New strategies to improve Natural Killer cells for “off-the-shelf” allogeneic immunotherapy

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Preface

The herein presented thesis consists of two original publications

The work presented in this thesis was carried out between April 2019 and March 2022 and was supervised by Dr. rer. nat. Stefan Heinrichs and Prof. Dr. med. Peter Horn at the Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen.

Ὅσον ζῇς φαίνου

As long as you live, shine!

First line of the “Seikilos Epitaph”
1st or 2nd century CE

Zusammenfassung

Natürliche Killerzellen (NK-Zellen) sind ein wesentlicher Teil der angeborenen Tumor-Immunüberwachung im menschlichen Körper. Im Gegensatz zu T-Zellen verursachen NK-Zellen keine Graft-versus-Host-Erkrankung, wenn sie in allogene, nicht HLA-identische Empfänger übertragen werden. Daher scheinen NK-Zellen optimal als universelle Spenderzellen für eine Immuntherapie "off-the-shelf" geeignet. Jedoch scheiterte die Infusion allogener NK-Zellen in klinischen Studien weitgehend an der Abstoßung der Zellen durch das Wirtsimmunsystem. Um dieses Problem zu umgehen, wurden HLA-Klasse-I-Moleküle in primären NK-Zellen ausgeschaltet, um sie vor allogenen T-Zellen zu schützen. Das Abschalten von HLA-Klasse-I-Molekülen führte jedoch zu Fratrizid in den NK-Zellkulturen. Überexpression eines einkettiges HLA-E-Moleküls konnte die Zellen schützen. Die so doppelt-modifizierten NK-Zellen waren immun gegen allogene T-Zellen, unterlagen nicht dem Fratrizid und behielten dennoch ihr zytotoxisches Potenzial gegen maligne Zellen.

Die NK-Zell-Immuntherapie könnte noch effizienter sein, würden CAR-modifizierte NK-Zellen eingesetzt. Dazu wurden CD33-CAR NK-Zellen gegen AML generiert. CD33-CAR NK-Zellen aber zeigten eine geringere Expansionskapazität als Kontroll-CAR-NK-Zellen. Phänotypisierungen ergaben eine starke Hochregulation von CD33 auf bis zu 50% der kultivierten NK-Zellen, was in Kultur mit CD33-CAR-NK-Zellen zu Fratrizid führte, der die Expansion der Zellen behinderte. Nicht alle NK-Zellspender wiesen jedoch eine hohe CD33-Expression auf. Genetische Studien ergaben, dass der Einzelnukleotid-Polymorphismus (engl. SNP) rs12459419 (C>T) in Exon 2 von CD33 prädiktiv für niedrige CD33-Expressionswerte auf kultivierten NK-Zellen war. NK-Zellkulturen von homozygoten Trägern des T-Allels am SNP rs12459419 wiesen niedrige Frequenzen von $CD33^+$ NK-Zellen auf und waren daher gut für die Produktion von CD33-CAR NK-Zellen geeignet. Außerdem zeigten $CD33^+$ NK-Zellen eine stärkere Zytotoxizität und eine höhere Zytokinproduktion, während $CD33^-$ NK-Zellen zu stärkerer antikörperabhängiger zellulärer Zytotoxizität fähig waren.

Wir haben erfolgreich eine Genom-Editierungsplattform für primäre menschliche NK-Zellen aufgebaut und HLA-defiziente NK-Zellen für eine allogene Therapie "off-the-shelf“ erzeugt. Außerdem haben wir eine neuartige funktionelle Dichotomie von kultivierten NK-Zellen definiert, die für klinische Zwecke genutzt werden kann. Diese Ergebnisse können dazu beitragen, künftige adoptive NK-Zell-Therapieansätze für bösartige Erkrankungen mitzugestalten.

Summary

Natural Killer (NK) cells are a main contributor to innate tumor-immune-surveillance in the human body. In contrast to T cells, NK cells do not cause Graft-versus-Host-Disease (GvHD) when transferred into allogeneic non-HLA-matched recipients. Therefore, NK cells appear optimally suited as universal donor cells for “off-the-shelf” immunotherapy. Yet, infusion of allogeneic NK cells largely failed in clinical trials, as the cells were rejection by the host’s immune system. To mitigate this shortcoming, HLA class I molecules in primary NK cells were knocked out to facilitate their evasion from alloreactive T cells. This knockout however led to fratricides of cultured NK cells. We therefore overexpressed a single-chain HLA-E molecule and to rescue the cells. Importantly, this double modified NK cells were shown to be immune to allogeneic T cells, were not subject to fratricide, yet still retained their cytotoxic potential against malignant cells.

NK cell immunotherapy could be even more efficient if CAR-modified NK cells were used. To this end, CD33-CAR NK cells were generated for AML. Curiously, CD33-CAR NK cell cultures showed reduced expansion capacity compared to control CAR NK cells. Phenotypical analysis revealed a strong upregulation of CD33 on up to 50% of the cultured NK cells, which then led to fratricide upon exposure to CD33-CAR NK cells, which in turn hampered expansion of the cells. Notably, not all NK cell donors showed high CD33 expression. Genetic studies revealed that the single-nucleotide polymorphism (SNP) rs12459419 (C>T) in exon 2 of *CD33* was predictive of low CD33 expression levels on cultured NK cells. NK cell cultures from homozygous carriers of the T allele at SNP rs12459419 had low frequencies of $CD33^+$ NK cells and therefore were well suited for production of CD33-CAR NK cells. Interestingly, $CD33^+$ NK cells harnessed stronger cytotoxicity and higher cytokine production, while $CD33^-$ NK cells exerted higher antibody-dependent cellular cytotoxicity (ADCC).

In conclusion, we have successfully created a genome editing platform for primary human NK cells and created HLA-deficient NK cells for “off-the-shelf” allogeneic therapy. We have also defined a novel functional dichotomy of cultured NK cells which can be harnessed for clinical purposes. These results can help to guide future NK cell adoptive therapy approaches for malignancies.

1 Introduction

Immunotherapy for cancer is one of the major promises of medicine in our times. Ever since the early contributions of William B. Coley in 1890 using live streptococcal bacteria injected into malignant lesions to induce an immunoreaction against sarcomas (1), immunotherapy has become increasingly fascinating (2–4). Based on the impressive treatment results of immune checkpoint inhibitors (ICIs) – especially for melanoma and other solid cancers (5) – and the initial successes of cluster of differentiation (CD)19-targeted autologous T cell therapies for B cell malignancies (6, 7), immunotherapy has come of age (8).

1.1 Natural Killer cells

Natural Killer (NK) cells are a central part of the innate cellular immune system and comprise between 5-20% of peripheral blood lymphocytes in healthy human individuals (9). Phenotypically, human NK cells can be defined as $CD19^{-}$, surface immunoglobulin $(sIg)^{-}$, $CD3^{-}$, T cell receptor $(TCR)^{-}$ and $CD56^{+}$ leukocytes. And as the name suggests, the most prominent feature of NK cells is their natural cytotoxic activity against malignant and distressed cells (10).

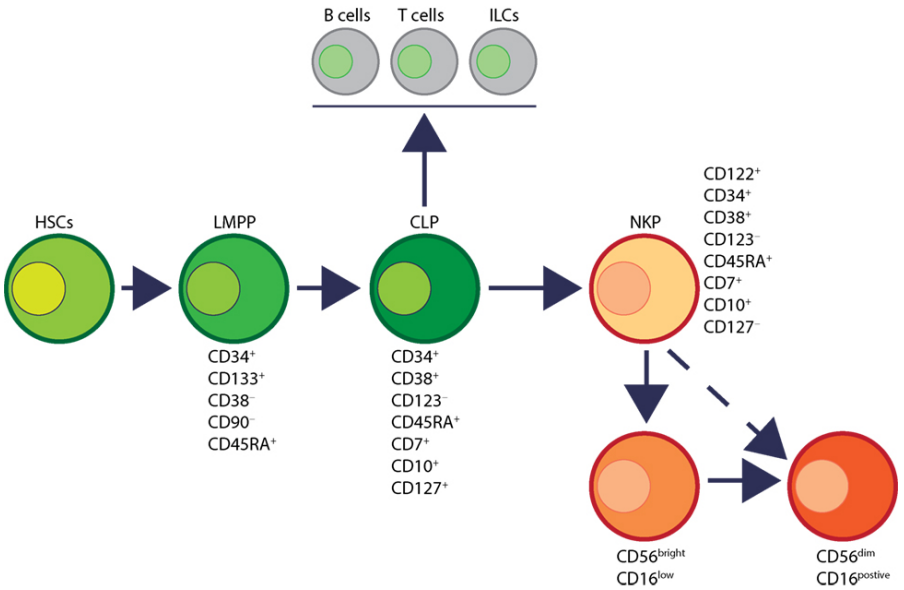

Figure 1: Linear model of NK cell development. NK cells share the common lymphoid progenitor with B cells, T cells and innate lymphoid cells (ILC). Acquisition of IL2RB/CD122 marks the progression to the NKP and makes the cells responsive towards IL-15. IL-15 now directs the differentiation towards the mature NK cell subsets (reprinted from Abel *et al.*, 2018 (11) under the terms of the CC BY license 4.0).

During development, NK cells share a common progenitor with T and B cells, the common lymphoid progenitor (CLP, **Figure 1** (11)). The acquisition of interleukin(IL)-2 receptor subunit β (IL2RB/CD122 in **Figure 1**) expression is thought to mark the step towards NK cell lineage commitment, concomitant with loss of CD127 (the receptor for

IL-7) expression (12). IL2RB is the shared subunit of the IL-2 and IL-15 receptors and facilitates responsiveness towards both cytokines in NK cell progenitors (NKP) (13). Especially IL-15 has been described as the main driver in the development, proliferation and activation of NK cells (14, 15). Upregulation of CD56 marks the transition from NKP to more mature blood NK cells (11).

In healthy individuals, all NK cells are $CD56^{+}$, yet they can be classified into $CD56^{bright}$ and $CD56^{dim}$ subpopulations (**Figure 2** (16)). Among those, the $CD56^{dim}$ population contains the more mature cells which arise from the $CD56^{bright}$ progenitor population (16). The $CD56^{bright}$ cells account for up to 90% of peripheral blood NK cells (17, 18). Some reports actually postulate a direct route of differentiation from the NKP towards the $CD56^{dim}$ subset (19). The demarcation of $CD56^{dim}$ and $CD56^{bright}$ NK cells is indicative of their functions: $CD56^{bright}$ NK cells are potent secretors of proinflammatory cytokines, while $CD56^{dim}$ NK cells harness more potent cytolytic capacities (20). Additionally to the downregulation of CD56 and loss of CD27 upon maturation, the $CD56^{dim}$ NK cell population expresses high levels of the low-affinity Fc γ receptor III (FcγRIII), also known as CD16a, and killer immunoglobulin-like receptors (KIR), and acquires CD57 expression after terminal maturation (16).

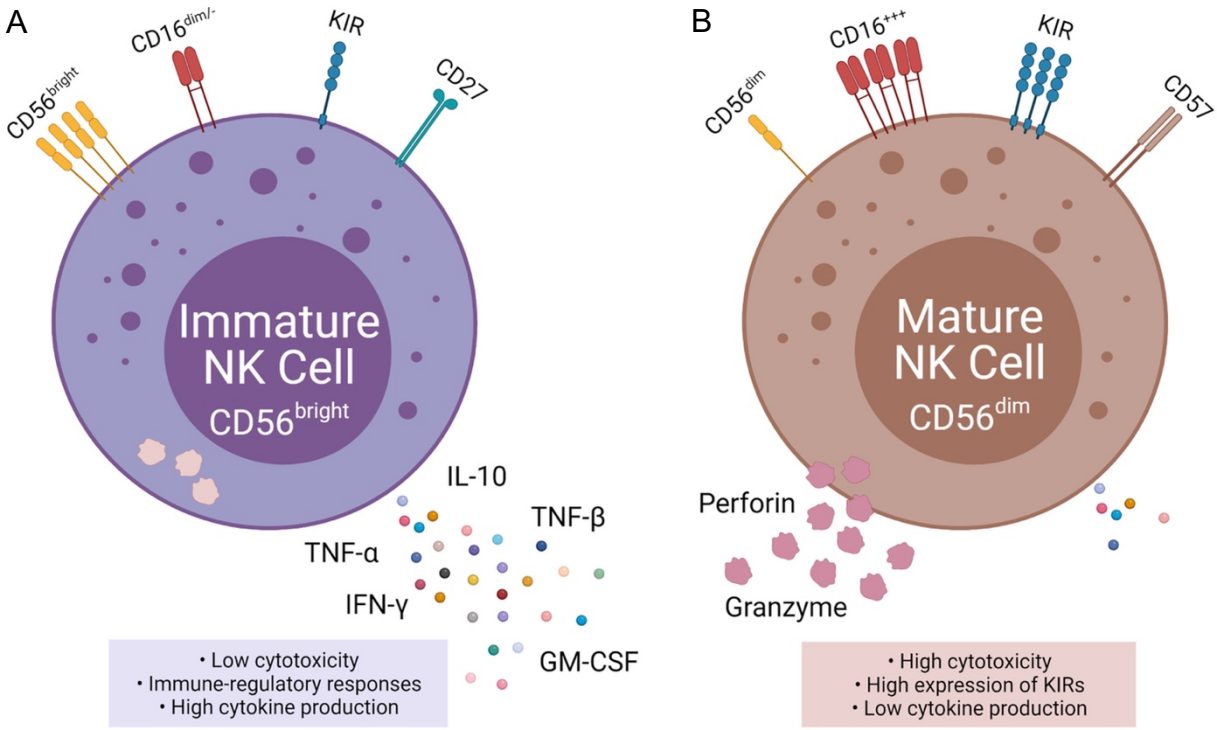

Figure 2: $CD56^{bright}$ and $CD56^{dim}$ NK cell phenotypes. A: The more immature NK cell subtype is $CD56^{bright}$ and $CD16^{dim/-}$ and is characterized by high cytokine production. Typical are low expression levels of KIRs and expression of CD27. B: The mature NK cell is $CD56^{dim}$ and expresses high levels of CD16. Mature NK cells are capable of strong cytotoxicity and express high levels of KIRs and CD57 (modified from Heipertz *et al*., 2021 (16) under the terms of the CC BY license 4.0).

1.2 NK cell receptors and education

So far, the best characterized function of NK cells is their innate cellular cytotoxicity (10). Upon encounter with virus-infected or transformed cells of various origins, NK cells exert potent lysis of these target cells. Importantly, a highly delicate balance between activating and inhibitory receptor signals governs whether a cytotoxic response is initiated upon probing a potential target cell or not (21).

1.2.1 NK cell activating receptors

After maturation in the bone marrow or secondary lymphoid organs, NK cells serve as immune effector cells without the need for positive or negative selection (11). The reason for this is that NK cells express germline-encoded natural cytotoxicity receptors which do not require genetic recombination like the TCR and the B cell receptor (BCR) (11). Also, while T cells via their TCRs only recognize peptides presented by Major Histocompatibility Class (MHC) antigens, the activating natural killer receptors (aNKR) of NK cells bind to ligands which are expressed directly on the surface of stressed, transformed or virus-infected cells (22). Hence, these activating receptors allow NK cells to recognize their targets in a non-MHC-restricted manner (23). Once bound to their cellular ligands, aNKRs incite signaling via motifs in their cytoplasmic domains or within the cytoplasmic domains of adapter molecules (24). The archetypical motif for immune cell signaling is the immunoreceptor tyrosine-based activation motif (ITAM) which is also used by TCRs and BCRs (25). After phosphorylation of the ITAMs, downstream effector molecules are recruited, and a cytotoxic response is initiated to kill the target cell.

As NK cells express several different types of aNKRs, a multitude of different target structures and ligands can be recognized (23, 26, 27). The most prominent aNKR is the C-type lectin-like receptor natural killer group 2 D (NKG2D) that recognizes the major histocompatibility complex class I-related chains A and B (MIC-A/B) (28), and all known six members of the UL16 binding protein (ULBP) family (29–32). Mechanistically, these NKG2D ligands are upregulated upon cellular stress or malignant transformation (33). Other aNKRs are the DNAX accessory molecule 1 (DNAM-1) which binds to members of the Nectin family (34), the natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46, which bind various viral epitopes (35), and CD16a which binds antibodies and allows the lysis of antibody-opsonized cells, a process called antibody-dependent cellular cytotoxicity (ADCC) (36). This diverse

repertoire allows a single NK cell to recognize various target structures and hence a multitude of target cell types, be it transformed or virus infected.

1.2.2 NK cell inhibitory receptors and the missing-self hypothesis

T cells are educated in the thymus to only recognize aberrant epitopes presented on autologous HLA molecules (37). Ligands for NK cell activating receptors, however, are ubiquitously expressed, although at low levels on healthy tissues (33). Yet, only increased levels of stress ligands elicit a cytotoxic NK cell response (33). This tolerance towards low levels of aNKR ligands is achieved via the expression of germline-encoded inhibitory natural killer receptors (iNKR). Under normal conditions, these iNKRs counteract the effects of the aNKRs and protect healthy cells from NK cell attack (38).

Among these inhibitory receptors, the family of KIRs is a major player in human NK cells (39). Although a few activating KIRs (aKIR) exist just like aNKRs, most of the KIRs are inhibitory (iKIR). Structurally, all inhibitory KIR genes encode for receptors with long cytoplasmic tails with immunotyrosine-based inhibitory motifs (ITIMs) which are responsible for mounting the inhibitory response upon ligation (40). On the other hand, all activating KIRs have short cytoplasmic tails without ITAMs, making them dependent on binding the ITAM-containing adaptor DNAX-activating protein 12 (DAP12) for signaling (41). KIRs bind the ubiquitously expressed Human Leukocyte Antigen (HLA) class I molecules on the surface or target cells and induce signaling that in case of iKIRs prevents the triggering of a cytotoxic response by counteracting or disabling the activating signaling of aNKRs. In contrast, the engagement of aKIRs leads to NK cell activation (39). In total, there are 16 different KIR genes within the KIR gene cluster on chromosome 19 (of which two are pseudogenes) (42). As these genes are highly polymorphic and the expression pattern of KIRs on NK cells is stochastically controlled, the KIR repertoire varies widely within the human population and even amongst NK cell clones within a single individual (42).

The identification of iKIRs led to a paradigm shift in our models on how NK cells find their targets (43): Instead of solely relying on the recognition of a single target structure, like cytotoxic T cells do through their TCRs, NK cells probe other cells for HLA molecules and have more than one mode of action. On healthy cells, HLA molecules should be present and therefore induce inhibitory signaling by the iKIRs, protecting the cell under surveillance even when ligands for aNKRs or aKIRs are present (**Figure 3**,

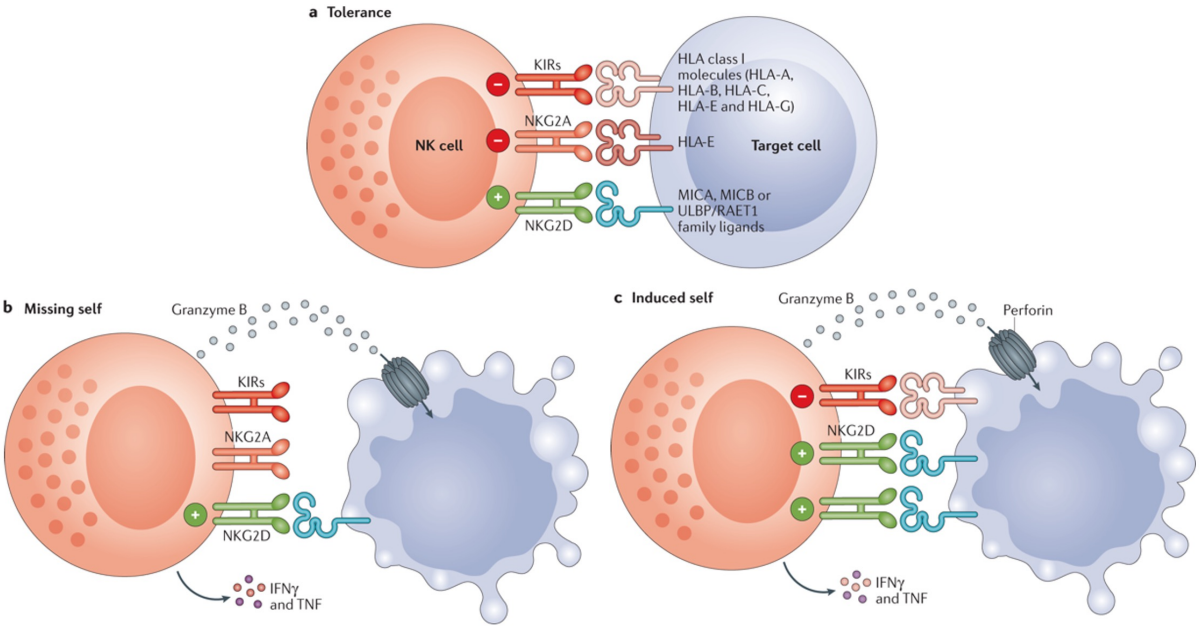

Figure 3: Principles of target cell recognition by NK cells. a) Balanced signals via activating and inhibitory receptors lead to tolerance towards the probed cell. b) When no inhibitory signal is relayed but activating receptors are engaged, the target cell is lysed. c) Prevailing activating signals lead to NK cell activation and subsequent lysis of the target cell. (Reprinted and modified by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Reviews Clinical Oncology, Myers *et al*., 2021 (44), Copyright © 2020, Springer Nature Limited).

a (44)). However, when HLA molecules are downregulated or missing, the inhibitory signals relayed by iKIRs are absent and the activating signals of activating receptors prevail, thus eliciting the cytotoxic response (**Figure 3**, b). This mechanism is the core of the missing-self hypothesis, postulated for the first time by Klas Kärre in 1981 (43). This model has been complete by the induced-self mode, where stress ligands are upregulated on the target cell which leads to sufficient activating signaling in the NK cell to overcome the inhibitory signaling and killing of the target cell (**Figure 3**, c) (45). As cancer cells often downregulate the expression of HLA molecules to evade recognition by T cells (46), KIRs have evolved to close the hole in our defense and render the malignant cells susceptible towards NK cells (39).

As NKR ligands on NK cells and HLA molecules on normal cells are both present in abundance, the initiation of killing is controlled by the balance of activating and inhibitory signals. Hence, even when aNKR ligands are expressed at high levels, a target cell could be saved from NK cell attack by an increased expression of HLA class I molecules which would be engaged by iKIRs. Next to KIRs, NK cells express a variety of other iNKRs. One of the best characterized iNKRs is natural killer group 2A (NKG2A) glycoprotein (47, 48). NKG2A belongs to the same C-type lectin-like family as NKG2D and natural killer group 2 C (NKG2C) (49). Interestingly, NKG2A and NKG2C both use CD94 as dimerization partner and both bind their cognate ligand HLA-E (50) (although NKG2A has a higher affinity for HLA-E (51)). In contrast to NKG2C, which is only

expressed by a minor subset of NK cells, NKG2A is present on all NK cells during the immature CD56^{bright} stage of development and also at lower levels on early CD56^{dim} NK cells (52). There it might play a pivotal role in NK cell education alongside KIRs (53). In the later CD56^{dim} state, NKG2A is lost while KIR expression is elevated (52).

Apart from KIRs and NKG2A, which bind HLA class I molecules, an array of other iNKRs is expressed on NK cells (54, 55). Curiously, many of them were first identified in T cells. Prominent examples are T-cell immunoreceptor with Ig and ITIM domains (TIGIT) (56), CD96, T cell immunoglobulin and mucin domain-3 (TIM-3) (57), and lymphocyte-activation gene 3 (LAG3) (58, 59). They structurally are all members of the immunoglobulin superfamily.

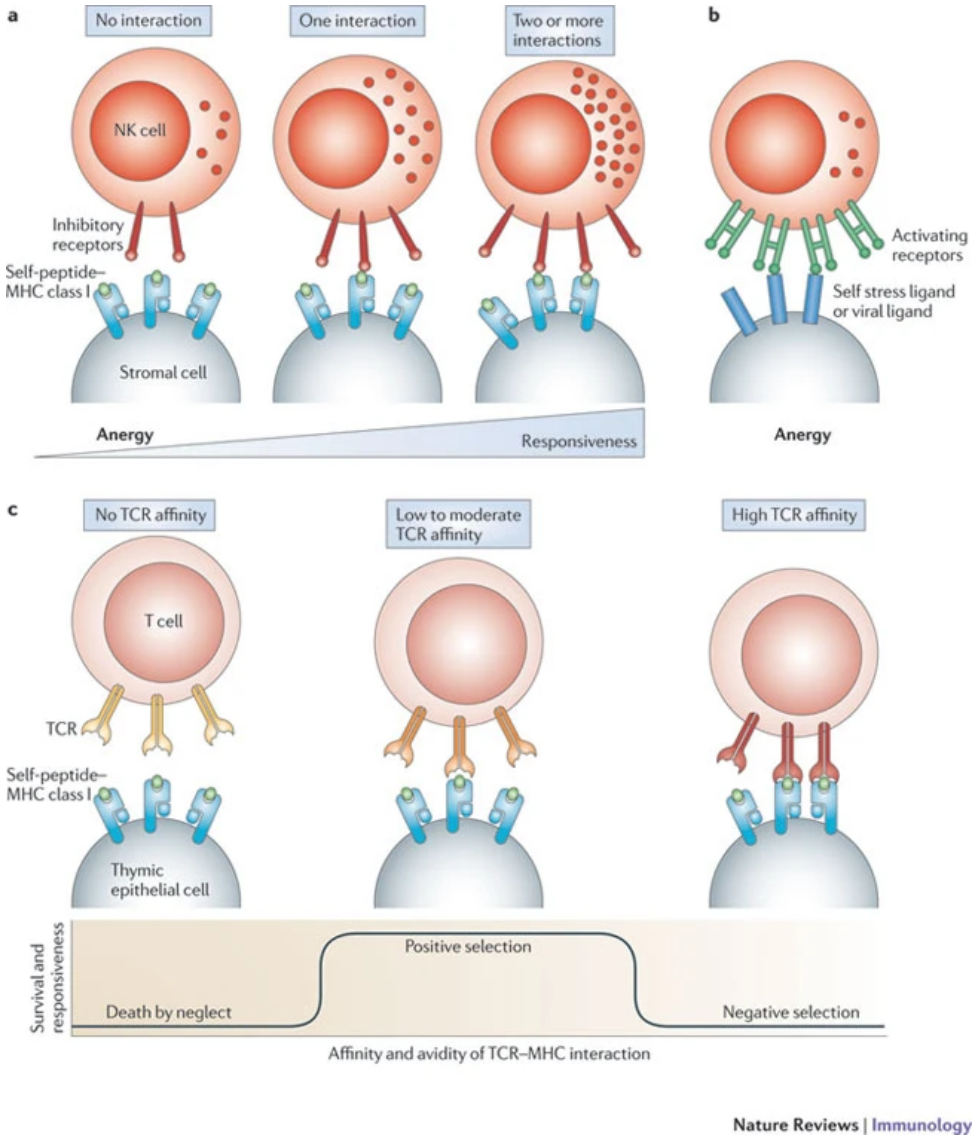

Figure 4: Functional maturation of T and NK cells in comparison. a) NK cell responsiveness to activating signaling is dependent on the strength of the negative stimulus presented by stromal cells during maturation. b) When receiving an activating stimulus during maturation, NK cells become anergic. c) T cells are being negatively selected in the thymus either when their TCR has no or too high affinity to self-MHC class I molecules. Only low to moderate MHC-affinity TCR bearing T cells are being positively selected (Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Reviews, Sun and Lanier, 2011 (60), Copyright © 2011, Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved).

1.2.3 NK cell education

While NK cells do not undergo negative and positive selection for their receptors, as T cells do in the thymus to suppress autoreactivity while still maintaining reactive T cells, NK cells still need to be educated to exert their cytotoxic functions (**Figure 4** (60)). According to the current model of NK cell education, NK cells need to receive a net negative stimulus during their development to enable their cytotoxic activity later in the periphery (60).

In conclusion, NK cells stay hyporesponsive and unable to kill HLA class I-deficient cells either when no KIRs are expressed or when no HLA class I molecules are present during their development in the bone marrow or when the signaling of activating stimuli prevails over the inhibitory ones. Basically, this implies that the educational process in the bone marrow is setting the baseline levels for the activity of mature NK cells later in the periphery. The cellular mechanisms behind this educational process are still unclear.

1.3 The cytotoxic response of immune effector cells

Cytotoxic lymphocytes kill their target cells by inducing apoptosis (61). To accomplish this, they need to physically interact and engage into what is called the immunological synapse. First, loose cell-to-cell interactions are facilitated by cell adhesion molecules. For NK cells, CD2 and L-selectin have been implicated in this early stage (62, 63). Lymphocyte function-associated antigen 1 (LFA-1) signaling then prepares the cell for subsequent polarization and lipid raft accumulation (64, 65). Subsequently, both activating and inhibitory receptors on NK cells engage with their respective ligands on the target cell. If the signaling through the inhibitor receptors prevails, cell polarization is counteracted, the immunological synapse is actively resolved and the cells drift apart (66–68). However, recognition by aNKRs that is not counteracted by inhibitory signals leads to the formation of cytotoxic immunological synapses with the target cell. The cell cytoskeleton of the NK cell is now being polarized towards the immunological synapse and the cytotoxic granules are mobilized towards the contact zone with the target cell (69, 70). These granules contain a cocktail of effector molecules that are then exocytosed into the synaptic cleft and can efficiently induce apoptosis in target cells (71). The main protagonists in the granules are perforin and a group of molecules called granzymes (72).

In the classical model (73, 74), perforin forms pores in the target cell membrane, reminiscent of the membrane attack complex of the complement system. These pores now facilitate the entry of granzymes into the target cells. Whether this process is solely due to passive diffusion of granzymes or whether the perforin pore serves a different mechanism of transport is still unclear (75, 76). Within the granzyme family, granzymes A and B are the most abundant in NK cells with granzyme B being the most prominent in human NK cells (77). Biochemically, granzymes are serine proteases that start cleaving their target proteins after entry into the target cells. Well known targets of granzyme B are molecules involved in apoptosis like caspases 3, 7, 8, and 10, thereby activating them and inducing apoptosis (78–80). In addition, also alpha-tubulin, the BH3 interacting domain death agonist (BID) of the B-cell lymphoma 2 (BCL-2) family and some viral proteins and also cellular proteins involved in viral replication have been identified as targets for granzymes (81–84). Interestingly, while this pathway is shared between cytotoxic T cells and NK cells, the timing is different: In T cells, cytotoxic granules are only formed after ligation of the TCR and therefore cytotoxic responses of T cells can take hours until death of the target cells occurs (85). NK cells possess preformed cytotoxic granules, which are at the ready as soon as a target cell is engaged (86, 87). NK cells can therefore release their cytotoxic cargo within minutes upon encountering the target cell (88). In addition, having preformed granules enables NK cells to quickly kill multiple targets, a process called serial killing, and even engaging into immunological synapses with several target cells at once (89).

1.4 Human leukocyte antigens

HLA are the human MHC molecules. The main function of HLA is the presentation of either endogenous or exogenous peptides to enable immune surveillance by lymphocytes, especially T cells, which can recognize HLA molecules and the presented peptides via their TCRs (90). Generally, HLA genes can be divided in three classes of which only the classes I and II are involved in peptide presentation. The HLA gene cluster is located on the short arm of chromosome 6 (91). Not only are HLA molecules of polygenic origin, but they also show an immense degree of polymorphism.

1.4.1 HLA class I

HLA class I comprises the classical *HLA-A*, *-B*, and *-C* genes and the non-classical *HLA-E*, *-F* and *-G* genes (91). HLA class I molecules are single-pass transmembrane proteins with 3 extracellular domains (α1, α2 and α3), hereafter referred to as heavy chains (92). The heavy chains all depend on the non-polymorphic β2-microglobulin (B2M) as invariant light chain for correct surface presentation (92). HLA class I molecules are expressed on all nucleated cells where they present peptides to $CD8^+$ T cells, depending on binding of the $CD8^+$ T cell coreceptor to the membrane-proximal α3 domain for stabilization of the HLA-TCR complex (93). The peptide binding groove is formed by the apical α1 and 2 domains (92). After binding of B2M to the heavy chain and release from the chaperone calnexin, a complex with calreticulin, antigen peptide transporters 1 and 2 (TAP complex), tapasin, endoplasmatic reticulum-resident protein 57 (ERp57) and protein disulfide isomerase (PDI) is formed to load proteasome-derived peptides onto the HLA molecule (94). Subsequently, it is released for trafficking to the cell membrane.

Generally, only classical HLA class I molecules are involved in antigen presentation towards cytotoxic T cells, although reports exist about HLA-E-specific $CD8^+$ T cells (95). Apart from peptide presentation, classical HLA class I molecules are engaged by KIRs on NK cells to maintain tolerance and facilitate education (39). Similarly, non-classical HLA molecules are involved in tolerance through other inhibitory receptors (66).

1.4.2 HLA class II

Adding to the complexity of HLA, class II molecules consist of two polymorphic chains (91). The *HLA-DP*, *-DQ* and *-DR* genes encode for proteins expressed on the cell surface (91). Each chain is a single-pass transmembrane protein consisting of two domains (α1 and α2 for the α chain, and β1 and β2 for the β chain) (92). Analogous to the situation with HLA class I, the membrane-distal α1 and β1 domains form the peptide binding grove for presentation to $CD4^+$ T cells, while the α2 and β2 domains each contain a motif for binding the CD4 T cell coreceptor (96). In contrast to HLA class I, HLA class II molecules do not present endogenously produced peptides but peptides taken up from the environment (97). HLA class II molecules are only expressed on so called antigen presenting cells (APC), like dendritic cells, macrophages, and B cells (97). Like HLA class I molecules binding to inhibitory

receptors, HLA class II molecules can have protective functions, either by serving as ligands for inhibitory receptors as well, in case of LAG3 (98), or by being recognized by regulatory $CD4^+$ T cells which then create an immunosuppressive environment (99).

1.4.3 The role of HLA molecules in transplantation

When a foreign (= non-self) peptide, for example stemming from a pathogen or from a cancer-derived neo-epitope, is presented on the surface of a cell by HLA, TCRs recognize this cell and elicit a cytotoxic response (100). This way, malignantly transformed, stressed and virus-infected cells are cleared from the organism before more harm can be done.

Apart from the recognition of foreign peptides, TCRs can also detect non-self HLA molecules from another individual, leading to an immune response (101, 102). The importance of HLA in organ transplantation, hematopoietic stem cell transfer (HSCT) or bone marrow transplantation (BMT) is without question: The HLA genes of the donor must match, at least to a certain degree, that of the recipient. The transplant could otherwise be rejected due to recognition as foreign by the host's immune system (103). In BMT or HSCT, the situation can even be reversed: Here, the transplanted immune cells can start attacking the recipient, leading to often fatal Graft-versus-Host-Disease (GvHD) (104). The main drivers of GvHD are the donor's T cells which recognize the foreign HLA-peptide complexes on the host's cells (105). Of all HLA genes, 6 are classically regarded for organ transplantation and HSCT (*HLA-A, -B, -C, -DR, -DQ, -DP*).

The mechanism of an allogeneic response towards foreign HLA is different compared to the physiological response towards self-HLA presenting a foreign peptide derived from a pathogen or a neo-epitope (106). MHC and TCRs have evolved towards a high binding affinity (107, 108). Upon T cell maturation in the thymus, the majority of T cells are negatively selected because their TCRs bind too strongly to self-HLA due to this co-evolution (109, 110). Only those few T cells are positively selected, which have TCRs with low affinity towards self-HLA in complex with self-peptides, insufficient to trigger T cell activation (60). TCRs with no binding affinity to self-HLA are also negatively selected. This delicate window of affinity enables the TCRs to even sense minor changes in structure of the HLA-peptide complex, for example a foreign peptide from a neo-epitope or a pathogen. In transplantation, however, the whole HLA-peptide complex of the donor organ or cells is different from self-HLA due to the abundant HLA

polymorphisms, regardless of the presented peptide. As the T cell with its TCR has not been selected for low affinity towards this foreign HLA-peptide complexes, there is a high probability that it has a high affinity towards the foreign HLA molecule due to their co-evolution (106).

1.5 Immunotherapy

Immunotherapy against existing cancers encompasses a huge variety of diverse strategies (2). The focus of the work in this thesis is the transfer of immune effector cells often referred to as adoptive transfer (ACT).

1.5.1 ACT using autologous immune effector cells

In principle, two different approaches for ACT of immune cells are possible: The autologous cellular therapy uses the patient's own immune cells – usually after *ex vivo* manipulations and expansions – for treatment and is in general not associated with autoimmune phenomena, as no differences due to mismatches of HLA loci between donor and recipient exist. The allogeneic cellular therapy uses immune cells from another donor. Lymphokine activated killer (LAK) cells were the first autologous immune effector cells to be used for the treatment of malignant disease in humans (111, 112). Yet, severe toxicities of the accompanying cytokine infusions and limited response rates hampered their clinical success (112–117).

Another strategy was to isolate tumor-infiltrating lymphocytes (TILs) from the patient's solid cancers which proved more efficacious due to the tumor antigen-targeting TCRs of the T cells (112). For this kind of therapy, TILs are isolated from resected tumor tissue and reactivated *in vitro* prior to reinfusion in order to overcome their tumor stroma-induced exhaustion.

NK cells, having a plethora of receptors for targeting transformed cells, seemed a promising cell type for ACT. However, infused autologous NK cells did not mediate meaningful anti-tumor effects (118, 119). To boost the efficacy of ACT, either allogeneic effector cells can be used to exploit their alloreactivity, or they can be genetically engineered.

1.5.2 Allogeneic ACT

In contrast to autologous cellular therapy, allogeneic therapy administers cells that are not derived from the patient. The reason to decide for the allogeneic setting can be twofold. First of all, it has practical reasons: Due to chemo- and radiotherapy treatments of the patients' and due to the disease itself, their immune cells are often compromised which hampers their viability and anti-tumor functions (120–124). Also, the manufacturing process for an autologous cell therapy product can take up to several weeks (e.g. CAR T cells, see 1.5.4) from apheresis to infusion, which can be critical in case of a rapidly progressing disease. In allogeneic ACT, multiple doses of the same cell product can be manufactured in large lots from either a single or a cohort of healthy donors ahead of time and stored appropriately, most probably frozen. When needed, the product is thawed and administered with minimized delay of mere hours. This approach is called "off-the-shelf". Hence turning towards healthy donors as allogeneic source can overcome the logistical problems of autologous ACT. Second and apart from the practical reasons, the allogeneic setting holds a biological advantage over the autologous approach: The HLA mismatch-induced alloreactivity, known from GvHD, helps the donor immune cells to clear out the malignant cells via the Graft-versus-Tumor (GvT) effect which has been shown after allogeneic HSCT (125–127) and after donor lymphocyte infusions (DLI) (128–131). At the same time, a pronounced GvT effect is accompanied by an increased risk for GvHD (131–133). Through the GvT effect, the tumor can be eradicated by the alloreactive T cells that bind to the recipient HLA on the tumor cells.

Later studies revealed that NK cell alloreactivity due to KIR-mismatch is a major contributing factor of the GvT effect (134–141). At first, this seems puzzling as NK cells do not cause GvHD (142–145). Yet, NK cells are capable of recognizing allogeneic cells via a mismatch between their KIRs and the recipient's HLA molecules. Essentially, this NK cell alloreactivity works through missing-self recognition as either one or multiple KIRs present on the allogeneic NK cells do not bind or only bind too weakly to the host's HLA molecules. This way, although the recipient's malignant cells express HLA molecules, the activating signals relayed by aNKRs on the NK cells prevail and trigger a cytotoxic attack. It has even been suggested that this KIR mismatch can prevent the onset of GvHD through eradication of the recipient's APCs and alloreactive donor T cells by the NK cells before they can trigger GvHD (146, 147). This presence of the GvT effect without any GvHD makes NK cells a superior cell type for allogeneic therapy over T cells, enabling use of healthy donor cells to create an

"off-the-shelf" cell therapy product that potentially can be used in every cancer patient irrespective of HLA constellation (44).

1.5.3 Allogeneic NK cell therapy approaches

For decades the culture of primary NK cells was limited by the lack of appropriate culture protocols. In mixed cultures from peripheral blood mononuclear cells (PBMCs), successful expansion could be reached with high percentages of NK cells, probably aided by the presence of accessory immune cells (148). Purified NK cells, as they would be needed for GvHD-safe allogeneic "off-the-shelf" therapy, however, could not be generated in high numbers, especially not for multiple doses (148). A promising tool to expand NK cells were so called artificial APCs (aAPC) (149, 150). These were cell lines, mostly K562-derived, that were engineered to express either one or a combination of membrane-bound (mb)IL-15, mbIL-21, 41BB-ligand and OX40. Using these as feeder cells for NK cell expansion dramatically boosted expansion rates.

Unhampered by the low expansion rates of pure NK cell cultures *in vitro*, various attempts were made to exploit the GvT effects in allogeneic NK cell therapy (143, 151–153). In 2005, Jeffrey Miller and colleagues treated 43 patients with various malignant diseases (10 metastatic melanomas, 14 metastatic renal cell carcinomas, 1 refractory Hodgkin disease, 19 poor-prognosis acute myeloid leukemias (AML)) with allogeneic NK cell infusions (143). Their main finding was the need for a lymphodepleting preconditioning to enable NK cell persistence beyond day 5 after infusion. The high-dose cyclophosphamide treatment led to a rise in endogenous IL-15 levels, a cytokine crucial for NK cell proliferation (14), and showed NK persistence for up to 28 days. Of the 19 AML patients that received the high-dose cyclophosphamide conditioning, 5 achieved complete remission (CR). Notably, the CR rate was 75% in those patients that received KIR-mismatched NK cells and only 13% in those without the KIR-mismatch. This prompted further studies to focus more on the KIR-mismatch than only on the allogeneic setting. In the study by Shi and colleagues, 10 multiple myeloma patients received KIR-mismatched NK cells before delayed autologous HSCT (151). Of note, the patients received daily infusion of IL-2 to support the infused NK cells. In this trial, however, only 50% of patients achieved near CR (nCR) compared to 40% nCR in the group without NK cells. Interestingly, despite a high-intensity preconditioning therapy and a confirmed rise of endogenous IL-15 levels in the patients, the donor NK cells were only detectable for about one week post infusion.

Mixed lymphocyte reactions confirmed rising frequencies of donor-specific alloreactive T cells which rejected the infused NK cells (151). Before, the HLA-mismatch has aided the therapy via the GvT effect. Here, the HLA barrier now posed a problem: The infused allogeneic cells were rejected due to HLA mismatch by the patients' T cell system. Curiously, the IL-2 infusions might have also hampered the NK cell expansion according to other studies (153, 154). Nevertheless, clinical trials using KIR-mismatched allogeneic NK cells continued. In 2011, in a trial with 13 elderly AML patients, 1/5 patients with active disease achieved CR, 3/6 patients in CR were maintained in CR and 2 patients with minimal residual disease (MRD) achieved CR after infusion. NK cell persistence was not measured (155). In 2016, Romee and colleagues showed an overall response rate of 5/9 in an elderly AML cohort that received KIR-mismatched cytokine-induced memory-like (CIML) NK cells (152). Persistence, however, was limited to approximately three weeks post infusion, again showing the limited lifespan of allogeneic NK cells. These are just exemplary studies. More are reviewed by Shimasaki and colleagues (156).

Summed up, allogeneic NK cells show promising results, especially in the treatment of AML, yet their short lifespan *in vivo* and their rejection by the host's immune system are limiting their anti-tumor functions and are potential starting points to improve their efficacy for future therapies (156).

1.5.4 Chimeric antigen receptor-redirected therapy

The success of TILs, expressing predefined TCRs, and the rise of viral gene transfer led to the exploration of transgenic T cells, expressing transgenic TCRs (157). Mispairing of transgenic TCR chains with endogenous chains, however, can lead to autoimmune reactions and the tumor cells can still evade TCR-transgenic T cells by downregulation of MHC molecules (46, 158). To enable the targeting of malignant cells in an MHC-non-restricted manner, chimeric antigen receptors (CAR) were developed (**Figure 5** (159)). When a TCR is engaged by a peptide-MHC complex, a multimeric complex is assembled to elicit signaling and the cytotoxic attack, whereas a CAR unifies all signaling moieties into one polypeptide chain (3): The antigen targeting domain can be either derived from a ligand, a receptor, a designed ankyrin repeat protein (DARPin) or the single chain variable fragment (scFv) of an antibody, linked via a transmembrane domain to one or more intracellular signaling domains of either T or

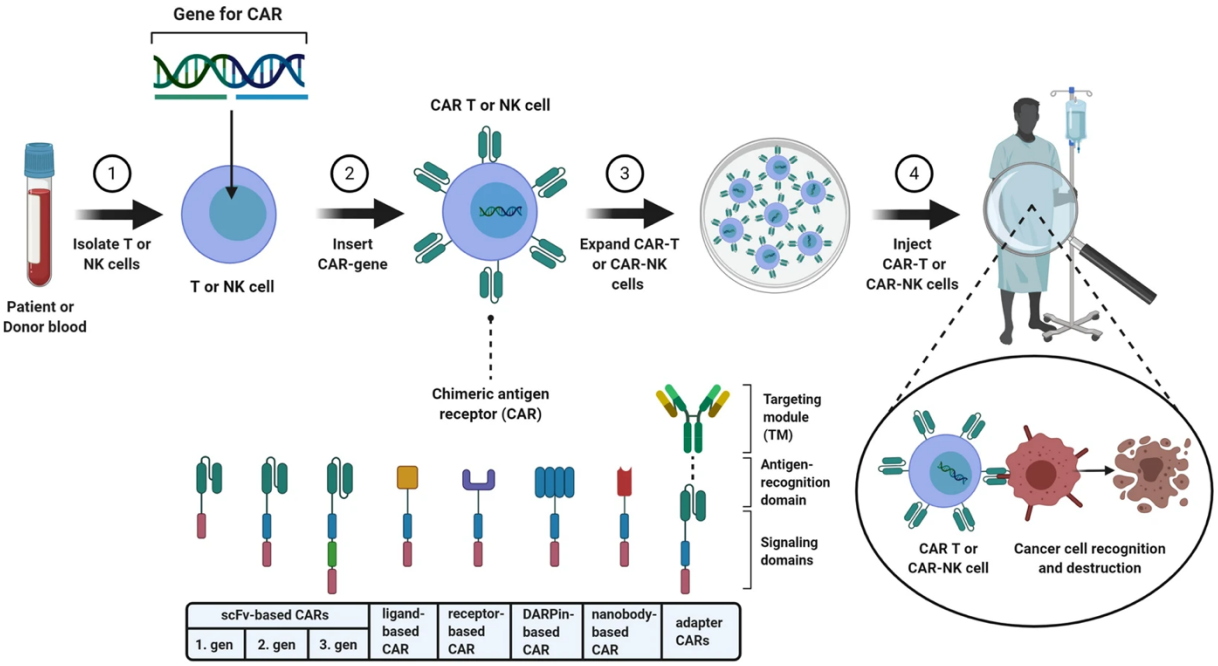

Figure 5: Procedure of CAR T or NK cell ACT. 1) T or NK cells are isolated from peripheral blood. 2) Subsequently, the coding sequence for the CAR is transferred into the cells before they are expanded (3). 4) Finally, the cells are being infused into the patient where they recognize and kill the malignant cells (under magnifying glass). Lower figure: CARs can be classified into generations based on how many intracellular signaling domains they contain: Only one domain CARs are first generations CARs, when bearing two domains they are called second generation CARs and when they carry three intracellular domains they are referred to as third generation CARs. The targeting domain of a CAR can be derived from multiple entities: A ligand, a receptor, a DARPin, a nanobody or it can be a modular approach where the targeting domain of the CAR itself only serves as an adapter that binds to another targeting moiety which can be an antibody (reprinted from Albinger *et al.*, 2021 (159) under the terms of the CC BY license 4.0).

NK cell activating receptors and coreceptors (**Figure 5**, **lower panel**) (3, 159). The targeting domain on the surface of the immune cell enables redirection of cytotoxicity towards theoretically any surface marker on another cell, enabling killing in a MHC-non-restricted manner (160). Figure 5 shows the classical manufacturing process for CAR lymphocytes. First, the donor T or NK cells are isolated and activated (**Figure 5, part 1**). Subsequently, the coding sequence for the CAR is retrovirally inserted into the T or NK cell's genome (**Figure 5, part 2)**. Following an expansion period, the therapeutic cells are reinfused into the patient to target the malignant cells (**Figure 5, parts 3, 4 and magnifying lens**).

The first generation of CAR molecules had only the CD3ζ chain for intracellular signaling and were insufficient for anti-tumor activity and *in vivo* expansion of the CAR T cells (161) (**Figure 5, lower panel)**. Incorporation of costimulatory domains yielded the second generation of CARs, comprising the CD28 or 4-1BB intracellular domains which markedly improved activation and persistence of the transduced T cells (162). Using CD19 as target antigen, this second generation CAR was successfully used for treating B cell malignancies (6, 7, 163–165). To date, CAR T cell products targeting CD19 on B cell malignancies have received market releases by the American Food

and Drug Administration (FDA) and European Medicines Agency (EMA) (3). A third generation of CARs contains two costimulatory domains in addition to the CD3ζ chain, which can be CD28 and 4-1BB, but also the intracellular domains of other costimulatory receptors have been described to provide sufficient signaling when incorporated into third generation CARs (166–168). However, third generation CARs have not been as extensively used in clinical trials so far.

For allogeneic CAR therapies, various approaches have been developed to mitigate the danger of GvHD while, as best as can, keeping the GvT effect. For T cells, this includes genome editing approaches to target the CAR expression cassette into the TCR locus or the selection of subsets that are devoid of alloreactivity (169–172). Using CARs in NK cells, which are devoid of GvHD, has so far been hampered by the low gene transfer efficiencies in NK cells. Currently, more targets for CAR-redirected therapy using CAR T and NK cells are being investigated, examples being CD123 and CD33 for the treatment of AML (173–175).

1.5.5 CD33 in AML immunotherapy

CD33 is widely expressed on immature myeloid cells and only lost during the differentiation of the progenitor cells towards the erythroid and thrombocytic lineage. Importantly, AML blasts express CD33 at high levels which makes it a suitable target for AML immunotherapy (176, 177). The earliest CD33 targeting approach for the treatment of AML used a radiolabeled CD33 antibody to specifically deliver radiation to the diseased hematopoietic organs (178). A lot of experience in CD33 immunotherapy is derived from the drug Gemtuzumab Ozogamicin (GO), sold under the name Mylotarg. GO consists of the humanized IgG4 CD33 antibody clone P67.6 conjugated to the bacterial toxin calicheamicin (179, 180). The toxin-antibody conjugate exploits the fact that the whole complex of antigen and antibody is internalized into endosomes after GO binding to CD33 due to downstream ITIM signaling (181) and the calicheamicin released from the antibody, causing severe DNA damage and cell death. In comparison to mere targeting antibodies like rituximab or ICIs, GO shows severe toxicities including myelosuppression, high liver transaminase levels, hyperbilirubinemia and can even lead to fatal veno-occlusive disease (VOD) (179, 182). Failure to detect clinical benefit in a major follow-up clinical trial led to a market withdrawal of Mylotarg in 2010 (183). Subsequent trials used up to three times lower dosage or fractionation, but still could show that the clinical benefit of GO

depends crucially on dosage regime and AML subtype, leading to re-release of the drug in 2017 (184). After its re-release, a splicing polymorphism in CD33 has gained attention with regard to treatment outcome: The single-nucleotide polymorphism (SNP) rs12459419 (C>T) influences splicing of exon 2. In case of cytosine, the protein serine and arginine rich splicing factor 2 (SRSF2) binds and splicing occurs normally (**Figure 6** (185), left). When a thymidine is present, however, binding of SRSF2 is weakened and the likelihood of skipping of exon 2 is increased (**Figure 6**, right), leading to an

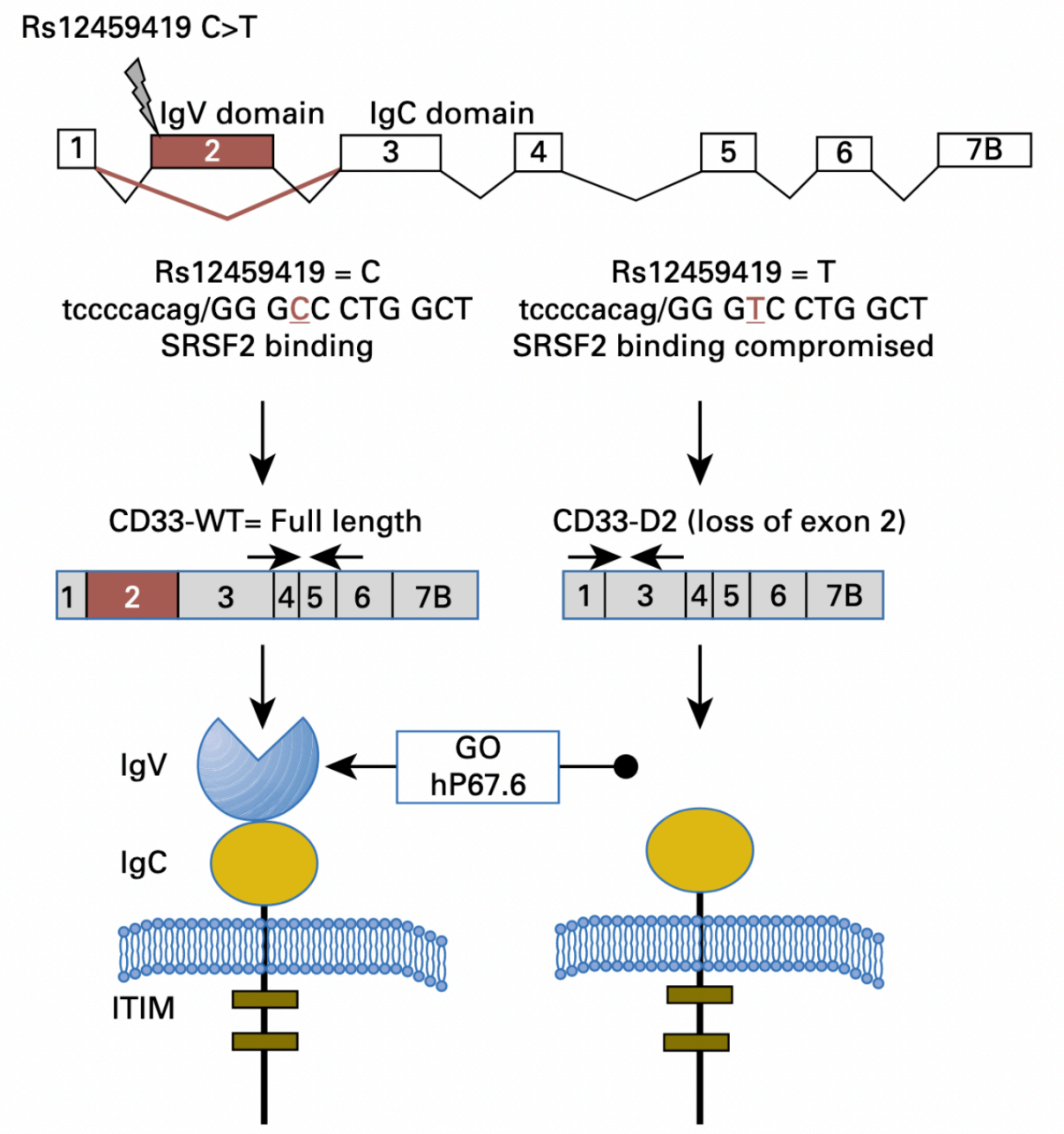

Figure 6: SNP-dependent alternative splicing in CD33. When the splice factor SRSF2 encounters a cytosin, splicing occurs normally and the full-length transcript is processed. In case of thymidin, binding of SRFS2 is compromised and the chance of exon 2 skipping is increased, leading to the truncated CD33-D2 transcript. The corresponding protein lacks the IgV domain which contains the binding site for the humanized antibody clone p67.6 which together with the bacterial toxin calicheamicin forms the compound Gemtuzumab Ozogamicin (Reprinted with permission from the American Society of Clinical Oncology: Wolters Kluwer Health, Inc, Journal of Clinical Oncology, Lamba *et al*., 2017 (185), Copyright © 2017, by the American Society of Clinical Oncology).

altered transcript (CD33-D2). Phenotypically, myeloid cells and AML blasts from individuals carrying the T allele, either hetero- or homozygously, show reduced expression levels of the full length CD33 molecules (186). A study in microglia shows that the CD33-D2-encoded molecule does not transit to the cell surface but resides within peroxisomes (187). Even if the truncated protein was to traffic to the cell surface, the epitope bound by GO lies within the spliced out V-set domain encoded by exon 2 and would not be present in the truncated protein (**Figure 6**, lower part). One study argues that the truncated CD33 is not made at all in peripheral blood myeloid cells and AML blasts (188). However, fitting to the reduced expression levels of full length CD33 in carriers of the T allele and the absence of the GO targeting epitope in the CD33-D2 molecule, the phase III clinical trial by Lamba and colleagues shows that only AML patients carrying the C allele benefit from GO treatment (185). Interestingly, even heterozygous individuals do not respond to GO treatment (185).

After the success of CD19-retargeted CAR T cells (6, 7, 163–165), the expertise from targeting CD33 with antibodies was transferred to the CAR technology to manufacture CD33-CAR T cells. In fact, the CD33-CAR used in this study uses the Gemtuzumab scFv (189). To date, two reports about a total of four patients exist which have been treated with CD33-CAR T cells (120, 190). Befitting the side effects of GO treatment, CD33-CAR T cell treatment in the study from Wang and colleagues led to pronounced pancytopenia due to on-target off-tumor effects on healthy myeloid host cells (190). Apart from this, a marked decrease in blasts was reported following CD33-CAR T cell infusion. Yet, the patient still relapsed and died 13 weeks after infusion (190). While the study by Tambaro and colleagues barely reported results on the CAR T cells' efficacy at all, they highlighted the difficulties of autologous ACT that they experienced firsthand: of the 10 patients enrolled in the study, only 8 were eligible for apheresis due to their medical condition, only 4 processes yielded a final T cell product that met the release criteria, and 1 of the 4 patients had died from the disease before they could receive the cells (120). The authors themselves state that overcoming logistical challenges as well as rapid manufacturing protocols and sufficient starting material are crucial points for successful ACT (120). When allogeneic NK cells are used, these challenges are obsolete as the cells can be manufactured ahead of time and from healthy donors. In addition, NK cells do not establish long-term immunological memory like it has been described for T cells (3, 191), alleviating the need to ablate them to enable normal myelopoiesis after therapy.

2 Aim of the thesis

My thesis was embedded in the framework of the iCAN33 project, funded by Europäischer Fonds für regionale Entwicklung (EFRE) North Rhine Westphalia (NRW).

The objective of this network was the development of an allogeneic NK cell product expressing a CD33-CAR for “off-the-shelf” immunotherapy for AML.

The main task of my thesis was to diminish the impact of the HLA class I expression on the CAR NK cells, thereby potentially improving CAR NK cell engraftment and expansion after infusion into recipients.

Thus, the aims were

a) the establishment of a genome-editing workflow in primary human NK cells from peripheral blood,
b) the functional knock-out of classical HLA class I expression in primary human NK cells and the subsequent testing of their functions and viability and,
c) the evaluation of HLA class I-deficient NK cell potential to evade allorecognition by primary human T cells and,
d) the evaluation of specificity and efficacy of CD33-CAR NK cells.

3 Publications

I. **HLA Class I Knockout Converts Allogeneic Primary NK Cells Into Suitable Effectors for "Off-the-Shelf" Immunotherapy**
Hoerster K, Uhrberg M, Wiek C, Horn PA, Hanenberg H and Heinrichs S (2021) Front. Immunol. 11:586168. doi: 10.3389/fimmu.2020.586168

II. **CD33 Delineates Two Functionally Distinct NK Cell Populations Divergent in Cytokine Production and Antibody-Mediated Cellular Cytotoxicity**
Hejazi M, Zhang C, Bennstein SB, Balz V, Reusing SB, Quadflieg M, **Hoerster K**, Heinrichs S, Hanenberg H, Oberbeck S, Nitsche M, Cramer S, Pfeifer R, Oberoi P, Rühl H, Oldenburg J, Brossart P, Horn PA, Babor F, Wels WS, Fischer JC, Möker N and Uhrberg M (2022). Front. Immunol. 12:798087. doi: 10.3389/fimmu.2021.798087

3.1 Publication I

HLA Class I Knockout Converts Allogeneic Primary NK Cells Into Suitable Effectors for "Off-the-Shelf" Immunotherapy

Hoerster K, Uhrberg M, Wiek C, Horn PA, Hanenberg H and Heinrichs S

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- Statistical analysis: 100%
- Writing the manuscript: 85%
- Revising the manuscript: 45%

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ORIGINAL RESEARCH
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HLA Class I Knockout Converts Allogeneic Primary NK Cells Into Suitable Effectors for "Off-the-Shelf" Immunotherapy

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Cellular immunotherapy using chimeric antigen receptors (CARs) so far has almost exclusively used autologous peripheral blood-derived T cells as immune effector cells. However, harvesting sufficient numbers of T cells is often challenging in heavily pre-treated patients with malignancies and perturbed hematopoiesis and perturbed hematopoiesis. Also, such a CAR product will always be specific for the individual patient. In contrast, NK cell infusions can be performed in non-HLA-matched settings due to the absence of alloreactivity of these innate immune cells. Still, the infused NK cells are subject to recognition and rejection by the patient's immune system, thereby limiting their life-span *in vivo* and undermining the possibility for multiple infusions. Here, we designed genome editing and advanced lentiviral transduction protocols to render primary human NK cells unsusceptible/resistant to an allogeneic response by the recipient's $CD8^+$ T cells. After knocking-out surface expression of HLA class I molecules by targeting the B2M gene *via* CRISPR/Cas9, we also co-expressed a single-chain HLA-E molecule, thereby preventing NK cell fratricide of B2M-knockout (KO) cells *via* "missing self"-induced lysis. Importantly, these genetically engineered NK cells were functionally indistinguishable from their unmodified counterparts with regard to their phenotype and their natural cytotoxicity towards different AML cell lines. In co-culture assays, B2M-KO NK cells neither induced immune responses of allogeneic T cells nor re-activated allogeneic T cells which had been expanded/primed using irradiated PBMNCs of the respective NK cell donor. Our study demonstrates the feasibility of genome editing in primary allogeneic NK cells to diminish their recognition and killing by mismatched T cells and is an important prerequisite for using non-HLA-matched primary human NK cells as readily available, "off-the-shelf" immune effectors for a variety of immunotherapy indications in human cancer.

Keywords: NK cells, B2M knockout, HLA class I, off-the-shelf, allogeneic, genome editing, immunotherapy, adoptive cell transfer

INTRODUCTION

Adoptive cell transfer (ACT) of autologous genetically modified immune cells has emerged as an attractive treatment option for various malignancies of hematologic origin. Yet, the highly personalized nature of these cell products generates extreme costs and patient-specific factors can still impede the manufacturing process due to large variabilities. For a significant number of patients, an autologous final product cannot be generated in time for treatment [reviewed in (1)].

To alleviate these problems, research in the field is moving towards "off-the-shelf" products, making use of immune effector cells from healthy donors. However, this endeavor is complicated by problems of alloreactivity and immune tolerance for mismatched HLA constellations. A severe side effect of allogeneic cellular therapy is Graft-versus-Host-Disease (GvHD), a life-threatening complication caused by the transplanted alloreactive T cells and known since the early days of hematopoietic stem cell transplantation (HSCT) (2–5). To circumvent this complication, several approaches have been developed. Virus-specific cytotoxic T (VST) cells, for example, have successfully been used to control latent infections post HSCT without causing GvHD (6, 7). Accordingly, they have been proposed as a potential T cell population to create "off-the-shelf" therapeutic products (8, 9). Another option is the selective depletion of T cell subsets alloreactive towards specific HLA (10, 11). However, this bias towards certain T cell subsets is again limiting the application potential of the products. An interesting approach to abrogate unwanted or alloreactive signaling from the endogenous T cell receptors (TCRs) in chimeric antigen receptor (CAR) T cells uses genome editing on common TCR domains (12, 13), however this genomic editing will require additional gene transfer systems and therefore will add several layers of complexities to CAR T cell clinical trials.

Thus, an obvious solution is to simply use another type of immune effector cells: natural killer (NK) cells. Importantly, even when infused at large quantities into immunocompromised patients, NK cells do not cause GvHD in the first place and can even prevent it (14, 15). In contrast to T and B cells, NK cells express germline-encoded activating and inhibitory receptors and integrate signals to distinguish between healthy and transformed or stressed cells (16). This *innate* recognition of transformed cells and absence of GvHD have proven to be of great potential for the treatment of malignancies in animal models and clinical trials (17–24). While the infusion of autologous NK cells is ineffective in various cancers, donor NK cell infusions after or through haploidentical HSCT demonstrated that NK cells with killer cell immunoglobulin-like receptor (KIR) mismatches with the recipient do not cause any damage to normal tissue, but still can eliminate residual malignant cells (25–32). Importantly, mature NK cells contained within the stem cell graft were shown to be responsible for the anti-tumor effects observed early after transplantation and therefore were unlikely to originate from the reconstituted NK cell compartment (33–35). The efficacy and apparent safety of NK cells in allogeneic adoptive cell therapies has made them an attractive cell type for the manufacturing of "off-the-shelf" cell-based products. However, two major aspects of human NK cells, the marked resistance to standard genetic modifications with lentiviral vectors and the limited *ex vivo* expansion capacities, have hampered their use for both allogeneic and also autologous CAR-redirected immunotherapies. Hence, researchers have resorted to stable NK cell lines, such as NK-92 (36–42), or to using NK cells differentiated from CD34+ hematopoietic stem and progenitor cells (HSPCs) or pluripotent stem cells (PSCs) (43–46) with subsequent expansion using feeder cells (47–50).

While alloreactivity and GvHD are activities initiated by the graft, graft rejection by the host's immune system is another factor to consider in allogeneic non-myeloablative therapies. To prevent rejection of the graft by the host immune system, a wide variety of concepts have been used, including the expression of the immune checkpoint inhibitors CTLA4-Ig and PD-L1 (51) or engagement of the "don't eat me"-signal CD47 (52). Rather than equipping cells with means to fend off attacking immune cells, some studies arm the therapeutic cells with receptors to fight back and lyse the approaching alloreactive host T cells (9, 53). Others have set out to disrupt the HLA barrier/antigens entirely, hiding the infused cells from recognition by alloreactive host T cells. The latter aim has been achieved either by targeting genes essential for the HLA processing machinery such as the class II transcriptional activator (CIITA) and beta-2-microglobulin (B2M) or by disrupting individual HLA genes (54–67). In most of these preclinical studies however, the starting material was either a transformed cell line or PSC cells that subsequently had to be differentiated into the tissue of choice in elaborated and time-consuming protocols.

In this study, we used recent advances in genome editing and HLA biology to generate NK cells ideally suited for adoptive cellular therapy. Based on the recent breakthrough for genetically modifying human NK cells (68), we constructed a chimeric envelope with the surface and transmembrane domains of the baboon endogenous retrovirus and the cytoplasmic tail of the amphotropic murine retrovirus for efficient gene transfer, similarly to constructs described before (69–71). We then disrupted HLA class I expression in human NK cells by targeting B2M *via* a CRISPR/Cas9 lentiviral vector (72) and finally equipped the HLA class I knockout NK cells with a modified single-chain HLA-E molecule (58, 73). Consequently, these double-modified NK cells neither activated nor expanded allogeneic T cells and were also protected from autolysis/fracticide by NK cells. Combined with novel NK cell culture expansion protocols for GMP settings (74, 75), highly cytotoxic, primary "off-the-shelf" human NK cells were generated in relevant amounts without the need of lengthy differentiation protocols, PSCs or feeder cells.

MATERIALS AND METHODS

Cells and Cell Lines

NK cells were purified from PBMC using a negative selection protocol with the NK cell isolation kit (#130-092-657, Miltenyi Biotec) and MACS LS columns (Miltenyi Biotec) according to

the manufacturer's protocol. NK cells were cultured in NK MACS medium (Miltenyi Biotec), supplemented with 1% of the enclosed NK MACS Supplement, 5% heat-inactivated human AB serum (Sigma-Aldrich, H4522), 1% Penicillin/Streptomycin (Sigma-Aldrich), 500 U/ml rhIL-2 and 140 U/ml rhIL-15 (both from Miltenyi Biotec) and termed NK MACS complete medium. T cells were purified from whole blood using the RosettaSep HLA T cell kit (Stemcell Technologies) according to the manufacturer's protocol and cultured in DMEM (Thermo Fisher Scientific) supplemented with 5% heat-inactivated human AB serum, 1% Penicillin/Streptomycin and 50 U/ml rhIL-2 if not stated otherwise, in the following termed T cell medium. Whole blood was obtained from healthy donors at the University of Düsseldorf after informed consent. PBMNCs were isolated by Ficoll density gradient centrifugation. SKM1, K562 and Kasumi-1 cell lines where maintained in RPMI medium (Thermo Fisher Scientific) supplemented with 10% heat-inactivated FCS, termed R10 medium.

Lentiviral Vectors

The CRISPR/Cas9 vector pLE38-Cas9-sgB2M/gNKG2A is a third-generation self-inactivating (SIN) lentiviral vector based on the pRRL SIN backbone (76). Expression cassettes for the U6-promoter/gRNA and the EFSns-promoter/Cas9 were derived from pLCv2 (72). The targeting sections of the gB2M sequence (5′-GAGTAGCGCGAGCACAGCTA-3′) and gNKG2A sequences (5′-TGAACAGGAAATAACCTATG-3′) were designed using the GPP sgRNA designer tool (Broad Institute, Cambridge, MA, USA) and cloned into the Esp3I sites of the pLE38-Cas9-stuffer vector using annealed oligonucleotides. The sc-HLA-E coding sequence was designed after Gornalusse et al. (58). Briefly, the fragment encoding the HLA-E*03:01 heavy chain was cloned from HEK293T cells with the forward-primer incorporating the last repeat of the $(G_4S)_4$ linker for the final sc-HLA-E sequence and a BamHI restriction site and the reverse-primer harboring a *MluI* restriction site for assembly into the vector. The fragment encoding the sequence for B2M-leader/HLA-G-leader/$(G_4S)_3$-linker/mutatedB2M-chain/$(G_4S)_4$-linker was synthesized by LGC genomics. The gB2M targeting site and the protospacer adjacent motif (PAM) site were mutated from 5′-CCTTAGCTGTGCTCGCGCTACTC-3′ to 5′-CACTGGCC GTGCTGGCCCTGCTG-3′ to avoid editing of the sc-HLA-E by gB2M. The HLA-G-leader-B2M-sequence was PCR amplified using primers harboring restriction sites for *Xba*I and *BamH*I and assembled together with the amplified HLA-E*03:01 heavy chain encoding fragment into a lentiviral transfer vector *via* the *Xba*I and the *Mlu*I restriction sites. Expression was driven from the SFFV promoter (77).

For construction of the pcoBaEVTM chimeric baboon envelope vector, the surface and transmembrane subunits of the wild-type sequence of the M7 strain of the Baboon endogenous virus (NC_022517) was fused to the cytoplasmic sequence of the amphotropic murine leukemia virus (AF411814), synthesized as a codon-optimized cDNA by GeneArt (ThermoFisher) according to our design and then cloned in our envelope expression plasmid using *EcoR*I and *Not*I (78).

Lentiviral particles were produced in HEK293T cells by cotransfection of pcoBaEVTM, pCMV-ΔR8.91 (76) and the lentiviral transfer vector. Supernatants were harvested 48 and 72 h after transfection, concentrated by high-speed centrifugation, resuspended in non-supplemented NK MACS medium supplemented with 20 mM HEPES and titered on K562 and SKM1.

NK Cell Transduction

Transductions were performed 7 days after the preparation of CD56+ CD3- cells and start of the NK cell expansion protocol. Briefly, lentiviral particles corresponding to an MOI of 1 (titered on K562/SKM1) were adjusted to a volume of 100 µl using plain NK MACS medium without additives, mixed with the equal volume of plain NK MACS supplemented with 5 µg/ml Vectofusin-1 (Miltenyi Biotec), incubated at room temperature for 8 min and mixed with 50 µl cell suspension containing 1×10^6 NK cells in NK MACS complete medium. For simultaneous double-transductions, both particle populations were used at MOIs of 1 and pooled prior to mixing with NK MACS and Vectofusin-1. Subsequently, the 250-µl cell/particle mix was transferred into 48-well plates and centrifuged for 90 min at 400*g*, 32°C. After spinoculation, cells were incubated at 37°C for additional 4 h before 500 µl NK MACS complete medium where carefully added to the cells.

Flow Cytometry and Phenotyping

Flow cytometric data where acquired using a CytoFLEX (Beckman Coulter). Antibodies from Thermo Fisher Scientific were anti-HLA-E (clone 3D12; Thermo Fisher). Antibodies from BioLegend were: anti-pan-HLA class I (clone W6/32), CD3 (clone HIT3a), CD4 (clone RPA-T4), CD8 (clone SK1), CD56 (clone 5.1H11), CD16 (clone 3G8), CD57 (clone HNK-1), KIR2DL2/3 (CD158b, clone DX27), KIR2DL1/S1/3/5 (CD158a,h, clone HP-MA4), CD107a (clone H4A3), CD137 (clone 4B4-1), anti-NKG2D (CD314, clone 1D11), anti-NKp30 (clone P30-15), anti-NKp44 (clone P44-8), anti-NKp46 (clone 9E2). Antibodies from Miltenyi Biotec were anti-NKG2A (CD159a, clone REA110), anti-NKG2C (CD159c, clone REA205). Antibody stainings were performed in PBS (Thermo Fisher Scientific) supplemented with 0.5% BSA (Sigma-Aldrich) and 2 mM EDTA (Sigma-Aldrich), termed MACS buffer. Of note, the clone W6/32 does not recognize the sc-HLA-E due to the covalently linked N-terminus of the incorporated B2M (79), enabling discrimination between endogenous HLA class I and the sc-HLA-E. Analysis was performed using the CytExpert software (Beckman Coulter) and FlowJo V10.6.2 (Becton Dickinson).

NK Cell Cytotoxicity Testing

NK cells were co-cultured for 6 h with K562 and Kasumi-1 cells in 100 µl R10 medium supplemented with 500 U/ml rhIL-2 and 140 U/ml rhIL-15 at the effector target ratios 4:1, 1:1 and 0.25:1. To allow for discrimination between NK cells and targets while avoiding to gate out dead target cells, a "no-wash" protocol was applied. Briefly, CD56-antibody was added to the wells at the end of the incubation period, mixed and stained at 4°C for 20 min.

Subsequently, the cell mixture was diluted in 300 µl of MACS buffer supplemented with 7AAD and incubated for 2 min at room temperature before data acquisition. The specific lysis of targets was determined by the percentage of $7AAD^+$ cells within the $CD56^-$ singlets.

Fratricide Assay

NKG2A-KO NK cells were generated using gNKG2A, which targets the *KLRC1* gene encoding for NKG2A, and the transduction protocol as described above. The mixture of sc-HLA-E only and sc-HLA-E/B2M-KO NK cells, obtained after double transduction, was co-cultured for 24 and 48 h with either untransduced, parental NK cells from the same donor or NK cells after knockout of NKG2A. Cells were stained with 7AAD and antibodies for pan-HLA class I, HLA-E and NKG2A before acquisition. Selective depletion of B2M-KO cells was evaluated by gating on all sc-HLA-E^+ target cells and then discriminating between HLA class I^+ and HLA class I^- cells.

T Cell Proliferation Assay

Proliferation of allogeneic T cells was evaluated by CFSE dilution. Briefly, 5×10^6 freshly isolated T cells were resuspended in 5 ml prewarmed PBS/0.1% BSA. 10 µl of a 5-mM CFSE solution were added and cells were incubated at 37°C, 5% CO_2 for 7 min, subsequently topped up with 16 ml cold DMEM/10% FCS, incubated at 4°C in the dark for 5 min and washed twice. For the assay, 200,000 T cells where co-cultured with 50,000 NK cells in 200 µl T cell medium supplemented with 2 U/ml rhIL-2 for 6 days. Cells stimulated with PMA/Ionomycin served as a qualitative positive control and medium controls were used as negative controls. On day 6, cells were stained for CD56, CD4, CD8 and 7AAD. CFSE dilution was analyzed in $CD4^+$ and $CD8^+$ T cells after gating on $7AAD^-/CD56^-$ singlets.

T Cell Reactivation and Degranulation Assay

Alloreactive T cells were expanded from isolated T cells by incubation for 14 days with 30-Gy irradiated PBMC of the respective NK cell donors. To test reactivation and degranulation of alloreactive T cells, 160,000 to 200,000 expanded T cells where incubated with 80,000 to 100,000 either parental or modified NK cells (Effector-Target ratio of 2:1) for 24 and 48 h in T cell medium. For degranulation assays, monensin and anti-CD107a antibody were added to the cultures 4 h before acquisition. For analysis, samples were stained with 7AAD and antibodies for CD3, CD4, CD8. For the reactivation assay, cells were additionally stained for CD137. Degranulation and reactivation were analyzed in $CD4^+$ and $CD8^+$ T cells after gating on $7AAD^-/CD3^+$ singlets. A baseline measurement was performed on the day the assay was set up and medium, as well as autologous and 3rd party NK cells, served as negative and specificity controls at the time points of analysis.

T Cell Cytotoxicity Assay

For T vs NK cell cytotoxicity assays, CFSE-stained T cells were cultured for 20 h with the NK cell lines at the effector target ratios 4:1, 2:1 and 1:1 (calculated on $CD8^+$ T cells) in 200 µl T cell medium. A "no-wash" protocol was applied to prevent loss of dead target cells: Before acquisition, the cell mixture was diluted with the same volume of MACS buffer supplemented with 7AAD and incubated at room temperature for 2 min before acquisition. Autologous NK cells served as negative controls and for gating purposes. NK cell lysis by T cells was determined as the percentage of $7AAD^+$ cells within the $CFSE^-$ singlets.

Statistics

Statistical analysis was performed using GraphPad Prism with the tests given in the figure legends. The level of statistical significance was set to $p < 0.05$. Statistically significant differences are reported in the figure legends.

RESULTS

Concomitant Single-Chain HLA-E Expression on Primary NK Cells Allows for a Functional Knockout of HLA Class I Surface Expression Without Leading to Fratricide

In primary NK cells, the efficient knockout of the classical HLA class I genes A, B and C by CRISPR/Cas9-based genome editing is challenging due to the extensive polymorphism and the presence of six genomic target sites. The functional elimination of all HLA class I proteins with a single hit can be achieved by using a single CRISPR/Cas9 lentiviral vector targeting the beta-2-microglobulin gene (B2M), the shared invariant light chain of all HLA class I molecules (**Figure 1A**). We first tested two distinct gRNAs targeting B2M (gB2M) in the pLE38-Cas9 vector for their knockout efficiencies by transducing the human diploid AML cell line SKM1. Analyzing the transduced cells after staining with the pan-HLA class I monoclonal antibody W6/32 revealed a decrease in surface expression of classical HLA class I molecules by flow cytometry, starting four days after transduction and generating stable knockouts with both guide sequences when analyzed 10 days later (**Figure 1B**). We decided to use gB2M#1 for further experiments as it yielded a higher gene editing rate.

Despite the high gene transfer efficiencies that can be achieved in NK cells with baboon envelope-pseudotyped lentiviral vectors (68), we observed only approximately 16% HLA class I-negative cells (**Figure 1C**) four days after transduction of primary NK cells with the CRISPR/Cas9 HLA class I targeting vector. This percentage of HLA class I-negative cells steadily decreased over time and dropped below 1% on day 14 post transduction (**Figure 1D**, red line). We hypothesized that this progressive loss of successfully targeted NK cells was most likely a consequence of the "missing self"-induced killing by neighboring NK cells, a phenomenon also called "fratricide". In parallel cultures, we therefore co-expressed a modified single-chain (sc-)HLA-E molecule (**Figure 1A**) on the surface of NK cells, as this chimeric protein can efficiently protect the HLA class I-negative NK cells from fratricide by engaging the inhibitory receptor dimer CD94/NKG2A (58). By itself, lentiviral overexpression of sc-HLA-E yielded a distinct positive

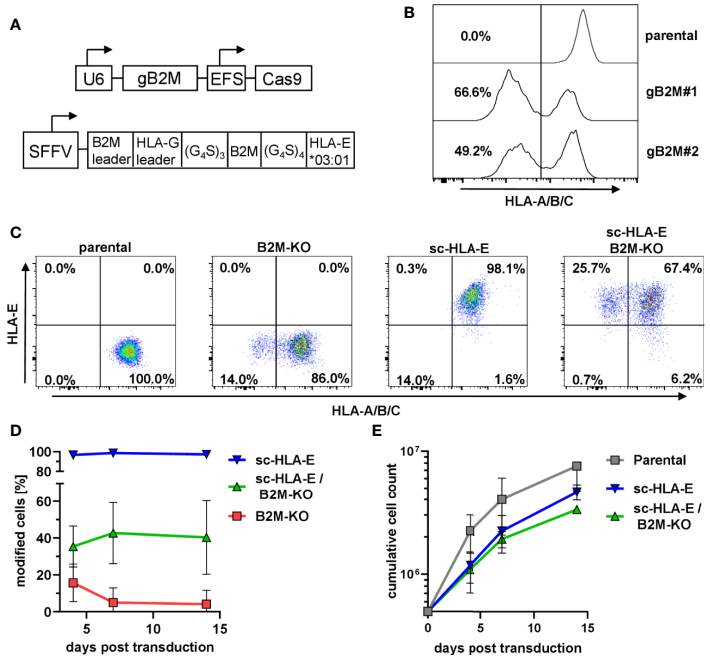

FIGURE 1 | Knockout of B2M and simultaneous overexpression of sc-HLA-E in primary NK cells **(A)** Outline of the lentiviral expression cassettes used for CRISPR/Cas9 mediated B2M-knockout and expression of the sc-HLA-E molecule. Cas9 and gB2M are expressed from the same lentiviral vector, driven by an EFS and an U6 promoter, respectively. The HLA-E*03:01 heavy chain is linked to B2M and the HLA-G leader peptide by G_4S linkers and expression is driven from an SFFV promoter. **(B)** Knockout of B2M in SKM1 cells abrogates surface expression of HLA class I detected *via* flow cytometry on day 10 after transduction. **(C)** Frequencies of HLA class I and sc-HLA-E expressing primary NK cells 4 days after transduction with lentiviral particles encoding gB2M and Cas9, sc-HLA-E or a combination of both compared to an untransduced control. **(D)** Frequencies of modified cells measured on day 4, 7 and 14 after transduction (mean ± SD; n = 6 for sc-HLA-E, n = 5 for sc-HLA-E/B2M-KO and B2M-KO). **(E)** Growth of NK cell cultures measured 4, 7 and 14 days after transduction. Shown is the cumulative cell count of all NK cells within the culture. The NK cells showed a fold-expansion from the day of transduction of 15.2 ± 5.72 for parental, 9.36 ± 1.29 for sc-HLA-E and 6.74 ± 0.31 for sc-HLA-E/B2M-KO NK cells (mean ± SD; n = 3).

population that was stable over time (**Figures 1C, D**, blue line). By pooling the two lentiviral supernatants encoding sc-HLA-E and sgB2M/Cas9, we achieved a mean gene editing/transduction frequency of 35.4% that remained stable around 40.4% on days 7 and 14 post transduction (**Figures 1C, D**, green line). NK cell expansion was documented for 14 days after transduction (3 weeks after isolation), demonstrating that sc-HLA-E and sc-HLA-E/B2M-KO modified NK cells indeed grew slower compared to the mock-transduced controls ("parental"), but still achieved an almost seven-fold expansion from the day of transduction (**Figure 1E**). NK cells were used for downstream experiments within 4 weeks from the day of preparation.

Protection From Fratricide Is Dependent on NKG2A

In order to prove that the HLA-E-mediated protection of HLA class I-negative NK cells in mixed cultures was due to the engagement of the inhibitory receptor CD94/NKG2A (73), we designed the corresponding guide RNAs against *KLRC1*, the gene encoding NKG2A, and used the same lentiviral expression system as above (**Figure 2A**). Flow cytometry analysis demonstrated that transduction of primary human NK cells with the pLE38-Cas9 vector successfully abrogated NKG2A expression seven days post transduction (**Figure 2B**). To test whether *NKG2A*-KO NK cells lead to the relative reduction of sc-HLA-E/B2M-KO NK cells, as they would no longer be tolerated by NKG2A-deficient NK cells, the bulk-transduced NKG2A-KO NK cell cultures were co-cultured with a mixture of sc-HLA-E/B2M-KO and sc-HLA-E NK cells. Importantly, addition of NKG2A expressing parental NK cells did not have any effect on the frequency of sc-HLA-E/B2M-KO NK cells in a mix with B2M competent sc-HLA-E-expressing NK cells when compared to the baseline controls (**Figure 2C**, upper panel and **Figure 2D**). In contrast, a co-culture containing NKG2A deficient NK cells showed a strong depletion of the sc-HLA-E/B2M-KO NK cells after 24 h, leading to elimination of more than 85% of B2M-KO cells (**Figure 2C**, lower panel and **Figure 2D**). In support of these observations, we also noted that B2M-KO NK cells did not persist in NK cell cultures with low NKG2A-expression levels despite the presence of sc-HLA-E (data not shown). These results

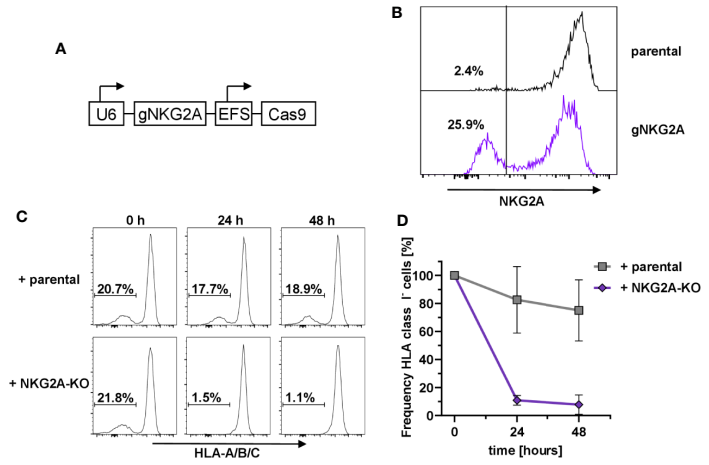

FIGURE 2 | Dependency on NKG2A expression for sc-HLA-E-mediated protection from NK cell fratricide **(A)** Outline of the lentiviral expression cassettes used for CRISPR/Cas9 mediated NKG2A-knockout. **(B)** Transduction with Cas9 and gNKG2A led to abrogation of NKG2A surface expression in primary NK cells 7 days post transduction. **(C)** Representative histograms showing the frequency of sc-HLA-E/B2M-KO NK cells in presence of B2M-competent sc-HLA-E NK cells upon addition of parental cells (upper panel) or NKG2A-KO NK cells (lower panel) at different time points. **(D)** Quantification of changes in sc-HLA-E/B2M-KO NK cell frequencies relative to the time point (0 h) when parental or NKG2A-KO NK cells were added. Statistical analysis was performed by two-way ANOVA with Geisser-Greenhouse correction (ε = 0.6972) for repeated measures and Holm-Sidak testing for multiple comparisons between each time point within both groups. For the samples containing NKG2A-KO NK cells, the decrease in frequency of HLA class I^- was statistically significant at both time points (mean ± SD; n = 4).

demonstrated that the prevention of fratricide is strongly dependent on the HLA-E/NKG2A signaling.

B2M-KO NK Cells Are Phenotypically Similar to Unmodified Cells and Retain Uncompromised Effector Functions

HLA molecules serve a vital role in the education of NK cells *via* KIRs and NKG2A (80, 81). As the forced expression of HLA-E on neighboring cells might lead to tonic engagement of CD94/NKG2A, which can signal *via* downstream targets (82, 83), we next investigated if the genetic modifications in HLA expression impacted the NK cell phenotype and functions. To this end, we performed multi-color flow cytometric analysis using a panel comprising various maturation markers as well as activating and inhibitory receptors. The results in **Figures 3A, B** demonstrated that the modified NK cells exhibited an immunophenotypic profile similar to their unmodified counterparts: The majority of cells was $CD56^{bright}$ and CD16 was expressed on almost all cells with a slight bias towards $CD16^{bright}$ cells. Only a minor fraction of NK cells expressed CD57 usually associated with terminal maturation and replicative senescence (84), while almost all cultured NK cells were positive for the activating receptors NKG2D and NKp30. NKp44 and NKp46 was present on about 60 – 80% of NK cells from three donors, but little to no $NKG2C^+$ cells were detected. KIR2DL/S1/2/3 expression was detectable on roughly 50% to 60% of NK cells while NKG2A expression was present in over 90% of cells. Interestingly, the expression levels of NKG2A were diminished in both sc-HLA-E-expressing NK cells, rendering these cells $NKG2A^{dim}$. In addition, the frequencies of CD16, NKG2C and KIR expressing NK cells were slightly lower in the sc-HLA-E-expressing NK cell cultures compared to parental NK cells while the frequency of NKp44 expressing NK cells in cultures expressing sc-HLA-E was slightly higher.

To test the cytotoxic effector cell functions, the genetically modified NK cells were co-incubated with the AML cell lines K562 (HLA class I^-) and Kasumi-1 (HLA class I^+). Flow cytometric analysis after 6 h of co-incubation revealed uncompromised natural cytotoxicity towards both AML cell lines in a dose-dependent fashion with no statistically significant differences detectable (**Figure 3C**). Therefore, the high cytotoxicity towards the HLA class I^+ Kasumi-1 cells highlighted that the remarkable cytotoxicity of the NK cells against AML blasts is not inhibited by the genetic modifications using either CRISPR/Cas9 technology or lentiviral overexpression of HLA molecules.

Expression of sc-HLA-E Suppresses Proliferation of Allogeneic T Cells

In the next set of experiments, we wanted to explore whether the modifications of HLA class I surface expression also conferred escape of immune recognition by allogeneic T cells. An allogeneic T cell response can be initiated *via* two different pathways, either a direct recognition by binding of the TCR to the foreign HLA proteins themselves or indirectly by donor peptides presented on self-HLA molecules by antigen-presenting cells (85). Through both pathways, T cells become activated, exert effector functions and undergo clonogenic expansion.

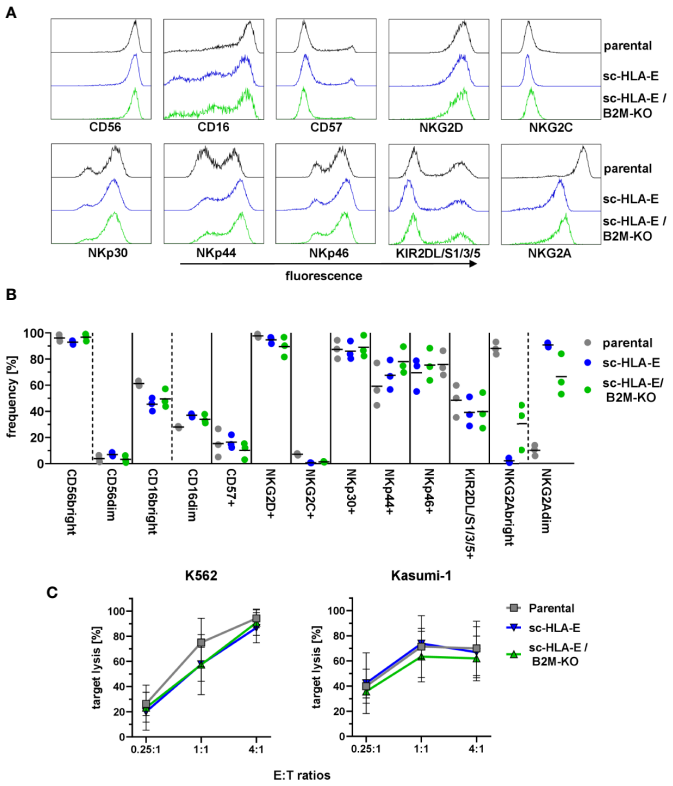

FIGURE 3 | Functional and phenotypical analysis of the gene modified NK cells. **(A)** Representative flow cytometry plots of the phenotypical analysis of the NK cell cultures. **(B)** Quantification of NK cell frequencies for phenotypic markers in parental, sc-HLA-E and sc-HLA-E/B2M-KO NK cell cultures from left to right for each marker (all data points shown, n = 3). **(C)** Quantification of natural NK cell cytotoxicity by lysis of the AML cell lines K562 and Kasumi-1 after co-incubation for 6 h. Statistical analysis was performed by two-way ANOVA with Holm-Sidak testing for multiple comparisons. No statistically significant differences could be detected (mean ± SD; K562: n = 6 for parental and sc-HLA-E/B2M-KO and n = 5 for sc-HLA-E NK cells; Kasumi: n = 4 for parental and sc-HLA-E/B2M-KO and n = 3 for sc-HLA-E NK cells).

We therefore measured the expansion of allogeneic T cells from healthy unrelated donors as a surrogate for immune recognition in a mixed lymphocyte culture (MLC) with the modified NK cells in comparison to parental NK cells and a medium control. To this end, purified T cells were labeled with the dye CFSE and co-cultured with NK cells. After six days, the proliferation of the T cells was assessed by flow cytometry as the frequency of $CFSE^{dim}$ cells. The data in **Figure 4** demonstrated that the $CD4^+$ T cells did not proliferate in response to HLA mismatched NK cells irrespectable whether these cells overexpressed sc-HLA-E or not and also independent of the B2M status. In contrast, co-culture with parental unmodified NK cells activated alloreactive $CD8^+$ T cells and induced their proliferation, visible as the increased percentage of $CFSE^{dim}$ cells (**Figure 4A** second panel top row and **Figure 4B** first panel). Surprisingly, sc-HLA-E only NK cells also did not induce allogeneic $CD8^+$ T cell proliferation, despite intact HLA class I expression (**Figure 4A**, lower panel). Quantification showed that, while allogeneic responses vary greatly between the individual pairs in the mixed MLCs, a significant allogeneic stimulus was only generated by unmodified NK cells and only for $CD8^+$ T cells (**Figure 4B**).

Only B2M-KO NK Cells Are Protected From Allogeneic $CD8^+$ T Cell Responses

As only unmodified allogeneic NK cells elicited a proliferative response in $CD8^+$ T cells, we hypothesized that the overexpression of sc-HLA-E can actively suppress T cell activation/proliferation and consequently cytotoxicity even after direct TCR-mediated recognition of the foreign HLA on the target NK cells. Therefore, in order to investigate whether mere overexpression of sc-HLA-E in NK cells is sufficient to protect them from alloreactive T cell cytotoxicty, we evaluated T cell degranulation and subsequent lysis of parental, sc-HLA-E or scHLA-E/B2M-KO NK cells by HLA-mismatched T cells. As only a fraction of T cells is capable of directly recognizing foreign HLA molecules for any given donor-recipient pair, expansion of the alloreactive T cells occured prior to

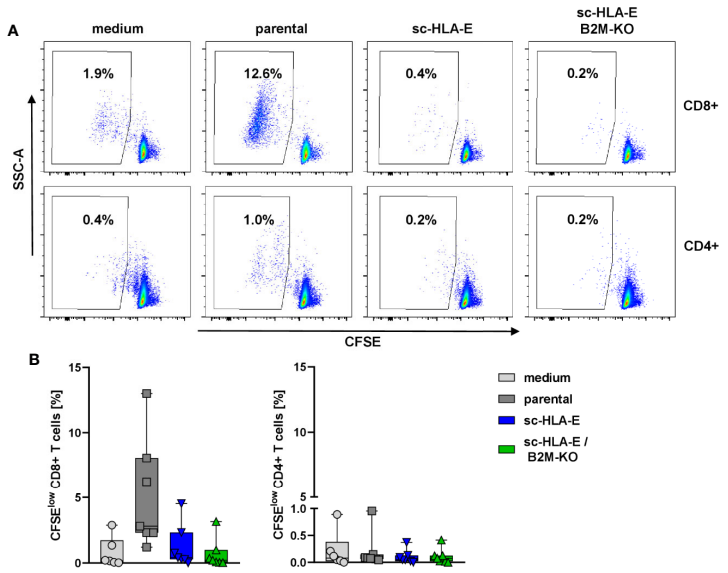

FIGURE 4 | CFSE proliferation assay to measure the immune response of allogeneic T cells towards the modified NK cells. **(A)** Representative flow plots showing the frequency of $CFSE^{low}$ $CD8^+$ (upper panel) and $CD4^+$ (lower panel) T cells after 6 days of co-incubation with NK cells carrying the modifications depicted above the plots. **(B)** Quantification of activated $CFSE^{low}$ $CD8^+$ (left) and $CD4^+$ (right) T cells after co-incubation with the modified NK cells. Statistical analysis was performed using one-way ANOVA with Holm-Sidak testing for multiple comparisons. The higher frequency of $CFSE^{low}$ $CD8^+$ T cells was statistically significant compared to sc-HLA-E and sc-HLA-E/B2M-KO NK cells (Box plots including median, quartiles and all data points, n = 7).

the experiments by co-culture ("priming") with 30 Gy-irradiated PBMCs of the specific NK cell donor for 14 days. Subsequently, these T cells were co-cultured with NK cells and then analyzed for expression of CD137 or CD107a as markers for activation and degranulation, respectively. Autologous and also HLA-disparate "3rd party" NK cells served as important controls.

These co-culture experiments with primed T cells revealed a specific activation of $CD8^+$ but not $CD4^+$ T cells in presence of parental as well as sc-HLA-E-expressing NK cells for up to 48 h as measured by CD137 expression (**Figures 5A, B**). In contrast, sc-HLA-E/B2M-KO NK cells did not induce expression of CD137 in a significant fraction of $CD8^+$ T cells, similarly to co-cultures with autologous and also 3rd party NK cells, thus confirming the donor specificity of the assay. Analysis of degranulation by CD107a staining at 24 h of co-culture revealed a similar pattern (**Figure 5C**), with degranulation in the presence of parental and sc-HLA-E-expressing NK cells, while the CD107a levels of T cells challenged with sc-HLA-E/B2M-KO NK cells was comparable to those using autologous and 3rd party controls. Finally, the specific cytotoxicity towards the different genetically modified NK cells was assessed with purified populations at effector to target ratios of 4:1, 2:1 and 1:1 (calculated on $CD8^+$ T cells). After 20 h of co-culture with primed T cells (**Figure 5D**), between 10 and 40% of parental and sc-HLA-E expressing NK cells were killed in a dose-dependent manner. In contrast, sc-HLA-E/B2M-KO NK cells were not lysed at E:T ratios of 1:1 and 2:1, and even E:T ratios of 4:1 resulted in only <10% lysis.

DISCUSSION

In this study, we have established a robust methodology to generate primary NK cells that are devoid of classical HLA class I molecule surface expression. Compared to their unmodified counterparts, the genome-edited NK cells escaped immune recognition by mismatched $CD8^+$ and $CD4^+$ T cells, thus making them suitable tools for "off-the-shelf" allogeneic immunotherapy. To achieve this, we first had to overcome the obstacle that NK cells are "hard-to-transduce" cells. This *relative* resistance of primary human NK cells to lentiviral and also alpha-retroviral vectors using VSV-G or RD114 pseudotypes was just recently documented again (86) and is simply due to the low expression levels of the cellular proteins that serve as surface receptors for entry of such pseudotyped vector particles (68). Based on the pioneering work of Els Verhoeyen and her colleagues establishing the envelope of the baboon endogenous virus (BaEV) as novel pseudotype for human primary cells (69), two recent studies demonstrated efficient NK cell transduction with the BaEVRless envelope using either CH296/retronectin-coated plates (87) or Vectofusin-1 as enhancers of viral uptake (68, 69, 88). However, as the BaEVRless envelope with the deletion of the

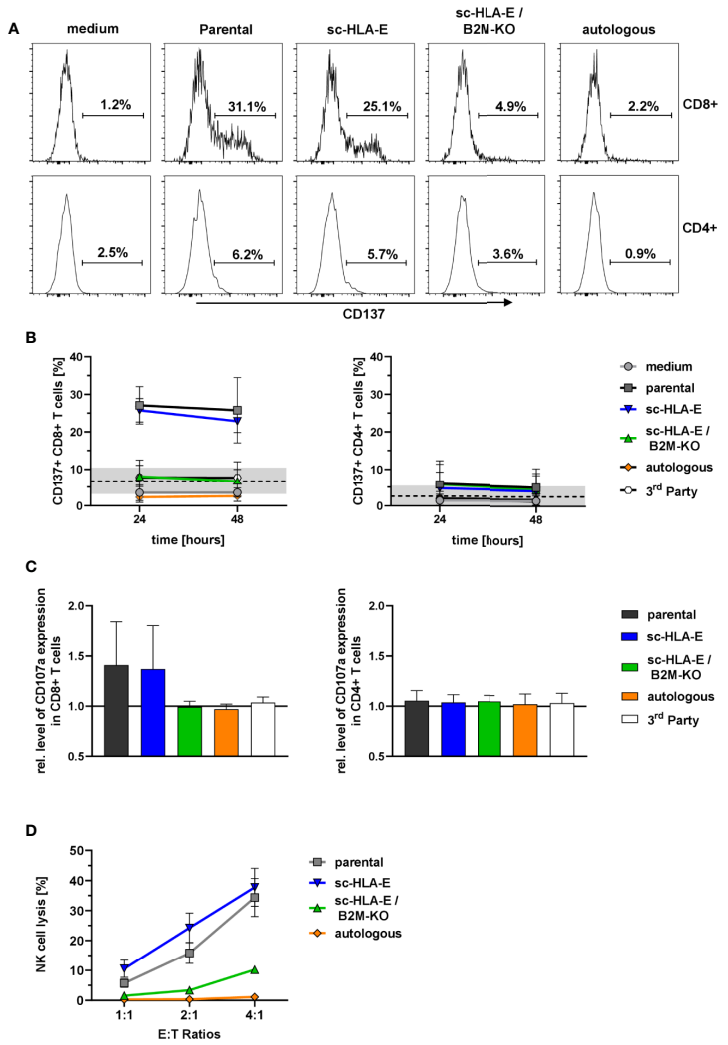

FIGURE 5 | Activation and degranulation of primed T cells in presence of modified NK cells. **(A)** Representative flow plots showing the frequency of $CD137^+$ cells within $CD8^+$ (upper panel) and $CD4^+$ (lower panel) T cell subsets after 24 h of co-incubation with the different NK cell cultures depicted above the plots. **(B)** Quantification of $CD137^+$ frequencies among $CD8^+$ (left) and $CD4^+$ (right) T cells at two time points. Statistical analysis was performed by two-way ANOVA with matching by time points and Holm-Sidak testing. At both time points, the frequency of $CD137^+$ cells within the $CD8^+$ T cells was significantly lower when challenged with sc-HLA-E/B2M-KO NK compared to parental and sc-HLA-E NK cell containing cultures (mean ± SD; n = 7 for medium control, parental, sc-HLA-E, sc-HLA-E/B2M-KO NK cells, n = 4 for autologous and 3rd party controls; black line and grey box indicate the mean of the baseline measurements ± 95% confidence interval). **(C)** Levels of CD107a normalized to the medium controls in $CD8^+$ (left) and $CD4^+$ (right) T cells 24 h after co-incubation with NK cells. Statistical analysis was performed using Friedman test with Dunn's correction for multiple comparisons. Levels of CD107a were significantly lower for $CD8^+$ T cells incubated with sc-HLA-E/B2M-KO NK cells compared to T cells incubated with parental or sc-HLA-E NK cells (mean ± SD; n = 7 for medium control, parental, sc-HLA-E, sc-HLA-E/B2M-KO NK cells, n = 4 for autologous and 3rd party controls). **(D)** Lysis of NK cells by primed T cells at the effector targets ratios 4:1, 2:1 and 1:1 (calculated on $CD8^+$ T cells) after 20 h of co-incubation. Statistical analysis was performed by two-way ANOVA with Holm-Sidak testing for multiple comparisons. The reduced lysis of sc-HLA-E/B2M-KO NK cells was statistically significant at E:T ratio 4:1 compared to parental and sc-HLA-E NK cells and at ratio 1:1 compared to sc-HLA-E NK cells but not to parental NK cells, yet the p value almost met the criterion with p = 0.0516 (mean ± SD; n = 5).

R protein is highly fusogenic already in the packaging cells, we constructed another version of the BaEV envelope featuring a fusion of the surface and transmembrane regions with the cytoplasmic tail of the amphotropic endogenous murine retrovirus, as described (69). This pseudotype for lentiviral vectors enabled us to reproducibly and efficiently perform genome editing of primary NK cells.

In past clinical trials, mainly genetically non-manipulated allogeneic NK cells were used for immunotherapy of malignancies including AML, myeloma and solid tumors (17, 20, 22, 23, 89). The clinical response rates were highly variable, ranging from 26 to 50% and often with only transient improvements (17, 20, 22, 23). Remarkably, no GvHD was observed in these trials despite the various HLA mismatch constellations, except for one study with higher T cell contaminations (89). All NK cell trials had two things in common: (i) preconditioning therapy using fludarabine and cyclophosphamide to deplete recipient lymphocytes in order to avert immunological rejection, and (ii) subcutaneous injections of IL-2 to facilitate NK cell engraftment and maintenance. The study from Miller et al. (20) showed that only high-intensity conditioning using fludarabine and high-dose cyclophosphamide was able to facilitate engraftment of NK cells beyond day 5 post infusion, compared to regimens that administered only fludarabine or low-dose cyclophosphamide and prednisolone, arguing that rigorous lymphodepletion is indispensable for successful engraftment and post-injection expansion. Importantly, the lymphodepleting conditioning was accompanied by a rise of endogenous IL-15 levels which roughly correlated with NK cell *in vivo* expansion. In all trials with high-dose conditioning (17, 20, 22, 23), donor-derived NK cells were detected in the patients by PCR for up to 28 days post infusion. During these four weeks, a decline in numbers was usually evident between 8 and 17 days (17, 20, 22, 23), which coincided with the patients' with hematopoietic recovery and rise in endogenous T cell counts. Additionally, Shi et al. (23) reported that T cells from patients treated with NK cells showed reactivity towards donor-derived PBMNCs in an *in vitro* MLR. This finding is bolstered by the observation by Curti et al. (17) that a second infusion of NK cells is rejected even quicker than the first one: 5 days vs. 17 days, respectively. Taken together, these results strongly suggest that the mounting of an alloreactive immunological T cell memory response is a major contributing factor for the short-term NK cell persistence. Shi and colleagues even argued that the regular IL-2 injections might have facilitated the quick establishment of an allogeneic T cell response (23).

Thus, these clinical studies highlight the potential benefit of a knockout of HLA class I for allogeneic NK cell therapy to avoid donor-specific alloreactions of the patient's T cells and extend the persistence of the transfused NK cells. In addition, the evasion from a pool of alloreactive patient T cells, whose numbers would inevitably build up due to indirect allorecognition after infusion, should readily enable multiple infusions and even has the potential to make lymphotoxic conditioning obsolete.

We achieved the functional deficiency of HLA class I molecules by a lentiviral CRISPR/Cas9-mediated knockout of B2M. However, given that HLA class I expression protects against NK cell recognition, it is not surprising that B2M-KO NK cells did not persist in culture, but were lysed by their neighboring NK cells based on the "missing self" activation. The phenomenon of NK cells killing each other, called fratricide, has been observed before, yet in other contexts. In murine NK cells, for example, trogocytosis of NKG2D ligands from tumor cells can trigger fratricide, which has been proposed as a negative feedback loop to control NK cell activation (90). Patients with multiple myeloma, who were treated with the monoclonal antibody daratumumab targeting CD38, clinically benefitted from the antibody treatment. However, an unexpected side effect was the loss of CD38+ autologous NK cells in the peripheral blood and even in the bone marrow of the patients *via* an antibody-dependent cellular cytotoxicity (ADCC) (91, 92). In an experimental setting, this fratricide of autologous CD38+ NK cells was overcome by a CRISPR/Cas9-mediated knockout of CD38 in *in vitro* expanded NK cells (91), thus providing a potential therapeutic strategy to enhance the efficacy of the antibody infusions further.

To avoid fratricide, we co-expressed a sc-HLA-E molecule as described by Gornalusse and colleagues (58) as an efficient approach to protect HLA-deficient PSC-derived cells from NK cell lysis. Despite the necessity to introduce two genetic modifications in the NK cells, the knockout of B2M and the overexpression of sc-HLA-E, we noted only a minor reduction in the expansion kinetics/characteristics of our NK cell cultures, when we transduced the cells simultaneously with the mixture of both concentrated supernatants. It seems likely that this reduction can be attributed to the higher vector doses used to achieve efficient transduction and editing frequencies. For clinical purposes however, the NK cells will be expanded for at least 21 days in a closed system such as the Prodigy (74, 75), thus sufficient opportunities for sequential genetic manipulations can be established in an optimal cell expansion protocol. Additionally, there is no need to purify the edited cells, as they would simply persist due to their immune evasive properties, thus facilitating a simple manufacturing process. To further validate the fratricide hypothesis and exclude that the loss of B2M directly led to NK cell death, we performed the fratricide assays using NK cells in which the *KLRC1* gene, coding for the inhibitory receptor NKG2A that recognizes sc-HLA-E, had been knocked out by genome editing. In these experiments, NKG2A-deficient NK cells eliminated the B2M-KO cells, regardless of whether sc-HLA-E was expressed or not.

The phenotypical and functional analyses revealed robust concordances between the parental and the genetically modified NK cells. While the killing of established target cells for NK cells such as K562 was comparable, the only notable difference between the parental NK cells and those expressing sc-HLA-E, regardless of the B2M-KO, was the lower expression level of NKG2A. One obvious explanation of the diminished NKG2A surface expression here is that the overexpressed sc-HLA-E already binds to NGK2A within the cells, thus leading to retention of the complex. This idea is clearly reminiscent of the approach developed by Kamiya and colleagues, in which they engineered NKG2A$^{dim/-}$ NK cells for immunotherapy by cytoplasmically targeting NKG2A with a scFv fused to an

endoplasmatic reticulum (ER) retention peptide, thereby retaining NKG2A in the ER (93).

Curiously, sc-HLA-E-expressing NK cells with intact HLA class I expression did not evoke allogeneic T cell proliferation, while the sc-HLA-E positive NK cells were still efficiently lysed when the same T cells were pre-activated in an MLR with irradiated feeder cells for 14 days. One obvious explanation is that the frequency of alloreactive T cells against a specific HLA type is relatively low and that these few T cells upon activation upregulate NKG2A. The newly expressed NKG2A is rapidly engaged by a sc-HLA-E molecule on neighboring NK cells, thus hampering the activation and the proliferation of the alloreactive T cells. Although this situation can readily occur in an *in vitro* setting in which the activated T cell is surrounded by sc-HLA-E expressing NK cells, *in vivo* the likelihood of such interactions is very low and one can expect numerous events of indirect immune recognition that will inevitably generate a large pool of alloreactive T cells capable of eliminating all HLA divergent cells.

In summary, we think that the universal "off-the-shelf" effector cell product for adoptive cellular therapies should be B2M-deficient NK cells overexpressing sc-HLA-E. These cells will be completely invisible for allogeneic T cell responses and will be protected from NKG2A+ recipient NK cells. Whether these modifications are sufficient for such modified NK cells to evade recognition and destruction of the patient's immune system needs to be explored in clinical trials. Nevertheless, our modifications appear to be highly valuable to enhance efficacy of CAR-modified NK cells. Indeed, a recently published seminal NK cell study for CD19-positive lymphoid tumors by Liu and colleagues used a single dose of partly matched (mostly 4/6 with regard to A, B and DRβ1) allogeneic NK cells that had been transduced with a retroviral vector encoding three different transgenes: a CD19 CAR, soluble IL-15 and the iCASP9 suicide gene (19). In eleven treated patients, neither GvHD nor a cytokine release syndrome occurred. Thus, the suicide gene was never employed (19). Independent of the cell doses infused, eight patients (75%) had a clear immune response against the CD19+ malignant cells, which was complete and lasting in seven out of the eight patients. Remarkably, the additionally expressed IL-15 appeared to promote the long-term expansion of the donor NK cells *in vivo* for up to 12 months (19). Although cellular alloreactions by the recipients' T cell systems subsequent to the infusions were not tested and probably strongly influenced by the lymphodepleting conditioning in these heavily pretreated patients, it cannot be ruled out that the high degree of HLA matching, the expression of IL-15 and the variety of additional treatments and substances that the patients received after the NK cell infusions all played major roles.

Our study adds the knockout of B2M in combination with sc-HLA-E expression as another building block to the development of "off-the-shelf" cellular NK cell therapies to enable manufacturing of safer and more efficient cell products to benefit a larger group of patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KH and SH conceived the experiments. KH and CW performed experiments and analyzed data. KH, MU, HH, PH and SH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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3.2 Publication II

CD33 Delineates Two Functionally Distinct NK Cell Populations Divergent in Cytokine Production and Antibody-Mediated Cellular Cytotoxicity

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CD33 Delineates Two Functionally Distinct NK Cell Populations Divergent in Cytokine Production and Antibody-Mediated Cellular Cytotoxicity

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The generation and expansion of functionally competent NK cells *in vitro* is of great interest for their application in immunotherapy of cancer. Since CD33 constitutes a promising target for immunotherapy of myeloid malignancies, NK cells expressing a CD33-specific chimeric antigen receptor (CAR) were generated. Unexpectedly, we noted that CD33-CAR NK cells could not be efficiently expanded *in vitro* due to a fratricide-like process in which CD33-CAR NK cells killed other CD33-CAR NK cells that had upregulated CD33 in culture. This upregulation was dependent on the stimulation protocol and encompassed up to 50% of NK cells including $CD56^{dim}$ NK cells that do generally not express CD33 *in vivo*. RNAseq analysis revealed that upregulation of $CD33^{+}$ NK cells was accompanied by a unique transcriptional signature combining features of canonical $CD56^{bright}$ ($CD117^{high}$, $CD16^{low}$) and $CD56^{dim}$ NK cells (high expression of granzyme B and perforin). $CD33^{+}$ NK cells exhibited significantly higher mobilization of cytotoxic granula and comparable levels of cytotoxicity against different leukemic target cells compared to the $CD33^{-}$ subset. Moreover, $CD33^{+}$ NK cells showed superior production of IFNγ and TNFα, whereas $CD33^{-}$ NK cells exerted increased antibody-dependent cellular cytotoxicity (ADCC). In summary, the study delineates a novel functional divergence between NK cell subsets upon *in vitro* stimulation that is marked by CD33 expression. By choosing suitable

stimulation protocols, it is possible to preferentially generate CD33$^+$ NK cells combining efficient target cell killing and cytokine production, or alternatively CD33$^-$ NK cells, which produce less cytokines but are more efficient in antibody-dependent applications.

Keywords: NK cell, CD33-CAR, cytokine production and cytotoxicity, RNAseq analysis, NK cell expansion

INTRODUCTION

Natural Killer (NK) cells are getting increasingly into the focus of allogeneic cell-based therapy due to their powerful effector mechanisms enabling eradication of tumor and virus-infected cells without eliciting graft-*versus*-host disease (1). Classically, circulating NK cells are divided into CD56^{bright} NK cells that are non-cytotoxic and primarily respond with IFNγ and TNFα production in response to exogenous cytokines and CD56^{dim} NK cells that are uniquely able to exert cytotoxic effector functions *via* CD16-mediated antibody-dependent cellular cytotoxicity (ADCC) as well as direct targeting of aberrant cells on the basis of missing self-recognition and expression of stress-induced ligands (2). Beyond these two major subsets, a multitude of less well-defined NK cell subpopulations with more subtle differences exist along a continuum of different functional states (3). Unfortunately, in NK cells stimulated and expanded *in vitro*, functional distinction of NK cell subsets on the basis of CD56 expression is not useful due to unspecific upregulation on virtually all NK cells. Similarly, other function-associated surface molecules are highly sensitive to shedding, such as CD16, or are quickly downregulated in culture, such as CD62L (4, 5). Thus, although it is apparent that the available protocols to expand NK cells do not lead to a homogenous pool of NK cells but rather result in a heterogeneous mixture of NK cell subsets with divergent functional capabilities, the tools to differentiate between functional subsets on the basis of surface molecules are scarce.

CD33 (siglec-3) is the smallest member of the sialic acid-binding immunoglobulin-like lectin (Siglec) family with only two Ig superfamily (IgSF)-like domains, a distal V domain mediating sialic acid binding and a membrane-proximal C2 domain (6). Besides the Siglec family-defining binding of CD33 to sialylated ligands, such as glycoproteins, glycolipids, and gangliosides, more specific protein ligands are currently unknown, with the exception of the recently defined complement component 1q (C1q) (7). The presence of an immunoreceptor tyrosine-based inhibitory motif (ITIM) and a second ITIM-like motif in the cytoplasmic domain suggests that CD33 is an inhibitory receptor, but the role of CD33 and its ITIM-mediated inhibition for regulation of the activation states of immune cells is elusive. Expression of CD33 is assumed to be largely restricted to the myeloid lineage, and with the exception of the CD56^{bright} NK cell subset, it seems to be absent from the lymphocytic lineages including mature T, B, and CD56^{dim} NK cells (8). CD33 is broadly expressed on various myeloid lineages and has gained therapeutic relevance as target on CD33-expressing myeloid and rare subsets of acute lymphoblastic leukemia (9).

Here, we studied the relevance of CD33 expression on *in vitro* stimulated NK cells, triggered by the initial observation of fratricide in cultures of CD33-CAR NK cells. We demonstrate that CD33 can be exploited to define two functionally distinct NK cell subsets representing CD33$^+$ polyfunctional NK cells capable of strong cytokine production and target-based cytotoxicity and a CD33$^-$ subset exhibiting efficient antibody-dependent cellular cytotoxicity (ADCC) due to strong expression of CD16. Notably, the CD33$^+$ subset becomes highly abundant when using a commercially available medium-based protocol, whereas it remains only a minor subset when employing protocols using established stimulator cell lines. Thus, the frequency of the CD33 subset in the expanded NK cell product can be controlled *a priori* by choosing a suitable protocol for NK cell stimulation.

MATERIALS AND METHODS

Human Samples

Buffy coats of healthy donors were kindly provided by the Blutspendezentrale at the University Hospital Düsseldorf. The protocol was accepted by the institutional review board at the University of Düsseldorf (study number 2019-383) and is in accordance with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMC) were isolated using density gradient centrifugation with Lymphocyte Separation Medium (PromoCell, Heidelberg, Germany).

Flow Cytometry

The following fluorescence-labeled monoclonal antibodies (mAb) were used: CD3-FITC or PE/Cy5 (clone UCHT1), CD11c-FITC (3.9), CD16-APC/Cy7 (3G8), CD30-PE/Cy7 (BY88), CD33-PE, BV605 or PE/Cy5 (P67.6), CD56-PE/Dazzle^{TM}594 (N901), CD57-FITC (HCD57), CD62L-PE/Cy7 (DREG56), CD107-BV510 (H4A3), CD117-BV421 (104D2), KLRG1-APC/Fire 750 (SA231A2), PD1-APC/Cy7 (EH12-2H7), NKG2D-PE (1D11), NKp46-BV510 (9E2), NKp44-APC (P44-8), NKp30-BV785 (P30-15), Granzyme B-Pacific Blue (GB11), IFNγ PE/Cy7 (B27), Perforin-PE (dG9), TNFα PE/Dazzle^{TM} 594 (all from Biolegend, CA, USA), CD3 PerCP-Vio700 (REA613), CD14-PerCP-Vio700 (REA599), CD33-APC, CD56 (REA196), anti-biotin-VioBright515 and 7-AAD (all Miltenyi Biotec), NKG2C-AF700 (134591) from R&D systems (MN, USA), and CD158b1,b2,j-PE/Cy5 (GL183), NKG2A-APC (Z199) purchased from Beckman Coulter (CA, USA). Flow cytometric analyses were performed on a CytoFLEX (Beckman Coulter) or MACSQuant 10 Analyzer (Miltenyi Biotec). Data analysis was performed on Kaluza 2.1.1 software.

Cell Lines

The HLA class I-deficient target cell line K562 was grown in Dulbecco's modified Eagle's medium (DMEM) 4.5 g/L Glucose with L-Glutamine (Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS, FBS, Merck) and 1% Gentamycin. Human Burkitt lymphoma cell line Raji was cultivated in RPMI-1640 (Thermo Fisher Scientific, Waltham, MA, USA), 1% Penicillin/Streptomycin (P/S) (Gibco), and 10% FBS. K562-mb15-41BBL (10) (kindly provided by D. Campana, National University of Singapore) and K562-mb15-mb21-41BBL (11) were cultured in RPMI-1640, 1% Penicillin/Streptomycin, and 10% FBS. Human RS4;11 B cell precursor acute lymphoblastic leukemia (B-ALL) cells ectopically expressing human CD33 and GFP (RS4;11-CD33) or GFP only (RS4;11-GFP) were cultured in RPMI 1640 supplemented with 10% FBS and 2 mmol/L L-glutamine (Gibco). All cell lines used were free of mycoplasma.

NK Cell Expansion

Isolation of pure NK cells was performed with MojoSort™ Human NK Cell Isolation Kit (Biolegend). PBMC or enriched NK cells were cultured in NK MACS medium [1% NK MACS supplement, 5% human AB serum (Sigma Aldrich), 500 U/ml IL-2 (Proleukin), and 25ng/ml IL-15 (Miltenyi Biotec)]. NK cell expansion with stimulator cells was performed as previously described (10). Briefly, PBMCs (1.5×10^6) were incubated in 24-well plates with 1×10^6 irradiated (40 Gy) K562-mb15-41BBL cells or K562-mb15-mb21-41BBL in SCGM CellGro Medium (CellGenix, Freiburg, Germany) supplemented with 10% FBS and 1% P/S and 40 U/ml human IL-2 (Proleukin).

Chimeric Antigen Receptor NK Cells

CAR constructs were designed *in silico* and consist of a human GM-CSFRα signal peptide, an antigen-specific scFv derived from Gemtuzumab (CD33-CAR) or FMC63 (CD19-CAR), a CD8α hinge and transmembrane (TM) domain, followed by 4-1BB and CD3ζ intracellular (IC) domains. Codon-optimized CAR constructs were inserted into a third-generation lentiviral plasmid backbone (Lentigen Technology Inc., Gaithersburg, MD, USA) under control of a human EF-1α promoter. Baboon envelope (BaEV) pseudotyped lentiviral vectors (LV) containing supernatants were generated by transient transfection of HEK 293T cells, as previously described (12). NK cell enrichment was performed with NK cell isolation kit for human cells (Miltenyi Biotec). NK cells stimulated with NK MACS medium and 80 ng/ml of IL-1ß (day 2) were transduced with BaEV-LV encoding CD33-CAR or CD19-CAR constructs in the presence of 10 µg/ml Vectofusin-1 for 24 h, after 2 h spinoculation at 400 × g, 32°C (13). CAR expression was flow cytometrically evaluated using biotinylated human recombinant CD33-Fc protein (R&D Systems) or CD19 detection reagent (Miltenyi Biotec), respectively, followed by VioBright515-conjugated antibiotin antibody (Miltenyi Biotec).

RNA Sequencing

$CD33^+$ and $CD33^-$ NK cells were flow cytometrically sorted (MoFlo XDP, Beckman Coulter) and stored in TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) for extraction of total RNA. Reverse transcription and library production were performed in the NGS Integrative Genomics (NIG) facility in Göttingen, Germany, with an Illumina Truseq RNA preparation kit. Sequencing of the libraries was performed on an Illumina HiSeq4000 (single-read 1 × 50 bp). Sequence reads were mapped to the human genome (hg38) and analyzed using DESeq2 software (v1.26.0) as described previously (14, 15). Heatmaps and volcano plots were performed with R packages pretty heatmap (pheatmap) (v1.0.12) (16) and EnhancedVulcano (1.4.0) (17), respectively.

NK Cell Function

For analysis of degranulation and cytokine production, NK cells and K562 target cells were mixed at an effector/target (E/T) ratio of 1:1 in a volume of 200 µl in a 96-well plate (round bottom). CD107a mAb was added prior to incubation. To determine spontaneous degranulation or cytokine production, control samples without target cells were included. After 1 h incubation time, 2 µl of 2 mM Monensin and 2 µl Brefeldin (1000×, Biolegend) were added and samples incubated for further 4 h. Subsequently, cells were stained for selected surface markers. Interferon-γ and TNF-α were intracellularly stained following treatment with fixation and permeabilization buffer (Biolegend). For cytotoxicity analysis, K562 target cells were stained with 5 mM CFDA-SE (Invitrogen) and mixed with NK cells at various E/T ratios. After 5 h incubation, propidium iodide (PI, Biolegend) was added shortly before flow cytometric analysis to quantify the viable target cells as described previously (18). In some experiments, cytotoxicity of CAR NK cells was assessed against GFP-expressing tumor cells. In order to determine CD33-CAR-induced NK cell fratricide, *in vitro* expanded NK cells were labeled with 1 µM CellTrace Violet (CV, Thermo Fisher Scientific, Waltham, MA, USA) and cocultured with unlabeled CD33-CAR NK cells at various E/T ratios for 24 h. For ADCC analysis, 1 µg/ml of rituximab (Truxima®, Celltrion Healthcare, South Korea) was added directly to the co-culture of NK cells and Raji target cells (ratio 1:1). NK cells without Raji and NK cells with Raji but without Rituximab served as controls.

Statistical Analyses

Normal distribution of the data was calculated with Shapiro-Wilk normality test. Depending on normality, paired/unpaired t-test or Mann-Whitney test was performed. The difference between more than two groups was analyzed with 1-Way ANOVA. All analyses were done using GraphPad Prism 8.0.0 (GraphPad Software, CA, USA).

RESULTS

$CD33^+$ NK Cells Are Recognized and Eliminated by CD33-CAR NK Cells

Our interest in $CD33^+$ NK cells was fueled by an observation made during the generation and expansion of CD33-specific

CAR NK cells for therapy of myeloid leukemia. To this end, PBMC-derived and magnetically enriched NK cells were transduced with a Baboon envelope (BaEV)-pseudotyped lentiviral vector (LV) carrying a CD33-specific CAR. The construct consisted of an antigen-specific scFv antibody domain derived from the CD33-specific mAb Gemtuzumab, CD8α hinge and transmembrane (TM) domains, and 4-1BB and CD3ζ intracellular (IC) domains (**Figure 1A**). Following lentiviral transduction, CD33-CAR NK cells were stimulated using NK MACS medium, supplemented with IL-2 and IL-15, for 2 weeks. As shown in **Figure 1B**, NK cells expanded much less in the transduced setting compared to non-transduced NK cells. Flow cytometric analyses revealed that this was due to selective depletion of $CD33^+$ NK cells, whereas $CD33^-$ NK cells remained unaffected (**Figure 1C**). Depletion of $CD33^+$ NK cells was dependent on the presence of CD33-CAR NK cells since in non-transduced controls, $CD33^+$ NK cells were present at high frequencies (>50%) (**Figure S1**). Furthermore, when employing control CAR constructs with specificity for CD19 instead of CD33 (but otherwise identical features), no such effects on the frequency of $CD33^+$ NK cells were noted, and no overt inhibition of NK cell expansion was observed for CD19-CAR-transduced compared to non-transduced NK cells (**Figure 1D**). The data suggested that $CD33^+$ NK cells arising in the cultures were targets for CD33-CAR NK cells. To more closely look into this possibility, we analyzed the cytotoxic activity of CD33-CAR NK cells against autologous NK cells, labeled with a cell tracing fluorescent reagent. Indeed, CD33-CAR NK cells exhibited substantial cytotoxicity against autologous NK cells that were stimulated for 2 weeks in order to induce CD33 expression, whereas non-transduced NK cells did not show cytotoxicity against the same autologous NK cell targets (**Figure 1E**). Finally, the specificity of CD33-CAR NK cells was tested against $CD33^+$ tumor cells: the RS4;11 tumor cell line was killed by CD33-CAR NK cells with high efficiency when expressing CD33, whereas a variant that did not express CD33 (GFP^+RS4;11) was not recognized (**Figure 1F**). Similarly, non-transduced NK cells did not kill the tumor cells regardless of

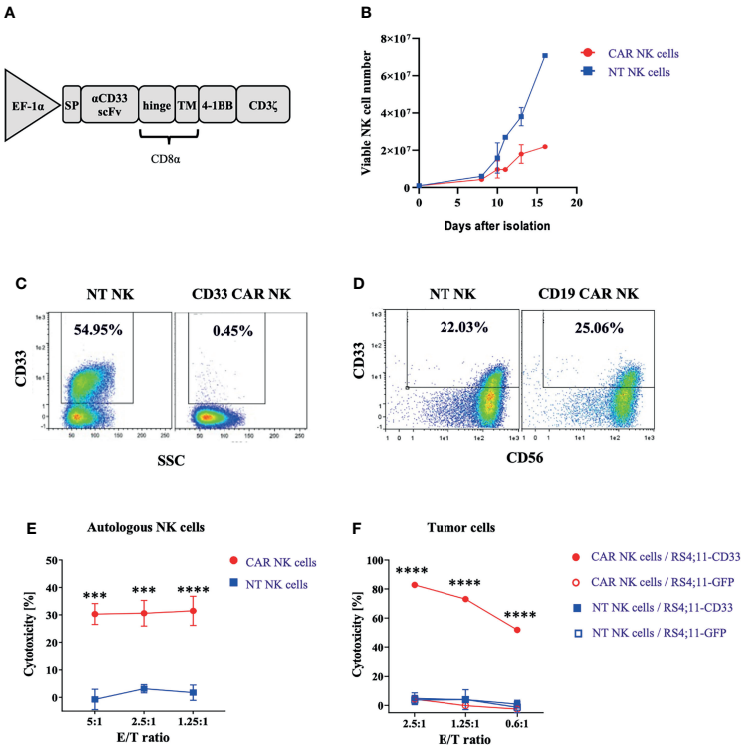

FIGURE 1 | CD33-CAR-mediated NK cell fratricide. **(A)** CD33-CAR construct design. **(B)** *In vitro* proliferation of non-transduced (NT) NK cells and CD33-CAR NK cells after isolation. CD33 expression on **(C)** CD33-CAR NK cells and **(D)** CD19-CAR NK cells was determined by flow cytometry (day 14) compared with NT NK cells **(E)** CD33-CAR-induced NK cell fratricide with *ex vivo* expanded autologous NK cells as targets. NT NK cells were included as control. **(F)** Cytotoxicity of CD33-CAR NK cells against CD33-expressing RS4;11 tumor cells. NT NK cells and CD33-negative RS4;11-GFP cells were included as controls. Data were analyzed by two-tailed unpaired t-test ***$p < 0.001$; ****$p < 0.0001$.

CD33 expression due to natural resistance of RS4;11 cells to NK cell-mediated killing. Thus, the observed killing of NK cells by other autologous NK cells, referred to as fratricide, was due to recognition of CD33 by the respective CD33-CAR NK cells.

CD33 Expression in Culture Is Primarily Due to Upregulation on CD56^{dim} NK cells and Depends on the Stimulation Protocol

CD33 is expressed *in vivo* on CD56^{bright} but not CD56^{dim} NK cells (8). We were thus wondering if the CD33^{+} NK cells developing in culture were also primarily due to expansion of CD56^{bright} NK cells. We thus flow cytometrically sorted the two respective CD56 subsets to high purity from peripheral blood and cultured them separately for 4 weeks in NK MACS medium. As shown in **Figure 2A**, both subsets contributed to the expansion of the CD33^{+} subset: whereas in CD56^{bright} NK cells the large majority of cells maintained CD33 expression, in CD56^{dim} NK cells the frequency of CD33 increased over time from basically no expression at the beginning of culture to around 50% at day 28 as shown in the exemplary experiment in **Figure 2A**. The CD56^{dim} data largely resembled the kinetics of CD33 expression in the control setup using unseparated NK cells, which was expected since CD56^{dim} NK cells typically constitute more than 90% of all NK cells and thus are the dominant subset contributing to the increase in CD33^{+} cells observed in the cultures (**Figure 2A**).

Of note, in about 10% of samples, CD33 expression remained low during stimulation, and this was accompanied by a lack of CD33 expression on CD56^{bright} NK cells *in vivo* (**Figure S2A**).

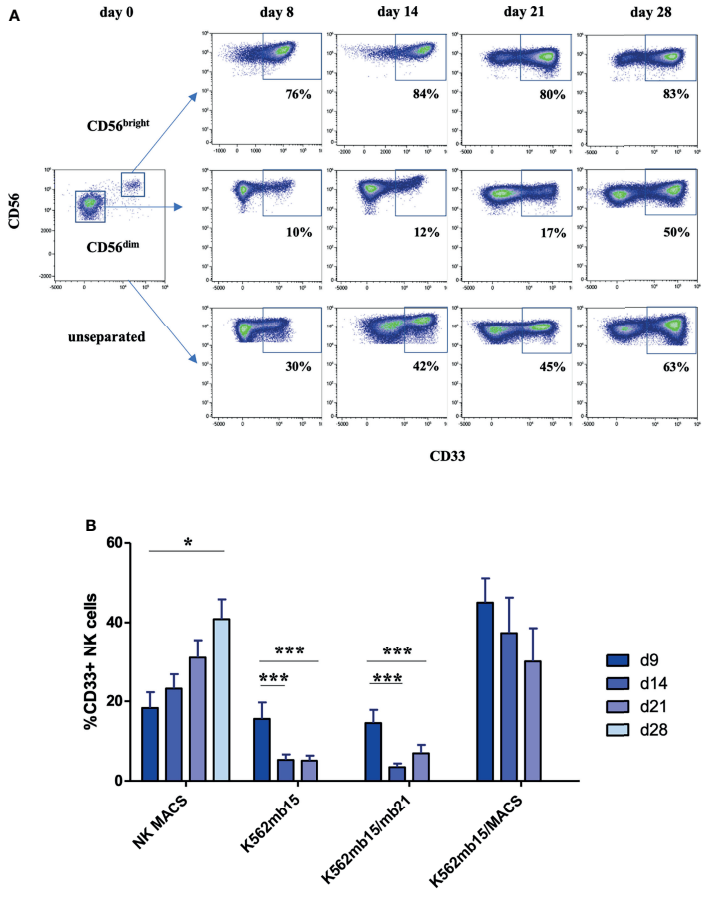

FIGURE 2 | Upregulation of CD33 on CD56^{dim} NK cells depends on stimulation protocol. **(A)** CD56^{dim} and CD56^{bright} NK cells were enriched by flow cytometric cell sorting and subsequently cultured in NK MACS medium for 28 days. Unsorted NK cells served as control. Flow cytometric dot plots of one representative donor of three show CD33 expression on CD56^{dim}, CD56^{bright}, and unseparated NK cells cultured in NK MACS medium on days 0, 8, 14, 21, and 28. **(B)** CD33 expression on NK cells stimulated with different stimulatory protocols using NK MACS medium (n=10), K562-mb15-41BBL (n=10), or K562-mb15-mb21-41BBL (n=8) cells and combination of K562-mb15-41BBL and NK MACS medium (n=3) on days 0, 8, 14, 21, and 28. Data were analyzed by 1-way ANOVA, *p < 0.05; ***p < 0.001.

Subsequent search for a putative underlying genetic polymorphism by targeted sequencing of CD33 (**Figures S2B, C**) revealed that all samples, which did not express CD33 on the $CD56^{bright}$ subset and failed to upregulate CD33 during culture, carried a previously described single nucleotide polymorphism (SNP, rs12459419 C>T) associated with skipping of exon 2 encoding the IgV domain of CD33 due to alternative splicing (19). The SNP occurred either in homozygous configuration or in one case in combination with a known, more rarely occurring null allele (**Figure S2C**). Since the IgV domain encodes the epitope recognized by the CD33-specific antibody used in the CAR (and other commercially available mAbs), failure to detect CD33 expression in culture seems to be largely due to this splicing polymorphism (20). This notion is also compatible with the allelic frequency of the SNP (rs12459419), which is found in homozygous configuration in approximately 10% of the Caucasoid population (https://gnomad.broadinstitute.org/variant/19-51728477-C-T?dataset=gnomad_r2_1).

Besides NK MACS medium, another established method for expansion of primary NK cells utilizes stimulator cells expressing the ligand for 4.1BB (CD137) together with a membrane-bound version of IL-15, IL-21, or both (10, 11). In order to understand how far the particular stimulatory protocol influences the upregulation of CD33, we comparatively analyzed the different NK cell stimulation protocols side by side. As shown in **Figure 2B**, upregulation of CD33 was significantly stronger when using NK MACS medium compared to protocols using K562 stimulator cells expressing 4.1BB and membrane-bound IL-15 (K562-mb15-41BBL) or additionally membrane-bound IL-21 (K562-mb15-mb21-41BBL). Analysis of the kinetics revealed that whereas NK MACS medium led to a continuous increase in CD33 expression over time as already outlined above, the K562-based protocols exhibited an initial moderate increase to 10–20% $CD33^+$ cells before significantly decreasing to <5% at day 14 and maintaining low levels until day 21 (the timepoint when NK cells are harvested according to the protocol). Mechanistically, upregulation of CD33 expression could be either a default process during *in vitro* stimulation of NK cells that is eventually inhibited by the stimulator cell lines or the NK MACS medium could provide a soluble stimulus for CD33 expression. We thus combined both protocols by using K562-mb15-41BBL cells together with NK MACS medium and found that CD33 expression is rescued by addition of the NK MACS medium (**Figure 2B**), suggesting that a soluble medium-derived factor is involved in induction of CD33 expression on NK cells.

CD33 Delineates Two NK Cell Subsets With Unique Transcriptional Signatures

In order to gain more insights into the phenotypic and transcriptional characteristics of $CD33^+$ and $CD33^-$ NK cell subsets, PBMC-derived NK cells were cultured for 2 weeks in NK MACS medium and subsequently sorted by flow cytometry into the two CD33 subsets for further analysis by RNAseq. Heatmap analyses demonstrated that the two CD33 subsets have divergent transcriptional signatures leading to separate clustering of $CD33^+$ and $CD33^-$ subsets (**Figure 3A**). More than 200 genes (adjusted $p < 0.05$) were found to be differentially expressed between the two subsets (**Figure S3**). Among the most significant differences were the transcription factors RORA, encoding the RAR-related orphan receptor α (RORα), which was upregulated in $CD33^-$ NK cells, and RORC, encoding RORγT, which was more abundant in $CD33^+$ NK cells (**Figure 3B**). RORα and RORγt are well-known for their involvement in regulation of innate lymphoid cell (ILC) 2 and ILC3 development, respectively, but their role in NK cell development is currently unknown (21). The third highly significant difference in TF expression was represented by the Ikaros family member Aiolos (IKZF3), which was downregulated in $CD33^+$ NK cells. Other significantly overexpressed genes in $CD33^+$ NK cells are receptors involved in cell-cell interaction such as the integrins ITGAX (CD11c), ITGAD (CD11d), and ITGB7. This is compatible with analysis of the underlying biological pathways showing highest significance for differences in the adhesion pathway (**Figure S4**).

The expression differences between $CD33^+$ and $CD33^-$ NK cells for several genes encoding cell surface receptors such as abundant transcripts for CD117 (c-kit) and low transcripts of KLRG1 and CD16 (FCGR3A) (**Figures 3A, B**) were reminiscent of the differences between $CD56^{bright}$ and $CD56^{dim}$ NK cells (22), suggesting that $CD33^+$ NK cells might be more similar to $CD56^{bright}$ NK cells. However, within principal component analysis (PCA), $CD33^+$ NK cells were not closer related to $CD56^{bright}$ NK cells than $CD33^-$ NK cells (data not shown). Moreover, this similarity did not extend to key cytotoxic molecules such as perforin and granzyme B, which were strongly expressed in both subsets on the mRNA (data not shown) and protein level (**Figure 3C**). Next, we analyzed surface expression of a panel of typical NK cell-related molecules: besides verifying the transcriptional differences in CD16 and CD117 expression, we noted a higher expression of KIR receptors and a small subset of $CD33^-$ cells that expressed CD57 and KLRG1, two markers of terminally differentiated NK cells (23). The expression of natural cytotoxicity receptors NKp44 and NKp46 was significantly higher in $CD33^+$ NK cells, whereas no significant differences were found for NKp30, the lectin-like family of NK cell receptors NKG2A, C, and D, or the checkpoint inhibitors PD-1, TIM3, and TIGIT (**Figure 3D**). Finally, we compared the data generated in small-scale tissue culture (24-well plate) to a single large-scale experiment using the CliniMACS Prodigy system. The phenotypes of $CD33^+$ and $CD33^-$ NK cell subsets generated with the Prodigy platform were highly similar to those seen in 24-well plate cultures, demonstrating that similar kinds of $CD33^+$ and $CD33^-$ NK cell subsets are generated when transferring the small-scale conditions to a large-scale GMP-compatible setup (**Figure S5A**). Only CD62L, a marker of naïve NK cells, appeared to be somewhat more frequent in the $CD33^+$ subset in the large-scale experiment.

$CD33^+$ NK Cells Are Polyfunctional, Combining Strong Cytokine Production and Cytotoxicity

Next, we assessed how far the distinct transcriptional and phenotypic properties translate into functional differences

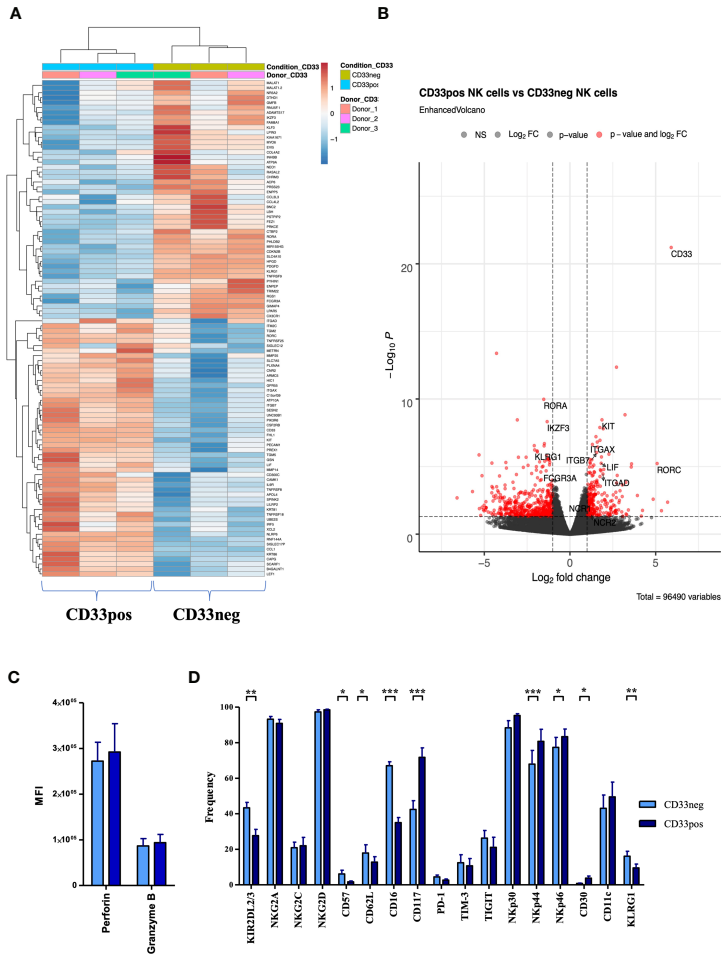

FIGURE 3 | Different transcriptional and phenotypical characteristics of CD33$^-$ and CD33$^+$ NK cells. **(A)** CD33$^-$ and CD33$^+$ NK cells were sorted on day 21 following culture with NK MACS medium (n=3). RNA sequencing was performed on the Illumina platform. The heatmap illustrates the top 100 differentially expressed genes between CD33$^-$ and CD33$^+$ NK cells. **(B)** The volcano plot indicates the RNA sequencing data based on 55,394 genes by plotting the logarithm of the fold change between CD33$^-$ and CD33$^+$ NK cells on the x axis and the negative logarithm of the p value on the y axis. The dashed lines indicate p values equaling 0.05. Red points represent the genes with highest statistical significance of fold change. Top genes of interest are labeled. Stimulated NK cells with NK MACS medium (days 14–21) were analyzed for **(C)** intracellular perforin and granzyme B (n=5) or **(D)** for the cell-surface expression of the indicated molecules (n=17) by flow cytometry. Expression was compared between CD33$^-$ and CD33$^+$ NK cells. Statistical significance was determined by paired t-test, *p < 0.05; **p < 0.01; ***p < 0.001.

between the two NK cell subsets. As shown in **Figure 4**, a significantly higher frequency of CD33$^+$ cells produced IFNγ and TNFα in response to K562 target cells compared to the CD33$^-$ subset (**Figures 4A, B**). Notably, CD33$^+$ NK cells also exhibited a higher frequency of CD107$^+$ cells, reflecting more effective mobilization of cytotoxic granules to the cell surface (**Figure 4C**). However, when measuring direct cytotoxicity against K562 cells, both subsets showed comparable lysis over a range of effector/target ratios (**Figure 4D**). Similar results were obtained when NK cells were expanded with the CliniMACS Prodigy system (**Figure S5B**). Finally, we assessed antibody-dependent cellular cytotoxicity (ADCC) employing the NK cell-resistant CD20$^+$ target cell line Raji in combination with the therapeutic anti-CD20 reagent Rituximab, which is a human IgG1 mAb that binds with its Fc part to the CD16 receptor on NK cells. In accordance with the significantly higher expression

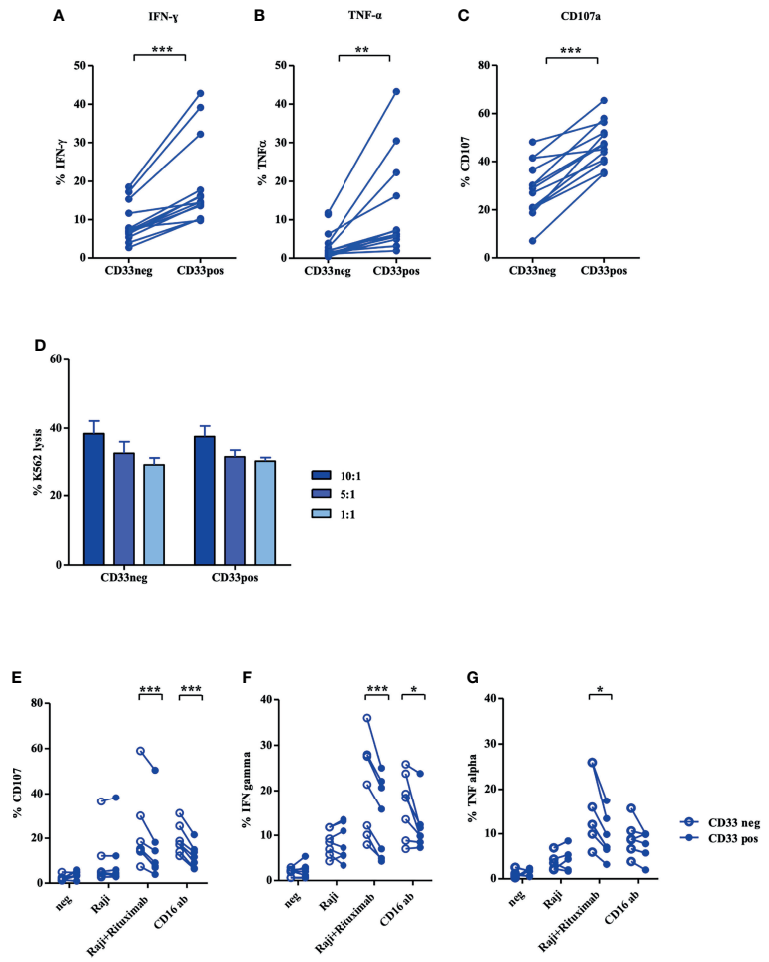

FIGURE 4 | CD33$^+$ NK cells display strong functionality in both cytokine production and cytotoxicity. Stimulated NK cells with NK MACS medium (day 14) were incubated with K562 cells at an effector/target ratio of 1:1. Intracellular **(A)** IFN-γ and **(B)** TNF-α production and **(C)** CD107a mobilization were evaluated by flow cytometry (n=13). **(D)** CFDA-SE stained K562 cells were incubated with CD33$^+$ and CD33$^-$ NK cells in effector/target ratios of 10:1, 5:1, and 1:1 (n=10). For ADCC, NK cells stimulated in NK MACS medium (day 14) were incubated with/without Raji and Rituximab, and **(E)** CD107a expression and intracellular **(F)** IFN-γ and **(G)** TNF-α production were measured by flow cytometry (n=8). Statistical significance was determined by paired t-test, *p < 0.05; **p < 0.01; ***p < 0.001.

of CD16, CD33$^-$ NK cells exhibited stronger mobilization of cytotoxic granules and higher cytokine responses to Raji compared to CD33$^+$ NK cells, with IFNγ showing more significant differences than TNFα (**Figures 4E–G**).

DISCUSSION

The expression of CD33 is largely restricted to the myeloid lineage and recently gained much interest as a target for immunotherapy of AML and other CD33-expressing malignancies, initially with the antibody-drug conjugate Gemtuzumab Ozagamicin, followed by bispecific antibody conjugates, antibody-cytokine conjugates, and lately CD33-CAR T cells (9, 24–26). Unexpectedly, we found that CD33 is upregulated *in vitro* on a significant subset of NK cells. Although *in vivo* CD33 expression is restricted to CD56^{bright} NK cells, which are a subset of non-cytotoxic, rather immature NK cells, this was not the case *in vitro*: CD33 could be efficiently induced on purified CD56^{dim} NK cells leading to frequencies of CD33$^+$ NK cells up and above 50% with NK MACS medium. The present work suggests that CD33 constitutes a marker to

distinguish between functionally divergent NK cell subsets *in vitro*. Expression of CD33 defines a polyfunctional NK cell subset combining cytotoxic and cytokine effector functions. On the other hand, due to lower expression of CD16, $CD33^+$ NK cells are less efficient in ADCC compared to $CD33^-$ NK cells, which in turn produce significantly less cytokines. Interestingly, CD33 seems to demarcate two separate cell states, which are either positive or negative with very few NK cells expressing intermediate levels of CD33 (see also **Figures 2**, **3**). Comparative transcriptional analysis by RNAseq revealed that the transcriptional programs of the two subsets are not simply mirroring $CD56^{bright}$ and $CD56^{dim}$ subsets but constitute quite independent cellular entities compatible with their unique functional properties, making both subsets potentially interesting tools for cancer therapy. It remains to be determined which of the differentially expressed TFs are involved in orchestrating these transcriptional changes, e.g., RORα and RORγT, which are so far described as master regulators of ILC2 and ILC3 development, respectively (21). Furthermore, downregulation of the Ikaros family member Aiolos in $CD33^+$ NK cells is interesting in this context since it was previously reported to be involved in shaping of the final NK cell maturation program in an Aiolos-deficient mouse model (27).

The upregulation of CD33 on NK cells *in vitro* is significant and strong enough to mediate recognition and subsequent fratricide by CD33-CAR NK cells. Whereas this principally hampers their expansion for cell therapeutic purposes, our study suggests that the problem can be circumvented in several ways: firstly, about 10% of the Caucasoid population are homozygous for an SNP (rs12459419) that leads to efficient alternative splicing and skipping of the IgV domain of CD33, which is the binding site for the CAR used in this study (19, 20). Due to the lack of the CD33 target site, those donors could be selected for expansion of CD33-CAR NK cells without inhibition by fratricide and stored for further clinical use such as allogeneic therapy of AML. Of note, due to the lack of GvH disease, application of CAR NK cells in the allogeneic setting constitutes a major advantage compared to CAR T cell therapies, which are presently only applicable in the autologous setting (28). Moreover, upregulation of CD33 was largely restricted to NK MACS medium, whereas an alternative protocol for expansion of NK cells based on K562 stimulator cells, which is already part of NK cell-based clinical protocols (29), did only lead to a transient wave of CD33 expression. Whether fading of the CD33 subset with stimulator cell-based protocols is due to downregulation of CD33 or possibly outperformance by $CD33^-$ NK cells is currently unknown. In any case, by adding NK MACS medium to stimulator cell cultures, expression of CD33 is again induced, suggesting a yet undefined component in the medium that supports expansion of the $CD33^+$ subset.

The biological role of CD33 expression on $CD56^{bright}$ NK cells is currently unclear. Generally, as a member of the Siglec family, CD33 is able to recognize sialylated ligands, but so far, no specific cell-bound glycoproteins or other glycosylated ligands were identified that are preferentially recognized by NK cells. In functional terms, the CD33 receptor contains intracytoplasmic ITIM motifs, and it was previously shown that crosslinking of CD33 leads to inhibition of effector functions (30). Notably, Siglecs were shown to bind not only in *trans* but also in *cis* to cell-bound ligands, which might lead to a constitutive inhibitory state (30). However, our study clearly shows that $CD33^+$ NK cells have polyfunctional characteristics marked by strong cytokine production and efficient killing. The association of ITIM-containing inhibitory receptors with gain of function is not without precedent in NK cells, since expression of ITIM-containing inhibitory KIR receptors mediate licensing of NK cells, an educational process associated with a strong gain of function (31). Similarly to CD33, inhibitory KIRs are providing a constitutive inhibitory state since they bind to ubiquitous HLA class I molecules present on all healthy nucleated cells. Moreover, both KIR and the Siglec family are not only structurally related but are also genetically linked in the extended leukocyte receptor complex (LRC) on chromosome 19q13.4 (32). At this time, it can only be speculated whether these similarities including the common signal transduction *via* SHP1 and SHP2 could be an indicator for a similar licensing-like function of CD33.

Altogether, expression of CD33 delineates a novel transcriptional and functional dichotomy that arises during *in vitro* expansion of NK cells. In terms of clinical translation, analysis of CD33 expression might give simple guidance on the composition of NK cell products during the *in vitro* expansion process for clinical use, with increased cytokine production and mobilization of cytotoxic granula on $CD33^+$ cells on the one hand and superior CD16-mediated functions on $CD33^-$ NK cells on the other hand.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in NCBI Project ID: PRJNA777044 (http://www.ncbi.nlm.nih.gov/bioproject/777044).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethikkommission Düsseldorf, Medical Faculty (study number 2019-383). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MH, CZ, MU, and NM conceived and planned the experiments. MH, CZ, KH, SO, and VB carried out the experiments. MH and SBB performed calculations. MH, CZ, SB, and VB conducted data analysis and interpretation. MU supervised the project. MH and MU wrote the manuscript. MU, SB, WW, and PH edited the manuscript. MN, SC, and RP provided data. SR, MQ, SH, HH,

PO, HR, JO, PB, PH, FB, WW, and JF provided critical feedback and commented on the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.798087/full#supplementary-material

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Conflict of Interest: CZ, MQ, MN, SC, RP, and NM are employees of Miltenyi Biotec.

The remaining 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.

Figure S1

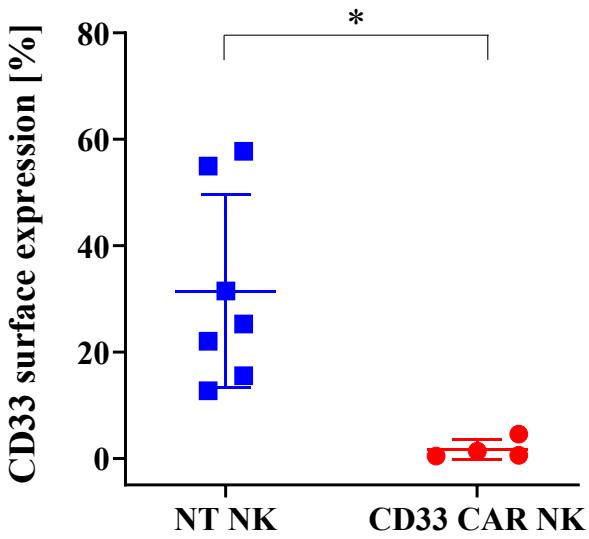
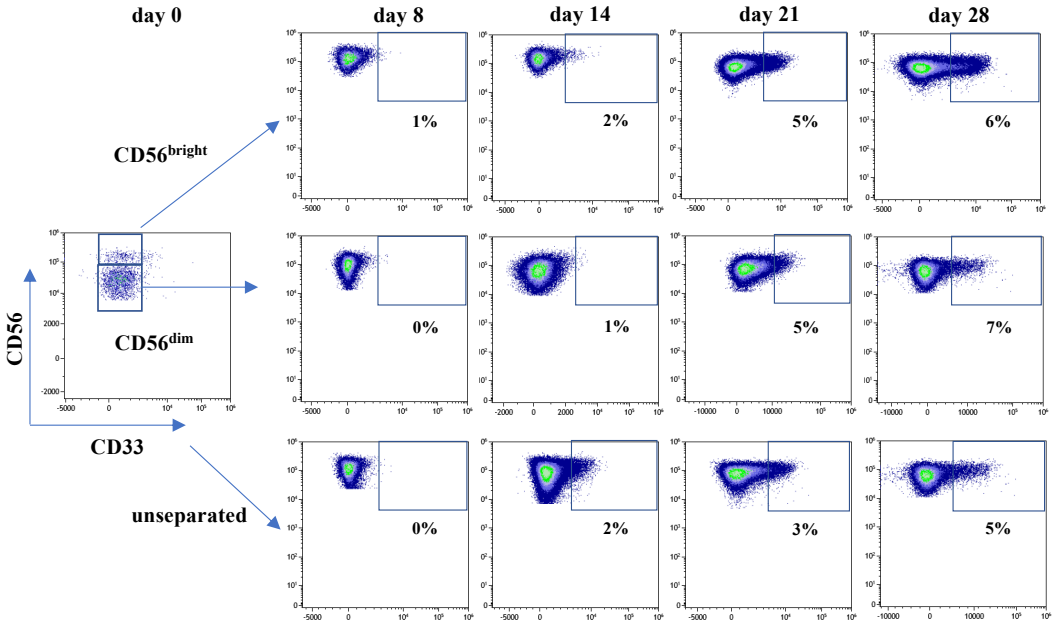


Figure S2

A



B

Target	Forward Primer	Reverse Primer
5´prime UTR	tacctccctctgtgcccgag	ttccaggagcaccagagcct
Exon 1+2	aggctctggtgctcctggaa	tgaactggggagttcttgtcgt
Exon 3	aagcctgtcgtgcttagcgg	aaatgtccccagcaccagcc
Exon 6	gggcagggggtgtgatgatg	atgacggtgggccacattgg

C

SampleID	Allele 1	Allele 2	CD33 in CD56bright	CD33 in culture
NK#40	c.41C>T (p.Ala14Val)	c.41C>T (p.Ala14Val)	neg	neg
NK#42	c.41C>T (p.Ala14Val)	c.41C>T (p.Ala14Val)	neg	neg
NK#43	c.41C>T (p.Ala14Val)	c.41C>T (p.Ala14Val)	neg	neg
D176	c.41C>T (p.Ala14Val)	c.41C>T (p.Ala14Val)	neg	neg
D222	c.41C>T (p.Ala14Val)	c.466_469del (p.Gly156ThrfsTer5)	neg	neg
NK#38	c.28C>T (p.Leu10Leu)	c.913T>C (p.Ser305Pro)	pos	pos
NK#39	WT	WT	pos	pos
D175	WT	c.41C>T (p.Ala14Val)	pos	pos
D177	WT	WT	pos	pos
D178	WT	c.41C>T (p.Ala14Val)	pos	pos
D179	WT	WT	pos	pos
D223	WT	WT	pos	pos

Figure S3

CD33pos NK cells					CD33neg NK cells				
Genes	baseMean	log2FoldChange	pvalue	padj	Genes	baseMean	log2FoldChange	pvalue	padj
CD33	261,75	5,90	6,21E-22	8,59E-18	FEZ1	186,41	-4,28	4,19E-14	2,90E-10
SIGLEC17P	337,25	2,71	4,44E-13	2,05E-09	RORA	939,50	-1,53	1,06E-10	3,67E-07
KRT86	116,81	3,21	1,47E-09	4,08E-06	KIAA1671	184,59	-3,07	3,51E-09	6,93E-06
KIT	1274,58	1,87	3,48E-09	6,93E-06	IKZF3	2455,56	-1,33	4,73E-09	8,19E-06
CAPG	3580,26	1,74	1,28E-08	1,97E-05	GIMAP4	1231,58	-1,49	2,01E-07	1,99E-04
ATP10A	4014,45	1,94	1,59E-08	2,20E-05	LBH	425,95	-2,08	2,84E-07	2,40E-04
CD300C	569,29	1,52	6,16E-08	7,75E-05	SLC4A10	386,96	-1,51	2,95E-07	2,40E-04
SPINK2	264,77	1,75	1,14E-07	1,32E-04	ENPEP	113,46	-2,06	5,88E-07	4,28E-04
IL9R	239,85	1,59	1,54E-07	1,64E-04	KLRG1	144,83	-1,92	7,00E-07	4,84E-04
TNFRSF8	873,92	1,31	2,31E-07	2,13E-04	PRKCE	777,43	-1,90	8,23E-07	5,43E-04
FHL1	521,86	1,61	3,59E-07	2,76E-04	INHBB	74,21	-5,31	1,38E-06	7,94E-04
LILRP2	696,49	1,56	9,10E-07	5,72E-04	CTBP2	317,33	-1,36	1,50E-06	8,30E-04
NLRP6	104,63	2,27	1,21E-06	7,30E-04	PRSS23	185,42	-3,01	2,06E-06	1,02E-03
CCL1	121,66	1,84	1,94E-06	1,02E-03	LPAR5	53,14	-2,56	2,04E-06	1,02E-03
ITGAX	3330,02	1,28	3,14E-06	1,30E-03	HPGD	1065,55	-1,27	2,63E-06	1,26E-03
GPR55	914,01	1,17	3,02E-06	1,30E-03	PYHIN1	965,33	-1,24	2,81E-06	1,30E-03
C15orf39	1693,75	1,17	4,91E-06	1,77E-03	RGS1	1101,79	-1,33	3,13E-06	1,30E-03
RORC	138,35	5,08	5,96E-06	1,92E-03	MIR155HG	1208,47	-1,19	3,20E-06	1,30E-03
LIF	2115,07	1,82	5,72E-06	1,92E-03	TNFRSF9	431,29	-1,47	4,01E-06	1,59E-03
TNFRSF18	2479,22	1,19	5,91E-06	1,92E-03	PDGFD	255,39	-1,77	4,47E-06	1,72E-03
SIGLEC12	155,52	3,59	6,35E-06	2,00E-03	COL4A2	111,00	-3,74	5,00E-06	1,77E-03
CSF2RB	1660,58	1,39	7,80E-06	2,35E-03	TRIM22	736,52	-1,18	4,92E-06	1,77E-03
PLXNA4	41,80	3,58	1,07E-05	2,82E-03	RASAL2	43,60	-4,56	5,53E-06	1,91E-03
ITGB7	4889,33	1,31	1,05E-05	2,82E-03	FAM8A1	439,99	-1,19	7,44E-06	2,29E-03
IRF5	1885,64	1,13	1,08E-05	2,82E-03	MYO6	132,51	-2,45	8,13E-06	2,39E-03
ITM2C	910,49	1,97	1,63E-05	4,02E-03	CX3CR1	159,48	-1,57	9,58E-06	2,76E-03
TGM5	223,76	1,77	1,73E-05	4,21E-03	CDKN2B	86,98	-1,96	1,02E-05	2,82E-03
SCARF1	461,20	1,81	2,06E-05	4,78E-03	CCL3L3	297,41	-1,62	1,06E-05	2,82E-03
PECAM1	3012,92	1,41	2,46E-05	5,57E-03	NEO1	79,53	-2,72	1,15E-05	2,95E-03
B4GALNT1	878,87	1,43	2,67E-05	5,78E-03	CHRM3	98,55	-3,71	1,29E-05	3,24E-03
XCL2	929,64	1,17	2,67E-05	5,78E-03	KLF3	116,14	-1,96	1,83E-05	4,37E-03
SLC7A5	2111,66	1,07	2,91E-05	6,19E-03	FCGR3A	3412,21	-1,39	2,07E-05	4,78E-03
MMP25	2978,45	1,21	3,19E-05	6,68E-03	PHLDB2	98,46	-1,70	2,52E-05	5,63E-03
ITGAD	342,09	1,93	3,56E-05	7,23E-03	DTHD1	109,25	-2,24	3,26E-05	6,73E-03
RNF144A	1025,00	0,95	3,83E-05	7,66E-03	EVI5	52,96	-3,12	3,88E-05	7,66E-03
CAMK1	296,76	1,35	4,29E-05	8,35E-03	BNC2	28,38	-5,13	4,75E-05	9,00E-03
KRT81	39,08	2,76	4,53E-05	8,70E-03	ENPP5	36,44	-4,11	4,97E-05	9,17E-03
APOL4	576,89	1,30	4,86E-05	9,09E-03	NR5A2	96,32	-1,89	6,76E-05	1,21E-02
CNR2	385,51	1,75	5,48E-05	9,98E-03	CCL4L2	424,00	-1,67	9,32E-05	1,65E-02
PREX1	9618,07	1,57	1,04E-04	1,81E-02	GMFB	1031,87	-1,03	1,02E-04	1,79E-02
UNC93B1	758,47	1,57	1,34E-04	2,21E-02	ADAMTS17	107,34	-1,74	1,06E-04	1,81E-02
HIC1	1243,51	1,43	1,37E-04	2,23E-02	MALAT1	8675,66	-0,87	1,12E-04	1,87E-02
TNFRSF25	64,93	3,26	1,47E-04	2,32E-02	MALAT1.2	8675,66	-0,87	1,12E-04	1,87E-02
ARMC5	924,18	1,55	1,47E-04	2,32E-02	ATP9A	141,53	-3,33	1,44E-04	2,32E-02
LEF1	259,20	1,28	1,50E-04	2,33E-02	ACP6	296,94	-1,10	1,82E-04	2,76E-02
MMP14	229,75	2,72	1,65E-04	2,53E-02	PSTPIP2	568,31	-1,19	2,22E-04	3,23E-02
GSN	1471,64	1,63	1,99E-04	2,99E-02	LPIN3	34,76	-3,79	2,35E-04	3,25E-02
UBE2S	785,83	1,05	2,04E-04	3,03E-02	RNU5F.1	50,37	-2,03	2,33E-04	3,25E-02
SESN2	1102,85	1,24	2,07E-04	3,05E-02	RTKN	274,40	-1,29	2,44E-04	3,35E-02
TGM2	48,80	3,06	2,26E-04	3,25E-02	C21orf91	1058,93	-1,03	2,56E-04	3,48E-02
PIK3R6	403,91	1,48	2,33E-04	3,25E-02	UTS2	24,61	-4,55	3,02E-04	3,94E-02
METRN	238,60	1,18	2,35E-04	3,25E-02	PCSK5	35,62	-4,28	3,11E-04	3,98E-02
CD7	4380,88	1,37	2,80E-04	3,77E-02	SPRY2	596,94	-1,10	3,23E-04	4,10E-02
RP11.345J18.2	231,77	1,77	2,83E-04	3,77E-02	ENPP4	495,14	-0,93	3,28E-04	4,12E-02
C1orf162	113,57	1,61	2,95E-04	3,89E-02	SRP9	1425,72	-0,81	3,44E-04	4,25E-02
IL17RA	819,16	1,39	3,09E-04	3,98E-02	LYST	6359,63	-0,79	3,76E-04	4,56E-02
GRAMD1B	2080,27	1,24	3,32E-04	4,13E-02	IL17RD	45,07	-4,50	3,83E-04	4,57E-02
GAS7	570,44	1,15	3,58E-04	4,38E-02	SPR	73,25	-3,27	4,20E-04	4,92E-02
XCL1	1025,32	0,84	3,81E-04	4,57E-02	CD164	3639,67	-0,75	4,42E-04	5,10E-02
CHST2	629,76	1,12	3,89E-04	4,60E-02	CLEC2D	1099,71	-0,88	4,50E-04	5,10E-02
PNPLA6	2337,23	1,36	4,28E-04	4,97E-02	LITAF	1586,25	-1,06	4,73E-04	5,16E-02
MIIP	1065,85	0,90	4,46E-04	5,10E-02	TGFBR3	616,76	-0,94	4,74E-04	5,16E-02
NOTCH1	3024,88	1,22	4,57E-04	5,14E-02	GNG2	3671,61	-0,81	4,83E-04	5,22E-02
GRAMD4	1385,24	0,96	4,62E-04	5,15E-02	SNRPE	676,58	-0,96	5,05E-04	5,41E-02
MXD3	719,66	0,96	4,73E-04	5,16E-02	ADGRG2	144,54	-1,56	5,09E-04	5,42E-02
TIAM1	1010,83	1,08	5,40E-04	5,61E-02	NT5E	98,47	-1,76	5,24E-04	5,54E-02
TXNDC5	110,86	1,46	5,47E-04	5,65E-02	LIM2	108,20	-2,13	5,31E-04	5,57E-02
CASS4	182,74	1,26	5,74E-04	5,86E-02	GLCE	233,17	-1,26	5,82E-04	5,86E-02
SLC16A3	5337,61	0,85	5,84E-04	5,86E-02	CCL5	6966,31	-0,77	5,83E-04	5,86E-02
RNF130	956,17	0,90	6,04E-04	5,97E-02	ITGB2.AS1	141,50	-1,31	5,97E-04	5,94E-02
DLL1	100,07	2,92	6,15E-04	5,99E-02	NUAK2	25,61	-4,22	6,10E-04	5,98E-02
NCF4	3552,77	0,80	6,66E-04	6,36E-02	LIN7C	862,16	-0,84	6,22E-04	6,02E-02
IMPDH1	1089,32	0,99	7,50E-04	6,92E-02	TCEAL1	89,54	-1,89	6,51E-04	6,26E-02
CYP1B1	113,18	2,68	7,91E-04	7,11E-02	IFNLR1	36,78	-2,59	6,86E-04	6,50E-02
PLEKHF1	551,08	0,99	8,21E-04	7,33E-02	ALG10B	286,14	-1,32	7,20E-04	6,78E-02
KCTD17	529,99	0,86	8,36E-04	7,42E-02	CLDN12	71,45	-2,22	7,31E-04	6,79E-02
ZTS1	194,04	1,65	9,00E-04	7,78E-02	S1PR5	76,05	-1,60	7,27E-04	6,79E-02
MB	48,67	2,01	9,05E-04	7,78E-02	SLC40A1	66,61	-2,87	7,73E-04	7,05E-02
ORAI2	3257,66	1,14	9,31E-04	7,95E-02	TIGIT	1988,17	-1,22	7,74E-04	7,05E-02
MBOAT7	579,98	0,95	9,64E-04	8,08E-02	NES	44,77	-4,17	7,84E-04	7,09E-02
OSM	1084,21	0,97	9,96E-04	8,25E-02	LNX1	31,10	-2,78	8,50E-04	7,49E-02
SAPCD2	692,30	1,21	1,04E-03	8,49E-02	PTK7	338,20	-2,92	8,67E-04	7,55E-02
MATK	3147,37	1,25	1,05E-03	8,49E-02	TMED7	932,20	-0,97	8,68E-04	7,55E-02
TRPM2	260,68	1,45	1,07E-03	8,51E-02	PGGT1B	561,08	-1,03	9,52E-04	8,08E-02
CCR8	152,35	1,49	1,13E-03	8,68E-02	FKBP10	82,26	-2,42	9,62E-04	8,08E-02
SDK2	243,41	1,46	1,13E-03	8,68E-02	SGK1	236,89	-1,01	9,96E-04	8,25E-02
PTGIS	411,04	1,42	1,13E-03	8,68E-02	TENM1	166,95	-1,26	1,01E-03	8,33E-02
TBL3	617,32	1,07	1,11E-03	8,68E-02	NEAT1	1675,91	-1,18	1,04E-03	8,49E-02
H1FX	1627,78	0,92	1,11E-03	8,68E-02	CTLA4	53,74	-3,00	1,05E-03	8,49E-02
GFM	252,07	1,48	1,15E-03	8,69E-02	NBPF15	29,92	-2,36	1,07E-03	8,51E-02
ARHGAP45	5890,81	1,22	1,15E-03	8,69E-02	MYB	388,33	-0,88	1,14E-03	8,68E-02
JAG2	175,88	1,87	1,21E-03	8,76E-02	LYPLA1	1094,02	-0,84	1,13E-03	8,68E-02
GPR35	233,22	1,50	1,17E-03	8,76E-02	TSKU	29,14	-4,06	1,21E-03	8,76E-02
ADAM19	4886,10	1,10	1,18E-03	8,76E-02	BCL2L15	123,98	-1,31	1,19E-03	8,76E-02
LRWD1	650,02	0,85	1,22E-03	8,76E-02	ZMAT3	397,06	-1,00	1,19E-03	8,76E-02
PPP1R14B	1070,08	0,83	1,18E-03	8,76E-02	RAP1A	767,10	-0,78	1,20E-03	8,76E-02
RP1.102K2.8	35,19	2,53	1,25E-03	8,83E-02	TAF1D	2045,29	-0,77	1,22E-03	8,76E-02
SPATA3.AS1	91,70	1,41	1,26E-03	8,83E-02	UBL3	306,14	-1,00	1,26E-03	8,83E-02
PSD4	10799,31	0,96	1,24E-03	8,83E-02	RALGPS2	42,65	-2,20	1,29E-03	8,98E-02
GPR68	2333,71	1,04	1,30E-03	8,99E-02	PPP1CB	3643,04	-0,76	1,29E-03	8,98E-02
RANGAP1	2215,07	0,75	1,33E-03	9,14E-02	NPAS2	36,53	-4,33	1,35E-03	9,18E-02
CCNF	2604,63	0,69	1,34E-03	9,17E-02	HNF1B	52,19	-1,70	1,44E-03	9,64E-02
CARD9	440,72	1,38	1,37E-03	9,27E-02	MSMO1	1636,24	-1,00	1,45E-03	9,67E-02
UBALD2	1170,88	0,92	1,40E-03	9,45E-02	HOOK1	90,27	-2,01	1,47E-03	9,68E-02
SAMD14	62,39	2,17	1,45E-03	9,67E-02	CHIC1	163,68	-1,17	1,49E-03	9,68E-02
MAFF	430,42	1,35	1,49E-03	9,68E-02	GCA	203,17	-1,14	1,49E-03	9,68E-02
MAML3	436,38	1,06	1,47E-03	9,68E-02	TRMT13	273,18	-0,98	1,53E-03	9,68E-02
KIAA0930	2482,80	1,15	1,52E-03	9,68E-02	PRELID3B	2017,18	-0,70	1,50E-03	9,68E-02
SSBP4	911,39	0,99	1,52E-03	9,68E-02	CLCN4	36,63	-4,46	1,59E-03	9,82E-02
C10orf54	1001,64	0,87	1,53E-03	9,68E-02	ZDHHC21	733,66	-1,06	1,64E-03	1,00E-01
MYBL2	2352,62	0,78	1,52E-03	9,68E-02	PERP	679,61	-1,33	1,66E-03	1,01E-01
ACY3	124,15	1,40	1,55E-03	9,73E-02	STARD4	1864,28	-0,72	1,66E-03	1,01E-01
RASAL3	2875,22	0,93	1,55E-03	9,73E-02	GRHL2	69,38	-2,36	1,71E-03	1,03E-01
GRK6	1250,26	0,82	1,57E-03	9,77E-02	RCHY1	366,03	-1,08	1,71E-03	1,03E-01
SUN2	4013,35	0,81	1,57E-03	9,77E-02	DTX4	40,42	-3,22	1,73E-03	1,03E-01
NPTX1	30,62	3,05	1,65E-03	1,00E-01	VCAM1	172,12	-1,09	1,77E-03	1,04E-01
INCENP	1694,56	0,89	1,65E-03	1,00E-01	YAF2	464,66	-0,80	1,77E-03	1,04E-01
C14orf80	461,57	1,00	1,73E-03	1,03E-01					
KIFC1	3210,65	0,81	1,74E-03	1,03E-01					
FZR1	844,75	0,98	1,75E-03	1,03E-01					
GPAT3	1025,64	1,17	1,79E-03	1,04E-01					

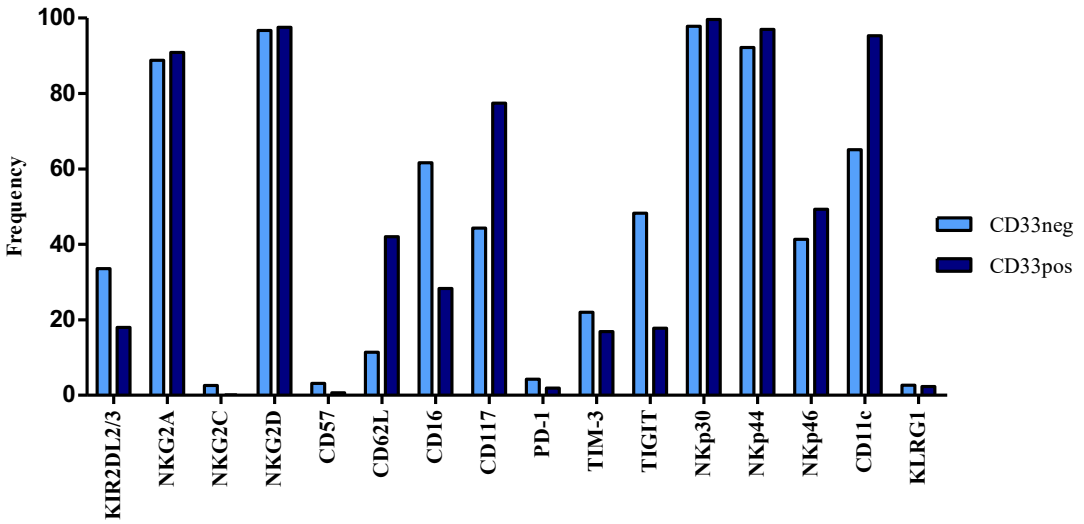
Figure S4

Top 10 Biological Processes

GO:BP		stats		
Term name	Term ID	p_{adj}	$-\log_{10}(p_{adj})$ 0 ≤16	T∩Q
cell adhesion	GO:0007155	5.709×10^{-7}		25
biological adhesion	GO:0022610	6.298×10^{-7}		25
regulation of multicellular organismal process	GO:0051239	9.371×10^{-5}		31
cell differentiation	GO:0030154	1.026×10^{-4}		39
T cell activation	GO:0042110	1.491×10^{-4}		13
cellular developmental process	GO:0048869	1.685×10^{-4}		39
multicellular organismal process	GO:0032501	2.220×10^{-4}		54
leukocyte activation	GO:0045321	3.603×10^{-4}		20
regulation of cell adhesion	GO:0030155	5.016×10^{-4}		15
system development	GO:0048731	6.091×10^{-4}		41

Figure S5

A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NKG2C
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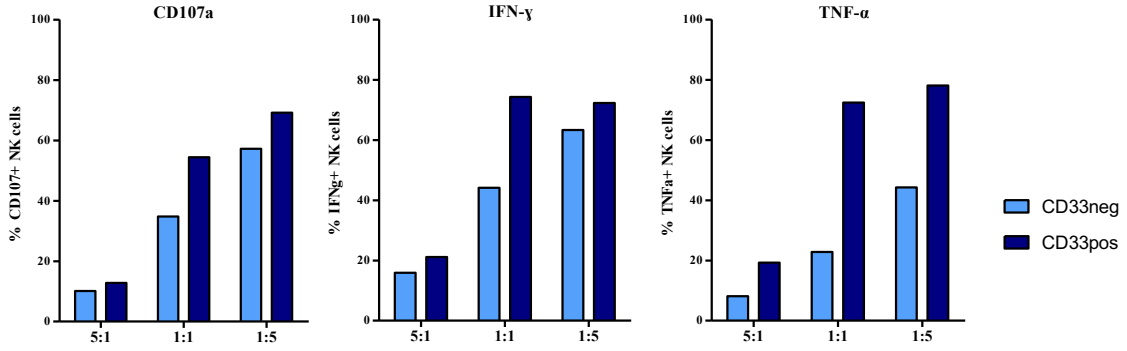
TIM-3
TIGIT
NKp30
NKp44
NKp46
CD11c
KLRG1
CD33neg
CD33pos

B


CD107a
% CD107+ NK cells
IFN-γ
% IFNg+ NK cells
TNF-α
% TNFa+ NK cells
100
80
60
40
20
0
5:1
1:1
1:5
CD33neg
CD33pos

Supplementary figure legends:

Figure S1: CD33 expression on non-transduced (NT) NK and CD33 CAR NK cells. CD33 expression on NT (n=7) and CD33 CAR NK cells (n=4) were determined by flow cytometry at the end of cell expansion. Data were analyzed by two-tailed unpaired t-test. *, $p < 0.05$.

Figure S2: (A) Flow cytometric dot plots of one representative donor with no CD33 expression on $CD56^{bright}$ NK cells *ex vivo*. Enriched NK cells were sorted for two subpopulations $CD56^{dim}$ and $CD56^{bright}$ NK cells and were cultured in NK MACS medium for 28 days. Unsorted NK cells served as control. (**B**) Primers for genotyping CD33. (**C**) CD33-coding SNP rs12459419-Ala14Val was genotyped using MiSeq sequencing system.

Figure S3: Most differentially expressed genes at the level of transcription in $CD33^+$ and $CD33^-$ NK cells. $CD33^+$ and $CD33^-$ NK cells were cultured for 14 days and sorted for RNAseq on the Illumina platform. Most differentially expressed genes were calculated comparing $CD33^+$ (left table) and $CD33^-$ NK cells (right table) with respect to donors in R and are shown with baseMean, log2FoldChange, p-value, and adjusted p-value (padj), n=3.

Figure S4: Top 10 Biological Process pathways within $CD33^+$ NK cells. Most differentially expressed genes of $CD33^+$ NK cells (see Sup Fig. 2) were analyzed using g:Profiler. Top 10 Gene Ontology Biological Processes (GO-BP) terms are displayed with Term name and ID, adjusted p-value (Padj), the -log10 Padj, and number of genes within the pathway ($T \cap Q$).

Figure S5: Phenotypical and functional characteristics of $CD33^-$ and $CD33^+$ NK cells expanded using the CliniMACS Prodigy system (n=1). **(A)** Cell-surface expression of the indicated molecules was analyzed by flow cytometry. (**B**) Expanded NK cells (day 14) were incubated with K562 cells at an effector/target ratio of 1:1. CD107a mobilization (left) and intracellular IFN-ɣ (middle) and TNF-α (right) production were evaluated by flow cytometry.

Supplementary Material and Methods:

CD33 sequencing

An amplicon-based NGS 2-step PCR protocol served for analysis of CD33 genomic DNA. First step PCR was performed using CD33 specific primer (Biolegio, Nijmegen, The Netherlands, Supplementary Table S1). The second PCR attached sample-specific barcodes and Illumina adapter-sequences. 6 pM of the resulting library was used for a 2 x 250 cycles run on an Illumina MiSeq instrument. Data analysis was performed by an in-house developed Visual Basic-Script software including assembling of paired-end reads with PANDA-Seq (1), an error-correction algorithm and alignment to the genomic CD33 reference sequence (GenBank Acc. NC_000019.10).

NK cell Expansion using the CliniMACS Prodigy system

Apheresis material of one donor was kindly provided, upon written informed consent, by the Institute of Experimental Hematology and Transfusion Medicine at the University Hospital Bonn. Mononuclear cell apheresis was performed using the Spectra Optia apheresis system (Terumo BCT, Lakewood, CO, USA). NK cell enrichment was conducted in a two-way process. First, T cells were depleted using CliniMACS CD3 Reagent (Miltenyi Biotec, Bergisch Gladbach) and the Tubing Set 310 (Miltenyi Biotec, Bergisch Gladbach). NK cells were enriched using CliniMACS CD56 Reagent (Miltenyi Biotec, Bergisch Gladbach) and the Tubing Set 520 (Miltenyi Biotec, Bergisch Gladbach). The NK cell expansion process was initiated on the Prodigy system with 1×10^8 NK cells using NK MACS GMP medium with 1% NK MACS supplement, 500 U/ml MACS GMP recombinant human IL-2, 140U/ml MACS GMP recombinant human IL-15 (all Miltenyi Biotec, Bergisch Gladbach), and 5% human AB serum (PAN Biotech, Aidenbach, Germany) in the TS510. Expansion was conducted over 14 days using a pre-designed process. Cell product was harvested in NaCl 0.9% infusion solution (Frisenius Kabi, Bad Homburg, Germany) supplemented with 0.5% Albutein human serum albumin (HSA; Grifols, Frankfurt am Main, Germany).

1. Masella AP, Bartram AK, Truszkowski JM, Brown DG, Neufeld JD. PANDAseq: paired-end assembler for illumina sequences. BMC Bioinformatics. 2012;13:31.

4 Discussion

The overall aim of this thesis within the iCAN33 framework was to develop strategies that could improve the *in vivo* persistence of allogeneic NK cells for for “off-the-shelf” immunotherapy. To this end, we performed a knockout (KO) of HLA molecules on primary NK cells via genome editing and showed that this KO can protect the NK cells from recognition and lysis by T cells (Publication I) (192). This strategy was designed to improve the persistence of allogeneic NK cell products in humans, by providing more time for the immune effector cells to combat the malignant cells before being rejected by the host’s immune system.

Another major topic in this thesis was to understand the observation that our *in vitro* enrichment and expansion of primary human NK cells induced CD33 expression on these cells that resulted in extensive fratricide under “good manufacturing practice (GMP)”-compatible conditions. In the publication by Hejazi *et al.* (Publication II) (189), we demonstrated that this aberrant high expression of CD33 predominantly occurred when the GMP-adapted commercial NK cell medium from Miltenyi Biotec was employed but not when the old Campana-method of expanding the NK cells with K562-derived feeder cells was employed. We also showed a potential solution for this problem: Homozygous carriers of the T allele of the SNP rs12459419 within exon 2 of *CD33* present with low frequencies of $CD33^{dim}$ NK cells and the CD33 expression levels are not greatly upregulated when creating CD33-CAR NK cells with the GMP-compatible medium. When chosen, these donors allowed to alleviate the high degree of fratricide observed when using normal donors with high expression levels of CD33 on the CAR NK cells. In addition, we demonstrated that CD33 can also be used as a functional marker for cultured NK cells from normal donors: $CD33^{+}$ NK cells show the higher cytotoxic potential, making them more suitable for a stand-alone therapy, while the $CD33^{-/dim}$ counterparts exert more potent ADCC. Therefore, expansion of this NK cell subset appears to be attractive for generating CAR NK cells targeting other antigens such as CD19 for B cell neoplasms or CD123 for AML blasts. These cells might also be better effector cells for combination immunotherapy with monoclonal antibodies (mAb) that operate by ADCC.

4.1 Utility of HLA class I-deficient NK cells

After the initial engraftment of therapeutic immune cells in allogeneic hosts, their persistence is largely dictated by their absolute lifespan, their *in vivo* expansion

capacity, and their resilience to recognition and destruction by the host immune system (143, 151, 193). In this thesis, we have shown that mere KO of *B2M* in primary NK cells leads to fratricide within the *in vitro* cultures of highly enriched NK cells due to missing-self-induced killing (192). We also provided a solution to this problem by protecting the HLA class I-deficient NK cells through overexpression of an artificial single-chain(sc)-HLA-E molecule that renders the cells immune to $CD8^+$ and $CD4^+$ T cell responses (192). Carbodyfluorescein succinimidyl ester (CFSE) dilution and cytotoxicity assays nicely demonstrated the protection against allogeneic T cells as well as against fratricide, as the NK cells grown for prolonged periods of time *in vitro* (in the GMP-compatible medium) almost all expressed the natural receptor for HLA-E, NKG2A, at high levels. *In vivo*, however, the infused NK cells are subjected to a more complex system of immune cells where only approximately 50% of NK cells actually express NKG2A (194). This invokes the discussion, whether mere HLA class I abrogation is really sufficient to protect the *in vitro* expanded HLA class I-deficient NK cells from alloreactions beyond artificial cell culture systems. It is important to realize here that several novel immunotherapy approaches do not aim to permanently engraft the therapeutic cells in the patient, often because of severe long-term toxicity of persisting T cells. In CD33-targeted immunotherapy for example, prolonged persistence of the cytotoxic effectors would lead to extended pancytopenia in the myeloid lineages with highly increased risk of infection for the patient (195). So, the aim of a HLA class I KO in CAR NK cells is no to entirely prevent the rejection of the allogeneic cellular product, but to prolong its persistence and short-term engraftment to maximize the anti-tumor effects, while keeping the toxicity in a clinically manageable time window.

Another major cellular factor for the rejection of allogeneic NK cells *in vivo* is the HLA class II expression. Our results have not shown a reactivation of $CD4^+$ T cells towards cultured NK cells. However, NK cells have been shown to obtain HLA class II expression either indirectly from dendritic cells via trogocytosis or *de novo* upon activation (196, 197). Therefore, NK cell activation and HLA class II acquisition *in vivo* after adoptive transfer can lead to engagement of $CD4^+$ T cells. Although it is questionable whether $CD4^+$ T cells can mount cytotoxic responses (191), a subset of circulating $CD28^-/CD4^+$ T cells has been found at low levels in healthy controls and elevated in various diseases that appears capable of direct cytotoxicity (198–201). In fact, $CD4^+$ T cells play a pivotal role in the rejection of solid organs and in GvHD via the produced cytokines and early studies on the contribution of conventional $CD4^+$ and

$CD8^+$ T cells in rejection of cardiac allografts in mice have revealed that $CD4^+$ T cells can mediate rejection on their own after depletion of $CD8^+$ T cells (104, 202). As shown in follow-up studies, this rejection was not a consequence of a population of directly cytotoxic $CD4^+$ T cells, but the result of a proinflammatory cytokine response attracting macrophages and granulocytes, thereby facilitating organ damage and loss of function (203). More importantly, even in the absence of MHC class II on the transplanted organ, indirect allorecognition by $CD4^+$ T cells was sufficient to trigger rejection via recruitment of inflammatory myeloid cells into the organ (204). As a bottom line, even without assuming direct cytotoxic activity, $CD4^+$ T cells can mediate rejection of allogeneic cells. More research is necessary to clarify whether disrupting of the HLA class II expression would further improve the *in vivo* persistence of allogeneic CAR NK cells in patients.

Although we showed that the sc-HLA-E presenting the HLA-G leader peptide can at least partially inhibit the fratricide of HLA class I negative NK cells, the artificial HLA-E molecule can theoretically promote the emergence of HLA-E-restricted T cells, which belong to a rare subset of $CD8^+$ T cells that recognizes viral peptides presented by HLA-E (95, 205). However, the low polymorphism rates of HLA-E (two dominating alleles in the caucasian population) and the presented HLA-G leader peptide limit this possibility.

In 2020, Liu *et al.* from the MD Anderson Cancer Center in Houston published a milestone study with at least partially HLA-matched (4 out of 6) NK cells from cord blood units which were expanded *in vitro* with genetically engineered K562 feeder cells and transduced with a retroviral vector expressing a CD19-CAR construct, soluble IL-15 and an inducible Caspase-9 (206). Prior to infusions, the patients received conditioning with cyclophosphamide and fludarabine. For relapsed or refractory B cell neoplasms, the authors reported a clinical response rate of 73% and identified 7 patients with CR (4 lymphomas and 3 chronic lymphocytic leukemias (CLL)). Although most of the patients received secondary treatments after the NK cell therapy, the genetically modified NK cells were detectable via qPCR for up to 12 months (206). Importantly, the NK cell persistence was never confirmed by additional methods such as flow cytometry. These results clearly raise the question whether the abrogation of HLA class I is really necessary for longer persistence of allogeneic NK cells and to maximize their efficacy. Interestingly, previous studies showed the rebounding alloreactive T cell compartment after conditioning to be causative for the short

persistence of haploidentical NK cells after infusion (151, 152), yet the study by Liu and colleagues seems to have circumvented the problem simply by using a CAR and the soluble IL-15. Surely, one can only cautiously compare the patient cohorts regarding disease and pretreatment regimens which both may impact the patients' immune competence and the kinetics of the rebounding T cell compartment.

In a very recent clinical trial, Berrien-Elliott and colleagues administered the IL-15 superagonist N-803 in conjunction with haploidentical NK cell infusions in relapsed/refractory AML patients (193). They observed reduced persistence of the NK cells and strongly reduced clinical benefits, compared to the group treated with recombinant IL-2 (193). As IL-15 is known to support NK cell persistence *in vitro* and *in vivo* in humans (14, 206, 207), this was rather puzzling. However, the group showed that N-803 administration efficiently expands the alloreactive $CD8^+$ T cells and that these cells accelerated the rejection of the allogeneic NK cells (193). This finding suggests that the clinical success of treating patients with N-803 in combination with allogeneic NK cells would strongly improve if the cells were devoid of HLA class I expression. Hence, pursuing HLA class I abrogation in primary NK cells to improve their persistence has potential for clinical translation. However, safety and regulatory measures surrounding genome editing of primary cells need to be addressed before such approaches can begin their journey into clinical routine.

4.2 Fratricide in CAR expressing cytotoxic lymphocytes

In the iCAN33 project, the CD33-CAR NK cell cultures did not expand as well as untransduced NK cells or NK cells expressing a CD19-CAR construct, when the cells were expanded with a GMP-compatible NK cell medium from Miltenyi Biotec (data unpublished and (189)). Phenotypic analysis of the untransduced and the CD19-CAR NK cells revealed approximately 50% (or even higher) of CD33 expressing cells, while the cultures with CD33-CAR NK cells showed very little CD33 expression (189). CD33 expression on human NK cells has been reported *in vivo*, as well as *in vitro* after activation, yet at low frequencies (208, 209). Thus, we postulated as a working hypothesis, that the CD33-CAR NK cells will engage in cytotoxic activity with CD33 expressing NK cells within the culture in a process called fratricide. Subsequently, we and other groups within the consortium could confirm the fratricide within the CAR expressing NK cell cultures by flow cytometry (189). Changing the target antigen from CD33 to CD123 would have been a possible solution to overcome the CD33-CAR NK

cell fratricide. CD123 is also expressed on AML blasts and a valid target antigen for AML immunotherapy (176). A study using CD123-CAR T cells, however, has shown complete myeloablation in humanized mice (175), while GO treatment and CD33-CAR T cell infusions in humans did not have this effect (120, 185, 190). This suggests CD33 as a more suitable target for AML immunotherapy without risking irreversible myeloablation.

A recently published study using a Mylotarg/My96-derived scFv stated that the CAR NK cell expansion was not negatively influenced by the CD33-CAR (173). Yet the overall culture expansion of the CD33-CAR NK cells shows a tremendous lag phase compared to unmodified NK cells and an approximately 3-fold lower overall expansion (173). Also, the CD33 expression on CD33-CAR NK cells was clearly lower compared to untransduced NK cells from the same donors (173), hinting towards fratricide by the CD33-CAR NK cells. These studies are not the first in which a CAR has been expressed in a cytotoxic lymphocyte against a target antigen which is expressed on the effector cell itself. Two prominent examples are CAR T cells against CD5 and against the signaling lymphocyte activation molecule family member 7 (SLAMF7), both of which are highly expressed on T cells (210, 211). In these studies, the researchers did not report major difficulties with CAR T cell expansion. Nevertheless, Gogishvili and colleagues also observed a loss of SLAMF7 expressing cells in their CAR T cell cultures, similarly to what was observed in the study by Hejazi and colleagues but without the reduction in proliferation (210). The study by Mamonkin *et al.*, however reporter a minor growth delay of the CAR expressing T cells which was compensated by the fact that all surviving T cells expressed the CD5-CAR and had downregulated their CD5 surface protein levels (211).

An interesting mechanism could be the steric nature of the CAR/antigen pair: If the CAR can bind the antigen in *cis* on the cell surface, the antigen would be masked from recognition by a CAR from another cell. Therefore, the cell would be protected from fratricide by other CAR T cells in the culture. This phenomenon has been observed before in a clinical trial: A single leukemic B cell had been accidentally transduced with a CD19-CAR construct during the manufacturing process for an autologous CAR T cell product (212). As a consequence, the CD19 epitope on this leukemic B cell was masked for the CD19-CAR T cells and led to resistance and relapse when re-infused back into the patient (212). A similar masking mechanism could save the CD5- and SLAMF7-CAR expressing T cells once all the cells not expressing a CAR have been

eradicated by fratricide. However, similar masking of the CD33-CAR and the CD33 molecule did not occur in our cultures with CD33-CAR NK cells due to unknown reasons.

4.3 Choosing the optimal NK cell culture protocol

Culture of human T cells for immunotherapy always follows a uniform principle: the concomitant stimulation of CD3 and CD28 on T cells to maximally activate them and drive their expansion. For NK cells, however, one has the choice between a multitude of different culture protocols, varying in media composition and different cytokine cocktails (213, 214). Many classical NK cell expansion protocols relied on the presence of genetically modified cytokine-presenting aAPCs (149, 150). The highly effective and attractive stimulation of human NK cells with genetically altered K562 leukemic cells, which has been used in clinical trials outside of Europe, e.g. by Liu *et al.* in their 2020 CD19-CAR NK cell trial (206), is not simply possible in Germany. Therefore, the newly available commercial NK cell culture medium from Miltenyi Biotec has enabled feeder cell-free GMP-grade manufacturing of human NK cell preparations from apheresis (174, 215). This is the main procedure that was used in the iCAN33 consortium *in vitro* and *in vivo.*

While all NK cell culture protocols are very different, a common denominator lies in the artificial phenotype of the resulting NK cells: Cultured NK cells are all $CD56^{superbright}$, tend to uniform expression of NKG2A while still expressing KIRs (216, 217). Despite the high CD56 and NKG2A expression levels, which normally mark the cytokine-secreting immature peripheral blood NK cells, these highly activated cultured NK cells exert profound cytotoxicity even towards target cell lines which are typically resistant towards lysis by freshly isolated, uncultured NK cells (207). With these characteristics, cultured NK cells do not match their physiological counterparts. Thus, the study results in this thesis apply to cultured highly activated NK cells.

The study conducted by Hejazi *et al*. is a good example of how important the choice of culture protocol is to manufacture a final cell therapy product that matches the release criteria: We could show that the new expansion protocol supports $CD33^{+}$ NK cells which do not arise in the K562 feeder cell-dependent culture systems (189). At first, the CD33 expression in NK cells in this study was a dramatic setback, as it severely hampered the expansion of CD33-CAR NK cells under the GMP-compatible culture conditions. Ultimately, however, it also paved the way to the solution, namely, that

homozygous carriers of the T allele at SNP rs12459419 are suitable donors to create “off-the-shelf” CD33-CAR NK cells. The SNP rs12459419 leads to efficient alternative splicing of *CD33* mRNA and largely skipping of exon 2, theoretically yielding a truncated protein lacking the IgV domain. This truncated protein has been described to be retained intracellularly in microglia (187), while its expression in myeloid blood cells and AML blasts could not be shown (188). However, almost all CD33 antibodies (218), including the antibody clone p67.6 I used in this thesis and also the Mylotarg/My96 scFv on which our CAR construct was based, detect an epitope located in exon 2 of *CD33.* Thus, we have not investigated whether the transcript lacking exon 2 is translated in NK cells.

Interestingly, rs12459419 is the very same SNP that has been reported to influence the CD33 expression levels on myeloid cells and thus also to predicts the outcome of GO treatment in AML patients (185, 186). For this SNP it has been shown that the truncated CD33 protein either does not traffic to the cell surface in microglia (187), or that it is not translated at all in myeloid cells and AML blasts (188). From our work and the literature, we concluded that the only way to produce sufficient numbers of CD33-CAR NK cells in a Prodigy device under GMP conditions is to use donors homozygous for the T allele in rs12459419 (189).

4.4 Alternatives to primary NK cells

From the beginning of cellular immunotherapy, using the natural cytotoxicity of innate immune effector cells has been an attractive strategy to potentially treat malignant diseases. Due to the difficulties in expanding NK cells *ex vivo* and their conceived resistance to genetic manipulations, researchers and clinicians in the field have resorted to T cells, which remained the main protagonists in the field of immunotherapy for decades. With the rise of feeder cell-based expansion protocols for NK cells, they reentered the arena of immunotherapy. However, successes were again limited and the obstacle of “resistance” to lentiviral transduction remained (219). To overcome this, three approaches have been developed: Either the use of cytotoxic NK cell lines, or the derivation of NK cells from easy to modify progenitor cells or (induced) pluripotent stem cells (PSC or iPSC), or the use of biological engager molecules to retarget the endogenous immune cells.

4.4.1 NK Cell lines

Although some NK cell lines mirror the hard-to-transduce nature of their primary counterparts, they have the advantage of being continuously growing stable cell lines. Hence, even low gene transfer efficiencies can be made up for by minute selection of clones. This way, many cell lines have been generated over the years that harbor genetic modifications to combat cancer (220, 221), of which NK92 is clearly the most popular. NK92 was derived from the peripheral blood of a non-Hodgkin lymphoma patient (222). NK92 cells exert cytotoxic capabilities similar to peripheral blood NK cells and are dependent in their *in vitro* growth on IL-2 signaling. Although NK92 cells did not initiate any malignant growth or disease in immunodeficient murine models, the regulatory measures world-wide require the irradiation of NK92 cells with 60 Gray prior to infusion into human subjects due to the malignant origin. While infusions of unmodified NK92 were entirely safe even with doses as high as $1x10^{10}/m^2$, clinical responses were limited, consisting predominantly of minor responses and at best stable disease, but rarely CR (223–226). One major advantage of using NK92 instead of primary NK cells are the reproducibly high doses that can be manufactured in short time. Limited successes prompted either the combination of NK92 cells with ICIs, fusion cytokines, or the use of CAR-modified NK92 cells. While many trials are still ongoing, the few available results do not show effects superior to unmodified NK92 cells (227, 228).

When looking at the major limitations of primary NK cell therapy, being the short lifespan and rejection after infusion, the limited successes using NK92 cells are to no surprise. Recent studies highlight again how crucial the *in vivo* lifespan is to therapy response (206). With NK cell lines that receive mandatory irradiation before infusion, the lifespan will be limited to a few days and cannot be prolonged per se. Hence, NK cell lines cannot even level with primary NK cells on this matter.

4.4.2 *In vitro* generation of NK cells from progenitor cells

Being able to efficiently genetically modify immune effector cells appears to be a prerequisite in the age of redirected cellular therapy. As NK cells were considered to be naturally resistant to standard retro- and lentiviral vectors (219), T cells took over as favored effector cell type, when originally NK cells were considered more attractive because of their natural cytotoxicity. While some resorted to NK cell lines, others investigated the production of NK cells *in vitro* from easy-to-transduce progenitor cells.

Bone marrow hematopoietic stem and progenitor cells (HSPCs) are genetically accessible and present the most obvious choice of progenitor cell for generation of NK cells (229, 230). Furthermore, culture and genetic manipulation of human HSPCs is already being performed in the clinics (231, 232), making this step feasible. Researchers had already started to explore the generation of NK cells from HSCs already back in the 1990s (233). Due to the invasive procedures necessary to obtain bone marrow HSCs directly, others resorted to umbilical cord blood (UCB) or mobilized peripheral blood progenitor cells as starting material for generating NK cells (230, 234). The major drawbacks are, even still today, the very time-consuming protocols, lasting for up to 5 weeks, and the necessity of feeder cell layers, which are often transformed murine or human cell lines. Attempts to omit the feeder cell layer or use human stromal cells also works, however yielded lower numbers of NK cells (235, 236).

To overcome the need for obtaining fresh starting primary cell material, (i)PSC lines have been utilized to create NK cells. In principle, PSCs are easy to modify and can effortlessly be subcloned and cultured almost indefinitely. This allows to introduce a whole array of genetic modifications over time and makes using these cells an intriguing approach towards “off-the-shelf” therapeutics. An often-addressed topic in this regard is the HLA barrier. Primary homozygous cells and especially KO of one or various HLA and HLA-associated genes has been proven to yield universal-donor cells in preclinical models (237–241). Using PSCs for the differentiation of NK cells also does not rely on feeder cell lines, as the embryoid bodies efficiently create their own feeder layer. The combination of both, namely NK cell derivation from PSCs and HLA genome editing to create universal donor NK cells, has not been attempted.

Irrespective of the source and the protocol used, these approaches are attractive for immunotherapy purposes, when the desired cell type cannot be genetically manipulated. The work in this thesis, however, showed that with new pseudotyping-envelopes NK cell transductions and genome editing are readily available for research as well as clinical work (192, 207, 242). Depending on the desired modification, the differentiated state of peripheral blood NK cells can also be an advantage: A recent study showed that the differentiation of cytokine-inducible SH2 containing protein(CISH)-deficient NK cells from *CISH*-KO iPSCs can be significantly delayed (243). Hence, when attempting genome editing in progenitor cells, one must take into account that the introduced modification might hamper the differentiation process. Such side effects would preclude the use of progenitor cells to generate NK cells with

the desired modification. Finally, considering the time and cost of several weeks of cultures under GMP-compliant conditions, deriving NK cells from progenitor cells or PSCs appears to be rather impractical compared to the use of primary NK cells for clinical indications.

4.4.3 Engager molecules

With the obstacles of CAR effector cell manufacturing in mind, researchers and clinicians have turned towards the construction of biologically active molecules to bolster the endogenous immune cell response without the need for GMP-compliant cell culture and transduction protocols. Engager molecules like bispecific T/killer cell engagers (BiTE/BiKE) are examples for such molecules. They are comprised of two targeting domains derived from scFvs that crosslink, for example, CD19 or CD33 and either CD3 on T cells or CD16 on NK cells and hence retarget their cytotoxicity (244–246). The most recent addition to the family of engager molecules for NK cells are trispecific killer cell engagers (TriKE), which contain an additional IL-15 molecule as linker between the antigen-targeting moiety and CD16 or NKG2C targeting domain. The simultaneous binding of the IL-15 receptor on the tethered NK cell induces downstream signaling to boost proliferation and cytotoxicity (247, 248).

4.5 Modifications for future NK cell therapies

CARs are currently the predominant genetic modifications that are performed with immune effector cells for immunotherapy. T cells can readily be equipped with CARs and be manufactured at high doses, generating large quantities of highly active CAR T cells capable of HLA-non-restricted killing. Since the initial description of the first generation CAR constructs around the 1990s (249, 249), several generations of CARs, differing in their signaling moieties and effector molecules, have been developed leading to a diversified arsenal of effector responses (3). Yet, redirecting the cytotoxicity of immune cells towards target antigen structures on the surface of tumor cells has always been the prime directive in CAR T cell immunotherapy. With the rather recently developed baboon endogenous retrovirus-derived pseudotype for lentiviral particles, transduction of primary NK cells has become readily possible and allowed for the transfer of CAR technology to NK cells. Several clinical trials are ongoing and the first clinical trial with allogeneic CD19-CAR NK cells showed promising results (206). The direct transfer of CAR technology from T cells to NK cells, however tends

to let us forget the major difference between the two cell types: A single T cell only recognizes one or at least a limited number of target antigen structures due to its unique TCR whereas a single NK cell can recognize malignant cells of multiple origins (23, 26). NK cells govern their cytotoxicity after engaging with the target cells with a plethora of activating and inhibitory receptors. NK cells therefore almost use Boolean logic to analyze signatures of surface molecules on target cells and then mount the adequate responses (21). In addition, when expanding NK cells from peripheral blood under GMP-compliant conditions with IL-2 and IL-15, these activated NK cells exhibit a tremendous boost in cytotoxicity towards virtually any cancer cell line (207, 250, 251). This is, at least in part, achieved by the massive upregulation of aNKRs (217, 252, 253). Therefore, in contrast to T cells, NK cells do not necessarily need a redirecting cytotoxicity receptor to fulfill their anti-tumoral functions, at least *in vitro.* Soldierer *et al.* showed that expression of CARs does not add to the cytotoxicity of activated NK cells against the majority of AML tumor cells *in vitro* (207). Only blocking of the aNKRs with monoclonal antibodies allowed to measure the CAR-mediated NK cell cytotoxicity (207). This evokes the question: Should CARs be the prime or at least the sole genetic modification that is needed for NK cells to become efficient effector cells for immunotherapy in humans? This question can ultimately only be answered with respect to the cancer entity that is targeted. Nevertheless, apart from CAR modifications, researchers have started to explore other options. As the major obstacle in allogeneic NK cell ACT so far has been the limited lifespan of the infused cells in patients, one of the major tasks to address here is to prolong NK cell lifespan *in vivo.* Here, the first human CAR NK cell study by Liu *et al*. (206) used soluble IL-15 expressed by the genetically modified NK cells to enhance their expansion and persistence *in vivo*. Arguably, this could be considered as the major contributing factor to the clinical outcome in these patients with B cell malignancies. As IL-15 can also directly enhance the cytotoxicity of NK cells (207, 217, 250–253), its effects on primary NK cells can even be beneficial without the signaling of a CAR (Hanenberg, iCAN33 network, personal communication 2021).

Within the context of IL-15 signaling in NK cell therapy, the janus kinase/signal transducer and activator of transcription(JAK/STAT)-axis regulating molecule CISH has been identified as fruitful target for NK cell immunotherapy (254). *CISH* expression, once induced by IL-2 or IL-15 signaling, establishes a negative feedback loop by degrading JAK, limiting further IL-15 signaling. Consequently, a *CISH*-KO has been shown to prolong and enhance IL-15-induced signaling in NK cells, thereby boosting

the expansion, life-span and cytotoxicity of NK cells and even improving their metabolic fitness (243, 255). A recent report also suggested a role for CISH as a negative regulator of activating receptor signaling in NK cells (256). Next to the introduction of CARs, the KO or knockdown (KD) of *CISH* has already gained wide attention in the field of NK cell immunotherapy and is by now frequently mentioned when future directions of the field are addressed (156, 243, 256). Another promising candidate for immunosuppressive growth factor signaling, that has been linked to the functional deficits of T and NK cells in the tumor microenvironment of solid tumors, is the transforming growth factor-β receptor II (TGF-βRII) (257). Expression of a dominant-negative TGF-βRII resulted in resistance to the TGF-β mediated suppression of T and NK cells and enhanced their killing efficacy (257–259). Yet, with genome editing of primary NK cells in hand, multiple attractive candidates can now be systemically tested.

Another opportunity for the extension of the lifespan extension of infused CAR lymphocytes could lie in manipulating epigenetic modifiers, as a recent publication suggested: In a CD19-CAR T cell trial, insertional mutagenesis of the CAR lentiviral provirus into the *tet methylcytosine deoxygenase 2* (*TET2)* locus led to the emergence of a hyperproliferative CAR T cell clone (260). As the second *TET2* allele in this patient was hypomorphic, the insertional inactivation by the CAR lentivirus led to complete loss of TET2 activity in this single T cell. The resulting epigenetic perturbation in turn favored the outgrowth of the single cell, which at its peak made up for 94% of the peripheral blood CAR T cells of the infused patient (260) and was considered as highly successful in eradicating the residual malignant B cells. Besides the excessive growth, this T cell clone showed a bias towards a central memory phenotype and potent cytokine secretion, leading to high-grade cytokine release syndrome and CR. Ever since this study, *TET2* has been implied as a potential target to enhance the immunotherapy with CAR T cells. In NK large granular lymphoma (LGL), *TET2* has in fact been observed to be either frequently mutated or its promoter to be methylated (261, 262). Thus, *TET2* could be an interesting target gene to promote primary NK cell proliferation beyond the physiological levels *ex vivo* upon KO or KD. Yet, no such study has been conducted up to this point. Another frequently underexpressed gene in NK cell lymphoma is receptor-type tyrosine-protein phosphatase κ (*PTPRK*) (263). As a negative regulator of phospho-STAT3, PTPRK normally represses proliferative signaling in NK cells. In nasal-type NK/T cell lymphoma, *PTPRK* is either heterozygously lost or hypermethylated, leading to aberrant STAT3 signaling which

promotes tumoral progression. *STAT3* itself has also been shown as a mutational hotspot in NK and T cell LGL, especially within the SH2 domain that is responsible for STAT3 dimerization (264).

4.6 Concluding remarks

Above, I have discussed the findings of this thesis along the lines of current immunotherapy approaches and ongoing research. Figure 7 represents an overview of the findings of this thesis, based on the aims and the outlook on future therapies to expand NK cell lifespan. Figure 7A emphasizes the need for application-dependent culture and selection protocols. Unlike the culture of T cells, which has already been standardized, NK cell cultures produce highly variable products depending on the protocol used. The example here in this thesis is the expression of CD33 on cultured NK cells following the feeder-free protocol used in our two studies (189, 192). Hejazi

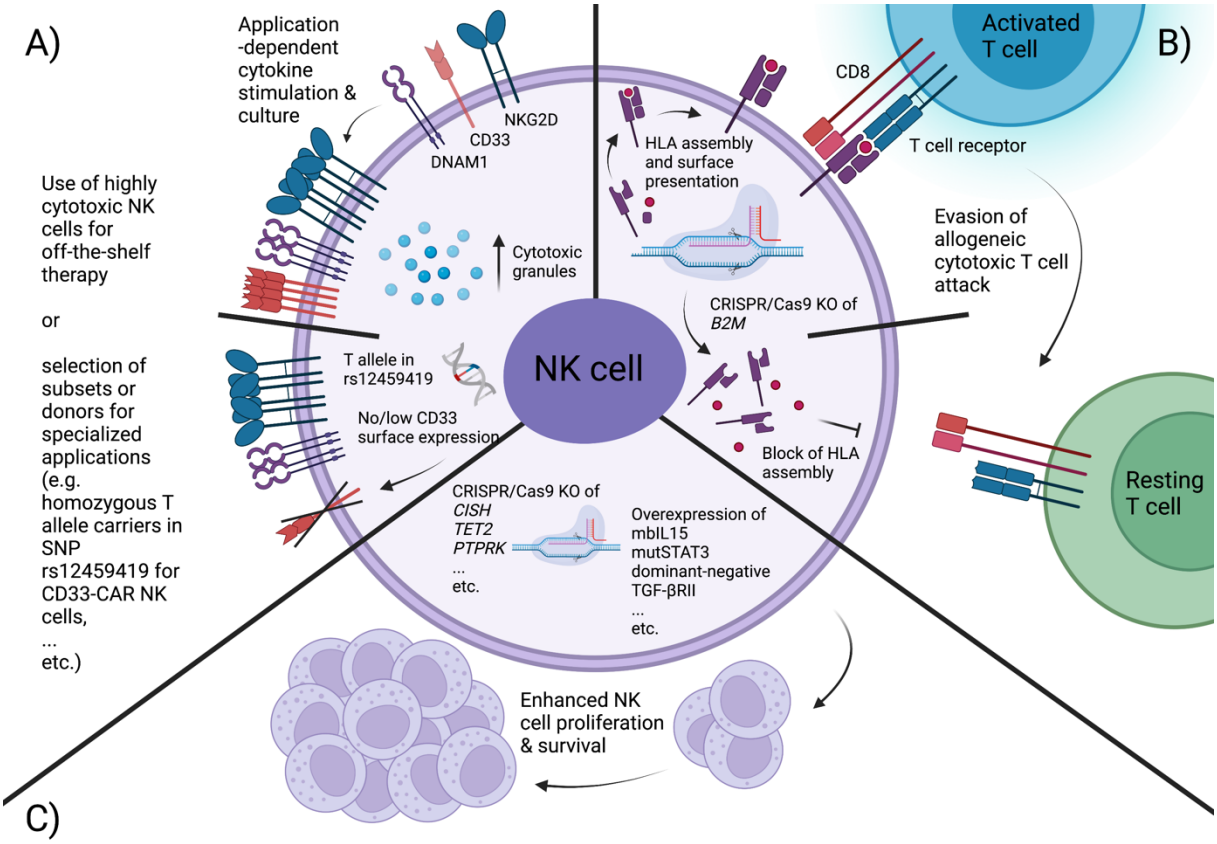


Figure 7: Future directions to optimize NK cell immunotherapy. A) Cytokine and culture conditions should be established to optimize the manufactured cell type to match the desired application. In this case, NKG2D, DNAM-1, CD33 and cytotoxic effector molecules in cytotoxic granules are upregulated by culture and result in a highly cytotoxic effector cell for "off-the-shelf" immunotherapy. In case of the homozygous T allele in SNP rs12459419, CD33 surface expression is low and enables CD33-CAR NK cell manufacturing as they do not target each other when expressing the CAR. B) Allogeneic NK cells are recognized by T cells by HLA class I molecules (T cell with blue halo; TCR binding to HLA on NK cell). When B2M is knocked out by genome editing, HLA class I assembly and hence surface expression is blocked. The NK cell is now evading the cytotoxic T cell (green resting T cell with TCR not bound to NK cell). C) Genome editing of known or proposed factors involved in NK cell proliferative senescence can help to enhance NK cell proliferation and survival (created with BioRender.com).

and colleagues have shown that the aberrant CD33 expression marks an especially cytotoxic NK cell subset which arises only when using the new NK cell medium from Miltenyi Biotec and not with the aAPC-based approaches that have been widely used before (189). Also, the finding is highlighted that NK cell cultures from individuals being homozygous for the T allele at SNP rs12459419 in *CD33* exon 2 can be harnessed to create CD33-CAR NK cells. In Figure 7B, the strategy of overcoming the HLA barrier by KO of *B2M* is outlined, which has been shown by us to avert cytotoxic T cells from attacking allogeneic NK cells to prolong their persistence and hence efficacy (192). A recently published study showed that administration of N-803 expands alloreactive $CD8^+$ T cells which rejected haploidentical NK cells infused in increasing doses in AML patients (193). Using the KO of *B2M* here could be an attractive countermeasure to enable use the of N-803 and haploidentical NK cell infusion without risking their rejection. Finally, Figure 7C depicts the yet unused potential of genetic engineering and genome editing in NK cells to boost their potential *in vivo*. I have stated that current immunotherapy approaches are designed in major part after T cell therapies, relying on CARs or engager molecules to retarget NK cell cytotoxicity. I conclude that future NK cell therapies will only succeed when leaving behind the concept of redirecting NK cell activity. A focus should rather be on the means to overcome NK cell specific limitations, such as their limited lifespan, susceptibility to the tumor microenvironment and cytokine-dependency. Exemplary studies using soluble IL-15, KO of *TET2* or *CISH* or overexpression of dominant-negative TGF-βRII were discussed and might just be the beginning for a new class of NK cell therapeutics (207, 256, 257, 260–262).

Looking back on the past years of immunotherapy, a shift in the field is imminent: Coming from the idea of personalized medicine, where an autologous CAR T cell product is designed specifically for each patient, we are now turning our eyes back to “off-the-shelf” solutions like with pills and other medicines in a pharmacy. While this strategy seemed unlikely to be achievable with T cells due to the HLA barrier and the dangers of GvHD, NK cells were always suited for the task. Yet, they have been hard to culture, expand and genetically modify. With these obstacles overcome, we are now expanding the toolbox of immunotherapy, hoping to be able to provide not only effective, but also affordable therapies for all of humanity. Yet, many hurdles remain to bolster the efficacy of NK cell therapy products, especially for treatment of, for example, solid tumors. I am certain that this thesis will contribute to help the field to move forward.

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List of abbreviations

A

(a/i)KIR	(activating/inhibitory) Killer immunoglobulin-like receptor
(a/i)NKR	(activating/inhibitory) Natural killer receptor
(a)APC	(artificial) Antigen presenting cell
ACT	Adoptive cell transfer
ADCC	Antibody dependent cellular cytotoxicity
AML	Acute myeloid leukemia

B

B2M	Beta(β)2-microglobulin
BCR	B cell receptor
BCL-2	B-cell lymphoma 2
BID	BH3 interacting domain death agonist
BiKE	Bispecific killer cell engager
BiTE	Bispecific T cell engager
BMT	Bone marrow transplant

C

CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CIML	Cytokine-induced memory-like
CISH	Cytokine-inducible SH2 containing protein
CLP	Common lymphoid progenitor
CLL	Chronic lymphocytic leukemia

D

DAP12	DNAX-activation protein 12
DARPin	Designed ankyrin repeat protein
DLI	Donor lymphocyte infusion
DNAM-1	DNAX accessory molecule 1

E

EFRE	Europäischer Fonds für regionale Entwicklung
EMA	European Medicines Agency
ERp57	Endoplasmic reticulum-resident protein 57

F

FcγRIII	FC γ receptor III
FDA	Food and Drug Administration

G

GMP	Good manufacturing practice
GO	Gemtuzumab Ozogamicin
GvHD	Graft-versus-host-disease
GvT	Graft-versus-tumor (effect)

H

HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transfer
HSPC	Hematopoietic stem and progenitor cell

I

ICI	Immune checkpoint inhibitor
IL	Interleukin
ILC	Innate lymphoid cell
IL2RB	Interleukin-2 receptor subunit β
(i)PSC	(Induced) pluripotent stem cell
ITAM	Immunotyrosine activating motif
ITIM	Immunotyrosine inhibitory motif

J

JAK	Janus kinase

K

KD	Knockdown
KO	Knockout

L

LAG3	Lymphocyte activation gene 3
LAK	Lymphokine-activated killer
LFA-1	Leukocyte function-associated antigen-1
LGL	Large granular lymphoma

M

mAB	Monoclonal antibody
mb	Membrane-bound
MHC	Major histocompatibility complex
MIC-A/B	Major histocompatibility complex class I-related chains A/B
MRD	Minimal residual disease

N

NCR	Natural cytotoxicty receptor
(n)CR	(near) Complete remission
NHL	Non-Hodgkin lymphoma
NK	Natural Killer
NKG2A	Natural killer group 2A
NKG2C	Natural killer group 2C
NKG2D	Natural killer group 2D
NKP	Natural killer cell progenitor
NRW	North Rhine Westphalia

P

PBMC	Peripheral blood mononuclear cell
PDI	Protein disulfide isomerase
PTPRK	Receptor-type tyrosine-protein phosphatase κ

S

sc	Single-chain
scFv	Single-chain variable fragment
sIg	Surface immunoglobulin
SLAMF7	Signaling lymphocyte activation marker family member 7
SNP	Single-nucleotide polymorphism

SRSF2	Serine and arginine rich splicing factor 2
STAT	Signal transducer and activator of transcription

T

TCR	T cell receptor
TET2	Tet methylcytosine deoxygenase 2
TIGIT	T-cell immunoreceptor with Ig and ITIM domains
TIL	Tumor infiltrating lymphocyte
TIM-3	T cell immunoglobulin and mucin domain-3
TGF-βRII	Transforming growth factor β receptor 2
TriKE	Trispecific killer cell engager

U

UCB	Umbilical cord blood
ULBP	UL16 binding protein

V

VOD	Veno-occlusive disease

List of figures

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Publications

CD33 Delineates Two Functionally Distinct NK Cell Populations Divergent in Cytokine Production and Antibody-Mediated Cellular Cytotoxicity

Hejazi M, Zhang C, Bennstein SB, Balz V, Reusing SB, Quadflieg M, **Hoerster K**, Heinrichs S, Hanenberg H, Oberbeck S, Nitsche M, Cramer S, Pfeifer R, Oberoi P, Rühl H, Oldenburg J, Brossart P, Horn PA, Babor F, Wels WS, Fischer JC, Möker N and Uhrberg M (2022). Front. Immunol. 12:798087. doi: 10.3389/fimmu.2021.798087

HLA Class I Knockout Converts Allogeneic Primary NK Cells Into Suitable Effectors for "Off-the-Shelf" Immunotherapy

Hoerster K, Uhrberg M, Wiek C, Horn PA, Hanenberg H and Heinrichs S (2021) Front. Immunol. 11:586168. doi: 10.3389/fimmu.2020.586168

Signaling properties of murine MPL and MPL mutants after stimulation with thrombopoietin and romiplostim

Rommel MGE, **Hoerster K**, Christian Milde C, Franziska Schenk F, Roser L, Kohlscheen S, Heinz N, and Modlich U (2020) Exp Hematol. 85: 33-46.e6. doi: 10.1016/j.exphem.2020.04.006

Hematopoietic-Extrinsic Cues Dictate Circadian Redistribution of Mature and Immature Hematopoietic Cells in Blood and Spleen

Stenzinger M, Karpova D, Unterrainer C, Harenkamp S, Wiercinska E, **Hoerster K**, Pfeffer M, Maronde E, Bonig H (2019). Cells. 5;8(9):1033. doi: 10.3390/cells8091033.

Curriculum vitae

The curriculum vitae is not included in the online version for data protection reasons.

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