KIR would not be inhibited by HLA in the same individual and the NK cell could become autoreactive. To ensure self-tolerance, NK cells have to undergo a 'licensing' process: NK cells with KIRs not encountering their ligand remain hyporeactive, whereas NK cells with KIRs that encounter their corresponding HLA ligand in the same individual become the more potent effector cells[59]. NK cells with licensed KIR are also termed educated NK cells. The mechanisms of NK cell education are not fully understood, but different metabolic pathways in educated and non-educated NK cells have been described[60]. Moreover, educated NK cells contain more and denser cytotoxic granules than non-educated NK cells, which could explain the higher killing capacity of educated NK cells[61].

NK cell-based immunotherapy

NK cells in the setting of hematopoietic stem cell transplantations

NK cell-based immunotherapy gained enthusiasm in the setting of hematopoietic stem cell transplantations (HSCT), when it was discovered that NK cells are key players in mediating anti-tumor effects in acute myeloid leukemia (AML)[62,63]. HSCT are used to treat certain types of cancers such as leukemia, lymphoma and myeloma to rescue the bone marrow damage after high-dose chemotherapy or radiation[64]. New bone marrow including platelets, red blood cells, and immune cells can subsequently develop from the transplanted stem cells. The stem cells can either be derived from the patient (autologous) before the chemotherapy treatment or from a healthy donor (allogeneic).
Allogeneic transplants provide the potential cure for patients with hematological malignancies because the donor-derived cells can mediate more potent anti-tumor

responses than autologous ones, a so-called graft-versus-tumor effect (GVT)[65]. Due to the benefit of GVT, allogeneic hematopoietic transplants are now generally used for patients with high risk disease (i.e. AML with bad prognostic factors) or in late disease stages when other treatment options have proven to be no longer successful[66]. The GVT effect is mainly mediated by NK cells and T cells. Donor NK cells are more easily triggered to kill those tumor cells on which they sense the absence of the HLA class I. Meanwhile, donor T cells react to non-donor antigens presented within HLA class I and mediate GVT when those antigens are presented on tumor cells. However, when non-donor antigens are expressed on the patient's healthy cells, donor T cells attack those healthy cells, too, which is known as T cell-mediated graft-versus-host disease (GVHD) and imposes a limitation of allogeneic transplants[65]. Non-donor antigens include the major histocompatibility antigens (HLA) and/or minor histocompatibility antigens, which are peptides derived from common proteins that differ slightly between donor and recipient due to genetic polymorphism outside of the HLA region[67]. To limit the risk of GVHD, allogeneic transplants can be derived from identical donors i.e., donors fully matched for both HLA haplotypes (maternal and paternal haplotype), which can be family members or matched-unrelated donors that were selected from the world donor bank. However, fully HLA-matched transplants are not available for everyone and particularly difficult to find for less common HLA genotypes and in increasingly small families[68]. To address the lack of fully matched donors, allogeneic HSCT protocols with different donor stem cell sources have been explored including haploidentical (half-matched) donors and stem cells derived from cord blood, which greatly enhanced the choices of donors. In the case of haploidentical donors, only one of the two HLA haplotypes is matched (either maternal or paternal haplotype) and the other is mismatched. Haploidentical HSCT come with the risk of the HLA mismatch leading to graft rejection and/or severe GVHD and to lower this risk, immunosuppressive therapy is given, making patients vulnerable for infections. But nowadays, haploidentical HSCT became a viable option due to T cell depletion from the graft and additional improved immunosuppressive therapy after transplantation, which better balances GVHD versus anti-tumor- and anti-pathogen immunity[65,69]. Haploidentical HSCT is now considered a reasonable choice with clinical results that are comparable to the use of matched-unrelated donors and that are possibly better than those achieved with cord-blood stem cells[70].

KIR-ligand mismatch principle

In the setting of haploidentical HSCT, it was demonstrated that NK cells are important mediators of GVT. Ruggeri et al. found that AML patients, who had received a haploidentical HSCT, had lower relapse rates when HLA differences between patient and donor were present that resulted in a 'missing-self' scenario of the donor NK cells[62,63]. Even more, AML cells in a xenograft mouse model where solely eliminated by those donor NK cells for which the 'missing-self' scenario occurred[62]. Other studies confirmed the findings by Ruggeri et al.[71-73]. Importantly,

NK cells did not cause GVHD in this setting, which is a great advantage of NK cells over T cells[62].

This concept is now known as "KIR-ligand mismatch" and describes donor NK cells with licensed inhibitory KIRs (due to expression of the cognate HLA ligand on donor cells) for which one or more HLA ligands are absent in the patient[74]. Consequently, these KIR-ligand mismatched NK cells do not encounter their cognate HLA ligand on patient cells and can respond more potently to target cells due to the lack of inhibitory signaling by the HLA ligand (NK cell alloreactivity) (**Figure 3**). Selection of KIR-ligand mismatched donor NK cells is possible for individuals that miss at least one of the three HLA allele groups C1, C2, Bw4, which is the case for circa 70% of the population[75]. **Figure 3** shows an example of an NK cell with 1 inhibitory KIR. Because KIRs are acquired in a stochastic manner, NK cells without KIR, with one KIR or with a combination of two or more inhibitory KIRs can exist in every individual[58]. This means that only a subpopulation of NK cells from one individual will be fully mismatched as indicated in **Figure 3B**, while other subpopulations might express one KIR that is HLA-matched and another that is HLA-mismatch. In the latter situation, the net balance of all integrated signals will determine the magnitude of the response. The receptor-ligand mismatch principle is not possible for NKG2A and its ligand HLA-E as HLA-E has only very limited polymorphism and all HLA-E molecules are recognized by the same NKG2A receptor[54].

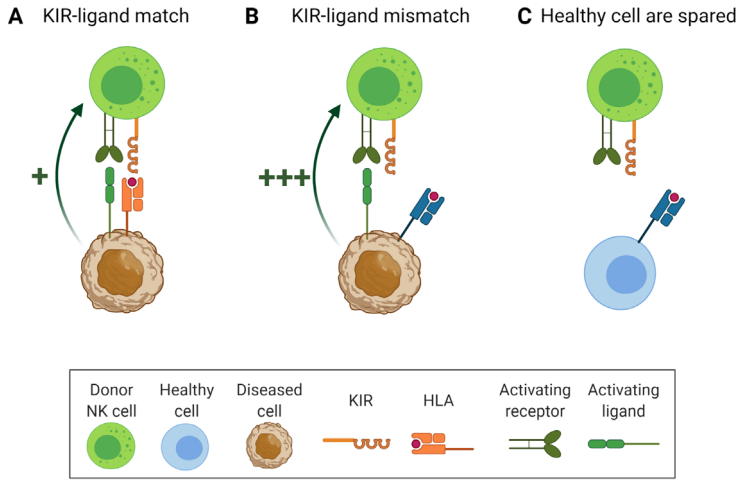


Figure 3: KIR-ligand mismatch concept between healthy donor NK cells and patient cells. A) The donor NK cell encounters a tumor cell that expresses the cognate HLA ligand for the NK cell's KIR (KIR-ligand match). When the tumor cell expresses activating ligands as well, the net balance of activating and inhibitory signals received by the NK cells will determine the NK cell response. **B)** The donor NK cell encounters a tumor cell, which does not express the cognate HLA ligand for the NK cell's KIR due to a genetic mismatch between donor and patient (KIR-ligand mismatch). This principle is similar to the 'missing-self' situation, where endogenous NK cells encounter tumor cells that downregulated HLA class I. Consequently, the NK cell depicted in B is responding more potently to the tumor cell than the NK cell

depicted in A (magnitude of NK cell responses is indicated by the + symbol). **C)** When donor NK cells encounter the patient's healthy cells, alloreactive NK cells generally do not attack the patient's healthy tissue due to the absence of NK cell activating signals on healthy cells. The figure was created using BioRender.com.

Different platforms for NK cell-based therapy

The above-introduced concept describes one possible platform for NK cell therapy in the setting of HSCT, in which autologous NK cells or NK cells from an (haplo-)identical donor can mediate anti-tumor effects. Because of the clinical evidence for their anti-tumor potential, NK cells have also been tested for adoptive cell therapy, which emerged as another attractive platform for NK cell-based therapies, either in combination with or without HSCT. Adoptive NK cell transfer can be performed with autologous or with allogeneic NK cells (**Figure 4**). Multiple studies demonstrated that the infusion of adoptive NK cells was safe in both the autologous and allogeneic setting for hematological malignancies as well as for solid tumors (reviewed in[76,77]). However, infusion of autologous NK cells failed to induce potent anti-tumor effects in several studies and more promising results were obtained with allogeneic NK cells, especially against AML, after both HSCT and infusions of allogeneic NK cells by themselves (reviewed in [77]). For example, one of the first clinical studies outside the HSCT setting found that administration of haploidentical NK cells was well-tolerated and achieved remission in 5 of 19 poor-risk AML patients[78].

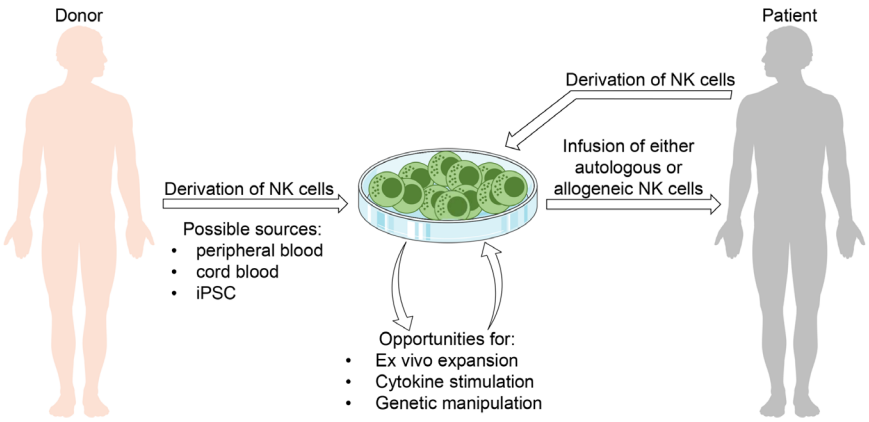


Figure 4. General concept for adoptive NK cell transfer. NK cells can either be derived from a healthy donor (allogeneic setting) or from the patient itself (autologous setting). Most frequently, NK cells are isolated from peripheral blood. Newer NK cell sources include NK cell isolation from cord blood and generation of NK cells from induced pluripotent stem cells (iPSC). Following NK cell derivation, the NK cells can be cultured in vitro to expand and to activate the NK cells. In vitro culture provides ample opportunities for NK cell modification to generate more potent NK cell products. Finally, the NK cells are infused into the patient.

To further improve clinical responses, especially against solid tumors, the development of NK cell-directed therapies focusses on several aspects including the optimal NK cell administration (NK cell source, dose, pre-conditioning) and enhancing the NK cell killing potential and persistence in vivo. Regarding the NK cell source, NK cells from peripheral blood are most commonly used in the clinical studies published so far, but other NK cell sources include umbilical cord blood, stem cells (embryonic or induced pluripotent stem cells) or NK cell lines[76]. Independently of the NK cell source, high doses of NK cells are used in most clinical trials. Although there is no consensus yet on the number of NK cells that should be infused, doses between $5x10^6$ - $1x10^8$ NK cells/kg body weight are used in most clinical trials[79]. Such numbers cannot be reached by isolation from peripheral blood and require NK cell expansion.

Several protocols have been developed for NK cell expansion using different approaches, for example performing the expansion in the presence of cytokines and/or feeder cells such as autologous PBMCs or irradiated K562 tumor cells[80,81]. A K562 feeder cell-based expansion method with membrane-bound IL-21 can for instance lead to an expansion of almost 50,000-fold more NK cells and methodologies are rapidly advancing to achieve higher yields[82]. While the optimal expansion protocol still needs to be determined, methodologies are rapidly advancing to achieve higher yields and allow sufficient numbers to perform several rounds of NK cell infusions, which could represent a strategy to counteract the relatively short in vivo persistence of NK cells[80]. The ex vivo expansion of NK cells additionally provides opportunities to increase the NK cell killing potential and persistence, for example by addition of cytokines or manipulating NK cells (**Figure 4**). Genetic manipulations of NK cells by transducing them with CAR receptors may improve NK cell anti-tumor efficacy and NK cell persistence[79]. Currently, over 100 clinical and preclinical studies are ongoing with CAR-NK cells derived from NK cell lines or primary cells[83]. Modifications of NK cells could further be utilized to improve recruitment of infused NK cells to the tumor e.g., by generating NK cells with specific chemokine receptors that are important in the targeted tumor type[84]. Especially for solid tumors, the recruitment of infused NK cells to the tumor site is another crucial factor that needs to be addressed because NK cell infiltration in solid tumors is generally low[85].
Overall, various platforms and strategies are explored to boost the anti-tumor efficacy of NK cells including ex vivo expansion, NK cell activation and manipulation. More research is needed to identify the most promising combinations of strategies that will generate optimal NK cells with sustained anti-tumor activity after infusion into patients.

Tumor cells hiding in suppressive tumor microenvironments - a challenge for effective NK cell therapy

Tumor cells are fitted with numerous features to ensure tumor growth and survival. These features are recognized as the hallmarks of cancer with one of them being 'evading immune destruction'[86]. To evade immune cells, tumors can for example alter their ligand expression e.g., by downregulating HLA class I to escape T cell recognition as described before. To also escape NK cells, tumor cells acquire additional mechanisms of resistance, for example to keep expression of inhibitory ligands for NK cells or specifically cleave off the activating NKG2D ligands MICA/MICB, which can subsequently occur as soluble forms[87,88].

Importantly, tumor cells do not exist in isolation, but are embedded in the tumor microenvironment (TME). The TME is comprised of many different cellular and non-cellular components, including extracellular matrix, stromal cells such as cancer-associated fibroblasts, angiogenic vascular cells and immune cells[89]. Together, the TME components form a complex network, of which the tumor takes advantage to support its survival and progression[89]. Even more, tumor cells can recruit and instruct other cells, including immune cells, to establish a tumor-supporting and immunosuppressive TME[89]. Within the TME, functional NK cells, $CD8^+$ T cells and $CD4^+$ T helper cells type 1 (Th1) can generally cooperate with dendritic cells and proinflammatory macrophages to perform anti-tumor immune responses, whereas $CD4^+$ $Foxp3^+$ regulatory T cells (Treg) and heterogenous populations of myeloid-derived cells, including tumor-associated macrophages (TAM), can support the tumor and impair anti-tumor immune responses, directly or indirectly by creating a immunosuppressive TME[90].

In the TME, anti-tumor effector cells, including NK cells, are often found to be dysfunctional. Tumor-infiltrating NK cells have been described to acquire an altered phenotype compared to blood NK cells, characterized by low expression of several activating receptors such as NKp46, DNAM1, NKG2D, and CD16, in both blood cancers[91-93] and solid cancers[94-97]. Additionally, high levels of the inhibitory receptor NKG2A or the inhibitory checkpoints TIGIT and TIM-3 were found on NK cells in tumors [93,98,99]. Such changes in NK cell phenotype can impair their anti-tumor effector functions, resulting in lower cytotoxic capacity and IFN-γ secretion, and have been associated with disease progression or poor response to therapy[93-95]. The altered phenotype and effector function of NK cells could be mediated by the tumor cells or tumor-associated immune cells in the TME, such as TAMs, as both tumor and other immune cells can interact with NK cells directly and indirectly through secretion of numerous factors that can compromise NK cell effector functions.

The TME can further influence NK cells indirectly through accumulation of immunosuppressive factors in the TME, which can be produced by other TME cells. Among these factors are TGF-β, IL-10, nitric oxide, arginine, as well as prostaglandin E2 (PGE2)[29]. Additionally, soluble MICA/MICB molecules, secreted by tumor cells, can act as suppressing rather than activating signals as binding of soluble MICA/MICB to the activating receptor NKG2D induces downregulation of the receptor[100]. Moreover, hypoxia is present in most tumors as the result of increased oxygen

consumption by the tumor cells paired with insufficient oxygen delivery by a dysfunctional vasculature[101]. We and others have previously shown that hypoxia affects NK cells and inhibits their anti-tumor cytotoxicity[102,103].
The cellular metabolism in the TME is another relevant factor that can affect NK cells negatively. Tumor cells undergo metabolic reprograming from oxidative phosphorylation towards enhanced aerobic glycolysis (also known as the Warburg effect), which supports them to thrive in a hypoxic TME[104,105]. The high rates of glycolysis in tumor cells limit the availability of nutrients like glucose in the TME and create metabolic competition between tumor and immune cells[106]. As for other immune cells, glucose is also a key fuel for NK cells[107,108]. Additionally, the TME can also contain high levels of lactate, a by-product of aerobic glycolysis, which can inhibit NK cell anti-tumor functions[109].
The numerous factors in the TME form a complex network and jointly create the immunosuppressive environment that can influence the potential of immunotherapies[110]. To create effective NK cell-based therapies, it is crucial to first, understand how the complex TME network affects the developed NK cell products and second, to find strategies that can overcome TME-mediated NK cell suppression.

NK cell-based therapy approaches for multiple myeloma (MM)
The use of alloreactive NK cells represent a promising therapy approach for MM. In a clinical study, infusion of KIR-ligand mismatched NK cells, followed by autologous HSCT, resulted in complete remission in around 50% of MM patients[111]. However, the anti-tumor functions of NK cells are often impaired in advanced MM, at least in part due to the immunosuppressive bone marrow. Already early on, the bone marrow of MM patients is infiltrated by highly heterogeneous cell populations, including pro-tumor immune cells, that foster MM tumor growth and largely inhibit anti-tumor immune responses including NK cells[8,112]. Such functional impairment of NK cells needs to be prevented to unfold the full anti-tumor potential of NK cells and to create effective NK cell-mediated immunotherapies. Previously, our group showed that cytotoxic impairment of NK cells through hypoxia could be restored by activating the NK cells with high doses of IL-2[102]. In a follow-up study, lactate and PGE2 were included next to hypoxia to better mimic the TME in MM and both factors were found to reduce NK cell cytotoxicity despite IL-2 activation[113]. This study underlines the importance of mimicking the in vivo situation as closely as possible when testing NK cell products. Due to the complexity of the TME, cytokine-activated NK cells likely need to be combined with other strategies such as monoclonal antibodies or immunomodulatory drugs to further potentiate the anti-tumor effects. In our above-described study, addition of the ADCC-mediating antibody Daratumumab could at least partially reverse overcome the resistance induced by TME factors and restore NK cell responses[113]. The monoclonal antibody Daratumumab targets $CD38^{+}$ myeloma cells and NK cell-mediated ADCC is recognized as one mechanism involved in the clinical success of this drug[114]. Additionally, our group demonstrated the functional relevance of KIR-ligand mismatching for potentiating NK cell anti-myeloma responses in combination with

Daratumumab[113]. However, as described above, the TME is characterized by numerous factors that might impose challenges to NK cells and further studies are therefore necessary get a better picture of how NK cell-based therapies might be affected by the complex TME and which combinational therapy will be most beneficial.

NK cell-based therapy approaches for breast cancer

Although NK cells are frequently found in low numbers in solid tumors, NK cell infiltration and activity have been associated with response to therapy in several recent studies[31,115-117]. For the subset of breast cancer patients with $HER2^+$ disease, tumor-infiltrating NK cells correlated with enhanced response to the $HER2^+$-targeting antibody Trastuzumab, which supports the hypothesis that NK cells play an important role in mediating ADCC when Trastuzumab is given[116]. However, the phenotype of circulating and tumor-infiltrating NK cells in breast cancer was characterized by low levels of many activating receptors (NKp30, NKG2D, DNAM1, CD16) and the described phenotype coincided with decreased NK cell cytotoxicity[97,118]. Such dysfunctional NK cells in breast cancer might be a consequence of the immunosuppressive TME[97,119]. Especially in breast cancer, the TME is highly heterogeneous in cellular- and spatial composition. In the past, breast cancer was considered immunologically 'cold' with low immune cell infiltration and mutational burden, but more recent profiling studies demonstrate that the breast cancer TME is highly heterogeneous and dynamic[120]. As the importance of the TME is increasingly recognized, better characterization of the TME, its composition and potential impact on NK cells will be necessary in order to develop new treatments.

NK cell-based therapies in the setting of HSCT or adoptive cell transfer could represent strategies to increase NK cell numbers and their effectiveness for breast cancer. That the concept of allogeneic HSCT is also a feasible approach for breast cancer and could induce a GVT effect, has been shown by our group and by others in mouse models as well as in a clinical study with metastatic breast cancer[121-123]. In a follow-up study, our group demonstrated that murine NK cells from a haploidentical, alloreactive donor are mediators of the anti-tumor effects as they cured mice from 4T1 breast cancer, whereas NK cells from a syngeneic donor could not achieve such responses[124]. Our findings are in line with the results of alloreactive NK cells in AML patients[63] and underline that, also against murine breast cancer, the GVT effect is facilitated by a KIR-HLA ligand mismatch (Ly49-MHC ligand mismatch in mice) between donor NK cells and tumor cells. Furthermore, administration of alloreactive NK cells alone could cure mice and were as effective as NK cells in combination with a bone marrow transplant, suggesting that it is worthwhile to test the use of alloreactive NK cell therapy in breast cancer patients[124]. One phase II clinical trial with allogeneic NK cells has been published, where 4 of 6 patients had stable disease 6 weeks after infusion and very poor in vivo persistence of NK cells[125]. That the TME directly affects adoptively transferred NK cells was apparent in another mouse study, where adoptive NK cells that reached the tumor site had an altered phenotype e.g., lower expression of the activating receptor NKp46, than NK cells that

migrated to the spleen[126]. The described studies support the notion that the TME is limiting the success of adoptively transferred NK cells and that NK cell effector functions and persistence need to be boosted. More research is required to understand in more detail which challenges the produced NK cells have to face in the breast cancer TME and to subsequently identify the best combination of strategies to push NK cell-based therapies towards success for breast cancer.

Outline of the thesis

Our research group aims to develop effective NK cell-based therapy by using NK cells derived from healthy donors. In order to optimize human NK cells in such a way that they remain functional in a suppressive TME, we need to understand in more detail which challenges NK cells encounter in the TME and how these challenges can be overcome. In this thesis, we describe strategies to potentiate the effector functions of cytokine-activated donor-derived NK cells against two cancer types, multiple myeloma and breast cancer, by using clinically-relevant models that represent TME characteristics. The **chapters 2-4** follow up on our group's previous work focusing on how to enhance NK cells against tumor cells, while the **chapters 5-8** address additional TME factors that might need to be considered to augment NK cell efficacy.

In **chapter 2**, we provide a literature overview of studies addressing the interaction between HLA class I and KIR or NKG2A receptors and the principle that interfering with HLA-mediated NK cell inhibition e.g., with KIR-ligand mismatching or with blocking antibodies, can enhance NK cell responses against multiple myeloma. In **chapter 3**, we investigated the interaction between NKG2A and HLA-E and the functional consequences for cytokine-activated donor NK cells. To represent the TME in MM, co-cultures of activated NK cells and MM target cells contained TME factors. We analyzed $NKG2A^+$ and $NKG2A^-$ NK cell subsets and their responses against $HLA\text{-}E^-$ and $HLA\text{-}E^+$ MM target cells in combination with an ADCC-triggering antibody. In **chapter 4**, we investigated whether the combination of two strategies, namely reducing NK cell inhibition through HLA class I and enhancing activation through ADCC, are also applicable for breast cancer. We tested the ADCC-mediating antibody Trastuzumab combined with KIR-ligand mismatching to enhance NK cell effector functions. As models we used breast cancer cell lines, cultured under hypoxia to represent the TME, as well as human primary breast cancer cells, which have been exposed to the TME in the patient.

Having studied a concept of how NK cell responses can be augmented against tumor cells, we further explored several TME characteristics that could contribute to limiting NK cell anti-tumor responses in an immunosuppressive TME. In particular for solid tumors, understanding the spatial distribution of NK cells in relation to tumor cells and other tumor-infiltrating leukocytes will be essential to improve clinical responses of NK cell-based immunotherapy approaches. In **chapter 5**, we therefore

profiled endogenous tumor-infiltrating NK cells in two human breast cancer cohorts to gain insights into NK cell density, phenotype and cellular distribution.
Since glucose is an important fuel for NK cells and the nutrient availability known to be reduced in many cancer types, but not yet studied for MM, we assessed the glucose concentration in the bone marrow of MM patients in **chapter 6**. Subsequently, we investigated whether the glucose concentrations, as found in MM bone marrow, affected the anti-tumor responses of activated donor-derived NK cells (**chapter 6**).
In **chapter 7**, we studied the interactions between donor NK cells and macrophages (TAM and M1 macrophages), as a proxy for tumor accessory cells, and assessed whether NK cell effector functions could be enhanced by an ADCC-triggering antibody directed against TAM, with the idea being that this approach could contribute to reshaping the TME towards a more immunostimulatory environment through NK cell activation and associated secretion of proinflammatory cytokines.
In addition to TME cells and metabolic factors, many soluble factors in the TME affect NK cell activity. In **chapter 8**, we studied alternative splicing of HLA-C and whether allele polymorphism influenced the occurrence of alternatively spliced HLA-C products, which might influence HLA-C expression levels and thus NK cell responses since HLA-C molecules are major inhibitory ligands for NK cells.
In **chapter 9**, the findings of the individual chapters are discussed and the implications of our findings for NK cell-based immunotherapies are described together with a future perspective for combined therapy approaches.

References

1. Abbas AK, Lichtman AH, Pillai S. *Basic immunology e-book: functions and disorders of the immune system.* Elsevier Health Sciences; 2019.
2. Couzin-Frankel J. Breakthrough of the year 2013. *Science.* 2013;342(6165):1432-1433.
3. Zhang Y, Zhang Z. The history and advances in cancer immunotherapy: understanding the characteristics of tumor-infiltrating immune cells and their therapeutic implications. *Cell. Mol. Immunol.* 2020;17(8):807-821.
4. Fares CM, Van Allen EM, Drake CG, Allison JP, Hu-Lieskovan S. Mechanisms of resistance to immune checkpoint blockade: why does checkpoint inhibitor immunotherapy not work for all patients? *American Society of Clinical Oncology Educational Book.* 2019;39:147-164.
5. Huntington ND, Cursons J, Rautela J. The cancer–natural killer cell immunity cycle. *Nature Reviews Cancer.* 2020:1-18.
6. Havel JJ, Chowell D, Chan TA. The evolving landscape of biomarkers for checkpoint inhibitor immunotherapy. *Nature Reviews Cancer.* 2019;19(3):133-150.
7. Rajkumar SV, Dimopoulos MA, Palumbo A, et al. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. *The lancet oncology.* 2014;15(12):e538-e548.
8. Nakamura K, Smyth MJ, Martinet L. Cancer immunoediting and immune dysregulation in multiple myeloma. *Blood, The Journal of the American Society of Hematology.* 2020;136(24):2731-2740.
9. Kumar SK, Dispenzieri A, Lacy MQ, et al. Continued improvement in survival in multiple myeloma: changes in early mortality and outcomes in older patients. *Leukemia.* 2014;28(5):1122-1128.
10. Martin T, Huff CA. Multiple myeloma: current advances and future directions. *Clinical Lymphoma Myeloma and Leukemia.* 2019;19(5):255-263.
11. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* 2018;68(6):394-424.
12. O'Shaughnessy J. Extending survival with chemotherapy in metastatic breast cancer. *The oncologist.* 2005;10:20-29.
13. Tokumaru Y, Joyce D, Takabe K. Current status and limitations of immunotherapy for breast cancer. *Surgery.* 2020;167(3):628-630.
14. Loibl S, Gianni L. HER2-positive breast cancer. *The Lancet.* 2017;389(10087):2415-2429.
15. Cerwenka A, Lanier LL. Natural killer cells, viruses and cancer. *Nature reviews immunology.* 2001;1(1):41-49.
16. Kiessling R, Klein E, Wigzell H. „Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur. J. Immunol.* 1975;5(2):112-117.
17. Herberman RB, Nunn ME, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. I. Distribution of reactivity and specificity. *Int. J. Cancer.* 1975;16(2):216-229.
18. Peng H, Tian Z. Diversity of tissue-resident NK cells. Paper presented at: Semin. Immunol.2017.
19. Long EO, Sik Kim H, Liu D, Peterson ME, Rajagopalan S. Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annu. Rev. Immunol.* 2013;31:227-258.
20. Poznanski SM, Ashkar AA. What defines NK cell functional fate: phenotype or metabolism? *Front. Immunol.* 2019;10.
21. Orange JS. Formation and function of the lytic NK-cell immunological synapse. *Nature Reviews Immunology.* 2008;8(9):713-725.
22. Prager I, Watzl C. Mechanisms of natural killer cell-mediated cellular cytotoxicity. *J. Leukoc. Biol.* 2019;105(6):1319-1329.
23. Thorburn A. Death receptor-induced cell killing. *Cell. Signal.* 2004;16(2):139-144.
24. Zhu Y, Huang B, Shi J. Fas ligand and lytic granule differentially control cytotoxic dynamics of natural killer cell against cancer target. *Oncotarget.* 2016;7(30):47163.

25. Vanherberghen B, Olofsson PE, Forslund E, et al. Classification of human natural killer cells based on migration behavior and cytotoxic response. *Blood, The Journal of the American Society of Hematology.* 2013;121(8):1326-1334.
26. Bernardini G, Gismondi A, Santoni A. Chemokines and NK cells: regulators of development, trafficking and functions. *Immunol. Lett.* 2012;145(1-2):39-46.
27. Fauriat C, Long EO, Ljunggren H-G, Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood, The Journal of the American Society of Hematology.* 2010;115(11):2167-2176.
28. Screpanti V, Wallin RP, Ljunggren H-G, Grandien A. A central role for death receptor-mediated apoptosis in the rejection of tumors by NK cells. *The Journal of Immunology.* 2001;167(4):2068-2073.
29. Gaggero S, Witt K, Carlsten M, Mitra S. Cytokines Orchestrating the Natural Killer-Myeloid Cell Crosstalk in the Tumor Microenvironment: Implications for Natural Killer Cell-Based Cancer Immunotherapy. *Front. Immunol.* 2020;11.
30. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. *Nat. Immunol.* 2008;9(5):503-510.
31. Barry KC, Hsu J, Broz ML, et al. A natural killer–dendritic cell axis defines checkpoint therapy–responsive tumor microenvironments. *Nat. Med.* 2018;24(8):1178-1191.
32. Corvino D, Kumar A, Bald T. Plasticity of NK cells in Cancer. *Front. Immunol.* 2022;13:888313.
33. Wang W, Erbe AK, Hank JA, Morris ZS, Sondel PM. NK cell-mediated antibody-dependent cellular cytotoxicity in cancer immunotherapy. *Front. Immunol.* 2015;6:368.
34. Ochoa MC, Minute L, Rodriguez I, et al. Antibody-dependent cell cytotoxicity: immunotherapy strategies enhancing effector NK cells. *Immunol. Cell Biol.* 2017;95(4):347-355.
35. Lanier LL. NK cell recognition. *Annu. Rev. Immunol.* 2005;23:225-274.
36. Konjević GM, Vuletić AM, Martinović KMM, Larsen AK, Jurišić VB. The role of cytokines in the regulation of NK cells in the tumor environment. *Cytokine.* 2019;117:30-40.
37. Walzer T, Dalod M, Robbins SH, Zitvogel L, Vivier E. Natural-killer cells and dendritic cells:"l'union fait la force". *Blood.* 2005;106(7):2252-2258.
38. Moretta A, Bottino C, Vitale M, et al. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu. Rev. Immunol.* 2001;19(1):197-223.
39. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, Mandelboim O. Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Eur. J. Immunol.* 2001;31(9):2680-2689.
40. Mandelboim O, Lieberman N, Lev M, et al. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature.* 2001;409(6823):1055-1060.
41. Kaifu T, Escalière B, Gastinel LN, Vivier E, Baratin M. B7-H6/NKp30 interaction: a mechanism of alerting NK cells against tumors. *Cell. Mol. Life Sci.* 2011;68(21):3531-3539.
42. Niehrs A, Garcia-Beltran WF, Norman PJ, et al. A subset of HLA-DP molecules serve as ligands for the natural cytotoxicity receptor NKp44. *Nat. Immunol.* 2019;20(9):1129-1137.
43. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol.* 2001;22(11):633-640.
44. Bryceson YT, March ME, Ljunggren H-G, Long EO. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood.* 2006;107(1):159-166.
45. Sanchez-Correa B, Lopez-Sejas N, Duran E, et al. Modulation of NK cells with checkpoint inhibitors in the context of cancer immunotherapy. *Cancer Immunol. Immunother.* 2019;68(5):861-870.
46. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: co-inhibitory receptors with specialized functions in immune regulation. *Immunity.* 2016;44(5):989-1004.
47. Campbell KS, Purdy AK. Structure/function of human killer cell immunoglobulin-like receptors: lessons from polymorphisms, evolution, crystal structures and mutations. *Immunology.* 2011;132(3):315-325.
48. Blunt MD, Khakoo SI. Activating killer cell immunoglobulin-like receptors: Detection, function and therapeutic use. *Int. J. Immunogenet.* 2020;47(1):1-12.
49. Kulkarni S, Martin MP, Carrington M. The Yin and Yang of HLA and KIR in human disease. Paper presented at: Semin. Immunol.2008.

50. Buhler S, Nunes JM, Sanchez-Mazas A. HLA class I molecular variation and peptide-binding properties suggest a model of joint divergent asymmetric selection. *Immunogenetics.* 2016;68(6):401-416.
51. Rene C, Lozano C, Eliaou JF. Expression of classical HLA class I molecules: regulation and clinical impacts: Julia Bodmer Award Review 2015. *Hla.* 2016;87(5):338-349.
52. Carey BS, Poulton KV, Poles A. Factors affecting HLA expression: a review. *Int. J. Immunogenet.* 2019;46(5):307-320.
53. Goodson-Gregg FJ, Krepel SA, Anderson SK. Tuning of human NK cells by endogenous HLA-C expression. *Immunogenetics.* 2020;72(4):205-215.
54. Wieten L, Mahaweni N, Voorter C, Bos G, Tilanus M. Clinical and immunological significance of HLA-E in stem cell transplantation and cancer. *Tissue Antigens.* 2014;84(6):523-535.
55. Borrego F, Masilamani M, Marusina AI, Tang X, Coligan JE. The CD94/NKG2 family of receptors. *Immunol. Res.* 2006;35(3):263-277.
56. Kaiser BK, Pizarro JC, Kerns J, Strong RK. Structural basis for NKG2A/CD94 recognition of HLA-E. *Proceedings of the National Academy of Sciences.* 2008;105(18):6696-6701.
57. Celik AA, Kraemer T, Huyton T, Blasczyk R, Bade-Döding C. The diversity of the HLA-E-restricted peptide repertoire explains the immunological impact of the Arg107Gly mismatch. *Immunogenetics.* 2016;68(1):29-41.
58. Fauriat C, Andersson S, Björklund AT, et al. Estimation of the size of the alloreactive NK cell repertoire: studies in individuals homozygous for the group A KIR haplotype. *The Journal of Immunology.* 2008;181(9):6010-6019.
59. Elliott JM, Yokoyama WM. Unifying concepts of MHC-dependent natural killer cell education. *Trends Immunol.* 2011;32(8):364-372.
60. Schafer JR, Salzillo TC, Chakravarti N, et al. Education-dependent activation of glycolysis promotes the cytolytic potency of licensed human natural killer cells. *J. Allergy Clin. Immunol.* 2019;143(1):346-358. e346.
61. Goodridge JP, Jacobs B, Saetersmoen ML, et al. Remodeling of secretory lysosomes during education tunes functional potential in NK cells. *Nat. Commun.* 2019;10(1):1-15.
62. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science.* 2002;295(5562):2097-2100.
63. Ruggeri L, Mancusi A, Capanni M, et al. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. *Blood.* 2007;110(1):433-440.
64. Garrett D, Yoder LH. An overview of stem cell transplant as a treatment for cancer. *Medsurg Nurs.* 2007;16(3):183.
65. Singh AK, McGuirk JP. Allogeneic stem cell transplantation: a historical and scientific overview. *Cancer Res.* 2016;76(22):6445-6451.
66. Ciurea SO, Al Malki MM, Kongtim P, et al. The European Society for Blood and Marrow Transplantation (EBMT) consensus recommendations for donor selection in haploidentical hematopoietic cell transplantation. *Bone Marrow Transplant.* 2020;55(1):12-24.
67. Spierings E. Minor histocompatibility antigens: past, present, and future. *Tissue Antigens.* 2014;84(4):374-360.
68. Gragert L, Eapen M, Williams E, et al. HLA match likelihoods for hematopoietic stem-cell grafts in the US registry. *N. Engl. J. Med.* 2014;371(4):339-348.
69. Fuchs EJ, O'Donnell PV, Eapen M, et al. Double unrelated umbilical cord blood vs HLA-haploidentical bone marrow transplantation: the BMT CTN 1101 trial. *Blood.* 2021;137(3):420-428.
70. van Gorkom G, Van Elssen C, Janssen I, Groothuis S, Evers S, Bos G. The impact of donor type on resource utilization and costs in allogeneic hematopoietic stem cell transplantation in the Netherlands. *Eur. J. Haematol.* 2021.
71. Giebel S, Locatelli F, Lamparelli T, et al. Survival advantage with KIR ligand incompatibility in hematopoietic stem cell transplantation from unrelated donors. *Blood.* 2003;102(3):814-819.
72. Cooley S, Weisdorf DJ, Guethlein LA, et al. Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. *Blood, The Journal of the American Society of Hematology.* 2010;116(14):2411-2419.

73. Gill S, Olson JA, Negrin RS. Natural killer cells in allogeneic transplantation: effect on engraftment, graft-versus-tumor, and graft-versus-host responses. *Biol. Blood Marrow Transplant.* 2009;15(7):765-776.
74. Ruggeri L, Mancusi A, Perruccio K, Burchielli E, Martelli MF, Velardi A. Natural killer cell alloreactivity for leukemia therapy. *J. Immunother.* 2005;28(3):175-182.
75. Ruggeri L, Mancusi A, Urbani E, Velardi A. Identifying NK alloreactive donors for haploidentical hematopoietic stem cell transplantation. In: *Tumor Immunology.* Springer; 2016:141-145.
76. Suen WC-W, Lee WY-W, Leung K-T, Pan X-H, Li G. Natural killer cell-based cancer immunotherapy: a review on 10 years completed clinical trials. *Cancer Invest.* 2018;36(8):431-457.
77. Myers JA, Miller JS. Exploring the NK cell platform for cancer immunotherapy. *Nature Reviews Clinical Oncology.* 2021;18(2):85-100.
78. Miller JS, Soignier Y, Panoskaltsis-Mortari A, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. *Blood.* 2005;105(8):3051-3057.
79. Heipertz E, Zynda E, Stav-Noraas TE, et al. Current perspectives on "Off-The-Shelf" allogeneic NK and CAR-NK cell therapies. *Front. Immunol.* 2021:4858.
80. Lamb MG, Rangarajan HG, Tullius BP, Lee DA. Natural killer cell therapy for hematologic malignancies: successes, challenges, and the future. *Stem Cell. Res. Ther.* 2021;12(1):1-19.
81. Granzin M, Wagner J, Köhl U, Cerwenka A, Huppert V, Ullrich E. Shaping of natural killer cell antitumor activity by ex vivo cultivation. *Front. Immunol.* 2017;8:458.
82. Denman CJ, Senyukov VV, Somanchi SS, et al. Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. *PLoS One.* 2012;7(1):e30264.
83. Gong Y, Klein Wolterink RG, Wang J, Bos GM, Germeraad WT. Chimeric antigen receptor natural killer (CAR-NK) cell design and engineering for cancer therapy. *J. Hematol. Oncol.* 2021;14(1):1-35.
84. Kremer V, Ligtenberg MA, Zendehdel R, et al. Genetic engineering of human NK cells to express CXCR2 improves migration to renal cell carcinoma. *Journal for immunotherapy of cancer.* 2017;5(1):1-13.
85. Bald T, Krummel MF, Smyth MJ, Barry KC. The NK cell–cancer cycle: advances and new challenges in NK cell–based immunotherapies. *Nat. Immunol.* 2020;21(8):835-847.
86. Hanahan D. Hallmarks of Cancer: New Dimensions. *Cancer Discov.* 2022;12(1):31-46.
87. Doubrovina ES, Doubrovin MM, Vider E, et al. Evasion from NK cell immunity by MHC class I chain-related molecules expressing colon adenocarcinoma. *The Journal of Immunology.* 2003;171(12):6891-6899.
88. Zhao Y, Chen N, Yu Y, et al. Prognostic value of MICA/B in cancers: a systematic review and meta-analysis. *Oncotarget.* 2017;8(56):96384.
89. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell.* 2012;21(3):309-322.
90. Labani-Motlagh A, Ashja-Mahdavi M, Loskog A. The tumor microenvironment: a milieu hindering and obstructing antitumor immune responses. *Front. Immunol.* 2020;11:940.
91. Stringaris K, Sekine T, Khoder A, et al. Leukemia-induced phenotypic and functional defects in natural killer cells predict failure to achieve remission in acute myeloid leukemia. *Haematologica.* 2014;99(5):836.
92. Chiu J, Ernst DM, Keating A. Acquired natural killer cell dysfunction in the tumor microenvironment of classic Hodgkin lymphoma. *Front. Immunol.* 2018;9:267.
93. Pazina T, MacFarlane AW, Bernabei L, et al. Alterations of NK Cell Phenotype in the Disease Course of Multiple Myeloma. *Cancers (Basel).* 2021;13(2):226.
94. Peng L-s, Zhang J-y, Teng Y-s, et al. Tumor-associated monocytes/macrophages impair NK-cell function via TGFβ1 in human gastric cancer. *Cancer immunology research.* 2017;5(3):248-256.
95. Han B, Mao F-y, Zhao Y-l, et al. Altered NKp30, NKp46, NKG2D, and DNAM-1 expression on circulating NK cells is associated with tumor progression in human gastric cancer. *Journal of immunology research.* 2018;2018.
96. Lavin Y, Kobayashi S, Leader A, et al. Innate immune landscape in early lung adenocarcinoma by paired single-cell analyses. *Cell.* 2017;169(4):750-765. e717.

97. Mamessier E, Sylvain A, Bertucci F, et al. Human breast tumor cells induce self-tolerance mechanisms to avoid NKG2D-mediated and DNAM-mediated NK cell recognition. *Cancer Res.* 2011;71(21):6621-6632.
98. Halama N, Braun M, Kahlert C, et al. Natural killer cells are scarce in colorectal carcinoma tissue despite high levels of chemokines and cytokines. *Clin. Cancer Res.* 2011;17(4):678-689.
99. Xu L, Huang Y, Tan L, et al. Increased Tim-3 expression in peripheral NK cells predicts a poorer prognosis and Tim-3 blockade improves NK cell-mediated cytotoxicity in human lung adenocarcinoma. *Int. Immunopharmacol.* 2015;29(2):635-641.
100. Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature.* 2002;419(6908):734-738.
101. Muz B, de la Puente P, Azab F, Azab AK. The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. *Hypoxia.* 2015;3:83.
102. Sarkar S, Germeraad WT, Rouschop KM, et al. Hypoxia induced impairment of NK cell cytotoxicity against multiple myeloma can be overcome by IL-2 activation of the NK cells. *PLoS One.* 2013;8(5):e64835.
103. Balsamo M, Manzini C, Pietra G, et al. Hypoxia downregulates the expression of activating receptors involved in NK-cell-mediated target cell killing without affecting ADCC. *Eur. J. Immunol.* 2013;43(10):2756-2764.
104. Romero-Garcia S, Lopez-Gonzalez JS, B´ ez-Viveros JL, Aguilar-Cazares D, Prado-Garcia H. Tumor cell metabolism: an integral view. *Cancer Biol. Ther.* 2011;12(11):939-948.
105. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell Metab.* 2008;7(1):11-20.
106. Chang C-H, Qiu J, O'Sullivan D, et al. Metabolic competition in the tumor microenvironment is a driver of cancer progression. *Cell.* 2015;162(6):1229-1241.
107. O'Brien KL, Finlay DK. Immunometabolism and natural killer cell responses. *Nature Reviews Immunology.* 2019;19(5):282-290.
108. Keating SE, Zaiatz-Bittencourt V, Loftus RM, et al. Metabolic reprogramming supports IFN-γ production by CD56bright NK cells. *The Journal of Immunology.* 2016;196(6):2552-2560.
109. Brand A, Singer K, Koehl GE, et al. LDHA-associated lactic acid production blunts tumor immunosurveillance by T and NK cells. *Cell Metab.* 2016;24(5):657-671.
110. Binnewies M, Roberts EW, Kersten K, et al. Understanding the tumor immune microenvironment (TIME) for effective therapy. *Nat. Med.* 2018;24(5):541-550.
111. Shi J, Tricot G, Szmania S, et al. Infusion of haplo-identical killer immunoglobulin-like receptor ligand mismatched NK cells for relapsed myeloma in the setting of autologous stem cell transplantation. *Br. J. Haematol.* 2008;143(5):641-653.
112. Zavidij O, Haradhvala NJ, Mouhieddine TH, et al. Single-cell RNA sequencing reveals compromised immune microenvironment in precursor stages of multiple myeloma. *Nature cancer.* 2020;1(5):493-506.
113. Mahaweni NM, Bos GM, Mitsiades CS, Tilanus MG, Wieten L. Daratumumab augments alloreactive natural killer cell cytotoxicity towards CD38+ multiple myeloma cell lines in a biochemical context mimicking tumour microenvironment conditions. *Cancer Immunol. Immunother.* 2018;67(6):861-872.
114. Frerichs KA, Nagy NA, Lindenbergh PL, et al. CD38-targeting antibodies in multiple myeloma: mechanisms of action and clinical experience. *Expert Rev. Clin. Immunol.* 2018;14(3):197-206.
115. Cursons J, Souza-Fonseca-Guimaraes F, Anderson A, et al. A natural killer cell gene signature predicts melanoma patient survival. *bioRxiv.* 2018:375253.
116. Muntasell A, Rojo F, Servitja S, et al. NK cell infiltrates and HLA class I expression in primary HER2+ breast cancer predict and uncouple pathological response and disease-free survival. *Clin. Cancer Res.* 2019;25(5):1535-1545.
117. Mazzaschi G, Facchinetti F, Missale G, et al. The circulating pool of functionally competent NK and CD8+ cells predicts the outcome of anti-PD1 treatment in advanced NSCLC. *Lung Cancer.* 2019;127:153-163.
118. Mamessier E, Sylvain A, Thibult M-L, et al. Human breast cancer cells enhance self tolerance by promoting evasion from NK cell antitumor immunity. *The Journal of clinical investigation.* 2011;121(9):3609-3622.

119. Wilson BE, Gorrini C, Cescon DW. Breast cancer immune microenvironment: from pre-clinical models to clinical therapies. *Breast Cancer Res. Treat.* 2021:1-11.
120. Gatti-Mays ME, Balko JM, Gameiro SR, et al. If we build it they will come: targeting the immune response to breast cancer. *NPJ breast cancer.* 2019;5(1):1-13.
121. Vanclee A, van Gelder M, Schouten H, Bos G. Graft-versus-tumor effects on murine mammary carcinoma in a model of nonmyeloablative haploidentical stem cell transplantation. *Bone Marrow Transplant.* 2006;37(11):1043-1049.
122. Fleskens A, Lalisang R, Bos G, Van Gelder M, Jansen R, Schouten H. HLA-matched allo-SCT after reduced intensity conditioning with fludarabine/CY in patients with metastatic breast cancer. *Bone Marrow Transplant.* 2010;45(3):464-467.
123. Ueno NT, Cheng YC, Rondón G, et al. Rapid induction of complete donor chimerism by the use of a reduced-intensity conditioning regimen composed of fludarabine and melphalan in allogeneic stem cell transplantation for metastatic solid tumors. *Blood.* 2003;102(10):3829-3836.
124. Frings PW, Van Elssen CH, Wieten L, et al. Elimination of the chemotherapy resistant subpopulation of 4T1 mouse breast cancer by haploidentical NK cells cures the vast majority of mice. *Breast Cancer Res. Treat.* 2011;130(3):773-781.
125. Geller MA, Cooley S, Judson PL, et al. A phase II study of allogeneic natural killer cell therapy to treat patients with recurrent ovarian and breast cancer. *Cytotherapy.* 2011;13(1):98-107.
126. Krneta T, Gillgrass A, Chew M, Ashkar AA. The breast tumor microenvironment alters the phenotype and function of natural killer cells. *Cell. Mol. Immunol.* 2016;13(5):628-639.

2

Tuning Natural Killer Cell Anti-Multiple Myeloma Reactivity by Targeting Inhibitory Signaling via KIR and NKG2A

Niken M. Mahaweni, Femke A. I. Ehlers, Gerard M. J. Bos, and Lotte Wieten

Frontiers in Immunology. 2018 Dec 4;9:2848.
doi: 10.3389/fimmu.2018.02848

Abstract
Natural killer (NK) cells are attractive candidates for allogeneic cell-based immunotherapy due to their potent antitumor effector function and good safety profile. NK cells express killer immunoglobulin-like receptors (KIRs) and the NKG2A receptor important for NK cells education as well as providing inhibitory signals upon encountering HLA-expressing target cells. Multiple myeloma (MM) is an example of a tumor expressing relatively high levels of HLA molecules. In this review, we discuss the functional relevance of inhibitory KIRs and NKG2A for NK cells anti-MM response and strategies to lower these inhibitory signaling to enhance clinical efficacy of allogeneic NK cells in MM.

Introduction
Over the past years, NK cells became popular candidates for immunotherapy against cancer due to their unique combination of a potent anti-tumor effector function and a very good safety profile (1). The capacity of NK cells to discriminate healthy- from diseased cells creates the opportunity to safely use NK cells in the allogeneic setting and to maximally benefit from their anti-tumor potential while not risking development of graft vs. host pathology. In the allogeneic setting, this latter feature is a great benefit over strategies using conventional T cells. Although both T cells and NK cells exploit major histocompatibility (MHC) molecules for immune surveillance, they do it in an intrinsically different manner. T cell activation occurs upon interaction between the T-cell receptor (TCR) and a foreign MHC-peptide complex which, in an allogeneic MHC mismatched setting, easily results in graft vs. host disease (GVHD). NK cells, on the other hand, rather sense the absence of MHC molecules, a phenomenon called “missing-self recognition” described first in the 1990's by Ljunggren and Karre (2). Even in the absence of MHC molecules, NK cells do not attack healthy cells because for activation a sufficient level of activating signals, provided by viral- or stress proteins, is required and these signals are usually not sufficiently present on healthy cells (3).

NK cells can sense self vs. missing-self via receptors belonging to the killer immunoglobulin-like receptor (KIR) family and NKG2A. In this review, we will provide an overview of the functional relevance of KIR and NKG2A for the anti-tumor response of NK cells in an allogeneic setting. We will specifically address the role of allogeneic NK cells in multiple myeloma (MM), a hematological malignancy characterized by the expansion of malignant plasma cells in the bone marrow. To date, MM remains incurable despite the greatly improved clinical perspective due to novel immunomodulatory agents like lenalidomide and pomalidomide and highly promising antibodies like daratumumab (anti-CD38) and elotuzumab (anti-CS-1/SLAMF7). Given their excellent safety and feasibility profiles, NK cells are interesting candidates to combine with these novel agents to enhance clinical efficacy and to ultimate achieve curative treatment for MM patients.

Killer Immunoglobulin-Like Receptors (KIRs) Biology

The KIR family consists of activating- and inhibitory receptors. Activating family members are characterized by a short cytoplasmic ITAM activating signaling domain and are called KIRxDS. Inhibitory family members have a long and inhibitory ITIM domain and are named KIRxDL. Both the activating and the inhibitory KIRs have two (KIR2DSx or KIR2DLx) or three (KIR3DSx or KIR3DLx) extracellular immunoglobulin-like domains for ligand interaction. Classical human leukocyte antigen (HLA) class I molecules are the most important ligands for both the activating- and the inhibitory KIRs. The best characterized inhibitory KIRs are: KIR2DL1, binding to HLA-C group 2 (C2) alleles having a lysine at position 80; KIR2DL2/3, interacting with HLA-C group 1 (C1) alleles having an asparagine at position 80 (4–6). KIR3DL1, binding HLA-B alleles bearing a Bw4 motif as well as HLA-A*23/*24/*32 (7, 8). KIR3DL2 has been shown to interact with HLA-A*3/*11 (9) and HLA-F (10). The activating KIR2DS1 and KIR2DS2 have been shown to bind with C2 and C1 alleles, respectively, and KIR2DS4 interacts with subsets of HLA-C alleles and with HLA-A*11 (11, 12). The ligands for the other KIRs remain elusive so far.

The genes encoding the KIRs are located in the KIR gene cluster in the leukocyte receptor region on chromosome 19, and so far, 27 different KIR haplotypes have been described (http://www.imgt.org/). KIR2DL4, KIR3DL2, KIR3DL3, and KIR3DP1 are so called framework genes and are present in all the haplotypes. Based on the additional presence/absence of the other KIRs, the haplotypes can be further grouped into haplotype-A and –B. While A haplotypes express only KIR2DS4 as activating KIR and eight other KIRs (KIR2DL1, KIR2DL3, KIR2DL4, KIR3DL1, KIR3DL2, KIR3DL3, KIR2DP1, and KIR3DP1), the B haplotypes express multiple activating receptors in combination with various other KIR genes (13). In the population, the A to B haplotype ratio is on average 1.8:1 (14) and in most populations B/x haplotypes are more common than A/A. A study comparing KIR haplotype A and B frequencies in MM demonstrated that there was no difference in distribution between MM patients and healthy individuals (14). Moreover, analysis of KIR repertoires of 182 MM patients revealed that the genotypic presence of KIR3DS1, most pronounced in Bw4 missing patients, was associated with reduced progression free survival after autologous SCT (15). Nonetheless, further extensive studies on the influence of the KIR genetic repertoire on development and progression of MM are missing.

Further variation in KIR repertoires between individuals results from the relatively polymorphic nature of the KIR genes and expression differences can occur due to null/low/high expression allele variants and copy number variation (16). Furthermore, KIRs are acquired in a stochastic manner leading to intra-individual diversity in KIR receptor expression between NK cells (17). Within the A haplotype four inhibitory KIRs, namely KIR2DL1, KIR2DL3, KIR3DL1, KIR3DL2 can be expressed. A combination of cell surface expression of all four inhibitory KIRs is rarely found within one healthy individual (< 5%). Co-expression of three inhibitory KIRs occurs also in rather few NK cells (about 10%), while co-expression of 2 KIRs and expression

of a single KIR occurs more frequently (30% and 35%, respectively). Functionally immature NK cells, lacking all KIRs, represent about 20% (18).

NKG2A Receptor Biology

NK cells of healthy individuals frequently express NKG2A (20–80%) (19, 20). NKG2A expression occurs more frequently on KIR-negative NK cells and decreases as NK cells acquire KIRs (18). NKG2A is an inhibitory member of the C-type lectin-like NKG2 receptor family that also comprises the inhibitory NKG2B and the activating NKG2C/E/H receptors (21). NKG2A engages HLA-E, a non-classical HLA class I molecule constitutively expressed at low levels on the cell surface of virtually every cell. In contrast to the classical HLA class I molecules, HLA-E displays only very limited polymorphism and only two common protein variants are known (HLA-E*01:01 and HLA-E*01:03) (21). These two HLA-E allelic variants differ in one amino acid at position 107 on the alpha2 domain of the HLA-E heavy chain, and HLA-E*01:01 has an Arginine at position 107 while HLA-E*01:03 has a Glycine (22). This amino acid difference results in a higher peptide binding affinity and consequently a higher surface expression for HLA-E*01:03. NKG2A binds to both HLA allotypes and so far, no obvious functional differences between the two HLA-E alleles have been described (23, 24). While the KIRs are highly polymorphic, NKG2A is well conserved with only a few known polymorphism (13, 25, 26).

NK Cell Education and Recognition of Missing Self

Inhibitory receptors for HLA play a pivotal role in the shaping of a functional NK cell repertoire. NK cells develop from the bone marrow and acquire inhibitory receptors in a stochastic manner (27). Mature NK cells can express no-, one- or a combination of inhibitory receptors. As the KIR and HLA genes are located on different chromosomes (KIR on chromosome 19 and HLA on chromosome 6) they can be inherited independently. Consequently, individuals can express KIRs for which the corresponding HLA ligand is missing. For example, an individual can express KIR3DL1 without being Bw4 positive. To warrant self-tolerance, even in the absence of a ligand, NK cells are continuously educated by their HLA environment in a process called “licensing” or “arming.” Although the mechanistic basis of this process is not fully understood, it is known that NK cell subsets expressing no inhibitory receptor or a receptor for which the HLA ligand is not endogenously expressed are hyporesponsive (28). On the other hand, NK cells expressing inhibitory receptors that can engage HLA become more responsive (29). Those so-called educated NK cells have been shown to hold higher density granules (30). Moreover, they are more potent cytokine producers and killers than non-educated NK cells (31). From previous studies it known that the more inhibitory receptors an NK cell expresses, the more potent its effector function (32, 33).

HLA Class I and HLA-E Expression in Multiple Myeloma

Many viruses and tumors have evolved strategies to reduce HLA expression presumably to escape from CD8 T cell immunity, and educated NK cells are excellent

in targeting these cells. However, while loss of expression of classical HLA class I is frequently seen in many types of cancer it is becoming more and more clear that numerous of these tumors remain positive for HLA-E (34). By doing so, these tumor cells can evade from CD8 T cells and, as the majority of the NK cells expresses NKG2A, the tumor also remains relatively protected against NK cells. Under physiological conditions, HLA-E expression is tightly linked to HLA class I expression. The reason is that HLA-E presents the leader peptides that are removed from HLA class I molecules before leaving the endoplasmic reticulum to travel to the cell surface (35). Consequently, a reduced expression of HLA class I can be expected to result in lower levels of HLA-E on the cell surface. But, apparently this is not necessarily the case, and tumors, as well as several viruses, have developed ways to maintain HLA-E expression even in the absence of HLA class I leader peptides. Why this is possible is not completely known. One option could be that HLA-E presents a TAP- and HLA class I independent peptide repertoire, as seen in TAP deficient LCL 721.221 cells (36). Alternatively, peptides from stress proteins like Hsp60 have been shown stabilize HLA-E on the cell membrane (37).

In MM, we observed that MM cell lines frequently express only low levels of HLA-E in vitro while HLA-E expression was much higher upon in vivo growth of the cells in the bone marrow of immunodeficient mice (38). Moreover, primary MM cells obtained from patients expressed relatively high levels of HLA class I as well as HLA-E (38). Furthermore, HLA expression has been shown to be related to disease status in MM, since MM cells isolated from late-stage pleural effusions expressed higher levels of HLA class I and reduced levels of activating NKG2D ligands as compared to earlier stage MM cells (39). A comparable observation was made by comparing MGUS vs. MM samples, showing higher levels of HLA class I and reduced levels of MICA on the MM samples (40). Given the presence of both HLA class I and HLA-E on MM, interfering with inhibitory signaling to lower the NK cell activation threshold, so basically creating missing-self, for NK cells in MM seems a good strategy. This could be perceived either by KIR-ligand mismatching based on genotypes in an allogeneic transplantation setting, the use of clinically available monoclonal antibodies to block KIR (e.g., lirilumumab) or NKG2A (e.g., monalizumab) (41, 42), or by agents such as the proteasome inhibitors lactacystin, bortezomib and carfilzomib that have been shown to reduce HLA class I expression in MM (43–45).

Creating Missing-Self for Multiple Myeloma by KIR-Ligand Mismatching in the Allo-SCT Setting

The potential of exploiting missing-self recognition to enhance the antitumor potential of NK cells in the allogeneic setting became most evident from the ground breaking work of Ruggeri et al. showing that a so called KIR-ligand mismatch improved clinical outcome after haploidentical stem cell transplantation (haplo-SCT) in patients with acute myeloid leukemia (AML) (46, 47). In the haplo-SCT setting, patient and donor are matched based on one of the HLA haplotypes meaning that half of the HLA genes is mismatched between patient and donor. This enables

incompatibility between inhibitory KIRs, expressed on the donor NK cells, and their HLA ligands on patient tumor cells. As the donor KIR-ligand mismatched NK cells in this setting will remain educated by the donor HLA background (48, 49), they can efficiently detect missing-self and mediate more potent responses against the tumor cells than the non-mismatched NK cells that receive inhibitory signals via HLA (**Figure 1**).

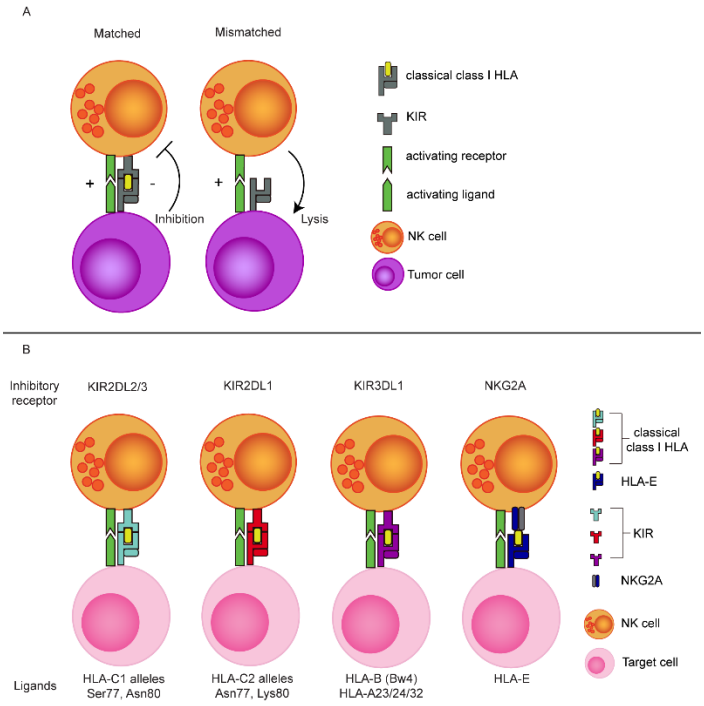


FIGURE 1. The concept of NK cell alloreactivity concept based on interaction with HLA class I. (A) When an inhibitory KIR binds to a "matched" classical class I HLA molecule, an NK cell receives inhibitory signal from this interaction. In the absence of the corresponding class I HLA molecule (mismatched situation), the inhibitory signal is absent, resulting in a reduced NK cell activation threshold. (B) Inhibitory KIRs and NKG2A and their corresponding class I HLA molecules. KIR, Killer immunoglobulin-like receptor; HLA, Human leukocyte antigen; Ser, Serine; Asn, Aspargine; Lys, Lysine.

Only very limited data on the potential benefit of KIR-ligand mismatching in allo-SCT in MM is available. Nevertheless, Kröger et al. showed that in HLA-C mismatched unrelated donor allo-SCT, patients receiving a KIR-ligand mismatched graft had longer progression free survival than patients receiving a matched graft (50). In general, there is still no real consensus on whether or not a KIR-ligand mismatch has a clinical benefit and presumably this is highly dependent on the exact conditioning- and transplantation protocol. In contrast to KIR-HLA class I, mismatching for HLA-E and NKG2A is not an option due to the limited polymorphism of HLA-E. However, early upon reconstitution, the relatively immature NK cells express NKG2A but not

KIRs and it can take up to 3 months till a fully mature KIR repertoire is present (51). As NKG2A could inhibit the anti-MM response of these reconstituting NK cells (38), it may be an interesting option to interfere with HLA-E NKG2A interaction using a monoclonal antibody like monalizumab in the context of allo-SCT.

For a long time, feasibility of haplo-SCT was limited by the high occurrence of post-transplant complications such as GVHD and infections. However, due to the recent successes of improved T cell depletion methods (e.g., by αβ-depletion) or by post-transplant administration of cyclophosphamide, haplo-SCT became a feasible approach with a good safety profile and the major advantage that a large number of donors is usually available within the family (52). To be eligible for KIR-ligand mismatched transplantation, patients should genotypically lack expression of at least one of the inhibitory KIR ligands, meaning that they should miss either HLA-C1, -C2, or –Bw4 or a combination thereof. Nonetheless, ~1/3 of the population express all three ligands (53). This was also observed in a large clinical study on leukemia and myelodysplastic syndrome (54), where around 34–36 % of the patients expressed all three ligands and the rest of the patients (64–66 %) lacked at least one ligand. In addition, to the absence of C1, C2, or Bw4 in the patient, the selected donor should express the HLA ligand that is missing in the patient to make sure the NK cells will be educated to sense the missing ligand and the corresponding KIR should be present on the cell surface. Especially for KIR3DL1 this is important to confirm, preferably by flow cytometry, as null alleles frequently occur (55).

Creating Missing-Self for NK Cell Adoptive Transfer in Multiple Myeloma

Various research groups have shown that NK cells played a major role in the elimination of tumor cells in the allogenic setting. Nonetheless, previous studies have also shown that NK-cell numbers and effector to target ratios are important for tumor cell clearance (56, 57). To enable infusion of high numbers of NK cells, large scale ex vivo expansion of NK cells for adoptive NK cell-based therapy is currently heavily investigated (58, 59). To date, several groups have infused NK cells into MM patients as a form of adoptive immunotherapy. Szmania et al. infused up to 1×10^8 (per kilogram) ex vivo expanded NK cells derived from MM patients or haploidentical family donors into 8 high-risk relapsed MM patients (60). These NK cell infusions were well tolerated and a significant in vivo expansion of the NK cells was observed in two subjects. Although in five patients the NK cell infusion did not affect the disease progression, in one patient it resulted in a partial response and in another patient in a delayed disease progression. In another study, umbilical cord blood-derived NK cells were infused into 12 high-risk relapsed MM patients (61). In this study, four different doses of NK cells were administered each to three patients; 5×10^6, 1×10^7, 5×10^7, and 1×10^8 demonstrating a good safety profile a partial response in 10 patients as their best response. In the 21st month of follow up, four patients progressed or relapsed.

Given the relatively high expression of HLA-class I on the MM cell surface (38, 39, 62), selection of KIR-ligand mismatched NK cell donors could be an approach to enhance clinical anti-MM responses of infused NK cells. As the potential benefit of a KIR-ligand mismatch is not very well established in MM, we recently addressed this question in a series of in vitro studies in which we were especially interested in the functional relevance of KIR-ligand mismatching for highly activated NK cells. The reason for this was that most current insight in the role of KIR and NKG2A comes from studies using unactivated NK cells or from NK cells that are reconstituting after allo-SCT. The situation might be very different for the highly activated NK cells that are typically used for adoptive NK cell therapy as their activation threshold could be changed by the activation. Our studies revealed that for unactivated NK cells as well as for highly activated (1000 U/mL IL-2) NK cells, KIR-ligand mismatched NK cells were better effector cells than KIR-ligand matched NK cells against various MM cell lines (38). This was also the case in the presence of immunosuppressive factors, like hypoxia, PGE2 and lactate, that are frequently found in tumor microenvironment (63). Even when we further potentiated the NK-cell anti-MM response via antibody-dependent cell-mediated cytotoxicity (ADCC), by combining NK cells and daratumumab (anti-CD38), KIR ligand mismatched NK cells degranulated more robustly than their matched counterparts (63). Although the difference between the matched and mismatched subsets was not very large, one can anticipate that in an immunosuppressive tumor microenvironment, where the NK cell receives and integrates a multitude of inhibitory signals, reduction of any extra inhibitory signals by KIR-ligand mismatching could help to potentiate the NK cell response against HLA class I competent MM cells. As many of the currently used ex vivo expanded clinical NK cell products harbor very high percentages of NKG2A+ NK cells, we also evaluated the relevance of NKG2A interaction for the anti-MM response of highly activated NK cells. This showed that, at least in vitro and on ex vivo primary MM cells, the level of HLA-E on the MM cells was not sufficient to trigger potent inhibitory signaling via CD94-NKG2A (64). Enhanced levels of HLA-E on the MM cells, by using an HLA-E stabilizing peptide, did, however, result in inhibition via NKG2A, and illustrated that the expression level of HLA-E influenced the inhibitory potential of NKG2A (64). Together these data emphasize the complexity of the NK cell antitumor response. Furthermore, they suggest that creating missing self by KIR-ligand mismatching for highly activated NK cells could help to potentiate clinical efficiency while creating missing-self based on interfering with NKG2A may only be beneficial for tumors expressing high levels of HLA-E.

Creating Missing-Self with Monoclonal Anti-KIR or Anti-NKG2A Antibodies

The use of currently clinically available antibodies that block KIR or NKG2A is an alternative interesting option to create missing-self in MM. Blocking antibodies would especially be helpful for the 30% of donors expressing all three KIR ligands. It may also be applied under conditions where NKG2A is mediating strong inhibitory effects (e.g., for tumors with very high levels of HLA-E or for unactivated NK cells).

Blocking KIR-ligand interaction using an anti-HLA antibody showed enhanced killing of primary MM by haploidentical KIR-ligand mismatched NK cells in an in vitro autologous transplantation setting (65). In other in vitro studies, the addition of IPH2101, a clinical anti-KIR antibody, increased NK cell cytotoxicity against HLA-C positive acute myeloid leukemia and lymphoma cells (66, 67). In spite of the in vitro successes, the clinical efficacy of the antibody still needs to be further elucidated. In a phase I clinical study in patients with relapsed/refractory MM, the IPH2101 antibody has been shown to be safe and well tolerated, however, it did not result in clear clinical responses despite an observed improvement in in vitro cytotoxicity against a MM cell line (41). A phase II trial with IPH2101 in patients with smoldering MM, was prematurely terminated due to lack of therapeutic benefit (68). To unravel the unexpected lack of benefit, a follow up study was performed which showed that infusion of IPH2101 had led to both reduced KIR2D surface expression on NK cells and reduced NK function (69). KIR2D removal of anti-KIR treated NK cells was mediated by trogocytosis, a mechanism by which monocytes remove antibody-bound molecules from the cell surface. These studies suggest that blocking KIR by anti-KIR antibodies could result in uneducated, hyporesponsive NK cells and subsequently in limited effects of the antibody in vivo. Also they illustrate that, despite its in vitro potential, a better understanding of how to use the blocking antibody in vivo is essential.

One way to improve clinical responses of IPH2101 may be by combinational therapies with drugs providing strong activating signals to the NK cells. In a phase I clinical trial with relapsed/refractory MM patients, the combination of the anti-KIR antibody with lenalidomide, an immunomodulatory agent, augmented NK cell function and resulted in objective responses (70). Moreover, combination of IPH2101 and the ADCC-triggering antibody daratumumab could also enhance NK cell cytotoxicity against MM cell lines and against primary myeloma cells in vitro while IPH2101 alone did not induce a significant antitumor effect in this setting (71).

Blocking NKG2A is another option to reduce inhibitory NK cell signaling aiming to improve the effector function of either endogenous NK cells or of donor NK cell in allo-SCT or adoptive transfer settings. In a preclinical mouse study, infusion of NKG2A+ NK cells mediated anti-leukemia effects when NK cells were pre-treated with an anti-NKG2A antibody and rescued the mice from developing leukemia (42). In another in vitro preclinical study, blocking NKG2A with the anti-NKG2A antibody monalizumab could restore the cytotoxic potential of NK cells derived from patients with chronic lymphocytic leukemia (72). However, thus far completed clinical trials testing safety and efficacy of monalizumab in MM patients are not available.

Future Perspective

To further enhance the NK-cell antitumor response, novel combination strategies are currently being explored and also there it may be relevant to evaluate the additive value of interfering with inhibitory signaling via HLA. Examples of such strategies are combinations with antibodies (monoclonal, bi- or tri-specific) targeting tumor-associated or –specific antigens to trigger ADCC. Also, genetic modification of NK cells during ex vivo NK cell expansion is frequently explored as a novel way to improve NK function for instance by creating NK cells expressing chimeric-antigen receptors (CAR) to potently trigger NK cell activation. Another interesting option is the combination of haplo-SCT and infusion of a high number of highly activated NK cells from the same donor. This combination would bypass the drawbacks of slow reconstitution of mature NK cells (up to 2–6 months) in haplo SCT (73, 74, 75) and lack of persistence for ex vivo expanded NK cells as they are short lived and not clonally expand upon activation (76). The combination setting would have the best of both worlds. First, the adoptively transferred NK cells could be manipulated during ex vivo expansion and they can mediate their potent antitumor effects in the first lymphopenic period after haplo-SCT while they simultaneously contribute to protection from viral infections. Second, the NK cells that reconstitute from the donor stem cells will provide persistence of donor NK cells. Third, the process of donor selection for both procedures needs to only be done once. The use of haplo-SCT in Europe is increasing since 2005 (77). Our group recently finished a phase I study performing haplo-SCT in MM, now continued as a phase II study (NL49476.000.14), which can serve as platform for haplo-SCT and NK-infusion combination therapy in MM. At MD Anderson such a combination study has already been in patients with AML showing feasibility and better disease-free survival and less infections (78).

Studies with sufficient power to demonstrate a potential clinical relevance of creating missing-self are currently lacking in MM. Moreover, since the benefit of interfering with inhibitory HLA-induced signaling seems to depend on the exact transplantation protocol, the activation status of the NK cell, and on the input via other activating- or inhibitory receptors, it will be important to test the clinical relevance for NK cells receiving very strong activating signal via a CAR or via potent bi- or even tri- specific antibodies and in the haplo-SCT NK infusion combination setting as well. The potential shown in especially in vitro, studies and the relatively high expression of classical HLA class I molecules as well as non-classical HLA-E on MM cells make it worthwhile to further explore the potential benefit of reducing inhibitory signaling via HLA by genetic mismatching or blocking antibodies.

Author Contributions

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Funding

This work was supported by a grant from the Kankeronderzoeksfonds Limburg. LW was supported by a grant from Dutch Cancer association (KWF kankerbestrijding; UM2012-5375).

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Dahlberg CIM, Sarhan D, Chrobok M, Duru AD, Alici E. Natural killer cell-based therapies targeting cancer: possible strategies to gain and sustain anti-tumor activity. Front Immunol. (2015) 6:605. doi: 10.3389/fimmu.2015.00605
2. Ljunggren HG, Karre K. In search of the missing self:MHC molecules and NK cell recognition. Immunol Today (1990) 11:237–44.
3. Moretta A, Biassoni R, Bottino C, Mingari MC, Moretta L. Natural cytotoxicity receptors that trigger human NK-cell-mediated cytolysis. Immunol Today (2000) 21:228–34. doi: 10.1016/S0167-5699(00)01596-6
4. Hilton HG, Vago L, Older Aguilar AM, Moesta AK, Graef T, Abi-Rached L, et al. Mutation at positively selected positions in the binding site for HLA-C shows that KIR2DL1 is a more refined but less adaptable NK cell receptor than KIR2DL3. J Immunol. (2012) 189:1418–30. doi: 10.4049/jimmunol.1100431
5. Falco M, Moretta L, Moretta A, Bottino C. KIR and KIR ligand polymorphism: a new area for clinical applications? Tissue Antigens (2013) 82:363–73. doi: 10.1111/tan.12262
6. Colonna M, Brooks EG, Falco M, Ferrara GB, Strominger JL. Generation of allospecific natural killer cells by stimulation across a polymorphism of HLA-C. Science (1993) 260:1121–4.
7. Peruzzi M, Wagtmann N, Long EO. A p70 killer cell inhibitory receptor specific for several HLA-B allotypes discriminates among peptides bound to HLA-B*2705. J Exp Med. (1996) 184:1585–90. doi: 10.1084/jem.184.4.1585
8. Stern M, Ruggeri L, Capanni M, Mancusi A, Velardi A. Human leukocyte antigens A23, A24, and A32 but not A25 are ligands for KIR3DL1. Blood (2008) 112:708–10. doi: 10.1182/blood-2008-02-137521
9. Döhring C, Scheidegger D, Samaridis J, Cella M, Colonna M. A human killer inhibitory receptor specific for HLA-A. J Immunol. (1996) 156:2–6.
10. Goodridge JP, Burian A, Lee N, Geraghty DE. HLA-F and MHC class I open conformers are ligands for NK cell Ig-like receptors. J Immunol. (2013) 191:3553–62. doi: 10.4049/jimmunol.1300081
11. Stewart CA, Laugier-Anfossi F, Vely F, Saulquin X, Riedmuller J, Tisserant A, et al. Recognition of peptide-MHC class I complexes by activating killer immunoglobulin-like receptors. Proc Natl Acad Sci USA. (2005) 102:13224–9. doi: 10.1073/pnas.0503594102
12. Graef T, Moesta AK, Norman PJ, Abi-Rached L, Vago L, Older Aguilar AM, et al. KIR2DS4 is a product of gene conversion with KIR3DL2 that introduced specificity for HLA-A * 11 while diminishing avidity for HLA-C. J Exp Med. (2009) 206:2557–72. doi: 10.1084/jem.20091010
13. Middleton D, Gonzelez F. The extensive polymorphism of KIR genes. Immunology (2010) 129:8–19. doi: 10.1111/j.1365-2567.2009.03208.x
14. Hoteit R, Bazarbachi A, Antar A, Salem Z, Shammaa D, Mahfouz R. KIR genotype distribution among patients with multiple myeloma: Higher prevalence of KIR 2DS4 and KIR 2DS5 genes. Meta Gene. (2014) 2:730–6. doi: 10.1016/j.mgene.2014.09.008
15. Gabriel IH, Sergeant R, Szydlo R, Apperley JF, DeLavallade H, Alsuliman A, et al. Interaction between KIR3DS1 and HLA-Bw4 predicts for progression-free survival after autologous stem cell transplantation in patients with multiple myeloma. Blood (2010) 116:2033–9. doi: 10.1182/blood-2010-03-273706
16. Norman PJ, Hollenbach JA, Nemat-Gorgani N, Marin WM, Norberg SJ, Ashouri E, et al. Defining KIR and HLA class I genotypes at highest resolution via high-throughput sequencing. Am J Hum Genet. (2016) 99:375–91. doi: 10.1016/j.ajhg.2016.06.023
17. Andersson S, Fauriat C, Malmberg JA, Ljunggren HG, Malmberg KJ. KIR acquisition probabilities are independent of self-HLA class I ligands and increase with cellular KIR expression. Blood (2009) 114:95–104. doi: 10.1182/blood-2008-10-184549
18. Fauriat C, Andersson S, Bjorklund AT, Carlsten M, Schaffer M, Bjorkstrom NK, et al. Estimation of the size of the alloreactive NK cell repertoire: studies in individuals homozygous for the group A KIR haplotype. J Immunol. (2008) 181:6010–9. doi: 10.4049/jimmunol.181.9.6010
19. Angelo LS, Banerjee PP, Monaco-Shawver L, Rosen JB, Makedonas G, Forbes LR, et al. Practical NK cell phenotyping and variability in healthy adults. Immunol Res. (2015) 62:341–56. doi: 10.1007/s12026-015-8664-y
20. Pascal V, Schleinitz N, Brunet C, Ravet S, Bonnet E, Lafarge X, et al. Comparative analysis of NK cell subset distribution in normal and lymphoproliferative disease of granular lymphocyte conditions. Eur J Immunol. (2004) 34:2930–40. doi: 10.1002/eji.200425146

21. Geraghty DE, Stockschleader M, Ishitani A, Hansen JA. Polymorphism at the HLA-E locus predates most HLA-A and -B polymorphism. Hum Immunol. (1992) 33:174–84. doi: 10.1016/0198-8859(92)90069-Y
22. Strong RK, Holmes MA, Li P, Braun L, Lee N, Geraghty DE. HLA-E allelic variants: Correlating differential expression, peptide affinities, crystal structures, and thermal stabilities. J Biol Chem. (2003) 278:5082–90. doi: 10.1074/jbc.M208268200
23. Lauterbach N, Wieten L, Popeijus HE, Vanderlocht J, van Zon PMH, Voorter CEM, et al. Peptide-induced HLA-E expression in human PBMCs is dependent on peptide sequence and the HLA-E genotype. Tissue Antigens (2015) 85:242–51. doi: 10.1111/tan.12525
24. Celik AA, Kraemer T, Huyton T, Blasczyk R, Bade-Döding C. The diversity of the HLA-E-restricted peptide repertoire explains the immunological impact of the Arg107Gly mismatch. Immunogenetics (2016) 68:29–41. doi: 10.1007/s00251-015-0880-z
25. Park KS, Park JH, Song YW. Inhibitory NKG2A and activating NKG2D and NKG2C natural killer cell receptor genes: susceptibility for rheumatoid arthritis. Tissue Antigens (2008) 72:342–6. doi: 10.1111/j.1399-0039.2008.01110.x
26. Iwaszko M, Swierkot J, Kolossa K, Jeka S, Wiland P, Bogunia-Kubik K. Influence of CD94 and NKG2A variants on susceptibility to rheumatoid arthritis and efficacy of anti-TNF treatment. Jt Bone Spine (2016) 83:75–9. doi: 10.1016/j.jbspin.2015.06.010
27. Manser AR, Weinhold S, Uhrberg M. Human KIR repertoires: shaped by genetic diversity and evolution. Immunol Rev. (2015) 267:178–96. doi: 10.1111/imr.12316
28. Anfossi N, André P, Guia S, Falk CS, Roetynck S, Stewart CA, et al. Human NK cell education by inhibitory receptors for MHC class I. Immunity (2006) 25:331–42. doi: 10.1016/j.immuni.2006.06.013
29. Kim S, Poursine-Laurent J, Truscott SM, Lybarger L, Song YJ, Yang L, et al. Licensing of natural killer cells by host major histocompatibility complex class I molecules. Nature (2005) 436:709–13. doi: 10.1038/nature03847
30. Goodridge JP, Jacobs B, Saetersmoen ML, Clement D, Clancy T, Skarp- E, et al. Modulation of secretory lysosomes during NK cell education leads to accumulation of granzyme B and enhanced functional potential. bioRxiv (2018) doi: 10.1101/305862
31. Tu MM, Mahmoud AB, Makrigiannis AP. Licensed and unlicensed NK cells: differential roles in cancer and viral control. Front Immunol. (2016) 7:166. doi: 10.3389/fimmu.2016.00166
32. Thomas LM. Current perspectives on natural killer cell education and tolerance: emerging roles for inhibitory receptors. Immunotargets Ther. (2015) 4:45–53. doi: 10.2147/ITT.S61498
33. Sim MJW, Stowell J, Sergeant R, Altmann DM, Long EO, Boyton RJ. KIR2DL3 and KIR2DL1 show similar impact on licensing of human NK cells. Eur J Immunol. (2016) 46:185–91. doi: 10.1002/eji.201545757
34. Wieten L, Mahaweni NM, Voorter CEM, Bos GMJ, Tilanus MGJ. Clinical and immunological significance of HLA-E in stem cell transplantation and cancer. Tissue Antigens (2014) 84:523–35. doi: 10.1111/tan.12478
35. Iwaszko M, Bogunia-Kubik K. Clinical significance of the HLA-E and CD94/NKG2 interaction. Arch Immunol Ther Exp. (2011) 59:353–67. doi: 10.1007/s00005-011-0137-y
36. Kraemer T, Celik AA, Huyton T, Kunze-Schumacher H, Blasczyk R, Bade-Döding C. HLA-E: Presentation of a broader peptide repertoire impacts the cellular immune response - Implications on HSCT outcome. Stem Cells Int. (2015) 2015:346714. doi: 10.1155/2015/346714
37. Michaëlsson J, Teixeira de Matos C, Achour A, Lanier LL, Kärre K, et al. A signal peptide derived from hsp60 binds HLA-E and interferes with CD94/NKG2A recognition. J Exp Med. (2002) 196:1403–14. doi: 10.1084/jem.20020797
38. Sarkar S, van Gelder M, Noort W, Xu Y, Rouschop KMA, Groen R, et al. Optimal selection of natural killer cells to kill myeloma: the role of HLA-E and NKG2A. Cancer Immunol Immunother. (2015) 64:951–63. doi: 10.1007/s00262-015-1694-4
39. Carbone E, Neri P, Mesuraca M, Fulciniti MT, Otsuki T, Pende D, et al. IMMUNOBIOLOGY HLA class I , NKG2D , and natural cytotoxicity receptors regulate multiple myeloma cell recognition by natural killer cells. Blood (2005) 105:251–8. doi: 10.1182/blood-2004-04-1422.Supported
40. Bernal M, Garrido P, Jiménez P, Carretero R, Almagro M, López P, et al Changes in activatory and inhibitory natural killer. (NK) receptors may induce progression to multiple myeloma: Implications for tumor evasion of T and NK cells. Hum Immunol. (2009) 70:854–7. doi: 10.1016/j.humimm.2009.07.004

41. Benson DM, Hofmeister CC, Padmanabhan S, Suvannasankha A, Jagannath S, Abonour R, et al. A phase 1 trial of the anti-KIR antibody IPH2101 in patients with relapsed / refractory multiple myeloma. Blood (2012) 120:4324–33. doi: 10.1182/blood-2012-06-438028.Portions
42. Ruggeri L, Urbani E, Andre P, Mancusi A, Tosti A, Topini F, et al. Effects of anti-NKG2A antibody administration on leukemia and normal hematopoietic cells. Haematologica (2016) 101:626–33. doi: 10.3324/haematol.2015.135301
43. Wu X, Shao Y, Tao Y, Ai G, Wei R, Meng X, et al. Proteasome inhibitor lactacystin augments natural killer cell cytotoxicity of myeloma via downregulation of HLA class I. Biochem Biophys Res Commun. (2011) 415:187–92. doi: 10.1016/j.bbrc.2011.10.057
44. Yang G, Gao M, Zhang Y, Kong Y, Gao L, Tao Y, et al. Carfilzomib enhances natural killer cell-mediated lysis of myeloma linked with decreasing expression of HLA class I. Oncotarget (2015) 6:26982–94. doi: 10.18632/oncotarget.4831
45. Shi J, Tricot GJ, Garg TK, Malaviarachchi PA, Szmania SM, Kellum RE, et al. Bortezomib down-regulates the cell-surface expression of HLA class I and enhances natural killer cell-mediated lysis of myeloma. Blood (2008) 111:1309–17. doi: 10.1182/blood-2007-03-078535
46. Ruggeri L, Mancusi A, Capanni M, Urbani E, Carotti A, Aloisi T, et al. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. Blood (2007) 110:433–40. doi: 10.1182/blood-2006-07-038687
47. Ruggeri L. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science (2002) 295:2097–100. doi: 10.1126/science.1068440
48. Björklund AT, Schaffer M, Fauriat C, Ringdén O, Remberger M, Hammarstedt C, et al. NK cells expressing inhibitory KIR for non-self-ligands remain tolerant in HLA-matched sibling stem cell transplantation. Blood (2010) 115:2686–94. doi: 10.1182/blood-2009-07-229740
49. Haas P, Loiseau P, Tamouza R, Cayuela JM, Moins-Teisserenc H, Busson M, et al. NK-cell education is shaped by donor HLA genotype after unrelated allogeneic hematopoietic stem cell transplantation. Blood (2011) 117:1021–9. doi: 10.1182/blood-2010-02-269381
50. Kröger N, Shaw B, Iacobelli S, Zabelina T, Peggs K, Shimoni A, et al. Comparison between antithymocyte globulin and alemtuzumab and the possible impact of KIR-ligand mismatch after dose-reduced conditioning and unrelated stem cell transplantation in patients with multiple myeloma. Br J Haematol. (2005) 129:631–43. doi: 10.1111/j.1365-2141.2005.05513.x
51. Russo A, Oliveira G, Berglund S, Greco R, Gambacorta V, Cieri N, et al. NK cell recovery after haploidentical HSCT with posttransplant cyclophosphamide: dynamics and clinical implications. Blood (2018) 131:247–62. doi: 10.1182/blood-2017-05-780668
52. Parmesar K, Raj K. Haploidentical stem cell transplantation in adult haematological malignancies. Adv Hematol. (2016) 2016:3905907. doi: 10.1155/2016/3905907
53. Ruggeri L, Mancusi A, Urbani E, Velardi A. Identifying NK alloreactive donors for haploidentical hematopoietic stem cell transplantation. In: Bondanza A, Casucci M, editors. Tumor Immunology. Methods in Molecular Biology. New York, NY: Humana Press. p. 141–145. doi: 10.1007/978-1-4939-3338-9_14
54. Miller JS, Cooley S, Parham P, Farag SS, Verneris MR, McQueen KL, et al. Missing KIR ligands are associated with less relapse and increased graft-versus-host disease. (GVHD) following unrelated donor allogeneic HCT. Blood (2007) 109:5058–61. doi: 10.1182/blood-2007-01-065383
55. Alicata C, Pende D, Meazza R, Canevali P, Loiacono F, Bertaina A, et al. Hematopoietic stem cell transplantation: Improving alloreactive Bw4 donor selection by genotyping codon 86 of KIR3DL1/S1. Eur J Immunol. (2016) 46:1511–7. doi: 10.1002/eji.201546236
56. Ilander M, Olsson-Strömberg U, Schlums H, Guilhot J, Brück O, Lähteenmäki H, et al. Increased proportion of mature NK cells is associated with successful imatinib discontinuation in chronic myeloid leukemia. Leukemia (2017) 31:1108–16. doi: 10.1038/leu.2016.360
57. Balsamo M, Vermi W, Parodi M, Pietra G, Manzini C, Queirolo P, et al. Melanoma cells become resistant to NK-cell-mediated killing when exposed to NK-cell numbers compatible with NK-cell infiltration in the tumor. Eur J Immunol. (2012) 42:1833–42. doi: 10.1002/eji.201142179
58. Suck G, Koh MBC. Emerging natural killer cell immunotherapies: Large-scale ex vivo production of highly potent anticancer effectors. Hematol Oncol Stem Cell Ther. (2010) 3:135–42. doi: 10.1016/S1658-3876(10)50024-4

59. Koehl U, Kalberer C, Spanholtz J, Lee DA, Miller JS, Cooley S, et al. Advances in clinical NK cell studies: Donor selection, manufacturing and quality control. Oncoimmunology (2016) 5:1–11. doi: 10.1080/2162402X.2015.1115178
60. Szmania S, Lapteva N, Garg T, Greenway A, Lingo J, Nair B, et al. Ex vivo–expanded natural killer cells demonstrate robust proliferation in vivo in high-risk relapsed multiple myeloma patients. J Immunother. (2015) 38:24–36. doi: 10.1097/CJI.0000000000000059
61. Shah N, Li L, McCarty J, Kaur I, Yvon E, Shaim H, et al. Phase I study of cord blood-derived natural killer cells combined with autologous stem cell transplantation in multiple myeloma. Br J Haematol. (2017) 177:457–66. doi: 10.1111/bjh.14570
62. Gao M, Gao L, Yang G, Tao Y, Hou J, Xu H, et al. Myeloma cells resistance to NK cell lysis mainly involves an HLA class I-dependent mechanism. Acta Biochim Biophys Sin. (2014) 46:597–604. doi: 10.1093/abbs/gmu041
63. Mahaweni NM, Bos GMJ, Mitsiades CS, Tilanus MGJ, Wieten L. Daratumumab augments alloreactive natural killer cell cytotoxicity towards CD38+ multiple myeloma cell lines in a biochemical context mimicking tumour microenvironment conditions. Cancer Immunol Immunother. (2018) 67:861–72. doi: 10.1007/s00262-018-2140-1
64. Mahaweni NM, Ehlers FA, Sarkar S, Janssen JW, Tilanus MG, Bos GM, et al. NKG2A expression is not per se detrimental for the anti-multiple myeloma activity of activated natural killer cells in an in vitro system mimicking the tumor microenvironment. Front Immunol. (2018) 9:1415. doi: 10.3389/fimmu.2018.01415
65. Shi J, Tricot G, Szmania S, Rosen N, Garg TK, Malaviarachchi PA, et al. Infusion of haplo-identical killer immunoglobulin-like receptor ligand mismatched NK cells for relapsed myeloma in the setting of autologous stem cell transplantation. Br J Haematol. (2008) 143:641–53. doi: 10.1111/j.1365-2141.2008.07340.x
66. Romagne F, Andre P, Spee P, Zahn S, Anfossi N, Gauthier L, et al. Preclinical characterization of 1-7F9, a novel human anti-KIR receptor therapeutic antibody that augments natural killer-mediated killing of tumor cells. Blood (2009) 114:2667–77. doi: 10.1182/blood-2009-02-206532
67. Kohrt HE, Thielens A, Marabelle A, Sagiv-Barfi I, Sola C, Chanuc F, et al. Anti-KIR antibody enhancement of anti-lymphoma activity of natural killer cells as monotherapy and in combination with anti-CD20 antibodies. Blood (2014) 123:678–86. doi: 10.1182/Blood-2013-08-519199
68. Korde N, Carlsten M, Lee M-J, Minter A, Tan E, Kwok M, et al. A phase II trial of pan-KIR2D blockade with IPH2101 in smoldering multiple myeloma. Haematologica (2014) 99:e81–3. doi: 10.3324/haematol.2013.103085
69. Carlsten M, Korde N, Kotecha R, Reger R, Bor S, Kazandjian D, et al. Checkpoint inhibition of KIR2D with the monoclonal antibody IPH2101 induces contraction and hyporesponsiveness of NK cells in patients with myeloma. Clin Cancer Res. (2016) 22:5211–22. doi: 10.1158/1078-0432.CCR-16-1108
70. Benson DM, Cohen AD, Jagannath S, Munshi NC, Spitzer G, Hofmeister CC, et al. A phase I trial of the anti-KIR antibody IPH2101 and lenalidomide in patients with relapsed/refractory multiple myeloma. Clin Cancer Res. (2015) 21:4055–61. doi: 10.1158/1078-0432.CCR-15-0304
71. Nijhof IS, Van Bueren JJL, Van Kessel B, Andre P, Morel Y, Lokhorst HM, et al. Daratumumab-Mediated lysis of primary multiple myeloma cells is enhanced in combination with the human Anti-KIR antibody IPH2102 and lenalidomide. Haematologica (2015) 100:263–8. doi: 10.3324/haematol.2014.117531
72. McWilliams EM, Mele JM, Cheney C, Timmerman EA, Fiazuddin F, Strattan EJ, et al. Therapeutic CD94/NKG2A blockade improves natural killer cell dysfunction in chronic lymphocytic leukemia. Oncoimmunology (2016) 5:1–9. doi: 10.1080/2162402X.2016.1226720
73. Nguyen S, Dhedin N, Vernant JP, Kuentz M, Al Jijakli A, Rouas-Freiss N, et al. NK-cell reconstitution after haploidentical hematopoietic stem-cell transplantations: immaturity of NK cells and inhibitory effect of NKG2A override GvL effect. Blood (2005) 105:4135–42. doi: 10.1182/blood-2004-10-4113
74. Rizzieri DA, Koh LP, Long GD, Gasparetto C, Sullivan KM, Horwitz M, et al. Partially matched, nonmyeloablative allogeneic transplantation: clinical outcomes and immune reconstitution. J Clin Oncol. (2007) 25:690–7. doi: 10.1200/JCO.2006.07.0953
75. Chang YJ, Zhao XY, Huang XJ. Immune reconstitution after haploidentical hematopoietic stem cell transplantation. Biol Blood Marrow Transplant. (2014) 20:440–9. doi: 10.1016/j.bbmt.2013.11.028
76. Martín-Antonio B, Suñe G, Perez-Amill L, Castella M, Urbano-Ispizua A. Natural killer cells: angels and devils for immunotherapy. Int J Mol Sci. (2017) 18:E1868. doi: 10.3390/ijms18091868

77. Passweg JR, Baldomero H, Bader P, Bonini C, Duarte RF, Dufour C, et al. Use of haploidentical stem cell transplantation continues to increase: the 2015 european society for blood and marrow transplant activity survey report. Bone Marrow Transplant. (2017) 52:811–7. doi: 10.1038/bmt.2017.34
78. Ciurea SO, Schafer JR, Bassett R, Denman CJ, Cao K, Willis D, et al. Phase 1 clinical trial using mbIL21 ex vivo – expanded donor-derived NK cells after haploidentical transplantation. Blood (2017) 130:1857–69. doi: 10.1182/blood-2017-05-7856

3

NKG2A expression is not per se detrimental for the anti-multiple myeloma activity of activated natural killer cells in an in vitro system mimicking the tumor microenvironment

Niken M. Mahaweni, Femke A. I. Ehlers, Subhashis Sarkar, Johanna W. H. Janssen, Marcel G. J. Tilanus, Gerard M. J. Bos, and Lotte Wieten

Frontiers in Immunology. 2018 Jun 22;9:1415.
doi: 10.3389/fimmu.2018.01415

Abstract
Natural killer (NK) cell-based immunotherapy is a promising therapy for cancer patients. Inhibitory killer immunoglobulin-like receptors (KIRs) and NKG2A are required for NK cell licensing, but can also inhibit NK cell effector function. Upon reconstitution in a stem cell transplantation setting or after ex vivo NK expansion with IL-2, NKG2A is expressed on a large percentage of NK cells. Since the functional consequences of NKG2A co-expression for activated NK cells are not well known, we compared NKG2A+ vs NKG2A− NK cell subsets in response to K562 cells, multiple myeloma (MM) cell lines and primary MM cells. NK cells were isolated from healthy donors (HLAC1+ C2+ Bw4+) and activated overnight with 1,000 U/ml IL-2. NK cell degranulation in subsets expressing KIRs and/or NKG2A was assessed at 21 or 0.6% O_2. Activated NKG2A+ NK cell subsets degranulated more vigorously than NKG2A− subsets both at 21 and 0.6% O_2. This was irrespective of the presence of KIR and occurred in response to HLA-deficient K562 cells as well as HLA competent, lowly expressing HLA-E MM cell lines. In response to primary MM cells, no inhibitory effects of NKG2A were observed, and NKG2A blockade did not enhance degranulation of NKG2A+ subsets. KIR− NK cells expressing NKG2A degranulated less than their NKG2A− counterparts in response to MM cells having high levels of peptide-induced membrane HLA-E, suggesting that high surface HLA-E levels are required for NKG2A to inhibit activated NK cells. Addition of daratumumab, an anti-CD38 to trigger antibody-dependent cell-mediated cytotoxicity, improved the anti-MM response for all subsets and degranulation of the KIR−NKG2A− "unlicensed" subset was comparable to KIR+ or NKG2A+ licensed subsets. This demonstrates that with potent activation, all subsets can contribute to tumor clearance. Additionally, subsets expressing KIRs mismatched with the HLA ligands on the target cell had the highest level of activation in response to MM cell lines as well as against primary MM. Our current study demonstrated that if NK cells are sufficiently activated, e.g., *via* cytokine or antibody activation, the (co-)expression of NKG2A receptor may not necessarily be a disadvantage for NK cell-based therapy.

Introduction

Natural killer (NK) cell-based immunotherapy is an attractive novel therapy against cancer owing to its target selectivity and killing potential (1). NK cells are armed with both activating and inhibitory receptors, and their activation is dependent on the balance between activating and inhibitory signals. The major inhibitory receptors, killer immunoglobulin-like receptors (KIRs) and the NKG2A receptor, provide NK cells with inhibitory signals and are involved in the education process of an NK cell (2, 3). This NK cell education process, also known as licensing, plays a pivotal role in shaping the NK cell ability to kill a target cell. Previous studies on murine NK cells have demonstrated that the number of inhibitory receptors for self-major histocompatibility complex (MHC) expressed on NK cells is proportionate to the strength of NK cell responsiveness against a target cell (4, 5). A more recent study has shown that this is also relevant for human NK cells (6). Moreover, they observed that NKG2A has a stronger licensing impact compared to the KIRs without a significant difference between KIR2DL2/3, KIR2DL1, and KIR3DL1. In the context of the NK cell response against tumor cells, inhibitory receptors have a dual role: on the one hand, having more inhibitory receptors, and thus better licensed and potentially more potent NK cells, could be advantageous for the NK cell response against MHC/HLA-class I-deficient tumor cells. On the other hand, a licensed NK cell could be inhibited when binding to its cognate ligand expressed on an MHC/HLA-class I competent tumor cell unless an excessive amount of activating signals is present (7). To reduce the inhibitory effects mediated by KIRs, donor-derived, alloreactive, KIR–ligand mismatched NK cells have been proposed as one of the solutions to achieve a better response against tumor cells. Such donor NK cells would namely be fully licensed, albeit, their anti-tumor reactivity would not be hampered due to the genetic incompatibility between donor KIR and patient HLA ligands (8, 9). In contrast to the KIRs, mismatching for NKG2A and its HLA-E ligand is not possible due to the limited polymorphism of HLA-E. NKG2A can, however, be an important inhibitory receptor for NK cells as it has been shown that NKG2A can inhibit anti-tumor reactivity of NKG2A+ NK cells and NKG2A blocking antibodies could improve the anti-tumor response (10). Moreover, NKG2A is expressed on a large fraction of the NK cells (20–80%) (11, 12), and this percentage is even higher on reconstituting relatively immature NK cells upon allogeneic stem cell transplantation (13). Also, NKG2A has been shown to be overexpressed on NK cells isolated from chronic lymphocytic leukemia patients (14). Our group aims to develop NK cell-based immunotherapy for multiple myeloma (MM), a hematological malignancy characterized by the growth of malignant plasma cells in the bone marrow for which curative treatment options are currently lacking. We previously reported that both primary MM and MM cell lines express HLA-ABC and HLA-E (15). Additionally, we demonstrated that NKG2A negative KIR–ligand mismatched NK cells were more effective against HLA class I competent MM cell lines compared to NKG2A negative KIR–ligand matched NK cells (15), also under a more suppressive tumor microenvironment (16). Also, we showed that an antibody-dependent cell-mediated

cytotoxicity (ADCC)-triggering antibody, like daratumumab, can enhance the NK anti-MM response and that having a KIR–ligand mismatch can further potentiate the response (16). Although several of the above-mentioned studies illustrate the functional relevance of either KIRs or NKG2A to set the NK cell activation threshold, the effect of co-expression of these receptors on the single NK cell level remains largely unexplored. As NKG2A is (co-)expressed on many NK cells, including KIR positive subsets, we follow up on our previous findings by investigating whether (co-)expression of NKG2A is beneficial, due to enhanced NK cell licensing, or detrimental due to inhibitory interactions with HLA-E for the NK cell anti-MM response. We compare NK subsets with vs without NKG2A in three different settings: 1. in response to HLA-deficient target cells, 2. in response to HLA competent target cells, and 3. in the presence of ADCC-triggering antibodies. As we intend to perform future clinical studies with activated NK cells and the effect of NKG2A co-expression on activated NK cells remains largely elusive, we activated the NK cells throughout the study with IL-2. Additionally, to explore the influence of tumor microenvironment on the process of NK cell activation, we performed the experiments in the presence of ambient air (21%) or low (0.6%) oxygen concentration. This oxygen concentration is selected from previous experiments (17) and relevant for tumor hypoxia setting.

Materials and Methods

Cell Lines and Culture

K562 cell line was cultured in IMDM and 10% fetal calf serum (FCS). UM-9, RPMI8226/s-luc, U266, and RPMI8226/s cell lines were cultured in RPMI1640 and 10% FCS. JJN-3 cell line was cultured in 40% IMDM, 40% low glucose DMEM, and 20% FCS. All cell culture media were supplemented with 100 U/ml penicillin (Gibco) and 100 µg/ml streptomycin (Gibco). K562 and U266 cell lines were purchased from American Type Culture Collection (ATCC, USA). UM-9 and RPMI8226/s-luc cell lines were gifts from Dr. A. Martens, Vrije Universiteit Medisch Centrum, The Netherlands. RPMI8226/s and JJN-3 cell lines were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany). All media were from Gibco, Breda, The Netherlands, and FCS was produced by Greiner Bio-One International, Gmbh. Cell lines were cultured at 37°C in humidified air containing 5% CO_2 with 21% O_2 (Sanyo MCO-20AIC, Sanyo Electric Co., Japan).

Primary MM Cells

Primary MM cells were obtained from the department of cytogenetics as leftover material from a patient subject to a cytogenetic examination. Under the Dutch law on Research Involving Human Subject (http://www.ccmo.nl/en/non-wmo-research), leftover materials from a patient can be used for research and are waived from individual patient's consent. MM cells were purified using CD138 beads positive selection according to manufacturer's instruction (Miltenyi Biotech). The purified

cells were resuspended in RPMI1640 and 10% FCS supplemented with 100 U/ml penicillin and immediately used in degranulation assay.

HLA Genotyping, NK Cell Donor Selection, and Analysis of KIR–Ligand Matched/ Mismatched Status

The genotypic expression of HLA epitopes relevant for KIR2DL1 (HLA group C2); KIR2DL2/3 (HLA group C1); or KIR3DL1 (HLA-Bw4 and HLA-A*23, -A*24, -A*32) in cell lines and healthy blood donors was determined using Luminex® sequence-specific oligonucleotides analysis (One Lambda). Based on the genotyping result: UM9, U266, and JJN-3 were HLA-C1+C2−Bw4− and RPMI8226/s was HLA-C1+C2+Bw4−. KIR–ligand matched NK cells for UM9, U266, and JJN-3 were, therefore, KIR2DL2/3 positive. KIR–ligand mismatched NK cells for UM9, U266, and JJN-3 were KIR2DL1 positive and/or KIR3DL1 positive. For RPMI8226/s, KIR–ligand matched NK cells were KIR2DL2/3 and/or KIR2DL1 positive. KIR–ligand mismatched NK cells for RPMI8226/s were KIR3DL1 positive. NK cell donors were healthy volunteers or buffy coats with genotype HLA-C1+C2+Bw4+ and phenotypically expressing KIR2DL1, KIR2DL2/3, and KIR3DL1. All donors signed informed consent forms. The use of buffy coats, being a by-product of a required Medical Ethical Review Committee (METC) procedure, does not need ethical approval in the Netherlands under the Dutch Code for Proper Secondary Use of Human Tissue.

NK Cell Isolation

Natural killer cells were isolated by negative selection of NK cells isolation kit using MACS beads and columns according to manufacturer's protocol (Miltenyi Biotec, GmbH). For all experiments, NK cells were activated overnight with 1,000 IU/ml recombinant human IL-2 (Proleukin, Novartis) in RPMI-1640 medium (Gibco) supplemented with 10% FCS (Greiner Bio-One), 100 U/ml penicillin (Gibco) and 100 µg/ml streptomycin (Gibco) at 37°C in humidified air containing 5% CO_2 with 21% O_2 (Sanyo MCO-20AIC, Sanyo Electric Co., Japan).

CD107a Degranulation Assay

To assess NK cell degranulation, CD107a expression on NK cells was analyzed using flow cytometry-based assay. For this, target cells (tumor cells) were plated in 24-well plate at a concentration of 2 × 106 cells/ml per well and incubated overnight at 37°C in humidified air containing 5% CO_2 with 21% O_2 (Sanyo MCO-20AIC, Sanyo Electric Co., Japan) or 0.6% O_2 (except experiment in **Figure 5**) (Invivo2, 1000 Ruskinn Technology Ltd., Bridgend, UK). Prior to the assay, IL-2-activated NK cells were harvested and washed and when indicated in the experiment, subjected to pre-incubation with 50 mM sodium l-lactate (Sigma) or 100 ng/ml prostaglandin E2 (Sigma) or medium (**Figure 1**; **Figure S1** in Supplementary Material), otherwise NK cells were immediately co-cultured with tumor cells in the assay without pre-incubation. For the NKG2A blocking assay, NK cells were incubated with 1 µg/ml anti-NKG2A antibody (clone: Z199, Beckman Coulter) (**Figure 3C**) or anti-NKG2A-PE-Cy7

(clone: REA110, Miltenyi Biotec) (**Figure 5D**) for 1 h in 37°C in humidified air containing 5% CO_2 with 21% O_2 or 0.6% O_2 when indicated in the figure. For the HLA-E blocking assay, target cells were incubated with 10 µg/ml anti-HLA-E antibody (clone: 3D12HLA-E, eBioscience) (**Figure 5C**; **Figure S7** in Supplementary Material) for 30 min in 37°C in humidified air containing 5% CO_2 with 21% O_2 or 0.6% O_2 when indicated in the figure. For the ADCC assay (**Figure 4**), tumor cells were pre-incubated for 30 min with 1 µg/ml daratumumab or medium at 21% O_2 or 0.6% O_2 before co-cultured with NK cells. NK cells exposed to tumor microenvironmental factors (TMEF) were then, in duplicate wells, co-cultured with the target cells and 2 µl anti-CD107a-Horizon V450 (clone: H4A3, BD) was added per well. After 1 h of co-culture, monensin (BD) was added. After another 3 h, the plate was placed on ice to stop the reaction. Cells were then stained on ice with anti-CD3-APC/H7 (clone: SK7, BD), anti-CD56-PeCy7 (clone: B159, BD), anti-KIR2DL1-APC (clone: 143211, R&D), anti-KIR2DL2/3/S2-PE (clone: DX27, Miltenyi Biotec), anti-KIR3DL1-FITC (clone: DX9, Miltenyi Biotec), and anti-NKG2A-PC5.5 (clone: Z199, Beckman Coulter). To analyze different subsets of NK cell, CD3−CD56+ cells were gated followed by gating of NKG2A− and NKG2A+ population and further gating based on the KIRs expressions.

Induction of HLA-E Expression Using HLA Leader Peptides

U266 cells were incubated with 500 µM of HLA-A1 (VMAPRTLLL), HLA-B7 (VMAPRTVLL), or a non HLA-E binding control peptide (RGPGRAFVTI) (Biosynthesis Inc.) overnight at 37°C, 21% O_2 as previously described (15, 18). Additional negative controls were included by incubating U266 cells in DMSO, the peptide's solvent or in the medium. After the incubation, HLA-E expression was determined by flow cytometry by staining the cells with an HLA-E antibody (clone: 3D12 HLA-E, eBioscience) or with a matched isotype control, mouse IgG1 kappa (clone: P3.6.2.8.1, eBioscience). Following the induction, U266 cells were used in the CD107a assay (**Figure 6**) as described in the previous section.

Flow Cytometry

Cells were washed with PBS (Gibco) and stained first for dead cells using Live/Dead® Fixable Aqua Dead Cell Stain Kit (Molecular Probes™, USA) for 30 min on ice in the dark. Cells were further washed with FACS buffer (PBS, 1% FCS) and stained with antibodies for 30 min on ice in the dark. All flow cytometric analyses were performed with BD FACS Canto II. Data were analyzed with FlowJo 10.1r5 64 bit software.

Statistical Analysis

All statistical analysis was performed with GraphPad Prism V software (Graphpad Software Inc., San Diego, CA, USA) using non-parametric t-test with repeated measure (Wilcoxon signed rank test). * indicates a p-value of <0.05 and ** indicates a p-value of <0.01, and *** indicates a p-value of <0.001.

Results

Expression of the Inhibitory NKG2A Receptor Could Be Advantageous for IL-2-Activated NK Cells Against HLA Negative Tumor Cells

To investigate the effect of NKG2A expression on the anti-tumor response of IL-2-activated NK cells against HLA-class I negative target cells, we performed a flow cytometry-based degranulation (CD107a) assay by co-culturing NK cells and HLA-class I negative K562 cells followed by staining for KIRs and NKG2A to enable NK subset analysis. Under normal laboratory conditions of 21% O_2, a slightly higher percentage of degranulating (CD107a+) NK cells was observed for the subsets expressing NKG2A as compared to NKG2A negative counterparts, and this was observed for both KIR+ (average increase 4.6%) and KIR− (average increase 18%) subsets ($p < 0.05$ and $p < 0.001$, respectively) (**Figure 1A**). As the tumor microenvironment could potentially impair cytolytic effector function of the NK cells, co-cultures were also performed in the presence of biochemical context mimicking tumor microenvironment, i.e., in the presence of 0.6% O_2, or in the combination of hypoxia with 50 mM lactate or 100 ng/ml prostaglandin E2 (PGE2). At 0.6% of O_2, we observed more degranulation in NKG2A-expressing NK cells than for the subsets not expressing KIRs (average increase 16.3%) (**Figure 1B**). However, for KIR+ subsets there was no difference in the percentage of degranulating NK cells with vs without NKG2A (average increase 4.5%) (**Figure 1B**).

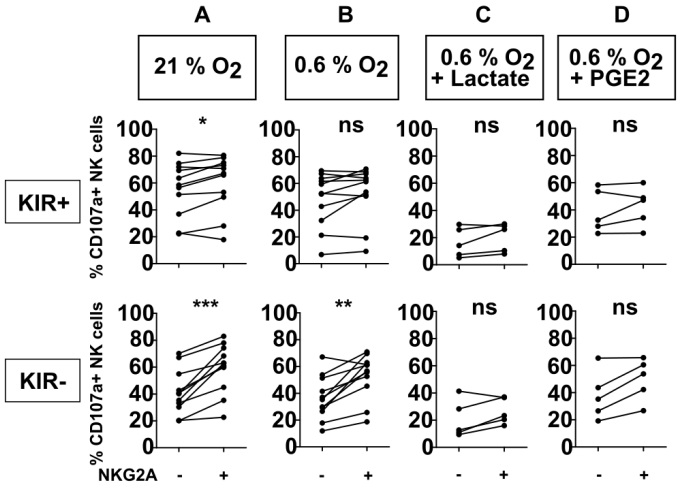


Figure 1. Effect of NKG2A on NK cell degranulation in the presence of different microenvironment factors. NK cells were co-cultured with target cells (K562 cells) in a 1:1 E:T ratio for 4 hours at 21 **(A)** or 0.6 % O_2 **(B)**, or combinations of 0.6 % O_2 and 50 mM lactate **(C)**, or 100 ng/mL PGE2 **(D)**. Flow cytometry was used to subtype NK cells based on their expression of NKG2A and KIRs. The percentage of degranulating NK cells is shown as % CD107a+ NK cells. Each dot represents an average of a technical replicate from an individual NK cell donor. **(A)** and **(B)** n = 11 donors, **(C)** and **(D)** n = 5 donors tested in independent experiments (ns = not significant, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$).

In the conditions where 0.6% O_2 was combined with PGE2 (average increase 11.8% for KIR− and 3.7 for KIR+) or lactate (average increase 6.1% for KIR− and 4.2% for KIR+), however, this did not reach significance (**Figures 1C,D**). In the absence of target cells, the percentage of NK cell degranulation was very low (**Figure S1** in Supplementary Material). Nonetheless, we also observed a similar pattern as in conditions with target cells with slightly higher percentages of degranulating NKG2A positive NK cell subsets. Of note, in none of the donors, NKG2A expression levels by the NK cells were clearly influenced by 4 h co-culture of NK cells and K562 in the presence of hypoxia, lactate, or PGE2 (**Figure S2** in Supplementary Material). Altogether, these results suggest that against an HLA negative tumor cell line, the presence of the NKG2A receptor, especially on KIR− subsets could be beneficial for IL-2-activated NK cells also in the presence of more suppressive microenvironmental factors, presumably because these NK cells were better licensed.

NKG2A Does Not Inhibit the Response of IL-2-Activated NK Cells Against Myeloma Cell Lines Expressing Low Levels of HLA-E

The interaction between the NKG2A receptor and its ligand, HLA-E, can have an inhibitory effect on the NK cell anti-tumor capacity and could outweigh the beneficial effect of improved licensing. Therefore, we investigated the effect of NKG2A on IL-2-activated NK cell degranulation in response to three MM cell lines (UM9, RPMI8226/s, and JJN-3) expressing both HLA-class I and HLA-E (**Figure S3** in Supplementary Material). Based on the HLA genotypes for classical class I of the cell lines, NK cells were divided into subsets expressing: 1) no KIRs, 2) KIRs that are KIR–ligand matched, or 3) KIRs that are mismatched for the HLA ligands on the target cells. We subsequently compared the response of NK cells (co-)expressing NKG2A vs NK cells lacking NKG2A for each of the three groups. In the absence of target cells, the percentage of degranulating NK cells was negligible (**Figure S4** in Supplementary Material). We observed that for the matched and the KIR negative subsets, the percentage of degranulating NKG2A positive cells was slightly higher than degranulation of the subsets lacking NKG2A in most donors. This difference reached significance when NK cells were co-cultured with RPMI8226/s both in the presence of 21% O_2 or 0.6% O_2 (**Figure 2**). For NK cells expressing mismatched KIRs, we did not observe a difference between NKG2A+ vs NKG2A− cells against all cell lines. Although the three cell lines tested in this study expressed HLA-E, albeit at low levels (**Figure S3** in Supplementary Material), in only 4 out of 45 samples we observed a lower percentage of degranulating NK cells in NK subsets expressing NKG2A (NKG2A+ matched; NKG2A+ mismatched; or NKG2A+ KIR−) as compared to their counterparts without NKG2A (NKG2A− matched; NKG2A− mismatched; or NKG2A− KIR−) and these were all in the group of NK cells expressing mismatched KIRs. These data demonstrated that the presence of NKG2A on NK cells did not seem to have an inhibitory effect on the response of IL-2-activated NK cells against HLA-class I competent cell lines expressing low levels of HLA-E. Moreover, for the subsets expressing no- or matched KIRs, the NKG2A positive cells performed even slightly better than their NKG2A negative counterparts.

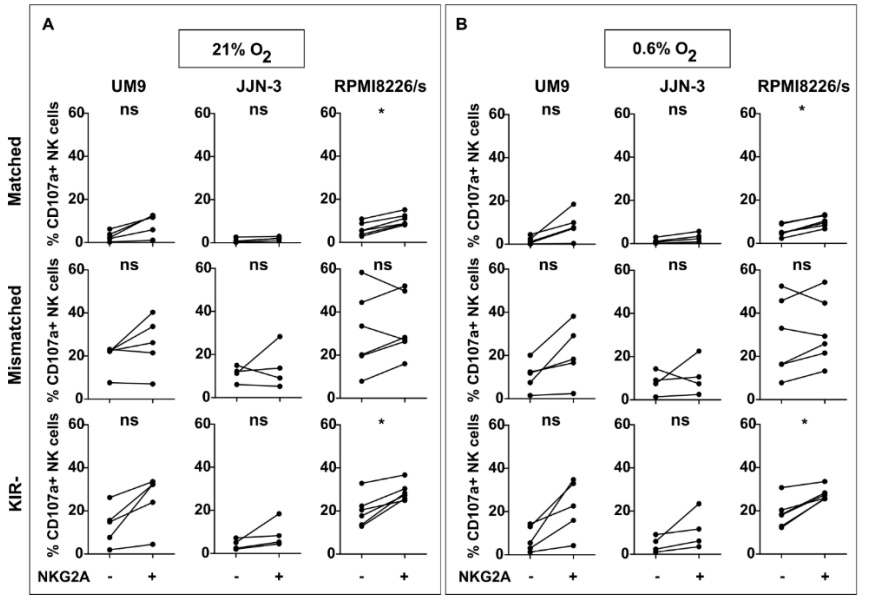


Figure 2. NKG2A and KIR subset analysis per cell line. NK cells were co-cultured with UM9, RPMI8226/s or JJN-3 cells in a 1:1 E:T ratio for 4 hours in 21 % O_2 **(A)** or 0.6% O_2 **(B)**. Flow cytometry was used to subtype NK cells based on their expression of NKG2A and KIRs. Degranulating NK cells were denoted as CD107a+ NK cells. Each dot represents an average of a technical replicate. n = 5 independent experiments with 5 different donors (UM9), 4 independent experiments with 4 different donors (JJN-3) and 6 independent experiments with 6 different donors (RPMI8226/s). (ns = not significant, * = $p<0.05$)

To further investigate the functional relevance of NKG2A, we incubated IL-2-activated NK cells with primary MM cells known to express relatively high levels of both the classical HLA-class I and nonclassical HLA-class I (HLA-E) molecules (15). This revealed that the NK cells were highly activated by K562 cell line used as positive control, but not by the primary MM cells or in the absence of target cells (**Figure 3A**). For both KIR positive and KIR negative subsets, we did not observe any difference in NK cell degranulation between NKG2A expressing vs non-expressing NK cells both in the presence of primary MM cells (**Figure 3B**) or in the absence of primary MM cells (**Figure S5** in Supplementary Material). As the level of degranulation in response to primary MM cells was very low and this could have blunted analysis of inhibitory effects by NKG2A, we blocked the HLA-E–NKG2A interaction with an NKG2A blocking antibody (**Figure 3C**) or with an anti HLA-E antibody (**Figure S6** in Supplementary Material). To study the effect in more detail, we analyzed the effect of blocking on different subsets of NK cells. However, because the blocking NKG2A antibody has the same epitope with the fluorochrome-labeled NKG2A antibody, we could not visualize the NKG2A+ population and, therefore, we subtyped our NK cells into KIR+ and KIR− subtypes. Our results demonstrated that there was no effect of NKG2A

blockade on the KIR+ subset and only a very small effect on the KIR− subset where we saw a small increase of CD107a+ NK cells in 2 out of 9 samples ($p < 0.05$) (**Figure 3C**). In this analysis, all KIRs were matched with the primary MM cells as the patients were C1+, C2+, and Bw4+. In two samples, there was a genetic discrepancy between KIRs and HLA on the primary MM cells enabling us to subgroup NK cells based on the KIR–ligand matched/mismatched status and to investigate whether KIR–ligand interaction played a bigger role than NKG2A–HLA-E interaction in inhibiting NK cells activity. Although the number of patients was not sufficient to perform statistical analysis, this suggested that the NKG2A− KIR–ligand mismatched cells degranulated more than the NKG2A− matched counterpart (**Figure 3D**). NKG2A+ KIR–ligand mismatched NK cells, however, were equally activated in one patient and more activated in one patient compared to the matched subset.

Altogether, the data from MM cell lines and primary MM cells demonstrated that NKG2A did not seem to play a major inhibitory role in the anti-MM response of high dose IL-2-activated NK cells.

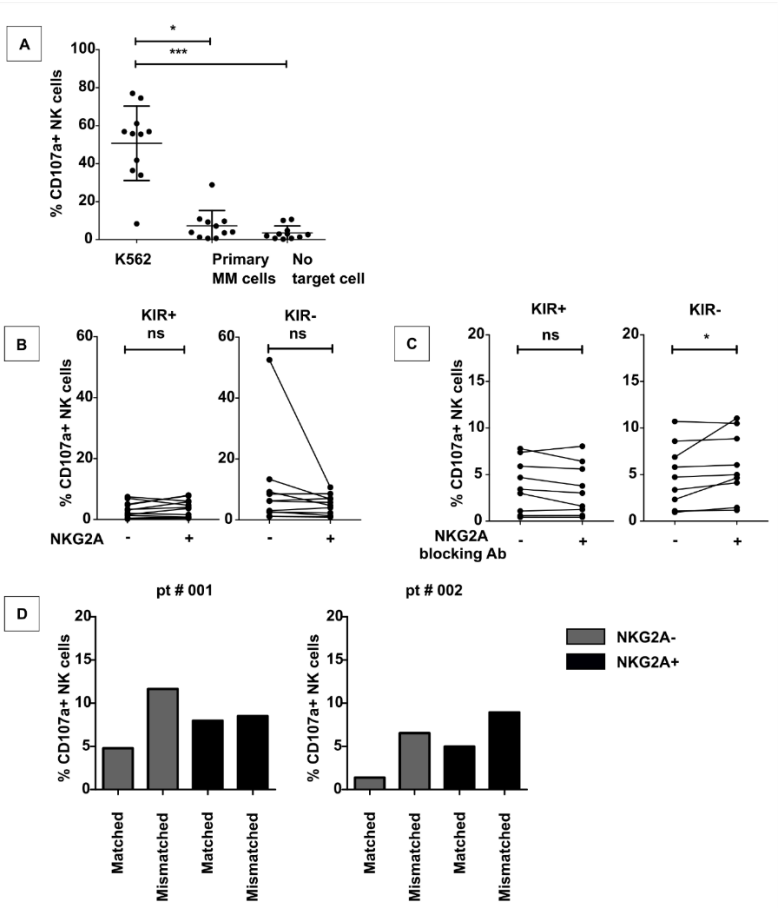


Figure 3. Effect of NKG2A expression on NK cells on NK cell activation against MM cell lines and primary MM cells. IL-2 activated NK cells were co-cultured with K562 cell line **(A)**, primary MM **(A, B, C, D)** or without target cells **(A)** for 4 hours in a degranulation assay with or without an NKG2A blocking antibody **(C)**. Flow cytometric analysis was used to subtype NK cells based on their expression of NKG2A and KIRs. Degranulating NK cells were denoted as CD107a+ NK cells. Each dot represents an average of a technical replicate. (A-C) n = 10 independent experiments with samples from 10 different MM patients as target cells (D) n = 2 different myeloma patients used as target cells (ns= not significant, * = p<0.05, ** = p<0.01, *** = p<0.001)

Daratumumab Triggers ADCC in All NK Cell Subsets Which Is Irrespective of the NKG2A Status

Myeloma-specific monoclonal antibodies that trigger NK cell-mediated ADCC are a potent way to boost the NK antitumor response, and also in this setting, we studied the role of NKG2A in controlling NK activation. To trigger ADCC, UM9, and RPMI8226/s, two MM cell lines that highly expressed CD38 were pre-incubated with

daratumumab followed by a CD107a assay with IL-2-activated NK cells at 21 or 0.6% O_2 and analysis of degranulation of individual NK cell subsets. This revealed that the addition of daratumumab clearly triggered NK cell degranulation for all subsets, at 21% O_2 as well as at 0.6% O_2 (**Figure S7** in Supplementary Material). For UM9, the median fold enhancement ranged from 2.5- to 19-fold at 21% O_2 and 2.0- to 44.5-fold at 0.6% O_2. For RPMI8226/s, the median fold enhancement ranged from 1.9- to 11.4-fold at 21% O_2 and 3.5- to 11.6-fold at 0.6% O_2. Analysis of CD107a+ NK cells per subset subsequently demonstrated that there was no difference in degranulation between subsets expressing NKG2A vs subsets lacking NKG2A (**Figure 4**).

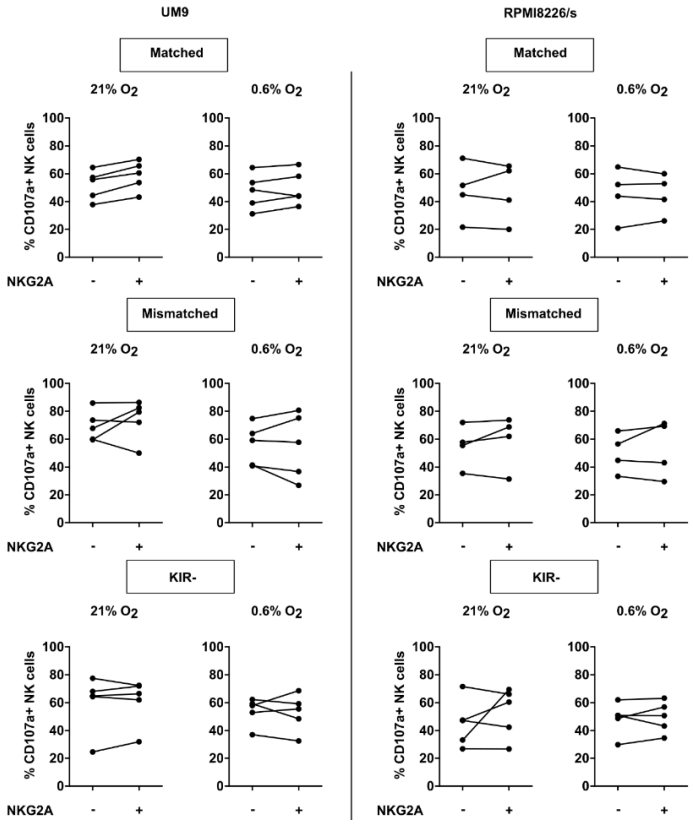


Figure 4. Effect of NKG2A co-expression on daratumumab-induced ADCC. UM9 or RPMI8226/s cells were pre-incubated with daratumumab for 30 minutes before adding IL-2 activated NK cells at a 1:1 E:T ratio. A degranulation assay was performed for 4 hours at 21% or 0.6% O_2. Flow cytometric analysis was used to subtype NK cells based on their expression of NKG2A and KIRs. Degranulating NK cells were denoted as CD107a+ NK cells. Each dot represents an average of a technical per donor. n = 5 independent experiments with 5 different donors and two different cell lines.

Natural killer cells also express CD38 on their surface and previous studies showed that NK cells could kill each other via ADCC triggered by NK cell-associated daratumumab. Therefore, we also compared the response of the NKG2A positive vs negative NK cells for the KIR+ and the KIR− subsets in the absence of tumor target cells. For this, IL-2-activated NK cells were incubated without (**Figure 5A**) or with daratumumab (**Figures 5B–D**) for 4 h followed by analysis of CD107a expression by NK cell subsets at 21% or 0.6% O_2. Without daratumumab, we showed that spontaneous NK cell degranulation was very low for all subsets. For KIR+ NK cells, both at 21% and 0.6% O_2, we observed a lower percentage of degranulating NK cells in subsets co-expressing NKG2A (**Figure 5B**). For KIR− subsets, we only saw this in the condition at 0.6% O_2. To determine whether this was truly due to NKG2A, we blocked HLA-E–NKG2A interaction with an antibody blocking either HLA-E or NKG2A. For all donors and in both the KIR+ and KIR− NK cell subsets, the level of degranulation of NKG2A positive subsets was higher than that of NKG2A negative subsets after blocking, except in one donor under hypoxia in the presence of anti HLA-E, NKGA+, KIR− showed lower percentage of degranulating NK cells (**Figure 5C,D**). This illustrates that NKG2A could inhibit daratumumab-induced fratricide.

As highly activated NK cells express higher levels of HLA-E than the MM cell lines (**Figure S3** in Supplementary Material), we hypothesized that the level of HLA-E might influence the potential of NKG2A to inhibit highly activated NK cells. To explore this, we performed a 4-h degranulation assay using IL-2-activated NK cells from three healthy donors against U266, a MM cell line expressing low levels of HLA-E. Prior to co-culture with NK cells, U266 cells were incubated with either medium, DMSO, control peptide, HLA-A1 peptide, or HLA-B7 leader peptide. The HLA-A1 or B7 peptides are derived from the leader sequence of HLA-class I and have been shown to bind HLA-E and enhance HLA-E surface expression (18). We observed that HLA-E was highly expressed on U266 cells upon peptide incubation, approximately sixfold (HLA-A1 peptide) and eightfold (B7 peptide) higher than the baseline expression (**Figure 6A**). In the absence of target cells (**Figure 6B**), NK cells subsets expressing NKG2A showed a higher degranulation compared to NK cell subsets not expressing NKG2A. For subsets expressing matched KIRs or no KIRs, NKG2A+ NK cells degranulated more than NKG2A− NK cells in the conditions where target cells were incubated without or with control peptide (**Figure 6C**). This was true for all three donors and in line with the data obtained with the other MM cell lines. For the KIR− subset, upregulation of HLA-E resulted in less degranulation in the NKG2A+ NK cells vs the NKG2A− cells, suggesting inhibition by NKG2A. For the matched KIR subset this effect was less pronounced. For the mismatch subset, we saw a lower percentage of degranulating NKG2A+ NK cells vs NKG2A− NK cells in all conditions. This supports the NK cell fratricide data (**Figure 5**) and together illustrates that NKG2A can inhibit high dose IL-2-activated NK cells but whether or not this occurs depends on the exact NK cell subset and presumably also on the type of target cells and the level of HLA-E on the target cells.

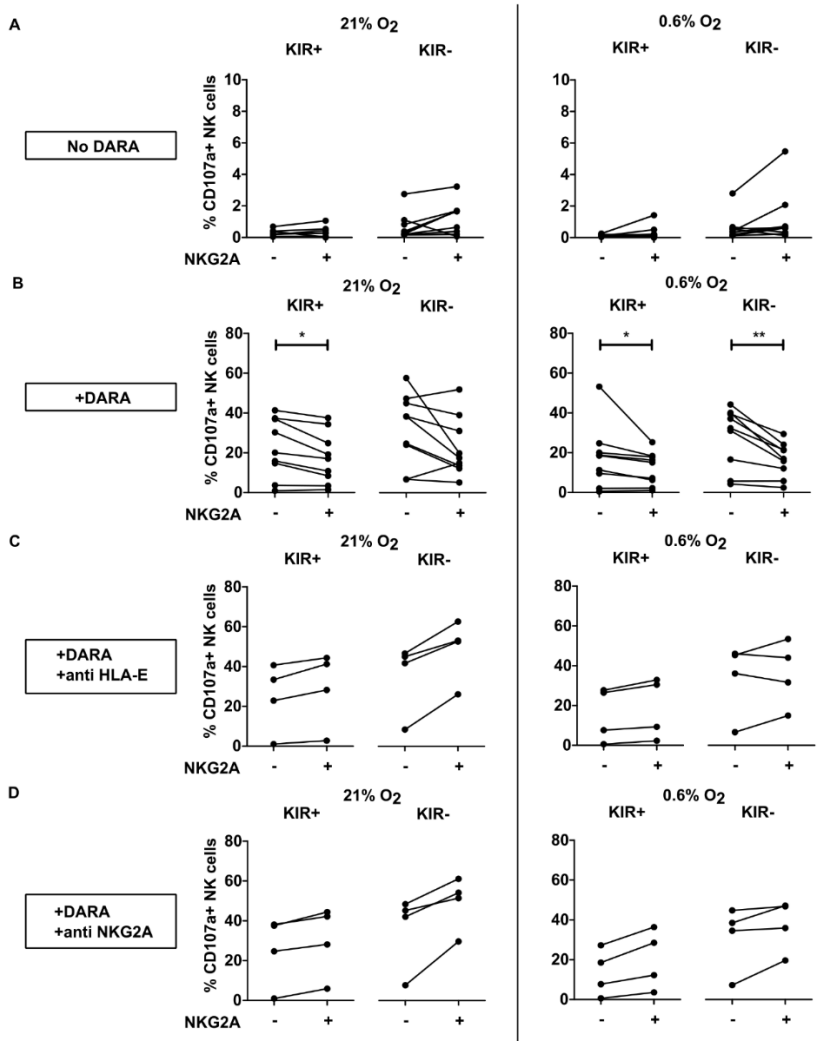


Figure 5. Effect of NKG2A and KIR expression on daratumumab-induced NK cell fratricide. NK cells were incubated at 21% or 0.6 % O_2 for 5 hours in the absence **(A)** or presence of daratumumab (DARA) **(B)**, or DARA and anti HLA-E **(C)** or DARA and anti NKG2A **(D)**. Flow cytometry was used to subtype NK cells based on their expression of NKG2A and KIRs. Degranulating NK cells were denoted as CD107a+ NK cells. Each dot represents the average of a technical replicate. (* = p<0.05, ** = p<0.01). n = 9 different donors (A&B) or 4 donors (C&D).

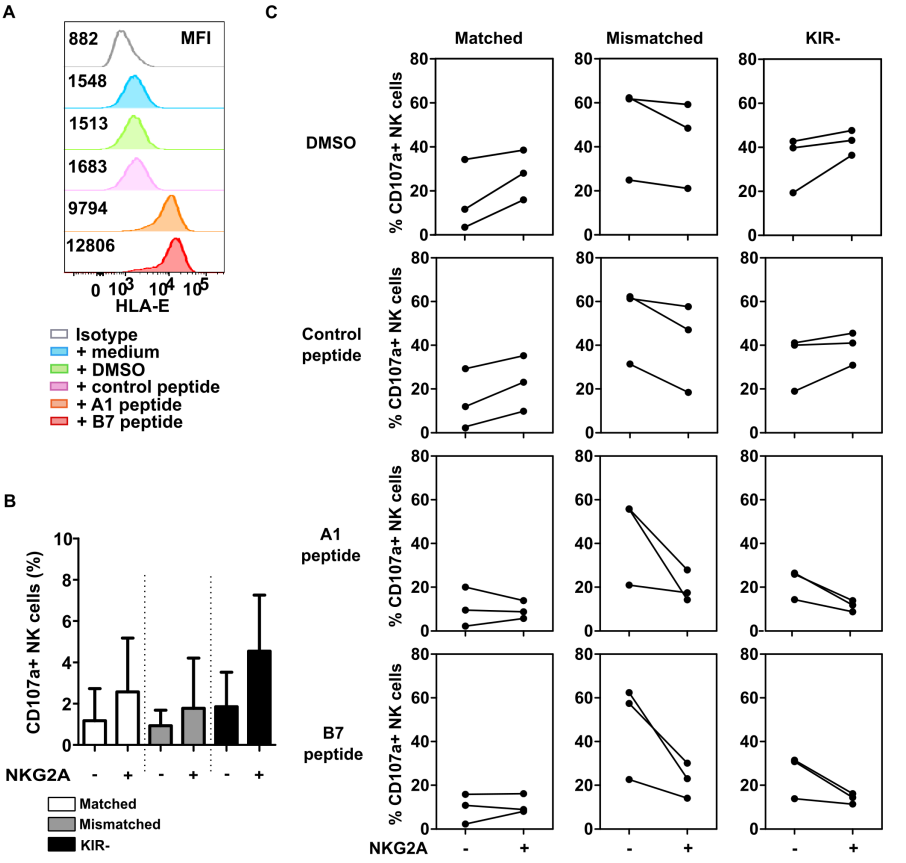


Figure 6. Inhibition via NKG2A is effective when a high level of HLA-E is present. (A) U266 cells were pre-incubated for 2 hours with HLA-B7 peptide, HLA-A1 peptide, DMSO, control peptide (non-HLA-E binding), or medium. HLA-E expression of U266 is depicted in the histogram, with its corresponding median fluorescence intensity (MFI). **(B)** Spontaneous degranulation of IL-2 activated natural killer (NK) cells cultured for 13 h in the absence of target cells. **(C)** Degranulation of NK cells upon 13 h co-culture with peptide- or control-incubated U266 target cells. Degranulating NK cells were denoted as CD107a+ NK cells. Each dot in the graphs represents the average of a technical replicate for an individual donor. Error bars in (B) indicate standard deviation. n = 3 different NK cell donors.

Discussion

We envision that the ideal NK cell product for cancer treatment would be a large number of highly activated NK cells which can withstand the suppressive tumor microenvironment. Therefore, to refine NK cell-based immunotherapy, we focus our investigations on NK cell ex vivo expansion and strategies to enhance activation and to reduce inhibition of NK cells. Since the IL-2-activated NK cell ex vivo expansion protocols could result in a higher percentage of NKG2A expressing NK cells (19, 20), we performed, in the current study, an in-depth analysis of the influence of NKG2A expression on different NK cell subsets in response to MM cells showing that the inhibitory potential of NKG2A, for activated NK cells, depends on the exact subset of NK cells and the HLA-E context of the target cell.

NKG2A has a dual function in NK cells, on the one hand, it is required for NK cell licensing, but it also acts as an inhibitory receptor to control the activation threshold of the NK cell and to avoid autoimmunity (3). Here, we show that high dose IL-2-activated NK cells expressing NKG2A degranulated more vigorously than subsets not expressing NKG2A. We observed this irrespective of the presence of KIR and in response to HLA-deficient K562 leukemia cells and to a lesser extent against HLA competent MM cell lines. For K562, this is in line with previous studies in both mice (4, 5) and human (6). These studies showed that for unactivated NK cells, the more inhibitory receptors an NK cell expresses, the better the NK cell is licensed, and the more potently it can respond to HLA-class I deficient tumors. However, for HLA competent tumors, the presence of NKG2A could be a disadvantage due to the inhibitory signaling resulting from the NKG2A–HLA-E interaction. Although some tumor cells downregulate surface expression of HLA-class I molecules, tumors can also maintain or even enhance HLA-class I (21, 22). We and others previously demonstrated that MM cell lines and primary MM cells express HLA-class I and HLA-E on their surface (15, 23). Nevertheless, in the present study, NKG2A expression on high dose IL-2-activated NK cells did not result in a reduced activation. On the contrary, the presence of NKG2A seemed to be more advantageous for the NK cell response, especially against MM cell lines. Although NKG2A expressing vs non-expressing subsets might differ in more features than only NKG2A, our data suggest that for this high dose IL-2-activated NK cells the benefit of better licensing due to NKG2A seemed stronger than the inhibitory effects provided by this receptor.

Even in response to primary MM cells, despite relatively high HLA-E levels, no inhibitory effects of NKG2A were observed. As this could have been caused by the very low level of NK cell degranulation, we also blocked the NKG2A receptor using monoclonal antibodies recognized for their capacity to block HLA-E or NKG2A. The effect of blocking was very minor and only present in KIR+ subsets which was in contrast to a previous study where Monalizumab, a clinical grade NKG2A blocking antibody, improved the cytotoxicity of low dose (250 U/ml) activated KIR− NKG2A+ NK cells against a variety of primary tumor cells (10). One of the differences with our study was that Ruggeri et al. used NKG2A+ KIR− NK cell clones, while we used a

heterogeneous population of NK cells. The percentages of NKG2A+ KIR− NK cells among our donors varied between 11.6 and 51.8% of total NK cell populations (**Table S1** in Supplementary Material) which might have influenced the blocking capacity of the antibody. In our previous study (15), also using whole NK cells but non-activated, we showed that there was an increased percentage of overall CD107a+ NK cells when we blocked HLA-E/NKG2A interaction using an HLA-E antibody. Therefore, another reason could be the difference in activation and or licensing status of the NK cells as we used in the current study healthy donor-derived NK cells pre-activated with a high dose of IL-2 (1,000 U/ml), while Ruggeri et al. used only 250 U/ml for activation. This suggests that the activation status of the NK cells is important for whether or not NKG2A can mediate strong inhibitory effects on NK cells. Importantly, this also suggests that if NK cells are sufficiently activated, e.g., via cytokine activation, the co-expression of NKG2A is not per se detrimental.

The potency of NKG2A to inhibit NK cells can also be influenced by the HLA-E expression level on the target cell. Previously, inhibition via KIR has been shown to have a linear relation with HLA-class I, meaning that the more of the ligand is expressed the more inhibition is mediated via the receptor (24). For NKG2A this seems different as the same group also showed that inhibition by NKG2A occurs only when HLA-E levels are above a certain threshold and the strength of the inhibitory signal could not be further amplified by increasing expression levels of the HLA-E ligand (24). Although the number of individuals was limited, in our experiments, peptide-induced HLA-E expression made NKG2A+ KIR− less responsive than their NKG2A− KIR+ counterparts. In line with this, blockade of the NKG2A co-receptor CD94 has been shown to enhance the response of highly activated NKG2A+ NK cells against a cell line transgenically expressing very high levels of HLA-E but not against primary ALL cells expressing an intermediate level of HLA-E (25). In addition, we observed that NKG2A positive NK cells, expressing high levels of HLA-E, mediated less daratumumab-induced fratricide than NKG2A negative NK cells which could be reversed by adding anti-HLA-E or anti-NKG2A. Highly activated T cells express increased levels of HLA-E and this protects them from killing by NKG2A positive NK cells (26). We now show that this is also true for highly activated NK cells. Furthermore, we show that NKG2A has the potential to inhibit highly activated NK cells but that this depends on the exact setting and that activated NK cells may have a different threshold for HLA-E than unactivated NK cells.

Another important point to take into account in the design of NK cell immunotherapy is that NK cells will have to function in a suppressive tumor microenvironment. TMEF, such as hypoxia, lactate, prostaglandin E2, and others have been shown to dampen NK cell anti-tumor responses through several mechanisms summarized in Ref. (27). We, therefore, evaluated the role of NKG2A also in the presence of factors from the TME but did not see very obvious differences with the data obtained under normal control conditions. In addition, we did not observe changes in NKG2A expression on

the NK cells, possibly because the 4 h incubation was relatively short to induce changes on IL-2-activated NK cells by hypoxia. We realize that our in vitro set up does not fully reflect the complexity of the in vivo TME, and several other studies in human or mice did show that the TMEF could lead to NK cell phenotypic change (28–30). Furthermore, we demonstrated in an earlier study that HLA-E levels on MM tumor cells are increased upon in vivo growth in the BM of immunodeficient mice as compared to in vitro passaged cells (15). Moreover, a recent paper has shown that under hypoxia and glucose deprivation, HLA-E can be upregulated in both human and mouse tumor cells as a result of microenvironmental stress (31). It would, therefore, be valuable to determine the effect of NKG2A on high dose IL-2-activated NK cells in an in vivo MM model or after longer exposure of hypoxia and or other TMEF.

Importantly, our data show that, for high dose IL-2-activated NK cells, all subsets can get activated by tumor cells in the context of laboratory setting mimicking tumor microenvironment. This is also true for the presumed “hyporesponsive” subset not expressing any licensing inhibitory receptor. Moreover, the addition of daratumumab, to trigger ADCC, even improved the response of this hyporesponsive subset to a level comparable to that of subsets expressing NKG2A or KIR. This is important as it illustrates that with potent activation all subsets could contribute to tumor clearance. Nevertheless, under all conditions, subsets expressing KIRs that were mismatched with the HLA ligands on the target cell had the highest level of activation, both in response to the MM cell lines as well as in response to primary MM. This emphasizes the relevance to select KIR–ligand mismatched NK cell donors or to use a KIR blocking antibody like lirilumumab. For NKG2A, selection of mismatched donors is not a feasible strategy, but, the interaction between NKG2A and HLA-E can be blocked with a clinically available monoclonal antibody (monalizumab). However, in case of an expanded NK cell product, where NK cells received a cocktail of cytokines, such as IL-2 or IL-15 providing strong activation signals, or in the situation where an ADCC-triggering antibody is used, this may not be very useful as these NK cells may be not severely inhibited by NKG2A. Additionally, blockade could even be detrimental. Therefore, a better understanding of the conditions leading to HLA-E expression in relation to the inhibitory effects via NKG2A, would be useful to predict for which patients blockade of NKG2A with monalizumab would be beneficial.

Ethics Statement

Blood from healthy volunteers was obtained under a general agreement with the university hospital for blood withdrawal from healthy volunteers used for reference values and not specified per study. All participants gave written informed consent. Primary MM cells were obtained from the department of cytogenetics as a leftover material from a patient subject to a cytogenetic examination. Under the Dutch law on Research Involving Human Subject, leftover materials from a patient can be used for research and are waived from individual patient’s consent. The use of buffy coats,

being a by-product of a required Medical Ethical Review Committee (METC) procedure, does not need ethical approval in the Netherlands under the Dutch Code for Proper Secondary Use of Human Tissue.

Author Contributions
All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of Interest Statement
GB is Chief Executive Officer/Chief Medical Officer/Co-founder of CiMaas, BV, Maastricht, The Netherlands. CiMaas is producing an ex vivo expanded NK cell product that will be used to treat myeloma patients. The other authors declare no conflict of interest.

Acknowledgments
The authors would like to thank Cytogenetic laboratory, MUMC+, Maastricht, The Netherlands for processing and providing the primary MM samples and Transplantation Immunology-Tissue Typing laboratory, MUMC+, Maastricht, The Netherlands for performing the HLA genotyping for the samples.

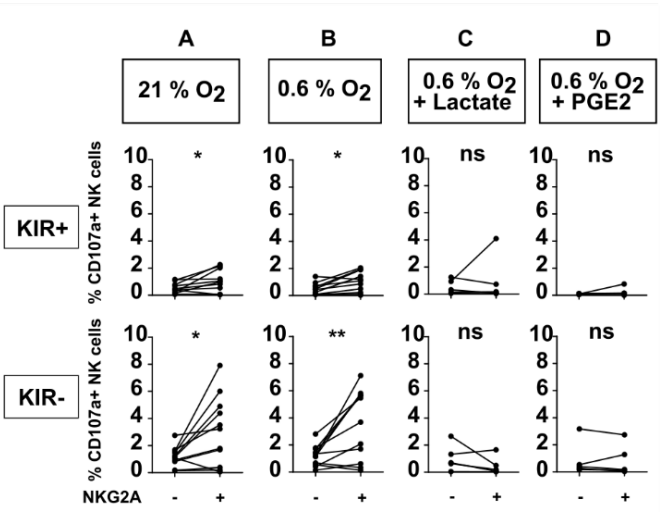
Funding
This study was funded by a grant from Kankeronderzoeksfonds Limburg (KOFL). LW was supported by a grant from Dutch Cancer Association (KWF kankerbestrijding; UM2012-5375).

References

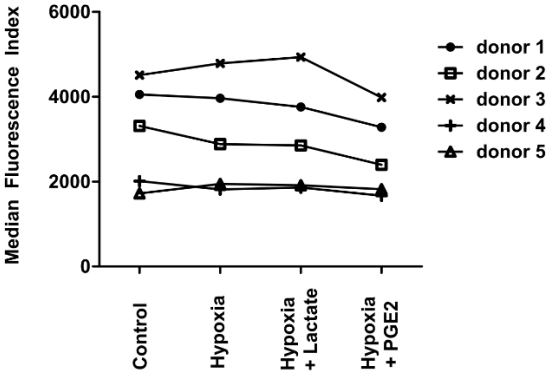
1. Vivier E, Ugolini S, Blaise D, et al (2012) Targeting natural killer cells and natural killer T cells in cancer. NatRevImmunol 12:239–252. doi: 10.1038/nri3174
2. Thomas LM (2015) Current perspectives on natural killer cell education and tolerance: emerging roles for inhibitory receptors. Immunotargets Ther 4:45–53. doi: 10.2147/ITT.S61498
3. He Y, Tian Z (2017) NK cell education via nonclassical MHC and non-MHC ligands. Cell Mol Immunol 14:321–330. doi: 10.1038/cmi.2016.26
4. Brodin P, Lakshmikanth T, Johansson S, et al (2009) The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells. Blood 113:2434–2441. doi: 10.1182/blood-2008-05-156836
5. Joncker NT, Fernandez NC, Treiner E, et al (2009) NK Cell Responsiveness Is Tuned Commensurate with the Number of Inhibitory Receptors for Self-MHC Class I: The Rheostat Model. J Immunol 182:4572–4580. doi: 10.4049/jimmunol.0803900
6. Sim MJW, Stowell J, Sergeant R, et al (2016) KIR2DL3 and KIR2DL1 show similar impact on licensing of human NK cells. Eur J Immunol 46:185–191. doi: 10.1002/eji.201545757
7. Tu MM, Mahmoud AB, Makrigiannis AP (2016) Licensed and Unlicensed NK Cells: Differential Roles in Cancer and Viral Control. Front Immunol 7:166. doi: 10.3389/fimmu.2016.00166
8. Ruggeri L, Capanni M, Urbani E, et al (2002) Effectiveness of Donor Natural Killer Cell Alloreactivity in Mismatched Hematopoietic Transplants. Science (80-) 295:2097–2100. doi: 10.1126/science.1068440
9. Curti A, Ruggeri L, Parisi S, et al (2016) Larger size of donor alloreactive NK cell repertoire correlates with better response to NK cell immunotherapy in elderly acute myeloid leukemia patients. Clin Cancer Res 22:1914–1921. doi: 10.1158/1078-0432.CCR-15-1604
10. Ruggeri L, Urbani E, Andre P, et al (2016) Effects of anti-NKG2A antibody administration on leukemia and normal hematopoietic cells. Haematologica 101:626–633. doi: 10.3324/haematol.2015.135301
11. Angelo LS, Banerjee PP, Monaco-Shawver L, et al (2015) Practical NK cell phenotyping and variability in healthy adults. Immunol Res 62:341–356. doi: 10.1007/s12026-015-8664-y
12. Pascal V, Schleinitz N, Brunet C, et al (2004) Comparative analysis of NK cell subset distribution in normal and lymphoproliferative disease of granular lymphocyte conditions. Eur J Immunol 34:2930–2940. doi: 10.1002/eji.200425146
13. Giebel S, Dziaczkowska J, Czerw T, et al (2010) Sequential recovery of NK cell receptor repertoire after allogeneic hematopoietic SCT. Bone Marrow Transplant 45:1022–1030. doi: 10.1038/bmt.2009.384
14. McWilliams EM, Mele JM, Cheney C, et al (2016) Therapeutic CD94/NKG2A blockade improves natural killer cell dysfunction in chronic lymphocytic leukemia. Oncoimmunology 5:1–9. doi: 10.1080/2162402X.2016.1226720
15. Sarkar S, van Gelder M, Noort W, et al (2015) Optimal selection of natural killer cells to kill myeloma: the role of HLA-E and NKG2A. Cancer Immunol Immunother 64:951–963. doi: 10.1007/s00262-015-1694-4
16. Mahaweni NM, Bos GMJ, Mitsiades CS, et al (2018) Daratumumab augments alloreactive natural killer cell cytotoxicity towards CD38+ multiple myeloma cell lines in a biochemical context mimicking tumour microenvironment conditions. Cancer Immunol Immunother 0:0. doi: 10.1007/s00262-018-2140-1
17. Sarkar S, Germeraad WT V, Rouschop KMA, et al (2013) Hypoxia induced impairment of NK cell cytotoxicity against multiple myeloma can be overcome by IL-2 activation of the NK cells. PLoS One 8:e64835. doi: 10.1371/journal.pone.0064835
18. Lauterbach N, Wieten L, Popeijus HE, et al (2015) HLA-E regulates NKG2C+ natural killer cell function through presentation of a restricted peptide repertoire. Hum Immunol 76:578–586. doi: 10.1016/j.humimm.2015.09.003
19. Oyer JL, Igarashi RY, Kulikowski AR, et al (2015) Generation of highly cytotoxic natural killer cells for treatment of acute myelogenous leukemia using a feeder-free, particle-based approach. Biol Blood Marrow Transplant 21:632–639. doi: 10.1016/j.bbmt.2014.12.037

20. Garg TK, Szmania SM, Khan JA, et al (2012) Highly activated and expanded natural killer cells for multiple myeloma immunotherapy. Haematologica 97:1348–1356. doi: 10.3324/haematol.2011.056747
21. Campoli M, Ferrone S (2008) HLA antigen changes in malignant cells: epigenetic mechanisms and biologic significance. Oncogene 27:5869–5885. doi: 10.1038/onc.2008.273
22. Wieten L, Mahaweni NM, Voorter CEM, et al (2014) Clinical and immunological significance of HLA-E in stem cell transplantation and cancer. Tissue Antigens 84:523–535. doi: 10.1111/tan.12478
23. Gao M, Gao L, Yang G, et al (2014) Myeloma cells resistance to NK cell lysis mainly involves an HLA class I-dependent mechanism. Acta Biochim Biophys Sin (Shanghai) 46:597–604. doi: 10.1093/abbs/gmu041
24. Cheent KS, Jamil KM, Cassidy S, et al (2013) Synergistic inhibition of natural killer cells by the nonsignaling molecule CD94. Proc Natl Acad Sci 110:16981–16986. doi: 10.1073/pnas.1304366110
25. Björkström NK, Riese P, Heuts F, et al (2010) Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK cell differentiation uncoupled from NK cell education. Blood 116:3853–3864. doi: 10.1182/blood-2010-04-281675
26. Takao S, Ishikawa T, Yamashita K, Uchiyama T (2010) The rapid induction of HLA-E is essential for the survival of antigen-activated naive CD4 T cells from attack by NK cells. J Immunol 185:6031–6040. doi: jimmunol.1000176 [pii]\r10.4049/jimmunol.1000176
27. Baginska J, Viry E, Paggetti J, et al (2013) The Critical Role of the Tumor Microenvironment in Shaping Natural Killer Cell-Mediated Anti-Tumor Immunity. Front Immunol 4:490. doi: 10.3389/fimmu.2013.00490
28. Mamessier E, Sylvain A, Thibult M-L, et al (2011) Human breast cancer cells enhance self tolerance by promoting evasion from NK cell antitumor immunity. J Clin Invest 121:3609–3622. doi: 10.1172/JCI45816
29. Krneta T, Gillgrass A, Chew M, Ashkar AA (2016) The breast tumor microenvironment alters the phenotype and function of natural killer cells. Cell Mol Immunol 13:628–639. doi: 10.1038/cmi.2015.42
30. Pasero C, Gravis G, Guerin M, et al (2016) Inherent and Tumor-Driven Immune Tolerance in the Prostate Microenvironment Impairs Natural Killer Cell Antitumor Activity. Cancer Res 76:2153–2165. doi: 10.1158/0008-5472.CAN-15-1965
31. Sasaki T, Kanaseki T, Shionoya Y, et al (2016) Microenvironmental stresses induce HLA-E/Qa-1 surface expression and thereby reduce CD8+ T-cell recognition of stressed cells. Eur J Immunol 46:929–940. doi: 10.1002/eji.201545835

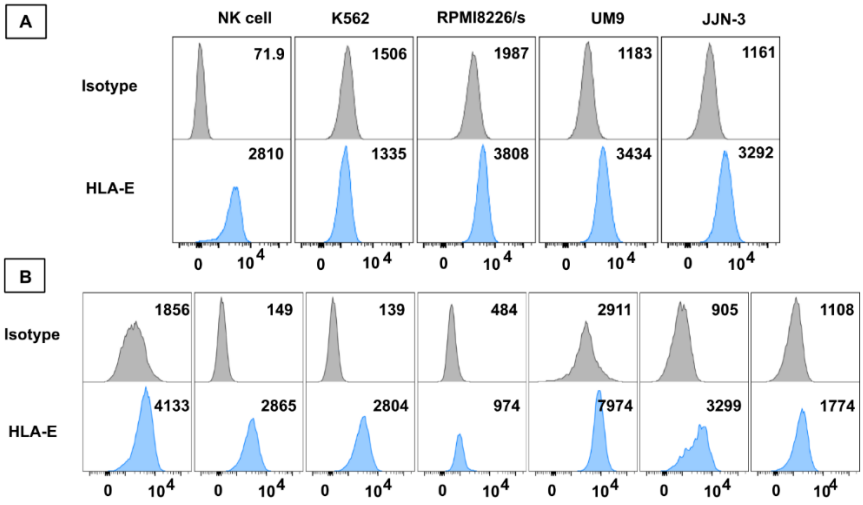
Supplementary Materials



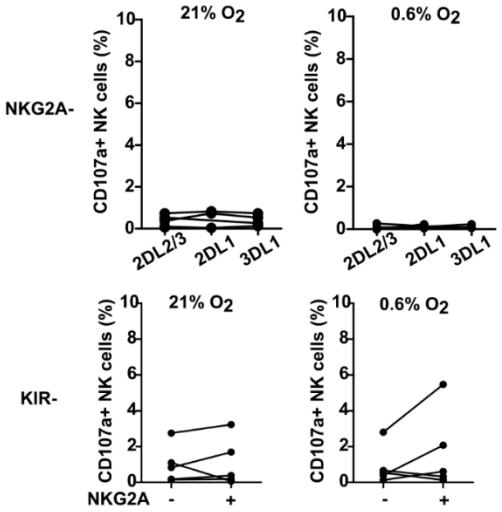
Supplementary Figure 1. Spontaneous NK cell degranulation in the presence of different microenvironment factors. NK cells were cultured without target cells for 4 hours at 21 **(A)** or 0.6 % O_2 **(B)**, or combinations of 0.6 % O_2 and 50 mM lactate **(C)**, or 100 ng/mL PGE2 **(D)**. Flow cytometry was used to subtype NK cells based on their expression of NKG2A and KIRs. The percentage of degranulating NK cells is shown as % CD107a+ NK cells. Each dot represents an average of a technical replicate from an individual NK cell donor. (A) and (B) n = 11 donors, (C) and (D) n = 5 donors tested in independent experiments (* = $p<0.05$, ** = $p<0.01$).



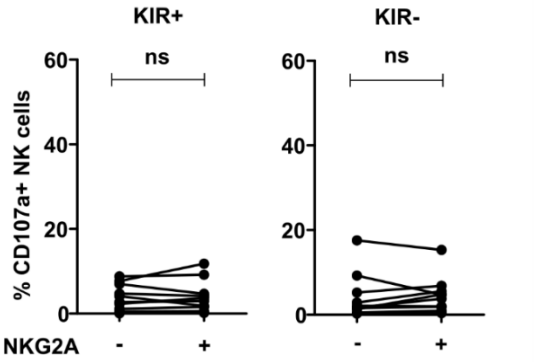
Supplementary Figure 2. NKG2A expression on NK cell is not affected by hypoxia, lactate, PGE2, or the combinations. NK cells were cultured for 4 hours in the presence of 21% or 0.6% O_2, or the combination of 0.6% O_2, and 50 mM lactate or 100 ng/mL PGE2. Each dot represents an average of a technical replicate for the different conditions. n = 5 independent experiments.



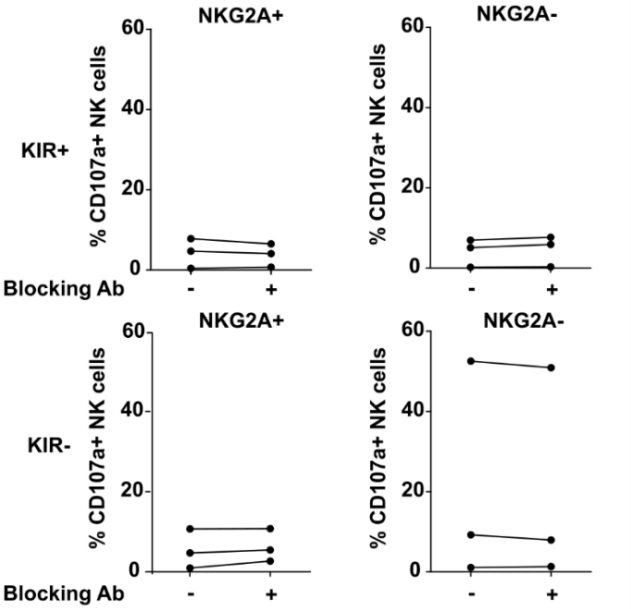
Supplementary Figure 3. HLA-E expression on different cell types. (A) IL-2 activated NK cells, K562 or MM cell lines (UM9, RPMI8226/s or JJN-3 cells) or **(B)** primary MM cells were stained with HLA-E antibody or isotypes as control or unstained. Each plot of primary MM cells represent 1 MM patient. Flow cytometric analysis was used to determine the expression of HLA-E. A median fluorescence intensity (MFI) is displayed in each plot.



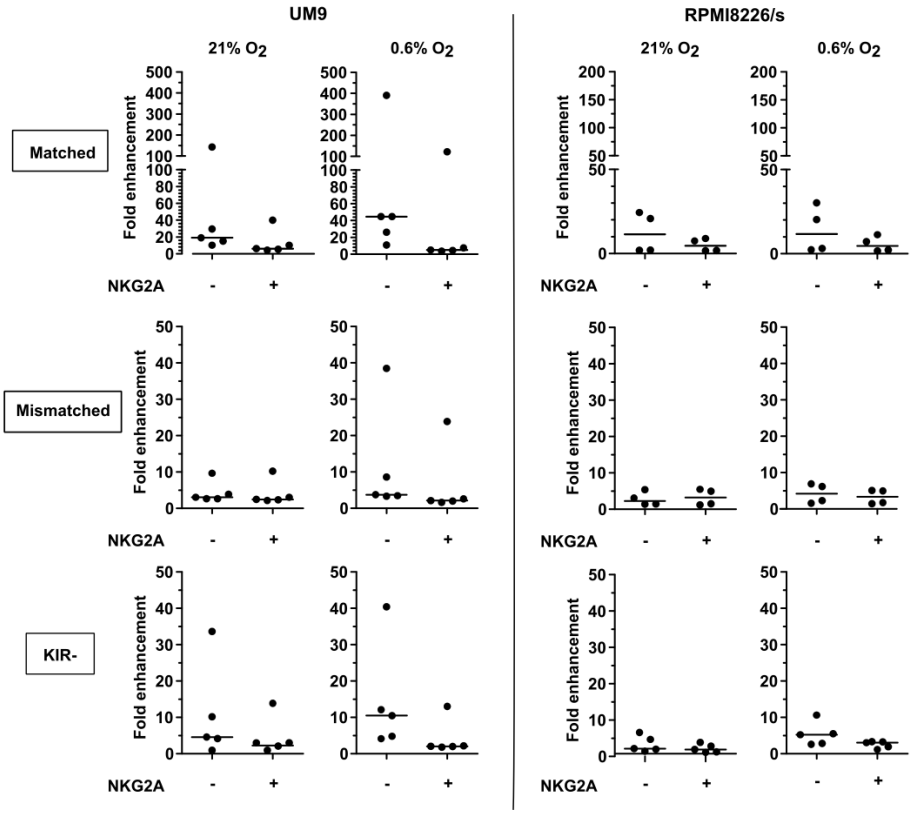
Supplementary Figure 4. Spontaneous NK cell degranulation per subset. NK cells were cultured without target cells (MM cell lines) for 4 hours in the presence of 21% or 0.6% O_2. The percentage of degranulating NK cells was denoted as CD107a+ NK cells. The subsets of 2DL2/3, 2DL1, and 3DL1 were gated based on NK cells expressing only one of the KIR receptors and lacking NKG2A expression. The subset of NKG2A+ KIR- was gated based on NK cells expressing only NKG2A receptor and missing all other KIR receptors. The subset of NKG2A- KIR- was gated based on NK cells expressing neither of NKG2A nor KIR receptors. n = 5 independent experiments.



Supplementary Figure 5. Spontaneous NK cell degranulation based on NKG2A expression. NK cells were cultured without target cells (primary MM cells from patients) for 4 hours in the presence of 21% O_2. The percentage of degranulating NK cells was denoted as CD107a+ NK cells. The subset of NKG2A+ KIR- was gated based on NK cells expressing only NKG2A receptor and missing all other KIR receptors. The subset of NKG2A- KIR- was gated based on NK cells expressing neither of NKG2A nor KIR receptors. n = 10 independent experiments.



Supplementary Figure 6. Effect of HLA-E blocking on NK cell degranulation against primary MM cells. IL-2 activated NK cells were co-cultured with primary MM cells in a 1:1 E;T ratio in the presence of 10 µg/mL anti-HLA-E antibody for 4 hours in the presence of 21% O_2. Flow cytometric analysis was used to determine the NK cell degranulation (denoted by %CD107a+ NK cell). n = 3 independent experiments with three different MM patients.



Supplementary Figure 7. Daratumumab enhanced degranulation of all NK subsets both at 21% or 0.6 % O_2. UM9 or RPMI8226/s cells were pre-incubated with daratumumab or medium for 30 minutes before adding IL-2 activated NK cells in a 1:1 E:T ratio. A degranulation assay was performed for 4 hours in the presence of 21 or 0.6% O_2. Flow cytometry was used to subtype NK cells based on their expression of NKG2A and KIRs. The fold increase in the percentage of CD107a+ NK cells in the presence of daratumumab for each subset was calculated by dividing the percentage of CD107a+ NK cells in the presence of daratumumab by the percentage of CD107a+ NK cells in the absence of daratumumab. Vertical bars shown in the plots are the median. n = 5 experiments with 5 donors

Supplementary Table 1. The proportion of NKG2A+ KIR- cells and NKG2A- KIR- cells from different donors.

Donor	NKG2A+ (%)		NKG2A- (%)	
	Total NKG2A+	NKG2A+ KIR-	Total NKG2A-	NKG2A- KIR-
A	42.9	30.3	54.4	14.5
B	29.4	16.1	68.0	15.4
C	81.4	51.8	16.7	5.3
D	27.7	18.4	62.4	26.2
E	54.3	42.5	37.9	18.1
F	42.8	31.7	49.8	35.2
G	54.7	18.9	39.1	4.5
H	52.1	34.3	33.6	13.4
I	66.2	36.6	29.3	3.1
J	19.2	11.6	77.8	14.0

NK cells were stained with fluorochrome-labeled antibodies targeting NKG2A, KIR2DL2/3, KIR2DL1, and KIR3DL1 receptor. The expression of receptors was measured by flow cytometry. The percentages presented in the table were calculated from total NK cells (CD3-CD56+). n = 10 donors

4

ADCC-Inducing Antibody Trastuzumab and Selection of KIR-HLA Ligand Mismatched Donors Enhance the NK Cell Anti-Breast Cancer Response

Femke A. I. Ehlers, Nicky A. Beelen, Michel van Gelder, Tom M. J. Evers, Marjolein L. Smidt, Loes F. S. Kooreman, Gerard M. J. Bos and Lotte Wieten

Cancers. 2021 Jun 28;13(13):3232.
doi: 10.3390/cancers13133232.

Simple Summary
Natural killer (NK) cells are potent killers of tumor cells. Many tumors, in-cluding breast cancers, develop mechanisms to suppress anti-tumor immune responses, requiring the development of strategies to overcome suppression. Here, we tested a combination therapy that aims to (1) enhance NK cell activation and (2) reduce NK cell inhibition mediated by sup-pressive factors in tumors or in the tumor microenvironment. We cultured cell lines under hy-poxia to mimic the tumor microenvironment or used patient-derived breast cancer cells that were primed by the patient's tumor environment. Our results demonstrated that cytokine-activated NK cells remained active under hypoxia and that tumor-targeting antibodies enhanced the NK cell anti-breast cancer response. Moreover, we observed that NK cell suppression by inhibitory ligands on the tumor cells can be reduced by the selection of NK cell donors with NK receptors that are incompatible with these ligands. Collectively, we present two powerful strategies to enhance the NK cell responses against breast cancer.

Abstract
Natural killer (NK)-cell-based immunotherapies are an attractive treatment option for cancer. We previously showed that alloreactive mouse NK cells cured mice of 4T1 breast cancer. However, the tumor microenvironment can inhibit immune responses, and these suppressive factors must be overcome to unfold the NK cells' full anti-tumor potential. Here, we investigated the combination of antibody-dependent cellular cytotoxicity (ADDC) and the selection of KIR-HLA-ligand mismatched NK cells to enhance NK cell anti-breast cancer responses in clini-cally relevant settings. Donor-derived and IL-2-activated NK cells were co-cultured with pa-tient-derived breast cancer cells or cell lines MCF7 or SKBR3 together with the anti-HER2 antibody trastuzumab. NK cells mediated anti-breast cancer cytotoxicity under normoxic and hypoxic conditions. Under both conditions, trastuzumab vigorously enhanced NK cell degranulation (CD107a) against HER2-overexpressing SKBR3 cells, but we observed a discrepancy between highly degranulating NK cells and a rather modest increase in cytotoxicity of SKBR3. Against patient-derived breast cancer cells, the anti-tumor efficacy was rather limited, and HLA class I expression seemed to contribute to inhibited NK cell functionality. KIR-ligand-mismatched NK cells degranulated stronger compared to the matched NK cells, further highlighting the role of HLA. In summary, trastuzumab and KIR-ligand-mismatched NK cells could be two strategies to potently enhance NK cell responses to breast cancer.

Keywords: alloreactive donor NK cells; HLA class I; antibody-dependent cellular cytotoxicity; breast cancer; tumor microenvironment

Introduction

Breast cancer is the most common cancer in women worldwide and remains a leading cause of death [1]. While newer therapies have improved survival rates over the past years, they still fail to cure metastatic disease. The effectiveness of standard therapies such as surgery, systemic therapy, and radiotherapy can be limited because tumor cells frequently develop resistance to therapy and subsequently progress [2]. Over the last years, immunotherapies, including monoclonal antibodies and cellular therapies, have emerged as promising treatment options. Trastuzumab (Herceptin) is a clinically approved antibody that targets human epidermal growth factor receptor 2 (HER2), a receptor that is overexpressed in 15–20% of breast cancer patients [3]. Next to direct anti-tumor effects, the mechanisms of trastuzumab include antibody-dependent cellular cytotoxicity (ADCC), which is mediated by CD16-expressing immune cells such as natural killer (NK) cells [4].

As NK cells are a crucial part of the first line of defense against tumors, they have gained increasing interest for cell-based immunotherapies, and ex vivo modifications can help to increase their effector functions in vivo in suppressive tumor microenvironments (TME) [5]. NK cells selectively kill tumor cells and, unlike T cells, do not require prior sensitization. They get activated when an excess of activation signals, such as stress signals, is received over inhibitory signals. The major inhibitory signals are mediated through human leukocyte antigens (HLA), which are expressed on all nucleated cells and bind to inhibitory killer-immunoglobulin-like receptors (KIRs) and NKG2A on NK cells. HLA ligands can be downregulated on tumor cells to escape T cells, but thereby tumor cells could become more susceptible to NK cells (missing-self hypothesis) [6]. HLA class I molecules are also critical for NK cell education, a process that is also known as NK cell licensing, and that requires the interaction between HLA ligands and the corresponding KIR or NKG2A receptors on the NK cell [7]. The more inhibitory receptors that find their ligand are expressed by NK cells, the higher the NK cell responsiveness against target cells, indicating that these receptors play a dual role in regulating NK cell effector functions by licensing NK cells on the one hand and inhibiting effector functions of previously licensed NK cells on the other hand [7].

In solid tumors, tumor-infiltrating NK cells are generally sparse, and they have been described as less cytotoxic than in healthy individuals [8]. To increase effectiveness, adoptive transfer of NK cells is tested. For this purpose, NK cells are either derived from the patient (autologous setting) or from healthy donors (allogeneic setting). Our group is focusing on developing effective donor-derived NK cell therapy for cancer. We have previously shown that murine NK cells from an HLA-haploidentical, alloreactive donor can cure mice of 4T1 breast cancer, while NK cells from a syngeneic donor failed to do so [9]. The concept of alloreactive NK cell donors, leading to an improved outcome compared to non-alloreactive donors, has previously been demonstrated in patients with acute myeloid leukemia that received haploidentical stem cell transplants [10]. NK cell alloreactive donors expressed licensed KIRs for which the corresponding HLA-ligands were missing in the recipient,

similar to endogenous NK cells encountering tumor cells that downregulated HLA; these NK cells are also termed KIR-HLA ligand mismatched NK cells [11]. Alloreactive NK cells do not attack the recipient tissues, as activating ligands are absent on healthy cells [11].

Despite much progress with improving their anti-tumor responses, adoptive NK cells are not always effective yet. Tumors themselves can escape immune responses and develop resistance to therapy. In addition, the TME plays a crucial role in suppressing anti-tumor responses because it is frequently an environment with immunosuppressive factors, such as hypoxia, that can mediate inhibitory effects on immune cells including NK cells [12–14]. Hypoxic areas with a pO_2 of 2.5 mm Hg (0.3% O_2) or lower were detected in solid malignancies including breast cancer [15]. In another study, hypoxia, identified by HIF1α expression, was measured in about 40% of breast cancers and associated with poor survival [16]. Suppressive TME factors such as hypoxia must be overcome to unleash the break in NK cells and unfold their full anti-tumor potential. Such strategies include both potent activation of NK cells and minimizing NK cell inhibition. In a previous study, we observed that hypoxia reduced cytotoxicity and degranulation of unactivated NK cells and demonstrated that the oxygen levels during the kill assay were the most critical influencers of the response [12]. Importantly, we demonstrated that activation of the NK cells with IL-2 could almost completely restore the NK cell responses, illustrating that IL-2 is a potent activator of NK cells and that NK cells can mediate anti-tumor responses in a hypoxic environment when sufficiently activated [12]. Another strategy to better activate NK cells is the use of monoclonal antibodies that trigger ADCC via the CD16 NK cell receptor that binds to Fc-fragments of IgG antibodies. We and others showed that NK cells can mediate ADCC in a hypoxic environment against hematological tumors [17,18]. Trastuzumab may be a clinically applicable manner to enhance donor NK cell responses against breast cancer. In addition, HLA-mediated NK cell inhibition can be reduced by blocking the interactions of inhibitory receptors and their corresponding HLA ligands through blocking antibodies (e.g., anti-NKG2A antibody Monalizumab or anti-KIR antibody Lirilumab) or by selecting genetically different NK cell donors with a KIR–HLA mismatch [11,19].

In this study, we investigated whether the combination of ADDC-triggering and the selection of KIR–ligand mismatched NK cells can enhance the NK cell anti-tumor response to human breast cancer in clinically relevant settings. To address our research question, we used the anti-HER2 antibody trastuzumab and determined the cytotoxic potential of IL-2-activated, donor-derived NK cells in breast cancer models in the presence of hypoxia, an immunosuppressive factor frequently present in solid tumors. In addition, we evaluated the degranulation potency of KIR–ligand mismatched NK cells in this setting.

Materials and Methods

Cell Culture and Animals

The breast cancer cell line MCF7, purchased from ATCC, was cultured in an EMEM medium (ATCC, Manassas, VA, USA) supplemented with 10 µg/mL insulin, 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 µg/mL streptomycin (1% Pen/Strep, Thermo Fisher Scientific, Waltham, MA, USA). The breast cancer cell line SKBR3, purchased from DSMZ, was cultured in McCoy's 5A medium (Gibco), supplemented with 20% FCS and 1% Pen/Strep. The HLA class I-negative cell line K562, purchased from ATCC, was used as a control cell line and cultured in IMDM medium (Gibco), supplemented with 10% FCS and 1% Pen/Strep. The cells were cultured at 37°C in an incubator containing 21% O_2 and 5% CO_2. For hypoxia exposure, the cells were cultured at 37 °C in a hypoxic chamber containing 0.2% O_2 and 5% CO_2 (Invivo$_2$ 1000, Ruskinn Technology Ltd., Bridgend, UK). NOD SCID gamma (NSG) mice were injected with 1×10^6 MCF7 cells subcutaneously into the flank. The local animal ethical committee had approved the experiments. Primary tumors were harvested and dissociated into single-cell suspension using the Tumor Dissociation Kit human (Miltenyi Biotec, Bergisch Gladbach, Germany) together with gentleMACS Dissociator (Miltenyi). Dissociated tumor cells were frozen until assays were performed.

NK Cell Culture

NK cells were isolated from healthy anonymous buffy coats (Sanquin blood bank, Maastricht, The Netherlands). The use of buffy coats does not need ethical approval in the Netherlands under the Dutch Code for Proper Secondary Use of Human Tissue. NK cell donors with an HLA $C1^+$ $C2^+$ $Bw4^+$ genotype and expression of KIR2DL1, KIR2DL2/3, and KIR3DL1 receptors were used to obtain NK cells licensed for all three KIRs. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Lymphoprep (Axis-Shield, Dundee, Scotland). From the PBMCs, NK cells were obtained by negative selection, utilizing an NK cell isolation kit and the MACS separation column system (Miltenyi Biotec) according to the manufacturer's protocol. The NK cells were subsequently activated with 1000 U/mL IL-2 (Proleukin, Novartis, Basel, Switzerland) and cultured overnight in RPMI-1640 medium (Gibco), supplemented with 10% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in an incubator containing 21% O_2 and 5% CO_2. For some of the primary breast cancer cells, expanded NK cells were used as effector cells. NK cells were expanded from CD3-depleted PBMCs in SGCM medium supplemented with 10% FCS, 1% Pen/Strep, and 1000 U/mL IL-2. After 17 days of expansion, NK cells were frozen and, prior to the experiment, thawed and recovered overnight in the presence of IL-2.

Cytotoxicity Assay

The cytotoxic potential of NK cells against breast cancer cell lines was determined in flow-cytometry based assays. The target cells SKBR3, MCF7, and K562 were either labeled with CellTracker™ CM-DiI Dye or with CellTracker™ Deep Red Dye (both

Thermo Fisher Scientific) and were incubated at 37 °C either with 21% O_2 or with 0.2% O_2. After 16 h incubation, the target cells were harvested and counted and 2 × 10^4 cells were plated per well in a 96-well plate. The target cells were pre-incubated with 1 µg/mL trastuzumab (Roche, Basel, Switzerland) or with a culture medium, as a control, for 30 min. The hypoxia-exposed cells were kept at 0.2% O_2 for all steps. IL-2-activated NK cells were harvested and washed before they were co-cultured with the target cells in a 1:1 or a 5:1 Effector:Target (E:T) ratio at 37 °C either with 21% O_2 or with 0.2% O_2. After 4 h of co-culture, plates were put on ice to stop the reaction. The cells were washed with PBS (Sigma-Aldrich, Munich, Germany) and stained for dead cells with Live/Dead® Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific) for 30 min on ice. The assay was analyzed by flow cytometry. Specific cytotoxicity was calculated as follows: (% dead tumor cells—% spontaneous tumor cell death)/(100%—% spontaneous tumor cell death)×100. Spontaneous tumor cell death in the presence of trastuzumab was used to calculate specific cytotoxicity in the conditions with trastuzumab.

CD107a Degranulation Assay

The target cell lines SKBR3, MCF7, and K562 were incubated for 16 h at 37 °C either with 21% O_2 or with 0.2% O_2 and subsequently harvested for the CD107a degranulation assay. In each well, 10^5 target cells were plated and pre-incubated with 1 µg/mL trastuzumab (Roche) or, as a control, with a culture medium for 30 min. IL-2-activated NK cells were harvested, washed, and subsequently co-cultured with the target cells in a 1:1 E:T ratio at 37 °C either with 21% O_2 or with 0.2% O_2. To each well, 5 µl of CD107a-Horizon V450 antibody (Miltenyi) was added. After 1 h of co-culture, Monensin (BD Biosciences, San Jose, CA, USA) was added to prevent reinternalization of CD107a, and after another 3 h of co-culture, the plates were put on ice to stop the reaction. The cells were washed with PBS and first stained with Live/Dead® Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific) for 30 min on ice before surface staining with the following antibodies was performed for 30 min on ice: anti-CD3-APC-Vio770 (BW264/56), anti-CD56-PerCP-Vio700 (REA196), anti-KIR2DL1-APC (143211), anti-KIR2DL2/3-PE (DX27), anti-KIR3DL1-FITC (DX9), and anti-NKG2A-PE-Vio770 (REA110). The assay was analyzed by flow cytometry.

KIR-Ligand Mismatched and Matched NK Cells

The HLA class I genotype of SKBR3 and MCF7 was determined by Luminex-SSO. The genotype for SKBR3 cells was HLA $C1^+$ $C2^-$ $Bw4^-$, and the genotype for MCF7 cells was HLA $C1^-$ $C2^+$ $Bw4^+$. Flow cytometry was performed to determine the phenotypic expression of HLA-C and Bw4 as described below. The matched and mismatched NK cell populations were identified based on the genotypic expression of HLA C1, HLA C2, and HLA Bw4, as well as the phenotypic expression of Bw4. For SKBR3, KIR-ligand-matched NK cells were KIR2DL2/3+ NK cells, and KIR-ligand-mismatched NK cells were $KIR2DL1^+$, $KIR3DL1^+$, and $KIR2DL1^+$ $KIR3DL1^+$ double-positive NK cells. For MCF7, KIR-ligand-matched NK cells were $KIR2DL1^+$ NK cells, and KIR-ligand-mismatched NK

cells were KIR2DL2/3$^+$, KIR3DL1$^+$, as well as KIR2DL2/3$^+$ KIR3DL1$^+$ double-positive NK cells.

Generation of F(ab')2 Fragment

The Pierce F(ab')$_2$ Preparation Kit (Thermo Fisher Scientific) was used according to the manufacturer's protocol to generate an F(ab')$_2$ fragment of the trastuzumab antibody. The following secondary antibody was used to stain for the F(ab')$_2$ fragment or trastuzumab: Alexa Fluor 647 AffiniPure F(ab')$_2$ Fragment Goat Anti-Human IgG, F(ab')$_2$ fragment specific (Jackson ImmunoResearch, Cambridgeshire, UK).

Primary Human Breast Cancer Cells

Primary human breast cancer tissue was obtained from the Maastricht Pathology Tissue Collection. Collection, storage, and use of tissue and patient data were performed in agreement with the "Code for Proper Secondary Use of Human Tissue in the Netherlands" and have been approved by the local ethics committee. The tissue was immediately stored in MACS Tissue Storage Solution (Miltenyi) until processing. To dissociate single cells, the Tumor Dissociation Kit human (Miltenyi) was used together with a gentleMACS Dissociator (Miltenyi) according to the manufacturer's instructions. Subsequently, the cell suspension was enriched for tumor cells by negative selection using the Tumor Cell Isolation Kit, human (Miltenyi). Tumor cells were identified by PanCytokeratin-AF488 (C11, ThermoFisher) and the purity of tumor cells was at least 70% PanCK$^+$ cells, with one exception of 44% PanCK$^+$ cells. For cytotoxicity and CD107a assays, either freshly isolated or IL-2-expanded NK cells were used, and co-cultures were performed for a duration of 16 h.

Flow Cytometry

To determine HER2 and HLA surface expression, SKBR3 and MCF7 cells were stained with Live/Dead® Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific) for 30 min on ice, followed by staining with HER2-APC (Neu24.7, BD), HLA-C-PE (DT9, BD), HLA-Bw4-PEVio770 (REA274, Miltenyi), HLA-E-PE (3D12, Thermo Fisher Scientific), HLA-ABC-APC (G46-2.6, BD), or HLA-ABC-PE (REA230, Miltenyi), or matched isotype controls for 30 min on ice. All analysis by flow cytometry was performed with BD FACS Canto II. Data were analyzed with FlowJo v10.6.1 64-bit software, (TreeStar, Ashland, OR, USA).

Statistics

The statistical analysis was performed with GraphPad Prism 8.4.3 software (Graphpad Software, San Diego, CA, USA) using paired, non-parametric *t*-tests (Wilcoxon matched-pairs signed-rank test).

Results

IL-2 Activated NK Cells Mediate Anti-Breast Cancer Responses under Hypoxia and Maintain the Potential to Mediate Trastuzumab-Induced ADCC

Since hypoxia can have inhibitory effects on NK cells and is frequently observed in breast cancer [14,15], we investigated the influence of hypoxia on NK cell anti-tumor responses either in the presence or absence of trastuzumab, an ADCC triggering therapeutic antibody. We used the HER2-non-amplified cell line MCF7 and the HER2-amplified cell line SKBR3 in NK cell functional assays together with trastuzumab. First, the influence of hypoxia on HER2 surface expression was detected by flow cytometry. Both cell lines expressed HER2 but the expression level of MCF7 was around 8-fold lower than of SKBR3, confirming their gene amplification status and hypoxia (0.2% O_2) did not influence HER2 expression of both cell lines (**Figure 1A,B**).

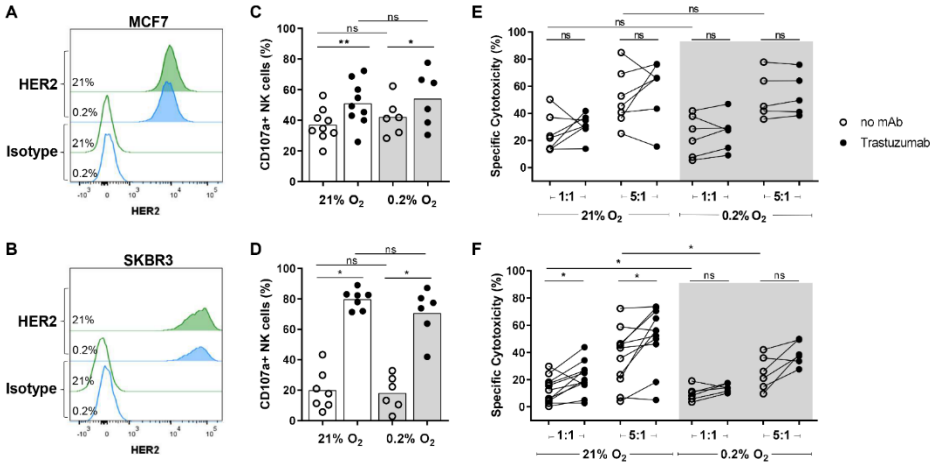


Figure 1. NK cell efficacy against non-amplified and HER2 amplified cell lines with or without trastuzumab under normoxia (21% O_2) and hypoxia (0.2% O_2). (**A**,**B**) HER2 surface expression levels were determined by flow cytometry on non-amplified MCF7 cells (**A**) and HER2-amplified SKBR3 cells (**B**). (**C**–**F**) For both degranulation (CD107a) and cytotoxicity assays, IL-2-activated NK cells were co-cultured with the target cells MCF7 or SKBR3 for 4 h either with 21% O_2 or 0.2% O_2 and analyzed by flow cytometry. CD107a assays were performed in 1:1 E:T ratios and % of $CD107a^+$ NK cells are shown per donor against MCF7 (**C**) or SKBR3 (**D**) with the bar height indicating the mean. Cytotoxicity assays were done in 1:1 or 5:1 E:T ratios and dead target cells are shown as % of specific cytotoxicity for MCF7 (**E**) and SKBR3 (**F**). The donors from Figure 2 are included in the conditions with 21% O_2 of (F). Each dot represents the average of duplicates from one NK cell donor. * $p < 0.05$, ** $p < 0.01$, ns = not significant.

To determine the influence of hypoxia on NK cell degranulation, either or not in the presence of trastuzumab, CD107a assays were performed. The cell lines were cultured either under normoxia or hypoxia for 16 h before IL-2-activated NK cells were added for a 4 h co-culture period. To confirm that the NK cells from all donors were highly activated and functional, K562 cells were included as control target cells against which NK cells from all donors could strongly degranulate and mediate cytotoxicity (**Figure S1**). With MCF7 as target cells under normoxic conditions, the

percentage of degranulating NK cells ranged from 20% to 56% without trastuzumab and from 26% to 72% with trastuzumab. The average degranulation increased from 37% without to 52% with trastuzumab (**Figure 1C**). Exposure of tumor cells to hypoxia did not reduce the degranulation of NK cells against MCF7 or the NK cell potentiating effect of trastuzumab (**Figure 1C**). Against the HER2-amplified SKBR3 cells, an average of 20% of NK cells degranulated under normoxia, and the addition of trastuzumab highly increased the percentage of degranulating NK cells to 80%, demonstrating vigorous degranulation in all donors (**Figure 1D**). Compared to normoxia, NK cell degranulation against SKBR3 was around 10% lower under hypoxic conditions, but this did not reach statistical significance, illustrating that degranulation levels of activated NK cells were not severely impaired by low oxygen (**Figure 1D**).

To assess the actual cytotoxic potential of NK cells in the presence of hypoxia and the ADCC-response mediated by trastuzumab, we performed 4 h cytotoxicity assays with IL-2-activated NK cells in different E:T ratios. Under normoxia, 25% of MCF7 cells were killed by NK cells in a 1:1 E:T ratio. As expected, the level of natural cytotoxicity was dependent on the E:T ratio and increased to an average of 52% in a 5:1 E:T ratio (**Figure 1E**). In line with NK cell degranulation, the cytotoxic capacity of MCF7 was not reduced by exposure to hypoxia (**Figure 1E**). With trastuzumab, cytotoxicity against the non-amplified MCF7 cells was increased in some donors, while it was decreased in other donors compared to natural cytotoxicity. Overall, trastuzumab did not enhance the average NK cell-mediated cytotoxicity against MCF7 under normoxic or hypoxic conditions (**Figure 1E**). The HER2-amplified SKBR3 cell line was more resistant to NK cell cytotoxicity. Under normoxic conditions, natural cytotoxicity ranged from 0–30% with an average of 11%, which was increased to 36% with a 5:1 E:T ratio (**Figure 1F**). The addition of trastuzumab increased the cytotoxicity in 9 out of 11 donors under normoxia and 5 out of 6 donors under hypoxia in both 1:1 and 5:1 E:T ratios. Although an ADCC effect by trastuzumab was observed, it was surprisingly small compared to the large increase in NK cell degranulation by trastuzumab. Increasing the concentration of trastuzumab up to 32 µg/mL did not further enhance the binding of the antibody to SKBR3 target cells (**Figure S2A**) or the ADCC response (**Figure S2C**). Compared to normoxia, NK-cell-mediated cytotoxicity was slightly reduced under hypoxic conditions, independent of trastuzumab and most evident in the 5:1 E:T ratio (36% under normoxia reduced to 25% under hypoxia) (**Figure 1F**).

We observed a large discrepancy between the level of trastuzumab-induced NK cell degranulation and the level of trastuzumab-induced killing of HER2-positive target cells. Because of this observation and because tumors can develop resistance to the direct cytotoxic effects of trastuzumab [18], we investigated whether the binding of trastuzumab to SKBR3 had induced resistance of SKBR3 cells to the cytotoxic machinery of NK cells. To do this, the cytotoxicity assays were performed with an $F(ab)_2$ fragment of trastuzumab, which can bind to HER2, but, due to the absence of an Fc-part, does not engage CD16 on the NK cell and therefore lacks the ADCC

potentiating effects. We observed that binding of the $F(ab)_2$ fragment to SKBR3 did not affect the killing of SKBR3 cells by NK cells (**Figure 2A**) or the level of NK cell degranulation (**Figure 2B**) as compared to the control condition without $F(ab)_2$. This illustrates that the binding of trastuzumab to the HER2 antigen did not result in tumor cell resistance to NK cells. To test if the relatively low levels of ADCC were caused by trastuzumab-mediated inhibition of NK cell cytotoxicity, cytotoxicity assays were performed with HER2-overexpressing SKBR3 cells and HER2-negative K562 cells combined as target cells in one well (**Figure 2C**, gating strategy in **Figure S3**). This revealed that NK cells killed K562 equally well when K562 cells were cocultured with SKBR3 cells, either with trastuzumab or not (**Figure 2C**).

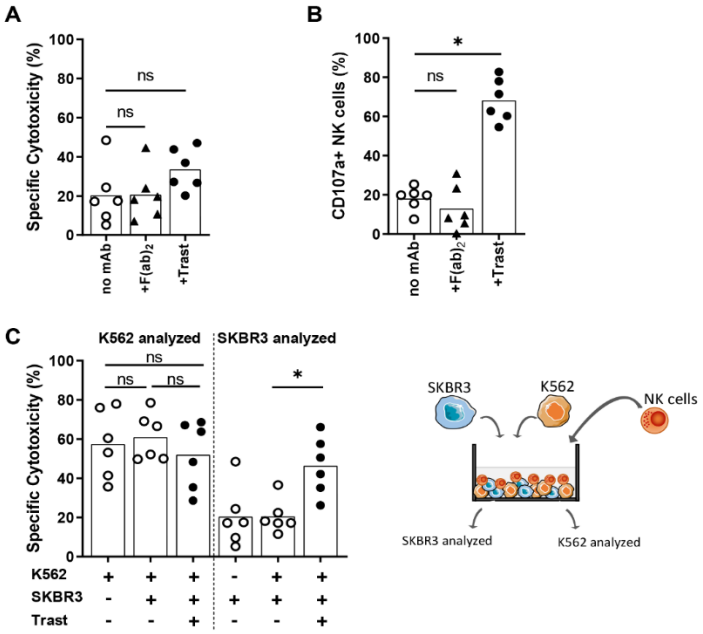


Figure 2. NK cell efficacy against the target cells SKBR3 in the presence of an $F(ab)_2$ fragment of trastuzumab. SKBR3 and K562 target cells were labeled with different dyes and co-cultured with IL-2-activated NK cells in a 1:1 E:T ratio with trastuzumab or with a $F(ab)_2$ fragment of trastuzumab for 4 h with 21% O_2. Flow cytometry was used to analyze specific cytotoxicity of tumor cells (**A**,**C**) and, in separate assays, degranulation of NK cells (**B**). (**C**) Target cells K562 and SKBR3 were combined in one well with or without trastuzumab and analyzed for specific cytotoxicity of K562 and SKBR3. The schematic setup is depicted on the right, and the full gating strategy is shown in Figure S3. Each dot represents one NK cell donor, and the average of duplicates is shown per donor with the bars indicating the mean. Data from three donors of (**A**) are also used in Figure 1F. * $p < 0.05$, ns = not significant.

Taken together, these results imply that IL-2-activated NK cells remained cytotoxic against the tested breast cancer cell lines under hypoxia. Moreover, despite the relatively small increase in killing of target cells, trastuzumab could potentiate the NK cell anti-tumor response by strongly enhancing NK cell degranulation under normoxic and hypoxic conditions, especially against HER2 overexpressing target cells.

NK Cell Anti-Tumor Efficacy against Primary Breast Cancer Cells Is Tumor Dependent and May Be Related to Tumor Cell Expression of HLA Class I

In addition to hypoxia, the TME consists of many other factors that can directly or indirectly suppress the NK cell anti-tumor response. To study NK cell efficacy against primary breast tumors derived from and potentially influenced by the clinically relevant TME in the patient, we set up a model utilizing human primary breast cancer cells that were isolated from patients. Leftover breast cancer material was included independently of the breast cancer subtype, but all samples were HER2-negative (non-amplified). Patients with $HER2^+$ breast cancer are usually treated with neoadjuvant therapy with very good response, and therefore we could not obtain sufficient remaining material from $HER2^+$ tumors to perform the assays. Where cell numbers allowed, trastuzumab was included to test if the level of HER2 expression on HER2 non-amplified breast cancer is sufficient to enhance NK cell activation. The patient-derived tumor tissues were dissociated into single-cell suspensions and subsequently enriched for tumor cells to specifically study the interaction of donor NK cells with tumor cells. Degranulation assays (CD107a) or cytotoxicity assays with the primary tumor cells as targets were performed by co-culturing these breast cancer cells with IL-2-activated NK cells as effector cells for 16 h. With an average of 10%, the overall NK cell degranulation against primary human breast cancer cells was rather low, and trastuzumab did not strongly increase NK cell degranulation against these HER2 non-amplified targets (**Figure 3A**). These data corresponded to our cell line data, indicating that HER2 expression as detected on non-amplified cells is not sufficient to trigger a strong increase in NK cell degranulation.

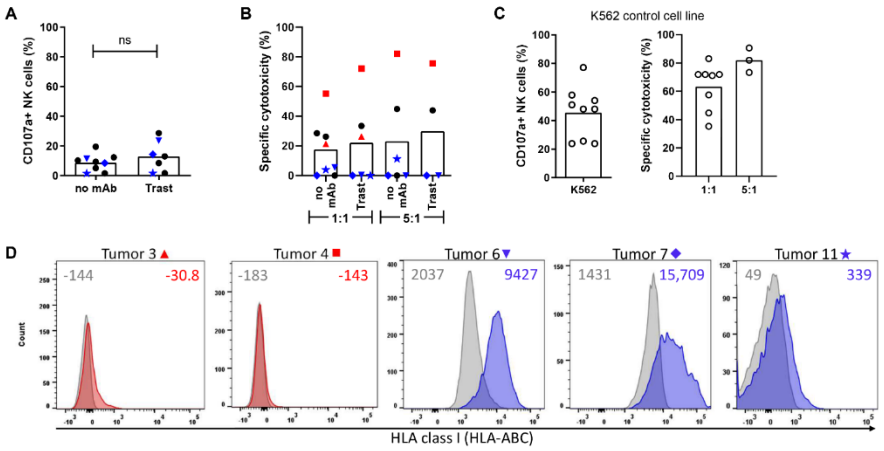


Figure 3. NK cell degranulation and cytotoxic potential against HER2 non-amplified primary breast cancer. (**A**,**B**) Patient-derived breast cancer cells were dissociated to single cells and incubated with or without trastuzumab for 30 min and subsequently co-cultured with IL-2-activated NK cells in 1:1 or 5:1 E:T ratios for 16 h at 21% O_2 and analyzed by flow cytometry. (**A**) Degranulating NK cells are shown as percentage $CD107a^+$ NK cells, and each symbol (black and blue) represents one tumor sample; the blue symbols correspond to the colors of the histograms in (**D**). (**B**) Dead tumor cells are shown as specific cytotoxicity. The bar graphs indicate the mean, while each dot represents an individual breast cancer specimen. The red and blue symbols correspond to the histograms in (**D**). (**C**) K562 cells were included as a control in CD107a and cytotoxicity assays to show that NK cells were potent killers. (**D**) Histograms show HLA class I staining of breast cancer cells (red for samples with kill, blue for samples with low kill), and grey histograms show isotype controls. Tumors 3 and 4 (red symbols) were not available for CD107a assays in A. Median fluorescent intensity is depicted for each staining. ns = not significant.

When analyzing the cytotoxic capacity of NK cells against the breast cancer cells, we observed a large variation between samples ranging from 0% to 60% specific cytotoxicity with 1:1 Effector:Target (E:T) ratios and from 0% to 80% in 5:1 E:T ratios (**Figure 3B**). In line with its effect on NK cell degranulation, trastuzumab did not mediate a clear ADCC effect (**Figure 3B**). K562 cells were included as a control to ensure that all NK cells were potent killers and, as expected, NK cells from all donors degranulated strongly and killed K562 cells (**Figure 3C**). Because HLA class I is an important inhibitory ligand for NK cells and its expression is frequently downregulated in breast cancer, either partially or completely [19], we stained for HLA class I expression on five tumors where we retrieved sufficient single cells to test the contribution of HLA class I to NK cell susceptibility. Two of those tumors were killed by NK cells (red histograms, corresponding to red symbols) and three were resistant to NK cell-mediated killing, resulting in low to no kill (blue histograms, corresponding to blue symbols) (**Figure 3B**). The tumors susceptible to NK cell-mediated killing (red symbols) expressed HLA class I at a very low level or not at all. Due to the low yield of tumor cells, we could not perform CD107a assays with the samples depicted in red symbols. The tumors resistant to NK cells (blue symbols) expressed HLA class I (**Figure 3D**). Our data illustrate that primary breast cancer cells can be relatively resistant to NK cells. Although the relative contribution of other

factors, including both activating and inhibitory ligands, needs to be addressed in more detail, our data also suggest that HLA class I expression is associated with NK cell resistance.

KIR ligand Mismatched NK Cells Degranulated More Vigorously Than Their HLA-Matched Counterparts, While Trastuzumab Activated All NK Cell Subsets

To further investigate the functional relevance of HLA class I as an inhibitor for NK cell anti-breast cancer responses under hypoxic conditions and in combination with trastuzumab, we investigated the degranulation level of NK cells that encounter their HLA class I ligand (KIR-ligand-matched) versus NK cells that do not encounter their ligand (KIR-ligand-mismatched). Such mismatch situations can occur either when endogenous NK cells encounter tumor cells that downregulated HLA or in the situation where patients receive donor NK cells that were selected based on the presence of a KIR-ligand mismatch to further potentiate NK cell anti-tumor responses. To enable analysis of the KIR-ligand-matching status, breast cancer cell lines were geno- and phenotyped for HLA class I. The genotype of MCF7 cells was determined as HLA $C1^-$, $C2^+$, and $Bw4^+$ and the genotype of SKBR3 as HLA $C1^+$, $C2^-$, and $Bw4^-$. HLA-C expression was confirmed by surface staining in both cell lines (**Figure 4A,B**). Despite the $Bw4^+$ genotype on MCF7, the Bw4 molecule was not clearly expressed on the cell surface (**Figure 4A**). The phenotypic expression of HLA-C and Bw4 was not altered by exposure to hypoxia in either of the two cell lines (**Figure 4A,B**). To determine the KIR-ligand-matched and mismatched NK cell subsets, $NKG2A^-$ NK cells that were single positive for either KIR2DL1, KIR2DL2/3, or KIR3DL1 were selected during analysis. Subsequently, the cell populations were grouped as the matched NK cell subset when the ligand was present on the target cells or as the mismatched NK cell subset when the corresponding HLA ligand was absent.

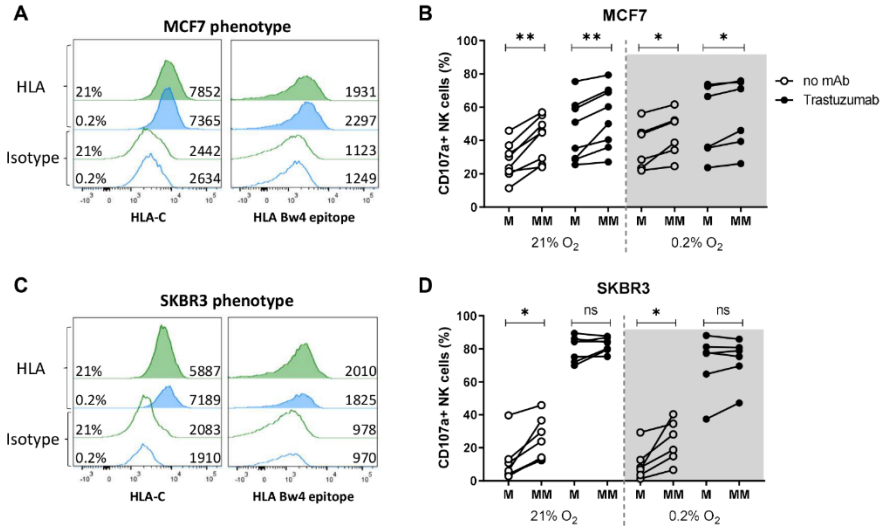


Figure 4. NK cell degranulation of KIR-ligand-matched and KIR-ligand-mismatched NK cells in response to breast cancer cells under normoxia or hypoxia with or without trastuzumab. (**A**,**B**) MCF7 and SKBR3 cells were exposed to 21% O_2 or 0.2% O_2 for 16 h and stained for HLA surface expression by flow cytometry. Histograms from normoxic conditions are indicated in green, with hypoxic conditions in blue. (**C**,**D**) Degranulation assays were performed by co-culturing NK wells either with MCF7 or SKBR3 target cells with or without trastuzumab for 4 h at 21% O_2 or 0.2% O_2. Based on the KIR expression of NK cells and HLA expression of target cells, KIR-ligand-matched (M) and -mismatched (MM) NK cell subsets were analyzed and the percentage of degranulating NK cells is shown per subset as % $CD107a^+$ NK cells. Each dot represents the average of duplicates from one NK cell donor. * $p < 0.05$, ** $p < 0.01$, ns = not significant.

The KIR-ligand-matched NK cell subset for MCF7 was $KIR2DL1^+$, while the KIR-ligand-mismatched NK cells for MCF7 were $KIR2DL2/3^+$, $KIR3DL1^+$, or $KIR2DL2/3^+$ $KIR3DL1^+$ double-positive cells. We included KIR3DL1 in the mismatched subset because of the very low Bw4 expression on MCF7. KIR-ligand-mismatched NK cells of all donors degranulated stronger against MCF7 cells than their KIR-ligand-matched counterparts (degranulation of seven out of eight donors under normoxia and five out of six donors under hypoxia increased at least 5%, **Figure 4B**). In the presence of trastuzumab, when total NK cell degranulation was slightly enhanced, the mismatched NK cell subset degranulated stronger than the matched NK cell subset in both normoxic and hypoxic conditions (**Figure 4B**, **Figure S4A**). With SKBR3 as target cells, the KIR-ligand-matched NK cell subset expressed KIR2DL2/3 and the KIR-ligand-mismatched subset expressed KIR2DL1, KIR3DL1, or a combination thereof. Compared to the matched population, NK cell degranulation of the KIR-ligand-mismatched population was enhanced more than 5% in seven out of seven donors under normoxia and five out of six donors under hypoxia (**Figure 4D**, **Figure S4B**). Trastuzumab by itself induced vigorous degranulation against SKBR3 in all NK cell subsets under normoxia and under hypoxia (**Figure 4D**). Due to the high degranulation, a potential additional effect of KIR-ligand mismatching with

trastuzumab was not detected (**Figure 4D**). NK cell degranulation of each subset against the HLA-deficient line K562 was analyzed to control for intrinsic difference between the subsets. A minor increase in degranulation from matched to mismatched NK cell subsets was observed against K562 cells; however, the increase was less consistent and much lower than the increase observed with MCF7 and SKBR3 as target cells (**Figure S5**). Altogether, these results demonstrated that KIR-ligand-mismatched NK cells degranulated more potently than their matched counterparts, indicating that HLA class I is a relevant inhibitory factor in normoxic as well as in hypoxic conditions, while trastuzumab alone resulted in strong NK cell degranulation of all subsets against HER2-amplified targets.

HLA expression levels are dynamically regulated by context-specific factors such as interferons [20] or 3D cell growth [21]. We therefore used a mouse model with MCF7 cells growing in immunodeficient mice to better mimic the three-dimensional growth of breast cancer in a TME. From these in vivo grown MCF7 tumors, single-cell suspensions were obtained and HLA class I expression levels and their impact on NK cell degranulation were evaluated in ex vivo CD107a assays. HLA-C expression levels on in vivo grown MCF7 cells were higher compared to MCF7 cultured in vitro (seven-fold expression vs. three-fold expression compared to isotype control in MCF7 ex vivo (**Figure 5A**) vs. in vitro (**Figure 4A**)), which underlines the importance of the environmental context. The KIR-HLA-ligand-matched KIR2DL1^{+} single-positive NK cell subset did not degranulate against those mouse-derived tumor cells, while the mismatched KIR2DL2/3^{+} and KIR3DL1^{+} single-positive populations degranulated (**Figure 5B**). Despite the Bw4-positive genotype, Bw4 expression remained negative on MCF7 ex vivo (**Figure 5A**). Consistent with this Bw4-negative phenotype, the corresponding KIR3DL1 receptor degranulated stronger than the matched KIR2DL1 against MCF7 ex vivo (**Figure 5B**). Overall, the ex vivo data confirm our in vitro results that stronger degranulation was observed in the KIR-ligand-mismatched NK cell subsets.

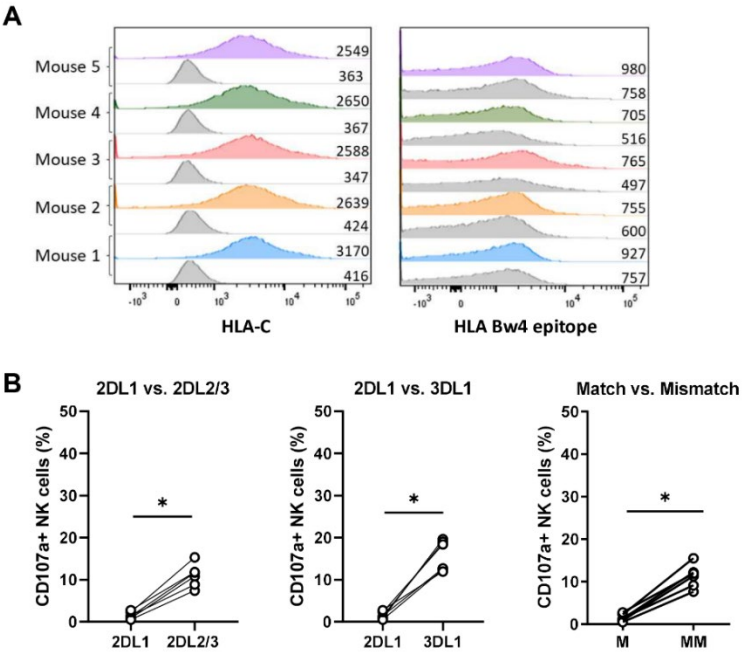


Figure 5. NK cell degranulation of KIR-ligand-matched and -mismatched NK cells in response to MCF7 grown in vivo. MCF7 tumors that were grown in mice were harvested and dissociated into single-cell suspension before performing HLA staining with *n* = 5 tumors (**A**) or 4 h CD107a assays with *n* = 6 tumors (**B**). Within the CD107a assay, degranulation is shown for each receptor (left and middle graph) and also as KIR-ligand-matched (M, 2DL1) vs. -mismatched (MM, 2DL2/3 and 3DL1) NK cell subsets as % $CD107a^+$ NK cells. Each dot represents a tumor isolated from one mouse and the average of duplicates is depicted. * $p < 0.05$.

<u>NK Cell Subsets Expressing NKG2A Degranulate More Potently against HLA-E Negative Tumors Than NK Cell Subsets without NKG2A</u>

NKG2A has, similar to KIR, a dual role in regulating NK cell functions; on the one hand by licensing NK cells, whereby NK cells can become more effective, and on the other hand by mediating inhibitory signals upon binding its ligand HLA-E. Both breast cancer cell lines used in this study did not express HLA-E (**Figure 6A**). We compared the anti-tumor response of $NKG2A^+$ vs. $NKG2A^-$ NK cell subsets in the CD107a assays to test whether licensing by NKG2A can have a positive effect on NK cell degranulation against HLA-E negative breast cancer targets.

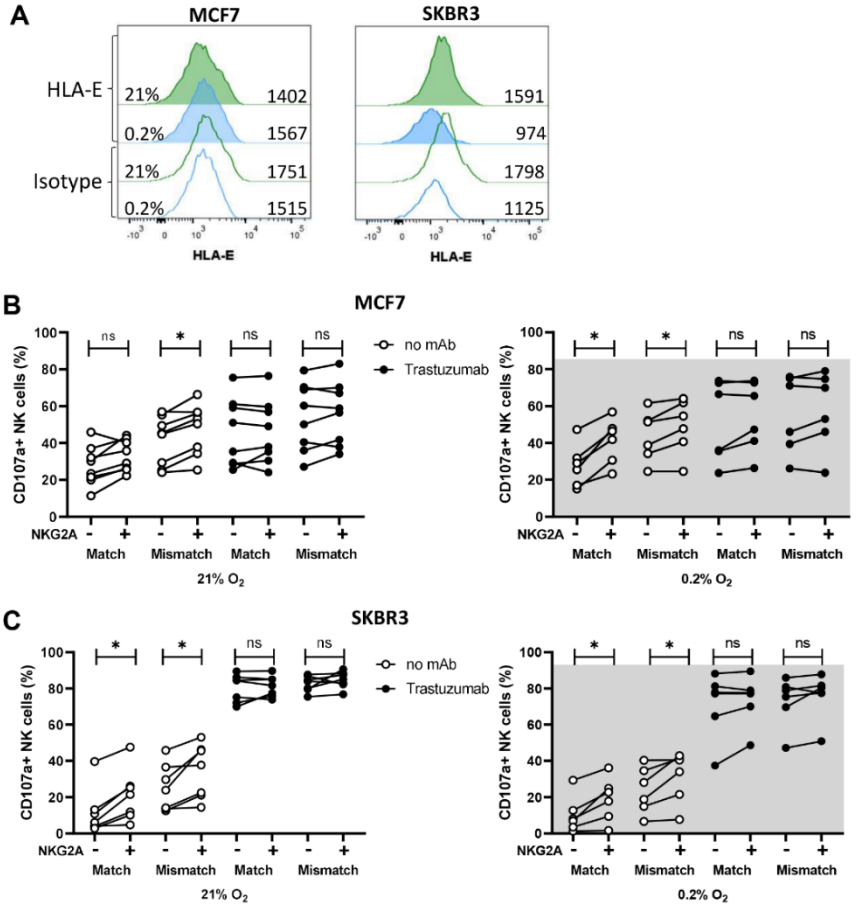


Figure 6. NK cell degranulation of the NKG2A$^+$ subsets in comparison to the NKG2A$^-$ subsets is slightly enhanced. (**A**) HLA-E expression of MCF7 and SKBR3 was determined by flow cytometry. (**B**,**C**) For degranulation assays, NK cells were co-cultured either with MCF7 or SKBR3 targets cells with or without trastuzumab for 4 h at 21%O_2 or 0.2% O_2. By flow cytometry analysis, NK cells were grouped in NKG2A$^-$ and NKG2A$^+$ subsets (indicated by − and + below graphs) and further divided into KIR-ligand-matched and -mismatched subsets based on their KIR expression. For each subset, NK cell degranulation (CD107a in %) is depicted in response to MCF7 (**B**) or SKBR3 (**C**). Each dot represents one NK cell donor and the average of duplicates. * $p < 0.05$, ns = not significant.

With MCF7 as target cells, NK cell degranulation was higher in the NKG2A$^+$ subsets compared to the NKG2A$^-$ subsets in all analyzed subsets without trastuzumab (matched and mismatched under normoxia and hypoxia), while degranulation in the presence of trastuzumab was comparable between NKG2A$^+$ and NKG2A$^-$ subsets (**Figure 6B**). In response to SKBR3, we also observed an increase in degranulation in the NKG2A$^+$ NK cell population compared to the NKG2A$^-$ population for all conditions without trastuzumab (**Figure 6C**). Since NK cell degranulation against SKBR3 was

already so vigorously augmented by trastuzumab, a potential further increase due to NKG2A could not be detected (**Figure 6C**). Spontaneous NK cell degranulation without target cells was below 10% in all conditions (**Figure S6**). Overall, the $NKG2A^+$ NK cell subsets responded slightly stronger than the $NKG2A^-$ NK cells to the HLA-E negative cell lines MCF7 and SKBR3.

Discussion

With the aim to develop effective NK-cell-based therapies against breast cancer, we investigated the combination of the monoclonal antibody trastuzumab and KIR-ligand-mismatched donor NK cells to improve the responses against breast cancer in an immunosuppressive environment. We found that KIR-ligand-mismatched NK cell subsets degranulated stronger against breast cancer than their matched subsets and that trastuzumab activated all NK subsets when HER2 was overexpressed. Importantly, our observations were consistent in a hypoxic environment, emphasizing that the combination of reducing the activation threshold for NK activation by the selection of KIR-ligand-mismatched donors and maximizing NK cell activation with an ADCC-inducing antibody can potentiate the NK cell anti-breast cancer response.

In our experiments with cell lines, we used hypoxia to mimic one of the important factors in the TME. Severe hypoxia has namely been observed in the core of tumors from breast cancer patients and has been associated with metastasis formation and thereby with severity of disease [15]. Moreover, we and others showed that hypoxia can reduce effector functions of unactivated NK cells [20] and that NK cell activation with high-dose IL-2 could restore NK cell cytotoxicity against multiple myeloma [12]. Here, we report that the IL-2-activated NK cell also remained functional against breast cancer.

For SKBR3, we observed a small reduction in NK cell killing potential under hypoxia. This reduction could suggest an adaption to hypoxia in the SKBR3 target cells contributing to resistance to NK cells, e.g., by a reduction in activating ligands or enhanced expression of inhibitory ligands. Resistance could be acquired by the NK cells, e.g., via altered receptor expression, which would result in less efficient NK cell activation. In our previous study on hypoxia, we observed a minor decrease in expression of CD16 and NKG2D but not in any of the other common NK cell receptors [12]. Moreover, on multiple myeloma cell lines, we did not see a change in the expression of stress-induced activating ligands MICA/B and ULBP1/2 [12]. In the present study, we observed that expression of HLA class I was not altered by hypoxia and that IL-2-activated NK cells were potently degranulating under hypoxia. We therefore anticipate that NK cells were activated under hypoxia in this breast cancer setting and that resistance would mainly occur inside SKBR3 target cells. Baginska et al. also demonstrated that hypoxia-induced resistance was not caused by defective recognition of targets cells but by autophagy, leading to the breakdown of NK cells' cytotoxic granules in hypoxic breast cancer cells [14]. To develop future strategies to improve the response in hypoxic tumors, it could be interesting to combine NK-cell-based strategies as proposed in this study with autophagy-reducing strategies such as chloroquine.

Although hypoxia is an important TME factor, our reductionist approach with cell lines did not take the full complexity of the TME into account, which may lead to an underestimation of the impact that hypoxia may have in combination with other TME factors. Our model with primary breast cancer samples showed considerable

variability in sensitivity to NK cells between the different patients, illustrating the potential importance of other TME factors. The exposure of the primary breast cancer cells to the TME in patients is therefore an important advantage of our model, as it enabled us to assess the effects of TME-induced resistance mechanisms to NK cells more comprehensively. Examples of such cell TME resistance mechanisms are changes in the level of autophagy or expression levels of activating or inhibitory ligands on the tumor cells. However, a limitation of the model could be the relatively harsh digestion procedure, which could affect tumor cell viability as well as surface expression of activating ligands MICA and MICB [21], leading to underestimation of the contribution of these ligands. Moreover, it does not predict the direct effects that soluble TME factors and other tumor-associated cells can have on NK cells, which illustrates the necessity to develop more complex in vitro or in vivo models mimicking the multifactorial TME in patients to further evaluate the impact of the TME on NK cell efficiency.

In our study, trastuzumab enhanced NK cell degranulation much more vigorously against HER2-amplified targets compared to non-amplified targets in vitro, and it did not enhance NK cell functions against non-amplified primary breast cancer cells, suggesting that the HER2 expression level is important for the potential of the ADCC response. Our observations are in agreement with previous studies reporting that the effect of monoclonal antibodies such as daratumumab and trastuzumab is specific to target cells expressing high levels of antigen [17,22,23]. HER2 expression can be modulated through receptor internalization [24]. Recently, a study elegantly showed that HER2 internalization could be prevented by endocytosis inhibitors, resulting in an improved ADCC response [25]. In our study, HER2 could be detected after 4 h incubation with trastuzumab, and NK cells were potently degranulating in response to HER2 amplified cells, indicating that HER2 endocytosis cannot be the only factor limiting the ADCC response we observed.

We report a large discrepancy between a potent increase in NK cell degranulation against HER2-amplified target cells but a less pronounced effect on the actual killing of the target cells induced by trastuzumab. We have not previously noticed such a large discrepancy between degranulation and cytotoxicity. When using the same experimental setup with multiple myeloma cells as targets, we observed an ADCC effect with the anti-CD38 antibody daratumumab that was more similar to the degranulation effect [17]. Although further studies are required to unravel the mechanisms behind this observation, the strong degranulation with trastuzumab could indicate a very potent cytokine release by the NK cells. A profound release of cytokines, such as IFN-γ and TNF-α, by NK cells can boost the overall anti-tumor response by stimulating antigen presentation, Th1 polarization, and CD8 effector functions [26]. Studies evaluating the relation between cytokine production and degranulation (CD107a) on a single-cell level demonstrated that NK cells either produce cytokines, express CD107a, or do both upon activation with target cells [27]. Cytokine production has also been shown to occur in an HLA-dependent manner, as KIR-ligand-mismatched NK cells had higher intracellular IFN-γ levels than KIR-ligand-matched cells upon activation with L721.221 target cells [28]. The trastuzumab-

induced degranulation of NK cells described in our study suggests that the combination of trastuzumab and IL-2-activated KIR-ligand-mismatched NK cells may also trigger a stronger production of cytokines, which could contribute to improved adaptive anti-tumor immunity.

In our study, we confirmed the functional relevance of HLA class I as an important inhibitory immune checkpoint for NK cell effector functions in breast cancer. As for many cancers, HLA class I expression can be partially or completely downregulated in breast cancer [29–31]. In our study, we mimicked the absence of HLA class I by using KIR-ligand-mismatched NK cells. In the presence of trastuzumab, KIR-ligand-mismatched NK cells remained the stronger degranulating subset against low HER2-expressing MCF7 cells, and when HER2 expression was high as in SKBR3, trastuzumab led to vigorous degranulation in all subsets, suggesting that the inhibitory ligand HLA class I matters less for the NK cell responses when trastuzumab is present. Muntasell et al. investigated predictive biomarkers for the response to trastuzumab treatment and found that patient stratification based on high HLA class I expression together with infiltrating NK cells improved prediction of better responses [32]. These data are in line with our results showing that NK cells can be effective against HLA class I^+ tumors in combination with trastuzumab. Muntasell's study also supports that both NK cells and T cells are major contributors to the anti-breast cancer response of $HER2^+$ patients.

Our results emphasize that selection of NK cell donors based on their KIR expression and HLA genotype can be an effective way to reduce inhibition in a setting with adoptive transfer of donor NK cells, which could be particularly useful for HER2-negative patients. However, since HER2 expression can be heterogeneously expressed within one tumor or be downregulated in response to trastuzumab treatment [33], KIR-ligand-mismatched NK cells may also be advantageous for HER2-positive patients treated with trastuzumab. Selection of KIR-ligand-mismatched donors is possible for patients that lack at least one of the three HLA epitope groups binding to inhibitory KIRs, which is the case for circa 70% of the population [34]. Although the here-evaluated KIR-ligand-mismatched subsets comprise a rather small percentage of total NK cells, KIR-ligand mismatching helps to reduce inhibition in these subsets, which can nonetheless be beneficial in a TME where many factors can limit NK cell anti-tumor responses. Our analysis indicated that the $NKG2A^+$ KIR-ligand-mismatched NK cells performed as well as or slightly better than their $NKG2A^-$ counterpart against HLA-E negative target cells. This observation implies that the $NKG2A^+$ KIR-ligand-mismatched subsets can also be considered fully mismatched against HLA-E negative targets, which can significantly enlarge the mismatched NK cell population since NKG2A is expressed on 20–80% of NK cells. In breast cancer patients, HLA-E expression was detected in 20–50% of samples [30,35]. High HLA-E expression can, however, inhibit NK cell responses [36]. We did not have breast cancer cells available that expressed HLA-E. However, in a previous study with the same experimental setup, we showed that high HLA-E levels in multiple myeloma cells inhibited NK cells, while low HLA-E levels were not sufficient to do so [36]. The

inhibitory potential of HLA-E has been established in multiple tumor models [37]. Therefore, it seems likely that high HLA-E expression will also limit NK cell anti-breast-cancer responses, and in those cases, blocking antibodies such as the anti-NKG2A antibody monalizumab might need to be considered. It would be relevant to further evaluate the additive effect of this approach on primary breast cancer with high levels of HLA-E.

Based on our results, we envision that alloreactive donors should be selected for NK cell-based therapies against HLA class I+ breast cancer. Multiple clinical trials showed that infusion of alloreactive NK cells is well tolerated when combined with lymphodepleting chemotherapy to suppress the host's immune response. It needs to be assessed whether KIR-ligand mismatching can further enhance trastuzumab-induced NK cell degranulation in a setup that better represents the complex TME where many factors can limit NK cell anti-tumor responses. Genetic manipulation of NK cells could be a second strategy to limit inhibitory signaling via HLA, which could be done by CRISPR/CAS9-mediated knock-out of inhibitory receptors such as NKG2A and KIR. Another attractive strategy could be temporarily reducing expression levels of the inhibitory receptors via silencing RNAs. Given the critical role of NKG2A and KIR in NK cell licensing, transient reduction of receptor expression may be especially relevant when full receptor knock-outs negatively influence NK cell potency.

Conclusions

In this study, we showed that IL-2-activated NK cells can mediate anti-breast cancer responses under hypoxic conditions. Our data also illustrated the relevance of HLA class I as an inhibitory immune-checkpoint for NK cells in breast cancer, prompting follow-up studies to enhance NK cell responses by the combination of ADCC-triggering antibodies and strategies to interfere with the interaction between HLA class I and KIR/NKG2A. Here, we demonstrated two strategies, ADCC-triggering by the anti-HER2 antibody trastuzumab and selection of KIR-ligand-mismatched NK cell donors, to induce strong NK cell degranulation. Other strategies to interfere with HLA class I inhibition could include monoclonal antibodies that block inhibitory receptors on NK cells, such as monalizumab, or genetically modified NK cells to reduce expression of inhibitory NK cell receptors, as this would reduce the threshold for NK cell activation and may contribute to the development of curative immunotherapeutic strategies for breast cancer patients.

Author Contributions: conceptualization, F.A.I.E., M.v.G., G.M.J.B. and L.W.; methodology, F.A.I.E., N.B., and L.F.S.K.; formal analysis, F.A.I.E., N.B., and T.M.J.E.; investigation, F.A.I.E., N.B., and T.M.J.E.; resources, L.F.S.K., M.L.S., and L.W.; writing—original draft preparation, F.A.I.E. and L.W.; writing—review and editing, N.B., M.v.G., T.M.J.E., M.L.S., L.F.S.K., and G.M.J.B.; supervision, L.W. and G.M.J.B.; funding acquisition, L.W. and G.M.J.B. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by Kankeronderzoeksfonds Limburg (KOFL).

Institutional Review Board Statement: The study using human leftover material was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethics Committee of Maastricht University Medical Center+ (METC 2019-1015, 01 July 2019). The animal experiments were approved by the Animal Ethics Committee of Maastricht University (DEC-UM 2014-095, 04-08-2015).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available from the corresponding author upon reasonable request.

Acknowledgements: The authors would like to thank the Department of Transplantation Immunology at Maastricht University Medical Center+ for performing the genotyping. The graphical abstract was created with BioRender.com (accessed on 7 June 2021).

Conflicts of Interest: G.M.J.B. is Chief Executive Officer/Chief Medical Officer/Cofounder of CiMaas, BV, Maastricht, The Netherlands. CiMaas is producing an ex vivo expanded NK cell product that will be used to treat myeloma patients.

References:

1. Torre, L.A.; Islami, F.; Siegel, R.L.; Ward, E.M.; Jemal, A. Global cancer in women: Burden and trends. Cancer Epidemiol. Prev. Biomark. 2017, 26, 444–457.
2. Waks, A.G.; Winer, E.P. Breast cancer treatment: A review. JAMA 2019, 321, 288–300.
3. Wolff, A.C.; Hammond, M.E.H.; Allison, K.H.; Harvey, B.E.; Mangu, P.B.; Bartlett, J.M.; Bilous, M.; Ellis, I.O.; Fitzgibbons, P.; Hanna, W. Human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncolo-gy/College of American Pathologists clinical practice guideline focused update. Arch. Pathol. Lab. Med. 2018, 142, 1364–1382.
4. Nahta, R.; Esteva, F.J. Herceptin: Mechanisms of action and resistance. Cancer Lett. 2006, 232, 123–138.
5. Shimasaki, N.; Jain, A.; Campana, D. NK cells for cancer immunotherapy. Nat. Rev. Drug Discov. 2020, 19, 200–218.
6. Vivier, E.; Ugolini, S.; Blaise, D.; Chabannon, C.; Brossay, L. Targeting natural killer cells and natural killer T cells in cancer. Nat. Rev. Immunol. 2012, 12, 239–252.
7. Kadri, N.; Wagner, A.K.; Ganesan, S.; Kärre, K.; Wickström, S.; Johansson, M.H.; Höglund, P. Dynamic regulation of NK cell responsiveness. Nat. Kill. Cells 2015, 95-114.
8. Habif, G.; Crinier, A.; André, P.; Vivier, E.; Narni-Mancinelli, E. Targeting natural killer cells in solid tumors. Cell. Mol. Im-munol. 2019, 16, 415–422.
9. Frings, P.W.; Van Elssen, C.H.; Wieten, L.; Matos, C.; Hupperets, P.S.; Schouten, H.C.; Bos, G.M.; van Gelder, M. Elimination of the chemotherapy resistant subpopulation of 4T1 mouse breast cancer by haploidentical NK cells cures the vast majority of mice. Breast Cancer Res. Treat. 2011, 130, 773–781.
10. Ruggeri, L.; Mancusi, A.; Capanni, M.; Urbani, E.; Carotti, A.; Aloisi, T.; Stern, M.; Pende, D.; Perruccio, K.; Burchielli, E.; et al. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: Challenging its predictive value. Blood 2007, 110, 433–440, doi:10.1182/blood-2006-07-038687.
11. Ruggeri, L.; Capanni, M.; Urbani, E.; Perruccio, K.; Shlomchik, W.D.; Tosti, A.; Posati, S.; Rogaia, D.; Frassoni, F.; Aversa, F. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science 2002, 295, 2097–2100.
12. Sarkar, S.; Germeraad, W.T.; Rouschop, K.M.; Steeghs, E.M.; van Gelder, M.; Bos, G.M.; Wieten, L. Hypoxia induced im-pairment of NK cell cytotoxicity against multiple myeloma can be overcome by IL-2 activation of the NK cells. PLoS ONE 2013, 8, e64835, doi:10.1371/journal.pone.0064835.
13. Ni, J.; Wang, X.; Stojanovic, A.; Zhang, Q.; Wincher, M.; Bühler, L.; Arnold, A.; Correia, M.P.; Winkler, M.; Koch, P.-S. Sin-gle-cell RNA sequencing of tumor-infiltrating NK cells reveals that inhibition of transcription factor HIF-1α unleashes NK cell activity. Immunity 2020, 52, 1075–1087.e1078.
14. Baginska, J.; Viry, E.; Paggetti, J.; Medves, S.; Berchem, G.; Moussay, E.; Janji, B. The critical role of the tumor microenvi-ronment in shaping natural killer cell-mediated anti-tumor immunity. Front. Immunol. 2013, 4, 490, doi:10.3389/fimmu.2013.00490.
15. Vaupel, P.; Höckel, M.; Mayer, A. Detection and characterization of tumor hypoxia using pO2 histography. Antioxid. Redox Signal. 2007, 9, 1221–1236.
16. Milani, M.; Harris, A.L. Targeting tumour hypoxia in breast cancer. Eur. J. Cancer 2008, 44, 2766–2773.
17. Mahaweni, N.M.; Bos, G.M.; Mitsiades, C.S.; Tilanus, M.G.; Wieten, L. Daratumumab augments alloreactive natural killer cell cytotoxicity towards CD38+ multiple myeloma cell lines in a biochemical context mimicking tumour microenvironment conditions. Cancer Immunol. Immunother. 2018, 67, 861–872, doi:10.1007/s00262-018-2140-1.
18. Balsamo, M.; Manzini, C.; Pietra, G.; Raggi, F.; Blengio, F.; Mingari, M.C.; Varesio, L.; Moretta, L.; Bosco, M.C.; Vitale, M. Hypoxia downregulates the expression of activating receptors involved in NK-cell-mediated target cell killing without af-fecting ADCC. Eur. J. Immunol. 2013, 43, 2756–2764, doi:10.1002/eji.201343448.

19. André, P.; Denis, C.; Soulas, C.; Bourbon-Caillet, C.; Lopez, J.; Arnoux, T.; Bléry, M.; Bonnafous, C.; Gauthier, L.; Morel, A. Anti-NKG2A mAb is a checkpoint inhibitor that promotes anti-tumor immunity by unleashing both T and NK cells. Cell 2018, 175, 1731–1743.e1713.
20. Parodi, M.; Raggi, F.; Cangelosi, D.; Manzini, C.; Balsamo, M.; Blengio, F.; Eva, A.; Varesio, L.; Pietra, G.; Moretta, L. Hypoxia modifies the transcriptome of human NK cells, modulates their immunoregulatory profile, and influences NK cell subset migration. Front. Immunol. 2018, 9, 2358.
21. MiltenyiBiotec. Epitope Preservation List TDK, Human. Available online: https://www.miltenyibiotec.com/US-en/products/tumor-dissociation-kit-human.html (accessed on 1 May 2021).
22. Boero, S.; Morabito, A.; Banelli, B.; Cardinali, B.; Dozin, B.; Lunardi, G.; Piccioli, P.; Lastraioli, S.; Carosio, R.; Salvi, S.; et al. Analysis of in vitro ADCC and clinical response to trastuzumab: Possible relevance of FcgammaRIIIA/FcgammaRIIA gene polymorphisms and HER-2 expression levels on breast cancer cell lines. J. Transl. Med. 2015, 13, 324, doi:10.1186/s12967-015-0680-0.
23. Tang, Y.; Lou, J.; Alpaugh, R.K.; Robinson, M.K.; Marks, J.D.; Weiner, L.M. Regulation of antibody-dependent cellular cy-totoxicity by IgG intrinsic and apparent affinity for target antigen. J. Immunol. 2007, 179, 2815–2823.
24. Bertelsen, V.; Stang, E. The mysterious ways of ErbB2/HER2 trafficking. Membranes 2014, 4, 424–446.
25. Chew, H.Y.; De Lima, P.O.; Cruz, J.L.G.; Banushi, B.; Echejoh, G.; Hu, L.; Joseph, S.R.; Lum, B.; Rae, J.; O'Donnell, J.S. Endocytosis inhibition in humans to improve responses to ADCC-mediating antibodies. Cell 2020, 180, 895–914.e827.
26. Mailliard, R.B.; Son, Y.-I.; Redlinger, R.; Coates, P.T.; Giermasz, A.; Morel, P.A.; Storkus, W.J.; Kalinski, P. Dendritic cells mediate NK cell help for Th1 and CTL responses: Two-signal requirement for the induction of NK cell helper function. J. Immunol. 2003, 171, 2366–2373, doi:10.4049/jimmunol.171.5.2366.
27. Bryceson, Y.T.; Fauriat, C.; Nunes, J.M.; Wood, S.M.; Björkström, N.K.; Long, E.O.; Ljunggren, H.-G. Functional analysis of human NK cells by flow cytometry. Methods Mol Bio 2010; pp. 335–352.
28. Yu, J.; Heller, G.; Chewning, J.; Kim, S.; Yokoyama, W.M.; Hsu, K.C. Hierarchy of the human natural killer cell response is determined by class and quantity of inhibitory receptors for self-HLA-B and HLA-C ligands. J. Immunol. 2007, 179, 5977–5989.
29. Garrido, M.A.; Rodriguez, T.; Zinchenko, S.; Maleno, I.; Ruiz-Cabello, F.; Concha, Á.; Olea, N.; Garrido, F.; Aptsiauri, N. HLA class I alterations in breast carcinoma are associated with a high frequency of the loss of heterozygosity at chromosomes 6 and 15. Immunogenetics 2018, 70, 647–659.
30. Silva, G.B.R.F.d.; Silva, T.G.A.; Duarte, R.A.; Neto, N.L.; Carrara, H.H.A.; Donadi, E.A.; Gonçalves, M.A.G.; Soares, E.G.; Soares, C.P. Expression of the classical and nonclassical HLA molecules in breast cancer. Int. J. Breast Cancer 2013, 2013, 250435.
31. Lee, H.J.; Song, I.H.; Park, I.A.; Heo, S.-H.; Kim, Y.-A.; Ahn, J.-H.; Gong, G. Differential expression of major histocompati-bility complex class I in subtypes of breast cancer is associated with estrogen receptor and interferon signaling. Oncotarget 2016, 7, 30119.
32. Muntasell, A.; Rojo, F.; Servitja, S.; Rubio-Perez, C.; Cabo, M.; Tamborero, D.; Costa-García, M.; Martínez-Garcia, M.; Menéndez, S.; Vazquez, I. NK cell infiltrates and HLA class I expression in primary HER2+ breast cancer predict and un-couple pathological response and disease-free survival. Clin. Cancer Res. 2019, 25, 1535–1545.
33. Seol, H.; Lee, H.J.; Choi, Y.; Lee, H.E.; Kim, Y.J.; Kim, J.H.; Kang, E.; Kim, S.-W.; Park, S.Y. Intratumoral heterogeneity of HER2 gene amplification in breast cancer: Its clinicopathological significance. Mod. Pathol. 2012, 25, 938–948.
34. Ruggeri, L.; Mancusi, A.; Urbani, E.; Velardi, A. Identifying NK alloreactive donors for haploidentical hematopoietic stem cell transplantation. In Tumor Immunology; Springer: 2016; pp. 141–145.

35. de Kruijf, E.M.; Sajet, A.; van Nes, J.G.; Natanov, R.; Putter, H.; Smit, V.T.; Liefers, G.J.; van den Elsen, P.J.; van de Velde, C.J.; Kuppen, P.J. HLA-E and HLA-G expression in classical HLA class I-negative tumors is of prognostic value for clinical out-come of early breast cancer patients. J. Immunol. 2010, 185, 7452–7459.
36. Mahaweni, N.M.; Ehlers, F.A.; Sarkar, S.; Janssen, J.W.; Tilanus, M.G.; Bos, G.M.; Wieten, L. NKG2A expression is not per se detrimental for the anti-multiple myeloma activity of activated natural killer cells in an in vitro system mimicking the tumor microenvironment. Front. Immunol. 2018, 9, 1415.
37. Wieten, L.; Mahaweni, N.; Voorter, C.; Bos, G.; Tilanus, M. Clinical and immunological significance of HLA-E in stem cell transplantation and cancer. Tissue Antigens 2014, 84, 523–535.

Supplementary Materials

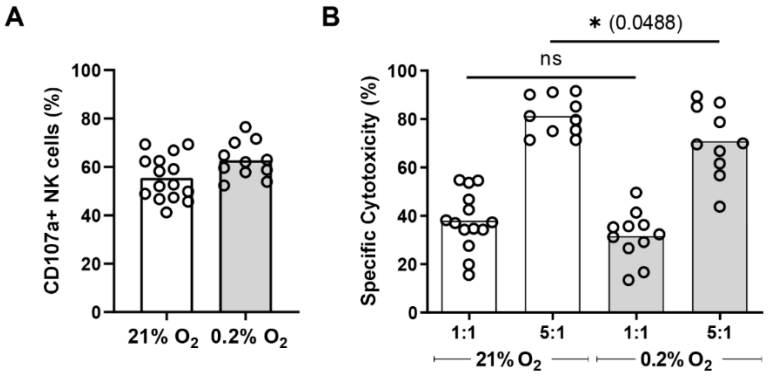


Figure S1. Anti-tumor efficacy of NK cell donors against the control target cell line K562. The HLA class I-negative cell line K562 was included as control target cells to test the NK cell killing potential of each donor. The degranulation (CD107a) and cytotoxicity assays were performed in the same way as described in Figure 1: IL-2 activated NK cells were co-cultured with the target cell line K562 for 4 h either with 21% O2 or 0.2% O2. Assays were measured by flow cytometry. (A) CD107a assays were performed in 1:1 E:T ratios and % of CD107a+ NK cells are shown per donor. (B) Cytotoxicity assays were done in 1:1 or 5:1 E:T ratios and dead target cells are shown as % of specific cytotoxicity. Each dot represents the average of duplicates from one NK cell donor and the bar height indicates the mean. * $p < 0.05$, ns = not significant.

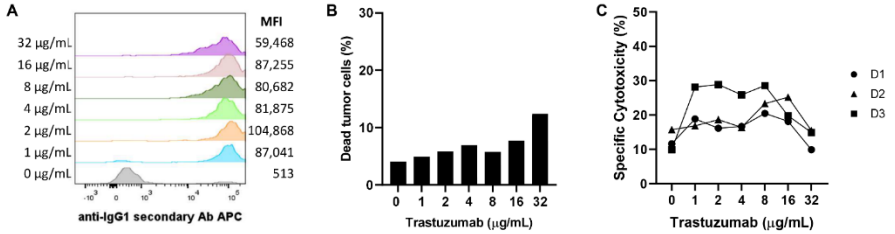


Figure S2. Titration of Trastuzumab antibody with target cell line SKBR3. SKBR3 cells were incubated with 0–32 µg/mL Trastuzumab for 30 min before either medium or IL-2 activated NK cells in a 1:1 E:T ratio were added for 4 h at 21% O_2. Analysis was performed by flow cytometry. (A) Binding of Trastuzumab to SKBR3 was detected by indirect staining with anti-IgG1 secondary antibody. (B) The spontaneous cell death of SKBR3 is reported as percentage of dead tumor cells. (C) Specific cytotoxicity of SKBR3 is shown. Each symbol represents one donor and the average of duplicates is depicted per donor.

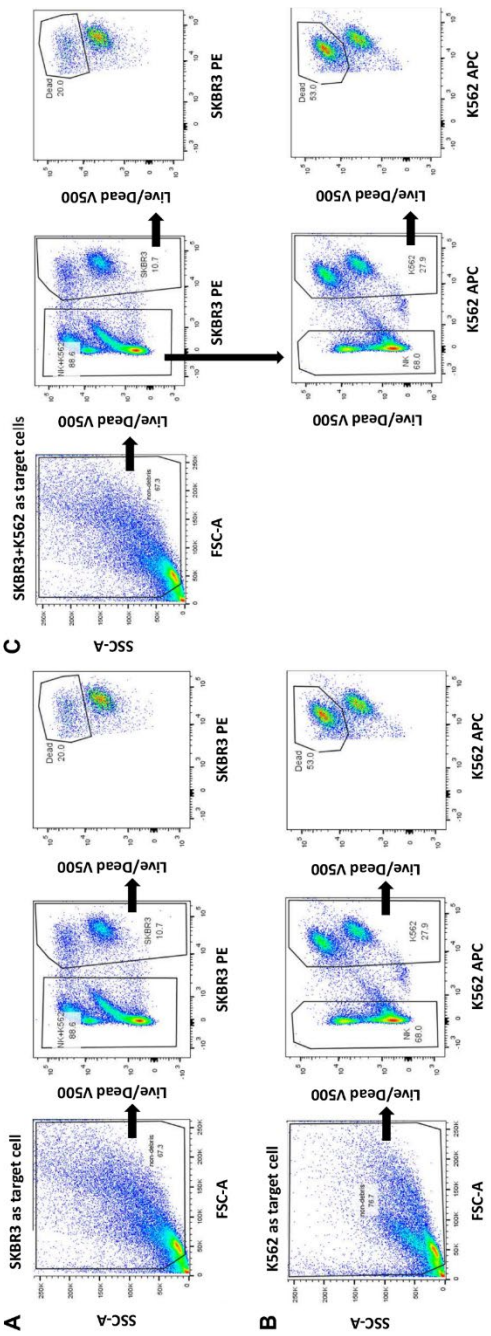


Figure S3. Gating strategy for the cytotoxicity assays with SKBR3 and K562 as target cells. First, debris was gated out, followed by gating on tumor cells that were labeled with a dye and subsequently dead tumor cells were gated. Representative example is shown for SKBR3 (A), K562 (B), SKBR3 and K562 combined (C).

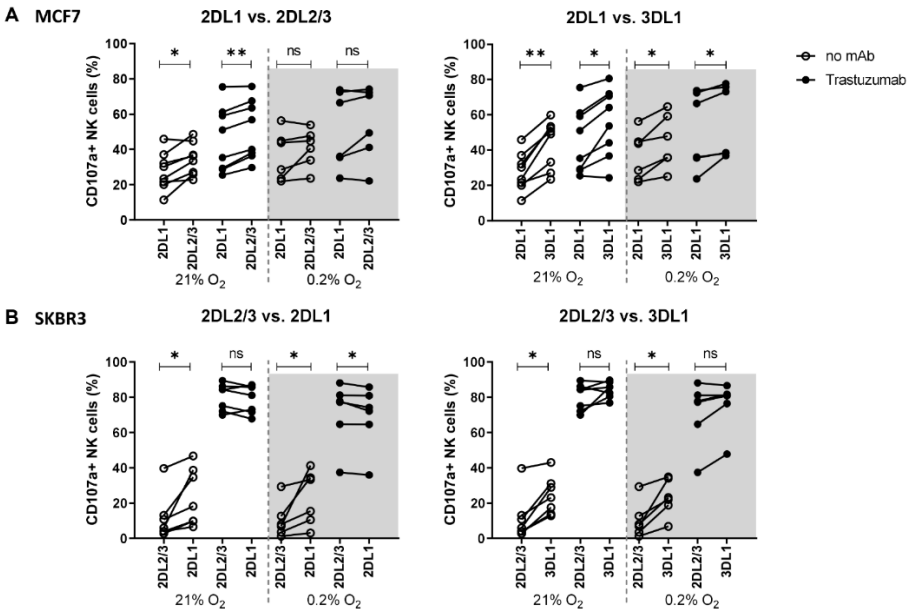


Figure S4. NK cell degranulation depicted per KIR receptor in response to MCF7 and SKBR3. Degranulation assays were performed by co-culturing NK wells either with MCF7 or SKBR3 target cells with or without Trastuzumab for 4 h at 21% O_2 or 0.2% O_2. NK cells that expressed one of the KIR receptors 2DL1, 2DL2/3, 3DL1 were selected by gating and the degranulation (% CD107a) is presented for each subset in response to MCF7 (A) or SKBR3 (B). Each dot represents the average of duplicates from one NK cell donor. * $p < 0.05$, ** $p < 0.01$, ns = not significant.

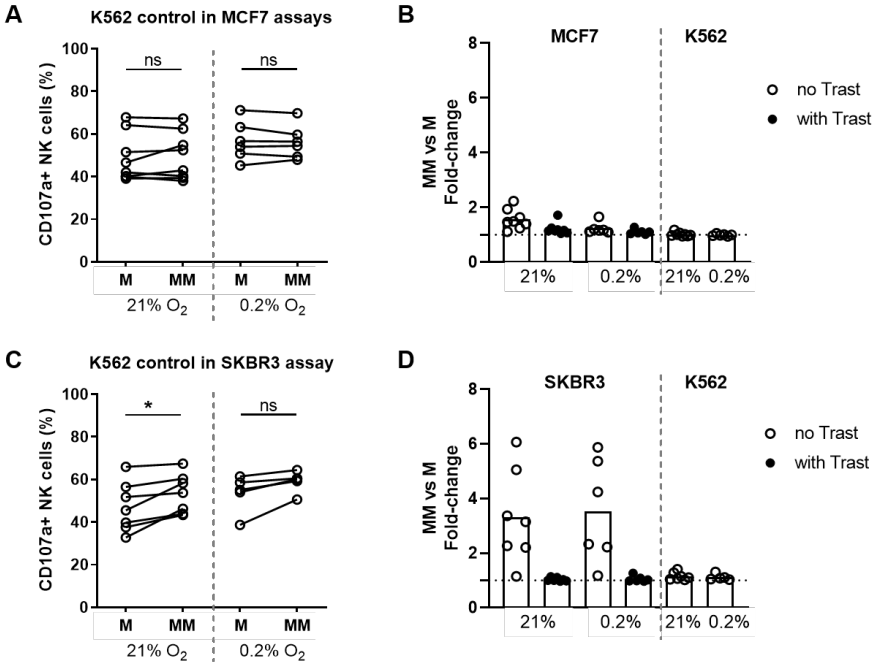


Figure S5. NK cell degranulation of NKG2A$^-$ KIR-ligand matched and mismatched NK cells depicted in response to K562 and depicted as fold-change compared to matched NK cells. (A, C) The degranulation potential of the same KIR-ligand matched (M) and -mismatched (MM) NK cell subsets that were determined in response to MCF7 and SKBR3 in Figure 4 were analyzed for the HLA-negative control cell line K562. The fold-change in degranulation of the mismatched subset compared to the matched subset was calculated for MCF7 and K562 control (B) and SKBR3 and K562 control (D) where a value of 1 indicates no change. Each dot represents the average of duplicates from one NK cell donor. * $p < 0.05$, ns = not significant.

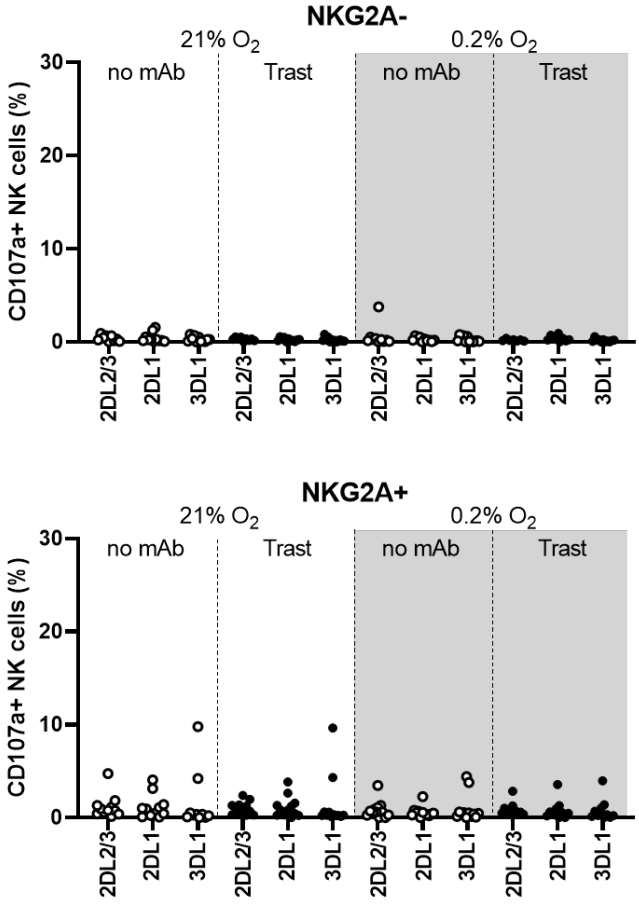


Figure S6. NKG2A and KIR subset analysis of spontaneous degranulation in NK cells without target cells. NK cells were cultured with or without Trastuzumab for 4 h at 21% O_2 or 0.2% O_2. By flow cytometry analysis, NK cells were grouped in NKG2A$^-$ subsets (above) and NKG2A$^+$ subsets (below) and the spontaneous degranulation of NK cells is presented as % CD107a+ cells for the three inhibitory KIRs 2DL2/3, 2DL1 and 3DL1. Each dot represents one NK cell donor.

Profiling NK cells within the tumor microenvironment of HER2$^-$ and HER2$^+$ human breast cancer

Femke A. I. Ehlers, Courtney B. Betts, Shamilene Sivagnanam, Loes F. S. Kooreman, E. Shelley Hwang, Gerard M. J. Bos, Lotte Wieten[#] and Lisa M. Coussens[#]
[#]Co-senior authors

Manuscript in preparation.

6

Activated natural killer cells withstand the relatively low glucose concentrations found in the bone marrow of multiple myeloma patients

Femke A. I. Ehlers*, Niken M. Mahaweni*, Timo I. Olieslagers, Gerard M. J. Bos and Lotte Wieten
* These authors contributed equally to this work

Frontiers in Oncology 2021 May 20;11:622896.
doi: 10.3389/fonc.2021.622896

Abstract
Infusion of ex vivo expanded and cytokine-activated natural killer (NK) cells is a promising alternative way to treat multiple myeloma (MM). However, the tumor microenvironment (TME) may suppress their function. While reduced glucose availability is a TME hallmark of many solid tumors, glucose levels within the TME of hematological malignancies residing in the bone marrow (BM) remain unknown. Here, we measured glucose levels in the BM of MM patients and tested the effect of different glucose levels on NK cells. BM glucose levels were measured using a biochemical analyzer. Compared to the normal range of blood glucose, BM glucose levels were lower in 6 of 9 patients (479-1231 mg/L; mean=731.8 mg/L). The effect of different glucose levels on NK cell cytotoxicity was tested in 4-hour cytotoxicity assays with tumor cells. 500 mg/L glucose (representing low range of MM BM) during the 4-hour cytotoxicity assay did not negatively affect cytotoxicity of activated NK cells, while higher glucose concentrations (4000 mg/L) diminished NK cell cytotoxicity. Since clinical application of NK cell therapy might require ex vivo expansion, expanded NK cells were exposed to a range of glucose concentrations from 500-4000 mg/L for a longer period (4 days). This did not reduce cytotoxicity or IFN-γ secretion nor affected their phenotypic profile. In summary, low glucose concentrations, as found in BM of MM patients, by itself did not compromise the anti-tumor potential of IL-2 activated NK cells *in vitro*. Although follow up studies in models with a more complex TME would be relevant, our data suggest that highly activated NK cells could be used to target tumors with a reduced glucose environment.

Keywords: NK cells, multiple myeloma, immunotherapy, tumor microenvironment, glucose.

Introduction

In the last decade, considerable effort has been put in the development of NK cell-based immunotherapy to treat cancer patients due to the clinical potential and good safety profile of NK cells. Multiple clinical trials using either autologous or allogeneic NK cells in various types of hematological- and solid cancers have demonstrated that NK cells could exert anti-tumor responses in patients without significant toxicity [1-3]. Mostly, ex vivo expanded and cytokine-activated NK cells are used to create highly cytotoxic NK cells. Nonetheless, despite these initially hopeful clinical outcomes, the therapeutic efficacy of NK cell-based immunotherapy could be improved by increasing NK-cell numbers, enhancing NK-cell activation, improving NK-cell tumor-targeting capacity, and improving in vivo NK-cell persistence [1].

The expansion and persistence of NK cells in vivo has been demonstrated to be positively correlated with the clearance of leukemic cells in patients receiving adoptive NK cell therapy [4]. However, the microenvironment of tumor cells could be unfavorable and even suppressive for NK cells allowing tumor cells to escape the NK-cell antitumor response. To be able to survive in the tumor microenvironment (TME), NK cells require cytokines such as IL-2 or IL-15 that can be produced by several cell types present in the TME, but the available amount might not be enough [5] [6]. The presence of other cytokines such as transforming growth factor-beta (TGF-ß) and IL-10 in the TME may play a role in the suppression of IL-2 production [7]. NK cell antitumor capacity can be hindered by TME factors such as myeloid derived suppressor cells [8], hypoxia [9, 10], or factors released by the tumor cells such as prostaglandin E2, TGF-ß, IL-10, reactive oxygen species, and arginase [11-13]. Additionally, the metabolic microenvironment of tumor cells could inhibit the antitumor response of immune cells such as cytotoxic T cells and NK cells [14]. To sustain their growth and survival, tumor cells frequently undergo metabolic reprogramming, allowing the enhancement of glucose uptake and metabolism. This process takes place not only within a hypoxic region but also in the area where sufficient oxygen is available, a phenomenon known as aerobic glycolysis or “the Warburg effect” [15]. Aerobic glycolysis is favorable for proliferating cells since it can provide both bioenergetics and biosynthesis requirements better than oxidative phosphorylation (OxPhos) [14]. Due to high rates of glycolysis, the glucose supply in the tumor microenvironment can be limited. In solid tumors, glucose availability is inversely correlated with the distance from the capillaries to the tumor and glucose levels as low as 20 mg/L have been reported for colon cancer with great intratumoral variability [16, 17]. To our knowledge, there is not much known about glucose levels within the microenvironment of hematological cancers.

Aerobic glycolysis appeared to be not only advantageous for tumor cells. Immune cells, such as cytotoxic T cells, have been shown to require a switch to aerobic glycolysis to exert their effector function [18]. Since both tumor cells and T cells are glycolytic, metabolic competition can occur within the tumor microenvironment. The glycolytic activity of the tumor cells can cause depletion of extracellular glucose thereby limiting the availability of glucose to T cells [19]. In mice, it has been

demonstrated that this metabolic competition hindered T-cell metabolism resulting in a defective IFN-ɣ production which is crucial for antitumor response [20, 21]. In NK cells, aerobic glycolysis has also been shown to be important for a potent NK-cell effector function. In mice, resting NK cells preferred OxPhos for their metabolism while highly activated NK cells enhanced especially glycolysis and to a lower extend OxPhos [22-24]. In humans, NK cells upregulated both glycolysis and OxPhos upon cytokine stimulation with IL-2 or IL-12/15 [25]. Additionally, $CD56^{bright}$ NK cells were found to be metabolically more active than $CD56^{dim}$ NK cells [25]. The same group also showed that elevated levels of OxPhos were essential for NK cell effector cytotoxicity and IFN-ɣ production.

Our group focuses on the development and refinement of NK-cell based immunotherapy to treat patients with cancer, especially multiple myeloma (MM) as there is no cure available to date for MM. We envision to inject a high number of highly activated NK cells to patients with MM. Glucose levels in the MM microenvironment remain unknown but may be important in controlling the anti-MM response of NK cells as NK cells use glucose as primary source of energy [26]. We, therefore, aimed to explore the possible consequences of MM metabolic microenvironment on the antitumor potential of activated and expanded NK cells. First, we investigated the glucose levels present in the microenvironment of patients having active MM to define the relevant in vitro experimental conditions. Second, based on these results, we performed 4-hour cytotoxicity assays in vitro to study the effect of short-term exposure to different glucose concentrations on NK cell cytotoxicity against tumor cells. Third, we studied the influence of longer exposure to the different glucose concentrations on expanded NK cells to evaluate whether NK cell effector functions could be optimized by adapting glucose levels during expansion. The results from this current study give us a better understanding whether in vivo glucose concentrations should be a concern for the NK-cell based immunotherapy and whether eventually an intervention might be needed to improve the therapy.

Materials and Methods

Glucose measurement

Leftover fresh BM samples were obtained from MM patients with active disease. The use of leftover material from clinical procedures did not require ethical approval in the Netherlands under the Dutch Code for Proper Secondary Use of Human Tissue. None of the patients objected to the use of leftover material. When feasible, samples were measured directly as a whole BM harvest. Otherwise, samples were centrifuged with speed 1170 g for 15 minutes at 4° Celsius, followed by harvesting of the "plasma" fraction which was stored in -20° Celsius before the glucose measurement was performed using YSI biochemical analyzer (Salm en Kipp, BV). Freezing the samples had minor influence on the glucose concentration, similar to variation between duplicates. Samples were measured in duplicate and the average per sample was reported.

Cell lines and culture media

The K562 cell line, purchased from ATCC, was cultured in IMDM (Gibco) and supplemented with 10% fetal calf serum (FCS) (Greiner Bio-One International, GmbH), 100 U/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco) (1% Pen/Strep). The RPMI-8226 cell line, purchased from DSMZ, was cultured in standard RPMI-1640 medium. Standard RPMI-1640 medium refers to RPMI-1640 medium containing 2000 mg/L glucose (Cat. 11554516, ThermoFisher) and was supplemented with 10% FCS and 1% Pen/Strep for all cultures. Glucose-free RPMI-1640 medium (Cat. 11560406, ThermoFisher) was supplemented with D-(+)-Glucose (Sigma) as indicated in the individual figures and with 10% FCS and 1% Pen/Strep. All cell culture experiments were performed at 37° C in an incubator with 5% CO_2 and 21% O_2 (Sanyo MCO-20AIC, Sanyo Electric Co, Japan).

NK cell culture and NK cell expansion

NK cells were isolated from anonymous buffy coats (Sanquin blood bank, Maastricht). The use of buffy coats does not need ethical approval in the Netherlands under the Dutch Code for Proper Secondary Use of Human Tissue. Peripheral blood mononuclear cells were isolated from the buffy coats by density centrifugation using Lymphoprep (Axis-Shield). Subsequently, NK cells were isolated using the NK cell isolation kit according to the manufacturer's protocol (Miltenyi Biotec, GmbH). For experiments in **Figure 2**, NK cells were activated overnight with 1000 U/mL recombinant human IL-2 (Proleukin, Novartis) either in standard RPMI-1640 medium containing 2000 mg/L glucose (Gibco, **Figure 2A**) or in glucose-free RPMI-1640 medium (Gibco) supplemented with the glucose concentration indicated in the **figure 2B** (named 'culture condition'). For experiments in **Figure 3-4**, NK cells were expanded from CD3-depleted PBMCs in the presence of 1000 U/mL IL-2 in alpha-medium (Biochrom, GmbH) supplemented with 10% human serum (Milan Analytica, AG), 2mM L-GlutaMax (Gibco), 1.3 g/L sodium bicarbonate (Biochrom), 2000 mg/L glucose (Sigma), and 0.5% Gentamycin-Sulphate (Gibco). The cells were expanded

with IL-2 for 16-22 days during which the NK cells expanded 10- to 20-fold. After expansion, the NK cells were subsequently cultured for 4 days in glucose-free RPMI-1640 medium supplemented with the glucose concentration indicated in the figure and with 1000 U/mL IL-2 (culture condition), followed by cytotoxicity assays and ELISA assays.

Labeling of tumor cells lines for cytotoxicity assays

One day prior to the cytotoxicity assay, 2 x 10^6 cells/ml K562 cells or RPMI-8226 cells were labeled with 3 µl Vybrant CM-DiI Cell-Labeling solution (Thermo Fisher) in PBS according to the manufacturer's instruction. After adding CM-DiI to the cell suspension, cells were incubated for 5 minutes at 37°C followed by 15 minutes at 4° C in the fridge. After the last incubation, cells were washed 2 times with PBS and centrifuged (280g for 8 minutes at room temperature). K562 or RPMI-8226 cells were then resuspended in IMDM or standard RPMI-1640 medium, respectively, supplemented with 10% FCS and 1% Penicillin/Streptomycin and incubated overnight at 37°C.

Cytotoxicity assay

On the day of the assay, the DiI-labeled tumor cells were harvested and 2x10^4 cells per well were plated in a 96-wells plate for the cytotoxicity assay. NK cells were harvested, washed and co-cultured with the tumor cells in 1:1 Effector:Target (E:T) ratio for 4 hours in the presence of different glucose concentrations (named 'killing condition') as indicated in the figures. See section 4.6 for subsequent Live/Dead staining. Specific cytotoxicity was calculated as follows: (% dead tumor cells - % spontaneous tumor cell death)/(100% - % spontaneous tumor cell death) x100.

Staining and flow cytometry

After a 4-hour cytotoxicity assay, cells were washed with PBS (Gibco) and stained for dead cells using Live/Dead® Fixable Aqua Dead Cell Stain Kit (Molecular Probes™) for 30 minutes on ice in the dark. Cells were further washed with PBS buffer (PBS, 1% FCS) and fixed with 1% paraformaldehyde in PBS solution. For NK cell phenotyping, expanded NK cells were harvested after culturing in either 500, 2000, or 4000 mg/L glucose for 4 days. Subsequently, NK cells were washed in PBS and first stained for dead cells using Live/Dead® Fixable Aqua Dead Cell Stain Kit (Molecular Probes™) for 30 minutes on ice in the dark, and subsequently stained for the following surface markers for 30 minutes on ice and in the dark: CD3-APC-Vio770, -VioBlue or -PerCP-Vio700; CD56-PE-Vio770 or -APC-Vio770; KIR2DL2/3-PE; KIR3DL1-PerCP; NKG2A-APC; KIR2DL1-FITC; NKp30-PE-Vio770; NKp44-VioBright; NKp46-PE; NKG2D-APC; PD1-VioBright; DNAM1-PE; CD96-PE-Vio770; NKG2C-APC; LAG3-VioBlue; TIM3-APC; TIGIT-PE. FMO controls were stained for Live/Dead, CD3 and CD56. All antibodies were purchased from Miltenyi Biotech. All flow cytometric analyses were performed with BD FACS Canto II. Data were analyzed with FlowJo 10.1r5 64-bit software.

ELISA assay

Supernatants of the cytotoxicity assays were collected and analyzed for IFN-ɣ levels using the PeliKine compactTM ELISA kit (Sanquin). The samples were thawed at room temperature and diluted 1:2 before use. ELISA assays were performed according to the manufacturer's instructions.

Statistical analysis

All statistical analysis was performed with GraphPad Prism 8.3 (GraphPad Software Inc, San Diego, CA, USA) using non-parametric paired t-test (Wilcoxon matched pairs test) for **figure 2A** or 2-way ANOVA when comparing multiple groups. * indicates a p-value of <0.05, ** indicates a p-value of <0.01.

Results

Glucose concentrations in the BM of MM patients are on average lower than normal blood glucose levels

To get an indication of glucose levels in the bone marrow (BM), glucose levels were measured in BM samples from MM patients. The glucose concentration in the BM of MM patients ranged between 479 to 1231 mg/L (mean = 731.8 mg/L, SD = 247.6). Compared to normal glucose levels in peripheral blood, which are < 1100 mg/L (<6.1 mmol/L) fasting or <1400 mg/L (<7.8 mmol/L) random [27], 6 of 9 MM patients had lower glucose levels with the lowest concentration reported here being twice as low as normal blood glucose levels (**Figure 1**).

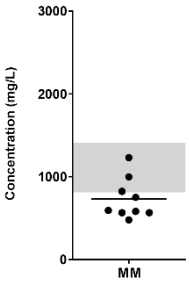


Figure 1. Glucose concentration in the BM of MM patients. BM samples from newly diagnosed MM patients were collected and glucose concentrations were determined using biochemical analyzer (YSI). Grey bar indicates the reference value range for normal fasting blood glucose (4.4 – 6.1 mmol/L or 820 – 1100 mg/L up to 1400 mg/L postprandial). n = 9 subjects.

Relatively low glucose concentrations present during killing do not affect NK cell killing capacity while high glucose reduced killing efficacy

Glucose has been described to be an important fuel for NK cells. Therefore, we tested the effect of the glucose levels as observed in BM of MM patients, the glucose levels in normal blood, and the glucose levels used in culture media on the cytotoxic capacity of NK cells derived from healthy donors. We activated NK cells overnight with 1000 U/mL IL-2 in standard RPMI-1640 medium containing 2000 mg/L glucose and then used the NK cells in a 4-hour cytotoxicity assay against K562 cells at 500, 1000, 2000, or 4000 mg/L glucose. In the presence of 1000 mg/L, which is representative for the glucose concentration in blood, NK cells killed 19% of K562 cells on average. The presence of 500 mg/L glucose (representing the low range of MM BM) during the cytotoxicity assay did not negatively affect the killing capacity of IL-2 activated NK cells as compared to conditions with 1000 mg/L, representing blood glucose, or 2000 mg/L, which is present in standard culture media (**Figure 2A**). Higher glucose concentration of 4000 mg/L, as used in high glucose culture media, reduced the cytotoxicity of NK cells to 5.5 % on average as compared to 500 mg/L, 1000 mg/L and 2000 mg/L glucose (p= 0.06) (**Figure 2A**).

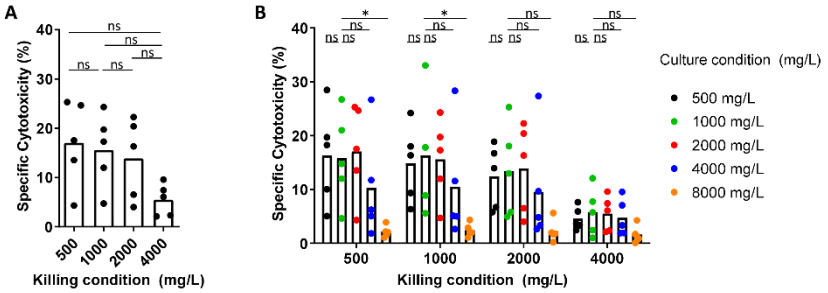


Figure 2. Short-term exposure to lower glucose concentrations does not reduce NK cell cytotoxicity while higher glucose levels reduce NK cell cytotoxicity. (A) NK cells were overnight activated with IL-2 in standard RPMI-1640 medium with 2000 mg/L glucose, followed by a 4h cytotoxicity assay with K562 cells at 1:1 E:T ratio in different glucose concentrations (killing condition). (B) NK cells were cultured in different glucose concentrations overnight (culture condition) in presence of IL-2, followed by a 4h cytotoxicity assay with K562 cells at 1:1 E:T ratio in different glucose concentrations (killing condition). Tumor cells killed by NK cells are denoted as percentage specific cytotoxicity. Bars show the average of n=5 donors in 3 independent experiments. Each dot represents the average of a technical duplicate. *p < 0.05. ns, not significant.

Because NK cells could be exposed for a longer period to the BM glucose levels while traveling through the BM, we investigated whether overnight exposure to different glucose concentrations affected NK cell cytotoxicity. The exposure to 500 mg/L up to 4000 mg/L glucose during overnight incubation (named 'culture condition') did not result in a lower cytotoxicity against K562 cells regardless of the glucose concentrations present during the killing process (named 'killing condition') (**Figure 2B**). After observing a reduction in NK cell-mediated killing with 4000 mg/L glucose in **figure 2A**, we included an even higher glucose concentration of 8000 mg/L for overnight culture. In the presence of this extremely high glucose, NK cell cytotoxicity was reduced to less than 5% in all killing conditions (**Figure 2B**). This reduction in NK cell cytotoxicity was unlikely due to high osmolarity caused by the high glucose levels as we did not see a reduction in NK cell cytotoxicity when NK cells were cultured in the presence of 1000 mg/L glucose and 7000 mg/L Mannitol (**Supplementary Figure 1**).

In summary, these results showed that the presence of 500 mg/L glucose, representative for the rather low glucose concentration in BM of MM patients, during the process of killing or during overnight incubation did not reduce the NK cell tumor-killing capacity in our in vitro setting. Quite the opposite, the presence of higher levels glucose concentration during killing or overnight activation (4000 and 8000 mg/L) diminished NK cell cytotoxic capacity.

Expanded NK cells exposed to relatively low glucose levels remain effective against tumor cells

Clinical application of NK cells might require ex vivo NK cell expansion to reach the large numbers of NK cells needed for NK cell infusions. As this expansion may also metabolically change the cells, we investigated how the killing capacity of expanded NK cells was affected by short-term exposure (4 hours, killing condition) or long-term exposure (4 days, culture condition) to glucose levels ranging between 500 mg/L and 4000 mg/L.

With 2000 mg/L glucose present during the cytotoxicity assay, expanded NK cells killed on average 49.5% K562 cells. RPMI-8226 cells, a MM cell line, were more resistant to NK cell-mediated killing than K562 cells and 26.6% RPMI-8226 cells were killed on average at 2000 mg/L glucose. Compared to this condition with 2000 mg/L glucose, the average cytotoxic potential of expanded NK cells against K562 and RPMI-8226 cells was not altered when NK cells were exposed to 500 mg/L or 4000 mg/L glucose during the killing process (**Figure 3A-B**). This result indicated that short exposure to varying glucose concentration did not influence the cytotoxic potential of expanded NK cells and differed from the freshly isolated NK cells that seemed to have reduced cytotoxicity with 4000 mg/L glucose during killing (**Figure 2**).

Next, we examined the effect of a four-day exposure to the different glucose concentrations on NK cell cytotoxicity. Compared to the culture condition of 2000 mg/L glucose, the average cytotoxicity of both K562 and RPMI-8226 was around 10% lower when NK cells were cultured in 500 mg/L glucose, however this did not reach significance. The average cytotoxicity of NK cells cultured in 4000 mg/L was not different from the culture condition with 2000 mg/L (**Figure 3A-B**). The NK cell viability remained the same in all conditions when NK cells were cultured in 500, 2000, or 4000 mg/L for 4 days (**Supplementary figure S2A**). Despite some donor variation, expanded NK cells of all donors maintained their cytotoxic capacity largely independent of the glucose concentration present during 4-day culture.

Besides production of cytotoxic granules, NK cells are known for their secretion of inflammatory cytokines such as IFN-ɣ and TNFα. NK cell-derived IFN-ɣ has multiple functions including support of antigen presentation and induction of a Th1 response, which is important for polarizing an adaptive immune response against tumor cells [28]. To investigate if the secretion of IFN-ɣ was influenced by the different glucose levels, the supernatants of NK- and tumor cell co-cultures were analyzed for IFN-ɣ secretion by ELISA. Cultured in the normal culture condition with 2000 mg/L glucose, expanded NK cells secreted on average 329 pg/mL IFN-ɣ with K562 cells as target cells and 23 pg/mL with RPMI-8226 as target cells, showing that K562 induced a much more potent IFN-ɣ response in NK cells than RPMI-8226 cells (**Figure 3C-D**). Without target cells, expanded NK cells did not secrete IFN-ɣ (**Supplementary figure S2B**). The average level of IFN-ɣ production was comparable for all the tested glucose levels during the short-term co-culture with tumor cells.

Compared to the 4-day culture condition with 2000 mg/L glucose, the average IFN-ɣ secretion remained constant when the expanded NK cells were exposed to 500 mg/L or 4000 mg/L for 4 days. This was found for both target cell lines K562 and RPMI-

8226. These data showed that a potent IFN-ɣ response, comparable to the amount secreted with 2000 mg/L glucose, was still observed independent of the glucose concentration during short- or long-term culture.

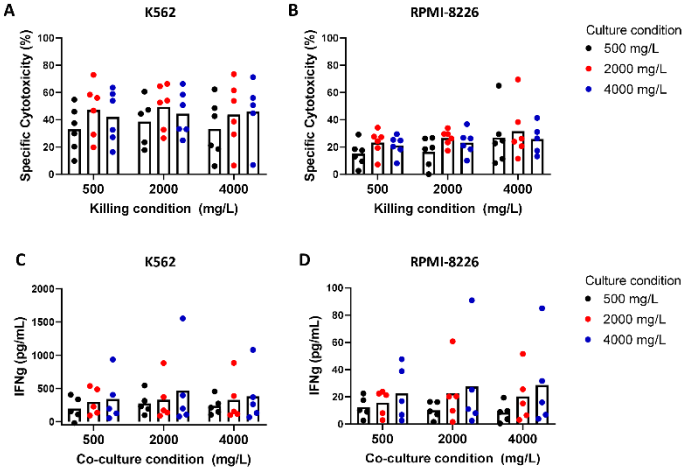


Figure 3. Short-term and long-term exposure to low or high glucose levels do not reduce effector functions of expanded NK cells. NK cells were expanded with 2000 mg/L glucose and subsequently cultured in different glucose concentrations for 4 days (culture condition) followed by a 4h cytotoxicity assay (killing condition) with K562 cells or RPMI8226 cells at 1:1 E:T ratio in different glucose concentrations. Tumor cells killed by NK cells are denoted as percentage specific cytotoxicity (A, B). After the co-culture with tumor cells, supernatants were collected and analyzed for secretion levels of IFN-γ by ELISA (C, D). Bars show the average of n=5-6 donors in individual experiments. For none of the conditions a p-value <0.05 was observed.

During ex vivo expansion, NK cells frequently alter their phenotype resulting in high NKG2A expression and low expression of killer-cell immunoglobulin-like receptors (KIRs). To test if the NK cell phenotype changed by culturing in low or high glucose during the 4-day culture period, NK cells were stained for several surface markers. The gating strategy is shown in **Figure S3**. The activating receptors NKp30, NKp46, DNAM1 and NKG2C are constitutively expressed on NK cells with NKG2C being expressed on a subset of NK cells. NKp44 and CD96 expression are induced upon activation [29]. After expansion of NK cells in the presence of IL-2, the NK cells had a highly activated phenotype and expressed all seven activating receptors (**Figure 4A-B**). With 86%, the majority of expanded NK cells was NKG2A positive and on average 10-18% of the NK cells expressed one or multiple KIR receptors (**Figure 4C-D**). Moreover, extensive cytokine activation of NK cells can lead to expression of exhaustion markers. The NK cells expressed TIM3 and TIGIT but only low levels of PD1 and LAG3 after expansion with IL-2 (**Figure 4E-F**). Expression of all investigated surface molecules was irrespective of the glucose concentrations present during culture, resulting in NK cells with a rather activating phenotypic profile.

In summary, the effector functions and the phenotypic profile of expanded NK cells were not influenced by variation in glucose levels during the killing process or during culture. Follow up studies that take multiple other TME factors and nutrient state into account, could be useful to further determine the impact of glucose on NK cells.

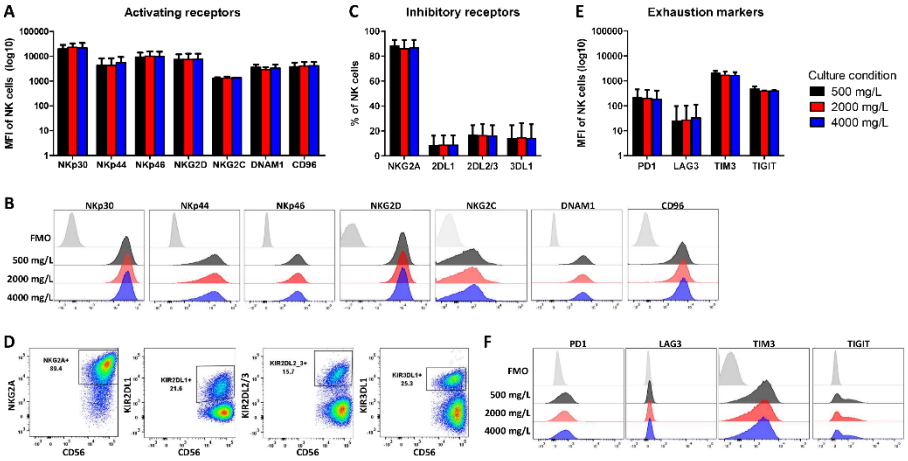


Figure 4. Phenotype profile of expanded NK cells is not altered by long-term exposure to low or high glucose levels. NK cells were expanded with 2000 mg/L glucose and subsequently cultured in different glucose concentrations for 4 days (culture condition). NK cells were stained with antibodies against activating receptors (A, B), inhibitory receptors (C, D) and exhaustion markers (E, F) and analyzed by flow cytometry. Bar graphs (A, C, E) depict the average expression of n=2 donors for NKG2C, TIM3, TIGIT and n=5 donors for all other markers, error bars indicate SD. FMO values were subtracted from MFI expression values. Representative histograms (B, F) or dot plots (D) of one donor are shown with the FMO of one of the three glucose concentrations as FMOs overlapped tightly (Supplementary Figure S3).

Discussion

To date, not much is known about glucose concentrations in the BM. As glucose is the primary fuel for NK cells, we aimed to gain more insight on the effect of glucose levels in the TME of MM on overnight cytokine-activated or ex vivo expanded NK cells and their antitumor response. First, we showed that glucose concentrations in the BM of MM patients with active disease were, in most cases, lower than normal blood glucose levels. Since we included only 9 patients in our study, our observation should be confirmed in a larger cohort. With this new insight on glucose concentrations in BM, we tested the effect of different glucose levels on freshly isolated- or expanded NK cells in vitro. As lowest glucose concentration we chose 500 mg/L since this represents the lower limit of BM glucose concentrations in MM patients. We observed that short-term exposure to 500 mg/L glucose did not have a detrimental effect on the killing capacity of overnight-activated NK cells. This may be because glucose was not completely depleted. 500 mg/L glucose is half of the normal concentration found in blood, where NK cells are known to be potent killers. Moreover, the majority of NK cells in the assays were $CD56^{dim}$ NK cells. Both human NK cell subsets are functionally different and have been shown to possess different metabolic requirements as well: $CD56^{bright}$ NK cells, which are the main cytokine producers have been shown to have a higher rate of glucose uptake and they appear to be metabolically more active than $CD56^{dim}$ NK cells upon cytokine stimulation [25]. Therefore, $CD56^{bright}$ peripheral blood NK cells may be more likely to suffer more from the restricted glucose in the environment than the $CD56^{dim}$ subset. In addition, during activation, NK cells are able to perform metabolic reprogramming and upregulate both glycolysis and OxPhos [30]. Since we used high dose IL-2 activated NK cells, these cells could have become more or less independent on the availability of glucose. Unlike T cells that are more dependent on glucose availability to become activated, NK cells might be the better tumor cell killers in the area where low glucose concentrations are located. Moreover, to fully conclude whether this would also be true for solid tumors, the glucose levels should be reduced even further as glucose levels in solid tumors can be much lower than 500 mg/L [17]. In addition, it would be interesting to further evaluate whether our findings are also true in a situation where not only glucose is reduced, but also other nutrients are limited.

Interestingly, high glucose concentrations of 4000 mg/L and even more obvious with 8000 mg/L glucose did result in a reduction of NK cell cytotoxicity in our study. This observation was highly unlikely due to high molarity since we did not observe the effect with mannitol. Our finding was in line with a previous study on unactivated human NK cells showing that short-term exposure of NK cells to 8000 mg/L glucose led to inhibition of NK cell cytotoxicity, probably due to an increase in intracellular calcium ion concentration to such high levels that it inhibited cytotoxic efficiency [31, 32].

Many clinical protocols, aiming at NK cell infusion as a mean for cancer immunotherapy, will require infusion of extremely high numbers of NK cells, which necessitates ex vivo NK cell expansion. We therefore anticipated that this expansion

period could provide an opportunity to either prime NK cells for the metabolic conditions in the tumor or enhance their function or persistence by interfering with their metabolic programming. For these experiments, we again chose 500 mg/L glucose as low, 2000 mg/L as normal, and 4000 mg/L as high glucose concentration, representing the in vivo BM concentrations in MM patients, glucose concentrations in standard-, and in high glucose culture media, respectively. Our data with IL-2 expanded NK cells implied that a period of acclimatization to a higher glucose concentration did not result in an altered NK cell cytotoxic capacity. Additionally, a period of acclimatization to a lower glucose level for 4 days after expansion did not reduce NK cell-mediated killing. For murine tumor-infiltrating T cells, it has been shown that inhibition of glycolysis during the ex vivo expansion could prime T cells towards enhanced persistence and overall anti-tumor response upon transfer into tumor-bearing immunodeficient mice [33]. In human NK cells expanded with membrane-bound IL-21 K562 feeder cells, highly functional licensed NK cells used both glycolysis and OxPhos and had a greater glycolytic capacity than less functional, unlicensed NK cells that relied on OxPhos alone [34]. Inhibition of NK cell cytotoxicity in highly functional NK cells was only observed after vigorous glucose deprivation and the use of metabolic inhibitors overnight [34]. This provides the functional NK cells with a greater flexibility to generate energy and indicates that the cytotoxic functions can be independent of the available glucose levels. We also found that the IFN-ɣ response of the expanded NK cells during a 4h cytotoxicity assay was largely unaffected by the glucose concentrations that we tested. IFN-ɣ production in murine NK cells was affected by inhibition of glycolysis when activation was receptor-mediated, but was not sensitive to glycolysis inhibition when activation occurred through cytokine signaling of IL-12 and IL-18, suggesting that the metabolic demand for IFN-ɣ is stimulus-dependent [23]. Similar to murine NK cells, human cytokine-activated NK cells continued to produce IFN-ɣ when exposed to short-term glucose deprivation of 4 hours [35] or long-term low glucose levels (as low as 0.01 mM) for two days [36]. However, $CD56^{bright}$ NK cells showed a defective IFN-ɣ production when the glycolysis rate was limited for a period of 18 hours [25] underlining the difference between $CD56^{dim}$ and $CD56^{bright}$ NK cells.

In our study, we observed a rather small reduction in glucose availability in the BM of MM patients as compared to normal peripheral blood values but we did not determine availability of other nutrients in the MM patients. In our in vitro assays, we reduced the amount of glucose, while other nutrients could contribute to NK cell efficacy as well. Glucose has been described as the key metabolic fuel for NK cells (summarized in [37]), but further research would be required to determine if a small reduction in glucose availability might have more impact in combination with other nutrient deficiencies, such as glutamine, or other TME factors that may suppress NK cell effector function or alter NK cell metabolism. While NK cells do not use glutamine as fuel for OxPhos, glutamine deficiency can impair NK cell functions due to the loss of the transcription factor cMYC [26]. Alternatively, the combination with TGF-β, frequently present in the MM TME [38], may enhance the effect of limited glucose as TGF-β has been shown to reduce the NK cells level of OxPhos and

glycolysis [39]. Hypoxia is another TME factor that can limit NK cell functions. We have previously shown that IL-2 activated NK cells retain their cytotoxic capacity when exposed to hypoxia [9]. However, a shift towards glycolysis is expected when oxygen is low and it is therefore important to study the effects of varying glucose concentrations on activated NK cells in models resembling a complex TME including factors like hypoxia and altered levels of nutrients other than glucose.
Even though we show that activated and expanded NK cells can cope with a low glucose environment, combination therapy should be considered to achieve better NK cell efficacy in MM patients. We have previously described several strategies how the NK cell potency could be enhanced e.g. by the combination with monoclonal antibodies such as Daratumumab [40]. Other options could include targeting of the TME to create a less NK cell suppressive TME. In MM, inhibitors of mechanistic target of rapamycin (mTOR) are tested to target the metabolism of MM cells and its TME cells that overexpress mTOR [41, 42]. Dual inhibitors targeting mTORC1/mTORC2 could potentially be an interesting drug to slow tumor cell growth in combination with NK cell transfer to kill tumor cells. However, mTOR inhibitors can also suppress immune cells [22, 43] and combination therapy approaches should be tested in carefully designed studies as timing of the drugs and NK cell infusion may be very important. Alternatively, it would be relevant to explore in further studies whether manipulating NK cell metabolism during ex vivo expansion could be used to potentiate NK cell effector function in the MM TME. In conclusion, our current findings showed that exposure to a relatively low glucose concentration, as found in the BM of MM patients, for either short-term or long-term culture did not have a detrimental effect on the NK cell cytotoxic capacity against tumor cells in our in vitro setup. Our data suggests that IL-2 activated and expanded NK cells could be well suited to function in a tumor environment where glucose availability is limited. Although this is positive news for NK-cell based immunotherapy, future studies are needed to investigate if these observations also hold true for the in vivo situation in patients with MM or other cancers.

Conflict of Interest

G. M. J. Bos is Chief Executive Officer/Chief Medical Officer/Co-founder of CiMaas, BV, Maastricht, The Netherlands. CiMaas is producing an ex vivo expanded NK cell product that will be used to treat myeloma patients. The other authors declare no conflict of interest.

Author Contributions

Conceptualization, N.M. and F.E.; methodology, N.M, F.E., T.O.; formal analysis, N.M and F.E..; investigation, N.M., F.E., T.O.; resources, N.M, G.B.; data curation, N.M. and F.E.; writing—original draft preparation, N.M. and F.E.; writing, review and editing, T.O., L.W. and G.B.; supervision, L.W. and G.B.; project administration, N.M. and F.E.; funding acquisition, L.W. and G.B. All authors have read and agreed to the published version of the manuscript.

Funding
This study was funded by a Grant from Kankeronderzoeksfonds Limburg (KOFL).

Acknowledgments
We thank Monique Grommé from Central Diagnostic Laboratory for helping with the sample handling.

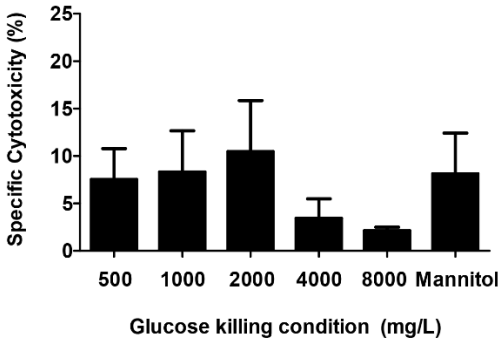
References

1. Fang, F., Xiao, W., and Tian, Z., NK cell-based immunotherapy for cancer. Seminars in Immunology, 2017. 31: p. 37-54 DOI: 10.1016/j.smim.2017.07.009.
2. Liang, S., Xu, K., Niu, L., Wang, X., Liang, Y., Zhang, M., et al., Comparison of autogeneic and allogeneic natural killer cells immunotherapy on the clinical outcome of recurrent breast cancer. OncoTargets and therapy, 2017. 10: p. 4273 DOI: 10.2147/OTT.S139986.
3. Veluchamy, J.P., Kok, N., van der Vliet, H.J., Verheul, H.M., de Gruijl, T.D., and Spanholtz, J., The rise of allogeneic natural killer cells as a platform for cancer immunotherapy: recent innovations and future developments. Frontiers in Immunology, 2017. 8: p. 631 DOI: 10.3389/fimmu.2017.00631.
4. Bachanova, V. and Miller, J.S., NK cells in therapy of cancer. Critical Reviews™ in Oncogenesis, 2014. 19(1-2): p. 133-41 DOI: 10.1615/critrevoncog.2014011091.
5. Larsen, S.K., Gao, Y., and Basse, P.H., NK cells in the tumor microenvironment. Critical Reviews™ in Oncogenesis, 2014. 19(1-2): p. 91-105 DOI: 10.1615/critrevoncog.2014011142.
6. Chiu, J., Ernst, D.M., and Keating, A., Acquired natural killer cell dysfunction in the tumor microenvironment of classic Hodgkin lymphoma. Frontiers in Immunology, 2018. 9: p. 267 DOI: 10.3389/fimmu.2018.00267.
7. Mocellin, S., Wang, E., and Marincola, F.M., Cytokines and immune response in the tumor microenvironment. Journal of immunotherapy, 2001. 24(5): p. 392-407 DOI: 10.1097/00002371-200109000-00002.
8. Jewett, A. and Tseng, H.-C., Tumor induced inactivation of natural killer cell cytotoxic function; implication in growth, expansion and differentiation of cancer stem cells. Journal of Cancer, 2011. 2: p. 443 DOI: 10.7150/jca.2.443.
9. Sarkar, S., Germeraad, W.T., Rouschop, K.M., Steeghs, E.M., van Gelder, M., Bos, G.M., et al., Hypoxia induced impairment of NK cell cytotoxicity against multiple myeloma can be overcome by IL-2 activation of the NK cells. PLoS One, 2013. 8(5): p. e64835 DOI: 10.1371/journal.pone.0064835.
10. Balsamo, M., Manzini, C., Pietra, G., Raggi, F., Blengio, F., Mingari, M.C., et al., Hypoxia downregulates the expression of activating receptors involved in NK-cell-mediated target cell killing without affecting ADCC. European journal of immunology, 2013. 43(10): p. 2756-2764 DOI: 10.1002/eji.201343448.
11. Baginska, J., Viry, E., Paggetti, J., Medves, S., Berchem, G., Moussay, E., et al., The critical role of the tumor microenvironment in shaping natural killer cell-mediated anti-tumor immunity. Frontiers in Immunology, 2013. 4: p. 490 DOI: 10.3389/fimmu.2013.00490.
12. Pietra, G., Manzini, C., Rivara, S., Vitale, M., Cantoni, C., Petretto, A., et al., Melanoma cells inhibit natural killer cell function by modulating the expression of activating receptors and cytolytic activity. Cancer research, 2012. 72(6): p. 1407-1415 DOI: 10.1158/0008-5472.CAN-11-2544.
13. Vitale, M., Cantoni, C., Pietra, G., Mingari, M.C., and Moretta, L., Effect of tumor cells and tumor microenvironment on NK-cell function. European journal of immunology, 2014. 44(6): p. 1582-1592.
14. Kroemer, G. and Pouyssegur, J., Tumor cell metabolism: cancer's Achilles' heel. Cancer cell, 2008. 13(6): p. 472-482 DOI: 10.1016/j.ccr.2008.05.005.

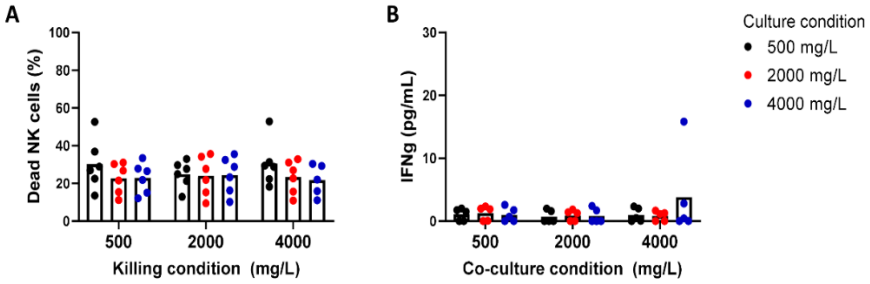
15. DeBerardinis, R.J., Lum, J.J., Hatzivassiliou, G., and Thompson, C.B., The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. Cell metabolism, 2008. 7(1): p. 11-20 DOI: 10.1016/j.cmet.2007.10.002.
16. Hu, X., Chao, M., and Wu, H., Central role of lactate and proton in cancer cell resistance to glucose deprivation and its clinical translation. Signal transduction and targeted therapy, 2017. 2(1): p. 1-8 DOI: 10.1038/sigtrans.2016.47.
17. Hirayama, A., Kami, K., Sugimoto, M., Sugawara, M., Toki, N., Onozuka, H., et al., Quantitative metabolome profiling of colon and stomach cancer microenvironment by capillary electrophoresis time-of-flight mass spectrometry. Cancer research, 2009. 69(11): p. 4918-4925 DOI: 10.1158/0008-5472.CAN-08-4806.
18. Pearce, E.L. and Pearce, E.J., Metabolic pathways in immune cell activation and quiescence. Immunity, 2013. 38(4): p. 633-643 DOI: 10.1016/j.immuni.2013.04.005.
19. Sukumar, M., Roychoudhuri, R., and Restifo, N.P., Nutrient competition: a new axis of tumor immunosuppression. Cell, 2015. 162(6): p. 1206-1208 DOI: 10.1016/j.cell.2015.08.064.
20. Chang, C.-H., Qiu, J., O'Sullivan, D., Buck, M.D., Noguchi, T., Curtis, J.D., et al., Metabolic competition in the tumor microenvironment is a driver of cancer progression. Cell, 2015. 162(6): p. 1229-1241 DOI: 10.1016/j.cell.2015.08.016.
21. Ho, P.-C., Bihuniak, J.D., Macintyre, A.N., Staron, M., Liu, X., Amezquita, R., et al., Phosphoenolpyruvate is a metabolic checkpoint of anti-tumor T cell responses. Cell, 2015. 162(6): p. 1217-1228 DOI: 10.1016/j.cell.2015.08.012.
22. Marçais, A., Cherfils-Vicini, J., Viant, C., Degouve, S., Viel, S., Fenis, A., et al., The metabolic checkpoint kinase mTOR is essential for IL-15 signaling during the development and activation of NK cells. Nature immunology, 2014. 15(8): p. 749 DOI: 10.1038/ni.2936.
23. Keppel, M.P., Saucier, N., Mah, A.Y., Vogel, T.P., and Cooper, M.A., Activation-specific metabolic requirements for NK Cell IFN-γ production. The Journal of Immunology, 2015. 194(4): p. 1954-1962 DOI: 10.4049/jimmunol.1402099.
24. Donnelly, R.P., Loftus, R.M., Keating, S.E., Liou, K.T., Biron, C.A., Gardiner, C.M., et al., mTORC1-dependent metabolic reprogramming is a prerequisite for NK cell effector function. The Journal of Immunology, 2014. 193(9): p. 4477-4484 DOI: 10.4049/jimmunol.1401558.
25. Keating, S.E., Zaiatz-Bittencourt, V., Loftus, R.M., Keane, C., Brennan, K., Finlay, D.K., et al., Metabolic reprogramming supports IFN-γ production by CD56bright NK cells. The Journal of Immunology, 2016. 196(6): p. 2552-2560 DOI: 10.4049/jimmunol.1501783.
26. O'Brien, K.L. and Finlay, D.K., Immunometabolism and natural killer cell responses. Nature Reviews Immunology, 2019. 19(5): p. 282-290 DOI: 10.1038/s41577-019-0139-2.
27. Organization, W.H., Definition and diagnosis of diabetes mellitus and intermediate hyperglycaemia: report of a WHO/IDF consultation. 2006.
28. Mailliard, R.B., Son, Y.-I., Redlinger, R., Coates, P.T., Giermasz, A., Morel, P.A., et al., Dendritic cells mediate NK cell help for Th1 and CTL responses: two-signal requirement for the induction of NK cell helper function. The Journal of Immunology, 2003. 171(5): p. 2366-2373 DOI: 10.4049/jimmunol.171.5.2366.
29. Sivori, S., Vacca, P., Del Zotto, G., Munari, E., Mingari, M.C., and Moretta, L., Human NK cells: surface receptors, inhibitory checkpoints, and translational applications. Cellular & molecular immunology, 2019. 16(5): p. 430-441 DOI: 10.1038/s41423-019-0206-4.
30. Gardiner, C.M. and Finlay, D.K., What fuels natural killers? Metabolism and NK cell responses. Frontiers in Immunology, 2017. 8: p. 367 DOI: 10.3389/fimmu.2017.00367.
31. Whalen, M.M., Inhibition of human natural killer cell function in vitro by glucose concentrations seen in poorly controlled diabetes. Cellular Physiology and Biochemistry, 1997. 7(1): p. 53-60 DOI: 10.1159/000154852.
32. Zhou, X., Friedmann, K.S., Lyrmann, H., Zhou, Y., Schoppmeyer, R., Knörck, A., et al., A calcium optimum for cytotoxic T lymphocyte and natural killer cell cytotoxicity. The Journal of physiology, 2018. 596(14): p. 2681-2698 DOI: 10.1113/JP274964.
33. Sukumar, M., Liu, J., Ji, Y., Subramanian, M., Crompton, J.G., Yu, Z., et al., Inhibiting glycolytic metabolism enhances CD8+ T cell memory and antitumor function. The Journal of clinical investigation, 2013. 123(10): p. 4479-4488 DOI: 10.1172/JCI69589.

34. Schafer, J.R., Salzillo, T.C., Chakravarti, N., Kararoudi, M.N., Trikha, P., Foltz, J.A., et al., Education-dependent activation of glycolysis promotes the cytolytic potency of licensed human natural killer cells. Journal of Allergy and Clinical Immunology, 2019. 143(1): p. 346-358. e6 DOI: 10.1016/j.jaci.2018.06.047.
35. Velásquez, S.Y., Himmelhan, B.S., Kassner, N., Coulibaly, A., Schulte, J., Brohm, K., et al., Innate Cytokine Induced Early Release of IFNγ and CC Chemokines from Hypoxic Human NK Cells Is Independent of Glucose. Cells, 2020. 9(3): p. 734 DOI: 10.3390/cells9030734.
36. Presnell, S.R., Spear, H.K., Durham, J., Riddle, T., Applegate, A., and Lutz, C.T., Differential Fuel Requirements of Human NK Cells and Human CD8 T Cells: Glutamine Regulates Glucose Uptake in Strongly Activated CD8 T Cells. ImmunoHorizons, 2020. 4(5): p. 231-244 DOI: 10.4049/immunohorizons.2000020.
37. Gardiner, C.M., NK cell metabolism. Journal of leukocyte biology, 2019. 105(6): p. 1235-1242 DOI: 10.1002/JLB.MR0718-260R.
38. Pittari, G., Vago, L., Festuccia, M., Bonini, C., Mudawi, D., Giaccone, L., et al., Restoring natural killer cell immunity against multiple myeloma in the era of new drugs. Frontiers in Immunology, 2017. 8: p. 1444 DOI: 10.3389/fimmu.2017.01444.
39. Zaiatz-Bittencourt, V., Finlay, D.K., and Gardiner, C.M., Canonical TGF-β signaling pathway represses human NK cell metabolism. The Journal of Immunology, 2018. 200(12): p. 3934-3941 DOI: 10.4049/jimmunol.1701461.
40. Mahaweni, N.M., Bos, G.M., Mitsiades, C.S., Tilanus, M.G., and Wieten, L., Daratumumab augments alloreactive natural killer cell cytotoxicity towards CD38+ multiple myeloma cell lines in a biochemical context mimicking tumour microenvironment conditions. Cancer Immunology, Immunotherapy, 2018. 67(6): p. 861-872 DOI: 10.1007/s00262-018-2140-1.
41. Lamanuzzi, A., Saltarella, I., Desantis, V., Frassanito, M.A., Leone, P., Racanelli, V., et al., Inhibition of mTOR complex 2 restrains tumor angiogenesis in multiple myeloma. Oncotarget, 2018. 9(29): p. 20563 DOI: 10.18632/oncotarget.25003.
42. Ramakrishnan, V. and Kumar, S., PI3K/AKT/mTOR pathway in multiple myeloma: from basic biology to clinical promise. Leukemia & lymphoma, 2018. 59(11): p. 2524-2534 DOI: 10.1080/10428194.2017.1421760.
43. Wang, F., Meng, M., Mo, B., Yang, Y., Ji, Y., Huang, P., et al., Crosstalks between mTORC1 and mTORC2 variagate cytokine signaling to control NK maturation and effector function. Nature communications, 2018. 9(1): p. 1-17 DOI: 10.1038/s41467-018-07277-9.

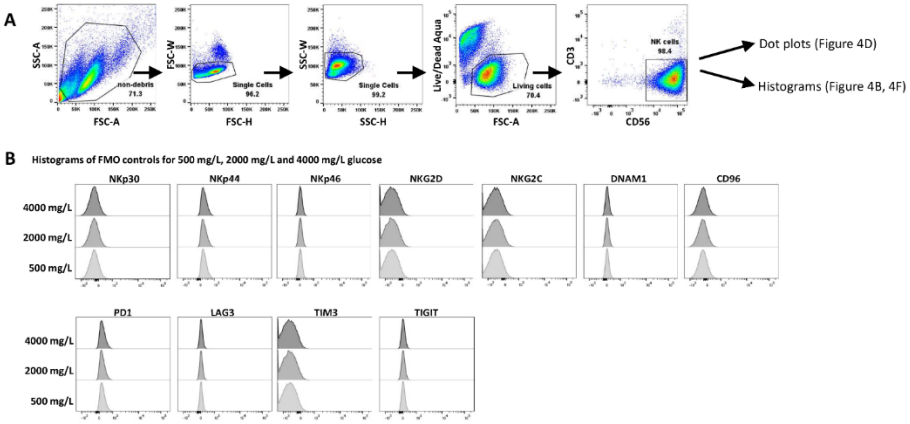
Supplementary Material



Supplementary Figure S1. High osmolarity is not the cause of a reduced cytotoxic capacity of NK cells due to high glucose concentration during culture. NK cells stimulated with 1000 U/ml IL-2 were cultured overnight in the presence of different glucose concentrations or high concentration of Mannitol equivalent to 8000 mg/L glucose. The following day, NK cells were co-cultured with K562 cells in 1:1 E:T ratio in a 4-hour cytotoxicity assay in different glucose concentrations as indicated. Dead cells were stained with Live/Dead Marker. Percentage of tumor cells killed by NK cells are denoted as percentage specific cytotoxicity. n = 2 donors in independent experiments.



Supplementary Figure S2. Effect of short- or long-term exposure to low or high glucose on NK cells. NK cells were expanded with 2000 mg/L glucose and subsequently cultured in different glucose concentrations for 4 days (culture condition). **(A)** NK cell viability was assessed by staining with a Live/Dead marker and analysis with flow cytometry. **(B)** IFN-γ secretion of expanded NK cells without presence of target cells, assessed by ELISA. Bars show average of n=5-6 donors in individual experiments.



Supplementary Figure S3: Analysis of the phenotypic profile of expanded NK cells. (A) Gating strategy was as follows: Debris was gated out and single living cells were selected before gating on CD3- CD56+ NK cells, followed by gating on the inhibitory receptors NKG2A, KIR2DL1, KIR2DL2/3, KIR3DL1 that present as subpopulations of NK cells (see Figure 4D) and activating receptors and exhaustion markers are presented as histograms (see Figure 4B and 4F). **(B)** Histograms of FMO controls for all glucose culture conditions (500 mg/L, 2000 mg/L, 4000 mg/L) are shown of a representative donor.

7

Exploring the potential of combining IL-2 activated NK cells with an anti-PDL1 monoclonal antibody to target multiple myeloma-associated macrophages

Femke A. I. Ehlers, Niken M. Mahaweni, Annet van de Waterweg Berends, Thara Saya, Gerard M. J. Bos and Lotte Wieten

In press at Cancer Immunology Immunotherapy.

Abstract
Multiple myeloma (MM) is an incurable disease, characterized by malignant plasma cells in the bone marrow. MM growth is largely dependent on the tumor microenvironment (TME), consisting of complex cellular networks and secreted factors that shape a tumor-permissive environment. Within the TME, tumor-associated cells (TAC) comprise heterogeneous cell populations that collectively support immunosuppression. Reshaping the TME towards an immunostimulatory environment may enhance effectiveness of immunotherapies. Here, we investigated interactions between donor-derived natural killer (NK) cells and TAC, like tumor-associated macrophages (TAM) and M1 macrophages, and assessed whether anti-tumor effector functions of NK cells could be enhanced by an ADCC-triggering antibody targeting macrophages. Monocytes were polarized *in vitro* towards either M1 or TAM before co-culture with high-dose IL-2 activated NK cells. NK cell responses were assessed by measuring degranulation (CD107a) and IFN-γ production. We found that NK cells degranulated and produced IFN-γ upon interaction with both macrophage types. NK cell responses against PD-L1^{+} M1 macrophages could be further enhanced by Avelumab, an anti-PD-L1- and ADCC-inducing antibody. Additionally, NK cell responses were influenced by HLA class I, shown by stronger degranulation in NK cell subsets for which the corresponding HLA ligand was absent on the macrophage target cells (KIR-ligand mismatch) compared to degranulation in the presence of the HLA ligand (KIR-ligand match). Our results suggest that NK cells could, next to killing tumor cells, get activated upon interaction with TAC, like M1 macrophages and TAMs, and that NK cells combined with PD-L1 blocking antibodies with ADCC potential could, through IFN-γ secretion, promote a more immune-favorable TME.

Keywords: NK cells, ADCC, tumor-associated cells, tumor microenvironment

Introduction

Multiple myeloma (MM) is an incurable hematological cancer, characterized by the accumulation of malignant plasma cells in the bone marrow (BM). Although therapeutic agents such as immunomodulatory drugs and monoclonal antibodies have substantially prolonged survival, tumor cells frequently develop resistance to therapy due to mutations and support from the tumor microenvironment (TME) (1). MM cells are dependent on the BM microenvironment to grow uncontrollably. Interaction with BM stromal cells and osteoclasts can for example support MM proliferation and survival through direct cell-cell contacts and soluble factors such as IL-6 (2). Moreover, the TME of MM patients is characterized by changes in the cellular composition compared to normal BM, already early on during disease development (3). BM-infiltrating cells represent highly heterogeneous cell populations and together they largely contribute to suppression of anti-tumor immune responses through various mechanisms such as secretion of suppressive cytokines, nitric oxide, reactive oxygen species, indoleamine-2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2), as well as lactate produced by MM cells (4, 5). Moreover, immature myeloid cells, which are often described as myeloid-derived suppressor cells, as well as regulatory T cells and tumor-associated macrophages (TAM) are recruited to the TME or acquire suppressive phenotypes in the TME (2). While they are often immunosuppressive, TAM are highly plastic cells that can, depending on the stimuli from the environment, acquire a large spectrum of activation states and functions. The simplistic classification of M1 and M2 macrophages with pro-tumor and anti-tumor functionality, respectively, represents the two extremes of the activation states that are typically used in in-vitro cultures (6). In the MM microenvironment, TAM are abundantly present and cytokines like IL-4, IL-10, and TGF-β promote their polarization towards a tumor-supporting phenotype (7-9). This can shape TAM to secrete proinflammatory cytokines, which in turn further support MM progression (10, 11). Additionally, TAM in MM are often described as M2-like and are for example a major source of the anti-inflammatory cytokine IL-10 (7, 10). Macrophage polarization is thus multifaceted to support both tumor proliferation and an immunosuppressive milieu (2).

The immunosuppressive TME in MM can result in defective T cell- and NK cell-mediated tumor killing (12-14). Hence, new therapy approaches aim to restore the dysfunctional antitumor responses e.g. by blocking immune checkpoint (IC) molecules such as TIGIT or the PD-1/PD-L1 pathway and by adoptive transfer of modified T cells or NK cells (15). IC are upregulated by inflammatory cytokines to prevent overactivation of the immune system, as well as by aberrant signalling pathways in tumor cells, including MM, to facilitate immune escape (16). Although advances have been made, IC immunotherapies do not always achieve durable responses and, next to restoring antitumor functions of exhausted immune cells, reprograming of the TME towards a proinflammatory, immunostimulatory TME may be crucial for sustained effectiveness of immunotherapies (17).

We and others focus on developing effective donor-derived NK cell-based therapy, which is considered a promising approach to restore impaired NK cell functionality in MM (18). NK cells can rapidly and effectively kill tumor cells. To recognize their targets, NK cells express an array of activating and inhibitory receptors and attack when the activating signals outweigh the inhibitory ones (19). Activating receptors for example bind to ligands upregulated in response to stress, while the major inhibitory receptors on NK cells are Killer-Immunoglobulin-like Receptors (KIRs) and NKG2A that bind to HLA class I molecules, which are expressed on all healthy cells. Next to natural cytotoxicity, the NK cells killing capacity can be enhanced through antibodies that induce antibody-dependent cellular cytotoxicity (ADCC) through binding to CD16 on NK cells and CD16 is one of the most potent activating receptors on NK cells (20). Additionally, NK cells can provide anti-tumor effects by promoting T helper type 1 (Th1) immune responses through secretion of proinflammatory cytokines including high amounts of IFN-γ (21). However, tumor cells have developed mechanisms to escape from NK cells, which can result in dysfunctional NK cell response in the MM environment. Therefore, different strategies have been exploited to create NK cells that remain functional in a suppressive TME. For tumor types like MM that are largely HLA class I+, donor NK cells can be used that possess KIRs for which the corresponding HLA ligands in the tumor are absent, resulting in a lower activation threshold and stronger NK cell anti-tumor cytotoxicity (22, 23). ADCC is another way to induce robust NK cell effector functions. We have previously shown that ADCC-triggering by the clinically-approved anti-CD38 antibody Daratumumab combined with KIR-ligand mismatched NK cells helped to amplify the NK cell responses against MM tumor cell lines (22).

So far, NK cell therapies have been mainly assessed for their direct anti-tumor effects i.e., killing tumor cells. In this study, we hypothesized that NK cells can further contribute to anti-tumor immunity through IFN-γ secretion by responding to myeloid-derived tumor associated cells (TAC) and that such NK cell responses can be amplified by ADCC-triggering antibodies binding to TAC. We tested the response of IL-2 activated donor-derived NK cells in combination with the anti-PD-L1 blocking antibody Avelumab, known to also trigger ADCC, on two types of in vitro macrophages that were either polarized towards a TAM-like phenotype or polarized towards a proinflammatory M1 phenotype. Moreover, we assessed the contribution of the inhibitory ligands HLA class I on TAM on NK cell functionality.

Materials and methods

Cell isolations and cell culture

Monocytes and NK cells were derived from buffy coats of healthy donors. The use of buffy coats does not require ethical approval in the Netherlands under the Dutch Code for Proper Secondary Use of Human Tissue. PBMCs were isolated by density gradient centrifugation with Lymphoprep (Axis Shield). Subsequently, monocytes were positively selected by CD14 MicroBeads (Miltenyi Biotec) and NK cells were isolated by negative selection using the NK cell isolation kit (Miltenyi Biotec) according to the manufacturer's protocol. NK cells were cultured overnight at a density of 1.5x106 cells/mL in RPMI1640 medium with Glutamax (Gibco), supplemented with 10% FCS and 1% P/S, plus 1000 U/mL recombinant human IL-2 (Proleukin, Novartis). If using fresh NK cells was not feasible, NK cells were frozen until further use and, prior to experiments, thawed and activated the same away as fresh NK cells. For the cytotoxicity assay, expanded NK cells were included: NK cells were expanded from CD3-depleted PBMCs in SGCM medium supplemented with 10% FCS, 1% Pen/Strep, and 1000 U/mL IL-2. After 17 days of expansion, NK cells were frozen and, prior to the experiment, thawed and recovered overnight in the presence of IL-2. The multiple myeloma cell line L363 was used to polarize TAM. L363 was purchased from DSMZ Germany and cultured in RPMI1640 medium with Glutamax (Gibco) and supplemented with 15% fetal calf serum (FCS, TICO Europe) and 1% penicillin/streptomycin (P/S, Gibco). The cells were cultured in an incubator with conditions of 37^{o}C, 21% O_2 and 5% CO_2.

Monocyte polarization towards macrophages

CD14+ monocytes were plated in serum-free X-VIVOTM 15 medium (Lonza) with either 100 ng/mL recombinant human macrophage colony-stimulating factor (M-CSF, Immunotools) or 100 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF, Miltenyi Biotec) to differentiate the monocytes towards TAM or M1, respectively. On day 4, half-medium change was performed. On day 7, the cells were polarized towards TAM or M1 for 48h as follows: To obtain TAM, 100 ng/mL M-CSF, 50 mM lactate (Sigma-Aldrich), 200 ng/mL prostaglandin E2 (PGE2; Sigma-Aldrich) were added together with either L363 cells in a 1:30 L363:monocytes ratio or with L363 supernatant. To obtain M1 macrophages, 100 ng/mL GM-CSF, 20 ng/mL recombinant human IFN-γ (rhIFN-γ, R&D Systems) and 10 ng/mL LPS (Sigma-Aldrich) were added to the medium.

To some conditions, supernatant from IL-2 activated NK cells or rhIFN-γ (R&D Systems) were added to the polarized macrophages for 24h, referred to as NK supernatant. Additionally, 5 µg/mL LEAF purified anti-human CD119/IFN-γ R alpha chain, mouse IgG1 κ blocking antibody (clone GIR-208) or 5 µg/mL LEAF purified mouse IgG1 κ isotype control (MGI-45/ MOPC-21, both Biolegend) were added 30 min prior to adding the NK supernatant.

Macrophage harvesting and flow cytometric analysis of macrophages

First, supernatant was removed and stored for CBA analysis, then wells were carefully washed with PBS and 10mM EDTA/PBS was added for 5 min at 37C. Cells were subsequently gently harvested using a cell lifter. For the FACS staining, macrophages were blocked with the FcR blocking reagent, human (Miltenyi) prior to surface staining for CD80 APC-H7 (L307.4, BD), CD86 FITC (FM95, Miltenyi), HLA-ABC PE (REA230, Miltenyi), HLA-DR APC (AC122, Miltenyi), CD163 PerCPCy5.5 (GHI/61, BD), CD206 APC (19.2, BD), CD209 PerCPCy5.5 (DCN46, BD), and PD-L1 PE-Cy7 (REA1197, Biolegend) for 30 min in the dark and on ice. Directly before measuring the sample on the flow cytometer, 30 ng DAPI (Sigma-Aldrich) was added. To control for background staining, cells stained only with 30 ng DAPI were used. All flow cytometric analyses were performed with BD Canto II and data were analyzed with FlowJo 10.1r5 64-bit software.

Cytokine profile via Cytometric Bead Assay (CBA)

The stored supernatant was thawed prior to the assay and 50 µl of the supernatant per condition was prepared according to the protocol of the BD CBA Flex Set kit to measure the concentrations of IFN-γ, IL1-β, IL-6, IL-10, VEGF and TNFα (BD Biosciences).

Cytotoxicity assay with L363 target cells

First, polarized macrophages were washed and co-cultured with IL-2 activated NK cells for 2 days. The NK cells were harvested by collecting the supernatant of the co-culture, while the macrophages adhered to the plates. Then, the macrophage-primed NK cells were co-cultured with the target cells L363, which were previously labeled with CellTrackerTM CM-DiI Dye (Invitrogen) according to the manufacturer's protocol. The co-culture was performed in 96-well plates with 2x104 NK-cells and 2x104 tumor cells per well (1:1 Effector:Target cell (E:T) ratio) for 4 hours. After co-culture, the cells were stained with Live/Dead® Fixable Aqua Dead Cell Stain Kit (Invitrogen) for 30 minutes on ice and then fixed in PBS with 1% paraformaldehyde. The samples were measured on a BD CANTO II. Specific cytotoxicity was calculated as follows: % specific cytotoxicity=((% dead cells-average % spontaneous death))/((100%-average % spontaneous death))*100 %

CD107a degranulation assay, intracellular staining and IncuCyte analysis

Polarized macrophages were harvested and re-seeded in 96 well plates. Macrophages were incubated with 2 ug/mL Avelumab (MedChemExpress) or medium for 30 min before IL-2 activated NK cells were added in a 1:1 E:T ratio for 4-hour co-culture. For the CD107a assays, CD107a-Horizon V450 antibody (H4A3, Miltenyi Biotec) was added immediately. After the first hour of co-culture, Monensin (BD Biosciences) was added and after another 3 hours, the plates were put on ice to stop the assay. The cells were washed with PBS and first stained with Live/Dead® Fixable Aqua Dead Cell Stain Kit (Molecular Probes™) for 30 min on ice before surface staining with the following antibodies was performed for 30 min on ice: anti-CD3-

APC-Vio770 (BW264/56), anti-CD56-PerCP-Vio700 (REA196), anti-KIR2DL1-APC (REA284), anti-KIR2DL2/3-PE (DX27), anti-KIR3DL1-FITC (DX9), and anti-NKG2A-PE-Vio770 (REA110). For IFN-γ staining, NK cells were subsequently treated with the Cytofix/Cytoperm™ Fixation/Permeabilization Kit from BD according to the manufacturer's protocol and stained with anti-IFN-γ (B27) for 30 min on ice. The assays were analyzed by flow cytometry with a BD CANTO II. For the image analysis using the Incucyte® S3 Live-Cell Analysis System, macrophages were labeled with CellTrackerTM CM-DiI Dye (ThermoFisher) and then co-cultured with NK cells as described above. Caspase-3/7 dye for apoptosis (Sartorius) was added according to the manufacturer's procedure to detect cell death.

Determination of KIR-ligand matched and mismatched NK cell subsets

The HLA class I genotype of the monocyte donors was determined by Luminex-SSO according to manufacturer's protocol. Donors with an HLA-$C1^+$ HLA-$C2^+$ HLA-$Bw4^+$ genotype were selected as NK cells donors for the assay to ensure that the analyzed KIRs are licensed. See table 1 for determination of KIR-ligand matched and -mismatched NK cell subsets.

Statistical analysis

All statistical analysis were performed with GraphPad Prism 9.2.0 (GraphPad Software Inc, San Diego, CA, USA) using non-parametric paired t-tests (Wilcoxon matched pairs tests). * indicates a p-value of <0.05, ** indicates a p-value of <0.01, *** indicates a p-value of <0.001.

Results

IL-2 activated NK cells degranulate and produce IFN-γ in response to in vitro polarized TAM and M1

To study NK cells responses against myeloid-derived TAC, we generated two types of macrophages, TAM and M1. TAM were generated by stimulating monocytes with M-CSF, PGE2, Lactate as well as the MM cell line L363 to enable interaction of TAM with MM cells, as is expected to occur in the TME. Because the presence of L363 cells after harvesting could influence NK cell responses, we also generated TAM with the same cocktail of M-CSF, PGE2 and lactate and with L363 supernatant (TAM-S) instead of L363 cells. Additionally, we polarized macrophages towards the opposing end with GM-CSF, rhIFN-γ, and LPS to generate proinflammatory M1. Upon polarization, M1 displayed the typical long-stretched morphology, while TAM appeared spindle-like shaped and less stretched (**Suppl. Fig. S1A**). Since macrophages are highly versatile in their phenotype, we assessed the phenotypic profile of the macrophages that we generated by staining for cell surface markers and measuring cytokines in the supernatant of polarized macrophages. While we observed differences between donors regarding the levels of expression of the different markers, all M1 expressed the co-stimulatory molecules CD80 and CD86 as well as high levels of the HLA class II molecule HLA-DR (**Fig. 1A-B**). Additionally, we assessed three receptors involved in host defense, of which CD206 and CD209 were expressed at low levels and CD163 was not expressed on M1 (Fig 1A-B). The proinflammatory cytokines IL-1β, TNF-α, IL-6 and IFN-γ were produced by M1 as well as the vascular endothelial growth factor (VEGF) and IL-10 (**Fig. 1C**). Compared to M1, TAM expressed lower levels of the co-stimulatory molecule CD80, while HLA-DR was also present on TAM and the scavenger receptors CD206 and CD163 as well as the CD209 receptor were expressed at low levels (**Fig. 1A-B**). All assessed cytokines were measured in the TAM supernatant at low levels with secretion of VEGF being the highest of the measured cytokines (**Fig. 1C**). TAM-S displayed a comparable morphology and phenotype as TAM polarized with L363 cells (**Suppl. Fig. S1A and S2**).

To test whether NK cell effector mechanisms could be triggered or influenced by the interaction with macrophages, IL-2 activated donor NK cells were co-cultured with M1 or TAM and NK cell degranulation (CD107a) was assessed. After 4 hours of co-culture, spontaneous degranulation of NK cells was below 5%, while up to 30% of NK cells degranulated in response to both M1 and TAM (mean of 11% against M1, 15% against TAM, **Fig. 2A**). Up to 20% of NK cell degranulation against TAM-S confirmed that NK cells responded to the macrophages (**Suppl. Fig. S3A**). To further study the effect of macrophages on the effector functions of NK cells, we determined IFN-γ levels in the supernatants after 24h co-cultures of macrophages and NK cells. This revealed an average of 180 pg/mL and 40 pg/mL IFN-γ in the co-cultures with M1 and with TAM, respectively (**Fig. 2B**). Moreover, there was no obvious difference in the IFN-γ production in co-cultures with TAM or TAM-S (**Suppl Fig 3B**). In supernatants from conditions with NK cells alone, TAM alone or M1 alone, IFN-γ was not produced, indicating that the IFN-γ secretion required the interaction between macrophages and NK cells (**Suppl Fig S3C**). While both macrophages and activated

NK cells could produce IFN-γ, intracellular staining for IFN-γ upon co-culture of macrophages and NK cells further confirmed that NK cells are producing IFN-γ in the co-cultures with macrophages (**Fig. 2C** and **Suppl Fig 3D-E**). Despite NK cells responding to M1 and TAM, both types of macrophages did not seem to be killed by NK cells (**Suppl Fig S3F**).
Because TAM have been shown to inhibit cytotoxic effector functions of NK cells which would compromise the NK cell anti-tumor responses (24, 25), we also determined whether co-cultures with macrophages reduced the tumor killing capacity of activated NK cells in our setup. For this, M1 and TAM were co-cultured with IL-2 activated donor NK cells for 24 hours. Afterwards, NK cells were harvested and co-cultured with the multiple myeloma cell line L363 in a 4-hour cytotoxicity assay. Without priming of macrophages, NK cells killed between 27% and 53% of L363 cells and this response was not strongly reduced when NK cells were primed by TAM or M1 (**Fig. 2D**). This illustrated that highly activated NK cells can be triggered to degranulate and produce IFN-γ by both macrophages types while leaving their direct cytotoxic anti-tumor potential remained intact.

NK cell degranulation and IFN-γ production in response to PD-L1+ M1 macrophage targets can be promoted with the ADCC-triggering antibody Avelumab
Since the production of IFN-γ could contribute to the overall anti-tumor effect of NK cells, we investigated whether the response could be enhanced by using clinically applicable ADCC-mediating antibodies that engage CD16, which is a powerful activating receptor on NK cells (20). We observed that both M1 and TAM expressed PD-L1 and that M1 expressed PD-L1 at higher levels, most likely due to the polarization with rhIFN-γ (**Fig. 3A**). Because we detected PD-L1 expression on the macrophages, we used the anti-PD-L1 antibody Avelumab to enhance the NK cell response against macrophages. Importantly, Avelumab has been used in clinical studies and can, next to blocking interaction of PD-L1 and its ligand PD-1, also mediate ADCC (26). To test NK cell degranulation against the macrophages with Avelumab, M1 or TAM were co-cultured for 4h with NK cells in the presence of the anti-PD-L1 antibody Avelumab. IL-2 activated NK cells did not express PD-1 (**Suppl. Fig. 4A**).
In the absence of macrophages, 0-4% of NK cells spontaneously degranulated without Avelumab and 1-14% of NK cells degranulated in presence of Avelumab (mean fold increase 4.5, **Fig. 3B**). Against M1, NK cells degranulation ranged from 2% to 12% without Avelumab (**Fig. 3B**). However, when Avelumab was added, degranulation against M1 was increased to 14 – 39% CD107a+ NK cells (mean fold increase 6.2, **Fig. 3B**). Against TAM, NK cell degranulation ranged from 3 – 26% and the addition of Avelumab resulted in a small increase in NK cell degranulation in 254 10 of 16 donors (mean fold increase 1.3, **Fig. 3B**). A comparable increase with Avelumab was also observed against TAM-S (**Suppl Fig S4C**).

To further assess the NK cell effector response, IFN-γ production was measured in the supernatant of 24h NK cell – macrophage co-cultures. Avelumab itself did not enhance IFN-γ levels in cultures with only NK cells or only macrophages (**Fig. 3C, Suppl. figure S4B**). In line with the avelumab-induced degranulation, more IFN-γ release was observed when Avelumab was added to M1 compared to conditions without Avelumab (**Fig. 3C**). Such a clear Avelumab-induced increase was not observed with TAM nor with TAM-S as target cells (**Fig. 3C, Suppl Fig S4D**).
Together, our results showed enhanced NK cell degranulation and IFN-γ production by Avelumab against M1 target cells with high PD-L1 expression and to some extent against TAM with lower PD-L1 expression.

PD-L1 expression on macrophages was upregulated by NK cells in an IFN-γ dependent manner but did not further enhance the Avelumab-induced NK cell responses against TAM

We and others previously showed that the magnitude of ADCC triggered by an ADCC-inducing antibody is related to the level of expression of the cognate antigen (22, 27). Since IFN-γ, produced by activated immune cells, has been shown to enhance PD-L1 expression (28), we assessed whether cytokines produced by activated NK cells could enhance PD-L1 expression on macrophages and whether this could improve the effect of Avelumab on TAM. To study this, macrophages were exposed to NK supernatant derived from NK cells that were activated with a high dose of IL-2 for 24h. No obvious changes in morphology were observed after treatment with NK supernatant (**Suppl Fig S1B**). PD-L1 expression was already high on M1 and was only slightly enhanced by addition of NK supernatant (1.3-fold on average), while PD-L1 expression on TAM was highly upregulated after incubation with NK supernatant (10-fold on average, **Fig. 3D**). The upregulation of PD-L1 on TAM was largely blocked by addition of an IFN-γ-receptor-blocking antibody, but not by an isotype control, demonstrating that the PD-L1 upregulation was largely mediated through IFN-γ (**Fig. 3D**). These results showed that highly activated NK cells produce IFN-γ in sufficient amounts to increase expression of the inhibitory checkpoint PD-L1 on macrophages. Additionally, we included conditions with two concentrations of rhIFN-γ to test if the PD-L1 expression on TAM could be enhanced further. Preincubation with both 5 ng/mL and 20 ng/mL rhIFN-γ enhanced PD-L1 expression to comparable levels as NK supernatant, which might suggest that the maximal expression under our in vitro culture conditions was reached (**Fig. 3E**).
Next, we tested whether the 10-fold increase in PD-L1 on TAM after incubation with NK supernatant or rhIFN-γ coincided with stronger Avelumab-induced NK cell responses. This was not the case; the fold-increase in degranulation with Avelumab (compared to control without Avelumab) was less than 2-fold for TAM with NK supernatant and for TAM with rhIFN-γ (**Fig. 3F**), which was comparable to the fold-increase for TAM in medium (**Fig. 3B**). Similarly, Avelumab-induced IFN-γ release was not further enhanced by the higher expression of PD-L1 on TAM preincubated with either NK supernatant or rhIFN-γ (**Fig. 3G**). Against TAM-S, we observed the same

responses as against TAM, supporting that the described responses are at least in part directed against the macrophages rather than against L363 (**Suppl Fig. S4E-G**). Overall, our results indicate that increased PD-L1 expression on TAM after exposure to NK supernatant or rhIFN-γ did not further amplify the effect of Avelumab on NK cell responses against TAM.

NK cell subgroups that do not encounter their HLA class I ligand degranulated stronger against macrophage target cells than NK cells that can interact with HLA class I

Since HLA class I is one of the most important inhibitors of NK cells, we studied whether HLA class I on macrophages is functionally relevant for NK cells. In our setup, both M1 and TAM expressed HLA class I (**Fig. 4A**). To investigate the relevance of HLA class I expression on macrophages, we analyzed the degranulation of NK cell subsets that encountered their cognate HLA ligand on macrophages (KIR-HLA ligand matched NK cells) and NK cell subsets not encountering their ligand (KIR-HLA ligand-mismatched NK cells) in CD107a assays. Table 1 provides an overview of the KIR-ligand matched and -mismatched NK cell populations.

Against M1 as target cells, the NK cell mismatched subgroup degranulated more vigorously than the matched subgroup in 16 of the 18 tested NK cell-target cell combinations (**Fig. 4B**). Against TAM, the difference in degranulation was less pronounced with some NK cell donors, but overall, the mismatched NK cell subsets degranulated more upon co-culture with TAM than the matched NK cells (12 of the 18 tested NK cell – target cell combinations) and this was also the case when TAM-S were used as target cells (**Fig. 4B**). The enhanced degranulation in the mismatched populations compared to the matched populations confirms that the HLA class I at least partly controls the magnitude of the NK cell response against macrophages.

HLA class I is known to be upregulated in response to IFN-γ (29). To further study the functional relevance of HLA on the Avelumab-induced NK cell responses, we determined expression levels of all HLA class I on TAM upon incubation with NK supernatant. As expected, total HLA-class I as well as all the HLA molecules most relevant for NK cells (i.e., HLA-C, Bw4, and HLA-E) were upregulated on TAM after incubation with NK supernatant (**Fig. 4C**). HLA expression levels could potentially counteract or limit the ADCC-response of NK cells with Avelumab. To test this, we selected three NK cell subsets either exclusively expressing NKG2A, KIR-ligand-matched, or KIR-ligand-mismatched inhibitory receptors. To confirm that the NK cell responses were directed against the macrophages, both TAM and TAM-S were analyzed. The enhanced expression of HLA class I in response to NK supernatant did not result in an obvious difference in degranulation patterns between the three NK cell subsets (**Fig. 4D-E**). If enhanced HLA expression was limiting the Avelumab-induced response, it would be expected that the KIR-ligand mismatched subset shows a stronger Avelumab effect than the other two subsets because the KIR-ligand mismatched subsets lack interaction with their inhibitory HLA-C ligands. However,

this was not the case and the increase in degranulation in combination with Avelumab was comparable for the subsets (**Fig. 4D-E**).
Our data therefore demonstrated that HLA class I is an inhibitor of NK cell responses against both M1 and TAM and that HLA expression at least partly mediates the magnitude of NK cell responses. Moreover, it suggests that enhanced HLA levels upon incubation with NK supernatant on TAM are not contributing to the absence of the Avelumab effect against TAM.

Discussion

Creating a proinflammatory and immunostimulatory TME may be crucial for long-lasting effects of immunotherapies. In this study, we observed that IL-2 activated donor-derived NK cells degranulated and produced IFN-γ upon interaction with macrophages in our in vitro setup. The extent of NK cell reactivity against macrophages was, at least partly, controlled by HLA class I and could be further enhanced in combination with the ADCC-inducing antibody Avelumab against PD-L1-high M1 target cells, but not against PD-L1$^+$ TAM. Importantly, we showed that the killing capacity of high-dose IL-2 activated NK cells remained comparable between macrophage-primed and unprimed NK cells. In previous studies, using a murine model or TAM derived from gastric cancer patients, NK cell responses such as degranulation and IFN-γ were shown to be impaired (24, 30). Our data provide evidence for the hypothesis that, even under suppressive conditions, activated NK cells can contribute to the overall anti-tumor immunity, on the one hand by direct cytotoxicity against tumor cells and on the other hand through production of IFN-γ which can enhance antigen presentation through HLA class I upregulation and stimulate Th1 and CD8$^+$ T cell responses (21, 31). This information is very valuable for strategies aiming to use adoptive transfer of donor NK cells that have been expanded and activated ex vivo.

Although NK cell-derived IFN-γ may promote type I immunity, IFN-γ is also known to upregulate multiple factors including inhibitory molecules and might thus have opposing effects on immune cells including NK cells (32). Moreover, IFN-γ can enhance expression of the immune checkpoint molecules PD-L1 on both tumor cells and other TME cells, hence, providing inhibition to tumor infiltrating T cells with an exhausted PD-1+ phenotype (33). To prevent this type of immune suppression, several clinically-approved antibodies are available to block the interaction between PD-1 and its ligand PD-L1. Of these antibodies, Avelumab is the only one that can also mediate ADCC by engaging with the FcγRIIIa CD16 and thereby a strong NK cell responses against tumor cells (26). In the present study, we showed that Avelumab-induced NK cell responses could also be directed against macrophages, most prominently against PD-L1-high M1. This demonstrates that the combination of NK cells and Avelumab may be also very relevant for tumors that lack expression of PD-L1 on tumor cells but that do contain PD-L1$^+$ TAC in their TME.

In our study, both TAM and M1 expressed PD-L1 while Avelumab boosted degranulation and IFN-γ production of NK cells in response to M1 but not in response to TAM (**Fig. 3**). This may have to with the higher degranulation level against TAM in the absence of Avelumab, or with the slightly lower expression levels of the PD-L1 antigen on TAM vs M1. In previous studies the relation between antigen expression levels and ADCC strength has been shown, for example responses to PD-L1 blockade have been positively correlated with PD-L1 expression and a higher PD-L1 expression was found in breast cancer patients with complete response, compared to patients

without complete response (34); breast cancer cell lines with high PD-L1 expression were more sensitive to Avelumab-mediated ADCC (27); and higher antigen expression and clustering on the cell surface have been correlated with stronger ADCC responses of Daratumumab (targeting CD38), Rituximab (targeting CD20), or Trastuzumab (targeting HER2) (22, 35-37).

We observed further upregulation of PD-L1 expression by NK-derived IFN-γ which was in line with a previous study showing that adoptively transferred expanded NK cells induced IFN-γ-mediated PD-L1 upregulation on tumor cells in xenograft models of lung cancer (38, 39). In our study, the increased PD-L1 expression on TAM upon incubation with rIFN-γ or NK cell supernatant did not translate in an increase in ADCC strength in the presence of Avelumab. In line with our data, a previous study evaluated PDL1 expression on a broad panel of cell lines and showed that IFN-γ increased PDL1 expression in all tested tumor cell lines. However, increased sensitivity to Avelumab-induced ADCC was observed only in some cell lines. Although no explanation for this effect was given, these data suggest that other factors may be influencing the overall result of Avelumab (26). In our study, the lack of Avelumab effect could not fully be explained by an increase in expression of inhibitory HLA molecules upon incubation with rhIFN-γ or NK cell supernatant. As the level of expression matters, one explanation may be that the PD-L1 level on TAM was still not high enough to trigger potent activation of NK cells via Avelumab. In addition, IFN-γ could lead to the enhanced expression of additional factors or molecules that have been shown to affect NK cell activation positively e.g., the adhesion molecules ICAM, or negatively, e.g. IDO (31, 40). In a future study, it would therefore be interesting to generate a comprehensive overview of the differences in non-HLA ligands for NK cells expressed by M1 vs TAM and how they are influenced by IFN-γ or other factors secreted by activated NK cells as this may help to explain the differences between M1 and TAM that we observed.

The receptor PD-1 is frequently expressed on exhausted T cells but in NK cells its role and functional relevance is still debated (41, 42). While PD-1+ NK cells have been found in MM patients (43), several studies showed that cytokine-activated NK cells express no or only low levels of PD-1 (27, 41, 44). In the situation where immune cells do express PD-1, Avelumab could thus have a dual effect: 1) block the inhibitory effects of PD-1/PD-L1 interactions, which will release PD-1+ T- or NK cells from inhibition, 2) mediate ADCC in NK cells against tumor or TME-associated PD-L1 expressing cells, and 3) activate NK cells by engagement of CD16 via the Fc domains of the free floating Avelumab antibody, potentially improving anti-tumor activity in a manner that is not depending on formation of a lytic junction and cytotoxicity. In our current set up, we observed some effect of Avelumab on NK cell degranulation in the absence of target cells. As we did not observe PD-1 expression on NK cells in our study, it is unlikely that the Avelumab effect was mediated through the mechanism of blocking the PD-1/PD-L1 axis, although we cannot fully exclude it. Moreover, the combination of effects may be highly relevant when other ADCC-

inducing antibodies are used that are directed against ligands of more abundantly expressed inhibitory receptors on NK cells.

In the current study we observed that KIR-ligand mismatched NK cell subsets, for which the HLA ligand on macrophages was absent, degranulated stronger than the corresponding KIR-ligand matched NK cell subsets, illustrating that HLA class I inhibited NK cell effector function. These results were in line with our previous findings with MM tumor cells, where we showed that selection of KIR-ligand mismatched donors represents a strategy to lower the NK cell activation threshold (22). The importance of HLA on macrophages has been highlighted in another study, where NK cells isolated from ovarian cancer patients could kill TAM that expressed low levels of HLA class I but not macrophages that expressed high levels of HLA class I (45). In another study, activated NK cells have been shown to attack macrophages differentiated towards M0 and M2, while M1 macrophages were resistant to NK cells due to their higher expression of HLA class I (46). In our study, M1 seemed to express higher levels of HLA class I than TAM. In agreement with this, the level of NK cell degranulation induced by M1 was slightly lower than the level of degranulation in response to TAM. Interestingly, IFN-γ production by NK cells seemed to be higher in the conditions with M1 than with TAM. This may be due to the difference in experimental set up and kinetics of both assays as degranulation was measured after 4h of co-culture and IFN-γ after 24h. It has been shown previously that NK cell degranulation and IFN-γ production do not always go in parallel (47). Since NK cell activation is determined by the net balance of a broad array of receptors, ligands other than HLA class I or soluble factors could be complementary regulators of the NK cell responses, and M1 and TAM may differ in the expression of such non-HLA regulators.

One of the limitations of our study is the fact that we were not able to comprehensively quantify cell death of macrophages in our flow cytometry-based cytotoxicity assays because macrophages did not detach by pipetting and more harsh isolation procedures resulted in a high level of spontaneous macrophages death and unreliable analysis of NK-cell mediated killing of macrophages. However, by evaluation of images, we observed that the macrophages in all conditions were still attached to the wells and did not undergo apoptosis, suggesting that both types of macrophages were not killed by the degranulating NK cells. Similar to lymphocytes, monocyte-derived cells can be equipped with protection mechanisms against misdirected granzyme B such as granzyme B antagonists, which could be one explanation for the absence of macrophage killing be observe despite the NK cell degranulation (48). In addition, we determined the response of NK cells against macrophages as proxy for tumor accessory cells in general. We used macrophages as target cells because they are abundantly present and are generally considered a central mediator of tumor progression and poor prognosis in MM (49, 50). In the TME, TAM can acquire profiles characterized by both proinflammatory and anti-

inflammatory cytokines including IL-6 and IL-10 (11). To mimic the interaction of TAM with MM cells, as it likely occurs in the TME, TAM in our study were generated with L363 cells or their supernatant. Tumor cell line-derived supernatant together with a cytokine-cocktail has been used previously to generate TAM in vitro (51). A specific marker to identify TAM is not yet known due to high plasticity of TAM depending on received signals and heterogeneity in TME in MM. Commonly used TAM or M2-like markers include CD163 and CD206, also in MM (50, 52). These markers are upregulated in response to stimulation with IL-10 and IL-4, which were not included in our cytokine cocktail and could thus be a reason why we observed rather low expression of these molecules on TAM in our study (53). As expected, pro-inflammatory cytokines were expressed by M1 as was the anti-inflammatory cytokine IL-10, which could be a response to the LPS stimulation (54). Compared to the other cytokines produced by TAM, we found high VEGF production, a factor known to be secreted by TAM in the MM BM and a main player of angiogenesis (52).

To include two ends of the macrophage spectrum, we generated M1 and TAM and the different NK cell responses suggest that the type of macrophage matters. In the TME of MM, the heterogeneity of TAM is however much more complex since they are exposed to the aberrant cytokine and signalling pathways of the TME (3). Therefore, our study should be followed up in more complex models that better mimic the complex network in the TME of MM as well as the 3D tumor growth, for instance with in vivo models or tumor organoids that reflect TAM plasticity (55, 56). In such models, it could also be assessed whether the NK cell degranulation against M1 and TAM is continuous and could eventually lead to NK cell exhaustion. Moreover, it would be also very interesting to determine the response of NK cells against other cells residing in the MM TME, e.g. stromal cells, that can be PD-L1 positive and that may promote the cytokine-mediated antitumor effects of NK cells in combination with Avelumab.

Collectively, our data support the idea that, even in a immune-suppressive TME such as MM BM, highly activated NK cells could be triggered by TAC and could thereby serve as adjuvants of immune responses through the production of pro-inflammatory cytokines, such as IFN-γ. This would be an important addition to their direct anti-tumor function i.e., killing tumor cells, and may contribute to improved long-lasting adaptive anti-tumor immunity.

Conflict of Interest

G. M. J. Bos is Chief Executive Officer/Chief Medical Officer/Co-founder of CiMaas, BV, Maastricht, The Netherlands. CiMaas is producing an ex vivo expanded NK cell product that will be used to treat myeloma patients. The other authors declare no conflict of interest.

Author Contributions

Conceptualization, F.E., N.M., L.W., G.B.; methodology, F.E., N.M., A.W., T.S.; data curation, F.E., N.M, A.W., T.S.; data analysis, F.E., N.M.; writing—original draft preparation, F.E. and L.W.; writing, review and editing, N.M., A.W., T.S., and G.B.; supervision, L.W. and G.B. All authors have read and agreed to the published version of the manuscript.

Funding

The study was supported by a grant from Kanderonderzoeksfonds Limburg (KOFL).

Acknowledgments

We thank Paul van den Driest and Johanna Bödder for their assistance in this project during their internships. We thank Nicky Beelen and Sabine Roersma for their laboratory assistance.

References

1. Martin T, Huff CA (2019) Multiple myeloma: current advances and future directions. Clinical Lymphoma Myeloma and Leukemia. 19: 255-63.
2. Holthof LC, Mutis T (2020) Challenges for immunotherapy in multiple myeloma: bone marrow microenvironment-mediated immune suppression and immune resistance. Cancers (Basel). 12: 988.
3. Zavidij O, Haradhvala NJ, Mouhieddine TH et al. (2020) Single-cell RNA sequencing reveals compromised immune microenvironment in precursor stages of multiple myeloma. Nature cancer. 1: 493-506.
4. Paolini L, Adam C, Beauvillain C et al. (2020) Lactic acidosis together with GM-CSF and M-CSF induces human macrophages toward an inflammatory protumor phenotype. Cancer immunology research. 8: 383-95.
5. Cetin M, Buyukberber S, Demir M et al. (2005) Overexpression of cyclooxygenase-2 in multiple myeloma: association with reduced survival. Am. J. Hematol. 80: 169-73.
6. Murray PJ, Allen JE, Biswas SK et al. (2014) Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity. 41: 14-20.
7. Gutiérrez-González A, Martínez-Moreno M, Samaniego R et al. (2016) Evaluation of the potential therapeutic benefits of macrophage reprogramming in multiple myeloma. Blood, The Journal of the American Society of Hematology. 128: 2241-52.
8. Akhtar S, Ali TA, Faiyaz A, Khan OS, Raza SS, Kulinski M, Omri HE, Bhat AA, Uddin S (2020) Cytokine-Mediated Dysregulation of Signaling Pathways in the Pathogenesis of Multiple Myeloma. Int. J. Mol. Sci. 21: 5002.
9. Zheng MM, Zhang Z, Bemis K, Belch AR, Pilarski LM, Shively JE, Kirshner J (2013) The systemic cytokine environment is permanently altered in multiple myeloma. PLoS One. 8: e58504.

10. Hope C, Ollar SJ, Heninger E et al. (2014) TPL2 kinase regulates the inflammatory milieu of the myeloma niche. Blood, The Journal of the American Society of Hematology. 123: 3305-15.
11. Kim J, Hematti P (2009) Mesenchymal stem cell–educated macrophages: A novel type of alternatively activated macrophages. Exp. Hematol. 37: 1445-53.
12. Beider K, Bitner H, Leiba M et al. (2014) Multiple myeloma cells recruit tumor-supportive macrophages through the CXCR4/CXCL12 axis and promote their polarization toward the M2 phenotype. Oncotarget. 5: 11283.
13. Wang J, De Veirman K, Faict S, Frassanito MA, Ribatti D, Vacca A, Menu E (2016) Multiple myeloma exosomes establish a favourable bone marrow microenvironment with enhanced angiogenesis and immunosuppression. The Journal of pathology. 239: 162-73.
14. Godfrey J, Benson Jr DM (2012) The role of natural killer cells in immunity against multiple myeloma. Leuk. Lymphoma. 53: 1666-76.
15. Minnie SA, Hill GR (2020) Immunotherapy of multiple myeloma. The Journal of clinical investigation. 130.
16. Oliva S, Troia R, D'Agostino M, Boccadoro M, Gay F (2018) Promises and pitfalls in the use of PD-1/PD-L1 inhibitors in multiple myeloma. Front. Immunol. 9: 2749.
17. Tang T, Huang X, Zhang G, Hong Z, Bai X, Liang T (2021) Advantages of targeting the tumor immune microenvironment over blocking immune checkpoint in cancer immunotherapy. Signal Transduction and Targeted Therapy. 6: 1-13.
18. Lamb MG, Rangarajan HG, Tullius BP, Lee DA (2021) Natural killer cell therapy for hematologic malignancies: successes, challenges, and the future. Stem Cell. Res. Ther. 12: 1-19.
19. Long EO, Sik Kim H, Liu D, Peterson ME, Rajagopalan S (2013) Controlling natural killer cell responses: integration of signals for activation and inhibition. Annu. Rev. Immunol. 31: 227-58.
20. Wang W, Erbe AK, Hank JA, Morris ZS, Sondel PM (2015) NK cell-mediated antibody-dependent cellular cytotoxicity in cancer immunotherapy. Front. Immunol. 6: 368.
21. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S (2008) Functions of natural killer cells. Nat. Immunol. 9: 503-10.
22. Mahaweni NM, Bos GM, Mitsiades CS, Tilanus MG, Wieten L (2018) Daratumumab augments alloreactive natural killer cell cytotoxicity towards CD38+ multiple myeloma cell lines in a biochemical context mimicking tumour microenvironment conditions. Cancer Immunol. Immunother. 67: 861-72. doi: 10.1007/s00262-018-2140-1
23. Ruggeri L, Mancusi A, Capanni M et al. (2007) Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. Blood. 110: 433-40. doi: 10.1182/blood-2006-07-038687
24. Krneta T, Gillgrass A, Poznanski S, Chew M, Lee AJ, Kolb M, Ashkar AA (2017) M2-polarized and tumor-associated macrophages alter NK cell phenotype and function in a contact-dependent manner. J. Leukoc. Biol. 101: 285-95.
25. Wu Y, Kuang DM, Pan WD, Wan YL, Lao XM, Wang D, Li XF, Zheng L (2013) Monocyte/macrophage-elicited natural killer cell dysfunction in hepatocellular carcinoma is mediated by CD48/2B4 interactions. Hepatology. 57: 1107-16.
26. Boyerinas B, Jochems C, Fantini M, Heery CR, Gulley JL, Tsang KY, Schlom J (2015) Antibody-dependent cellular cytotoxicity activity of a novel anti–PD-L1 antibody avelumab (MSB0010718C) on human tumor cells. Cancer immunology research. 3: 1148-57.
27. Juliá EP, Amante A, Pampena MB, Mordoh J, Levy EM (2018) Avelumab, an IgG1 anti-PD-L1 immune checkpoint inhibitor, triggers NK cell-mediated cytotoxicity and cytokine production against triple negative breast cancer cells. Front. Immunol. 9: 2140.
28. Mimura K, Teh JL, Okayama H et al. (2018) PD-L1 expression is mainly regulated by interferon gamma associated with JAK-STAT pathway in gastric cancer. Cancer Sci. 109: 43-53.
29. Méndez R, Rodríguez T, Del Campo A et al. (2008) Characterization of HLA class I altered phenotypes in a panel of human melanoma cell lines. Cancer Immunol. Immunother. 57: 719-29.
30. Peng L-s, Zhang J-y, Teng Y-s et al. (2017) Tumor-associated monocytes/macrophages impair NK-cell function via TGFβ1 in human gastric cancer. Cancer immunology research. 5: 248-56.

31. Martín-Fontecha A, Thomsen LL, Brett S, Gerard C, Lipp M, Lanzavecchia A, Sallusto F (2004) Induced recruitment of NK cells to lymph nodes provides IFN-γ for TH 1 priming. Nat. Immunol. 5: 1260-5.
32. Gocher AM, Workman CJ, Vignali DA (2022) Interferon-γ: teammate or opponent in the tumour microenvironment? Nature Reviews Immunology. 22: 158-72.
33. Herbst RS, Soria J-C, Kowanetz M et al. (2014) Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature. 515: 563-7.
34. Ahmed FS, Gaule P, McGuire J, Patel K, Blenman K, Pusztai L, Rimm DL (2020) PD-L1 protein expression on both tumor cells and macrophages are associated with response to neoadjuvant durvalumab with chemotherapy in triple-negative breast cancer. Clin. Cancer Res. 26: 5456-61.
35. Roghanian A, Teige I, Mårtensson L et al. (2015) Antagonistic human FcγRIIB (CD32B) antibodies have anti-tumor activity and overcome resistance to antibody therapy in vivo. Cancer Cell. 27: 473-88.
36. Chew HY, De Lima PO, Cruz JLG et al. (2020) Endocytosis inhibition in humans to improve responses to ADCC-mediating antibodies. Cell. 180: 895-914. e27.
37. Ehlers FA, Beelen NA, van Gelder M, Evers TM, Smidt ML, Kooreman LF, Bos GM, Wieten L (2021) ADCC-Inducing Antibody Trastuzumab and Selection of KIR-HLA Ligand Mismatched Donors Enhance the NK Cell Anti-Breast Cancer Response. Cancers (Basel). 13: 3232.
38. Poznanski SM, Ritchie TM, Fan IY et al. (2021) Expanded human NK cells from lung cancer patients sensitize patients' PDL1− negative tumors to PD1-blockade therapy. Journal for immunotherapy of cancer. 9.
39. Oyer JL, Gitto SB, Altomare DA, Copik AJ (2018) PD-L1 blockade enhances anti-tumor efficacy of NK cells. Oncoimmunology. 7: e1509819.
40. Aquino-López A, Senyukov VV, Vlasic Z, Kleinerman ES, Lee DA (2017) Interferon gamma induces changes in natural killer (NK) cell ligand expression and alters NK cell-mediated lysis of pediatric cancer cell lines. Front. Immunol. 8: 391.
41. Judge SJ, Dunai C, Aguilar EG et al. (2020) Minimal PD-1 expression in mouse and human NK cells under diverse conditions. The Journal of clinical investigation. 130: 3051-68.
42. Lanuza PM, Pesini C, Arias MA, Calvo C, Ramirez-Labrada A, Pardo J (2020) Recalling the biological significance of immune checkpoints on NK cells: a chance to overcome LAG3, PD1, and CTLA4 inhibitory pathways by adoptive NK cell transfer? Front. Immunol.: 3010.
43. Benson Jr DM, Bakan CE, Mishra A et al. (2010) The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti–PD-1 antibody. Blood, The Journal of the American Society of Hematology. 116: 2286-94.
44. Ahmadzadeh M, Johnson LA, Heemskerk B, Wunderlich JR, Dudley ME, White DE, Rosenberg SA (2009) Tumor antigen–specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. Blood, The Journal of the American Society of Hematology. 114: 1537-44.
45. Bellora F, Castriconi R, Dondero A et al. (2014) TLR activation of tumor-associated macrophages from ovarian cancer patients triggers cytolytic activity of NK cells. European journal of immunology. 44: 1814-22.
46. Bellora F, Castriconi R, Dondero A, Reggiardo G, Moretta L, Mantovani A, Moretta A, Bottino C (2010) The interaction of human natural killer cells with either unpolarized or polarized macrophages results in different functional outcomes. Proceedings of the National Academy of Sciences. 107: 21659-64.
47. Bryceson YT, Fauriat C, Nunes JM, Wood SM, Björkström NK, Long EO, Ljunggren H-G (2010) Functional analysis of human NK cells by flow cytometry. Natural Killer Cell Protocols. Springer. pp. 335-52
48. Classen C, Bird PI, Debatin K-M (2006) Modulation of the granzyme B inhibitor proteinase inhibitor 9 (PI-9) by activation of lymphocytes and monocytes in vitro and by Epstein–Barr virus and bacterial infection. Clin. Exp. Immunol. 143: 534-42.
49. Roussou M, Tasidou A, Dimopoulos M et al. (2009) Increased expression of macrophage inflammatory protein-1α on trephine biopsies correlates with extensive bone disease,

increased angiogenesis and advanced stage in newly diagnosed patients with multiple myeloma. Leukemia. 23: 2177-81.
50. Suyanı E, Sucak GT, Akyürek N, Şahin S, Baysal NA, Yağcı M, Haznedar R (2013) Tumor-associated macrophages as a prognostic parameter in multiple myeloma. Ann. Hematol. 92: 669-77.
51. Benner B, Scarberry L, Suarez-Kelly LP et al. (2019) Generation of monocyte-derived tumor-associated macrophages using tumor-conditioned media provides a novel method to study tumor-associated macrophages in vitro. Journal for immunotherapy of cancer. 7: 1-14.
52. Sun J, Park C, Guenthner N et al. (2022) Tumor-associated macrophages in multiple myeloma: advances in biology and therapy. Journal for Immunotherapy of Cancer. 10.
53. Ambarus CA, Krausz S, van Eijk M, Hamann J, Radstake T, Reedquist KA, Tak PP, Baeten D (2012) Systematic validation of specific phenotypic markers for in vitro polarized human macrophages. J. Immunol. Methods. 375: 196-206.
54. Mosser DM (2003) The many faces of macrophage activation. J. Leukoc. Biol. 73: 209-12.
55. Rebelo SP, Pinto C, Martins TR et al. (2018) 3D-3-culture: A tool to unveil macrophage plasticity in the tumour microenvironment. Biomaterials. 163: 185-97.
56. Papadimitriou K, Kostopoulos IV, Tsopanidou A, Orologas-Stavrou N, Kastritis E, Tsitsilonis OE, Dimopoulos MA, Terpos E (2020) Ex vivo models simulating the bone marrow environment and predicting response to therapy in multiple myeloma. Cancers (Basel). 12: 2006.

Table and Figures

Table 1. KIR-ligand matched and -mismatched NK cell subsets per macrophage donor.

Macrophage donor 1, 4, 5	**$C1^+$**	**$C2^-$**	**$Bw4^-$**
NK cell donors	KIR2DL2/3 = M	KIR2DL1 = MM	KIR3DL1 = MM
Macrophage donor 2	**$C1^-$**	**$C2^+$**	**$Bw4^+$**
NK cell donors	KIR2DL2/3 = MM	KIR2DL1 = M	KIR3DL1 = M
Macrophage donor 3	**$C1^+$**	**$C2^+$**	**$Bw4^-$**
NK cell donors	KIR2DL2/3 = M	KIR2DL1 = M	KIR3DL1 = MM

Macrophage donors were genotyped for HLA class I and classified as positive or negative for the HLA C1, C2 or Bw4 epitope groups. NK cells were derived from different donors, of which HLA class I genotype was determined to ensure that KIRs were licensed. $NKG2A^-$ NK cell subsets were grouped into KIR-ligand-matched (M) or -mismatched (MM) subsets based on the HLA typing of the macrophage targets, e.g., for macrophage donor 1, 4, 5, the KIR2DL2/3 single-positive NK cell subset was considered matched, while KIR2DL1 single-positive, KIR3DL1 single-positive, and $KIR2DL1^+$ $KIR3DL1^+$ double-positive NK cell populations were considered mismatched.

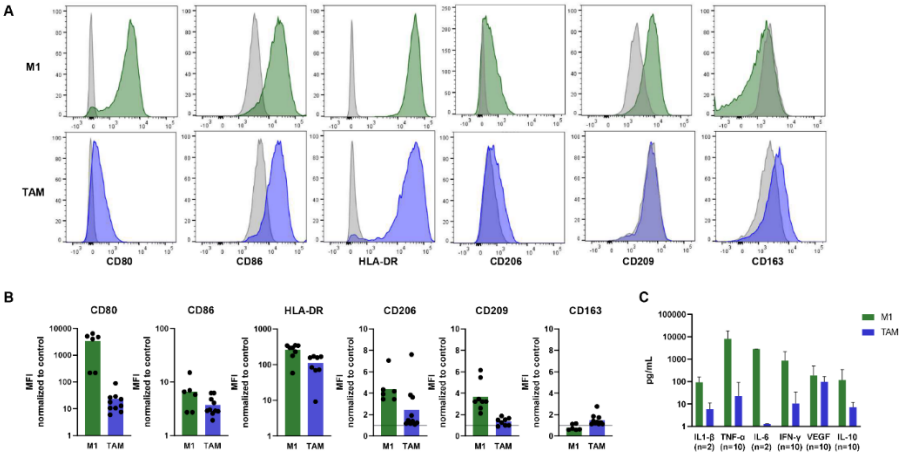


Figure 1. Phenotype of the macrophages polarized towards M1 and TAM. Monocyte-derived macrophages were polarized towards M1 or TAM phenotype. **A)** After harvesting, the cells were stained for expression levels of the indicated markers and measured by flow cytometry. Representative histograms are shown. Grey histograms depict FMO and green (M1) or blue (TAM) histograms depict staining with an antibody that is specific for the indicated marker. **B)** Quantification of A. Expression on all donors depicted as normalized MFI (MFI of marker/MFI of FMO). Each dot represents one donor in monoplo and bars indicate the mean of all donors (n=6-10). **C)** Cytokine production was assessed in the supernatant of polarized M1 and TAM by CBA (n=2 in duplo for all cytokines, additional n=8 in monoplo for 4 cytokines). The values were corrected for the concentrations measured in the medium used for polarization. Bars indicate means + SD. TAM = tumor-associated macrophages, MFI = median fluorescence intensity, FMO = Fluorescence-minus-one (unstained living cells).

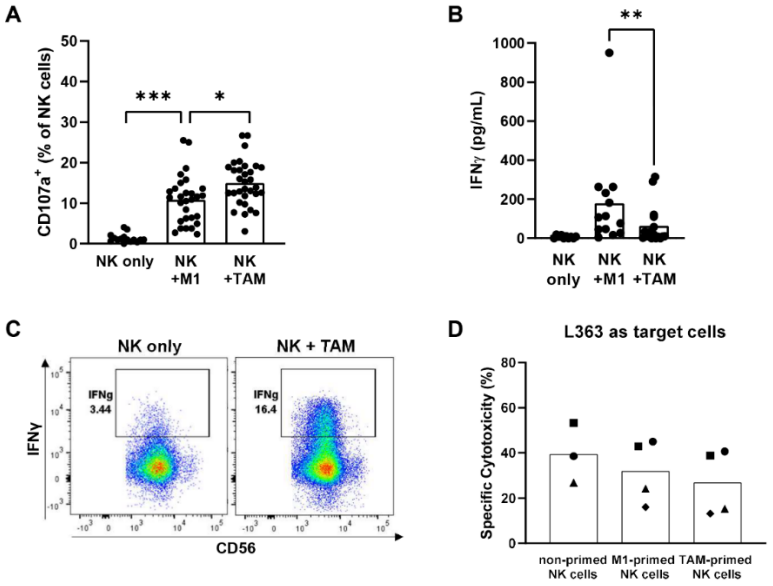


Figure 2. Cytokine-activated NK cells are triggered by in vitro-polarized macrophages. Monocyte-derived macrophages were polarized towards M1 or TAM phenotype and subsequently co-cultured with IL-2 activated NK cells. **A)** Macrophages (8 donors) and NK cells (12 donors) in different combinations were co-cultured for 4h, resulting in 29-32 unique macrophage-NK cell donor combinations depicted by the dots. NK cell degranulation (CD107a) against the macrophages as target cells was measured by flow cytometry in monoplo. **B)** IFN-γ production in the supernatant upon 24h co-culture of macrophages (4 donors) and NK cells (8 donors) was assessed by CBA in duplo. Dots show unique macrophage-NK cell donor combinations. **C)** IFN-γ production by NK cells was determined by intracellular staining in the same setup as in B. Representative plots are shown and quantification is shown in Suppl. Fig. S3E. **D)** Macrophages and NK cells were co-cultured for 24h. The macrophage-primed NK cells were harvested and co-cultured with L363 target cells for 4h in a 1:1 E:T ratio in duplo (cytotoxicity assay). Symbols represent the different NK cell donors. The percentage of dead L363 tumor cells is depicted as % specific cytotoxicity. In all graphs, bars represent means. Wilcoxon matched pairs tests were performed to determine statistical significance. Some of the donors in (**A,B**) are also used for Figure 3 and 4.

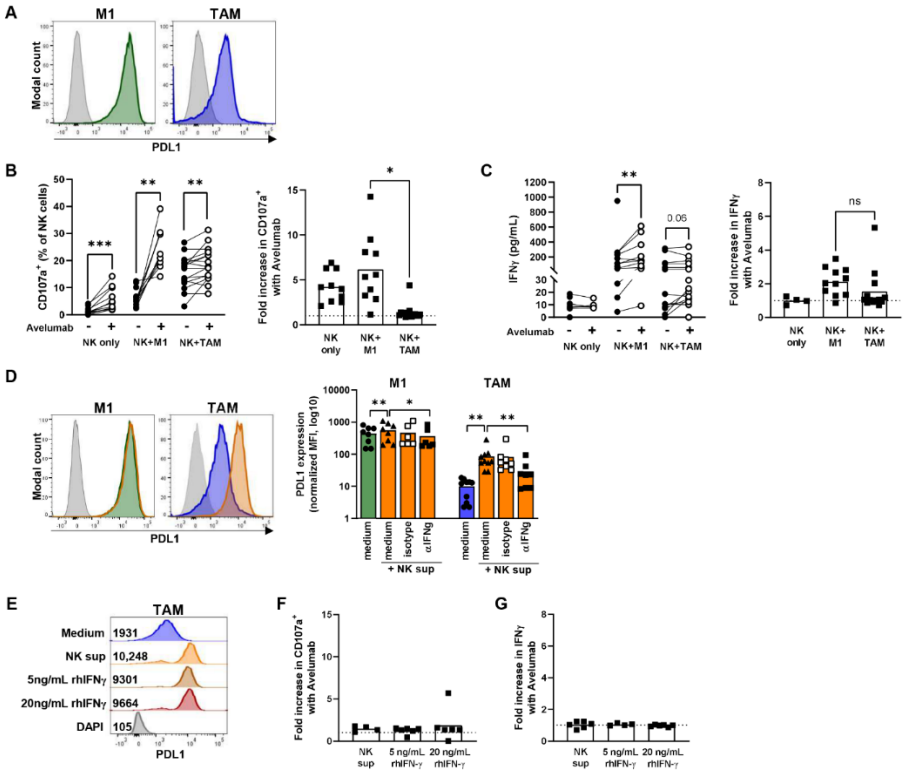


Figure 3. NK cell responses to PD-L1^{+} macrophage target cells can be promoted by Avelumab. A) PD-L1 expression on M1 and TAM of a representative donor with the PD-L1 expression in green (M1), blue (TAM) and grey (FMO, unstained living cells). **B-C)** Macrophages and activated NK cells were co-cultured for 4h **(B)** or 24h **(C)**. Macrophages (4 donors) were co-cultured with NK cells (8 donors) in different macrophage-NK cell donor combinations, resulting in up to 16 unique combinations (depicted as dots). The lines connect paired data points from the same donor with and without Avelumab. NK cell degranulation (CD107a) was measured by flow cytometry in monoplo **(B)**. IFN-γ levels were measured in the supernatants of the co-cultures by CBA in duplo **(C)**. **D)** PD-L1 expression on M1 and TAMs after 24h co-incubation with NK cell sup alone or in combination with IFN-γ receptor-blocking antibody or isotype control. Representative histograms are shown (orange = with NK cell sup) and quantification of PD-L1 expression is shown as normalized MFI (MFI of PD-L1-stained cells/MFI of FMO). Each dot represents one macrophage donor (n=8-10 donors). **E-G)** TAM (2 donors) were co-incubated with either NK sup or rhIFN-γ for 24h. Subsequently, PD-L1 expression was assessed **(E)** and TAMs were co-cultured with NK cells (5 donors) for 4h **(F)** or 24h **(G)** and NK cell degranulation (CD107a, monoplo) and IFN-γ secretion (duplo) were assessed as described above. The data points without Avelumab are also included in figure 2. Fold increase with Avelumab compared to without Avelumab is depicted. Wilcoxon matched pairs tests were performed to determine statistical significance. NK sup = NK cell supernatant. rhIFN-γ = recombinant human IFN-γ.

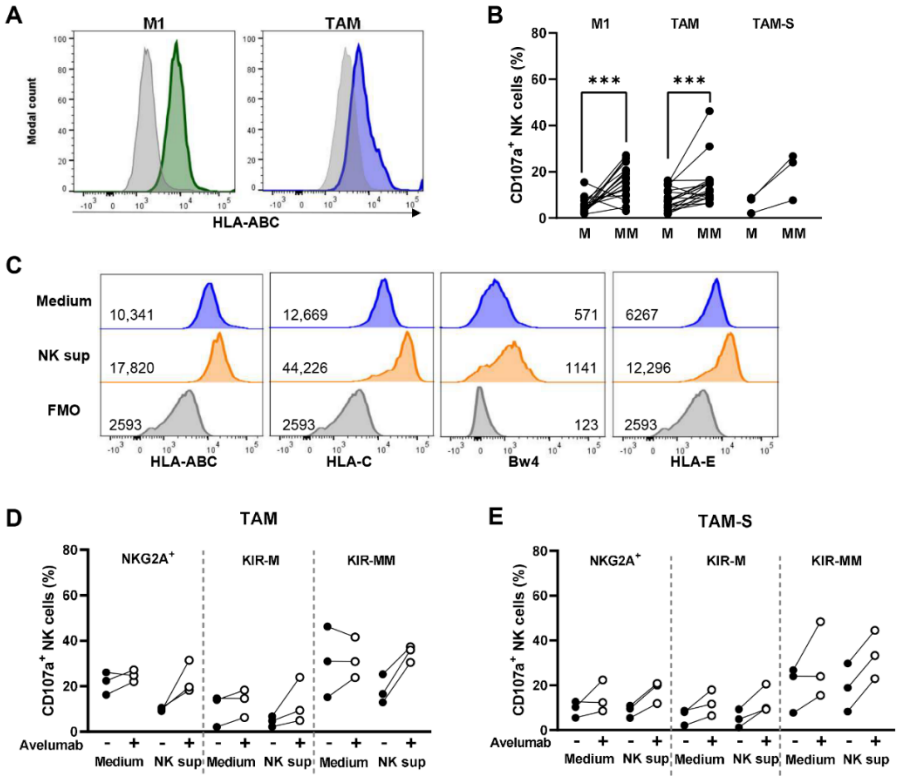
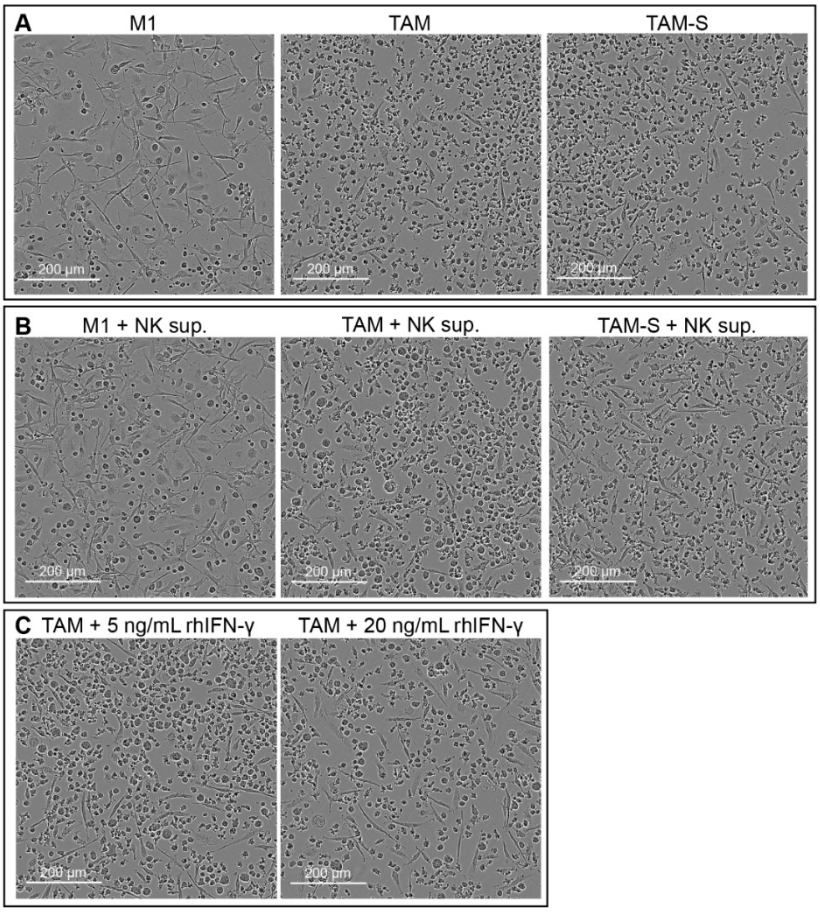
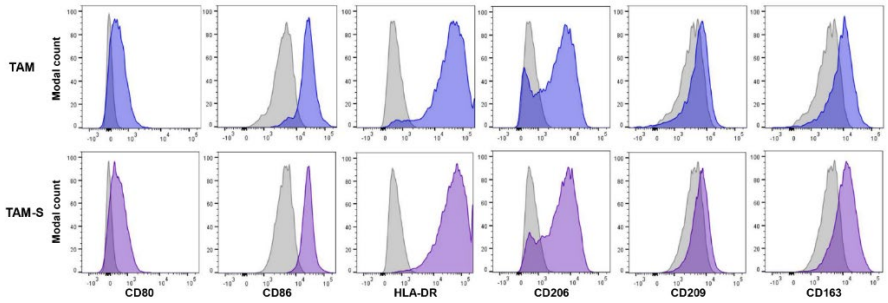


Figure 4. NK cell degranulation against macrophage targets was more pronounced in the KIR-ligand mismatched NK cell subset than in the matched NK cell subset. A) HLA-expression on M1 and TAM macrophages. **B-E)** NK cell subpopulations were analyzed from the degranulation assay: $CD56^+$ $CD3^-$ NK cells were further divided in NK cell subgroups that were KIR-HLA ligand-matched (M) or –mismatched (MM). Degranulation of both subgroups is shown. **C)** HLA expression on TAM in control medium or after 24h incubation with NK sup. Living macrophages served as FMO. **D-E)** $NKG2A^+$ KIR^-, $NKG2A^-$ KIR-matched, $NKG2A^-$ KIR-mismatched NK cell subsets were selected and their degranulation potential against TAM **(D)** or TAM-S **(E)** in medium vs. NK sup was compared with or without Avelumab (3 NK cell donors, 1 macrophage donor). Wilcoxon matched pairs tests were performed to determine statistical significance. NK sup = NK cell supernatant, FMO= Fluorescent-minus-one.

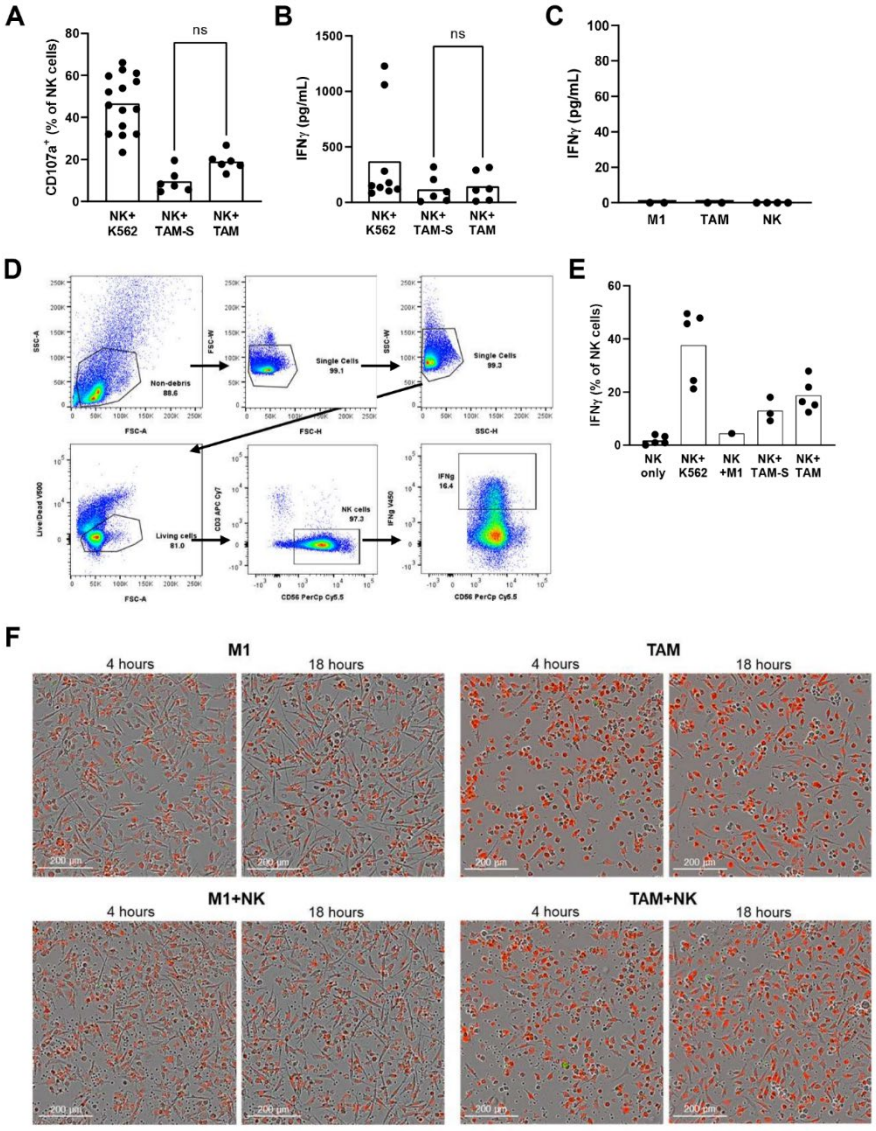
Supplementary Figures



Supplementary figure S1. Morphology of macrophages polarized towards M1 or TAM. A-C) Macrophages were polarized towards M1, TAM or TAM-S and incubated in medium **(A)**, NK sup **(B)** or rhIFN-γ **(C)** for 24h. Conditions were performed in duplo. Images were taken with a IncuCyte ® S3 Live-Cell Analysis System before harvesting the cells. NK sup = NK cell supernatant, rhIFN-γ = recombinant human interferon-γ, TAM = TAMs generated with L363 cells, TAM-S = TAMs generated with L363 supernatant.

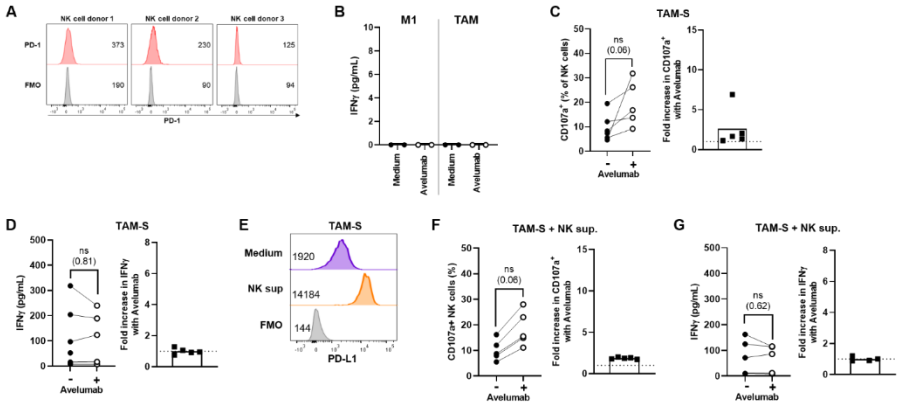


Supplementary figure S2. Phenotype of TAM polarized with L363 cells or with L363 supernatant. TAM-S were generated from 2 donors in monoplo and stained for the indicated cell surface markers and measured by flow cytometry. Grey histograms depict FMO and colored histograms depict staining with an antibody that was specific for the indicated marker. Histograms for TAM-S and TAM were generated from the same donor. TAM = TAMs generated with L363 cells, TAM-S = TAMs generated with L363 supernatant. FMO= Fluorescence-minus-one (unstained living cells).



Supplementary figure S3. NK cell responses against the universal positive control cell line K562 and against TAM polarized with L363 supernatant. A) NK cell degranulation (CD107a) against the universal positive control cell line K562 or against TAMs that were polarized with L363 supernatant (TAM-S) in a 1:1 Effector:Target cell ratio. As comparison, corresponding data from TAM polarized with L363 cells, from Fig. 2A, is depicted. One dot = one NK cells donor, performed in monoplo. **B)** IFN-γ production was assessed in the supernatant of NK cells that were cocultured for 24h with K562 or with TAM-S. IFN-γ was measured by CBA on a flow cytometer. One dot = one NK cells donor, performed in duplo. **C)** IFN-γ production as described in (B) in conditions with M1 alone, TAM alone or NK cells alone. **D)** Gating strategy to identify IFN-γ-producing NK cells by intracellular staining. **E)** Quantification of IFN-γ production by NK cells (5 donors total) against macrophages (2 donors) detected by intracellular staining. **F)** M1 and TAM

were labeled with CM-DiI (red fluorescence), seeded and co-cultured with NK cells (unlabeled) in the same conditions as for CD107a experiments. Caspase-Glo 3/7 (green fluorescent) was added to all conditions. Images were taken with a IncuCyte® S3 Live-Cell Analysis System at the indicated timepoints. Wilcoxon matched pairs tests were performed to determine statistical significance in (A) and (B).



Supplementary figure S4. NK cell responses in combination with Avelumab. (A) PD-1 expression on IL-2 activated NK cells, derived from 3 donors. **(B)** IFN-γ levels detected in the cultures with macrophages only, measured in duplo. **(C-G)** NK cell responses against TAM-S: **(B, F)** NK cell degranulation (CD107a) against TAM-S after 4h co-culture, performed in monoplo. **(C, G)** IFN-γ production after 24h co-cultures measured by CBA in duplo. E**)** PD-L1 expression on TAM-S after culture in medium alone or after NK sup (in monoplo), with FMO depicted in grey. In all graph with lines, the lines connect paired data points of one macrophage-NK donor combination with and without Avelumab. Bars depict means of all donors.

8

Polymorphic differences within HLA-C alleles contribute to alternatively spliced transcripts lacking exon 5

Femke A. I. Ehlers*, Timo I. Olieslagers*, Mathijs Groeneweg, Gerard M. J. Bos, Marcel G. J. Tilanus, Christina E. M. Voorter, Lotte Wieten

*Femke A. I. Ehlers and Timo I. Olieslagers are shared first authors.

HLA. 2022 Sep; 100(3):232-243.
doi: 10.1111/tan.14695

Abstract
The human leukocyte antigen (HLA) genes are amongst the most polymorphic in the human genome. Alternative splicing could add an extra layer of complexity, but has not been studied extensively. Here, we applied an RNA based approach to study the influence of allele polymorphism on alternative splicing of HLA-C in peripheral blood. RNA was isolated from these peripheral cells, converted into cDNA and amplified specifically for twelve common HLA-C allele groups. Through subsequent sequencing of HLA-C, we observed alternative splicing variants of HLA-C*04 and *16 that resulted in exon 5 skipping and were co-expressed with the mature transcript. Investigation of intron 4 sequences of HLA-C*04 and *16 compared to other HLA-C alleles demonstrated no effect on predicted splice sites and branch point. To further investigate if the unique polymorphic positions in exon 5 of HLA-C*04 or *16 may facilitate alternative splicing by acting on splicing regulatory elements (SRE), in-silico splicing analysis was performed. While the HLA-C*04 specific SNP in exon 5 had no effect on predicted exonic SRE, the HLA-C*16 specific exon 5 SNP did alter exonic SRE. Our findings provide experimental and theoretical support for the concept that polymorphisms within the HLA-C alleles influence the alternative splicing of HLA-C.

Keywords:
HLA-C, alternative splicing, exon skipping, splicing regulatory elements

Introduction

HLA-C belongs to the classical HLA class I molecules and its cell-surface expression is generally about 10-fold lower than expression of HLA-A and HLA-B molecules[1]. Despite its relatively low expression, HLA-C surface expression is critical for immunological reactions, for example in the setting of kidney- or stem cell transplantation where an HLA-C mismatch between patient and donor is associated with acute rejection and graft-versus-host disease, respectively[2-5].

The genes encoding classical HLA class I molecules including HLA-C are notoriously polymorphic and single nucleotide polymorphism (SNP) in the HLA-C genes have been shown to impact HLA-C expression levels: For example, polymorphisms in the transcription factor binding sites have been associated with differences in HLA-C promoter activity and cell type- or tissue specific expression[6]. In addition, a SNP located 35kB upstream of the HLA-C gene (-35C/T) has been associated with high HLA-C mRNA and cell-surface expression levels[7]. In a follow up study, evidence was acquired showing that this -35 SNP, presumably, marks a functional 3'UTR insertion/deletion variant influencing HLA-C expression levels through its effects on binding of microRNA-148[8].

Alternative splicing may be another mechanism leading to differences in HLA cell surface expression as it may impact protein expression both quantitatively and qualitatively. During alternative splicing of precursor mRNA (pre-mRNA), several alternative mRNA isoforms can be formed and the resulting proteins can be expressed at different levels on the cell surface or appear as soluble proteins[9]. Alternative splicing can take place in parallel to mature splicing and the alternative variants can be co-expressed with the normal variant[10]. The splicing process is catalysed by small nuclear ribonucleoproteins (snRNPs), which together form the spliceosome, and splicing is initiated when snRNPs attach to the four regions of the pre-mRNA involved in the splicing process: the 3' splice site, the branch point, the 5' splice site and the polypyrimidine tract[11]. Splicing activity can further be regulated by SREs. These SREs are found in both intron and exon regions and are classified as splicing enhancers or splicing silencers. Splicing enhancer elements are recognized by specific splicing activators that enhance exon inclusion, while splicing silencer elements are recognized by specific repressors that inhibit exon inclusion[11]. Nucleotide variation in these cis elements may result in alternative splicing such as exon skipping, intron retention, alternative 5' splice sites, alternative 3' splice sites and mutually exclusive exons[12].

Alternative splicing has been estimated to occur in more than 90% of the human genes[13]. For HLA genes, the number of alternative transcripts is probably strongly underestimated because the genes have been primarily studied by analysis of genomic DNA and not by mRNA analysis. So far, alternative splicing of HLA is best known for the non-classical HLA-G molecule. Seven variants of HLA-G have been identified (HLA-G1 to -G7), of which only HLA-G1 has the typical beta-2 microglobulin associated cell membrane appearance. HLA-G5 is the soluble counterpart of HLA-G1 as a result of a stop codon leading to exclusion of exon 5 encoding the

transmembrane region[10,14,15]. Both HLA-G1 and -G5 are functional proteins that can exert inhibitory functions on T and NK cells[16].

For classical HLA class I molecules, a limited number of alternatively spliced variants has been observed. Two studies identified soluble HLA-A and HLA-B molecules in human plasma and cell line supernatant, which was suspected to occur due to alternative splicing of exon 5[17,18]. Other studies also reported alternative splicing in HLA-A and -B, one of them showing that a common alternative splice site is contained in all alleles of HLA-A*11, resulting in co-expression of a mature and alternative transcript[19]. This alternative transcript is the result of a unique polymorphism in intron 5 and encodes a protein with 6 additional amino acids in the cytoplasmic tail[19]. The HLA-B*44:02:01:02S allele has been described as a soluble form of B44 due to a substitution of a single basepair (A>G) at the end of intron 4 that led to skipping of the complete exon 5[20].

Also for HLA-C alternative splicing has been observed: Already in 1989, Cianetti et al observed in two different clones from a cDNA library from a SV40 transformed human fibroblast line (GM637) two alternative ways of splicing, since both clones had identical HLA-C sequences (later identified as HLA-C*16:01:01:01), but one clone was devoid of exon 5[21]. Moreover, Vilches et al have described the occurrence of HLA-C*12 and *15 variants lacking exon 5 in HLA-C specific clones prepared from cDNA of B-LCL from two individuals from a cohort of Spanish gypsies[22]. Yang et al identified an HLA-C*02 variant lacking exon 5 in RPMI1788 cells secreting soluble HLA molecules in their culture supernatants[23]. They used a targeted approach to specifically amplify the alternative splice product since the quantities of alternatively spliced products may be much lower than that of the mature mRNA variants which may lead to poor amplification in the PCR reaction of the rare variant[23]. More recently by using an RNA-based sequencing approach, we identified an alternative transcript that includes 18 additional nucleotides from intron 5 for the HLA-C*03 allele group which may result from a SNP unique to HLA-C*03 at position 2499 within intron 5, that may cause an alternative branch point site and thus an alternative transcript[10]. In addition, a NK cell-specific promoter in the HLA-C gene has been identified, which resulted in many alternatively spliced HLA-C mRNAs in NK cells but not in other cells[24]. The alternative transcripts had varying ability to be translated into proteins and allele-specific differences in the 5' untranslated region could further modulate expression levels of HLA-C in NK cells, which were functionally relevant for regulating NK cell activity[24,25].

In the present study, we used a full-length sequencing approach to study possible additional alternative splice variants of HLA-C in peripheral blood cells covering all the common allele groups, except for HLA-C*17 and *18. By using this methodology, we observed an alternatively spliced variant of HLA-C lacking exon 5 in samples with HLA-C*04 or *16, that was co-expressed with the normal mature variant. We followed up on this observation by an in-silico analysis to study the impact of the SNPs distinguishing HLA-C*04 or *16 from the other HLA-C alleles on the strength of the intronic and exonic SREs.

Materials and Methods

RNA panel

A panel of RNA samples with known HLA-C high resolution typing was used for alternative splicing analysis. The panel covered twelve of the fourteen HLA-C allele groups with one to six samples per allele group (Table 1). Samples with HLA-C*17 and *18 alleles were not available and therefore not included in the panel.

Table 1: RNA panel covering the twelve common HLA-C allele groups.

HLA-C allele group	Allele	Number of samples	Alternative splicing of exon 5
*01	01:02:01	1	No
*02	02:02:02	3	No
*03	03:03:01	3	No
	03:04:01	1	No
*04	04:01:01	6	Yes (6 of 6)
*05	05:01:01	5	No
*06	06:02:01	2	No
*07	07:01:01	6	No
	07:02:01	5	No
	07:04:01	1	No
*08	08:01:01	3	No
	08:02:01	1	No
*12	12:03:01	3	No
*14	14:02:01	2	No
*15	15:02:01	4	No
*16	16:01:01	3	Yes (3 of 3)
*17	not available	0	unknown
*18	not available	0	unknown

cDNA was amplified from RNA samples with HLA-C allele-specific primers to investigate alternative splicing of exon 5 in all allele groups.

cDNA synthesis and PCR amplification

RNA was purified and isolated from peripheral blood samples with the RNeasy mini kit (Qiagen, Hilden, Germany), followed by a Dnase treatment (Thermo Fisher Scientific, Waltham, USA). cDNA was synthesized from 8 µl RNA using the Superscript III first-strand synthesis system for reverse transcriptase (RT)-PCR according to the manufacturer's protocol (Thermo Fisher Scientific). Amplification of cDNA was performed using gene-specific primers for HLA-C or allele-group specific primers for each HLA-C allele group (Table 2). For PCR reactions, 3 µl of cDNA were used in a total volume of 30 µl. The PCR reaction mix consisted of 16.6 mM ammonium sulphate (Merck, Darmstadt, Germany), 0.1 µg/µl cresol red (Sigma-Aldrich, St. Louis, USA), 0.2 mM of each dNTP (GE Healthcare, Diegem, Belgium), 1.4 U Expand High Fidelity Enzyme Mix (Roche, Basel, Switzerland), 5 % Glycerol (Alfa-Aesar, Karlsruhe,

Germany), 1.5 mM $MgCl_2$ (Thermo Fisher Scientific), 67 mM Tris-HCl with pH 8.8, 0.01 % Tween 20 (both from Merck, Darmstadt, Germany), and 15 pmol of each primer (Sigma-Adrich, St. Louis, USA).
The initial denaturation step was performed at 94°C for 2 min, followed by 10 cycles of 15 sec at 94°C, 30 sec at 63°C, 4 min at 68°C; 10 cycles of 15 sec at 94°C, 30 sec at 60°C, 6 min at 68°C; 10 cycles of 15 sec at 94°C, 30 sec at 60°C, 10 min at 68°C, and the final elongation step was performed for 7 min at 68°C. After the PCR products were checked by electrophoresis on a 1.5 % agarose gel, the amplicons were purified using ExoSAP-IT according to the manufacturer's protocol (Affymetrix, Santa Clara, USA).

Table 2: Overview of primers used for allele-specific amplification (A), gene-specific amplification (B) and primers used for sequencing of allele-specific products (C).

Allele group	Direc-tion	Primer sequence (5'-3')	Loca-tion	Position cDNA	Position gDNA
(A)					
01	FW	GGGTCTCACACCCTCCAGT	Exon 3	I2-361	719-737
02	FW	AGCACGAGGGGCTGCCG	Exon 4	857-873	1820-1836
03	FW	AGGACCAAACTCAGGACACT	Exon 4	737-756	1700-1719
04	FW	CTCCGCGGGTATAACCAGTT	Exon 3	400-419	776-795
05/06/08	FW	AGCACGAGGGGCTGCCA	Exon 4	857-873	1820-1836
07	FW	GCAGAGATACACGTGCCATA	Exon 4	834-853	1797-1816
12	FW	CGTGAGGCGGAGCAGTG	Exon 3	523-539	900-915
14	FW	GGGTCTCACACCCTCCAGT	Exon 4	I2-361	719-737
15	FW	CGCCTCCTCCGCGGGC	Exon 3	394-409	770-785
16	FW	CGTGCGGCGGAGCAGCA	Exon 3	523-539	900-915
all	REV	CACAGGCAGCTGTCTCAGG	3'UTR	1097-1115	2891-2909
(B)					
all	FW	GAGCTTGTGGAGACCAGG	Exon 4	757-774	1720-1737
all	REV	GCATCTCAGTCCCACACAG	3'UTR	1111-1128	2905-2923
(C)					
15	FW	CGCCTCCTCCGCGGGC	Exon 3	394-409	770-785
01/04/14	FW	GACGGCAAGGATTACA	Exon 3	427-442	803-818
12/16	FW	CGTGCGGCGGAGCAGCA	Exon 3	523-539	900-915
03	FW	AGGACCAAACTCAGGACACT	Exon 4	737-756	1700-1719
07	FW	GCAGAGATACACGTGCCATA	Exon 4	834-853	1797-1816
05/06/08	FW	AGCACGAGGGGCTGCCA	Exon 4	857-873	1820-1836
02	FW	AGCACGAGGGGCTGCCG	Exon 4	857-873	1820-1836
all	REV	AGAGCAGCTCCCTCCTTTTC	Exon 6	1019-1038	2543-2561

Low melting point agarose gel

The PCR products were separated on a 0.6% low melting point agarose gel (Thermo Fisher Scientific) at 50V for 6.5 hours. The PCR fragments were purified from the low melting gel using the MinElute gel extraction kit (Qiagen) and were used for sequencing.

RNA-based Sanger sequencing

Samples, either PCR products or gel extracted PCR fragments, were sequenced with forward and reverse primers as described in Table 2 using the ABI BigDye Terminator Chemistry (Thermo Fisher Scientific). The sequencing reaction contained 1 µl purified amplification product, 0.5 µl sequencing primer (5 pmol, Sigma-Aldrich), 1 µl of BigDye Terminator v1.1 mix, 1.5 µl 5x BigDye Terminator sequencing buffer and 6 µl water. Cycle sequencing was performed as follows: 1 minute at 96°C; followed by 25 cycles of 10 seconds at 96°C, 5 seconds at 50°C and 4 minutes at 60°C. Sequencing reactions were purified by Sephadex G-50 Fine (GE Healthcare Life Sciences, Little Chalfont, UK) and analysed on the ABI3730 DNA analyzer (Applied Biosystems, Foster City, USA). DNASTAR Lasergene software was used for sequence analysis (DNASTAR Lasergene 12 Core Suite, Madison, USA).

In-silico splicing analysis

The online available Human Splicing Finder (HSF) tool was used (HSF pro system, https://www.genomnis.com/, version 2.23.6[26]) for splicing analysis. This tool combines multiple algorithms to identify and predict sequence effects on splicing motifs, including splice sites and branch point sites. In this study, the HSF and the MaxEnt prediction algorithm was used to calculate the branch point and splice site strength of the different HLA-C alleles.

Additional to the HSF tool, the EX-SKIP tool (http://ex-skip.img.cas.cz/[27]) was used to predict exon skipping of HLA-C alleles by comparing ESE/ESS motif differences. EX-SKIP analyses exon sequences using integrated algorithms to identify the number of 8-mer ESEs and ESSs (PESE/PESS[28]), ESS decamers (FAS-ESS[29]), exon- and intron-identity elements (EIE/IIE[30]), RESCUE-ESE hexamers (RESCUE-ESE[31]) and neighbourhood inference (NI-ESE/NI-ESS[32]). The ESS/ESE ratio is determined based on the total ESE and ESS count; a higher ratio is predicted to increase chance to skip an exon.

Results

Alternative splicing of exon 5 in HLA-C alleles

To investigate alternative splicing in HLA-C, mRNA was transcribed into cDNA which was subsequently amplified using HLA-C gene-specific primers. By sequencing the amplified product covering exon 4 until 3'UTR, we observed an alternatively spliced variant of HLA-C in some samples and this alternative transcript lacked exon 5. For example, a sample with an HLA-C*07:02:01 and *08:01:01 typing did not express the alternatively spliced variant and the sequences matched the expected sequences of HLA-C*07:02:01 and *08:01:01 (**Figure 1A**). In contrast, a sample with an HLA-C*07:01:01 and *16:01:01 typing expresses both the mature and the alternative transcript, revealed by the presence of two PCR products of 716 bp and 596 bp (**Figure 1B**) and the overlapping peaks in the sequences after exon 4, with one peak representing the exon 5 sequence and the other peak the exon 6 sequence (**Figure 1A**). By using allele-specific amplification primers we determined in which of the two alleles exon 5 skipping was detected. In the sequence of HLA-C*07:01:01 no alternative transcript was observed, but in the sequence of HLA-C*16:01:01 both the mature and the alternative transcript were present, represented by the double peaks after exon 4 (**Figure 1C**).

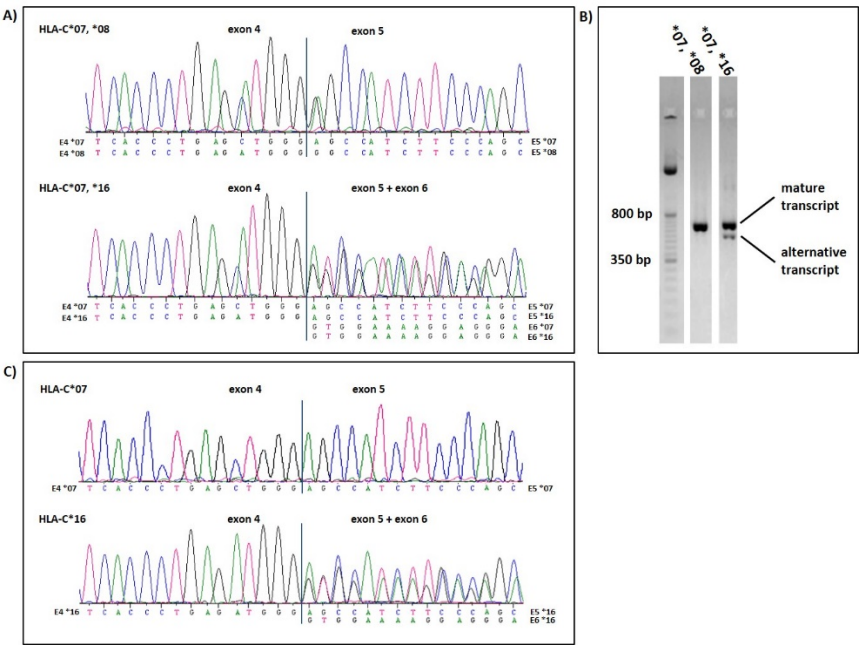


Figure 1: Identification of alternative HLA-C transcript missing exon 5. Two cDNA samples either with the HLA-C*07:02:01 and *08:01:01 alleles or HLA-C*07:01:01 and *16:01:01 was amplified with HLA-C gene- and allele-specific primers. A) Gene-specific sequence results of both samples. B) Electrophoresis result of gene-specific PCR product of both samples. C) Allele-specific sequence results of a sample with HLA-C*07:01:01 and *16:01:01.

Evidence for the role of HLA polymorphism in alternative splicing of HLA-C

To examine whether alternative splicing differs between HLA-C allele groups, we used the samples from our RNA panel, which covered twelve of the fourteen HLA-C allele groups (Table 1). cDNA from each sample was amplified with HLA-C allele-specific primers to detect alternative splicing in both alleles individually. We observed the alternatively spliced variant by gel electrophoresis and/or sequencing in all samples with HLA-C*04 and *16 alleles, but not in any of the other HLA-C allele groups (Table 1). In all samples where the alternative variant was present, it was co-expressed with the mature full-length transcript (data not shown).

To confirm that exon 5 was skipped in the alternative variants, the amplified products of HLA-C*04:01:01 and *16:01:01 were run on a low melting gel to separate the alternatively spliced product from the mature product. The alternative product was isolated from the gel and sequenced. For both HLA-C*04:01:01 and *16:01:01, the sequencing results of the alternative transcript showed a single sequence, in which exon 4 was directly followed by the exon 6 sequence (**Figure 2**). These results confirmed that the alternative product was the result of exon 5 skipping. The reading frame was maintained in the alternative transcript since exon 5 is 120 nucleotides long.

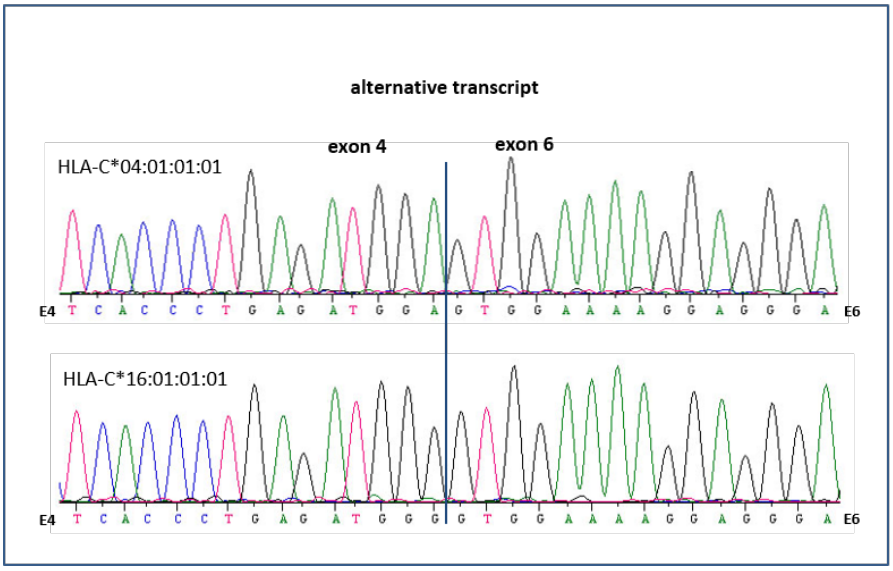


Figure 2: Exon 5 skipping in HLA-C*04 and HLA-C*16. The alternatively spliced products of C*04:01:01:01 and *16:01:01:01 were separated from the mature product by using a low melting gel. The isolated alternative products were sequenced and the resulting nucleotide sequences are shown.

In-silico analysis of the effect of intronic HLA-C variation on the branch point and splice site strength

Splicing specificity is mainly determined by the 5' splice site (donor), the branch point and 3' splice site (acceptor) sequences in the intron regions[10]. SNPs in the splice sites downstream and upstream exon 5 could potentially affect the splicing process by alteration of the complementary U1 snRNA binding sequence at the 5' splice site or the complementary U2AF1 snRNA binding sequence at the 3' splice site. SNPs introducing potentially new branch point sequences or branch point breaks might affect splicing via the attachment of splicing factor 1. We therefore hypothesized that intronic differences between HLA-C*04 or *16 and the other HLA-C allele groups may influence the branch point strength and/or the 5'- or 3'splice sites resulting in differences in exon 5 splicing specificity or efficacy.

To test this hypothesis on an in-silico basis we used the human splicing finder (HSF), an online tool from genomnis (https://www.genomnis.com/,[26]). First, we compared the branch point strengths within the intron 4 region of all HLA-C reference alleles. The tool predicted multiple branch point motifs in the intron 4 sequence of the different HLA-C alleles, but the branch point with the highest score was identical for all alleles (Table 3), indicating that exon 5 skipping in HLA-C*04 and *16 alleles is probably not the result of branch point differences.

Secondly, the variation in and strength of the splice sites downstream and upstream of exon 5 were compared for all the different HLA-C reference alleles using both the HSF algorithm as well as the MaxEnt algorithm. These regions were analyzed since exon 5 skipping could occur in case of acceptor- or donor splice site loss or when the acceptor splice site of exon 6 is preferred over the acceptor splice site of exon 5. For the acceptor splice site of exon 5, two (HSF) or three (MaxEnt) different splice site motifs were calculated (Table 3). Both algorithms predicted a different splice site for HLA-C*05 and *08 compared to the other HLA-C alleles and the MaxEnt algorithm additionally predicted a different splice site for HLA-C*07. Importantly, HLA-C*04 and *16 had splice sites that were comparable to the alleles that did not show exon 5 skipping in our assays (i.e. HLA-C*01, -*02, -*03, -*06, -*12, -*14, -*15). The splice site motif for the donor splice site of exon 5 and the acceptor splice site of exon 6 were identical for all HLA-C allele groups (Table 3). Based on this in-silico analysis, the calculated splice strength of HLA-C*04 and *16 was comparable to the other HLA-C alleles, in which no alternative splicing was detected. Therefore, these results do not support the hypothesis that variation in the splice site sequences is responsible for the observed exon 5 skipping in HLA-C*04 and *16 alleles.

Table 3: Analysis of intronic branch points and splice site motifs in HLA-C.

Branch point analysis	**HLA-C allele group**	**Strongest branch point motif**	**Strength**
Intron 4	All HLA-C groups	CCCTCAC	99.76
HSF	**HLA-C allele group**	**Splice site motif**	**Strength**
Acceptor splice site exon 5	*01, *02, *03, *04, *06, *07, *12, *14, *15, *16, *17, *18	CTCCTTTCCCAGAG	87.86
	*05, *08	CTCCTTTCCCAG**G**G	89.77
Donor splice site exon 5	All HLA-C groups	CAGGTAGGG	90.21
Acceptor splice site exon 6	All HLA-C groups	TTCTTCCCACAGGT	92.21
MaxEnt	**HLA-C allele group**	**Splice site motif**	**Strength**
Acceptor splice site exon 5	*01, *02, *03, *04, *06, *12, *14, *15, *16, *17, *18	ACCTTCCCCTCCTTTCCCAGAGC	11.26
	*05, *08	ACCTTCCCCTCCTTTCCCAG**G**GC	11.91
	*07	ACCTTC**T**CCTCCTTTCCCAGAGC	10.77
Donor splice site exon 5	All HLA-C groups	CAGGTAGGG	9.46
Acceptor splice site exon 6	All HLA-C groups	AGGGCATTTTCTTCCCACAGGTG	9.73

The Human Splicing Finder (HSF) tool from Genomnis was used to analyze branch point- and splice site motifs. For the branch point analysis of intron 4, the branch point with the highest score is depicted. To assess splice sites of exon 5 and 6, the HSF tool used the HSF and the MaxEnt algorithm to determine the splice site motifs and strength of the different HLA-C reference alleles: HLA-C*01:02:01:01, *02:02:02:01, *03:02:01, *04:01:01:01, *05:01:01:01, *06:02:01:01, *07:01:01:01, *08:01:01:01, *12:02:02:01, *14:02:01:01, *15:02:01:01, *16:01:01:01, *17:01:01:02 and *18:01:01:01.

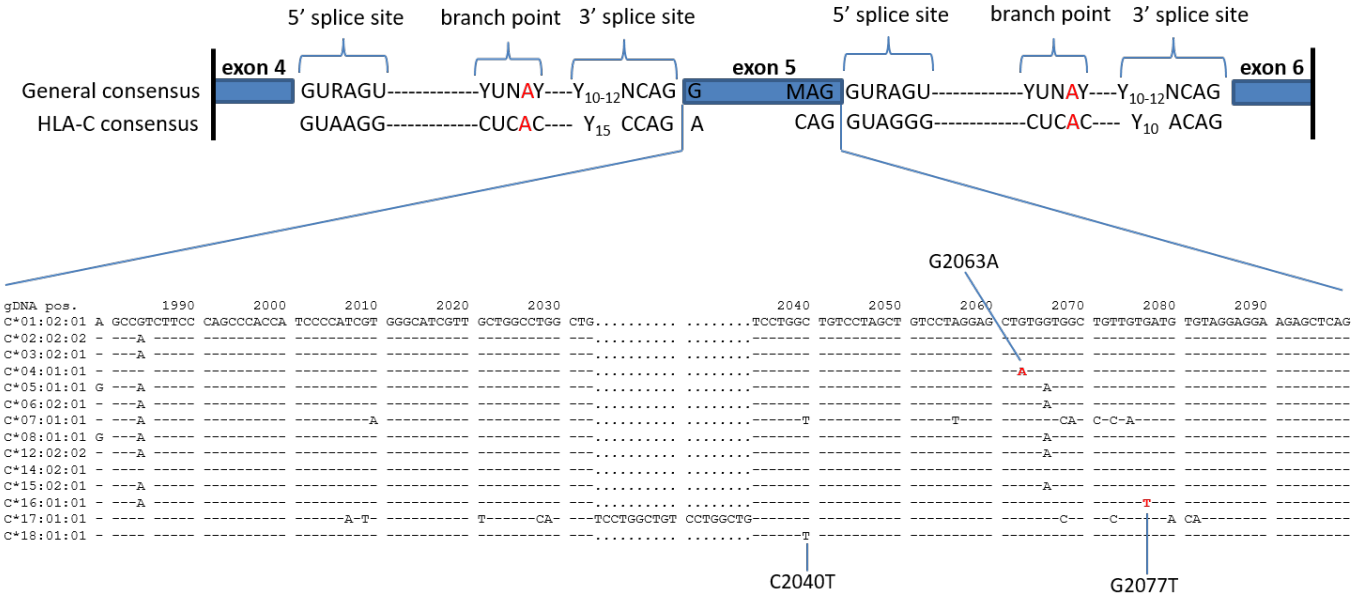


Figure 3: HLA-C polymorphism analysis. The consensus HLA-C sequence of the splice site and branch site regions surrounding exon 5 are shown on top. The HLA-C sequence variation within the exon 5 region is shown for each HLA-C allele group and single nucleotide differences in C*04, C*16 and C*18 are highlighted.

In-silico analysis of the effect of exonic HLA-C variation on splicing regulatory elements

SREs are short stretches (4-18 nucleotides) of DNA that act as cis-regulating elements by recruiting regulatory proteins that interact with components of the splicing machinery and enhance or suppress splicing[1]. SNPs in SRE may influence binding of those regulatory proteins consequently leading to alternative splicing. Four types of SRE exist: 1) exonic splicing enhancers (ESEs), 2) exonic splicing silencers (ESSs), 3) intronic splicing enhancers (ISE) and 4) intronic splicing silencers (ISS).

To provide a theoretical basis for the potential influence of exon SNPs in HLA-C*04 or *16 alleles on exon 5 skipping, we compared the polymorphic content of exon 5 of the different HLA-C alleles and studied the effect of exonic HLA-C polymorphism on ESE and ESS motifs in-silico. Unfortunately, it was not possible to test the effect on intronic motifs, since there are no reliable algorithms available to perform this in-silico analysis. The IPD-IMGT/HLA database (V3.44.0) describes 741 different HLA-C*04 alleles, of which 475 have a known sequence for exon 5[2]. With the exception of HLA-C*04:03, *04:06 and *04:82, all common and well-documented HLA-C*04 allele sequences have identical exon 5 sequences with an HLA-C*04 specific non-synonymous nucleotide substitution (G2063A) that results in an amino acid change from Valine to Methionine (**Figure 3**)[3]. For the HLA-C*16 allele group, the IPD-IMGT/HLA database describes 273 different alleles, of which 156 have a known sequence for exon 5. All the common and well-documented HLA-C*16 allele sequences are identical in this region and display an HLA-C*16 specific synonymous nucleotide substitution in exon 5 (G2077T, **Figure 3**).

To test whether these exonic polymorphisms could alter sequence motifs of ESE/ESS and affect exon 5 skipping, the prediction tool EX-SKIP was used. The tool determines the total number of ESE and ESS motifs using the PESE/PESS-[4], FAS-ESS-[5], EIE/IIE-[6], RESCUE-ESE-[7] and the neighborhood inference (NI)[8] algorithms. Based on the calculated ESS/ESE ratio it predicts which of two exon sequences has a higher chance of exon skipping. To determine the effect of the HLA-C*04 and *16 specific substitutions, we compared the ESS/ESE prediction results of the exon 5 sequences of HLA-C*04:01:01:01 and *16:01:01:01 to the exon 5 sequences of *01:02:01:01 and *02:02:02:01 respectively, because in this region the sequences are identical, except for substitutions G2063A and G2077T. EX-SKIP predicted that C*04 has 1 ESS and 5 ESE's less than C*01 changing the ESS/ESE ratio from 0.58 to 0.60 (3%) (Table 4). Although the tool predicted a higher change of exon skipping for HLA-C*04, the effect of G2063A on ESS/ESE ratio is minimal. For HLA-C*16, EX-SKIP predicted 10 ESS and 1 ESE more than C*02 and the ESS/ESE ratio changed from 0.56 to 0.66 (18%) (Table 4). By comparing the ESS/ESE ratio of all the HLA-C reference alleles, we observed that the HLA-C alleles for which we did not find exon 5 skipping had lower ESS/ESE ratios than HLA-C*16. Interestingly, the ratio for HLA-C*18 was identical to *16, but this HLA-C allele was not included in our sequencing panel and therefore

we could not relate this ratio to the actual presence of the alternatively spliced variant.
Overall, exon analysis by EX-SKIP predicted ESE and ESS motif changes in HLA-C*04 and *16 alleles. But, since the effect was only very small for HLA-C*04, we assume that only for *16 these changes may contribute to altered splicing processes and eventually result in the exon 5 skipping we observed.

Table 4: EX-SKIP analysis of the exon 5 sequence of HLA-C alleles.

HLA-C allele group	PESS (count)	FAS-ESS hex2 (count)	FAS-ESS hex3 (count)	IIE (count)	NI-ESS trusted (count)	PESE (count)	RESCUE -ESE (count)	EIE (count)	NI-ESE trusted (count)	ESS (total)	ESE (total)	ESS/ESE (ratio)
*01	0	4	3	42	4	12	5	32	42	53	91	0.58
*02	0	4	3	41	4	12	6	32	43	52	93	0.56
*03	0	4	3	41	4	12	6	32	43	52	93	0.56
*04	0	4	3	40	5	10	5	32	39	52	86	0.60
*05	1	3	2	39	3	12	6	30	42	48	90	0.53
*06	1	3	2	39	3	12	6	30	42	48	90	0.53
*07	1	3	2	36	6	12	7	34	40	48	93	0.52
*08	1	3	2	39	3	12	6	30	42	48	90	0.53
*12	1	3	2	39	3	12	6	30	42	48	90	0.53
*14	0	4	3	42	4	12	5	32	42	53	91	0.58
*15	1	3	2	39	3	12	6	30	42	48	90	0.53
*16	1	6	4	41	10	12	6	33	43	62	94	0.66
*17	0	5	3	41	6	16	6	30	61	55	113	0.49
*18	0	4	3	44	6	12	5	32	38	57	87	0.66

The exon 5 sequences of HLA-C*01:02:01:01, *02:02:02:01, *03:02:01, *04:01:01:01, *05:01:01:01, *06:02:01:01, *07:01:01:01, *08:01:01:01, *12:02:02:01, *14:02:01:01, *15:02:01:01, *16:01:01:01, *17:01:01:02 and *18:01:01:01 alleles were entered into the online EX-SKIP tool and the ESE/ESS results of the different algorithms are shown: 8-mer putative ESSs and ESEs (PESS/PESE), fluorescence-activated screen for ESS (FAS-ESS hex2, FAS-ESS hex3), intron- and exon-identity elements (IIE/EIE), Relative Enhancer and Silencer Classification by Unanimous Enrichment ESEs (RESCUE-ESE), neighborhood interference ESS and ESEs (NI-ESS/NI-ESE).

Discussion

HLA class I genes are highly polymorphic, but only a limited number of alternatively spliced transcripts has been identified[9]. In this study, sequencing of mRNA revealed an alternatively spliced transcript missing exon 5 in HLA-C*04 and *16 alleles. Alternative splicing of exon 5 has been show before in cell lines[10-12]. The strength of our study is that we studied alternative exon splicing in peripheral blood samples covering the most common HLA-C allele groups that were processed and analyzed with the same methodology. Hence, our data suggest that in peripheral blood samples splicing of exon 5 occurs more frequently in HLA-C*04 and *16 alleles than in the other allele groups. Variability in the nucleotide sequence can affect exon and intron recognition during the splicing process by altering the splice sites, branch point or SREs and this may eventually result in the formation of an alternatively spliced transcript. We therefore assessed how the specific nucleotide variability in HLA-C*04 and *16 alleles affected the splice sites, branch point and SREs by using an in-silico approach with several existing prediction algorithms[13,14]. This suggested that HLA-C*04 and *16 specific nucleotide variants in intron 4 did not affect splice site and branch site sequences. Furthermore, the specific SNP in exon 5 observed in HLA-C*04 compared to other HLA-C allele groups did not seem to have an effect on exonic SRE, while the HLA-C*16 specific SNP in exon 5 did alter exonic SRE. Although the results of these in-silico analysis are only predictive, they may provide a theoretical basis for the alternative splicing observed for the HLA-C*16:01:01 allele. For the alternative splicing of HLA-C*04:01:01 it is not clear why this occurs, although a role for intronic SREs could not be excluded based on our data.

Splice sites have been extensively characterized and in-silico tools like HSF are able to reliably predict alterations in splice site strength[15,16]. SREs are less well understood and are more difficult to predict by using in-silico approaches. The best way to accurately predict the effect of nucleotide variability on SREs and alternative splicing is the use of a combination of algorithms. In this study, we used the EX-SKIP tool to predict SRE alterations, since several of the available prediction algorithms have been combined in this tool. Multiple studies have evaluated EX-SKIP and showed promising results with a sensitivity of around 75%[17,18]. Although no tool can ensure 100% accuracy and experimental validation is necessary to draw a clinically relevant conclusion, these tools are very useful and commonly used to predict and understand the mechanisms of alternative splicing.

In our study, we investigated, on an in-silico basis, whether SNPs in the introns of the different HLA-C allele groups influenced branch point or splice site sequences and we observed no major differences between the groups. Intronic SNPs could, at least in theory, also influence intronic silencing and enhancer motifs by creating novel regulatory elements that in case of intron splicing silencers might promote exon skipping[14]. Although studies have identified more than 100 potential intronic splicing regulatory elements (ISREs) in other genes, the ISREs are less well understood[19,20]

and good prediction algorithms for ISREs are limited. Hence, we have not included the intron regions in our in-silico analysis of intronic regulatory elements and more research on the identification of ISREs is required to predict the effect of intronic variants on exon skipping.

When using the EX-SKIP tool to study the impact of exon 5 SNPs on alternative splicing of HLA-C, EX-SKIP predicted the same ESE/ESS ratio for HLA-C*16 and *18 alleles (Table 4), which suggests that exon 5 skipping might also occur in the reference allele HLA-C*18:01. Unfortunately, we were not able to test this because the HLA-C*18 genotype is rarely present in the Caucasian population (0.03%,[21]) and therefore a sample with HLA-C*18 could not be included in our test panel. In comparison, HLA-C*04 and *16 alleles are found in the Caucasian population with a frequency of 10.6% and 3.4%, respectively, and were thus present in our RNA panel. Next to HLA-C*18:01, *18:02 is the only other well-documented HLA-C allele with known exon 5 sequence in the IPD-IMGT/HLA database. In the exon 5 region, the sequence of HLA-C*18:02 differs from *18:01 by one nucleotide (T2040C) and is identical to the exon 5 sequence of HLA-C*01, with an EX-SKIP ESS/ESE ratio of 0.58 (Table 4). This observation suggests that exon 5 skipping might occur more frequently in HLA-C*18:01 than in *18:02 alleles. The C2040T substitution in HLA-C*18:01 is also present in the HLA-C*07 alleles (**Figure 3**), but in HLA-C*07 alleles it is not predicted to have an impact on exon 5 skipping since additional variation in exon 5 of HLA-C*07 seems to result in more enhancing and less suppressing elements, as is shown by the EX-SKIP analysis with an ESS/ESE ratio of 0.52 for HLA-C*07 (Table 4).

We found that the complete exon 5 region was spliced out in the alternative transcript of HLA-C*04 and *16 alleles. It remains to be determined whether the alternative transcripts of HLA-C*04 and *16 are translated into a mature and functional protein. However, because the reading frame was maintained, no premature stop codon was created, and therefore it is expected that the alternative transcripts can be fully translated into a protein. To analyze the protein expression, cells lacking HLA class I expression could be transfected with constructs encoding the mature and alternative sequence of HLA-C*04 and *16 and subsequently be analyzed for HLA-C protein expression for instance with western blot or flow cytometry.

Here, we studied alternative splicing of HLA-C in peripheral blood samples and we demonstrated that allele polymorphism in HLA-C*04 and *16 alleles correlate with occurrence of exon 5 skipping. We did not observe alternative splicing in the other allele groups in peripheral blood cells in a standardized setting measuring all HLA-C groups in the same experimental conditions. Also, our in-silico models support the hypothesis that polymorphism in the HLA-C alleles has an influence on alternative splicing leading to exon 5 skipping. Since the process of alternative splicing can be influenced by cell intrinsic and cell extrinsic factors, it could be relevant to assess

whether these allele-related differences in exon 5 skipping we observed in peripheral blood cells are also relevant under other conditions. This could be especially interesting because two studies described transcripts lacking exon 5 for HLA-C*02, *12:02 and *15:02 in transformed B-cell lines[10,12], whereas a third study showed exon 5 skipping of HLA-C*16 in transformed B cell lines[11]. Since transformation of cells or other environmental conditions could influence HLA expression levels and alternative splicing processes it would be interesting to structurally study the impact of polymorphisms in these settings as well[22].

Our study provides new perspectives for the analysis of alternative splicing products, not restricted to exon 5 skipping in HLA-C, for instance by using RNAseq data sets to ultimately understand their occurrence and functional role. Additionally, it will be interesting to further examine if the alternative variants occur in a tissue-specific or cell type-specific manner. One research group discovered alternative splicing of HLA-C specifically in NK cells[23]. Interestingly, alternative splicing occurred in the promotor region of HLA-C, resulting in different promoters and differential HLA-C expression on NK cells[23,24]. The differential expression of HLA-C on NK cells themselves regulated NK cell functions and the authors suggest that NK cell-intrinsic regulation of HLA-C evolved to fine-tune NK cell responses.

In summary, we reported alternative splicing of HLA-C in peripheral blood cells, which resulted in a transcript without the exon 5 region. The alternative transcript was only detected in HLA-C*04 and *16 alleles. In-silico splicing analysis predicted that nucleotide variability in HLA-C*16 alleles altered exonic SREs. Hence, our collective data support the concept that specific SNPs in the HLA-C genes can influence the process of alternative exon 5 splicing.

Acknowledgement
The Authors thank Tom Jansen for his assistance in sequencing and data analysis.

Conflict of interest
The authors have nothing to declare.

Author contribution
Femke A.I. Ehlers and Timo I. Olieslagers performed the experiments, analyzed the data and wrote the paper. Mathijs Groeneweg participated in data visualization. Mathijs Groeneweg, Gerard M.J. Bos, Marcel G.J. Tilanus, Christina E.M. Voorter and Lotte Wieten reviewed and edited the paper. Timo I. Olieslagers, Marcel G.J. Tilanus, Christina E. M. Voorter and Lotte Wieten conceptualized the study.

References

1. Apps R, Meng Z, Del Prete GQ, Lifson JD, Zhou M, Carrington M. Relative expression levels of the HLA class-I proteins in normal and HIV-infected cells. J. Immunol. 2015;194(8):3594-3600.
2. Flomenberg N, Baxter-Lowe LA, Confer D, et al. Impact of HLA class I and class II high-resolution matching on outcomes of unrelated donor bone marrow transplantation: HLA-C mismatching is associated with a strong adverse effect on transplantation outcome. Blood. 2004;104(7):1923-1930.
3. Pidala J, Wang T, Haagenson M, et al. Amino acid substitution at peptide-binding pockets of HLA class I molecules increases risk of severe acute GVHD and mortality. Blood. 2013:blood-2013-2005-501510.
4. Woolfrey A, Klein JP, Haagenson M, et al. HLA-C antigen mismatch is associated with worse outcome in unrelated donor peripheral blood stem cell transplantation. Biol. Blood Marrow Transplant. 2011;17(6):885-892.
5. Frohn C, Fricke L, Puchta JC, Kirchner H. The effect of HLA-C matching on acute renal transplant rejection. Neprol. Dial. Transplant. 2001;16(2):355-360.
6. Vince N, Li H, Ramsuran V, et al. HLA-C level is regulated by a polymorphic Oct1 binding site in the HLA-C promoter region. Am. J. Hum. Genet. 2016;99(6):1353-1358.
7. Thomas R, Apps R, Qi Y, et al. HLA-C cell surface expression and control of HIV/AIDS correlate with a variant upstream of HLA-C. Nat. Genet. 2009;41(12):1290.
8. Kulkarni S, Savan R, Qi Y, et al. Differential microRNA regulation of HLA-C expression and its association with HIV control. Nature. 2011;472(7344):495.
9. Black DL. Mechanisms of alternative pre-messenger RNA splicing. Annu. Rev. Biochem. 2003;72(1):291-336.
10. Voorter CE, Gerritsen KE, Groeneweg M, Wieten L, Tilanus MG. The role of gene polymorphism in HLA class I splicing. International journal of immunogenetics. 2016;43(2):65-78.
11. Matera AG, Wang Z. A day in the life of the spliceosome. Nat. Rev. Mol. Cell Biol. 2014;15:108-121.
12. Cartegni L, Chew SL, Krainer AR. Listening to silence and understanding nonsense: exonic mutations that affect splicing. Nat. Rev. Genet. 2002;3(4):285-298.
13. Pan Q, Shai O, Lee LJ, Frey BJ, Blencowe BJ. Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. Nat. Genet. 2008;40(12):1413.
14. Moreau P, Carosella E, Teyssier M, et al. Soluble HLA-G molecule: an alternatively spliced HLA-mRNA form candidate to encode it in peripheral blood mononuclear cells and human trophoblasts. Hum. Immunol. 1995;43(3):231-236.
15. Paul P, Cabestre FA, Lefebvre S, et al. Identification of HLA-G7 as a new splice variant of the HLA-G mRNA and expression of soluble HLA-G5,-G6, and-G7 transcripts in human transfected cells. Hum. Immunol. 2000;61(11):1138-1149.
16. Carosella ED, Rouas-Freiss N, Tronik-Le Roux D, Moreau P, LeMaoult J. HLA-G: an immune checkpoint molecule. Adv. Immunol. 2015;127:33-144.
17. Krangel MS. Two forms of HLA class I molecules in human plasma. Hum. Immunol. 1987;20(2):155-165.
18. Krangel MS. Secretion of HLA-A and-B antigens via an alternative RNA splicing pathway. The Journal of experimental medicine. 1986;163(5):1173-1190.
19. Tijssen H, Sistermans E, Joosten I. A unique second donor splice site in the intron 5 sequence of the HLA-A* 11 alleles results in a class I transcript encoding a molecule with an elongated cytoplasmic domain. Tissue Antigens. 2000;55(5):422-428.

20. Dubois V, Tiercy J, Labonne M, Dormoy A, Gebuhrer L. A new HLA-B44 allele (B* 44020102S) with a splicing mutation leading to a complete deletion of exon 5. Tissue Antigens. 2004;63(2):173-180.
21. Cianetti L, Testa U, Scotto L, et al. Three new class I HLA alleles: structure of mRNAs and alternative mechanisms of processing. Immunogenetics. 1989;29(2):80-91.
22. Vilches C, de Pablo R, Herrero MJ, Moreno ME, Kreisler M. Molecular cloning and polymerase chain reaction-sequence-specific oligonucleotide detection of the allele encoding the novel allospecificity HLA-Cw6. 2 (Cw∗ 1502) in Spanish gypsies. Hum. Immunol. 1993;37(4):259-263.
23. Yang D, Le J. Targeted amplification of alternatively spliced transcripts of major histocompatibility complex class I heavy chain. J. Immunol. Methods. 1994;176(2):265-270.
24. Li H, Ivarsson MA, Walker-Sperling VE, et al. Identification of an elaborate NK-specific system HLA-C expression. PLoS Genet. 2018;14(1):e1007163.
25. Goodson-Gregg FJ, Rothbard B, Zhang A, et al. Tuning of NK-specific HLA-C expression by alternative mRNA splicing. Front. Immunol. 2019;10.
26. Desmet FO, Hamroun D, Lalande M, Collod-Beroud G, Claustres M, Beroud C. Human Splicing: an online bioinformatics tool to predict splicing signals. Nucleic acids research. 2009;37(9):e67.
27. Raponi M, Kralovicova J, Copson E, et al. Prediction of single-nucleotide substitutions that in exon skipping: identification of a splicing silencer in BRCA1 exon 6. Hum Mutat. ;32(4):436-444.
28. Zhang XH, Chasin LA. Computational definition of sequence motifs governing constitutive exon splicing. Genes Dev. 2004;18(11):1241-1250.
29. Wang Z, Rolish ME, Yeo G, Tung V, Mawson M, Burge CB. Systematic identification and analysis of exonic splicing silencers. Cell. 2004;119(6):831-845.
30. Zhang C, Li W-H, Krainer AR, Zhang MQ. RNA landscape of evolution for optimal exon and intron discrimination. Proc. Natl. Acad. Sci. U. S. A. 2008;105(15):5797-5802.
31. Fairbrother WG, Yeo GW, Yeh R, et al. RESCUE-ESE identifies candidate exonic splicing enhancers in vertebrate exons. Nucleic Acids Res. 2004;32(suppl_2):W187-W190.
32. Stadler MB, Shomron N, Yeo GW, Schneider A, Xiao X, Burge CB. Inference of splicing regulatory activities by sequence neighborhood analysis. PLoS Genet. 2006;2(11):e191.
33. Lee Y, Rio DC. Mechanisms and regulation of alternative pre-mRNA splicing. Annu. Rev. Biochem. 2015;84:291-323.
34. Robinson J, Barker DJ, Georgiou X, Cooper MA, Flicek P, Marsh SGE. IPD-IMGT/HLA Database. Nucleic acids research. 2020;48(D1):D948-D955.
35. Mack SJ, Cano P, Hollenbach JA, et al. Common and well-documented HLA alleles: 2012 update to the CWD catalogue. Tissue antigens. 2013;81(4):194-203.
36. Badr E, ElHefnawi M, Heath LS. Computational Identification of Tissue-Specific Splicing Regulatory Elements in Human Genes from RNA-Seq Data. PloS one. 2016;11(11):e0166978.
37. Anna A, Monika G. Splicing mutations in human genetic disorders: examples, detection, and confirmation. J Appl Genet. 2018;59(3):253-268.
38. Moles-Fernández A, Duran-Lozano L, Montalban G, et al. Computational tools for splicing defect prediction in breast/ovarian cancer genes: how efficient are they at predicting RNA alterations? Front. Genet. 2018;9:366.
39. Jian X, Boerwinkle E, Liu X. In silico tools for splicing defect prediction: a survey from the viewpoint of end users. Genet. Med. 2014;16(7):497-503.
40. Grodecká L, Buratti E, Freiberger T. Mutations of pre-mRNA splicing regulatory elements: are predictions moving forward to clinical diagnostics? Int. J. Mol. Sci. 2017;18(8):1668.

41. Soukarieh O, Gaildrat P, Hamieh M, et al. Exonic splicing mutations are more prevalent than currently estimated and can be predicted by using in silico tools. PLoS Genet. 2016;12(1):e1005756.
42. Wang Y, Ma M, Xiao X, Wang Z. Intronic splicing enhancers, cognate splicing factors and context-dependent regulation rules. Nat Struct Mol Biol. 2012;19(10):1044-1052.
43. Voelker RB, Berglund JA. A comprehensive computational characterization of conserved mammalian intronic sequences reveals conserved motifs associated with constitutive and alternative splicing. Genome Res. 2007;17(7):1023-1033.
44. Gragert L, Madbouly A, Freeman J, Maiers M. Six-locus high resolution HLA haplotype frequencies derived from mixed-resolution DNA typing for the entire US donor registry. Hum. Immunol. 2013;74(10):1313-1320.
45. Stamm S, Ben-Ari S, Rafalska I, et al. Function of alternative splicing. Gene. 2005;344:1-20.

9

General discussion

General discussion

NK cell-based immunotherapies represent a promising approach to improve cancer therapies. However, the efficacy of NK cell-based treatment approaches for MM and breast cancer is currently limited because NK cell anti-tumor responses are restricted by several resistance mechanisms in the immunosuppressive TME, resulting in tumor escape from NK cells. In order to develop effective anti-tumor killers, it is essential to better understand how NK cells are impacted by the tumor and by the TME to subsequently design therapy approaches that can overcome NK cell inhibition and create potent anti-tumor responses.

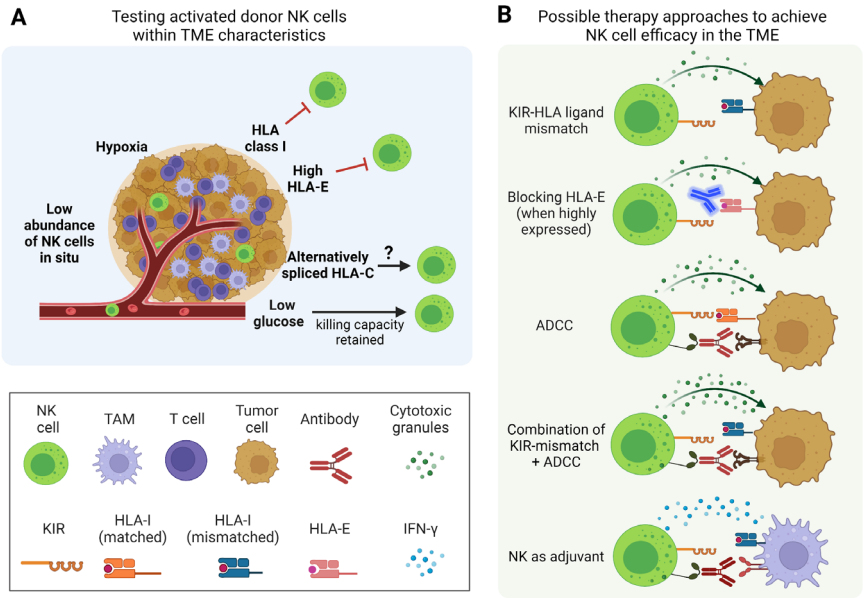


Figure 1. Optimizing NK cells for cancer immunotherapy. (**A**) In this thesis, we investigated how anti-tumor responses of IL-2 activated donor NK cells were impacted by classical HLA class I (chapter 3-4, 7), and by HLA-E (chapter 3), in conditions representing the TME, namely hypoxia (chapter 3-4) or reduced glucose levels (chapter 6). It will further be interesting to determine whether alternatively spliced HLA-C variants (chapter 8) would influence NK cell responses. Additionally, we profiled tumor-infiltrating NK cells in human breast cancer specimens and described low NK cell abundance (chapter 5), which highlights a remaining issue of increasing NK cell infiltration, survival and/or persistence in breast cancer. (**B**) Strategies developed in this thesis to boost the NK cell anti-tumor potential by enhancing their direct tumor-killing functions. Inhibitory signaling through classical HLA class I could be prevented by selecting KIR-HLA ligand mismatched donors. Inhibition through HLA-E could be prevented using blocking antibodies, but this might only be needed when HLA-E levels are highly expressed (chapter 3). Promoting the proinflammatory cytokine secretion of NK cells might be a complementary strategy to stimulate anti-tumor immunity, for instance by directing NK cells towards TME cells such as TAM (chapter 7). The figure was created using BioRender.com and (**B**) is adapted from chapter 4. TAM = tumor-associated macrophages.

This thesis explores which mechanisms and TME factors could limit NK cell responses and describes strategies to enhance NK cell effector functions with the aim to optimize NK cells in such a way that they achieve and retain their anti-tumor potency in an immunosuppressive TME (**Figure 1**). We investigated effector functions of healthy donor-derived NK cells against multiple myeloma and breast cancer cells in models that include clinically-relevant TME characteristics. Despite being two very different diseases, multiple myeloma and breast cancer have in common that the TME represents an immunosuppressive environment in which it is challenging for NK cells to exert their anti-tumor functions[1,2]. Previously, we have demonstrated that stimulation with the cytokine IL-2 can enhance NK cell activating signals and result in enhanced tumor killing also under hypoxic conditions[3]. In this thesis, we therefore mostly used donor-derived NK cells that were activated with high-dose IL-2 and assessed which challenges IL-2 activated NK cells encounter in the TME. Moreover, hypoxia has been included in many chapters of this thesis because hypoxia is present in both the bone marrow of multiple myeloma patients and in more than 50% of solid tumors, including breast cancer, we know from previous research that hypoxia is an important TME characteristic that can diminish unactivated NK cell functions[3-6]. In this thesis, hypoxia did not largely affect the outcome of the strategies explored with IL-2 activated NK cells and is therefore not separately discussed.

9.1 Magnitude of NK cell responses are tuned by HLA class I expression

To enhance NK cell effector functions, we need to understand to which extent the anti-tumor responses of cytokine-activated NK cells are controlled by inhibitory signaling. Inhibitory signals for NK cells are predominantly mediated through the inhibitory KIR and NKG2A receptors that bind to the classical HLA class I molecules HLA-ABC and to HLA-E, respectively. To permit attack of damaged cells but prevent autoreactivity, KIR and NKG2A are also involved in the functional development of NK cells by licensing those KIR^+ and/or $NKG2A^+$ NK cells, for which the cognate HLA ligand is present in the same individual[7,8]. NK cell licensing is a complex and dynamic process resulting in more potent NK cells after licensing, demonstrated by stronger anti-tumor responses of licensed NK cells compared to unlicensed NK cells[9,10]. Most studies have investigated licensing in resting NK cells and the functional relevance of the licensing status for highly activated NK cells remains relatively unexplored. When we studied the receptor co-expression of NKG2A and KIR and their functional consequences for cytokine-activated NK cells against normoxic or hypoxic target cells in multiple myeloma, we found that activated $NKG2A^+$ NK cells degranulated stronger than $NKG2A^-$ NK cells against $HLA\text{-}E^-$ or $HLA\text{-}E^{low}$ target cells in all conditions (**chapter 3**). These findings indicate that the $NKG2A^+$ NK cells are better licensed than their $NKG2A^-$ counterparts, irrespective of the NK cells' KIR expression. That NKG2A and KIR can act synergistically during NK cell licensing has been shown in a mouse model where combined deletion of the NKG2A and KIR genes affected NK cell function more severely than deletion of one of the two[11]. We observed that $NKG2A^+$ NK cells performed not as good as their $NKG2A^-$ counterparts, when high levels of HLA-E on target cells were induced by peptides, suggesting that high HLA-E

expression is necessary to mediate inhibition through NKG2A and outweigh the beneficial licensing effect of NKG2A (**chapter 3**).
Our results of **chapter 3** might be particularly relevant for expanded NK cells, where the majority of cells express NKG2A. While the repertoire of immature and mature NK cell subsets is maintained during expansion, the relatively immature NKG2A$^+$ CD56^{bright} NK cells were shown to expand the most in response to cytokines and therefore represent the dominant NK cell subset at the end of expansion[12,13]. Our study supports that NKG2A expression is not always detrimental for NK cells and it highlights the relevance of determining HLA-E expression levels on the target cells to predict responses of NKG2A$^+$ expanded NK cells.

Importantly, high levels of HLA-E on multiple myeloma cells inhibited activated NKG2A$^+$ NK cells in **chapter 3.** We and others described heterogeneous levels of HLA-E on both patient-derived primary cells and cell lines in multiple myeloma, in combination with high HLA-ABC expression (**chapter 3**,[14,15]). In a previous publication and in **chapter 2**, we described the functional consequences for NK cells, namely that HLA class I expression on multiple myeloma limited the efficacy of IL-2 activated NK cells[14,16]. The relevance of HLA class I as an inhibitory checkpoint for NK cells is further supported by a study showing that HLA class I-deficient melanoma cells were highly sensitive to NK cell cytotoxicity[17]. For multiple myeloma, it has been reported that higher HLA class I expression positively correlated with later disease stages, which could indicate that myeloma cells are especially resistant to NK cells during advanced multiple myeloma[15].
In line with our findings in multiple myeloma, we demonstrated that classical HLA class I (HLA-ABC) was an important inhibitory checkpoint on breast cancer, shown by lower NK cell degranulation against breast cancer cell lines in the presence of HLA class I compared to its absence (**chapter 4**). When using patient-derived breast cancer cells, activated NK cells did not kill the three HLA class I-positive tumors and killed the two HLA class I-negative tumors. Although resistance could be mediated by other inhibitory ligands or by the lack of activating ligands[18], these results suggest that HLA class I can be a critical inhibitory checkpoint on primary breast cancer cells as well (**chapter 4**). Classical HLA class I and HLA-E expression is maintained in about half of the breast cancer cases but with high intra- and inter-heterogeneity[19,20]. In line with those studies, we observed high heterogeneity in HLA class I expression by multiplex immunohistochemistry in both breast cancer cohorts of **chapter 5**, where we found HLA class I-positive tissue areas next to HLA class I-negative ones, suggesting that HLA class I loss occurs locally. Importantly, HLA-E expression in otherwise HLA class I-negative breast cancers has previously been shown to correlate with worse clinical outcome compared to tumors lacking both HLA-E and HLA class I[21]. In this scenario, tumor cells could probably escape both T cells through HLA class I downregulation and NK cells through sufficient expression of HLA-E to inhibit NK cells. While most healthy tissues usually express low levels of HLA-E, high HLA-E expression is also frequently observed in other cancer types than breast cancer (up to 90% of gynecologic tumors, up to 50% of melanoma, lung and

gastrointestinal cancers[22]), suggesting that HLA-E could be a mechanism to evade NKG2A$^+$ NK cells and NKG2A$^+$ CD8$^+$ T cells[23].
These aspects illustrate that both classical HLA class I and HLA-E on target cells, especially at high levels of expression, are important inhibitory checkpoints for cytokine-activated NK cells. Interfering with such inhibitory signaling could be a promising strategy to create more potent NK cells against HLA class I$^+$ positive targets.

9.1.1 Reducing NK cell inhibition by interfering with HLA class I augments NK cell responses

Since NK cell activation is determined by the sum of all activating and inhibitory signals an NK cell receives, reducing the inhibitory signals can represent a strategy to lower the NK cell activation threshold. One strategy, we previously published, involves selection of KIR-ligand mismatched donors, meaning donors with licensed KIRs for which the cognate HLA ligand is absent on the target cells (creating 'missing-self' based on genetic differences)[14,16]. There we showed that, for NK cells activated with high dose IL-2, KIR-ligand mismatched NK cells had a superior anti-myeloma capacity compared to their KIR-ligand mismatched counterparts, also in the presence of TME factors[16]. In this thesis, we extended the principle of selecting NK cell donors with a KIR-ligand mismatch to breast cancer, where it also proved to be beneficial at 21% O_2 as well as at hypoxic levels with 0.2% O_2 (**chapter 4**). Because KIR are acquired in a stochastic manner, every individual has NK cells without KIR, with one or with multiple KIRs[24]. This means that only a fraction of all obtained donor NK cells is KIR-ligand mismatched, while other NK cells are KIR-matched or KIR^{neg}. Although the NK cell subset with only mismatched KIRs can be small, we anticipate that every reduction of inhibition can be favorable, especially in a suppressive TME where many factors can contribute to NK cell inhibition. Moreover, the NKG2A$^+$ KIR-mismatched NK cells can be counted as KIR-ligand mismatched cells when the target cells lack HLA-E or express very low levels of HLA-E. This is illustrated by the fact that NKG2A$^+$ KIR-ligand mismatched NK cells performed as well as those without NKG2A expression against HLA-E negative breast cancer cells and K562 cells (**chapter 3 & 4**). Together, our studies demonstrated that selection of NK cell donors with KIR-ligand mismatches can be beneficial as it enhanced NK cell responses.
Blocking the KIR-HLA interaction with blocking antibodies could be an alternative strategy, when the selection of KIR-ligand mismatched NK cell donors is not possible. This concerns around 30% of the population that express HLA ligands for all three main KIRs (KIR2DL1, KIR2DL2/3, KIR3DL1)[25]. However, as discussed in **chapter 2**, using the KIR blocking antibodies IPH2101 and IPH2102 for multiple myeloma didn't result in clinical efficacy; it turned out that blocking KIR resulted in hypofunctional NK cells[26]. Targeting NKG2A, either by gene knockout or antibodies, to block the receptors' interaction with HLA-E seems to be more successful, potentially due to the frequent expression of HLA-E on tumor cells and NKG2A on both NK cells as well as tumor-infiltrating CD8$^+$ T cells[22]. The NKG2A-blocking antibody Monalizumab showed strong efficacy in initial results of clinical trials, especially when combined

with checkpoint blockade e.g., targeting the PD-1/PD-L1 axis, to activate $CD8^+$ T cells[22].

Interfering with NK cell inhibition could be extended beyond KIR and NKG2A to blocking other inhibitory checkpoints expressed on NK cells such as TIM-3, TIGIT, PD-1 or LAG-3, but the expression levels and more importantly the functional consequences of blocking these receptors on NK cells are insufficiently explored so far[27,28]. For example, TIGIT blocking antibodies are tested in clinical trials, but the effects of TIGIT as a negative regulator of cytotoxic lymphocytes have mostly been evaluated on T cells so far. On tumor-infiltrating NK cells, opposing results of TIGIT expression levels have been reported with low and high TIGIT expression on NK cells in melanoma and in colon cancer, respectively[29,30], which might indicate a dynamic expression of the molecules. In one study, $TIGIT^+$ NK cells were described to be highly functional[29]. On the contrary, blocking TIGIT was demonstrated to enhance NK cell functionality in ovarian cancer.[31] Overall, blocking these immune checkpoint molecules on NK cells requires more in-depth exploration to better understand the consequences for NK cells and the interplay between NK cells and T cells in response to checkpoint blockade.

9.2 Boosting NK cell responses by providing additional NK cell activation

Next to lowering inhibition, promoting NK cell activation is another principle to enhance NK cell potency. Activation by an ADCC-triggering antibody through the CD16A (FcγRIIIA) receptor is considered a potent way to enhance NK cell responses[32]. Because NK cells are the main effector cells of ADCC in humans, NK cells contribute significantly to the clinical success of those monoclonal antibodies that are able to induce ADCC[33]. Antibody-based therapies have started a new exiting era in cancer treatment and are nowadays widely used for a variety of solid and hematological cancer types since monoclonal antibodies can be directed against any antigen, for instance tumor-antigens[34]. Besides ADCC, mechanisms of action can, depending on the antibody, include direct effects on tumor cells such as induction of apoptosis or signaling inhibition, as well as other immune-mediated mechanisms such as antibody-dependent cellular phagocytosis and complement-dependent cytotoxicity[35].

Since CD16 is considered one of the most potent activating receptors for NK cells, we tested whether enhancing NK cell activation through ADCC-triggering antibodies can create more potent NK cells when combined with KIR-ligand mismatched NK cells to lower NK cell inhibition. In this thesis, we tested the clinically-approved ADCC-triggering antibody Trastuzumab that targets $HER2^+$ breast cancer (**chapter 4**). We found that IL-2 activated NK cells degranulated very strongly against the $HER2^+$ cell line SKBR3 when combined with Trastuzumab. The strong degranulation was observed in all NK cell subsets, also in the presence of HLA class I, and it was so high (70 to above 90%) that a potential further increase by KIR-ligand mismatching might have not been detected due to a technical limitation (**chapter 4**). However, in multiple myeloma, we observed that addition of the CD38-targeting ADCC-inducing

antibody Daratumumab further enhanced responses of the KIR-ligand mismatched NK cell subsets, illustrating that combination of the two strategies looks promising[16]. The ADCC response we observed seemed to depend on the expression level of HER2 as we saw an increase in NK cell degranulation and cytotoxicity against HER2^{high} SKBR3 cells, but not against HER2^{low} MCF7 cells. The growth factor receptor HER2 is expressed on normal epithelial and mesenchymal cells at low levels, but it is amplified on certain tumors including 15-20% of breast cancer patients[36]. Our results are in agreement with previously published articles showing that tumors with high HER2 expression were more susceptible to ADCC, as were tumor cells with high CD38 expression more susceptible to ADCC through Daratumumab[16,37,38]. To increase antigen expression levels at the cell surface and boost the ADCC effect, one study suggested temporary inhibition of endocytosis, which lead to stronger ADCC responses[39]. Other strategies to further enhance the ADCC response include increasing the antibody affinity to its target or to the NK cell. The latter can for instance be achieved by defucosylation of the antibody i.e., the removal of the sugar fucose, to effectively mediate ADCC at low antigen density, which has shown promising results in vitro but not yet in vivo[40-42]. For safety, however, it could also be an advantage that high levels of the target antigen are required for potent ADCC effects to spare healthy cells with low levels of antigens from NK cell attacks. This is supported by a recent study with HER2-targeting CAR-NK cells that were highly cytotoxic against HER2^{+} tumor cells, but did not kill non-malignant HER2^{low} epithelial cells[43]. The NK cells selectivity for high antigen levels could present an advantage over CAR-T cells, which also lysed non-malignant cells with low antigen levels[43]. In **chapter 7**, we also observed that the ADCC response of the anti-PDL1 antibody Avelumab was dependent on high PDL1 levels, which could potentially be an advantage when it would spare healthy PDL1^{+} immune cells from ADCC attacks. Another challenge of ADCC-mediating antibodies could be antigens that are also strongly expressed on normal cells such as CD38, which is commonly expressed on multiple myeloma but also on NK cells at high levels. Consequently, with Daratumumab, CD38^{+} NK cells can be killed by other NK cells, referred to as NK cell fratricide (**chapter 3**,[44]). To prevent such off-target toxicity, CD38 knock-out NK cells could be engineered to treat CD38^{+} hematological malignancies[45]. Whether high antigen expression is desired, should thus be carefully assessed.

To maximize the efficacy of antibody-based therapies for NK cells, novel constructs are in development such as bispecific and tri-specific antibodies, in some studies also called bispecific and tri-specific killer engagers (BiKEs and TriKEs). Instead of monoclonal antibodies that are directed against one target, bi- or tri-specific antibodies are small molecule constructs that can engage two or three different types of epitopes[27]. Due to the success of ADCC, many bi- and tri-specific antibodies constructs contain one anti-CD16 component with high affinity, while the other one or two domains can be directed against tumor antigens[46]. In preclinical studies, bispecific antibodies achieved promising results for hematological malignancies as well as for solid tumors and are currently tested in clinical trials[47,48]. Newer generations of tri-specific antibodies incorporate a cytokine to additionally enhance

survival and proliferation of NK cells, for example a tri-specific antibody with anti-CD16, anti-CD33 as tumor target, and IL-15 was engineered and mediated higher cytotoxicity and robust NK cell expansion in preclinical AML models compared to bispecific antibodies without cytokines[27]. One concern of this approach is that continuous stimulation with IL-15 might lead to NK cell exhaustion[49]. Another research group developed NK cell engagers that target one tumor antigen and two activating receptors, NKp46 and CD16, because the latter can be lost on tumor-infiltrating NK cells, which can diminish the efficacy of constructs only targeting CD16 on NK cells[50]. In **chapter 5**, in which we profiled endogenous NK cells in situ in breast cancer cohorts, we indeed also observed NK cells with low CD16 expression in some specimens, suggesting that enhancing NK cells through CD16 alone might not be very effective for these particular patients. Co-engagement of NKp46 and CD16 resulted in stronger NK cell activation than engaging both receptors individually and first promising results were obtained in vitro and in mice[50]. For both multiple myeloma and breast cancer, results from ongoing clinical trials are awaited to see whether the results with bispecific or tri-specific antibodies will be as promising as the ones from preclinical models[51,52].

An alternative strategy to augment NK cells includes the design of NK cells genetically engineered with chimeric antigen receptors (CAR-NK cells). Similar to monoclonal antibodies, CARs can be directed against the antigen of interest and current designs are most often directed against HER2 in solid tumors and against the CD19 antigen in hematological cancers (for B cell malignancies) [53]. Most studies with CAR-NK cells are still in very early phases of clinical trials and it will be interesting to see whether CAR engineering is sufficient to overcome immunosuppression[54].

9.3 NK cells must overcome multiple tumor resistance mechanisms to exert their anti-tumor effects

In the first paragraphs, I have discussed that the anti-tumor potency of IL-2 activated NK cells can be further enhanced by selecting KIR-ligand mismatched NK cell donors to reduce inhibition on the one hand, and by combining the mismatched NK cells with ADCC-triggering antibodies to boost activation on the other hand. In addition to designing powerful NK cells, it is crucial that NK cells are fully functional within the immunosuppressive TME and overcome tumor resistance. Multiple resistance mechanisms can occur in tumor cells that can be of tumor-intrinsic nature and/or mediated by the immunosuppressive TME. The TME is much more complex than solely the presence of hypoxic regions and is further characterized by low pH, high lactate, prostaglandins, suppressive cytokines and others[48]. In the following paragraphs, I describe how some of these TME factors could affect NK cell efficacy and how NK cell efficacy might be restored. And while I discuss one resistance mechanism at a time, it is more likely that NK cells encounter a combination of mechanisms and hence require a combination of strategies to counteract resistance and remain effective in the TME.

9.3.1 Tumor-intrinsic resistance mechanisms

Besides avoiding NK cell recognition, tumor cells can develop other cell-intrinsic resistance mechanisms to avoid being killed by NK cells. When studying the effect of Trastuzumab in breast cancer, we observed a relatively small ADCC effect in HER2^{high} SKBR3 cells; the cytotoxicity was only slightly increased compared to conditions without Trastuzumab (**chapter 4**). This was striking in comparison to the strong increase in NK cell degranulation by Trastuzumab, showing that the NK cells were activated but that degranulation did not proportionally correlate with tumor cell lysis. We did not detect such big discrepancy between high NK cell degranulation and moderate cytotoxicity in our previous studies [3,16]. The NK cells we used seemed fully functional because they potently degranulated, and they lysed SKBR3 cells, only with a smaller ADCC effect than expected based on the potent degranulation. Therefore, it seems more likely that the tumor cells were partially resistant to the NK cell attacks. Since NK cells can kill target cells through cytotoxic granules and/or death receptors (FasL or TRAIL), we question whether ADCC may be mainly mediated through one of the two cytotoxic pathways, either granules or death receptors, and whether SKBR3 cells are resistant to that pathway. Granule-mediated killing is the faster and generally the more dominant killing mechanism that can occur within minutes to hours, while killing through death receptors is the slower process of the two[55]. IL-2 activation of NK cells has been demonstrated to upregulate FasL expression on NK cells and to enhance both cytotoxic mechanisms by increased frequency of NK cell-cancer cell interactions[55,56]. Another recent study described that the killing events of NK cells were first almost exclusively mediated through granules, and while granule content decreased, Fas ligand expression increased and a switch to death receptor-mediated cytotoxicity was observed to mediate the final kill of an NK cell[57]. This switch in killing mechanism was noted for NK cells with and without IL-2 activation[57]. Regarding the killing mechanism during ADCC, one study described that ADCC could be enhanced by the immunomodulatory drug Lenalidomide in rituximab-treated lymphoma or leukemia cell lines, and that the effect was mainly mediated through granzyme B because NK cell-mediated ADCC was totally prevented by a granzyme B inhibitor and partially prevented by blocking Fas ligand expression[58]. Together, these studies could suggest that granule-mediated killing is the dominant pathway during NK cell-mediated ADCC. Whether SKBR3 cells are resistant to granule-mediated killing, remains to be studied. Such cell-intrinsic resistance to cytotoxic granules could be mediated by inactivation of the cytotoxic granules. Many tumors express molecules to degrade either perforins e.g., through Cathepsin B, or granzymes e.g., through the serpin proteinase inhibitor B9 (Serpin B9) that inhibits granzyme B[59]. Serpin B9 is commonly expressed in lymphocytes to protect them from their own active granzyme B and tumor cells seem to have hijacked this mechanism[60]. The occurrence of Serpin B9 in healthy cells as well as in tumor cells could, however, complicate therapeutic approaches to target such resistance.

Tumor cells can further counteract the effects of cytotoxic granules by upregulation of anti-apoptotic proteins. In multiple myeloma for instance, evading apoptosis is a

well-known mechanism of drug resistance and characterized by upregulation of the anti-apoptotic Bcl-2 molecules and/or the inhibitors of apoptosis (IAP) proteins such as survivin[61]. These anti-apoptotic proteins can either be induced cell-intrinsic or by the TME e.g., through soluble factors like IL-6 or VEGF produced by BM stromal cells[61]. To reverse apoptotic resistance, anti-apoptotic inhibitors could be used. Interestingly, small-molecule inhibitors to block anti-apoptotic proteins such as survivin (e.g. YM155 or FL118), also revealed synergetic effects with NK cells as the inhibitor improved Daratumumab-mediated ADCC responses in multiple myeloma[62,63].

Under hypoxia, another resistance mechanism can come into play, namely autophagy, which can degrade granzyme B molecules. Autophagy is a process, in which the cell self-digests components to recycle them or to prevent accumulation of waste products, and which can be induced in response to cellular stress, including hypoxia[64]. When we observed that the NK cell-mediated lysis of MCF7 breast cancer cells was reduced under hypoxic conditions, compared to MCF7 lysis with a normal oxygen concentration (**chapter 4**), autophagy could have potentially mediated the reduction in killing. One article described that hypoxic MCF7 breast cancer cells activated autophagy, which lead to granzyme B degradation and protected the tumor cells from killing by resting NK cells[65]. In the same study, it was demonstrated that the granzyme B levels in target cells could be restored by the autophagy inhibitor chloroquine[65]. However, autophagy is a highly regulated and multistep lysosomal degradation pathway and can, depending on the context, have both pro- and anti-tumor tumor effects which complicates therapy approaches and requires a more detailed understanding of its effects in the TME[66].

Modulations at the immunological synapse could be another way how tumor cells can interfere with NK cell-mediated killing. The immunological synapse describes the area of contact between NK cells (or T cells) and tumor cells, which is required to enable directed release of cytotoxic granules towards target cells. The formation of an immunological synapse is a finely regulated process that involves specific receptor-ligand interactions and spatiotemporal rearrangements of the NK cells cytoskeleton[67]. Compared to immune cells, in which rearrangements of the actin cytoskeleton are quite well studied, remodeling of the cytoskeleton in tumor cells only gained attention quite recently as another possible immune escape mechanism[68]. Interestingly, subpopulations of breast cancer and chronic lymphocytic leukemia cells rapidly clustered actin filaments near the immunological synapse, which acted as a protective shield and correlated with NK cell resistance and lower granzyme B levels inside tumor cells[69,70]. Since we observed in **chapter 4** that some SKBR3 breast cancer cells were quite resistant to NK cells in combination with Trastuzumab, it would be interesting to determine whether such actin accumulation near the immunological synapse also occurs in a subpopulation of SKBR3 and whether SKBR3 cells with such an “actin shield” are protected against NK cell-mediated ADCC. If so, it might be desirable to block cytoskeletal remodeling in breast cancer cells. Drugs to target the actin cytoskeleton must specifically target cancer cells to circumvent the extreme toxicity that is seen with general targeting of

the actin cytoskeleton; such tumor-specific drugs are in the experimental stage only and could be a future possibility for combination with potent NK cells if they prove to be clinically applicable[71].

9.3.2 Soluble mediators in the TME can hinder NK cells

Many cell surface receptors or ligands can additionally occur as soluble forms. Soluble (s) HLA-E molecules are one example of HLA molecules that can affect NK cell functions in its soluble form. sHLA-E have been found in sera of melanoma and neuroblastoma patients at significantly higher levels than in healthy controls, as well as in supernatant of a variety of cancer cell lines including breast cancer[72,73]. The generation of sHLA-E is presumably mediated by the cleavage of HLA-E from the cell membrane by proteases ADAM10 and ADAM17[23]. That sHLA-E molecules had immunomodulatory effects was demonstrated by the fact that sHLA-E led to decreased NK cell-mediated lysis of target cells[74]. As we demonstrated in **chapter 4** that low HLA-E expression on the tumor cell surface did not negatively affect activated NK cells, it would be interesting to determine whether sHLA-E effects on NK cells are also depending on expression levels. For the classical molecules HLA-ABC, limited studies have described the occurrence of soluble forms and additional research is needed to study whether these also have immunomodulatory features[75]. The most frequently studied soluble HLA molecule is HLA-G. Similar to HLA-E, sHLA-G molecules can be generated by proteolytic shedding of the membrane-bound HLA-G form[76]. Additionally, seven HLA-G isoforms, identified as G1-G7, can be generated through alternative splicing from the normal HLA-G variant[76]. Three of the seven HLA-G variants (G5-G7) lack the transmembrane region and subsequently occur as a soluble protein[77]. HLA-G5 has moreover been detected in its secreted form in cell supernatants and body fluids and has been shown to have similar immunomodulatory functions as it's membrane-bound counterpart G1, namely inhibiting NK cells and T cells[78,79]. Such effects of sHLA-G might be particularly relevant for tumors residing in hypoxic TMEs because HLA-G transcription was demonstrated to be induced in HLA-G-negative tumor cells in response to hypoxic stress[80].

In **chapter 8**, we studied alternative splicing in HLA-C and we reported HLA-C transcripts without exon 5 (encoding the transmembrane region of HLA) in peripheral blood cells. The occurrence of HLA-C transcripts we detected precisely lacked the exon 5 region and thus resemble the occurrence of the soluble HLA-G variant HLA-G5. It needs to be determined if the described HLA-C transcripts will be translated to functional proteins. If this is the case, the alternative HLA-C isoforms might be secreted as soluble forms, similar to sHLA-G5. Theoretically, sHLA-C could be released by tumor cells to escape T cells, and still mediate inhibition of NK cells through its soluble form. It would be interesting to further investigate if sHLA-C represents an escape mechanism by tumor cells, similar to sHLA-E and sHLA-G. Moreover, we found the HLA-C transcripts without exon 5 specifically in the two allele groups HLA-C*04 and *16, suggesting that genetic polymorphisms contribute to the occurrence of alternative splice variants in HLA-C (**chapter 8**). This finding adds

another layer of complexity to the diversity of HLA-C and indicates that HLA-C expression levels can be further modulated by genetic diversity. Since all HLA-C alleles serve as ligands for NK cells, different HLA-C expression levels could potentially have functional consequences for NK cells.

Other soluble molecules that can diminish NK cell effector functions are the MHC class I chain-related proteins A and B, MICA and MICB, which are frequently upregulated on tumor cells and serve as activating ligands for NK cells via NKG2D. Proteolytic shedding of MICA and MICB from the membrane is, however, recognized as an escape strategy of tumor cells as it results in reduced surface expression of these activating ligands, which can prevent tumor cell recognition through NKG2D[81,82]. Additionally, soluble forms of MICA and MICB can bind to NKG2D on NK cells, where they induce downregulation of the activating receptor NKG2D[83]. To counteract such effects and sustain cell surface expression of MICA and MICB, therapeutic antibodies have been developed that are directed against the site that is responsible for the shedding, and this novel strategy to prevent shedding of MICA and MICB has demonstrated efficacy in multiple preclinical tumor models[84].

Tumor cells can further evade immune cells through an excess of immunosuppressive cytokines present in the TME. Promoting NK cell dysfunction or inhibition in the TME through such cytokines including TGF-β, IL1-β, and IL-10, can prevent NK cells from recruiting other immune cells that are crucial for mediating a proper anti-tumor response[85]. TGF-β is one example for a key factor that suppresses NK cells in multiple ways. Direct effects of TGF-β on NK cells include downregulating the activating receptors NKp30 and NKG2D on NK cells, the NKG2D ligand MICA on tumor cells and reducing NK cell effector functions such as cytotoxicity and IFN-γ production[48]. Blocking suppressive cytokine signaling such as TGF-β might reverse the unfavorable cytokine milieu in the TME and thus represents an attractive option to restore NK cell efficacy in the TME. Small-molecule inhibitors or blocking antibodies targeting the TGF-β receptor are in development, as are therapeutic bi-specific antibodies that include a TGF-β-receptor-blocking domain[27,86]. In AML and in colon cancer models, it has for example been demonstrated that a TGF-β receptor inhibitor at least partially restored the TGF-β-mediated impairment of in vitro-expanded NK cells[87].

Overall, on the basis of a few examples, this section demonstrates that soluble mediators in the TME should be considered when designing NK cell-based immunotherapies.

9.3.3 Unfavorable nutrient availability in the TME might not be harmful for potently activated NK cells such as expanded NK cells

Tumor cells undergo metabolic reprograming from oxidative phosphorylation towards high rates of aerobic glycolysis (Warburg metabolism) to fulfill the energy needs required for tumor growth and its progression in the TME[88]. As a consequence of high glucose consumption by tumor cells, the TME is characterized by poor availability of glucose and by an excess of metabolic waste products[89]. Because immune cells also heavily rely on glucose as metabolic fuel, low glucose levels can

impair immune cell metabolism, including T cells, which was associated with cancer progression[90,91]. Moreover, there is convincing data that disturbed NK cell metabolism can hamper NK cell effector functions[92]. In a murine lung cancer model for example, the glycolytic capacity of NK cells and NK cell function inversely correlated with tumor progression[93]. Furthermore, the TME of ovarian cancer ascites was shown to significantly impair metabolic pathways (glycolysis and oxidative phosphorylation) in NK cells in an ex vivo model[94]. In several solid tumor types, extremely low glucose levels reaching 20 mg/L were reported with great variability between patients[95]. In the bone marrow of multiple myeloma patients, we measured glucose levels ranging from 479-1231 mg/L in nine patients, of which glucose values in six patients were lower than the normal range of blood glucose (820 – 1100 mg/L fasting blood glucose levels and up to 1400 mg/L postprandial) (**chapter 6**). Since glucose is considered a primary fuel for NK cells, we subsequently investigated how reduced glucose concentrations of 500 mg/L glucose, representing the lower end of levels measured in multiple myeloma bone marrow, affected IL-2 activated NK cell responses (**chapter 6**). Cytokines, such as IL-2, IL-12 and IL-15, have been described to strongly upregulate the metabolic pathways of both glycolysis and oxidative phosphorylation[96]. In our hands, reduced glucose concentrations of 500 mg/L glucose did neither negatively affect the cytotoxic potential of overnight activated NK cells, nor the potential of ex vivo expanded NK cells, which were exposed to the low glucose concentration for 4 days before the cytotoxicity assay was performed (**chapter 6**). Our finding of activated NK cells not being inhibited by low glucose were in line with a recent study describing that IL-21 feeder-cell-expanded NK cells were not impaired by nutrient deprivation in an ovarian cancer model[94]. In the referenced study by Poznanski et al., the expanded NK cells showed metabolic changes that were similar to those of tumor cells and showed sustained anti-tumor effector functions in a TME-tumor model with ascites fluid derived from ovarian cancer patients. Even more, the expanded NK cells had a stronger tumor killing potential than peripheral blood NK cells because they were not limited to utilizing glucose as fuel, but were equipped with a high flexibility as to which substrate to use (e.g. glucose, glutamine, fatty acids)[94]. Metabolic flexibility had before been reported in licensed NK cells, in which high capacity of both oxidative phosphorylation and glycolysis was observed, compared to unlicensed NK cells that primarily depended on OxPhos[97]. Cytotoxicity of the licensed NK cells was only decreased when both metabolic pathways were blocked[97].

Although further proof in vivo is required, the discovery of metabolic substrate flexibility in expanded NK cells provides promising news for adoptive transfer of NK cells and could be a promising strategy for improved NK cell efficacy. Moreover, the metabolic flexibility in NK cells could potentially be an advantage over T cell-based therapies, for which the nutrient availability in the TME seems to be a barrier that severely impaired CD8 T cell functions[89,91].

9.3.4 NK cell recruitment to the tumor is essential for potent anti-tumor effects

Improving NK cell recruitment to the tumor is especially relevant for solid tumors where NK cells are usually scarce. Although variable between specimens, we quantified low NK cell density compared to $CD3^+$ and $CD11b^+$ immune cell densities in all specimens when profiling NK cells in two independent breast cancer cohorts (**chapter 5**). Our finding supports that especially T cells are predominantly present in tumor areas, while NK cells might be restricted in entering the tumor.

Given that NK cells in solid tumors have been associated with improved patient survival[98-101], higher NK cell infiltration is expected to lead to better anti-tumor immune responses and should be promoted. Another aspect to consider for NK cell recruitment are the cytokine and chemokine axes in tumor tissues because they might favor infiltration of certain NK cell subsets: While the mature $CD56^{dim}$ NK cells are predominant in peripheral blood, $CD56^{bright}$ NK cells were the dominant NK cell subset in the TME of many solid tumors including breast cancer[102,103]. $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets are characterized by different chemokine receptor profiles, and tumors have been described to preferentially express ligands for chemokines that stimulate migration of $CD56^{bright}$ NK cells to the tumor[104]. However, it has also been reported that NK cell infiltration can be low despite high expression of NK cell-attracting chemokines in tumors[104]. The latter could suggest that NK cells do not stay or survive in the TME or that other factors prevent NK cell infiltration into solid tumors.

To improve homing to the tumor, NK cells could be genetically modified to express chemokine receptors that are known to be important in specific cancer types. An example is the CXCR2 receptor because its ligands can be secreted by solid tumors to promote angiogenesis and metastasis; and CXCR2 is expressed on $CD56^{dim}$ NK cells in blood, but rapidly lost during in vitro expansion[105]. Genetically engineered $CXCR2^+$ NK cells were able to migrate along the tumor-derived chemokine gradient and mediated better tumor lysis in vitro[105]. Another strategy to enhance NK cell homing to tumors could be through stimulation of chemokine ligands at the tumor site, for example did local administration of IFN-γ stimulate production of CXCL10, which attracted $CXCR3^+$ ex vivo expanded NK cells in a melanoma model[106].

Besides the chemokine axes, immune cell infiltration into solid tumor can be restricted by physical barriers, such as stroma and abnormal vasculature. Blood vessels in tumors frequently appear disorganized and heavily branched, which can lead to irregular blood flow and hinder infiltration of immune cells from the blood stream[107]. Growing evidence shows that continuous angiogenic stimulation can additionally lead to decreased expression of adhesion molecules on the endothelium[107]. Since adhesion molecules, like integrins and selectins, are used by leukocytes to adhere and subsequently reach the tumor parenchyma, the downregulation of endothelial adhesion molecules can further hamper immune cell infiltration and subsequent anti-tumor responses[107]. That physical barriers might also affect NK cells is suggested because tumor-infiltrating NK cells are predominantly reported in the stromal areas of the tumor[103,108]. To target tumor angiogenesis, angiogenic inhibitors, most commonly targeting VEGF or VEGF

receptors, are widely used as cancer treatments to counteract tumor growth, but with limited clinical success so far, e.g. due to drug resistance[109]. One recent study demonstrated a novel strategy to target angiogenesis, namely a vaccination approach directed against tumor endothelium, which resulted in effective and safe inhibition of angiogenesis in preclinical models[110]. It would be interesting to study how targeting tumor endothelium could be combined with NK cell-based immunotherapies.

9.4 NK cells do more than killing: NK cell-based therapy to orchestrate adaptive immune responses

Although the NK cells' ability to kill tumors is often highlighted, the NK cell subsets and their phenotypes and functions are much more diverse as reviewed in [111,112]. Next to their cytotoxic capacity, the NK cells' potential to secrete large amounts of proinflammatory cytokines, such as IFN-γ and TNF-α, can be highly relevant for the anti-tumor immune responses, as they can promote adaptive immune responses[113]. It is well established that the bi-directional crosstalk between NK cells and DCs can result in profound adaptive immune responses in cancer: NK cells have an important role in inducing DC maturation and subsequent T cell priming by mature DCs, while DCs can in turn further stimulate NK cell activation, proliferation and effector functions[114]. Additionally, a recent study described that NK cells, through the release of chemokines, also support DC recruitment into the TME, which positively correlated with overall patient survival[115]. Similarly, NK cell infiltration and functional DC populations were important for responses to anti-PD1 immune checkpoint blockade in melanoma patients[116].

Proinflammatory cytokines produced by NK cells could furthermore contribute to reshaping the generally very immunosuppressive TME towards a more proinflammatory and immune stimulatory TME, which is considered to be crucial to achieve sustained effectiveness of immunotherapies[117]. In **chapter 7**, we described that donor-derived IL-2 activated NK cells were triggered by tumor-associated cells, such as TAM, to degranulate and to produce IFN-γ. Through the IFN-γ production, NK cells could be able to contribute to a more proinflammatory TME. NK cell-derived IFN-γ could for example polarize TAM towards pro-inflammatory macrophages that are associated with increased phagocytosis and antigen presentation[118]. As described above in section 9.4.3, IFN-γ has been shown to also enhance recruitment of expanded NK cells to melanoma through the CXCL10-CXCR3 axis[106]. IFN-γ can further regulate immune responses through many different effects and mechanisms, depending on the timing and spatial distribution of the cytokine[119]. IFN-γ drives for instance upregulation of HLA class I expression on tumor cells, that provides inhibition to NK cells, but can present tumor antigens, which in turn can be recognized by tumor-specific $CD8^+$ T cells and initiate $CD8^+$ T cell mediated killing[120]. And although IFN-γ mainly mediates anti-tumor immunity, it can also lead to upregulation of immunosuppressive molecules such as PD-L1, which can inhibit PD-1^+ immune cells including PD-1^+ $CD8^+$ T cells and thereby contribute to tumor evasion

from immune responses[121]. In the event of IFN-γ-mediated PD-L1 upregulation, immune checkpoint blockade of the PD-1/PD-L1 pathway might be used to counteract the immunosuppression. In **chapter 7**, we observed that macrophages also upregulated PD-L1 in response to NK cell-derived IFN-γ. Subsequently, we studied whether we could take advantage of the high PD-L1 expression on macrophages by combining NK cells with the anti-PD-L1 antibody Avelumab. Of all clinically-approved PD-1/PD-L1-targeting antibodies, Avelumab is the only one that can mediate ADCC[122]. We found that the NK cell responses against PD-L1^{high} macrophages was further enhanced by Avelumab. These results suggest that NK cells could get activated in response to tumor-associated cells in the TME, especially in combination with Avelumab, and that NK cells could promote a more immunostimulatory TME through IFN-γ secretion, as an additional function to killing tumor cells. In line with our findings, two other studies observed PD-L1 upregulation on lung cancer cells after administration of ex vivo expanded NK cells, which sensitized the originally PD-L1^{neg} tumors to PD1-blockade therapy[123,124]. These studies and ours in **chapter 7** present an additional strategy how NK cell-derived IFN-γ could be used for improved immunotherapy responses.
Through secretion of proinflammatory cytokines, NK cell immunotherapies could thus serve as "adjuvants" for subsequent adaptive anti-tumor immune responses.

9.5 Successful NK cell-based therapy approaches will require combination therapy
Nowadays, we understand that NK cells are much more complex in their phenotype and their functions than initially thought when NK cells were identified as tumor killers. Their complexity makes it a lot more challenging than anticipated to develop NK cell therapies, but it also creates many opportunities when it comes to the selection of NK cell sources and to manipulating NK cells to create superior effector cells. During this discussion, I have presented several of the challenges that come with NK cell-based therapies and potential strategies how to counteract the challenge. For successful NK cell-based therapies, it will likely require a combination of multiple strategies because NK cells must be able to do the following: 1. infiltrate tumor areas, 2. recognize the tumor cells, 3. exert their tumor killing in a suppressive TME, for which NK cells should be sufficiently boosted and, 4. secrete proinflammatory cytokines, which will be desirable to orchestrate a proper adaptive immune response against the tumor[85]. The first step of NK cell recruitment to tumors remains a problem to solve for solid tumors including breast cancer. To enhance step 2-4 for solid tumors, we might instead be able to adapt strategies that have already been exploited for hematological malignancies, which have taught the research community a lot about NK cell biology, and for example the combinational strategy of KIR-HLA ligand mismatch and ADCC-triggering antibodies promoted NK cell efficacy for both MM and breast cancer in our hands.

9.5.1 Combining the power of NK cells and CD8$^+$ T cells for immunotherapies

One possible combination that should not be neglected are NK cells and CD8$^+$ T cells. Both NK cells and CD8$^+$ T cells kill tumor cells via the same cytotoxic mechanisms, namely via granules or death receptors, but the way they recognize target cells is fundamentally different with CD8$^+$ T cells requiring neoantigen presentation by HLA class I, and with NK cells responding to an array of activating signals expressed by malignant cells, while being inhibited by HLA class I. These differences in tumor cell recognition could potentially guide whether NK cells or T cells should be used for immunotherapy. Moreover, it would be interesting to explore the potential additive values of combining NK cells to strategies using other immune cells such as gamma-delta T cells or NKT cells[125,126].

In this thesis, we extensively discussed the relevance of HLA class I expression for NK cell inhibition. Together with studies describing that NK cell-sensitive tumors are HLA class I-negative[17], this could suggest that NK cell-based therapies could be primarily relevant for HLA class I-negative tumors, for which CD8$^+$ T cell-based immunotherapies are not suitable. In a study that used several genomic screening approaches, a transcriptional signature of NK cell-sensitive tumors was identified and correlated with resistance to immune checkpoint inhibitors[127], suggesting an opportunity for NK cell-based therapies for tumors that developed resistance to immune checkpoint therapy. Such resistance to checkpoint inhibitors was associated with defects in the IFN-γ- and antigen-presentation pathway e.g., loss of HLA class I[128]. Loss of HLA class I in tumor cells can be mediated by various underlying mechanisms and is either irreversible if the HLA class I gene is defect, or reversible if the HLA class I molecule is solely downregulated, for example due to alterations in the antigen-processing machinery[129]. The latter reversible loss of HLA class I expression can be upregulated by Th1-type cytokines like IFN-γ. Given that expanded NK cells may be highly effective in the suppressive TME[94], NK cells could thus potentially kill the HLA class I-negative tumors and, through IFN-γ, mediate HLA class I upregulation, if the HLA expression is reversible. For CD8$^+$ T cells to join fight against the cancer cells, immune checkpoint blockade might need to be considered to unleash the cytotoxic CD8$^+$ T cells if they are held in check by immune checkpoints such as the PD-1/PD-L1 axis. That NK cell-derived IFN-γ lead to upregulated MHC class I on tumor cells and enabled tumor recognition and killing by CD8$^+$ T cells has been shown in a B16 melanoma mouse model[130].

However, NK cells can also have a role in HLA class I-positive tumors. One study, in which predictive biomarkers for response to Trastuzumab were investigated in breast cancer, described that high HLA class I expression together with NK cell infiltration were predictors for better Trastuzumab responses[100]. The results of this study are in agreement with our observations in **chapter 4**, where Trastuzumab induced very strong degranulation in all NK cells, suggesting that NK cells, when sufficiently activated, can also mediate responses against HLA class I$^+$ tumors either by killing tumor cells and/or by promoting adaptive immunity through cytokine secretion. Moreover, the study by Muntasell et al. supports that both Trastuzumab-activated NK cells and CD8$^+$ T cells are important for mediating anti-breast cancer

responses[100]. Therefore, rather than deciding for either NK cells or T cells, the strength of both cell types should be kept in mind.

9.5.2 Combination of strategies to augment NK cells

Which combinational strategies should be combined to achieve potent NK cells in an immunosuppressive TME, still needs to be further investigated and likely depends on the tumor. When adoptive NK cell therapy is anticipated, the ideal NK cell donor and NK cell source should be selected in the beginning. NK cells can be derived from peripheral blood, umbilical cord blood, stem cells or NK cell lines[131]. In this thesis, we presented that those donors with a KIR-ligand mismatch can be beneficial over donors without. Irrespective of the source, large number of NK cells are thought to be required to achieve an anti-tumor effect in vivo and while the optimal number still needs to be determined, protocols used in clinical trials use up to $1x10^8$ NK cells/kg body weight[132]. To reach these numbers from donors (allogeneic NK cells) or from the patient (autologous NK cells), expansion protocols have been developed to perform large scale expansions of NK cells in the presence of cytokines, which can simultaneously enhance the potency of NK cells[47]. Subsequently, expanded NK cells can be administered alone or combined with a HSCT as discussed in **chapter 2**. The heterogeneity of strategies tested in clinical trials (various expansion protocols, infusion rounds, NK cell manipulations) complicates the comparison of clinical trials. But one example of recent success is demonstrated by adoptive transfer of feeder cell-expanded NK cells achieving good clinical responses without adverse effects in hematological malignancies[133,134].

Expanded NK cells likely need to be further combined with one or several approaches that prevent NK cell dysfunction in the TME. Which approach this should be, will depend on the TME characteristics of the particular tumor type. To decide which combination therapy should be applied for which tumor, we require more extensive sets of biomarkers to better predict therapy responses. Moreover, to improved understand the challenges NK cells will encounter, profiling of the tumor and its TME can be beneficial to know which factors or NK cell subsets should be targeted. Extensive profiling of tumor and immune cells was made possible by single-cell RNA-sequencing, and could be combined with multiplexed imaging methods and/or spatial transcriptomics to assess the spatial distribution of cells in the TME[135-137]. To improve prediction of combinational therapy approaches, it will be beneficial to test the approaches in different sets of tumor models that closely mimic the TME as well as the 3D structure of tumors e.g., patient-derived tumor organoids[138]. Together, the increasing number of available methodologies and various therapy approaches, including the ones investigated in this thesis, should provide the necessary tools to bring NK cell-based therapies to the next level.

References

1. Nakamura K, Smyth MJ, Martinet L. Cancer immunoediting and immune dysregulation in multiple myeloma. *Blood, The Journal of the American Society of Hematology.* 2020;136(24):2731-2740.
2. Gatti-Mays ME, Balko JM, Gameiro SR, et al. If we build it they will come: targeting the immune response to breast cancer. *NPJ breast cancer.* 2019;5(1):1-13.
3. Sarkar S, Germeraad WT, Rouschop KM, et al. Hypoxia induced impairment of NK cell cytotoxicity against multiple myeloma can be overcome by IL-2 activation of the NK cells. *PLoS One.* 2013;8(5):e64835.
4. Azab AK, Hu J, Quang P, et al. Hypoxia promotes dissemination of multiple myeloma through acquisition of epithelial to mesenchymal transition-like features. *Blood, The Journal of the American Society of Hematology.* 2012;119(24):5782-5794.
5. Colla S, Storti P, Donofrio G, et al. Low bone marrow oxygen tension and hypoxia-inducible factor-1α overexpression characterize patients with multiple myeloma: role on the transcriptional and proangiogenic profiles of CD138+ cells. *Leukemia.* 2010;24(11):1967-1970.
6. Vaupel P, Mayer A. Hypoxia in cancer: significance and impact on clinical outcome. *Cancer Metastasis Rev.* 2007;26(2):225-239.
7. He Y, Tian Z. NK cell education via nonclassical MHC and non-MHC ligands. *Cell. Mol. Immunol.* 2017;14(4):321-330.
8. Sim MJ, Stowell J, Sergeant R, Altmann DM, Long EO, Boyton RJ. KIR2DL3 and KIR2DL1 show similar impact on licensing of human NK cells. *Eur. J. Immunol.* 2016;46(1):185-191.
9. Joncker NT, Fernandez NC, Treiner E, Vivier E, Raulet DH. NK cell responsiveness is tuned commensurate with the number of inhibitory receptors for self-MHC class I: the rheostat model. *The Journal of Immunology.* 2009;182(8):4572-4580.
10. Brodin P, Lakshmikanth T, Johansson S, Kärre K, Höglund P. The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells. *Blood, The Journal of the American Society of Hematology.* 2009;113(11):2434-2441.
11. Zhang X, Feng J, Chen S, Yang H, Dong Z. Synergized regulation of NK cell education by NKG2A and specific Ly49 family members. *Nat. Commun.* 2019;10(1):1-12.
12. Liu LL, Pfefferle A, Sheng VOY, et al. Harnessing adaptive natural killer cells in cancer immunotherapy. *Mol. Oncol.* 2015;9(10):1904-1917.
13. Pfefferle A, Jacobs B, Netskar H, et al. Intra-lineage plasticity and functional reprogramming maintain natural killer cell repertoire diversity. *Cell Rep.* 2019;29(8):2284-2294. e2284.
14. Sarkar S, van Gelder M, Noort W, et al. Optimal selection of natural killer cells to kill myeloma: the role of HLA-E and NKG2A. *Cancer Immunol. Immunother.* 2015;64(8):951-963.
15. Carbone E, Neri P, Mesuraca M, et al. HLA class I, NKG2D, and natural cytotoxicity receptors regulate multiple myeloma cell recognition by natural killer cells. *Blood.* 2005;105(1):251-258.
16. Mahaweni NM, Bos GM, Mitsiades CS, Tilanus MG, Wieten L. Daratumumab augments alloreactive natural killer cell cytotoxicity towards CD38+ multiple myeloma cell lines in a biochemical context mimicking tumour microenvironment conditions. *Cancer Immunol. Immunother.* 2018;67(6):861-872.
17. Freeman AJ, Vervoort SJ, Ramsbottom KM, et al. Natural killer cells suppress T cell-associated tumor immune evasion. *Cell Rep.* 2019;28(11):2784-2794. e2785.
18. Wang B, Wang Q, Wang Z, et al. Metastatic consequences of immune escape from NK cell cytotoxicity by human breast cancer stem cells. *Cancer Res.* 2014;74(20):5746-5757.
19. Garrido MA, Rodriguez T, Zinchenko S, et al. HLA class I alterations in breast carcinoma are associated with a high frequency of the loss of heterozygosity at chromosomes 6 and 15. *Immunogenetics.* 2018;70(10):647-659.
20. Silva GBRFd, Silva TGA, Duarte RA, et al. Expression of the classical and nonclassical HLA molecules in breast cancer. *Int. J. Breast Cancer.* 2013;2013.
21. de Kruijf EM, Sajet A, van Nes JG, et al. HLA-E and HLA-G expression in classical HLA class I-negative tumors is of prognostic value for clinical outcome of early breast cancer patients. *The Journal of Immunology.* 2010:1002629.

22. Borst L, van der Burg SH, van Hall T. The NKG2A–HLA-E axis as a novel checkpoint in the tumor microenvironment. *Clin. Cancer Res.* 2020;26(21):5549-5556.
23. Wieten L, Mahaweni N, Voorter C, Bos G, Tilanus M. Clinical and immunological significance of HLA-E in stem cell transplantation and cancer. *Tissue Antigens.* 2014;84(6):523-535.
24. Fauriat C, Andersson S, Björklund AT, et al. Estimation of the size of the alloreactive NK cell repertoire: studies in individuals homozygous for the group A KIR haplotype. *The Journal of Immunology.* 2008;181(9):6010-6019.
25. Ruggeri L, Mancusi A, Urbani E, Velardi A. Identifying NK alloreactive donors for haploidentical hematopoietic stem cell transplantation. In: *Tumor Immunology.* Springer; 2016:141-145.
26. Carlsten M, Korde N, Kotecha R, et al. Checkpoint inhibition of KIR2D with the monoclonal antibody IPH2101 induces contraction and hyporesponsiveness of NK cells in patients with myeloma. *Clin. Cancer Res.* 2016;22(21):5211-5222.
27. Davis ZB, Vallera DA, Miller JS, Felices M. Natural killer cells unleashed: checkpoint receptor blockade and BiKE/TriKE utilization in NK-mediated anti-tumor immunotherapy. Paper presented at: Semin. Immunol.2017.
28. Alfarra H, Weir J, Grieve S, Reiman T. Targeting NK Cell Inhibitory Receptors for Precision Multiple Myeloma Immunotherapy. *Front. Immunol.* 2020;11.
29. Chauvin J-M, Ka M, Pagliano O, et al. IL15 stimulation with TIGIT blockade reverses CD155-mediated NK-cell dysfunction in melanoma. *Clin. Cancer Res.* 2020;26(20):5520-5533.
30. Zhang Q, Bi J, Zheng X, et al. Blockade of the checkpoint receptor TIGIT prevents NK cell exhaustion and elicits potent anti-tumor immunity. *Nat. Immunol.* 2018;19(7):723-732.
31. Maas RJ, Hoogstad-van Evert JS, Van der Meer JM, et al. TIGIT blockade enhances functionality of peritoneal NK cells with altered expression of DNAM-1/TIGIT/CD96 checkpoint molecules in ovarian cancer. *Oncoimmunology.* 2020;9(1):1843247.
32. Wang W, Erbe AK, Hank JA, Morris ZS, Sondel PM. NK cell-mediated antibody-dependent cellular cytotoxicity in cancer immunotherapy. *Front. Immunol.* 2015;6:368.
33. Ochoa MC, Minute L, Rodriguez I, et al. Antibody-dependent cell cytotoxicity: immunotherapy strategies enhancing effector NK cells. *Immunol. Cell Biol.* 2017;95(4):347-355.
34. Adams GP, Weiner LM. Monoclonal antibody therapy of cancer. *Nat. Biotechnol.* 2005;23(9):1147-1157.
35. Goydel RS, Rader C. Antibody-based cancer therapy. *Oncogene.* 2021;40(21):3655-3664.
36. Wolff AC, Hammond MEH, Allison KH, et al. Human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline focused update. *Arch. Pathol. Lab. Med.* 2018;142(11):1364-1382.
37. Tang Y, Lou J, Alpaugh RK, Robinson MK, Marks JD, Weiner LM. Regulation of antibody-dependent cellular cytotoxicity by IgG intrinsic and apparent affinity for target antigen. *The Journal of Immunology.* 2007;179(5):2815-2823.
38. Boero S, Morabito A, Banelli B, et al. Analysis of in vitro ADCC and clinical response to trastuzumab: possible relevance of FcgammaRIIIA/FcgammaRIIA gene polymorphisms and HER-2 expression levels on breast cancer cell lines. *J. Transl. Med.* 2015;13:324.
39. Chew HY, De Lima PO, Cruz JLG, et al. Endocytosis inhibition in humans to improve responses to ADCC-mediating antibodies. *Cell.* 2020;180(5):895-914. e827.
40. Pereira NA, Chan KF, Lin PC, Song Z. The "less-is-more" in therapeutic antibodies: Afucosylated anti-cancer antibodies with enhanced antibody-dependent cellular cytotoxicity. Paper presented at: MAbs2018.
41. Gong Y, Klein Wolterink RG, Gulaia V, et al. Defucosylation of Tumor-Specific Humanized Anti-MUC1 Monoclonal Antibody Enhances NK Cell-Mediated Anti-Tumor Cell Cytotoxicity. *Cancers (Basel).* 2021;13(11):2579.
42. Temming AR, de Taeye SW, de Graaf EL, et al. Functional attributes of antibodies, effector cells, and target cells affecting NK cell–mediated antibody-dependent cellular cytotoxicity. *The Journal of Immunology.* 2019;203(12):3126-3135.
43. Portillo AL, Hogg R, Poznanski SM, et al. Expanded human NK cells armed with CAR uncouple potent anti-tumor activity from off-tumor toxicity against solid tumors. *Iscience.* 2021:102619.

44. Wang Y, Zhang Y, Hughes T, et al. Fratricide of NK cells in daratumumab therapy for multiple myeloma overcome by ex vivo–expanded autologous NK Cells. *Clin. Cancer Res.* 2018;24(16):4006-4017.
45. Naeimi Kararoudi M, Nagai Y, Elmas E, et al. CD38 deletion of human primary NK cells eliminates daratumumab-induced fratricide and boosts their effector activity. *Blood.* 2020;136(21):2416-2427.
46. Phung SK, Miller JS, Felices M. Bi-specific and tri-specific NK cell engagers: the new avenue of targeted NK cell immunotherapy. *Mol. Diagn. Ther.* 2021;25(5):577-592.
47. Lamb MG, Rangarajan HG, Tullius BP, Lee DA. Natural killer cell therapy for hematologic malignancies: successes, challenges, and the future. *Stem Cell. Res. Ther.* 2021;12(1):1-19.
48. Melaiu O, Lucarini V, Cifaldi L, Fruci D. Influence of the tumor microenvironment on NK cell function in solid tumors. *Front. Immunol.* 2020;10:3038.
49. Felices M, Lenvik AJ, McElmurry R, et al. Continuous treatment with IL-15 exhausts human NK cells via a metabolic defect. *JCI insight.* 2018;3(3).
50. Gauthier L, Morel A, Anceriz N, et al. Multifunctional natural killer cell engagers targeting NKp46 trigger protective tumor immunity. *Cell.* 2019;177(7):1701-1713. e1716.
51. Caraccio C, Krishna S, Phillips DJ, Schürch CM. Bispecific antibodies for multiple myeloma: a review of targets, drugs, clinical trials, and future directions. *Front. Immunol.* 2020;11:501.
52. Hosseini SS, Khalili S, Baradaran B, et al. Bispecific monoclonal antibodies for targeted immunotherapy of solid tumors: Recent advances and clinical trials. *Int. J. Biol. Macromol.* 2021;167:1030-1047.
53. Gong Y, Klein Wolterink RG, Wang J, Bos GM, Germeraad WT. Chimeric antigen receptor natural killer (CAR-NK) cell design and engineering for cancer therapy. *J. Hematol. Oncol.* 2021;14(1):1-35.
54. Ruppel KE, Fricke S, Kohl U, Schmiedel D. Taking Lessons from CAR-T Cells and Going Beyond: Tailoring Design and Signaling for CAR-NK Cells in Cancer Therapy. *Front. Immunol.* 2022;13:822298.
55. Zhu Y, Huang B, Shi J. Fas ligand and lytic granule differentially control cytotoxic dynamics of natural killer cell against cancer target. *Oncotarget.* 2016;7(30):47163.
56. Medvedev AE, Johnsen A-C, Haux J, et al. Regulation of Fas and Fas-ligand expression in NK cells by cytokines and the involvement of Fas-ligand in NK/LAK cell-mediated cytotoxicity. *Cytokine.* 1997;9(6):394-404.
57. Prager I, Liesche C, Van Ooijen H, et al. NK cells switch from granzyme B to death receptor–mediated cytotoxicity during serial killing. *J. Exp. Med.* 2019;216(9):2113-2127.
58. Wu L, Adams M, Carter T, et al. Lenalidomide enhances natural killer cell and monocyte-mediated antibody-dependent cellular cytotoxicity of rituximab-treated CD20+ tumor cells. *Clin. Cancer Res.* 2008;14(14):4650-4657.
59. Cantoni C, Wurzer H, Thomas C, Vitale M. Escape of tumor cells from the NK cell cytotoxic activity. *J. Leukoc. Biol.* 2020;108(4):1339-1360.
60. Jiang L, Wang Y-J, Zhao J, et al. Direct tumor killing and immunotherapy through anti-SerpinB9 therapy. *Cell.* 2020;183(5):1219-1233. e1218.
61. Di Marzo L, Desantis V, Solimando AG, et al. Microenvironment drug resistance in multiple myeloma: emerging new players. *Oncotarget.* 2016;7(37):60698.
62. Holthof LC, van der Horst HJ, van Hal-van Veen SE, et al. Preclinical evidence for an effective therapeutic activity of FL118, a novel survivin inhibitor, in patients with relapsed/refractory multiple myeloma. *Haematologica.* 2020;105(2):e80.
63. Sanne J, Holthof L, Noort WA, et al. Sepantronium bromide (YM155) improves daratumumab-mediated cellular lysis of multiple myeloma cells by abrogation of bone marrow stromal cell-induced resistance. *Haematologica.* 2016;101(8):e339.
64. Mathew R, Karantza-Wadsworth V, White E. Role of autophagy in cancer. *Nature Reviews Cancer.* 2007;7(12):961-967.
65. Baginska J, Viry E, Berchem G, et al. Granzyme B degradation by autophagy decreases tumor cell susceptibility to natural killer-mediated lysis under hypoxia. *Proceedings of the National Academy of Sciences.* 2013;110(43):17450-17455.

66. Amaravadi RK, Kimmelman AC, Debnath J. Targeting autophagy in cancer: recent advances and future directions. *Cancer Discov.* 2019;9(9):1167-1181.
67. Orange JS. Formation and function of the lytic NK-cell immunological synapse. *Nature Reviews Immunology.* 2008;8(9):713-725.
68. Blumenthal D, Burkhardt JK. Multiple actin networks coordinate mechanotransduction at the immunological synapse. *J. Cell Biol.* 2020;219(2).
69. Al Absi A, Wurzer H, Guerin C, et al. Actin cytoskeleton remodeling drives breast cancer cell escape from natural killer–mediated cytotoxicity. *Cancer Res.* 2018;78(19):5631-5643.
70. Wurzer H, Filali L, Hoffmann C, et al. Intrinsic Resistance of Chronic Lymphocytic Leukemia Cells to NK Cell-Mediated Lysis Can Be Overcome In Vitro by Pharmacological Inhibition of Cdc42-Induced Actin Cytoskeleton Remodeling. *Front. Immunol.* 2021;12.
71. Foerster F, Braig S, Moser C, et al. Targeting the actin cytoskeleton: selective antitumor action via trapping PKC ε. *Cell Death Dis.* 2014;5(8):e1398-e1398.
72. Allard M, Oger R, Vignard V, et al. Serum soluble HLA-E in melanoma: a new potential immune-related marker in cancer. *PLoS One.* 2011;6(6):e21118.
73. Morandi F, Cangemi G, Barco S, et al. Plasma levels of soluble HLA-E and HLA-F at diagnosis may predict overall survival of neuroblastoma patients. *BioMed Research International.* 2013;2013.
74. Coupel Sp, Moreau A, Hamidou M, Horejsi V, Soulillou J-P, Charreau B. Expression and release of soluble HLA-E is an immunoregulatory feature of endothelial cell activation. *Blood.* 2007;109(7):2806-2814.
75. Voorter C, Gerritsen K, Groeneweg M, Wieten L, Tilanus M. The role of gene polymorphism in HLA class I splicing. *Int. J. Immunogenet.* 2016;43(2):65-78.
76. Carosella ED, Moreau P, LeMaoult J, Rouas-Freiss N. HLA-G: from biology to clinical benefits. *Trends Immunol.* 2008;29(3):125-132.
77. Paul P, Cabestre FA, Lefebvre S, et al. Identification of HLA-G7 as a new splice variant of the HLA-G mRNA and expression of soluble HLA-G5,-G6, and-G7 transcripts in human transfected cells. *Hum. Immunol.* 2000;61(11):1138-1149.
78. Zhang W-Q, Xu D-P, Liu D, et al. HLA-G1 and HLA-G5 isoforms have an additive effect on NK cytolysis. *Hum. Immunol.* 2014;75(2):182-189.
79. Bahri R, Hirsch F, Josse A, et al. Soluble HLA-G inhibits cell cycle progression in human alloreactive T lymphocytes. *The Journal of Immunology.* 2006;176(3):1331-1339.
80. Mouillot G, Marcou C, Zidi I, et al. Hypoxia modulates HLA-G gene expression in tumor cells. *Hum. Immunol.* 2007;68(4):277-285.
81. Waldhauer I, Goehlsdorf D, Gieseke F, et al. Tumor-associated MICA is shed by ADAM proteases. *Cancer Res.* 2008;68(15):6368-6376.
82. Boutet P, Agüera-González S, Atkinson S, et al. Cutting edge: the metalloproteinase ADAM17/TNF-α-converting enzyme regulates proteolytic shedding of the MHC class I-related chain B protein. *The Journal of Immunology.* 2009;182(1):49-53.
83. Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature.* 2002;419(6908):734-738.
84. de Andrade LF, Tay RE, Pan D, et al. Antibody-mediated inhibition of MICA and MICB shedding promotes NK cell–driven tumor immunity. *Science.* 2018;359(6383):1537-1542.
85. Bald T, Krummel MF, Smyth MJ, Barry KC. The NK cell–cancer cycle: advances and new challenges in NK cell–based immunotherapies. *Nat. Immunol.* 2020;21(8):835-847.
86. Ravi R, Noonan KA, Pham V, et al. Bifunctional immune checkpoint-targeted antibody-ligand traps that simultaneously disable TGFβ enhance the efficacy of cancer immunotherapy. *Nat. Commun.* 2018;9(1):1-14.
87. Otegbeye F, Ojo E, Moreton S, et al. Inhibiting TGF-beta signaling preserves the function of highly activated, in vitro expanded natural killer cells in AML and colon cancer models. *PLoS One.* 2018;13(1):e0191358.
88. Romero-Garcia S, Lopez-Gonzalez JS, B´ ez-Viveros JL, Aguilar-Cazares D, Prado-Garcia H. Tumor cell metabolism: an integral view. *Cancer Biol. Ther.* 2011;12(11):939-948.
89. Liu X, Hoft DF, Peng G. Tumor microenvironment metabolites directing T cell differentiation and function. *Trends Immunol.* 2021.

90. Chang C-H, Qiu J, O'Sullivan D, et al. Metabolic competition in the tumor microenvironment is a driver of cancer progression. *Cell.* 2015;162(6):1229-1241.
91. Scharping NE, Menk AV, Moreci RS, et al. The tumor microenvironment represses T cell mitochondrial biogenesis to drive intratumoral T cell metabolic insufficiency and dysfunction. *Immunity.* 2016;45(2):374-388.
92. Gardiner CM. NK cell metabolism. *J. Leukoc. Biol.* 2019;105(6):1235-1242.
93. Cong J, Wang X, Zheng X, et al. Dysfunction of natural killer cells by FBP1-induced inhibition of glycolysis during lung cancer progression. *Cell Metab.* 2018;28(2):243-255. e245.
94. Poznanski SM, Singh K, Ritchie TM, et al. Metabolic flexibility determines human NK cell functional fate in the tumor microenvironment. *Cell Metab.* 2021;33(6):1205-1220. e1205.
95. Hirayama A, Kami K, Sugimoto M, et al. Quantitative metabolome profiling of colon and stomach cancer microenvironment by capillary electrophoresis time-of-flight mass spectrometry. *Cancer Res.* 2009;69(11):4918-4925.
96. O'Brien KL, Finlay DK. Immunometabolism and natural killer cell responses. *Nature Reviews Immunology.* 2019;19(5):282-290.
97. Schafer JR, Salzillo TC, Chakravarti N, et al. Education-dependent activation of glycolysis promotes the cytolytic potency of licensed human natural killer cells. *J. Allergy Clin. Immunol.* 2019;143(1):346-358. e346.
98. Cursons J, Souza-Fonseca-Guimaraes F, Foroutan M, et al. A gene signature predicting natural killer cell infiltration and improved survival in melanoma patients. *Cancer immunology research.* 2019;7(7):1162-1174.
99. Li B, Jiang Y, Li G, Fisher Jr GA, Li R. Natural killer cell and stroma abundance are independently prognostic and predict gastric cancer chemotherapy benefit. *JCI insight.* 2020;5(9).
100. Muntasell A, Rojo F, Servitja S, et al. NK cell infiltrates and HLA class I expression in primary HER2+ breast cancer predict and uncouple pathological response and disease-free survival. *Clin. Cancer Res.* 2019;25(5):1535-1545.
101. Soo RA, Chen Z, Teng RSY, et al. Prognostic significance of immune cells in non-small cell lung cancer: meta-analysis. *Oncotarget.* 2018;9(37):24801.
102. Carrega P, Bonaccorsi I, Di Carlo E, et al. CD56brightperforinlow noncytotoxic human NK cells are abundant in both healthy and neoplastic solid tissues and recirculate to secondary lymphoid organs via afferent lymph. *The Journal of Immunology.* 2014;192(8):3805-3815.
103. Carrega P, Morandi B, Costa R, et al. Natural killer cells infiltrating human nonsmall-cell lung cancer are enriched in CD56brightCD16− cells and display an impaired capability to kill tumor cells. *Cancer.* 2008;112(4):863-875.
104. Castriconi R, Carrega P, Dondero A, et al. Molecular mechanisms directing migration and retention of natural killer cells in human tissues. *Front. Immunol.* 2018;9:2324.
105. Kremer V, Ligtenberg MA, Zendehdel R, et al. Genetic engineering of human NK cells to express CXCR2 improves migration to renal cell carcinoma. *Journal for immunotherapy of cancer.* 2017;5(1):1-13.
106. Wennerberg E, Kremer V, Childs R, Lundqvist A. CXCL10-induced migration of adoptively transferred human natural killer cells toward solid tumors causes regression of tumor growth in vivo. *Cancer Immunol. Immunother.* 2015;64(2):225-235.
107. Huijbers EJ, Khan KA, Kerbel RS, Griffioen AW. Tumors resurrect an embryonic vascular program to escape immunity. *Science Immunology.* 2022;7(67):eabm6388.
108. Pasero C, Gravis G, Guerin M, et al. Inherent and tumor-driven immune tolerance in the prostate microenvironment impairs natural killer cell antitumor activity. *Cancer Res.* 2016;76(8):2153-2165.
109. Haibe Y, Kreidieh M, El Hajj H, et al. Resistance mechanisms to anti-angiogenic therapies in cancer. *Front. Oncol.* 2020;10:221.
110. van Beijnum JR, Huijbers EJM, van Loon K, et al. Extracellular vimentin mimics VEGF and is a target for anti-angiogenic immunotherapy. *Nat. Commun.* 2022;13(1):2842.
111. Freud AG, Mundy-Bosse BL, Yu J, Caligiuri MA. The broad spectrum of human natural killer cell diversity. *Immunity.* 2017;47(5):820-833.
112. Peng H, Tian Z. Diversity of tissue-resident NK cells. Paper presented at: Semin. Immunol.2017.

113. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. *Nat. Immunol.* 2008;9(5):503-510.
114. Walzer T, Dalod M, Robbins SH, Zitvogel L, Vivier E. Natural-killer cells and dendritic cells:"l'union fait la force". *Blood.* 2005;106(7):2252-2258.
115. Böttcher JP, Bonavita E, Chakravarty P, et al. NK cells stimulate recruitment of cDC1 into the tumor microenvironment promoting cancer immune control. *Cell.* 2018;172(5):1022-1037. e1014.
116. Barry KC, Hsu J, Broz ML, et al. A natural killer–dendritic cell axis defines checkpoint therapy–responsive tumor microenvironments. *Nat. Med.* 2018;24(8):1178-1191.
117. Tang T, Huang X, Zhang G, Hong Z, Bai X, Liang T. Advantages of targeting the tumor immune microenvironment over blocking immune checkpoint in cancer immunotherapy. *Signal Transduction and Targeted Therapy.* 2021;6(1):1-13.
118. Gaggero S, Witt K, Carlsten M, Mitra S. Cytokines Orchestrating the Natural Killer-Myeloid Cell Crosstalk in the Tumor Microenvironment: Implications for Natural Killer Cell-Based Cancer Immunotherapy. *Front. Immunol.* 2020;11.
119. Burke JD, Young HA. IFN-γ: a cytokine at the right time, is in the right place. Paper presented at: Semin. Immunol.2019.
120. Méndez R, Rodríguez T, Del Campo A, et al. Characterization of HLA class I altered phenotypes in a panel of human melanoma cell lines. *Cancer Immunol. Immunother.* 2008;57(5):719-729.
121. Mimura K, Teh JL, Okayama H, et al. PD-L1 expression is mainly regulated by interferon gamma associated with JAK-STAT pathway in gastric cancer. *Cancer Sci.* 2018;109(1):43-53.
122. Boyerinas B, Jochems C, Fantini M, et al. Antibody-dependent cellular cytotoxicity activity of a novel anti–PD-L1 antibody avelumab (MSB0010718C) on human tumor cells. *Cancer immunology research.* 2015;3(10):1148-1157.
123. Oyer JL, Gitto SB, Altomare DA, Copik AJ. PD-L1 blockade enhances anti-tumor efficacy of NK cells. *Oncoimmunology.* 2018;7(11):e1509819.
124. Poznanski SM, Ritchie TM, Fan IY, et al. Expanded human NK cells from lung cancer patients sensitize patients' PDL1− negative tumors to PD1-blockade therapy. *Journal for immunotherapy of cancer.* 2021;9(1).
125. Basar R, Daher M, Rezvani K. Next-generation cell therapies: the emerging role of CAR-NK cells. *Blood Adv.* 2020;4(22):5868-5876.
126. Kabelitz D, Serrano R, Kouakanou L, Peters C, Kalyan S. Cancer immunotherapy with gammadelta T cells: many paths ahead of us. *Cell. Mol. Immunol.* 2020;17(9):925-939.
127. Sheffer M, Lowry E, Beelen N, et al. Genome-scale screens identify factors regulating tumor cell responses to natural killer cells. *Nat. Genet.* 2021;53(8):1196-1206.
128. Zaretsky JM, Garcia-Diaz A, Shin DS, et al. Mutations associated with acquired resistance to PD-1 blockade in melanoma. *N. Engl. J. Med.* 2016;375(9):819-829.
129. Garrido F, Ruiz-Cabello F, Aptsiauri N. Rejection versus escape: the tumor MHC dilemma. *Cancer Immunol. Immunother.* 2017;66(2):259-271.
130. Goding SR, Yu S, Bailey LM, Lotze MT, Basse PH. Adoptive transfer of natural killer cells promotes the anti-tumor efficacy of T cells. *Clin. Immunol.* 2017;177:76-86.
131. Suen WC-W, Lee WY-W, Leung K-T, Pan X-H, Li G. Natural killer cell-based cancer immunotherapy: a review on 10 years completed clinical trials. *Cancer Invest.* 2018;36(8):431-457.
132. Heipertz E, Zynda E, Stav-Noraas TE, et al. Current perspectives on "Off-The-Shelf" allogeneic NK and CAR-NK cell therapies. *Front. Immunol.* 2021:4858.
133. Ciurea SO, Schafer JR, Bassett R, et al. Phase 1 clinical trial using mbIL21 ex vivo–expanded donor-derived NK cells after haploidentical transplantation. *Blood, The Journal of the American Society of Hematology.* 2017;130(16):1857-1868.
134. Ciurea SO, Kongtim P, Soebbing D, et al. Decrease post-transplant relapse using donor-derived expanded NK-cells. *Leukemia.* 2022;36(1):155-164.
135. Papalexi E, Satija R. Single-cell RNA sequencing to explore immune cell heterogeneity. *Nature Reviews Immunology.* 2018;18(1):35-45.

136. Tsujikawa T, Kumar S, Borkar RN, et al. Quantitative multiplex immunohistochemistry reveals myeloid-inflamed tumor-immune complexity associated with poor prognosis. *Cell Rep.* 2017;19(1):203-217.
137. Rao A, Barkley D, França GS, Yanai I. Exploring tissue architecture using spatial transcriptomics. *Nature.* 2021;596(7871):211-220.
138. Christin JR, Shen MM. Modeling tumor plasticity in organoid models of human cancer. *Trends in Cancer.* 2022;8(3):161-163.

Appendices

Impact paragraph

Summary/Samenvatting/Zusammenfassung

Acknowledgements/Dankwoord/Danksagung

Curriculum Vitae & List of publications

Abbreviation list

Impact paragraph

Cancer is a leading cause of death worldwide. Although improvements have been made including earlier diagnosis and better treatment, cancer remains a high burden for society. One in four men and one in five women were diagnosed with cancer in 2018 and about 60% of cancer patients die from the disease [1]. Therefore, it is highly desirable to design therapies that will lead to higher cure rates of cancer. Immunotherapies are considered one of the major breakthroughs in the treatment of cancer and present promising approaches for cancers that are currently incurable [2]. Immunotherapies comprise various strategies that harness and strengthen the power of immune cells to better recognize and destroy tumor cells. But even within one patient, tumor cells can be highly heterogeneous cell populations and some tumor cells evade the destruction by immune cells and continue to grow. Tumor growth is further supported by the tissue surrounding the tumor, the TME. Therefore, one challenge of immunotherapies is to stimulate the immune cells sufficiently to detect and eliminate all tumor cells without off-target toxicity. NK cells are the immune cell type that we focused on in this thesis. In our research group, NK cell-based cancer immunotherapies have been studied for more than a decade and, continuing on our research groups' experiences, we assessed in this thesis how donor-derived NK cells can be further optimized to improve their anti-tumor responses against two cancer types, multiple myeloma and breast cancer.

Scientific impact

To enhance the anti-tumor efficacy of cytokine-activated NK cells, we described the following main principles in this thesis:

1. Selection of NK cell donors with a KIR-HLA ligand mismatch to prevent inhibitory signaling of NK cells through HLA class I
2. Combine KIR-HLA ligand mismatched NK cells with monoclonal antibodies that target tumor cells and that trigger ADCC in NK cells to potentiate NK cell activation
3. Increase NK cell activation and IFN-γ production through ADCC-triggering antibodies that are directed against tumor-supporting cells in the TME, such as macrophages

Importantly, the anti-tumor efficacy of highly activated NK cells was not diminished either by hypoxia or by low glucose levels, both of which are factors known to occur in the TME. As the TME is highly complex and contains multiple mechanisms that can reduce NK cell efficacy, it is desirable for future research to include more complex models that reflect the patient situation as closely as possible.

For solid tumors, we additionally need to acquire more knowledge about the spatial distribution of NK cells because increasing evidence supports that the complexity and diversity of the TME influences immunotherapy responses. As a first step towards NK cell distribution in breast cancer, we quantified and profiled NK cells in situ in breast cancer cohorts and found low infiltration of endogenous NK cells in

comparison to other immune cell subsets, suggesting that NK cell-based therapies need to include strategies that enhance NK cell infiltration into tumors.
During the years of my PhD studies, I have presented and discussed the scientific results on several national and internal conferences. Moreover, the scientific findings of this thesis are published in peer-reviewed and open-access journals and are thus available to the scientific community. The research presented in this thesis was supported by local donations through the Kankeronderzoeksfonds Limburg (Cancer Research Foundation Limburg), illustrating which positive impact local donations are having on advancing cancer research. And partly based on the findings in this thesis, a research grant of about 1.5 million Euros was recently obtained from Health~Holland and KWF, allowing new PhD students to further explore the development of effective NK cell treatment in breast cancer. For this goal, our institute will closely collaborate with the University of Utrecht to combine knowledge and work towards the goal of higher cure rates for breast cancer.

Target group
The scientific studies were performed with the goal to improve therapy approaches for cancer patients. While the results presented in this thesis were specifically tested for multiple myeloma and breast cancer, the findings could be relevant for a broader spectrum of cancer types and patients: NK cell-based therapies have potential for blood cancers other than multiple myeloma, in which their effectiveness has been demonstrated in preclinical and clinical studies, and recently, advances with NK cell-based approaches have also been made for solid tumors [3,4]. In this thesis, we for example demonstrated that the principle of combining KIR-HLA ligand mismatched donor NK cells with monoclonal antibodies could be extended from MM to breast cancer. Since it resulted in more potent NK cells in both MM and BC, the concept may be beneficial for patients with other tumor types that retain HLA class I expression.
However, we also observed differences in the level of NK cell responses, which might depend on the NK cell donor or the tumor cell or most likely a combination thereof. To select the optimal immunotherapy approach for each patient, better patient stratification will be required and can be supported by extensive profiling of both tumor and the TME. Good patient stratification will ensure that patients only receive treatments they respond to and consequently, money will not be wasted on ineffective treatments and, importantly, the quality of life for cancer patients will improve as they do not need to suffer from unnecessary treatments that often come with severe side effects.

Innovation
NK cells are most commonly derived from peripheral blood, either from the patient or from a healthy donor and the infusion of donor-derived NK cells has been demonstrated to be safe in several clinical trials. Moreover, donor NK cells might be advantageous as demonstrated by the enhanced NK cell activity of KIR-ligand mismatched NK cells in this thesis. Independent of the source, NK cells need to be

expanded to reach the high number used in clinical trials (up to $1x10^8$ NK cells/kg bodyweight). Producing the required numbers of NK cells for clinical trials needs to be performed in laboratories that fulfill the guidelines of good manufacturing practices. The fact that donor-derived NK cells are safe provides the opportunity for the generation of an "off-the-shelf" NK cell product, derived from large scale expansions of one donor. Such "off-the-shelf" NK cell products are considered much more cost effective than isolating NK cells from the patient itself and therefore represent a major advantage over T cell-based therapies, where donor-derived therapies are much more challenging due to GVHD mediated by donor T cells.

The results of this thesis might be relevant for guiding the design of clinical trials e.g., the selection of the best NK cell product for the patient, and can additionally be relevant for biotechnology companies that focus on the development, production, and medical application of NK cell products. The expansion process of NK cells provides a wide range of opportunities to improve the final NK cell product, for instance through cytokine stimulation, small interfering RNAs, or genetic modifications of NK cells. Based on the results of this thesis, it seems relevant to ensure sufficient IFN-γ production, sufficient expression of activating receptors including CD16 expression, and to interfere with inhibitory signaling. Moreover, the expansion process can provide possibilities to promote NK cell migration to solid tumors, which seems to be required based on the results of this thesis. In our research group, Prof. Dr. Gerard Bos and Dr. Wilfred Germeraad have founded CiMaas, a spin-off company from Maastricht University, with the goal to achieve better cure for cancer patients. One of the clinical product lines at CiMaas is the generation of donor-derived NK cells for cellular therapy. Protocols are available at CiMaas to produce NK cell products in accordance with the applicable Good Manufacturing Practices and to expand up to 10^{10} NK cells. To translate the novel therapy approaches into clinical practice, CiMaas closely collaborates with Maastricht University and the Maastricht University Medical Center to start clinical trials. Such collaborative networks of researchers and clinicians in university, hospital, and biotechnological companies are very valuable to support the translation of new therapeutic approaches into clinical practice.

Overall, with various approaches being in development and clinical trials showing promising results, immunotherapies are on the way to becoming more prominent therapeutic approaches in the future. Our research results provided small building blocks to create effective NK cell-based immunotherapies and we envision that our findings can eventually contribute to improved cancer immunotherapies.

References

1. TheCancerAtlas. Available online: https://canceratlas.cancer.org/the-burden/the-burden-of-cancer/ (accessed on February 6, 2022).
2. Couzin-Frankel, J. Breakthrough of the year 2013. Science 2013, 342, 1432-1433, doi:10.1126/science.342.6165.1432.
3. Lamb, M.G.; Rangarajan, H.G.; Tullius, B.P.; Lee, D.A. Natural killer cell therapy for hematologic malignancies: successes, challenges, and the future. Stem Cell. Res. Ther. 2021, 12, 1-19.
4. Melaiu, O.; Lucarini, V.; Cifaldi, L.; Fruci, D. Influence of the tumor microenvironment on NK cell function in solid tumors. Front. Immunol. 2020, 10, 3038.

Summary

Natural killer (NK) cells are part of the first line immune defense against malignant tumor cells and can readily kill damaged cells. NK cells can distinguish damaged cells from healthy cells through an extensive array of activating and inhibitory receptors and they react to target cells when the net balance of all integrated signals is shifted towards more activation than inhibition. This way, NK cell responses are regulated to allow killing of damaged cells without overactivity against healthy cells. An important group of inhibitory receptors are Human Leukocyte Antigen (HLA) class I molecules, whereas one of the most potent activating receptors is CD16A (FcγRIIIA). The latter can induce strong NK cell activation and subsequent tumor killing by binding to IgG antibodies and mediating antibody-dependent cellular cytotoxicity (ADCC).

As NK cells are potent tumor killers without major off-target toxicity against healthy cells, NK cell-based immunotherapies are considered a promising approach to improve cancer treatments. Clinical studies demonstrated that NK cell infusions, derived either from the patient or from a healthy donor, are well tolerated by the patients and can achieve promising anti-tumor results. Despite being equipped with powerful cytotoxic mechanisms, the NK cells' anti-tumor functions are, however, often inhibited by the tumor or the tumor microenvironments (TME). Immunosuppressive TME impose a challenge for many ongoing treatment approaches, including but not limited to immunotherapies, and the NK cell dysfunction inside tumor areas remains a challenge to be solved. In our research group, we focus on developing effective NK cell-based therapies by using NK cells derived from healthy donors and by utilizing two cancer types as models, namely the hematological malignancy multiple myeloma (MM) and breast cancer.

This thesis describes several strategies how to potentiate the NK cell responses against multiple myeloma and breast cancer cells with the aim to develop NK cells that retain their anti-tumor effector functions in an immunosuppressive TME. In **chapter 2**, we summarized the functional relevance of the inhibitory receptors KIR and NKG2A for NK cells in multiple myeloma, a tumor type that largely expresses the HLA class I molecules HLA-ABC and HLA-E, which are the corresponding ligands to the KIR and NKG2A receptors, respectively. Moreover, we described strategies that could interfere with inhibitory NK cell signals to improve the NK cell efficacy against multiple myeloma (**chapter 2**). In the following chapters, we tested the relevance of interfering with HLA-mediated NK cell inhibition in multiple myeloma and breast cancer to lower the NK cell activation threshold.

In most of the following experimental chapters, we isolated NK cells from peripheral blood of healthy donors and activated them with a high dose of the cytokine IL-2. After overnight activation with IL-2, NK cells were co-cultured with tumor cells to assess the NK cell anti-tumor functions, namely NK cell activation status

(degranulation by CD107a), cytokine secretion (IFN-γ), and tumor killing efficacy (cytotoxicity assays). Following up on previous research by our group about the role of KIR receptors on NK cells to target multiple myeloma cells, we investigated in **chapter 3** to which extent the NKG2A receptor influenced the anti-tumor responses of cytokine-activated NK cells against multiple myeloma. Next to NK cell inhibition, KIR and NKG2A receptors are also involved in NK cell licensing, a maturation process that results in more potent NK cells. Therefore, KIR and NKG2A play a dual role in NK cell responses; first by creating more potent NK cells through licensing, and second by inhibiting NK cell responses when the cognate ligand HLA class I is expressed. In **chapter 3**, we found that expression of NKG2A inhibited NK cell degranulation of $NKG2A^{+}$ NK cells only against HLA-E^{high} target cells, but it did not impair NK cell responses against target cells that expressed low levels of HLA-E. NKG2A expression even slightly enhanced NK cell activity against HLA-E^{low} target cells. The ADCC-mediating antibody Daratumumab enhanced NK cell degranulation of all NK cell subsets. These findings imply that NKG2A expression can have a beneficial effect on NK cells, presumably due to licensing, and that interfering with NKG2A, to create less inhibition, can be important for targeting HLA-E^{high} tumors, but might not be necessary when HLA-E expression is low or absent.

In **chapter 4**, patient-derived primary breast cancer cells were found to be relative resistant to NK cells, which seemed to be at least partially driven by HLA class I inhibition. Moreover, we presented that reducing HLA class I inhibition through selection of NK cell donors with a genetic mismatch between KIR on NK cells and HLA on tumor cells (KIR-HLA ligand mismatched NK cells) strongly enhanced NK cell degranulation. Additionally, all NK cells were more potent effector cells when combined with the antibody Trastuzumab. Trastuzumab is a clinically-approved anti-HER2 antibody that is known to mediate ADCC, which can provide very potent NK cell activation. Importantly, the combination of KIR-HLA ligand mismatched NK cell donors and ADCC-inducing antibody Trastuzumab remained effective in a hypoxic environment, which is frequently observed in breast cancer.

To achieve successful NK cell-based therapies, the complex and often immunosuppressive TME must be considered. Particularly for solid tumors, NK cell infiltration into tumor tissues is a crucial first step. In **chapter 5**, we assessed NK cell density, phenotype, and cellular distribution in two breast cancer cohorts by a multiplexed imaging technique to gain more in-depth knowledge about NK cells in situ. We reported that NK cells were the least abundant cell type of all evaluated leukocyte subsets and appeared not highly cytotoxic based on low granzyme B expression, supporting that NK cell infiltration must be improved (**chapter 5**).

Another factor to examine in the TME are low levels of nutrients including glucose, which can occur as a consequence of tumor growth. Since glucose is an important fuel for NK cells, we measured the glucose concentration in the bone marrow of multiple myeloma patients in **chapter 6**. Compared to the average glucose levels in

blood, glucose levels in the bone marrow of most patients were reduced. Our study further demonstrated that such reduced glucose concentrations (500 mg/L) did not impair the anti-tumor responses neither of cytokine-activated NK cells nor of expanded NK cells (**chapter 6**), suggesting that activated NK cells can withstand a low glucose TME.

Within the TME, tumor-associated cells such as immunosuppressive myeloid cells have been reported in literature to limit the anti-tumor potential of NK cells. To study the interaction between activated donor NK cells and tumor-associated cells, we generated two in vitro-polarized macrophage cell types, M1 and tumor-associated macrophages (TAM), and co-cultured these with NK cells (**chapter 7**). We found that NK cells responded to these macrophage target cells, measured by degranulation and IFN-γ secretion. With the aim to boost NK cells against the immunosuppressive TME, we tested whether an ADCC-triggering antibody could promote NK cell responses against the M1 and TAM macrophages and we found that NK cells degranulated stronger and produced more IFN-γ in combination with Avelumab directed against PD-L1^{high} macrophages. By secreting pro-inflammatory cytokines such as IFN-γ in response to tumor-associated cells, NK cells may serve as adjuvants by triggering anti-tumor responses in other immune cell types. This effect would be an important additional function next to killing tumor directly.

To escape immune responses, tumor cells can alter their ligand expression for example by shedding NK cell activating ligands or by altering HLA class I expression. In **chapter 8**, we described a role for genetic polymorphism in the occurrence of alternatively spliced RNA variants of HLA-C. If translated to a functional protein, the HLA-C variants might occur as soluble forms as they precisely lacked the transmembrane region, encoded by exon 5. Since all HLA-C molecules are recognized by NK cell receptors, it will be interesting to further determine whether the observed role of polymorphism results in altered HLA-C expression levels and thus influences NK cell responses, like it has previously been described for HLA-E and HLA-G.

In **chapter 9**, the findings of this thesis are discussed in the context of current challenges and advancements for NK cell-based cancer therapies. In summary, the strategies presented in this thesis provide small buildings blocks towards developing effective NK cell-based immunotherapies that retain their potency in an immunosuppressive TME.

Samenvatting

Natural killer cellen (NK-cellen) maken deel uit van het aangeboren immuunsysteem, dat de eerste verdedigingslinie vormt. NK-cellen circuleren in ons bloed en kunnen beschadigde cellen, bijvoorbeeld kwaadaardige kankercellen of virus geïnfecteerde cellen, binnen korte tijd doden. Het snelle doden van cellen is mogelijk omdat NK-cellen gewapend zijn met een "license to kill", dit betekend dat zij zijn geladen met toxische moleculen die worden uitgestoten zodra NK-cellen op beschadigde cellen stuiten. Om beschadigde van gezonde cellen te kunnen onderscheiden, beschikken NK-cellen over een uitgebreid assortiment van activerende en remmende receptoren (kleine antennes op het celoppervlak). Remmende signalen worden door alle gezonde cellen verzonden om NK-cellen af te remmen, terwijl activerende signalen vooral door beschadigde cellen worden verzonden. NK-cellen vallen cellen aan wanneer zij van de cel meer activerende dan remmende signalen ontvangen. Op deze manier wordt de reactie van de NK-cellen gereguleerd, zodat beschadigde cellen kunnen worden gedood zonder overactiviteit ten opzichte van gezonde cellen. Tot de remmende receptoren behoren de klasse I HLA-moleculen, terwijl CD16A (FcγRIIIA) een van de sterkste activerende receptoren is. CD16A-receptoren kunnen binden aan IgG-antilichamen, die op hun beurt tumorcellen binden. Deze binding van antilichamen aan NK-cellen kan een sterke activering van NK-cellen teweegbrengen en de celdood van de tumorcel initiëren. Dit mechanisme van cel doding wordt ADCC genoemd.

Op NK-cellen gebaseerde immunotherapieën worden beschouwd als een veelbelovende aanpak om kankertherapieën te verbeteren, omdat NK-cellen zijn uitgerust met krachtige mechanismen om specifiek kankercellen te doden zonder gezonde cellen te beschadigen. Klinische proeven hebben aangetoond dat infusies van NK-cellen goed door patiënten wordt verdragen en veelbelovende resultaten kunnen opleveren in de strijd tegen kanker. De voor de infusie gebruikte NK-cellen kunnen afkomstig zijn van de patiënt of worden gedoneerd door een gezonde persoon. Hoewel NK-cellen zijn uitgerust met krachtige verdedigingsmechanismen, zijn ze niet altijd succesvol in het bestrijden van kanker. Tumorcellen kunnen bijvoorbeeld ontsnappen aan de aanval van NK-cellen en de tumoromgeving (TME) kan NK-cellen ook afremmen. TME's die het immuunsysteem remmen, vormen een grote uitdaging voor veel van de huidige therapeutische benaderingen. Het algemene doel van op NK-cellen gebaseerde kankertherapieën is de remmende signalen van de tumor en de tumoromgeving te stoppen en de activiteit van de NK-cellen te verhogen, zodat de NK-cellen de kankercellen beter kunnen bestrijden. Om deze doelstellingen te bevorderen, ontwikkelen wij in onze onderzoeksgroep verschillende strategieën. Wij gebruiken NK-cellen afkomstig van gezonde donoren en richten ons op twee soorten kanker: multipel myeloom, een vorm van bloedkanker die het beenmerg aantast, en borstkanker.

In dit proefschrift beschrijven we verschillende strategieën om NK-cellen te stimuleren tegen multipel myeloom en borstkanker, met als doel therapieën te ontwikkelen waarbij NK-cellen functioneel blijven ondanks uitdagingen in de TME. In **hoofdstuk 2** hebben wij het belang van de remmende receptoren KIR en NKG2A voor NK-cellen in multipel myeloom samengevat. Multipel myeloom is een tumortype met veel HLA-klasse I moleculen, die remmende signalen afgeven aan de receptoren KIR en NKG2A. Bovendien hebben wij in **hoofdstuk 2** strategieën beschreven die de overdracht van remmende signalen naar NK-cellen kunnen onderbreken om de werkzaamheid van NK-cellen tegen multipel myeloom te verbeteren.
In de meeste van de volgende hoofdstukken hebben wij NK-cellen geïsoleerd uit het bloed van gezonde donoren en deze NK-cellen geactiveerd met de cytokine IL-2. Na activering met IL-2 werden de NK-cellen samengevoegd met tumorcellen om de anti-tumorfuncties van de NK-cellen in de verschillende testscenario's te onderzoeken. De volgende tests werden uitgevoerd: activeringsstatus van NK-cellen (degranulatie door CD107a), cytokinesecretie (IFN-γ) en tumordodende werkzaamheid (cytotoxiciteitstests). In aansluiting op eerder onderzoek van onze groep naar de rol van KIR-receptoren op NK-cellen, hebben wij in **hoofdstuk 3** onderzocht in hoeverre de remmende receptor NKG2A de anti-tumorfuncties van geactiveerde NK-cellen beïnvloedt. Naast NK-cel remming zijn ook KIR- en NKG2A-receptoren betrokken bij NK-cel licentiëring. Dit is een rijpingsproces dat NK-cellen moeten ondergaan om hun "license to kill" te verkrijgen. Daarom spelen de KIR- en NKG2A-receptoren een dubbele rol in NK-cellen: ten eerste door de vorming van sterkere NK-cellen via licenties, en ten tweede door de remming van NK-cel reacties wanneer klasse I HLA-moleculen aanwezig zijn en de passende remmende signalen naar de overeenkomstige KIR-receptoren sturen. NKG2A-receptoren ontvangen signalen van HLA-E. In **hoofdstuk 3** vonden wij dat de receptor NKG2A de activering van NKG2A-positieve NK-cellen alleen remde wanneer grote hoeveelheden HLA-E aanwezig waren op de doelcellen, maar niet wanneer kleine hoeveelheden HLA-E aanwezig waren. Expressie van NKG2A verhoogde de NK-cel activiteit tegen cellen met weinig HLA-E juist licht. Het ADCC-inducerende antilichaam Daratumumab verhoogde de activering van alle NK-cellen. Deze resultaten suggereren dat expressie van NKG2A een positief effect kan hebben op NK-cellen wanneer er weinig HLA-E op tumorcellen aanwezig is, vermoedelijk door licentiëring. Mogelijk leidt het bokkeren van NKG2A dus niet tot een sterkere NK-cel respons en is een blokkade niet nodig is wanneer HLA-E-expressie laag of afwezig is.

In **hoofdstuk 4** hebben wij geconstateerd dat primaire borstkankercellen van patiënten relatief resistent zijn tegen NK-cellen, hetgeen ten minste gedeeltelijk te wijten lijkt te zijn aan remmende signalering door HLA-klasse I moleculen. Bovendien konden wij aantonen dat de activering van NK-cellen sterk werd bevorderd wanneer NK-cellen werden geselecteerd uit specifieke donoren die door genetische verschillen tussen mensen niet in staat zijn de remmende signalen van de tumor-HLA-moleculen te ontvangen. Bovendien waren alle NK-cellen effectiever wanneer zij werden gecombineerd met het antilichaam Trastuzumab. Trastuzumab is een

klinisch goedgekeurd anti-HER2-antilichaam waarvan bekend is dat het ADCC induceert, hetgeen kan leiden tot zeer sterke NK-cel activering. Belangrijk is dat de combinatie van NK-cel donoren met genetische verschillen in hun KIR-HLA-repertoire en het ADCC-inducerende antilichaam Trastuzumab ook effectief bleef in een omgeving met weinig zuurstof, hetgeen vaak voorkomt bij borstkanker.

Om succesvolle op NK-cellen gebaseerde therapieën te ontwikkelen, moet rekening worden gehouden met de complexe TME, aangezien deze vaak een remmend effect heeft op immuun cellen. Vooral bij solide tumoren is de infiltratie van NK-cellen in het tumorweefsel een cruciale eerste stap. In **hoofdstuk 5** hebben wij de NK-cel dichtheid, het fenotype en de distributie van NK-cellen in twee groepen patiënten met borstkanker onderzocht en vastgesteld dat NK-cellen het minst aanwezig waren in de tumor in vergelijking met alle andere onderzochte immuun cellen en dat zij weinig toxische moleculen bevatten. Dit suggereert dat de infiltratie en functie van NK-cellen bij borstkankerpatiënten verder moet worden versterkt.

Een ander omgevingsfactor die veel voorkomt in tumoren zijn lage concentraties van voedingsstoffen, waaronder glucose, die kunnen optreden als gevolg van tumorgroei. Aangezien glucose een belangrijke brandstof voor NK-cellen is, hebben wij in **hoofdstuk 6** de glucoseconcentratie in het beenmerg van multipel myeloompatiënten gemeten. Vergeleken met het gemiddelde glucosegehalte in het bloed, was het glucosegehalte in het beenmerg van de meeste patiënten verlaagd. Onze studie toonde ook aan dat dergelijke verlaagde glucoseconcentraties (500 mg/L) geen invloed hadden op de antitumorrespons van geactiveerde NK-cellen of geëxpandeerde NK-cellen (**hoofdstuk 6**). Deze resultaten wijzen erop dat geactiveerde NK-cellen kunnen functioneren in een tumoromgeving met een laag glucosegehalte.

Het is bekend dat bepaalde immuun cellen in de TME het potentieel van NK-cellen beperken. Om de interactie tussen geactiveerde NK-cellen en tumorgeassocieerde immuun cellen te onderzoeken, hebben wij twee in vitro gepolariseerde macrofaagceltypen gegenereerd, namelijk ontstekingsmacrofagen (M1) en tumorgeassocieerde macrofagen (TAM), en deze samen met NK-cellen gekweekt (**hoofdstuk 7**). Wij vonden dat NK-cellen reageerden op deze macrofagen (gebaseerd op NK-cel activering en IFN-γ uitscheiding). Om NK-cellen te stimuleren tegen de immuun remmende TME, testten wij of een ADCC-activerend antilichaam de NK-cel respons tegen de M1- en TAM-macrofagen kon bevorderen, en wij vonden dat NK-cellen meer geactiveerd waren en meer IFN-γ produceerden wanneer zij gecombineerd werden met het antilichaam Avelumab. Avelumab richt zich tegen het molecuul PD-L1, dat in ons geval tot expressie komt op macrofagen. Door pro-inflammatoire cytokinen zoals IFN-γ af te scheiden in reactie op tumorgeassocieerde cellen, kunnen NK-cellen dienen als versterkers van immuunreacties, omdat zij via IFN-γ anti-tumorreacties in andere immuun cellen kunnen uitlokken. Dit effect zou

een belangrijke aanvullende functie van NK-cellen zijn naast het rechtstreeks doden van tumorcellen.

Om immuunreacties te omzeilen, kunnen tumorcellen hun signalering wijzigen, bijvoorbeeld door niet langer signalen uit te zenden die NK-cellen activeren of door de expressie van HLA-klasse I te wijzigen. In **hoofdstuk 8** hebben wij het HLA-molecuul HLA-C nader bestudeerd en beschreven dat genetische polymorfismen (varianten van genen) een rol spelen bij het ontstaan van alternatieve RNA-varianten van HLA-C. Bij vertaling in een functioneel eiwit zouden de HLA-C-varianten als oplosbare vormen kunnen voorkomen, omdat zij het transmembraangebied missen. Aangezien alle HLA-C-moleculen door NK-cel receptoren worden herkend, zal het interessant zijn verder te onderzoeken of de waargenomen rol van het polymorfisme leidt tot gewijzigde niveaus van HLA-C op het celoppervlak en zo de NK-cel reacties beïnvloedt, zoals eerder is beschreven voor HLA-E en HLA-G.

In **hoofdstuk 9** worden de resultaten van dit werk besproken in de context van de huidige uitdagingen en vorderingen bij de ontwikkeling van op NK-cellen gebaseerde kankertherapieën. Samengevat hebben wij in dit proefschrift verschillende strategieën gepresenteerd die kleine bouwstenen vormen voor de ontwikkeling van effectieve, op NK-cellen gebaseerde immunotherapieën die hun werkzaamheid behouden in een immuun-remmend TME.

Zusammenfassung

Natürliche Killerzellen (NK-Zellen) sind Teil der angeborenen Immunabwehr, welches die erste Verteidigungslinie bildet. NK-Zellen zirkulieren in unserem Blut und können geschädigte Zellen, z. B. Krebszellen oder virusinfizierte Zellen, innerhalb kürzester Zeit abtöten. Die schnelle Zelltötung ist möglich, da NK-Zellen gewappnet sind mit einer „Lizenz zum Töten", d.h. sie sind mit toxischen Molekülen beladen, welche ausgestoßen werden, sobald NK-Zellen auf geschädigte Zellen treffen. Um geschädigte von gesunden Zellen unterscheiden zu können, besitzen NK-Zellen ein umfangreiches Sortiment an aktivierenden und hemmenden Rezeptoren (kleine Antennen auf der Zell-Oberfläche). Hemmende Signale werden von allen gesunden Zellen gesendet, um die NK-Zellen zu bremsen, während aktivierende Signale vor allem von geschädigten Zellen gesendet werden. NK-Zellen greifen die Zielzellen an, wenn sie mehr aktivierende als hemmende Signale von der Zielzelle erhalten. Auf diese Weise wird die Reaktion der NK-Zellen so reguliert, dass geschädigte Zellen abgetötet werden können, ohne dass es zu einer Überaktivität gegenüber gesunden Zellen kommt. Zu den hemmenden Rezeptoren gehören u.a. die HLA-Moleküle der Klasse I, während CD16A (FcγRIIIA) einer der stärksten aktivierenden Rezeptoren ist. CD16A Rezeptoren können an IgG-Antikörper binden, die wiederum Tumorzellen binden. Durch diese Bindung von Antikörpern an NK-Zellen kann eine starke Aktivierung der NK-Zellen ausgelöst und der Zelltod der Tumorzelle initiiert werden. Dieser Mechanismus der Zelltötung wird ADCC genannt.

NK-Zell-basierte Immuntherapien gelten als vielversprechender Ansatz zur Verbesserung von Krebstherapien, weil NK-Zellen mit leistungsstarken Mechanismen ausgestattet sind um Tumorzellen gezielt zu töten ohne dabei gesunde Zellen zu beschädigen. Klinische Studien haben gezeigt, dass NK-Zell-Infusionen von Patienten gut vertragen werden und vielversprechende Ergebnisse in der Krebsbekämpfung erzielen können. Die für die Infusion genutzten NK-Zellen können entweder vom Patienten selbst stammen oder von einem gesunden Menschen gespendet werden. Obwohl die NK-Zellen mit leistungsstarken Mechanismen ausgestattet sind, sind sie nicht immer erfolgreich in der Krebsbekämpfung. So können z.B. Tumorzellen dem Angriff von NK-Zellen entkommen und auch die Tumormikroumgebung (TME) kann die NK-Zellen lahmlegen. TME, die das Immunsystem hemmen, stellen eine große Herausforderung für viele aktuelle Therapieansätze dar. Das allgemeine Ziel der NK-Zell-basierten Krebstherapien ist es, die hemmenden Signale des Tumors und der Tumorumgebung zu stoppen und die Aktivität der NK-Zellen zu erhöhen, sodass NK-Zellen die Krebszellen besser bekämpfen können. Um diese Ziele zu voranzubringen, entwickeln wir in unserer Forschungsgruppe verschiedene Strategien. Dabei verwenden wir NK-Zellen, die von gesunden Spendern stammen, und wir konzentrieren uns auf zwei Krebsarten: Multiples Myelom, eine Form von Blutkrebs, die das Knochenmark befällt, und Brustkrebs.

In dieser Arbeit werden verschiedene Strategien zur Verstärkung der NK-Zellen gegen die Krebsarten Multiples Myelom und Brustkrebs beschrieben, mit dem Ziel Therapieansätze zu entwickeln, bei denen die NK-Zellen trotz aller Herausforderungen in der Tumorumgebung funktionsfähig bleiben. In **Kapitel 2** haben wir die Bedeutung der hemmenden Rezeptoren KIR und NKG2A für NK-Zellen beim Multiplen Myelom zusammengefasst. Multiples Myelom ist ein Tumortyp, der in hohem Maße die Moleküle HLA Klasse I aufweist, welche hemmende Signale zu den Rezeptoren KIR und NKG2A senden. Darüber hinaus haben wir in **Kapitel 2** Strategien beschrieben, die die Weitergabe von hemmenden Signalen an NK-Zellen unterbrechen können, um die Wirksamkeit der NK-Zellen gegen das Multiple Myelom zu verbessern.
In den meisten der folgenden Kapitel haben wir NK-Zellen aus dem Blut gesunder Spender isoliert und diese NK-Zellen mit dem Botenstoff IL-2 aktiviert. Nach der Aktivierung mit IL-2 über Nacht wurden die NK-Zellen mit Tumorzellen zusammengebracht, um die Anti-Tumor-Funktionen der NK-Zellen in den verschiedenen Test-Szenarios zu untersuchen. Die folgenden Tests wurden durchgeführt: Aktivierungsstatus der NK-Zellen (Degranulation durch CD107a), die Zytokin-Sekretion (IFN-γ) und die Wirksamkeit der Tumorabtötung (Zytotoxizitäts-Tests). Anknüpfend an frühere Forschungsarbeiten unserer Gruppe über die Rolle von KIR-Rezeptoren auf NK-Zellen untersuchten wir in **Kapitel 3**, inwieweit der hemmende Rezeptor NKG2A die Anti-Tumor-Funktionen von aktivierten NK-Zellen beeinflusst. Neben der NK-Zell-Hemmung sind KIR- und NKG2A-Rezeptoren auch an der NK-Zell-Lizenzierung beteiligt. Dies ist ein Reifungsprozess, den NK-Zellen durchlaufen müssen, um ihre „Lizenz zum Töten“ zu erhalten. Daher spielen die Rezeptoren KIR und NKG2A eine doppelte Rolle bei NK-Zellen: erstens durch die Bildung stärkeren NK-Zellen durch Lizenzierung und zweitens durch die Hemmung von NK-Zellantworten, wenn HLA-Moleküle der Klasse I vorhanden sind und diese die entsprechenden hemmenden Signale an die passenden KIR-Rezeptoren senden. NKG2A-Rezeptoren empfangen Signale von HLA-E. In **Kapitel 3** fanden wir heraus, dass der Rezeptor NKG2A die NK-Zell-Aktivierung von NKG2A-positiven NK-Zellen nur hemmte, wenn große Mengen an HLA-E auf den Zielzellen vorhanden waren, aber nicht, wenn geringe Mengen an HLA-E vorhanden waren. Die Expression von NKG2A verstärkte die NK-Zellaktivität gegen Zellen mit wenig HLA-E sogar leicht. Der ADCC-vermittelnde Antikörper Daratumumab erhöhte die NK-Zell-Aktivierung aller NK-Zellen. Diese Ergebnisse deuten darauf hin, dass die Expression von NKG2A eine positive Wirkung auf NK-Zellen haben kann, wenn wenig HLA-E auf den Tumorzellen vorhanden ist, vermutlich aufgrund von Lizenzierung. Außerdem zeigen die Ergebnisse, dass eine Blockade von NKG2A möglicherweise nicht zu einer stärkeren NK-Zell Reaktion führen würde und deswegen nicht notwendig ist, wenn die HLA-E Expression gering oder nicht vorhanden ist.

In **Kapitel 4** haben wir festgestellt, dass primäre Brustkrebszellen, die von Patienten stammen, relativ resistent sind gegen NK-Zellen, was zumindest teilweise auf die hemmenden Signale der Moleküle HLA-Klasse I zurückzuführen zu sein scheint.

Darüber hinaus konnten wir zeigen, dass die NK-Zell Aktivierung stark verbessert wurde, wenn NK-Zellen von bestimmten Spendern ausgewählt wurden, die durch die genetischen Unterschiede zwischen Menschen die hemmenden Signale von den HLA-Molekülen des Tumors nicht empfangen können. Darüber hinaus waren alle NK-Zellen effektiver, wenn sie mit dem Antikörper Trastuzumab kombiniert wurden. Trastuzumab ist ein klinisch zugelassener Anti-HER2-Antikörper, von dem bekannt ist, dass er ADCC vermittelt, was zu einer sehr starken NK-Zellaktivierung führen kann. Wichtig ist, dass die Kombination von NK-Zellspendern mit genetischen Unterschieden in ihrem KIR-HLA Repertoire, und dem ADCC-induzierenden Antikörper Trastuzumab auch wirksam blieb in einer Umgebung mit niedrigem Sauerstoffgehalt, wie sie bei Brustkrebs häufig zu beobachten ist.

Um erfolgreiche NK-Zell-basierte Therapien zu entwickeln, muss die komplexe Tumormikroumgebung berücksichtigt werden, da diese häufig eine hemmende Wirkung auf Immunzellen hat. Insbesondere bei soliden Tumoren ist das Eindringen von NK-Zellen in das Tumorgewebe ein entscheidender erster Schritt. In **Kapitel 5** untersuchten wir die NK-Zelldichte, den Phänotyp und die Verteilung von NK-Zellen in zwei Patientengruppen mit Brustkrebs und wir fanden, dass NK-Zellen am wenigsten häufig vorkamen im Vergleich zu allen untersuchten Immunzell-Gruppen und dass sie mit wenig toxischen Molekülen beladen waren. Dies deutet darauf hin, dass das Eindringen und die Funktion von NK-Zellen in Brustkrebs-Patienten weiter verstärkt werden müssen.

Ein weiterer Faktor, der in Tumoren häufig vorkommt, ist eine niedrige Konzentration von verschiedenen Nährstoffen, z.B. Glukose, welche als Folge des Tumorwachstums auftreten können. Da Glukose ein wichtiger Brennstoff für NK-Zellen ist, haben wir in **Kapitel 6** die Glukosekonzentration im Knochenmark von Patienten mit Multiplem Myelom gemessen. Im Vergleich zu dem durchschnittlichen Glukosespiegel im Blut waren die Glukosespiegel im Knochenmark der meisten Patienten reduziert. Unsere Studie zeigte außerdem, dass solche reduzierten Glukosekonzentrationen (500 mg/L) weder die Anti-Tumor-Antwort von aktivierten NK-Zellen noch von expandierten NK-Zellen beeinträchtigten (**Kapitel 6**). Diese Ergebnisse deuten darauf hin, dass aktivierte NK-Zellen in einer Tumorumgebung mit niedriger Glukose funktionieren können.

Es ist bekannt, dass bestimmte Immunzellen innerhalb der Tumormikroumgebung das Anti-Tumor-Potenzial von NK-Zellen einschränken. Um die Interaktion zwischen aktivierten NK-Zellen und tumor-assoziierten Immunzellen zu untersuchen, erzeugten wir zwei in vitro-polarisierte Makrophagen-Zelltypen, nämlich M1 und tumorassoziierte Makrophagen (TAM), und kultivierten diese zusammen mit NK-Zellen (**Kapitel 7**). Wir fanden heraus, dass NK-Zellen auf diese Makrophagen-Zielzellen reagierten (gemessen durch NK-Zell-Aktivierung und IFN-γ-Sekretion). Mit dem Ziel, die NK-Zellen gegen das immun-hemmende TME zu stärken, testeten wir, ob ein ADCC-auslösender Antikörper die Reaktionen der NK-Zellen gegen die M1-

und TAM-Makrophagen fördern könnte, und wir stellten fest, dass die NK-Zellen in Kombination mit dem Antikörper Avelumab stärker aktiviert waren und mehr IFN-γ produzierten. Avelumab ist gegen das Molekül PD-L1 gerichtet, was in unserem Fall auf den Makrophagen exprimiert ist. Indem NK-Zellen als Reaktion auf tumor-assoziierte Zellen proinflammatorische Zytokine wie IFN-γ absondern, können NK-Zellen als Verstärker von Immunreaktionen dienen, weil sie durch IFN-γ anti-Tumor-Reaktionen in anderen Immunzellen auslösen können. Dieser Effekt wäre eine wichtige zusätzliche Funktion von NK-Zellen neben der direkten Abtötung von Tumorzellen.

Um Immunreaktionen zu entgehen, können Tumorzellen ihre Signalgebung verändern, indem sie beispielsweise Signale, die NK-Zellen aktivieren, nicht mehr versenden oder indem sie die HLA-Klasse-I-Expression verändern. In **Kapitel 8** haben wir das HLA-Molekül HLA-C genauer studiert und beschrieben, dass genetische Polymorphismen (Varianten von Genen) für das Vorkommen von alternativen RNA-Varianten von HLA-C eine Rolle spielen. Wenn sie in ein funktionelles Protein übersetzt werden, könnten die HLA-C-Varianten als lösliche Formen auftreten, weil diesen Varianten die Transmembranregion fehlt, welche von Exon 5 kodiert wird. Da alle HLA-C-Moleküle von NK-Zellrezeptoren erkannt werden, ist es interessant, weiter zu untersuchen, ob die beobachtete Rolle des Polymorphismus zu veränderten Niveaus von HLA-C auf der Zelloberfläche führt und somit die NK-Zellreaktionen beeinflusst, wie es zuvor bereits für HLA-E und HLA-G beschrieben wurde.

In **Kapitel 9** werden die Ergebnisse dieser Arbeit im Zusammenhang mit den aktuellen Herausforderungen und Fortschritten in der Entwicklung von NK-Zell-basierten Krebstherapien diskutiert. Zusammenfassend haben wir in dieser Arbeit verschiedene Strategien vorgestellt, die kleine Bausteine für die Entwicklung wirksamer NK-Zell-basierter Immuntherapien darstellen und die ihre Wirksamkeit in einer immun-hemmenden TME beibehalten.

Acknowledgements/Dankwoord/Danksagung

The work in this thesis would have not been possible without the support of many people including the great teams at Tumor Immunology and Transplantation Immunology, and everyone who has contributed to my projects and supported me along the way. I would like to thank you all!

My supervisors Dr. Lotte Wieten and Prof. Gerard Bos.
Dear **Lotte**, thank you for the opportunity to get my PhD and your help and support throughout. Your passion and enthusiasm for science have been an inspiration and you have always stimulated me to acquire new skills that allowed me to further grow in my position. During the last years, I have learned a lot from you and want to thank you for your excellent supervision and the chances you gave me. Thank you also for the opportunity to attend the many conferences, which have always been a lot of fun scientifically and socially.
Dear **Gerard**, thank you for your guidance during my PhD. You always provided a critical opinion and challenged me to get the best out of my projects. Even though your questions sometimes left me puzzled, I am grateful because they made me think out of the box but also brought the goal of the project back into the focus of my work. I also want to thank you for allowing me to put all the chapters in my thesis that I wanted; it took some time but I am happy with the result.

Dear members of the assessment committee, **Prof. Axel zur Hausen, Prof. Dirk De Ruysscher, Prof. Patrick Derksen, Dr. Eva König, Dr. Sander Rensen**, I much appreciate that you evaluated my PhD thesis and I would like to thank you all for your time and positive feedback.

My Paranymphs, Nicky and Timo.
Nicky, when you joined the lab for your internship, you were my first student and I am glad that you became my PhD colleague and friend afterwards. We shared quite a few experiments, and just as many fun moments outside the lab. I am happy we got to attend the EFI and NK conferences in Lisbon and Luxemburg together, which even got topped by our adventures in Portland! Never did we think that we would both get the one TEFAF fellowship, but so we did. We have experienced so many things together, and I would not want to miss any of those. Thank you for the great times and good luck with the final stretch of your PhD!
Timo, together we first obtained the Master degree and as colleagues we have managed a number of successful projects, including huge experiments with too many plates for the glucose paper, and of course the HLA-C paper, which has finally been published this year (and celebrated with a beer in Marseille, so cool that we managed this!). Thank you for your positive attitude and the fun moments we had in the lab and outside. You always knew how to organize more wine or how to start a party ;) It has been a pleasure to have you as a colleague and of course as my paranymph. Now get your PhD too, you got this!

Dank aan mijn collega's van Transplantatie Immunologie. **Ilse, Jacqueline, Filiz, Lize, Stefan, Dorien, Simone, Esther, Maud, Fausto, Carmen, Tom, Bert, Christel, Robert, Jeroen, Levi, Coline, Lisette, Marjolein, Veerle, Annette, Sandra**, ook kwam ik in het begin niet zo vaak op jullie lab, heb ik veel van jullie gevraagd de afgelopen jaren: Regelmatig had ik bloed nodig voor mijn experiment, vroeg ik naar HLA-typeringen met zo minst mogelijk cellen of een extra snelle typering zodat ik verder kon met mijn experiment - jullie hebben het allemaal mogelijk gemaakt. Heel erg bedankt daarvoor en zeker ook voor de gezelligheid op het lab zoals tijdens lab uitjes en kerstfeesten!

Christien en **Mathijs**, ik wil jullie bedanken voor de wetenschappelijke input, vooral voor het HLA-C paper, jullie interesse in mijn onderzoek, en de leuke sfeer.

Denise, als geen ander weet je een glimlach op iedereens gezicht te toveren. Een vrolijk hallo op de gang, een (mislukte) rijm indien je wat K562 cellen nodig had, of een spontane NK cell dance – you always know how to brighten the situation. Thank you for the cheerful atmosphere and for your support, especially during the last years of my/our PhD. Merci aussi pour ta visite à Marseille, ma chérie. I much enjoyed having you there.

Burcu, my non-Dutch but Dutch-speaking colleague, I well remember how we talked in the first year about conversations held in Dutch and how we are too slow to participate because by the time we formulated in our head what we want to say, the topic has changed already, haha. Respect how quickly and well you adapted! I have much enjoyed the positive atmosphere that you bring with you. Congresses or nights out were much more fun with you, you were always so motivated to dance that you even managed to get the rest of us on the dancefloor!

Ben, thank you for bringing the kind American sphere and the Minnesota mindset to the lab - I won't forget how the teacher of my writing class reminded me so much of you, just by the way he spoke and acted. Thank you for your kindness and fun conversations we had.

Also, big thanks to the **EFI crew**, these conferences have created some unforgettable moments with Venice as the cherry on the top ;)

Lieve dames van het secretariaat, **Brigitte, Audrey, en Diana**. Jullie stonden altijd klaar om iets voor mij te regelen, was het een afspraak met Lotte (geen eenvoudige taak), mijn declaraties, en allerlei letters die verstuurd moesten worden tijdens de laatste fase van mijn PhD. Dank jullie wel voor alle hulp en jullie interesse hoe het met mij en mijn proefschrift ging.

Prof. Marcel Tilanus, beste Marcel, dank u wel voor uw inzet tijdens het begin van mijn PhD. Ik heb veel van u geleerd over de belangrijke rol van HLA en deze opgedane kennis heeft mij veel geholpen tijdens mijn PhD traject.

Thank you to the past and present members of the Hemato group.
Birgit, dankjewel voor jouw steun aan mij en iedereen in het lab. Jij was er altijd om mij te helpen en je regelde het, als ik iets nodig had. Ook wil ik jou bedanken voor de leuke gesprekken en de gezelligheid tijdens koffiepauzen, lunches of diners. Ik heb ervan genoten om met jou samen te werken.
Wilfred, thanks to you I learned about this PhD position and I am very happy I joined the lab. Thank you for your guidance during my master thesis as well as during my PhD when a little chat with you would bring me a new idea and help me to continue.
Niken, you were the one to show me all the experiments from culturing your 'babies' to all sort of tips and tricks how to best combine Cytotox and CD107a assays in parallel in our lab and in the hypoxic chamber. Thank you for your input and your explanations, as well as for the fun conversations and most importantly for your friendship. I have enjoyed our late lunches or spontaneous dinners in the MUMC and especially our payday dinners, through which we discovered quite a few delicious dinner places in Maastricht!
Ying, you are such a hard-working person and a real gentleman, as everyone in the lab agrees. Thank you for helping me with my projects wherever you could. I still remember how you send me a message at 1 am on a Sunday morning that we finally have the correct vector to create the Luciferase-positive cell lines. Thanks for all your input and I wish you lots of success and happiness in your future career!
Lynn, deine lustige Art und das Multikulti der Sprachen (Gibt's das Wort? Egal, wir verstehen uns!) haben mir so manchen Arbeitstag versüßt. Vielen Dank für deine Hilfe mit meinen Projekten!
Sabine, Erik, Melis, we hebben het kantoor slechts korte tijd gedeeld, maar het was altijd gezellig met jullie. Dank jullie wel voor de leuke sfeer in de groep.
Antonia, nur kurz waren wir Kollegen und dann auch noch in der Covid-Zeit. Umso mehr habe ich es genossen auch außerhalb der Arbeit Zeit mit dir zu verbringen in der Kletterhalle oder in der Kaas-Bar. Viel Erfolg mit deinem neuen Kapitel!
Roel, it has been tremendously valuable for me to have you in the group during my first year of PhD. Thank you for your explanations, interesting discussions, and advice.
Silvie & **Marijn**, thank you for the warm welcome to the team when I just started my PhD. Every day you reminded everyone when it is time to take a little break for coffee or lunch. I have much enjoyed the time we spend together in the lab and in the office.
Janine, your dedication to research and the tasks you manage in one day are an inspiration for me. Thank you for your input and good luck with your career!
Michel, jij bent diegene die vanaf het begin het meest consequent Nederlands met mij heeft gesproken. Ook was mijn hoofd in de eersten maanden vaak een beetje zwaarder naar een wetenschappelijke discussie met jou, zo heeft het zeker geholpen om mijn vocabulaire te verbeteren. Dankjewel voor de fijne samenwerking aan het borstkanker project en ook voor de koffiepauzes in de jaren daarna.

Loes Kooreman, dank je wel voor de fijne samenwerking. Het was niet makkelijk om de NK – borstkanker studie op te zetten maar met jouw enthousiasme en inzet is het gelukt om alle (logistische) uitdagingen te overkomen.

Ik wil ook onze buren van **interne geneeskunde** en **MDL** bedanken voor jullie hulp in het lab en voor de gezelligheid in de keuken of op de gang. **Maria** & **Jose**, bij jullie kon ik altijd terecht om iets te vragen of te lenen, dank jullie wel.
Kristiaan, you were just next door, which facilitated the collaboration on your paper, the organization of immunology seminars as well as setting up a FACS course. Thank you for the opportunity to participate in all of these and for the great time. I wish you a lot of success with your future career!
Heike, vielen Dank für die gemeinsame Zeit und Unterhaltungen im Zellkultur Labor, wo wir so einige Stunden verbracht haben, weil mal eben Zellen splitten eben doch Zeit braucht.

My students **Tom**, **Annet**, **Amber**, I had a great time supervising each of you and learned a lot from and with you. Thank you for your contributions to my projects and the best of luck with finishing your PhDs!

During the last year of my PhD, I got to enjoy a special adventure in the lab of Lisa Coussens. Thank you to **GROW** for awarding me the TEFAF fellowship.
Dear **Lisa**, thank you for the opportunity to join your lab and for mentoring me during this time and even after I left your lab. Your guidance has been invaluable for me to expand my knowledge and to develop as a research scientist.
Thank you to all members of the **Coussens' lab**, especially **Courtney**, **Sam**, **Sushil**, **Amanda**, **Eivind**, **Meghan**, for the warm welcome to the lab, the scientific discussions, potluck dinners and other fun activities. And **Katie**, thank you for the fruitful collaboration. I have much enjoyed my time at OHSU thanks to all of you!

Thank you to my friends outside of work.
Myrthe and **Luise**, thank you for the fun times during basketball training, other workout sessions or coffee breaks, which have always been a great change from work.
Lea, von Studienkollegen zu Urlaubs-Partnern und Mitbewohnern haben wir so einiges zusammen erlebt. Danke für die gemeinsame Zeit!
Nina, du hast es geschafft mich selbst bei schlechtem Wetter aus dem Haus zu kriegen für unsere Joggingrunde, das hat jedes Mal wieder gutgetan. Danke dafür, sowie für deine so positive Art!
Hansje, dankjewel voor de gezelligheid in ons appartement. Ich freu mich schon sehr darauf, dass wir bald wieder dichter beieinander wohnen!
Katlijn, you have been my kind and helpful neighbor for all the years through my PhD. Thank you for always helping out and for the great Aperos, dinners, and BBQs we had in the house.

Elke, dank dir bin ich in Maastricht gelandet und ich geb dir recht, es ist eine äußerst schöne Stadt um zu studieren. Danke für deine Gastfreiheit und Hilfe bei meiner ersten Zimmersuche, sowie auch beim ersten Auszug - da wurde eure Familienfeier in Dithmarschen mal eben damit kombiniert, meine Möbel zurück zu meinen Eltern zu fahren, bei dir bzw. euch ist alles möglich ;)
Clara, seit unserem Praktikum in 2013 haben wir quasi immer in einer ähnlichen Lebensphase gesteckt und ich konnte mir bei dir immer einen Rat abholen. Danke für die Zeit als infernal trio zusammen mit Thea, sowie alle Treffen danach. Komm mal wieder zu Besuch!
Frida, du warst Teil der fantastischen Vårberg crew und ich freu mich, dass so viele andere Erlebnisse gefolgt sind. Es wird Zeit, dass du mir die echten Berge zeigst!
Liebe **Freunde von zu Hause**, danke für die jahrelange Freundschaft!

Thank you to my **Veracyte colleagues** for the great time in Marseille!

Lieve **familie Bijnen**, dank jullie wel dat ik altijd welkom ben bij jullie thuis.

Liebe Familie, jetzt bin ich dann auch mal fertig mit der Uni ;)
Liebe Mama, lieber Papa, ihr habt so viel beigetragen zu dieser Arbeit, indem ihr mich immer ermutigt meine Ideen zu verwirklichen und meinen eigenen Weg zu gehen. Danke vielmals für eure unermüdliche Unterstützung und für die offenen Arme, wenn ich nach Hause komme.
Liebe Oma, lieber Opa, unzählige Male habt ihr nachgefragt und nun ist es endlich soweit: Die Doktorarbeit ist geschafft! Danke für euer Interesse und eure Herzlichkeit.
Liebe Geschwister, lieber Stefan, auf euch kann ich immer zählen. **Marti**, von dir und deiner lockeren Art kann ich immer wieder lernen, dass man Dinge auch entspannter angehen kann und es trotzdem funktionieren wird. **Madita**, **Cello**, **Tobi**, **Stefan**, dank euch war und ist immer Leben in der Bude :)

Mitchell, thank you is not enough in return for your relentless support. You know how to encourage me to push through the last steps, and when I have a question about no matter what, you are always there to help me in the best way possible. I am lucky to share my life with you and *lucky we're in love in every way*. Ik hou van jou en dat zal altijd zo blijven.

THANK YOU ALL!

Curriculum Vitae

Femke Ehlers was born on 8 April 1991 in Bremen, Germany. In 2011, she enrolled at the Christian-Albrechts-Universität zu Kiel to study Biology and obtained the bachelor's degree in 2014. To continue her education in the biomedical field, Femke studied the MSc Biomedical Science at Maastricht University from 2014 to 2016. During the master studies, Femke performed the junior internship at the Central Diagnostic Laboratory, Maastricht University Medical Center (MUMC+), and the senior internship at the Department of Microbiology, Tumor and Cell Biology at the Karolinska Institute in Stockholm, Sweden.
In September 2016, Femke started her PhD trajectory under supervision of Dr. Lotte Wieten and Prof. Gerard Bos in the department of Transplantation Immunology and the department of Internal Medicine, Hematology Division, at MUMC+ and Maastricht University. During the fourth year of her PhD studies, she was awarded with the TEFAF fellowship from GROW – School for Oncology and Reproduction, which allowed her the unique opportunity to pursue research in the laboratory of Prof. Lisa Coussens at the Oregon Health & Science University, located in Portland, USA. Since February 2022, Femke works as a scientist in the immuno-oncology field at Veracyte in Marseille, France.

List of publications

Ehlers FAI, Mahaweni NM, van de Waterweg-Berends A, Bos GMJ, Wieten L. Targeting PDL1 positive macrophages with the anti-PDL1 monoclonal antibody Avelumab to enhance the anti-multiple myeloma potential of NK cells.
In press at Cancer Immunology Immunotherapy

Beelen NA, **Ehlers FAI**, Bos GM, Wieten L. Inhibitory receptors for HLA class I as immune checkpoints for natural killer cell-mediated antibody-dependent cellular cytotoxicity in cancer immunotherapy. Cancer Immunology, Immunotherapy. 2022 Oct 19:1-8.

Ehlers FAI, Olieslagers TI, Groeneweg M, Bos GMJ, Tilanus MGJ, Voorter CEM, Wieten L. Polymorphic differences within HLA-C alleles contribute to alternatively spliced transcripts lacking exon 5. Hla. 2022 Sep;100(3):232-43.

Ehlers FAI, Beelen NA, van Gelder M, Evers TM, Smidt ML, Kooreman LFS, Bos GMJ, Wieten L. ADCC-Inducing Antibody Trastuzumab and Selection of KIR-HLA Ligand Mismatched Donors Enhance the NK Cell Anti-Breast Cancer Response. Cancers. 2021 Jun 28;13(13):3232.

Ehlers FAI, Mahaweni NM, Olieslagers TI, Bos GMJ, Wieten L. Activated natural killer cells withstand the relatively low glucose concentrations found in the bone marrow of multiple myeloma patients. Frontiers in oncology. 2021 May 20;11:622896.

Kumar S, Poissonnier A, Betts CB, Murugan D, Valen Egeland E, **Ehlers F**, Woods J, Beelen N, Coussens LM. Tumor-infiltrating myeloid cells in cancer progression and therapy response. In: Cancer Immunotherapy Principles and Practice (Lisa H. Butterfield, Howard L. Kaufman, Francesco M. Marincola, Eds), Springer Publishing Company. Second Edition; 2021. Chapter 34, pp 444-461.

Gong Y, Klein Wolterink RGJ, Gulaia V, Cloosen S, **Ehlers FAI**, Wieten L, Graus YF, Bos GMJ, Germeraad WTV. Defucosylation of tumor-specific humanized anti-MUC1 monoclonal antibody enhances NK cell-mediated anti-tumor cell cytotoxicity. Cancers. 2021 May 25;13(11):2579

Wouters K, Kusters YH, Bijnen M, Wetzels S, Zhang X, Linssen PB, Gaens K, Houben AJ, Joris PJ, Plat J, Kooi ME, van der Kallen CJH, Mensink RP, Verboven K, Jocken J, Hansen D, Blaak EE, **Ehlers FAI**, Wieten L, Greve JW, Rensen S, Stehouwer CDA, Schalkwijk CG. NK cells in human visceral adipose tissue contribute to obesity-associated insulin resistance through low-grade inflammation. Clinical and translational medicine. 2020 Oct;10(6).

Mahaweni NM, **Ehlers FAI**, Sarkar S, Janssen JW, Tilanus MGJ, Bos GMJ, Wieten L. NKG2A expression is not per se detrimental for the anti-multiple myeloma activity of activated natural killer cells in an in vitro system mimicking the tumor microenvironment. Frontiers in immunology. 2018 Jun 22;9:1415.

Mahaweni NM, **Ehlers FAI**, Bos GMJ, Wieten L. Tuning natural killer cell anti-multiple myeloma reactivity by targeting inhibitory signaling via KIR and NKG2A. Frontiers in immunology. 2018 Dec 4;9:2848.

Manuscript in preparation:

Ehlers FAI, Blise K, Betts CBB, Sivagnanam S, Kooreman LF, Hwang ES, Bos GMJ, Wieten L, Coussens LM. Profiling NK cells within the tumor microenvironment of HER2- and HER2+ human breast cancer.

Abbreviation list

ADCC	Antibody-dependent cellular cytotoxicity
AML	Acute myeloid leukemia
BM	Bone marrow
CAIX	Carbonic anhydrase IX
CAR	Chimeric antigen receptors
ESE	Exonic splicing enhancers
ESS	Exonic splicing silencers
FasL	Fas ligand
FCS	Fetal calf serum
FcγRIIIA	Fc gamma receptor IIIA
FISH	Fluorescent in situ hybridization
GM-CSF	Granulocyte-macrophage colony stimulating factor
GVHD	Graft-versus-host disease
GVT	Graft-versus-tumor
Haplo SCT	Haploidentical stem cell transplantation
HER2	Human epithelial growth factor receptor
HLA	Human Leukocyte Antigen
HSCT	Hematopoietic stem cell transplantations
IC	Immune checkpoint
IFN-γ	Interferon-γ
IgG	Immunoglobulin G
IL	Interleukin
ILC	Innate lymphoid cells
ISE	Intronic splicing enhancers
ISRE	Intronic splicing regulatory elements
ISS	Intronic splicing silencers
KIR	Killer Immunoglobulin-like receptors
mAb	monoclonal antibodies
M-CSF	Macrophage colony-stimulating factor
MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major histocompatibility complex
mIHC	multiplex immunohistochemistry
MM	Multiple myeloma
mTOR	mechanistic target of rapamycin
NI	Neighborhood inference
NK cells	Natural killer cells

OxPhos	Oxidative phosphorylation
PanCK	PanCytokeratin
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed Death-1
PD-L1	Programmed Death-Ligand 1
PGE2	Prostaglandin E2
rhIFN-γ	recombinant human IFN-γ
SNP	Single nucleoid polymorphism
snRNPs	Small nuclear ribonucleoproteins
SRE	splicing regulatory elements
TAC	Tumor-associated cells
TAM	Tumor-associated macrophages
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th1	T helper cells type 1
TIGIT	T cell immunoglobulin and ITIM domain
TIM-3	T cell immunoglobulin and mucin domain containing molecule 3
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor-α
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cells
VEGF	Vascular endothelial growth factor