NATURAL KILLER CELLS
FROM UMBILICAL CORD BLOOD STEM CELLS:
A NOVEL IMMUNOTHERAPY PLATFORM FOR SOLID TUMORS

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CONTENTS

CHAPTER 1

General introduction and Scope of this Thesis

Adapted in part from the publication
"The rise of allogeneic Natural Killer cells as a platform for cancer immunotherapy: Recent innovations and future developments"

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GENERAL INTRODUCTION

Cancer comprises a class of diseases characterized by uncontrolled cell growth and invasion. Well known external factors contributing to cancer in humans are mainly related to unhealthy diet patterns, tobacco use, heavy consumption of alcohol, and prolonged exposure to carcinogenic chemicals and ionizing radiations[1]. Further, hereditary defects in the genetic make-up, viral infections and chronic inflammation can also predispose to cancer. Although advances in cancer research have contributed to better survival over recent years, certain challenges remain to be addressed[2,3]. Tumors are broadly classified into hematological (cancers of the blood) and solid tumors (affects tissues/organs). Tumors can either be benign (not cancer), or malignant (cancer)[4]. Upon diagnosis, malignant solid tumors are treated by conventional methods (surgery, chemotherapy and radiotherapy) to reduce the tumor mass. However, once metastatic disease has developed these cancers are generally not curable. Therefore, concerted research is required to develop novel treatments that will improve patients' outcome. Our immune system is a collection of immune effector subsets and molecules employed by our body to fight several diseases, including cancer. In a healthy individual, cells of the immune system such as B cells, T cells, and Natural Killer (NK) cells are thymus and/or bone marrow derived and distributed throughout the body providing specific and non-specific protection against viruses, bacteria and cancer cells[5]. B cells differentiate into plasma cells and produce antibodies that can direct immunity to specific targets (i.e. adaptive humoral immunity). T cells are the principal effectors responsible for cell mediated adaptive immunity and require antigen presentation by professional antigen presenting cells such as macrophages and dendritic cells. NK cells are part of the innate immune defense. They can identify stressed cells and provide a first line of protection against various infections but also against cancer cells[6, 7].

Natural killer cells in oncology

So far T cells have been the mainstay of cancer immunotherapy approaches that focus on adoptive cell therapy. It is generally recognized that NK cells play an essential role in anti-tumor immunity. Certainly, in case of the prevention of metastases through the elimination of circulating cancer stem cells with a high metastatic potential, NK cells are recognized to be the main immune effector cells[8]. Moreover, as solid tumors have a propensity to down-regulate MHC-I, NK cells provide a failsafe mechanism in these circumstances where cytotoxic T cells, which depend on MHC-I for tumor recognition and elimination, are debilitated. NK cells have recently been more intensely explored as a viable therapeutic platform next to T cell-based approaches.

Natural killer cells and their activating and inhibitory receptors

Human NK cells are generally categorized by their level of CD56 and CD16 expression into two subsets: $CD56^{bright}CD16^{dim}$ and $CD56^{dim}CD16^{bright}$ NK cells. Most NK cells in the peripheral blood and spleen are $CD56^{dim}CD16^{bright}$ and are cytotoxic against a variety of tumor cells, whereas $CD56^{bright}CD16^{dim}$ NK cells are immune regulatory in function and constitute the majority of NK cells in secondary lymphoid tissues, producing abundant cytokines but exerting weak cytotoxicity compared to $CD56^{dim}CD16^{bright}$ NK cells[9]. The ability of NK cells to discriminate between a cancer cell and a healthy cell is regulated by a balance between activating and inhibitory receptors. NK activating receptors like DNAM-1, NKG2D, Natural Cytotoxicity Receptors (NCRs) NKp30, NKp44, NKp46, CD94/NKG2C, CD94/NKG2E, CD16a and activating killer cell-immunoglobulin like receptors (KIRs) contribute to NK cell activation, triggering the release of cytotoxic granules and pro-inflammatory cytokines such as interferon-gamma (IFNγ) to fight cancer cells[10]. The NK cell activating receptor NKG2D (CD314) recognizes MHC class-I-chain related protein A and B (MICA and MICB) and ULBPs (1-6)[11], while DNAM-1 binds to CD112 (Nectin-2) and CD155 (poliovirus receptor)[12] on stressed, infected, and cancer cells. The ligands for NCRs are widely expressed on cells infected by viruses or by intracellular bacteria and on tumor cells, but their exact modes of action and roles in NK cytotoxicity are yet to be characterized[13]. In addition to this, the heterodimers of the NKG2 family; CD94/NKG2C and CD94/NKG2E recognize the non-classical MHC class I molecule HLA-E and associate with DAP-12 molecule to trigger an NK activation signal[14,15]. Another very important activation mechanism of NK cells is through the interaction of CD16a (FcγRIIIa, a low affinity Fc receptor) with the Fc portion of IgG_1 antibodies, forming an immunological synapse to engage antibody opsonized targets for NK cell mediated antibody dependent cell mediated cytotoxicity (ADCC)[16]. In addition to this, NK cells can also lyse tumor targets using Tumor Necrosis Factor α (TNF-α), Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL)[17]. The most prominent NK cell inhibitory receptors include inhibitory KIRs that recognize MHC class I (HLA-ABC) molecules, which are expressed on healthy tissues. Similarly, CD94/NKG2A, an inhibitory receptor from the NKG2 family, binds to HLA-E and induces NK cell tolerance through the activation of an intracellular immunoreceptor tyrosine-based inhibitory motif (ITIMs)[15]. Hence, knowing that NK cell functions are determined by an array of receptors, which can transmit either an activating or an inhibitory signal, depending on different ligand interactions on the surface of tumor cells, it is critical to shift the balance in a therapeutic setting towards an activating NK phenotype to expedite enhanced NK tumor killing mechanisms (Figure 1).

NK cell dysfunctionality in cancer

NK cells can control circulating tumor cells and prevent formation of tumor metastases[18]. However, tumors employ different strategies to evade killing by NK cells. Up-regulation of

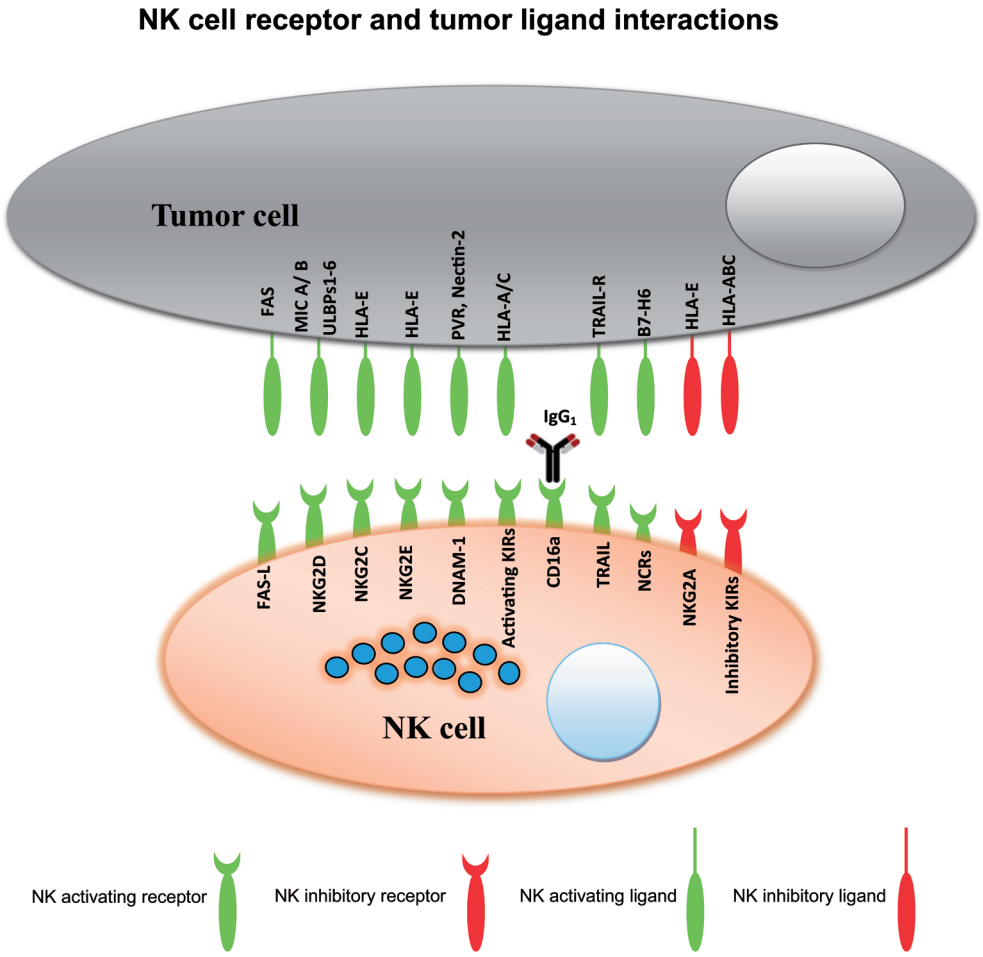

FIGURE 1: An overview of major NK cell activating and inhibitory receptors and their corresponding ligands expressed on tumor cells.

inhibitory ligands such as MHC class I molecules (HLA-ABC, HLA-G and HLA-E) has been associated with inhibition of NK cell activation[19-22]. Furthermore, as reported in renal cell carcinoma, increased expression of the inhibitory NKG2A receptor resulted in decreased functionality of tumor infiltrating NK cells[23]. On the other hand, down-regulation of NK activating ligands for NKG2D such as MICA and MICB, and increased shedding of tumor derived soluble MIC also impair NKG2D mediated NK cell tumor recognition[24]. Another important necessity for optimal NK cell function is the ability to home and migrate to tumor sites. Several studies have correlated increased homing of NK cells to tumor tissues with improved treatment outcomes in solid tumors[25-29]. However, the immunosuppressive tumor

infiltrate, comprising regulatory T cells (T-regs)[30], myeloid derived suppressor cells (MDSCs)[31], M2 macrophages[32] and immature dendritic cells, severely restricts NK cell functionality and possibly their entry into solid tumors. In chronic viral diseases, such as those associated with human immunodeficiency virus and cytomegalovirus infections, mainly exhausted NK cells with decreased cytokine production and reduced cytolytic activity are observed[33,34]. In cancer, similar observations have been made. In a study with breast cancer patients, the NK cell expression levels of activating receptors (NKG2D, DNAM, CD16 and NKp30) were decreased, whereas inhibitory receptor (NKG2A) expression levels were increased and this apparent dysfunctionality of NK cells was found to directly affect NK cell cytotoxicity[35]. Moreover, the effector subset of NK cells ($CD56^{dim}CD16^{+}$) from head and neck and breast cancer patients, when tested *in vitro*, was highly prone to apoptosis, which in part may also explain the low NK cell activity observed in these patients[36]. Impaired NK cell functionality may result from tumor-imposed suppressive mechanisms and presents a major hurdle for NK cell targeted immunotherapies. Therefore, approaches to restore or replace impaired NK cell cytotoxicity may prove essential for an effective host defense against cancer.

NK cells in the clinic

Novel NK cell based immunotherapeutic strategies are being developed to overcome the functional limitations of the use of cancer patient's autologous NK cells. To increase the number of functional NK cells even in case of a high tumor load, adoptive transfer of autologous NK cells served as a feasible approach, as this ruled out the need for immunosuppression, HLA-matching, and prevented the risk of graft versus host disease (GvHD). These advantages sparked the initiation of large-scale expansion protocols and clinical trials using autologous NK cells as a treatment modality for cancer. Though adoptive transfer of autologous NK cells resulted in an increased number of circulating NK cells in peripheral blood, it failed to produce significant therapeutic effects in hematological malignancies, metastatic melanoma and renal cell carcinoma, likely due to the inhibition by self-HLA molecules[37-39]. Moreover, the expansion efficiency and functional status of autologous NK cells were still limited when compared to allogeneic NK cells, as autologous cells were often obtained from heavily pre-treated patients[40]. In addition to this, it was difficult to track infused autologous NK cells in patients and to study their anti-tumor effects from peripheral blood analyses due to the inability to differentiate *ex vivo* manipulated and *in vivo* transferred autologous NK cells from the non-manipulated circulating NK cells. These limitations motivated researchers to shift their focus to allogeneic NK cells to treat cancer.

In patients with leukemia undergoing allogeneic Hematopoietic Stem Cell Transplantation (HSCT), NK cells, being the first lymphoid subset to appear after allogeneic HSCT[41], play a crucial role in controlling host defense against infections and residual cancer cells before T cells are reconstituted[42]. These donor T cells are prime mediators of GvHD[43] and the

life-threatening complications which arise due to GvHD have completely overshadowed the beneficial effects of alloreactive NK and T cells, fueling efforts to use T cell depleted grafts[44]. Further, this led to the development of NK cell-based therapies coupled with T cell depleted HSCs to enhance the graft versus tumor effect (GvT) without causing GvHD. Unlike autologous NK cells, allogeneic NK cells are not restricted by the patient's tumor's HLA expression, which is an added advantage to mount an improved anti-tumor effect[45,46]. Current translational efforts that are explored as anti-cancer therapies include adoptive transfer of *ex vivo* activated and/or expanded allogeneic NK cells, either alone or in combination with HSCT.

Sources of allogeneic NK cells used in the clinic

Commonly used allogeneic NK cells are apheresis products collected from haploidentical and unrelated donor PBMC[47]. Another source is umbilical cord blood (UCB), from which NK cells are generated from CD34+ progenitor cells that undergo expansion and differentiation using cytokines and growth factors and thereby mature into cytolytic NK cells[48]. Apart from PBMC and UCB, allogeneic NK cells have also been obtained from the clonal cell line NK-92, derived from immortalized lymphoma NK cells[49,50].

Allogeneic NK cell therapy in a transplant setting

Autologous or allogeneic HSCT serves as a curative regimen by reconstituting the immune system in hematological malignancies. At an earlier stage post HSCT, NK and T cells developing from the graft are immature and less in number with reduced functionality. Under those circumstances, the infusion of purified allogeneic NK cells was explored as a viable option to target minimal residual disease (MRD) and so prevent graft failure and relapse. Grafts for allogeneic HSCT and allogeneic NK cell treatments were obtained from HLA matched/mismatched and related/unrelated donors[45,46]. Earlier clinical trials performed by Passweg et al (2004)[51], Koehl et al (2005)[52], Shi et al (2008)[53], Yoon et al (2010)[54], Rizzieri et al (2010)[55] and Brehm et al (2011)[56] have shown that NK cells can be safely administered prior to or post-HSCT in patients with different types of hematological diseases. Immune suppression is a pre-requisite for most of the allogeneic HSCT and NK cell infusions. A non-myeloablative conditioning regimen usually consisting of cyclophosphamide (Cy) and fludarabine (Flu) was found to facilitate NK cell persistence and expansion *in vivo* [57]. High doses of Cy/Flu caused pancytopenia and resulted in high plasma IL-15 levels which also correlated with the detection of adoptively transferred NK cells up to 14 days after infusion, thus suggesting that excess IL-15 was probably utilized by the NK cells to proliferate and persist longer *in vivo*[58]. A summary of clinical trials with allogeneic NK cell infusions in a HSCT setting with published results[59] is summarized in Table 1. Taken together, it is evident from these studies, as well as from many others, that GvHD, which is mainly caused by T

cells from transplanted grafts, is a major concern in the field of allogeneic HSCT. Under these circumstances, it is difficult to reliably study the safety of allogeneic NK cell infusions. The timing of NK cell infusion, NK cell dosage and NK cell promoting conditioning regimens are critical factors that need to be more extensively studied to assess the safety and efficacy of allogeneic NK cell infusions.

Adoptive NK cell therapy in a non-transplant setting

To gain a better understanding of the safety and efficacy of allogeneic NK cell transfer, investigators started to study NK cells in a non-transplant setting. Landmark clinical trials were performed by Miller et al[57], Iliopoulou et al[60], Rubnitz et al[61], Bachanova et al[62] Curti et al[63] and Geller et al[40], predominantly in hematological malignancies, but also in various solid tumors. These studies demonstrated the safety and in part the efficacy of allogeneic NK cell infusions in the absence of GvHD. A summary of allogeneic NK cell clinical trials in a non-transplant setting with published results[59] is presented in Table 2. Overall, analyzing the data from adoptive allogeneic NK cell therapy trials in a non-transplant setting, we conclude that such treatments are safe and well tolerated and efficacious in hematological malignancies, especially in AML, but as yet relatively ineffective in solid tumors. Trials using allogeneic NK cells alone yielded valuable information on the *in vivo* persistence, donor chimerism and anti-tumor potential in different indications. Furthermore, unlike combined approaches with HSCT, the absence of life threatening GvHD and major treatment related toxicities makes this method advantageous and provides an opportunity to further enhance the cytotoxic effects of allogeneic NK cells.

TABLE 1: Summary of allogeneic NK cell clinical trials in a transplantation setting

Study	Malignancy	Clinical Trial design	Culture method*
Phase I (NCT01729091) *Shah et al* (2017) ref[64]	MM (n=12)	Conditioning with Mel on day -7 and Lnd from days -8 to -2 prior to UCB-NK cell infusion (day -5), followed by autologous-HSCT on day 0	*Ex vivo* expanded MNCs from unrelated UCB donors. Culture duration: 14 days with irradiated K562 clone 9.mbIL-21 aAPCs and IL-2 *CD3 depleted (on day 7)
Phase I (NCT01795378) *Choi et al (2016)* ref[65]	AML (n=45) and ALL (n=6)	Haplo-HSCT followed by DNKI from the same donor on day 6, 9, 13 and 20 post HSCT	*Ex vivo* expanded and activated PBNK-cells from haploidentical donors. Culture duration :2-3 weeks with IL-15 and IL-21
Phase I (NCT00402558) *Lee et al (2016)* Phase II (NCT01390402) ref[66]	AML (n=8), MDS (n=6) and CML (n=7)	Conditioning with Flu/Bu prior to haplo- allo NK-cell infusion, followed by IL-2 therapy (5x, daily); conditioning with Thy/Tac prior to HLA matched related unrelated allo-HSCT	*Ex vivo* expanded and activated PBNK-cells from haploidentical donors. Culture duration: o/n with IL-2. *CD3 depleted and CD56 selected (in 3 infusions)
Phase I (NCT01287104) *Shah et al (2015)* ref[67]	EWS (n=5), DSRCT (n=3), RMS (n=1)	HLA matched haplo- or unrelated allo-HSCT followed by aNK-DLI from the same donor on day 7 and 35 post HSCT	*Ex vivo* expanded and activated PBNK-cells from haploidentical donors. Culture duration :9-11 days with KT64.4-BBL artificial antigen presenting cells. *CD3 depleted and CD56 selected
Phase I/II (NCT01220544) *Killig et al (2014)* ref[68]	AML (n=24)	Haplo-HSCT followed by NK-cell infusion from same donor and OKT3 treatment from days -5 to+3	PBNK-cells from haploidentical donors. *CD3 depleted and CD56 selected
Phase I/II (NCT00823524) *Choi et al (2014)* ref[69]	AML (n=32), ALL (n=7), MDS (n=1), DLBCL (n=1)	HLA haplo-HSCT followed by DNKI from the same donor, 14 days and 21 days after HSCT	*Ex vivo* expanded and activated PBNK-cells from haploidentical donors. Culture duration :13-20 days with IL-15, IL-21 and hydrocortisone.
Phase I (IND # 12971) *Klingemann et al (2013)* ref[70]	NHL (n=6), MM (n=5) and HL (n=2)	MHC-mismatched haploidentical NK-MC infusion, 49-191 days post auto-HSCT	*Ex vivo* expanded and activated PBNK-cells from haploidentical donors. Culture duration: o/n with IL-2
Phase II (NCT01386619) *Stern et al (2012)* ref[71]	AML (n=8), ALL (n=5), HL (n=2) sarcoma(n=1)	Haplo-HSCT followed by NK-DLI from the same donor, +day 3, +day 40 and +day 100 post HSCT	PBNK-cells from haploidentical donors. *CD3 depleted and CD56 selected
Phase I/II (NCT01386619) *Brehm et al (2011)* ref[56]	AML (n=6), ALL (n=5), NB (n=5), RMS (n=1) HL (n=1)	Haplo-HSCT followed by IL-2 stimulated NK-cell infusion (cryo) or unstimulated NK-cell infusion (fresh) from the same donor, +day 3, +day 40 and +day 100 post HSCT	*Ex vivo* expanded and activated PBNK-cells from haploidentical donors. Culture duration :9-14 days with (group II) or without (group I) IL-2 (fresh or cryo). *CD3 depleted and CD56 selected

Infused dose NK-cells	Final product characteristics	Outcome
4 Escalating doses: 5×10^6, 1×10^7, 5×10^7 and 1×10^8 cells/kg	Mean purity: 98.9% CD56+/CD3- cells	Well tolerated. No GvHD. 4/12 progressed or relapsed. (median of 21 months follow-up)
4 Escalating doses: Median DNKIs are 5×10^7, 5×10^7, 1×10^8and 2×10^8 cells/kg	Median viability: 80%. Purity: 48-98% CD56+CD122+ cells. 0-22% CD3+CD56+ cells. 0-10.4% CD3+CD56- cells.	Toxicity observed in 73% of patients, 9/45 aGvHD. 29/51 CR (9.3-34.7 months follow-up), 35/51 PD
4 Escalating doses: 1×10^6, 5×10^6, 3×10^7 and 3×10^7 cells/kg in Phase I study. 4 escalating doses of 5x10^6 cells/kg in Phase II study.	Median purity: 0.02% CD3+ cells. 11.41% CD14+ cells. 21.84% CD19+ cells. 14.1% CD56+CD3- cells.	Well tolerated, no GvHD. 5/21 CR, 5/21 died of transplantation related issues and 11/21 died of relapse
Repeated doses (2x dose 1, 2 and 3): 1x10^5 cells/kg (dose 1), 1x10^6 cells/kg (dose 2) and 1x10^7 cells/kg (dose 3)	Median purity: CD3+ cells 1.4x10^4 cells/kg. CD56+ cells≥90%. Viability: ≥70%.	5/9 aGvHD. 2/9 SD, 7/9 CR. 4/9 are still alive (12.5-27.4 months after treatment)
Single dose: 1.61-32.2x10^6 CD56+/CD3- cells/kg	Purity: CD56+CD3- cells 99.97%. CD3+ cells 0.95-7.4x10^4 cells/kg	Toxicity correlated with haplo-HSCT. Deaths: 2/24 GvHD, 6/24 infections and 7/24 died of relapse. 9/24 CR (0.1-8.6year follow-up)
Escalating doses (2x): 0.2x10^8 cells/kg (3 pts), 0.5x10^8 cells/kg (3 pts), 1.0x10^8 cells/kg (8 pts) and ≥1.0x10^8 cells/kg (27 pts)	Viabillity:71-85%. Median purity: CD56+CD122+ cells >90%. CD3+CD56+ cells <3%. Fold expansion: 0.8-70 (after 13-20 days of culture)	Well tolerated. 9/41 aGvHD, 10/41 cGVHD. In total 11 patients died of TRM. In AML (21/29) (4/8) ALL/ Lymphoma are in CR
4 Escalating doses: 1×10^5 , 1×10^6 , 1×10^7 and 2×10^7 MC/kg	Median purity: 26% CD56+CD3- cells. 0.15% CD3+ cells. Median viability: 95% post wash.	Well tolerated. No GvHD. 6/13relapsed and 7/13 in remission.
Repeated doses (2-3): 0.3-3.8x10^7 cells/kg	Median purity: CD3+ cells 0.03x10^5 cells/kg. Median viability: 84%.	Safe and feasible. 4/16 aGvHD. Median follow-up of 5.8 years 4/16 are alive. 3/16 died from graft failure-
Repeated doses (1-3 doses): Group I: 3.2-38.3x10^6 cells/kg Group II: 6.0-45.1x10^6 cells/kg	Purity: CD56+CD3- cells 84.4-98.6%. CD3+ cells group I: 0.4-53.4x10^3 cells/kg. CD3+ cells group II: 7.7-98.3x10^3 cells/kg. Viability: freshly NK-cell unstimulated median 93%. Cryo NK-cell IL-2 stim 30-70%	Well tolerated without GvHD > grade II. Group I: 5/9 died (126-498 days post SCT), 3/9 CR (742-2218 days). Group II: 5/9 died (27-373), 2/9 CR and 2/9 in remission

Study	Malignancy	Clinical Trial design	Culture method*
Phase I (NCT00586690) *Rizzieri et al (2010)* ref[55]	Lymphoma (n=30)	3-6/6 HLA-matched haploidentical NK-cell infusion, 6 to 8 weeks post haplo-HSCT from the same donor	PBNK-cells from haploidentical donors. * Only CD56 selected
Phase I (NCT00569283) *Yoon et al (2010)* ref[54]	AML (n=12) MDS (n=2)	HLA mismatched HSCT followed by allo NK-cell infusion from the same donor	*Ex vivo* expanded, differentiated and activated CD34+ progenitor-cells (PB-derived) from haploidentical donors. Culture duration: 21 days with FLt3, IL-7 and hydrocortisone followed by 21 days with IL-15, IL-21 and hydrocortisone
(BB-IND-11347) *Shi et al (2008)* ref[53]	MM (n=10)	Conditioning with Flu/Dex/Mel followed by haplo- KIR-ligand mismatched NK-cell infusion on day 0 and day +2; IL-2 therapy daily (11x) starting on day +1 after NK-cell infusion; auto-HSCT on day +14	*Ex vivo* expanded and activated PBNK-cells from haploidentical donors. Culture duration: o/n with IL-2 (pts 1-5) and brief incubation with IL-2 and anti-CD3 beads (pts 5-10).
Pilot study *Koehl et al (2004)* ref[52]	AML (n=1) ALL (n=2)	Haplo-HSCT followed by KIR mismatched NK-cell infusion on day +1 or +2 post HSCT and additional infusions every 4-6 weeks; IL-2 therapy +2 days post HSCT, every second day for 2-4 weeks	*Ex vivo* expanded and activated PBNK-cells from haploidentical donors. Culture duration :>12 days with IL-2. *CD3 depleted and CD56 selected (in 3 infusions)
Pilot study *Passweg et al (2004)* ref[51]	AML (n=4), CML (n=1)	Haplo-HSCT followed by NK-DLI from the same donor 3-12 months post HSCT	PBNK-cells from haploidentical donors. *CD3 depleted and CD56 selected

Abbreviations Malignancies: Central Nervous System (CNS), Myeloproliferative Disorders (MPD), Lymphoproliferative Disorder (LPD), Multiple Myeloma (MM), Myelodysplastic Syndromes (MDS), Myelodysplastic Neoplasms (MDN) Myeloproliferative Neoplasms (MPN), Acute Myeloid Leukemia (AML), Lymphoblastic Leukemia-Lymphoma (LBLL), Acute Lymphoblastic Leukemia (ALL), Neuroblastoma (NB), Rhabdomyosarcoma (RMS), Chronic Myelogenous Leukemia (CML), Non-Hodgkin's Lymphoma (NHL), Mantle Cell Lymphoma (MCL), Diffuse Large B Cell Lymphoma (DLBCL), Follicular Lymphoma (FL), Anaplastic Large Cell Lymphoma (ALCL), Hodgkin's Lymphoma (HL), Renal Cell Cancer (RCC), Small Cell Lung Cancer (SCLC), Chronic Lymphocytic Leukemia (CLL), Hepatocellular Carcinoma (HCC), Primitive Neuroectodermal Tumor (PNET), Adrenal Cortical Carcinoma (ACC), Mantle Cell Lymphoma (MCL), Diffuse Large B Cell Lymphoma (DLBCL), Anaplastic Large Cell Lymphoma (ALCL), Ewing sarcoma (EWS), Desmoplastic Small Round Cell Tumor (DSRCT).

Infused dose NK-cells	Final product characteristics	Outcome
Repeated dose (1-3): median dose in 3-5/6 HLA match: 9,21x10^6 CD3+/CD56- cells/kg, median dose 6/6 HLA match: 10.6x10^6 CD3+/CD56- cells/kg	6/6 HLA-matched: Purity: 87-100% CD56+ cells. 0.53 +/- 1.1x10^6 cells/kg CD3+CD56-. 3-5/6 HLA-matched: Purity: 86-100% CD56+ cells. 0.27 +/- 0.78x10^6 cells/kg CD3+CD56-	Safe. Low toxicity. 6/6 HLA-matched: 6/14 aGvHD (1 severe) and median OS 12 months. 3-5/6 HLA-matched: 8/16 aGvHD and median OS 27 months
Single dose: 0.33-24.5x10^6 cells/kg	Mean purity: CD56+CD122+ cells 64%. CD3+ cells 1.0%. Mean viability: 88%.	1/14 aGvHD and 4/14 cGvHD. 9/14 died (between 1.7-15.5 months), 4/14 CR (between 16.2-21.6 months) 1/14 PD (25.9 months)
Combined dose (day 0 and day +2): 2.7-92x10^6 cells/kg	Purity: Median CD3+ cells 5.5x10^4 cells/kg. Viability: 95%.	Safe and no GvHD. 5/10 CR, 1/10 PR, 1/10 MR, 1/10 SD and 2/10 PD. 4/10 are alive at 1.4, 1.5, 2.3 and 3 years post NK-cell therapy
Repeated doses (1-3): 8.9-29.5x10^6 cells/kg (1st infusion), 3.3 and 11.1x10^6 cells/kg (2nd infusion), 33.8x10^6 cells/kg (3rd infusion)	Purity: CD56+CD3- cells 95%. Median CD3+ cells 0.04%, 45-1100x10^3 cells. Viability: 95%. Fold expansion: median 5 (after 14 days of culture)	Well tolerated, no GvHD. 1/3 CR (152 days), 2/3 died (80 days and 45 days after NK-cell infusion)
Single dose: 0.21-1.41x10^7 cells/kg	Median purity: CD56+CD3- cells 97.3%. T-cell 0.22x10^5 cells/kg	Well tolerated and feasible. 4/5 continuous remission (8-18 months), 1/5 PD

Abbreviations drugs: Cyclophosphamide (Cy), Fludarabine (Flu), Bortezomib (Bor), Dexamethasone (Dex), Clofarabine (Clo), Etoposide (Eto), Cisplatin (Cis), Paclitaxel (Pac), Docetaxel (Doc), Vinorelbine (Vin), Gemcitabine (Gem), Carboplatin (Car), Pemetrexed (Pem), Total Body Irradiation (TBI), Tacrolimus (Tac), Prednisolone (Pred), Methylprednisolone (mPred), Thymoglobulin (Thy), Vincristine (Vin), Adriamycin (Adr), Prednisone (Predn), Melphalan (Mel), Lenalidomide (Lnd),Muromonab-CD3 (OKT3).

Abbreviations others: Human Stem and Progenitor Cells (HSPC), acute Graft versus Host Disease (aGvHD), chronic Graft versus Host Disease (cGvHD), Donor NK cell Infusion (DNKI), Stimulated (Stim), Unstimulated (Unstim), Complete Remission (CR), DFS : Disease Free Survival , Partial Response (PR), Minimal Response (MR), Stable Disease (SD), Progressive Disease (PD), Donor derived IL-15/4-1BBL activated NK cell infusion (aNK-DLI), Transplant-related Mortality (TRM), Mononuclear Cell (MC), Tumor Lysis Syndrome (TLS), Passenger Lymphocyte Syndrome (PLS). *Culture method displays CD3 depleted PBMC's, otherwise deviated selection method is mentioned.

TABLE 2: Summary of allogeneic NK cell clinical trials in a non-transplantation setting

Study	Malignancy	Clinical Trial design	Culture method*
Phase I (EudraCT number: 2010-018988-41) *Dolstra et al* ref[72]	AML (n=10)	Conditioning with Cy/Flu followed by KIR mismatched UCB-NK-cell infusion	*Ex vivo* expanded, differentiated and activated UCB-NK cells from unrelated donors. Culture duration: 42 days with GM-CSF, G-SCF, IL-6, SCF, Flt3L, TPO, IL-7, IL-2 and IL-15 *CD34+ selected HSPC's
Phase I (NCT01898793) *Romee et al (2016)* ref[73]	AML (n=13)	Conditioning with Cy/Flu followed by cytokine induced memory-like NK-cell infusion and subsequent IL-2 therapy (every other day, 6x)	*Ex vivo* expanded and activated PBNK-cells from haploidentical donors. Culture duration :12-16h with IL-15, IL-12 and IL-18. *CD3 depleted and CD56 selected
Phase I (NCT00799799) *Curti et al (2016)* ref[74]	AML (n=16)	Conditioning with Cy/ Flu followed by KIR ligand mismatched NK-cell infusion; IL-2 therapy (3x weekly for 2 weeks)	PBNK-cells from haploidentical donors. *CD3 depleted and CD56 selected
Phase II (NCT00526292) *Shaffer et al (2016)* ref[75]	AML (n=6) and MDS (n=2)	Conditioning with Cy/Flu followed by HLA-mismatched NK-cell infusion; IL-2 therapy (6x) starting 1 day before and after NK-cell infusion	PBNK-cells from haploidentical donors. *CD3 depleted and CD56 selected
Phase I (NCT01212341) *Yang et al (2016)* ref[76]	Lymphoma (n=2) and solid tumor (n=18)	KIR ligand mismatched NK cell infusion	*Ex vivo* expanded and activated PBNK-cells from unrelated donors. Culture duration: 14 days with irradiated auto-PBMCs, OKT3 and IL-2
Phase I (NKAML: NCT00697671) Pilot study (NKHEM: NCT00187096) *Rubnitz et al (2015)* ref[77]	Relapsed Leukemia post HSCT (n=15) Refractory/relapsed leukemia (no prior HSCT) (n=14)	Conditioning with Clo/Eto/Cy followed by KIR matched or mismatched NK-cell infusion; IL-2 therapy (6x) starting 1 day before and after NK-cell infusion	*Ex vivo* expanded PBNK-cells from haploidentical donors. Culture duration :>12h. *CD3 depleted and CD56 selected
Phase I (EudracT number: 2005-006087-62) *Kottaridis et al (2015)* ref[78]	AML (n=7)	Conditioning with Flu and TBI followed by haploidentical tumor primed NK-cell infusion	*Ex vivo* expanded and activated PBNK-cells from haploidentical donors. Culture duration: o/n with CTV-1 lysate and cryopreserved for infusion. *Only CD56 selected

Infused dose NK-cells	Final product characteristics	Outcome
Escalating doses: 3x10^6 cells/kg (cohort 1), 10x10^6 cells/kg (cohort 2) and 30x10^6 cells/kg (cohort 3)	Mean purity: 74+/-13% CD34+ cell product. 75+/-12% generated CD56+CD3- NK-cells. 0.03+/-0.04% CD3+ cells. 0.16+/-0.21% CD19+ cells. Mean viability: 94%	Well tolerated, no GvHD nor toxicity. 4/10 DFS for 55, 47, 17 and 12 months after infusion
Repeated dose: level 1: 0.5 x 10^6 NK cells /kg level 2: 1 x 10^6 NK cells /kg level 3: 10x10^6 NK cells /kg	Purity: >90% CD56+CD3- cells	Well tolerated, no GvHD. 4/13 NE, 4/13 TF-PD, 3/13 CR, 1/13 Cri and 1/13 MLFS
Single dose: 1.29-5.53x10^6 cells/kg	Median purity: infused CD3+ cells: 0.65x10^5 cells/kg. Mean viability: 95%	Feasible study, moderate toxicity. 9/16 DFS, 7/16 in relapse (3-51 months), 1/16 died of bacterial pneumonia
Single dose: 4,3-22,4x10^6 cells/kg	Purity: ≥90% CD3-CD56+ cells. CD3+ cells <0.1%. Viability: 82-100%	No GvHD. 3/8 PR, 5/8 no response. Median survival is 12,9 months
Single dose: 1X10^6 cells/kg (cohort 1) 1x10^7 cells/kg (cohort 2) Repeated dose: 1x10^6 cells/kg (cohort 3) 3x10^6 cells/kg (cohort 4) 1x10^7cells/kg (cohort 5) 3x10^7 cells/kg (cohort 6)	Purity: CD16+/CD56+ cells: 98.13 +/- 1.98%; CD3+ cells: 0.41 +/- 0.43%; CD14+ cells: 0.40 +/- 0.37%; CD19+ cells: 0.15 +/- 0.25%. Fold expansion: 757.5 +/- 232.2. Viability: 92.9 +/- 2.1%	No GvHD nor severe toxicities. 8/20 SD, 9/20 PD, 3/20 NE. Median PFS in SD patients: 4 months (2-18 months)
Single dose: 3.5-103x10^6 cells/kg	Median purity: 98.4% CD56+ cells.0% CD3+CD56- T-cells. 0.31% CD19+ B-cells	Well tolerated, no GvHD. 6/29 PR, 14/29 CR, 8/29 no response and 1/29 NE. 4/29 are alive and DFS
Single dose: 1x10^6 cells/kg	Purity: CD56+ cells 97.17% of which 80% CD56+CD3- cells	Serious adverse reactions, no GvHD. 3/7 in CR remained in remission, 1/7 in PR achieved CR, 2/7 relapsed and 1/7 died (6 months follow up). Median OS: 141-910 days

Study	Malignancy	Clinical Trial design	Culture method*
Phase I (BB-IND-14560) *Szmania et al (2015)* ref[79]	MM (n=8)	Conditioning with Bor (+/- Cy/ Flu/Dex) followed by fresh haplo-(n=6) or cryopreserved auto (n=2) NK cells.	*Ex vivo* expanded and activated PBNK cells from haploidentical (fresh) and autologous (cryopreserved) donors. Culture: 8-9 days with K562-mb15-41BBL stimulator cells and IL-2
Phase II (NCT00274846) *Bachanova et al (2014)* ref[80]	AML (n=57)	Conditioning with Cy/Flu; IL2DT in cohort 3 followed by haploidentical NK-cell infusion 1 day later; IL-2 therapy (14x, daily)	*Ex vivo* expanded and activated PBNK-cells from haploidentical donors. Culture duration: o/n with IL-2. *CD3 depleted (cohort 1) or CD3 depleted/CD56 selected (cohort 2) or CD3/CD19 depleted (cohort 3)
Tonn et al (2013) ref[50]	Solid tumors/ sarcoma (n=12) Leukemia/ lymphoma (n=2)	Pre-treatment with mPred following NK-92 cell infusion	*Ex vivo* expanded and activated allogeneic NK-92 cells. Culture duration :100-300h with IL-2. *no selection
Pilot study (NCT00799799) *Curti et al (2011)* ref[63]	AML (n=13)	Conditioning with Cy/ Flu followed by KIR ligand mismatched NK-cell infusion; IL-2 therapy (3x weekly for 2 weeks)	PBNK-cells from haploidentical donors. *CD3 depleted and CD56 selected
Phase II (BB-IND 8847) *Geller et al (2011)* ref[40]	Refractory Metastatic Breast Cancer (n=14) Ovarian Cancer (n=6)	Conditioning with Cy/Flu with or without TBI followed by allogeneic NK-cell infusion; IL-2 therapy (3x weekly for 2 weeks)	*Ex vivo* expanded and activated PBNK-cells from haploidentical donors. Culture duration: o/n with IL-2
Pilot study *Bachanova et al (2010)* ref[62]	B-cell NHL (n=6)	Conditioning with Cy/Flu and mAb (Rituximab, 4x) before and after haplo NK-cell infusion followed by IL-2 therapy (6x, every other day)	*Ex vivo* expanded and activated PBNK-cells from haploidentical donors. Culture duration :8-16h with IL-2
Pilot study NKAML *Rubnitz et al (2010)* ref[61]	AML (n=10)	Conditioning with Cy/Flu followed by KIR mismatched NK-cell infusion; IL-2 therapy (6x) starting 1 day before and after NK-cell infusion	PBNK-cells from haploidentical donors. *CD3 depleted and CD56 selected
Phase I (EudraCT number: 2005-005125-58) I*liopoulou et al (2010)* ref[60]	Non-SCLC (n=16)	Haploidentical NK cell infusion after chemotherapy	*Ex vivo* expanded and activated PBNK-cells from haploidentical donors. Culture duration :21-23 days with IL-15 followed by 1h with IL-15 and hydrocortisone. *Only CD56 selected

Infused dose NK-cells	Final product characteristics	Outcome
Single dose: 2x10^7-1x10^8 cells/kg	Median purity: 78% CD3-CD56+ cells. CD3+/CD56- 0.1%. Viability cryopreserved: 94%. Viability fresh:93%. Recovery cryopreserved: 16%. Recovery fresh: 119%	Feasible and safe. 1/8 PR, 6/8 PD, 1/8 NE and 3/8 died between day 11-98 after NK-cell infusion
Single dose: 0.96 +/- 0.3x10^7 cells/kg (cohort 1) 0.34 +/- 0.05x10^7 cells/kg (cohort 2) 2.6 +/- 1.5x10^7 cells/kg (cohort 3)	Purity: NK cells 39 +/- 9%, T cells: 0.7% (cohort 1) NK-cells 75 +/- 6%, T-cells: 1.3% (cohort 2) NK-cells 54 +/- 16%, T cells: 0.3% (cohort 3)	Well tolerated, no GvHD and mild toxicities. 9/42 in remission (1.8-15 months) (cohort 1 and 2, n=42). 8/15 in remission (1-32 months) (cohort 3, n=15). DFS: 5% (cohort 1 and 2) and 33% in cohort 3
Repeated doses (2x 48h apart): 1x10^9 (cohort 1), 3x10^9 (cohort 2) and 1x10^6 (cohort 3) cells/m2 and additional dose level of 10^10 cells/m2 in some patients	Viability: >80%. Fold expansion: 32	Infusion of 10^10 NK-92 cells/m2 were well tolerated. 12/15 PD, 2/15 MR, 1/15 SD for 2 years, OS: 13-801 days
Single dose: 1.11- 5x10^6 CD3-CD56+ cells/kg	Mean viability: 95%. Median purity: 93.5% NK-cells. Maximum T-cell dose 10^5 cells/kg	Feasible and safe, no GvHD. 5/13 active disease: 1/5 CR (6 months), 4/5 died of PD. 3/6 treated in CR are DFS (34, 32, and 18 months), 2/13 in MR in CR (4 and 9 months)
Single dose: 8,33x10^6-3,94x10^7 cells/kg	Viability: >70%. Median T cells: 0.11% CD3+ cells.	TLS and PLS and limited infusion or IL-2 related toxicities. 1/20 died due to grade 5 toxicity. 4/20 PR, 12/20 SD and 3/20 PD (between 31–109 days)
Single dose: 21+/- 19 x 10^6 NK cells/kg	Purity: 43 +/- 11% NK-cells. 0.16 +/- 0.12% T-cells	Feasible and safe. 2/6 CR, 2/6 relapsed at 6 months, 2/6 died
Single dose: 5-81x10^6 cells/kg	Median purity: B-cells 0.097x10^6 cells/kg. T-cells 1x10^3 cells/kg	Feasible and safe. 10/10 in remission (569-1162 days)
Repeated doses (2-4): 0.2-29x10^6 cells/kg per dose	Median purity: (T-cells) CD3+CD56+CD28- 0.12x10^6 cells/kg. CD56+CD3- cells 97.9% (after20 days culture). Fold expansion: 23	Safe, no GvHD. 2/16 PR, 6/16 SD, 7/16 PD, 1/16 not treated. 1-year OS 56% (9/16), 2-year OS 19% (4/16)

Study	Malignancy	Clinical Trial design	Culture method*
Phase I *Arai et al (2008)* ref[49]	Metastatic RCC (n=11) or Malignant Melanoma (n=1)	NK-92 cell infusion	*Ex vivo* expanded and activated allogeneic NK-92 cells. Culture duration :15-17 days with or without IL-2. *no selection
Phase I (BB-IND 8847) *Miller et al (2005)* ref[57]	Metastatic Melanoma (n=10), Metastatic RCC (n=13), Refractory HL (n=1) and AML (n=19)	Conditioning with Low Cy/ mPred or Flu or high-Cy/Flu followed by NK-cell infusion; IL-2 therapy (14x, daily)	*Ex vivo* expanded and activated PBNK-cells from haploidentical donors. Culture duration: o/n with IL-2.

Abbreviations Malignancies: Central Nervous System (CNS), Myeloproliferative Disorders (MPD), Lymphoproliferative Disorder (LPD), Multiple Myeloma (MM), Myelodysplastic Syndromes (MDS), Myelodysplastic Neoplasms (MDN) Myeloproliferative Neoplasms (MPN), Acute Myeloid Leukemia (AML), Lymphoblastic Leukemia-Lymphoma (LBLL), Acute Lymphoblastic Leukemia (ALL), Neuroblastoma (NB), Rhabdomyosarcoma (RMS), Chronic Myelogenous Leukemia (CML), Non-Hodgkin's Lymphoma (NHL), Mantle Cell Lymphoma (MCL), Diffuse Large B Cell Lymphoma (DLBCL), Follicular Lymphoma (FL), Anaplastic Large Cell Lymphoma (ALCL), Hodgkin's Lymphoma (HL), Renal Cell Cancer (RCC), Small Cell Lung Cancer (SCLC), Chronic Lymphocytic Leukemia (CLL), Hepatocellular Carcinoma (HCC), Primitive Neuroectodermal Tumor (PNET), Adrenal Cortical Carcinoma (ACC), Mantle Cell Lymphoma (MCL), Diffuse Large B Cell Lymphoma (DLBCL), Anaplastic Large Cell Lymphoma (ALCL), Ewing sarcoma (EWS), Desmoplastic Small Round Cell Tumor (DSRCT).

Infused dose NK-cells	Final product characteristics	Outcome
Repeated doses (3x in cohort): 1x10^8 (cohort 1), 3x10^8 (cohort 2), 1x10^9 (cohort 3) and 3x10^9 (cohort 4) cells/m2	Fold expansion: 200 over 15-17 days. Viability: ≥80%	Safe and feasible, mild toxicities (1 grade 4, hypoglycaemia). 10/12 PD (died between day 101-1059), 1/12 alive (1450 days) and 1/12 died of bronchopneumonia (day 832)
Escalating doses: Low cy/mPred: 1x10^5, 1x10^6, 1x10^7 or 2x10^7 cells/kg (at least 3 per cohort). Flu or High-Cy/Flu: 2x10^7 cells/kg	Viability: >70%. Purity: NK-cells 40 +/- 2%. T-cells 1.75 +/- 0.3x10^5 cells/kg is 0.9 +/-0.1%. Monocytes 25 +/- 1.6% and B-cells 19 +/-2%	Feasible and tolerated without toxicities. Low-Cy/mPred: 2/17 with MRCC SD for 20 and 21 months. 4/17 with MM SD for 4-9 months. (n=17) High-Cy/Flu: 5/19 AML pts in CR (n=19)

Abbreviations drugs: Cyclophosphamide (Cy), Fludarabine (Flu), Bortezomib (Bor), Dexamethasone (Dex), Clofarabine (Clo), Etoposide (Eto), Cisplatin (Cis), Paclitaxel (Pac), Docetaxel (Doc), Vinorelbine (Vin), Gemcitabine (Gem), Carboplatin (Car), Pemetrexed (Pem), Total Body Irradiation (TBI), Tacrolimus (Tac), Prednisolone (Pred), Methylprednisolone (mPred), Thymoglobulin (Thy), Vincristine (Vin), Adriamycin (Adr), Prednisone (Predn), Melphalan (Mel), Muromonab-CD3 (OKT3).
Abbreviations others: Human Stem and Progenitor Cells (HSPC), acute Graft versus Host Disease (aGvHD), Donor NK cell Infusion (DNKI), Stimulated (Stim), Unstimulated (Unstim), DFS : Disease Free Survival , Complete Remission (CR), Partial Response (PR), Minimal Response (MR), Stable Disease (SD), Progressive Disease (PD), Donor derived IL-15/4-1BBL activated NK cell infusion (aNK-DLI), Transplant-related Mortality (TRM), Mononuclear Cell (MC), Tumor Lysis Syndrome (TLS), Passenger Lymphocyte Syndrome (PLS), Not Evaluable (NE). *Culture method displays CD3 depleted PBMC's, otherwise deviated selection method is mentioned in product characteristics.

SCOPE OF THIS THESIS

In this thesis, we explore the potential benefits of allogeneic NK cells either alone or in combination with the therapeutic monoclonal antibody cetuximab in the fight against solid cancer. We set out to study NK cell killing mechanisms and the effect of ADCC in solid epithelial malignancies, functionally comparing two clinically applicable NK cell products, i.e. peripheral blood NK cells (PBNK) and umbilical cord blood stem cell derived NK cells (UCB-NK), against epidermoid, colorectal and cervical tumors.

In chapter 2 we report on the development of two standardized eight color NK cell specific flowcytometry panels that enable monitoring of NK cell subset frequency, functionality, and phenotype in peripheral blood mononuclear cells under cytokine activated/non-activated and target cell stimulated/non-stimulated conditions in both fresh and cryopreserved samples. Using these standardized NK cell flowcytometry panels we conclude that cryopreserved PBMC samples are optimal for the assessment of NK cell functionality and NK cell receptor expression studies across multiple centers in both healthy donors and cancer patients.

In advanced cervical cancer, targeted intervention therapies have limited success in controlling tumor growth. About 80% of the cervical cancer cases express epidermal growth factor receptor (EGFR), thus making EGFR an attractive surface antigen to target cervical tumors. However, treatment with the anti-EGFR monoclonal antibody cetuximab failed to demonstrate therapeutic efficacy. Further, apparent down-regulation of HLA class-I surface expression levels also makes tumor cells escape T cell mediated killing. In these cases, NK cell-based therapies may offer a viable alternative to T cell-based approaches. Therefore, in this study, the anti-tumor effects of two different allogeneic NK cell products, i.e. UCB-NK cells and activated PBNK cells, were studied either alone or in combination with the anti-EGFR mAb cetuximab in cervical cancer (Chapter 3).

Similarly to cervical cancer, cetuximab monotherapy was also proven ineffective in $EGFR^{+}$ RAS^{mut} advanced colorectal cancer (CRC). About 40-50% of CRC patients have mutations in the RAS gene, thus leaving nearly half of the CRC patients with no standard treatment option. In the study described in Chapter 4, we tested whether activated allogeneic peripheral blood NK cells (PBNK) together with cetuximab could overcome these limitations.

In most of the patients with advanced CRC the NK cell response is highly impaired, as previously reported[81,82]. These findings were further substantiated by our data, the results of which revealed that CRC patients NK cell percentages and functionality were significantly lower than their healthy counterparts with a further reduction post-chemotherapy. In Chapter 5 we therefore investigated whether adoptively transferred allogeneic UCB-NK cells could effectively target colon cancer cells *in vivo.* Altogether, our *in vitro* and *in vivo*

studies provide a clear rationale to clinically explore UCB-NK cells as a generally applicable immunotherapeutic NK cell platform for solid tumors.

In Chapter 6 we discuss various therapeutic approaches that have the capacity to enhance NK cell-based cancer immunotherapy against solid tumors, focusing on the potential of promising lead products from various NK cell biotech industries and main findings from this thesis are summarized in Chapter 7.

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CHAPTER 2

Harmonized NK FACS panels to study NK cell phenotype and function

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"Standardized and flexible eight colour flow cytometry panels harmonized between different laboratories to study human NK cell phenotype and function"

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ABSTRACT

Advancements in multi-colour fluorescence activated cell sorting (FACS) panel warrant harmonized procedures to obtain comparable data between various laboratories. The intensifying clinical exploration of Natural Killer (NK) cell-based immunotherapy demands standardized and harmonized NK cell FACS panels and acquisition protocols. Eight-colour FACS panels were designed to study human NK cell phenotype and function within peripheral blood mononuclear cells (PBMC). The panels were designed around fixed backbone markers and channels, covering antigens for non-NK lineage exclusion (CD3, TCRγδ, CD19, CD14, SYTOX® Blue) and NK cell selection (CD45, CD56, CD16), complemented with variable drop-in markers/channels to study NK-cell phenotype (NKG2A, NKG2C, NKG2D and KIR2D) or NK cell function and activation (CD25, NKp44 and CD107a). Harmonized FACS set-up and data analysis for three different flow cytometers has been established, leading to highly comparable and reproducible data sets using the same PBMC reference samples (n=6). Further studies of NK cells in fresh or cryopreserved PBMC samples (n=12) confirmed that freezing and thawing of PBMC samples did not significantly affect NK phenotype or function. In conclusion, our data demonstrate that cryopreserved PBMC samples analysed by standardized FACS panels and harmonized analysis protocols will generate highly reliable data sets for multi-center clinical trials under validated conditions.

INTRODUCTION

Flow cytometry serves as a powerful analytical platform for rapid measurement, characterization and functional analysis of individual cells within heterogenic cell populations[1]. The ability to simultaneously detect multiple parameters in different cell types, promoted fluorescent activated cell sorting (FACS) analysis as a crucial tool to study the complexity of the immune system[2]. Recent advances in flow cytometry instruments and reagents have increased the possibilities for development of more complex multi-colour FACS panels, resulting in their extended use in research and clinical studies[3]. Multi-colour FACS panels facilitate a deeper understanding of the biology, distribution and interaction of different immune cell types, offering valuable information to more accurately diagnose, monitor and treat various immunological disorders and malignancies[4,5]. There is an ever-increasing number of multi-center clinical trials studying cellular therapy approaches. Thus, immune monitoring of patients should be eased using harmonized multi-colour FACS panels to yield reliable and reproducible data. However, despite the routine use of multi-colour FACS panels in such trials, limitations of implementing standardized methodologies and data analysis protocols have led to a high degree of variation, severely limiting data interpretation from different centers[6,7].

Extensive work done by several groups has identified the main issues that need to be carefully considered when developing multi-colour flow cytometry panels for harmonized use[8-10], which involve sample type, sample handling, panel design, selection of reagents, instrument set-up, and data analysis. They have also created a series of guidelines recommended to harmonize those processes. Briefly, the design of optimal multi-colour FACS panels requires careful selection of the most appropriate fluorochrome-conjugated antibodies to identify and characterize rare cell populations[11]. Prior to sample acquisition, it is crucial to optimize instrument settings, involving fine-tuning of the light scatters and photomultiplier tube (PMT) voltages for each detector, followed by accurate compensation for spectral overlap of all fluorochromes used. Furthermore, standard operating procedures (SOPs) for sample preparation, staining, acquisition, gating strategy and data analysis methods are essential to reduce data variability of multi-center FACS monitoring. Most of the available multi-colour FACS panels for immune subset analysis are designed for general characterization of major leukocyte populations[2,3,12]. There is an obvious need for similarly standardized and harmonized multi-colour FACS panels for specific subsets such as for instance natural killer (NK) cells. In particular, their increased use in cellular therapy approaches, as they are perceived as a safer option for targeted anti-cancer therapy than T cells[13], calls for the development of NK specific polychromatic FACS panels.

NK cells are innate lymphocytes mediating cytotoxic responses against virally infected or tumour cells. The vast majority of peripheral blood NK cells are CD56+CD16+ effector cells

and only a small subset represents CD56+CD16- regulatory cells[14]. Their function is tightly regulated by a delicate balance between inhibitory and activating receptors, among which CD16, a low affinity receptor for the Fc fragment of IgG_1, enables NK cell mediated cytotoxicity of IgG_1-coated cells, a phenomenon known as antibody dependent cellular cytotoxicity (ADCC)[15]. Although NK cells are involved in the outcome of important clinical interventions that are frequently monitored by multi-colour flow cytometry, such as transplantation[16-18] or immunotherapy[19], the existing multi-colour FACS panels for NK cell analysis are either restricted to detect antigens associated with malignant transformation[12] or if they include an extended immunophenotyping panel, their standardized implementation is limited by the fact that measurements have not been validated through harmonized procedures across multiple centers[20].

In this article, we describe the design and harmonization of two eight-colour NK FACS panels, allowing the generation of reproducible similar data sets across multiple centers, highlighting the advantages of using cryopreserved PBMC for phenotypic and functional immune monitoring studies of NK cells[21,22].

RESULTS

NK FACS panel establishment based on backbone and drop-in concept

To harmonize multicolour flow cytometry analysis for studying NK cell phenotype and function, three independent research centers using different flow cytometers equipped with compatible laser and detector/filter settings (Table 1) tested comparability and reproducibility of obtained data sets between centers. To this end, instrument set-up, sample preparation, acquisition and data analysis were performed independently using standardized protocols, which were commonly agreed on and followed in all three centers as described in the materials and methods section (Figure 1). Acquisition protocols were set up in each center using single stains, complete mixture of all antibody-fluorochrome combinations and fluorescence minus one (FMO) controls (Supplementary Fig. S1 online). Compensation matrices of the NK phenotype and function panels were generated for the three flow cytometers and these settings were used through the whole study (Supplementary Tables S1, S2, and S3 online). FACS panels were designed using a backbone and drop-in concept. Antigens of the backbone specifically discriminated the viable NK cell subset from PBMC, leaving four other detectors available to characterize the specific NK cell phenotype or function.

The backbone of the NK phenotype panel (summarized in Table 2) comprises a combination of CD3, TCRγδ, CD14 and CD19 lineage-specific antibodies (for negative selection of the NK cells), and CD45 and CD56 (for positive selection). In this respect it is important to remark on the extensive overlap between NK cells and γδT cells in terms of the

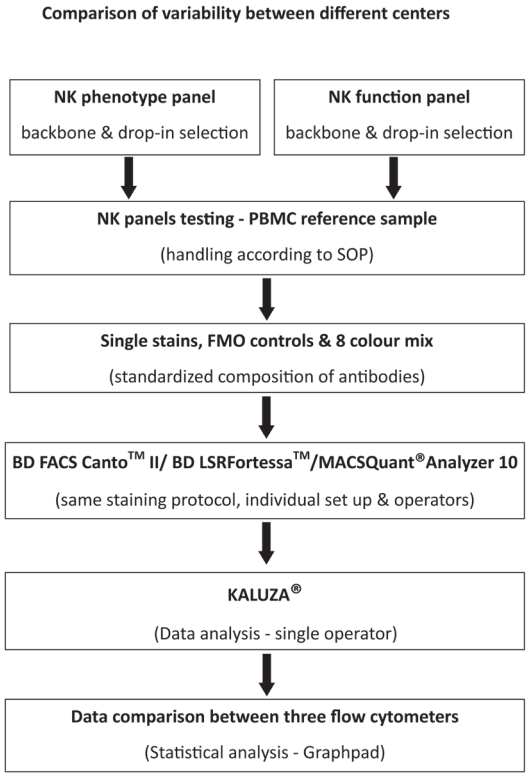

FIGURE 1: Experimental design to compare variability between flow cytometers
Flow chart of the experimental set up outlining the establishment and verification of the NK cell phenotype and function panels to compare variability between flow cytometers. Following selection of backbone and drop in antibodies, reference samples were used for optimizing and checking fluorochrome intensities using appropriate single stains and fluorescence minus one (FMO) controls to establish a protocol. Further, the data generated from three instruments were analysed using KALUZA® software and results were tabulated using Graphpad Prism for statistical differences for validation using the same reference sample.

expression of several NK cell receptors[23]; therefore, we included an anti-TCRγδ antibody in the lineage cocktail to avoid misinterpretation of results. Viable $CD45^{+}CD56^{+}$ NK cells were gated and CD16 APC, PanKIR2D FITC, NKG2A PE-Vio770, NKG2C PE and NKG2D PerCP-Cy5.5 (drop ins) were plotted against CD56 to quantify the percentage of NK cells positive for activating (CD16, NKG2C and NKG2D) and inhibitory (KIR2D and NKG2A) receptors (Table 2 and Supplementary Fig. S2 online). The backbone of the NK cell function panel, was slightly modified to acquire simultaneous information on NK cell, T cell and CD3+CD56+ non-conventional T cell subsets. Here, a combination of CD3 and TCRγδ conjugated to PerCP-Vio700 was used in one of the drop-in channels and removed from the exclusion channel covering CD14, CD19 conjugated to VioBlue and SYTOX® Blue for dead cell exclusion (Table 3).

TABLE 1: Comparison of instrument settings between three flow cytometers from three centers participated in the study

Fluoro chromes	Filter settings - Band pass & Long pass filters			PMT voltages		
	BD FACS Canto™ II	BD LSR Fortessa™	MACS Quant® Analyzer X	BD FACS Canto™ II	BD LSR Fortessa™	MACS Quant® Analyzer X
FSC	488/10	488/10	488/10	319	489	328
SSC	488 /10	488/10	488/10	462	266	476
FL1	530/30 & 502LP	530/30 & 505LP	525/50	496	484	417
FL2	585/42 & 556LP	575/26 & 550LP	585/40	435	473	416
FL3	780/60& 735LP	780/60& 750LP	750LP	540	549	487
FL4	670LP& 655LP	695/40& 685LP	655-730	507	681	594
FL5	660/20	670/14& 655LP	655-730	588	484	522
FL6	780/60& 735LP	780/60& 750LP	750LP	474	451	579
FL7	450/50	450/50	450/50	375	423	444
FL8	510/50& 502LP	525/50& 505LP	525/50	403	453	560

Three flow cytometers (BD FACS Canto™ II, BD LSR Fortessa™ and MACS Quant® Analyzer X) with compatible optical configuration were used for NK phenotype and NK function FACS panel design and optimization studies. Forward scatter (FSC), side scatter (SSC) and eight fluorescence emission channels (FL1-FL8) were used. Further, their corresponding band pass and long pass (LP) filter settings were compared and the photomultiplier tube (PMT) voltages obtained were mentioned in table 1.

Separately gated NK cells (CD56+/CD3-), T cells (CD3+CD56-) and CD3+CD56+ cells (within the $CD45^+$ gate) were then analysed for degranulation[24] (by CD107a), ADCC potential (by CD16)[25], and activation status (by CD25, NKp44)[26,27] (Table 3 and Supplementary Table S3 online). By developing this standardized and harmonized gating strategy a solid basis was set to check the individually developed acquisition protocols of all three participating centers for inter-center variability.

TABLE 2: Antibody specifications - NK phenotype panel

Laser	Antibody	Fluorochrome	Clone	Titration	Manufacturer	Catalogue No
Violet 405nm	CD45	VioGreen	5B1	1:11	Miltenyi	130-096-906
	CD3	VioBlue	BW264/56	1:11	Miltenyi	130-094-363
	TCRγδ	VioBlue	11F2	1:11	Miltenyi	130-101-557
	CD14	VioBlue	TÜK4	1:11	Miltenyi	130-094-364
	CD19	VioBlue	LT19	1:11	Miltenyi	130-098-598
	Sytox® Blue	Dead cell marker		1:1000	Life technologies	S11348

Laser	Antibody	Fluorochrome	Clone	Titration	Manufacturer	Catalogue No
Blue 488nm	PanKIR2D	FITC	NKVFS1	1:11	Miltenyi	130-098-689
	NKG2A	PE-Vio770	REA110	1:11	Miltenyi	130-105-647
	NKG2C	PE	REA205	1:11	Miltenyi	130-103-635
	NKG2D	PerCP-Cy5.5	1D11	1:11	Biolegend	320818
Red 633nm	CD16	APC	VEP13	1:11	Miltenyi	130-091-246
	CD56	APC-Vio770	REA196	1:11	Miltenyi	130-100-694

Antibody-fluorochrome conjugates for NK phenotype panel were distributed across Violet, Blue and Red lasers. Back bone antibodies (grey shades) were assigned to Violet and Red lasers. VioGreen was used to gate on CD45+ cells and VioBlue in the Violet laser was used as a dump channel to exclude non-NK lymphocytes including dead cells. Red laser was used to gate on viable NK cells, followed by NK phenotype panel drop in markers (white shades) in the Blue laser. All antibodies except Sytox Blue (1:1000) were used at a dilution of 1:11.

TABLE 3: Antibody specifications - NK function panel

Laser	Antibody	Fluorochrome	Clone	Titration	Manufacturer	Catalogue No
Violet 405nm	CD45	VioGreen	5B1	1:11	Miltenyi	130-096-906
	CD14	VioBlue	TÜK4	1:11	Miltenyi	130-094-364
	CD19	VioBlue	LT19	1:11	Miltenyi	130-098-598
	Sytox® Blue	Dead cell marker		1:1000	Life technologies	S11348
Blue 488nm	CD25	VioBrightFITC	4E3	1:11	Miltenyi	130-104-274
	CD107a	PE	H4A3	1:11	Miltenyi	130-095-515
	NKp44	PE-Vio770	2.29	1:11	Miltenyi	130-104-195
	CD3	PerCP-Vio700	BW264/56	1:11	Miltenyi	130-097-582
	TCRγδ	PerCP-Vio700	11F2	1:11	Miltenyi	130-103-784
Red 633nm	CD16	APC	VEP13	1:11	Miltenyi	130-091-246
	CD56	APC-Vio770	REA196	1:11	Miltenyi	130-100-694

Antibody-fluorochrome conjugates for NK function panel were distributed across Violet, Blue and Red lasers. Back bone antibodies (grey shades) were assigned to Violet and Red lasers. VioGreen was used to gate on CD45+ cells and VioBlue in the Violet laser was used as a dump channel to exclude CD14+, CD19+ and dead cells. Red laser was used to gate on viable NK cells. Blue laser was used to gate on CD3+, TCRγδ+ cells, non-conventional T cell subsets and NK function panel drop in markers (white shades). All antibodies except Sytox Blue (1:1000) were used at a dilution of 1:11.

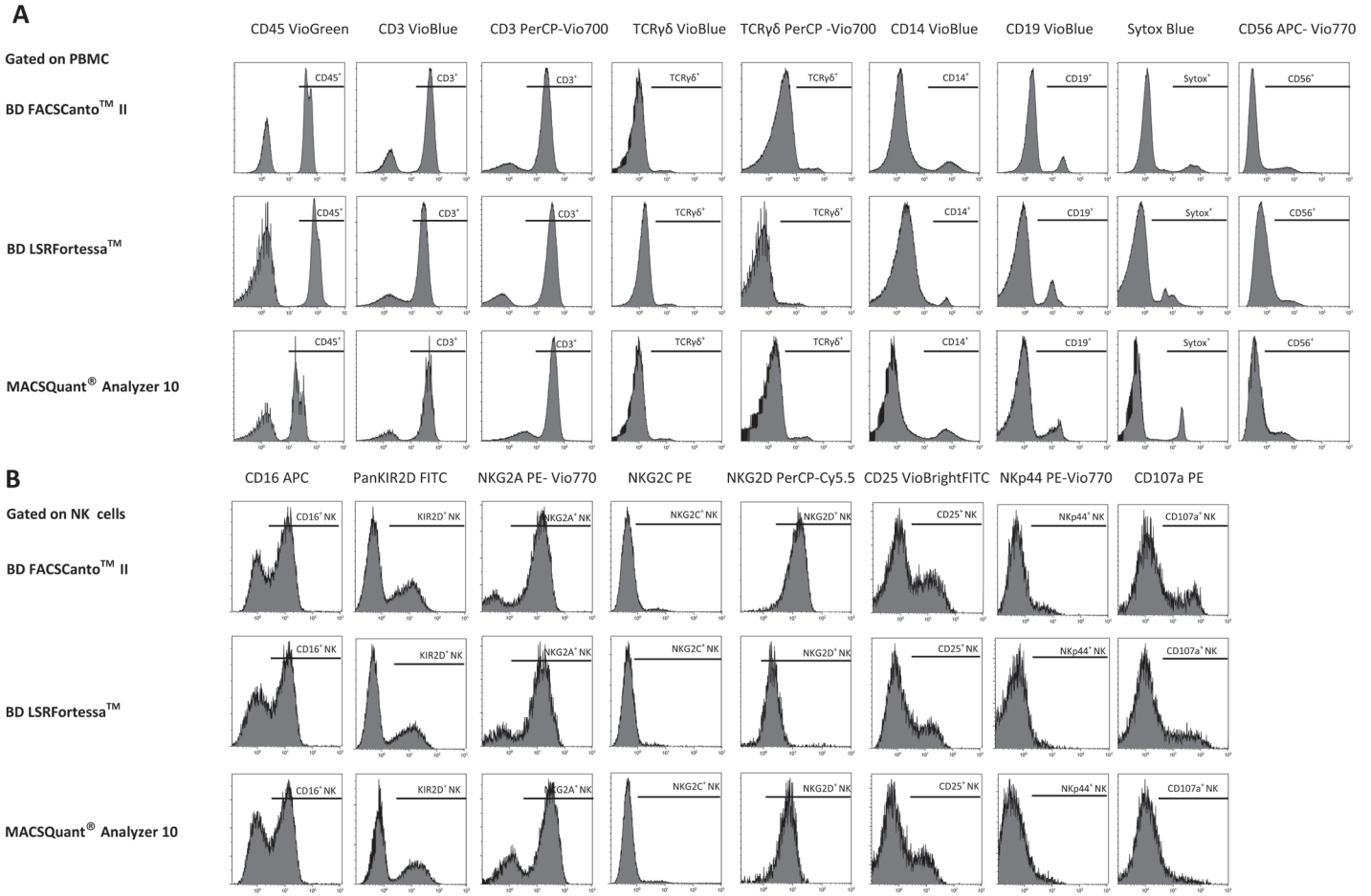
A
CD45 VioGreen
CD3 VioBlue
CD3 PerCP-Vio700
TCRγδ VioBlue
TCRγδ PerCP -Vio700
CD14 VioBlue
CD19 VioBlue
Sytox Blue
CD56 APC- Vio770
Gated on PBMC
BD FACSCanto™ II
BD LSRFortessa™
MACSQuant® Analyzer 10
CD45+
CD3+
TCRγδ+
CD14+
CD19+
Sytox+
CD56+
B
CD16 APC
PanKIR2D FITC
NKG2A PE- Vio770
NKG2C PE
NKG2D PerCP-Cy5.5
CD25 VioBrightFITC
NKp44 PE-Vio770
CD107a PE
Gated on NK cells
CD16+ NK
KIR2D+ NK
NKG2A+ NK
NKG2C+ NK
NKG2D+ NK
CD25+ NK
NKp44+ NK
CD107a+ NK

FIGURE 2: Comparison of FACS panels antibody fluorochrome intensities between three flow cytometers

All antibodies in the NK cell phenotype and function panel were tested for their fluorochrome intensities using the same staining and acquisition protocols on three different flow cytometers. Single stains from back bone antigens were evaluated for their antigen expression levels. PBMC were gated on NK cells for detecting NK receptors and NK functional antigens in the drop-in channels. One representative set of histograms (n=3) is shown for each marker tested at three different centers using three different flow cytometers. Antigen expression is expressed as percentage of positive cells for back bone antibodies in figure A and for drop-ins in figure B.

Standardized FACS panels and protocols overcome variability between flow cytometers and centers

The initial selection of antibodies to design NK cell FACS panels, handling of samples and development of a common gating strategy, was used to harmonize data acquisition and analysis between the participating centers. To verify that use of the individual instrument settings and optimized protocols would lead to comparable data sets, experiments were performed in three different centers with different flow cytometers using the same reference PBMC samples (n=6). First, single-stain antigen expression levels, acquired from each center, were compared for the NK cell phenotype and function panel (Figure 2). Data from all FCS files, collected from individual experiments, were analysed using the harmonized analysis protocol based on a fixed gating strategy. Using a predefined gating strategy, average frequencies (technical replicates divided by 2) for each parameter were compared between the three flow cytometers (BD FACSCanto™ II, BD LSRFortessa™ or MACSQuant® Analyzer X) at three different sites using a non-parametric Kruskal-Wallis test. With exception of CD14 VioBlue in BD LSRFortessa™ (Reference sample III, **p-value=0.001) and CD19 VioBlue in MACSQuant® Analyzer X (Reference sample III, *p-value=0.03), the rest of the measured parameters was comparable across different centers (Figure 2 and Table 4). Specifically looking at the variability of more sensitive antigens used for discrimination of NK phenotype, no differences were detected for expression of NKG2A, NKG2C, NKG2D, KIR2D (Figure 3A) between the three flow cytometers (p value range 0.09- 0.13). In order to detect the NK function-associated antigens, PBMC were exposed to target cells (A431) for 4hr and expression of CD107a, CD25, NKp44 and CD16 levels were analysed (Figure 3B).

Standardized FACS panels, sample handling, individual acquisition protocols for the three different flow cytometers, as well as harmonized gating strategies for the analysis of the same reference PBMC samples, resulted in a dataset without inter-center variability. These findings thus support the use of these protocols and NK cell FACS panels for use in the monitoring of multi-center trials.

TABLE 4: Comparison between instruments of the increment in average cell frequencies obtained after staining with the panels components.

	Reference sample I				Reference sample II				Reference sample III			
Antibodies	**Canto**	**Fortessa**	**MQ**	**p- value**	**Canto**	**Fortessa**	**MQ**	**p- value**	**Canto**	**Fortessa**	**MQ**	**p- value**
CD45 VioGreen	98.3	97.2	98.3	ns	99.5	94.0	98.5	ns	97.0	97.0	99.0	ns
CD3 VioBlue	75.6	73.0	76.7	ns	75.3	73.4	77.5	ns	67.2	69.0	71.7	ns
CD14 VioBlue	1.13	2.5	1.0	ns	1.11	3.7	1.0	ns	1.7	12.92	1.1	**
CD19 VioBlue	16.0	16.4	14.5	ns	7.7	7.7	12.5	ns	4.19	9.76	20.2	*
Sytox Blue	17.0	12.4	18.5	ns	16.2	13.1	14.5	ns	13.6	12.3	19.0	ns
CD56 APC-Vio770	2.9	2.5	2.4	ns	2.2	3.0	2.7	ns	9.29	9.8	8.3	ns
CD3 PerCP-Vio700	76.3	74.0	77.5	ns	74.6	77.5	77.4	ns	68.4	69.7	72.1	ns
TCRγδ PerCP-Vio700	1.9	2.5	2.9	ns	1.7	2.7	3.23	ns	2.6	2.5	2.9	ns
TCRγδ VioBlue	1.6	1.6	2.4	ns	1.7	1.4	2.71	ns	2.5	2.7	2.4	ns
CD56$^+$CD16 APC	33.0	28.0	31.6	ns	33.4	30.0	33.7	ns	36.0	34.5	31.5	ns
CD56$^+$NKG2A PE-Vio770	59.0	63.4	57.5	ns	59.6	63.8	61.0	ns	48.8	51.6	62.9	ns
CD56$^+$NKG2C PE	6.3	6.2	6.4	ns	7.9	4.9	6.0	ns	11.0	10.7	12.7	ns
CD56$^+$NKG2D PerCP-Cy5.5	92.6	91.3	92.9	ns	90.8	94.9	92.8	ns	97.8	95.5	93.5	ns
CD56$^+$PanKIR2D FITC	35.1	33.9	36.0	ns	34.2	33.9	29.2	ns	18.1	19.3	21.4	ns
CD56$^+$CD25 VioBrightFITC	7.2	7.8	8.0	ns	6.9	7.2	7.4	ns	22.3	23.4	25.2	ns
CD56$^+$NKp44 PE-Vio770	1.0	0.8	1.2	ns	5.6	4.4	6.6	ns	6.1	6.0	7.5	ns
CD56$^+$CD107a PE	23.7	24.2	19.5	ns	24.0	25.2	26.3	ns	31.2	28.2	33.8	ns

Canto: BD FACSCanto™ II; Fortessa: BD LSRFortessa™; MQ: MACS Quant Analyzer® X; p-value (Kruskal Wallis test) *p=0.03, **p=0.001.

Three reference samples (Reference sample I-III) were analysed using three different flow cytometers at three different centers by independent operators using an optimized protocol. The performance of the backbone antibodies (white shades) was evaluated using single stain controls for each measured parameter with background subtracted from corresponding negative controls. The drop-in markers for NK phenotype panel (light grey shades) were analysed, when gated on NK cells (CD45+CD3-CD56+). Background staining in NK phenotype panel drop in channels were eliminated using appropriate fluorescence minus one (FMO) controls. For the NK function panel drop in markers; CD107a, CD25 and NKp44, NK cells were stimulated with A431 cells and unstimulated values were subtracted before analysis (dark grey shades). The values shown for each parameter correspond to the average frequency of stained cells from two technical replicates measured in triplicates, following subtraction of appropriate background signal. The statistical comparisons were performed using non-parametric Kruskal-Wallis. Only significant p-values (<0.05) are shown.

NK cell phenotype and function is highly preserved in cryopreserved peripheral blood samples

With these standardized and harmonized protocols and panels, we initiated a study across the participating partners to compare different handling procedures or culture conditions for PBMC samples to identify the most optimal conditions to study NK cell phenotype and function. For this study, PBMC samples from 12 healthy human blood donors were stained with NK cell phenotype and function panel antibody mixes and were analysed using the BD LSRFortessa™ (6 donors) at VUmc and the MACSQuant® Analyzer X (6 donors) at Miltenyi (Figure 4). Different PBMC conditions, i.e. freshly isolated PBMC versus cryopreserved PBMC, and cytokine stimulated versus not cytokine stimulated, were compared. To test the ADCC effector function, NK cells were exposed to A431 (EGFR positive cell line) cells and A431 cells coated with cetuximab (CET). The expression levels of NK cell phenotype markers were comparatively stable following cryopreservation and no significant difference were observed between fresh and cryopreserved NK cell phenotypes. (Figure 5, supplementary figures S4 and S5 online, and Tables 5 and 6). Upon stimulation with IL-2 and IL-15, though NKG2D expression levels increased resulting in a more homogenous expression level in fresh NK cells compared to their cryopreserved counterparts, however the increase was not significant (Figure 5C and supplementary figure S5C online).

NK cell activation and function was studied by exposure of NK cells to A431 cells in the presence or absence of CET. The NK function FACS panel was used to detect NK cell degranulation (CD107a levels) and ADCC mediated cytotoxicity comparing differences in CD107a and CD16 cell surface levels between PBMC+A431+CET and PBMC+A431 conditions. Results show that neither the overall CD107a expression levels upon exposure to A431 target cells or A431 target cells coated with cetuximab, nor the increase in CD107a expression in the cetuximab condition were affected by cryopreservation (Figure 6A and Supplementary Fig S6A and S7A online and Tables 5 and 6). Total NK cell CD16 expression levels (Figure 6B and Supplementary Fig S6B and S7B online and Tables 5 and 6) and CD16 expression levels in the CD56dim CD16+ NK cell subset (Supplementary Fig S8 online and Tables 5

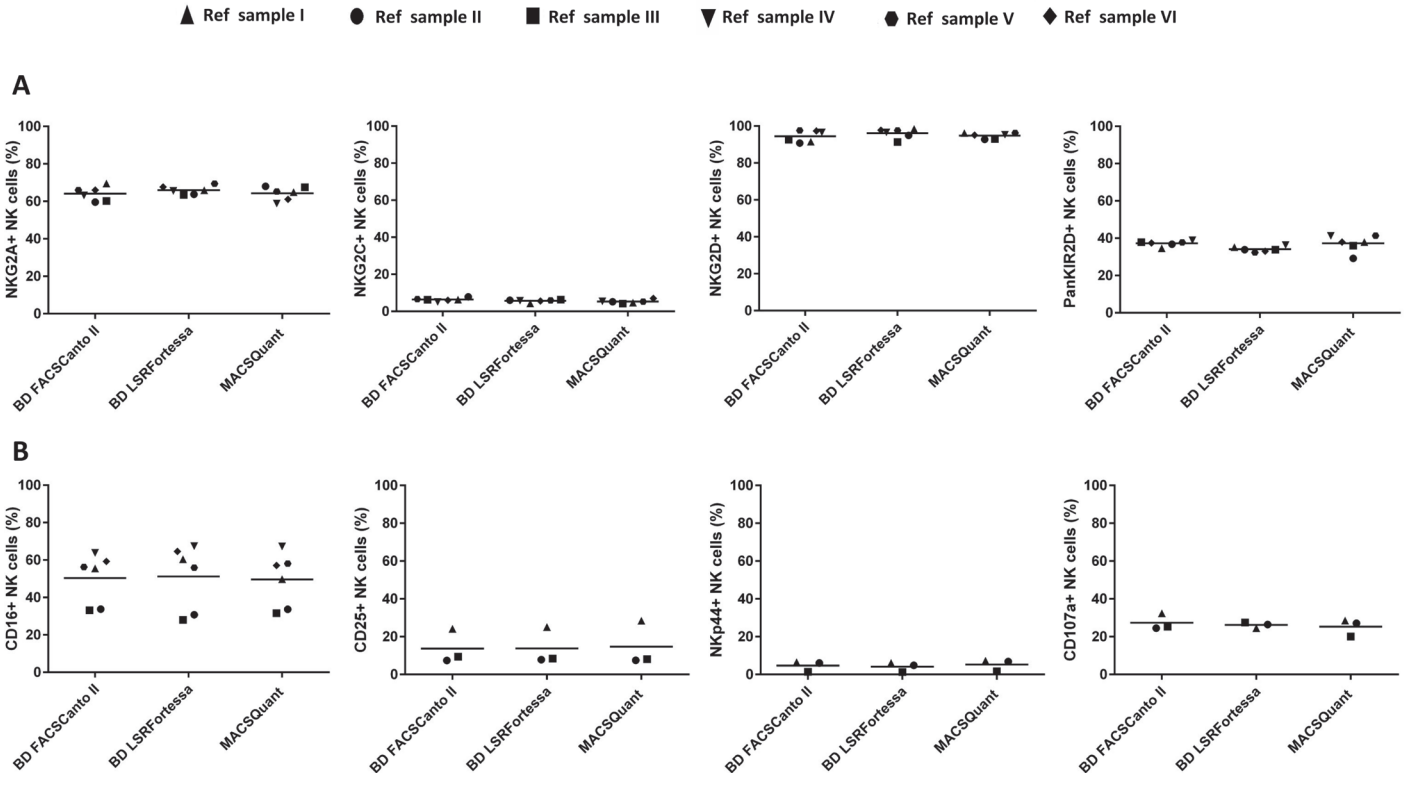

FIGURE 3: Evaluation of NK cell phenotypes and function marker expression between different flow cytometers

Expression levels for drop-in antigens in the NK cell phenotype (A) and function panel (B) were analysed and compared across different centers using the same reference samples (n=6). NKG2A, NKG2C, NKG2D, KIR2D and CD16 expression in BD LSRFortessa™, BD FACSCanto™ II and MACSQuant® Analyzer X were measured for NK phenotype panel (A). Similarly, for NK cell function panel analysis, three-time points of reference sample PBMC were stimulated with A431 cells and their percentage of NK cells positive for CD107a, CD25 and NKp44 levels were measured (B). Statistical analysis was performed using the non-parametric Kruskal-Wallis test.

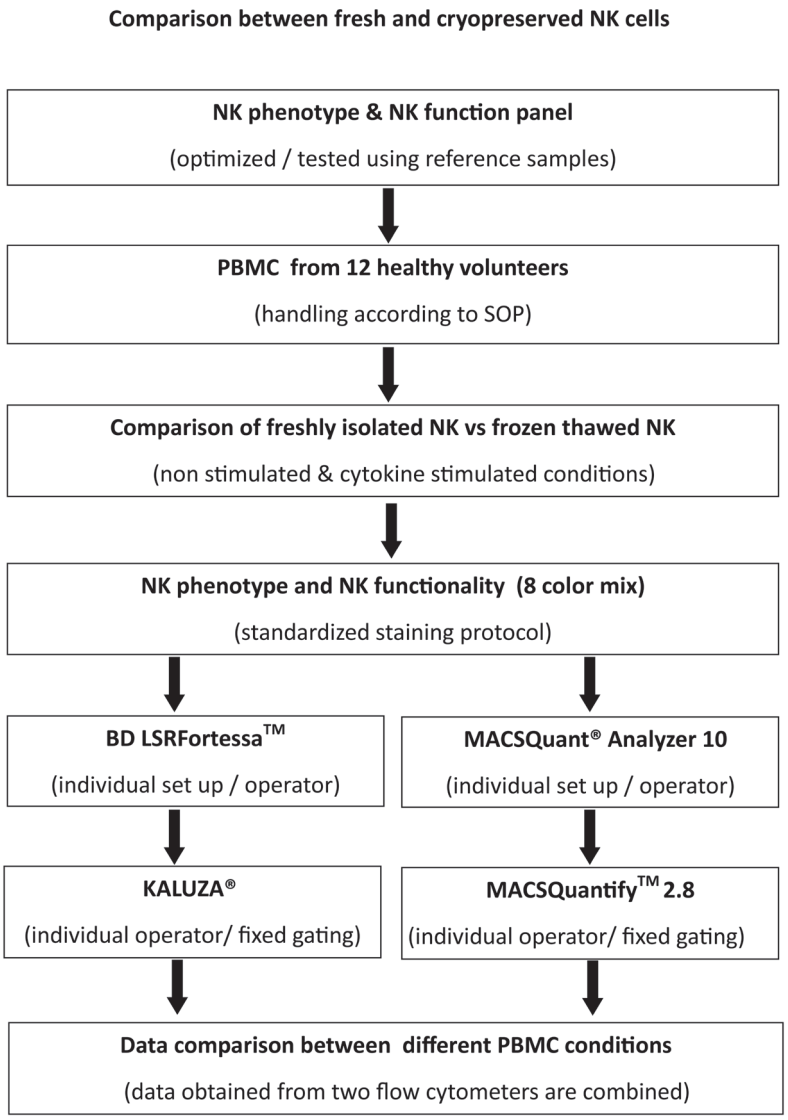

FIGURE 4: Experimental design to compare different PBMC conditions

The experimental set up describes the work flow to compare NK phenotype and function changes under different PBMC (non-stimulated and stimulated) conditions. PBMC from 12 healthy donors were stained with NK cell phenotype and NK function panel cocktails. Data was acquired for 6 donors using BD LSRFortessa™ and for other 6 donors in MACSQuant® Analyzer X. Further, FCS files from LSRFortessa™ were analysed on KALUZA® and for MACSQuant® Analyzer X in MACSQuantify™ 2.8. Data obtained from both devices were combined and examined for statistical significance between fresh and cryopreserved NK cells.

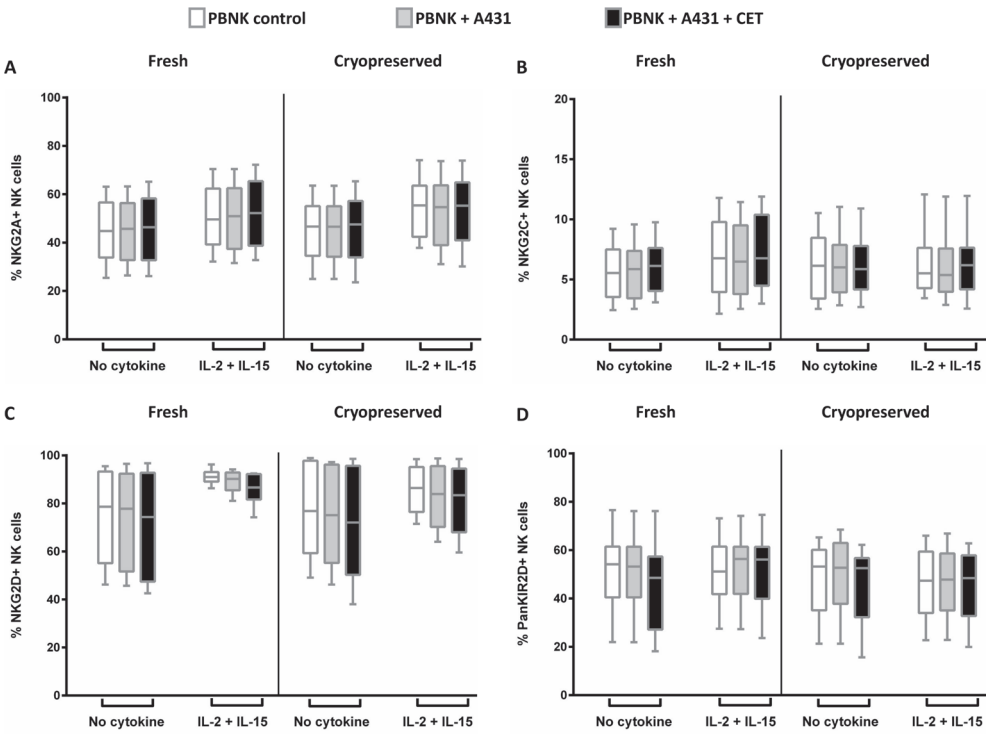

FIGURE 5: Comparison of NK cell phenotypes between fresh and cryopreserved NK cells
NK cell phenotypes between freshly isolated and cryopreserved thawed PBMC samples were compared. NK cells were either activated or non-activated with cytokines (IL-2+IL-15) and target cells (A431) alone or targets coated with cetuximab (CET). Expression levels of NKG2A (A), NKG2C (B), NKG2D (C), KIR2D (D) were compared for the following conditions: i) NK only ii) NK + A431 and iii) NK + A431 + CET conditions. NK only conditions are depicted as open rectangles, followed by NK + A431 with grey shades and NK + A431 + CET conditions represented as black rectangles. Columns represent data from12 donors, from each donor the mean of triplicate values was used; with bars showing SEM. Data are from independent experiments performed in triplicates from 12 PBMC donors (6 donors: BD LSRFortessa + 6 donors: MACSQuant). Statistical analysis was performed using the Wilcoxon test.

and 6) were significantly reduced (***p-value=0.0010) when cryopreserved samples were exposed to A431 alone. When comparing CD16 expression levels in PBNK exposed to A431 and CET, no significant differences were noted between fresh and cryopreserved samples. Further, no significant changes in NK cell expression of the activation marker NKp44 could be detected between fresh and cryopreserved nor between non-activated and cytokine activated NK cells27(Figure 6C). Upon exposure to A431 cells, freshly isolated NK cells that were stimulated with cytokines expressed significantly higher (*p-value=0.04) levels of CD25 when compared to NK cells from cryopreserved samples (Figure 6D and Supplementary Fig S6D and S7D online and Table 5 and 6).

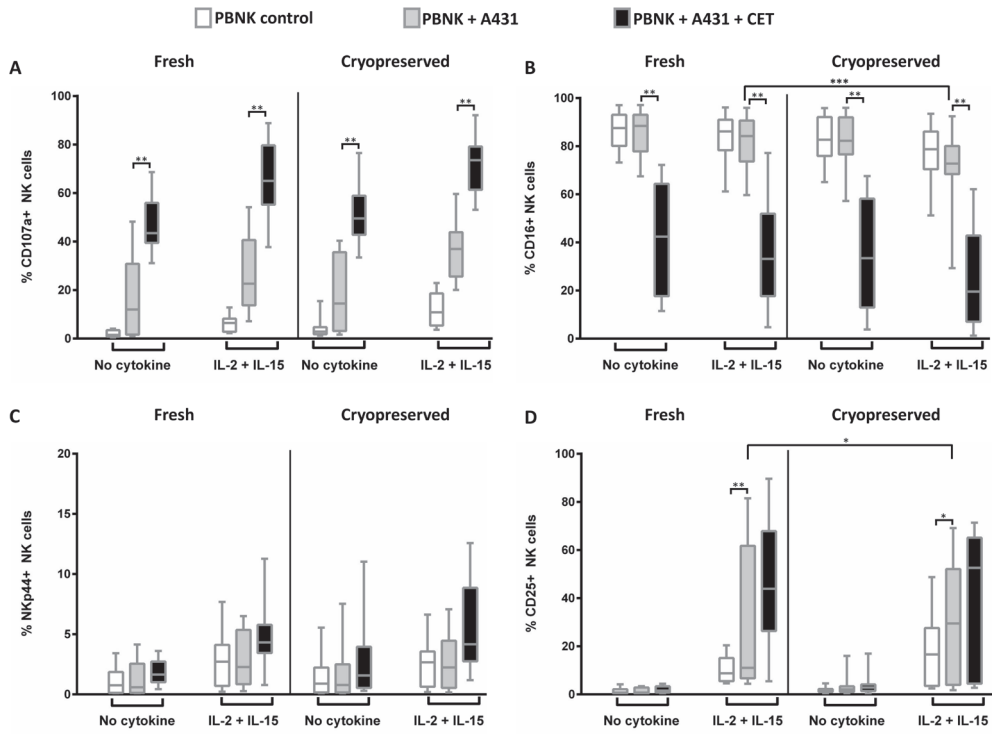

FIGURE 6: Comparison of NK cell function between fresh and cryopreserved NK cells
NK cell functions between freshly isolated and cryopreserved thawed PBMC samples were compared. NK cells were either activated or non-activated with cytokines (IL-2+IL-15) and target cells (A431) alone or targets coated with cetuximab (CET). Expression levels of CD107a (A), CD16 (B), NKp44 (C), CD25 (D) were compared for the following conditions: i) NK only ii) NK + A431 and iii) NK + A431 + CET conditions. NK only conditions are depicted as open rectangles, followed by NK + A431 with grey shades and NK + A431 + CET conditions represented as black rectangles. Columns represent data from12 donors, from each donor the mean of triplicate values was used; with bars showing SEM. Data are from independent experiments performed in triplicates from 12 PBMC donors (6 donors: BD LSRFortessa + 6 donors: MACSQuant). Statistical analysis was performed using the Wilcoxon test.

TABLE 5: Comparison of NK phenotype and function parameters between non-activated fresh and cryopreserved samples.

Non-activated fresh versus cryopreserved NK samples (Wilcoxon test)			
NK FACS panel drop ins	NK only	NK + A431	NK + A431 + CET
CD56$^+$ NKG2A PE-Vio770	ns	ns	ns
CD56$^+$ NKG2C PE	ns	ns	ns
CD56$^+$ NKG2D PerCP-Cy5.5	ns	ns	ns

Non-activated fresh versus cryopreserved NK samples (Wilcoxon test)			
$CD56^+$ PanKIR2D FITC	ns	ns	ns
$CD56^+$ CD107a PE	ns	ns	ns
$CD56^+$ CD16 APC	ns	ns	ns
$CD56^+$ NKp44 PE Vio770	ns	ns	ns
$CD56^+$ CD25 VioBrightFITC	ns	ns	ns
$CD56^{dim}$ CD16 APC	ns	ns	ns

Non-activated freshly isolated NK cell samples from 12 healthy donors and their corresponding cryopreserved counterparts were stained with the NK phenotype and NK function antibody mix and compared under different stimulation conditions (NK only, NK + A431, NK + A431 + CET). All samples were measured in triplicates. Statistical analysis to detect differences between non-activated fresh and cryopreserved samples was performed using the Wilcoxon test. No significant p-values (<0.05) were obtained for the assessed NK phenotype and function panel drop-in parameters. Marker expression level of individual donors are shown in supplementary figures S4, S5 and S8B online.

TABLE 6: Comparison of NK phenotype and function parameters between cytokine activated fresh and cryopreserved samples.

Activated fresh versus cryopreserved NK samples (Wilcoxon test)			
NK FACS panel drop ins	NK only	NK + A431	NK + A431 + CET
$CD56^+$ NKG2A PE-Vio770	ns	ns	ns
$CD56^+$ NKG2C PE	ns	ns	ns
$CD56^+$ NKG2D PerCP-Cy5.5	ns	ns	ns
$CD56^+$ PanKIR2D FITC	ns	ns	ns
$CD56^+$ CD107a PE	ns	ns p=0.06	ns
$CD56^+$ CD16 APC	ns	***p=0.0010	ns
$CD56^+$ NKp44 PE Vio770	ns	ns	ns
$CD56^+$ CD25 VioBrightFITC	ns	* p=0.03	ns
$CD56^{dim}$ CD16 APC	ns	***p=0.0010	ns

Cytokine activated freshly isolated NK cell samples from 12 healthy donors and their corresponding cryopreserved counterparts were stained with the NK phenotype and NK function antibody mix and compared under different stimulation conditions (NK only, NK + A431, NK + A431 + CET). All samples were measured in triplicates. Statistical analysis to detect differences between non-activated fresh and cryopreserved samples was performed using the Wilcoxon test. No significant p-values (<0.05) were obtained for the assessed NK phenotype and function panel drop-in parameters. Marker expression level of individual donors are shown in supplementary figures S6 and S7 and S8C online.

DISCUSSION

Recent advances in multiparametric flow cytometry offer new and exciting opportunities for the in-depth characterization of immune cell subsets in research, diagnosis and treatment[28]. However, insufficient standardization in sample handling, multi-colour panel design, and data analysis often hinders data interpretation in longitudinal studies performed by different laboratories. With this study, we aimed to develop harmonized FACS panels for the phenotypic and functional assessment of NK cells. Standardized methods for instrument set-up, sample preparation, gating strategies, and data analysis have been developed, including the testing of the best suitable antibody-fluorochrome combinations, compatible with the optical configuration of three different flow cytometers.

The FACS panels were designed with a backbone concept, using lineage antigens and a live/dead dye to effectively exclude the non-NK leukocyte populations and dead cells. By allocating the red laser exclusively for CD56 and CD16 detection, the measurement of an array of additional key NK cell antigens from PBMC was facilitated in panels designed to assess either NK phenotype or functions[29]. Moreover, by separating T and NK cell populations in the NK cell function panel, a precise identification and comparison of NK, T and non-conventional T cells (CD3+CD56+) frequencies and their phenotype and functions could be correlated from a single antibody mix. To enumerate NK cells, T cells and non-conventional T cell subset, we made use of a volumetric flow cytometric counting method or, alternatively, counting beads. The availability of three lasers with the flexibility to use eight colours avoided complexities involving fluorochrome spill over[30]. The TCRγδ antibody was included with the aim of simplifying the identification of NK cell populations from CD3dim or negative subsets under diseased conditions and in αβ-T cell depleted grafts. Moreover, several receptors originally identified in NK cells, are also expressed in γδT cells, such as NKG2D, NKp44, NKp30 or DNAM-1[23], and regulate in great part the cytotoxic responses that also γδT cells mediate against tumors[31]. Both NK panels had a built in dead cell marker which served as an internal control ensuring the quality and viability of PBMC, besides offering investigators to perform simultaneous cell counting on PBMC samples.

The inclusion of CD16 in the panel enables users to gate specifically on the CD56dimCD16+ and CD56brightCD16- subsets in parallel[14]. Further, CD45+CD3-CD56- cells can also be studied. This series of options allows users to further study changes in NK cell subsets in e.g. chronic viral infections including human immunodeficiency virus-1 and human cytomegalovirus infections, where NK cell subsets are redistributed with expansion of CD56 negative cells but also make this panel useful in conditions where there is either an increase of CD56+CD16- cells or a decrease in CD56 expression levels[32]. Furthermore, changes in CD16 and CD107a cell surface levels can be used to determine the occurrence of natural killing and ADCC killing of target cells. Generally, after NK cell activation upon target

cell encounter, CD16 surface levels are reduced and CD107a appears on the cell surface as a consequence of the release of the content of cytotoxic granules. Binding of CD16 to IgG_1 coated targets enhances NK cell activation and subsequent cytotoxic responses which is reflected by a more pronounced reduction in CD16 surface levels and increased levels of CD107a on the cell surface; both changes have been well reported in the literature[33] and also specifically in the presence of e.g. the EGFR-binding monoclonal antibody cetuximab[34]. The role of NK cells in eliminating metastasizing cancer cells is well acknowledged in the field of cancer immunotherapy, resulting in an ever-increasing number of clinical trials exploring their therapeutic potential[35]. Over the past decade, the development of several therapeutic IgG_1 monoclonal antibodies (mAbs) targeting different types of cancer[36], and the recent demonstration of their clinical efficacy, has enhanced the interest in better understanding their mode of action[37]. Information on NK CD16 levels is critical for clinical trials monitoring efficacy of therapeutic ADCC mAbs. Baseline and post mAb treated PBMC samples can be compared for NK CD16 levels, and this could indicate if NK cells were able to recognize and bind therapeutic mAbs, as an indirect measure of ADCC. In addition, activation status of NK cells can be monitored measuring CD25 and NKp44 levels as included in our NK function FACS panel. The analysis of CD25 and NKp44 expression is useful for instance, to check whether NK cells are pre-activated or sensitized in peripheral blood by exposure to targets or soluble NK activating ligands[38-40]. Furthermore, NKp44 levels can also be useful for follow up in patients who had adoptive transfer of pre-activated NK cells[41].

The NK cell phenotype panel offers information on major NK cell activating and inhibitory receptors NKG2A, NKG2C, NKG2D and KIR2D alleles, which primarily regulate NK cell activity. In different autoimmune diseases as well as in several cancer types, down regulation of NK cell receptors such as CD16, NKp46, NKp30, NKp44 or KLRB1 and a reduction in NK cell number and functional activity are quite common occurences[42-44]. Though these alterations are not necessarily restricted to NK cells, they underscore the relevance of monitoring immune cell functions in these patients. Our NK cell phenotyping panel will prove useful in monitoring NK cells receptor changes that may affect their ability to lyse tumours.

The most interesting and well-known aspect of NK cells is their ability to lyse target cells via the release of cytotoxic granules, or through Fas/FasL and TRAIL receptor interactions[45,46]. The flexible design of our panels provides a unique opportunity to measure different NK killing mechanisms, e.g. IFNγ, granzyme B and perforin, by modifying drop-in channel combinations. Similarly, natural cytotoxicity receptors (NKp46, NKp44, NKp30), DNAM-1 and other inhibitory/activating receptors can be analysed, suiting the user's needs.

We compared fresh and cryopreserved PBMC from healthy volunteers in BD LSRFortessa™ and MACSQuant® Analyzer X. Though a small decrease in NK cell percentages was observed within living CD45+ populations post cryopreservation, it was minor allowing the acquisition of sufficient events to measure NK cell immune functions. Overall cryopreservation did

not have a significant effect on the NK cell phenotype in both non-activated and cytokine activated PBMC samples. On the other hand, the decrease in the NK cell marker CD16 was more prominent in A431 stimulated cryopreserved NK cells. Importantly however, in the ADCC condition (NK+A431+CET), the decrease in CD16 expression levels was comparable between fresh and cryopreserved NK cells. Our study also indicated that there is no need for IL-2 and IL-15 cytokine stimulation preceding *in vitro* NK cytotoxicity experiments in this setting, as the influence of these cytokines was restricted to increase in CD25 expression on NK cells, with significantly higher CD25 levels in A431 stimulated fresh NK cells, but did not translate into significantly higher degranulation. In general, NK cell cytotoxicity assays are performed using fresh specimens, whereas our results here support the feasibility to use cryopreserved NK cells for this type of assays. The use of cryopreserved PBMC further limits the drawbacks involved in real time analysis of fresh samples over different time points, thus reducing instrument variability and allows generating more reproducible data sets in longitudinal analysis.

Multicenter trials should aim for harmonized panels and reproducible data generation, but have faced challenges in this respect due to variability in flow cytometers and antibody-fluorochrome conjugates resulting in inconsistent data sets[47-49]. With our panel design, uniform gating strategy and implementation of standardized procedures, we were able to obtain reproducible data in three different centers, thereby overcoming inter-laboratory variability issues. Further, differences related to instrument set-up or operators, did not significantly influence the data set. The unique ability to obtain highly reproducible data between three flow cytometers independent of operators and machines, offers an ideal opportunity to use these FACS panels in multicenter trials for monitoring of clinical specimens. The generation of comparable results between different centers could further help in designing cost effective clinical trials by reducing shipment costs effectively.

MATERIAL & METHODS

PBMC isolation and activation

Whole blood samples were collected from healthy donors after obtaining informed consent in accordance with the ‘‘Code for Proper Use of Human Tissues’’ as formulated by the Dutch Federation of Medical Scientific Organizations (www.fmwv.nl)[50]. PBMC were isolated from whole blood using Lymphoprep™ (STEMCELL Technologies, Vancouver, Canada) density gradient centrifugation. PBMC were incubated at a concentration of 5 x 10^6/ml in 6 well plates in Roswell Park Memorial Institute (RPMI) medium (Sigma Aldrich, Zwijndrecht, The Netherlands) containing 2% human serum albumin (HSA) overnight at 37°C, 95% humidity, 5% CO2 atmosphere. PBMC viability, numbers and NK cell content were counted

volumetrically by FACS using a cocktail of 7-AAD (1:200) (Sigma Aldrich, Zwijndrecht, The Netherlands), CD3 VioBlue (1:11), CD56 APC-Vio 770 (1:11), CD16 APC (1:11), CD45 VioGreen (1:11), and CD25 VioBrightFITC (1:11) antibodies from Miltenyi Biotec GmbH. PBMC viability (7AAD- CD45+) and NK cell (7AAD- CD45+ CD3- CD56+) and NK CD16 (7AAD- CD3- CD56+ CD16+) percentages measured before and after overnight incubation were 7AAD- CD45+ (lymphocytes): 91% ± 4% versus 84 ± 2%, %; 7AAD- CD45+ CD3- CD56+ (NK cells): 7.1% ± 3% versus 6.8% ± 6% and CD3- CD56+ CD16+ (CD16 positive cells within NK cells): 89% ± 3% & 82% ± 9%.

Cryopreservation and thawing of PBMC

PBMC were suspended at 1x 10^7 viable cells/ml in 500µl human serum albumin (HSA) per tube and 500µl of freezing medium containing 14% Dimethyl Sulfoxide (DMSO), (Sigma Aldrich, Zwijndrecht, The Netherlands) in Roswell Memorial Park Institute RPMI medium (Sigma Aldrich, Zwijndrecht, The Netherlands) was added to the cells. Isolated PBMC were frozen at a final concentration of 7% DMSO in pre-cooled Nalgene® Mr. Frosty freezing containers (Thermo Scientific, Landsmeer, The Netherlands) overnight at -80°C and transferred after 24h to liquid nitrogen for longer storage. To thaw PBMC, cryovials were transferred from liquid nitrogen into a pre-warmed 37°C water bath. Cells were thawed and washed twice in 500µl of PBS + 0.5% HSA to remove toxic DMSO and suspended in RPMI medium containing 2% HSA for further studies.

Sample handling

In the first phase of the study, peripheral blood mononuclear cells (PBMC) from one healthy donor were collected at different time points as reference samples (n=6) to establish harmonized data acquisition and analysis between three participating centers. All reference samples were isolated and cryopreserved at one center and shipped to other participating centers (n=3). The cryopreserved samples were thawed and analysed at least one month after PBMC collection at all participating centers. At all centers same 3-time points of reference samples were used to optimize the instrument settings, staining protocols and panels. An additional 3-time points of the same reference sample were stained with the NK cell phenotype panel antibody mix to show the obtention of reproducible data sets across the 3 centers.

In the second phase of the study in which the NK cell phenotype and function was compared between fresh and cryopreserved NK samples, PBMC were collected from different healthy donors (n=12; 6 samples were processed at Miltenyi Biotech and analysed using a MACSQuant® Analyzer X device, and other 6 were processed at VU medisch centrum and analysed using BD LSRFortessa™. One fraction of the isolated PBMC was cryopreserved and another fraction was used directly for NK cell analysis. Further, for testing differences

in the NK cell phenotype and function between non-activated and cytokine activated samples, both fresh and cryopreserved samples were split into 2 fractions. One fraction was activated overnight with 1000U/ml of IL-2 (Proleukin®; Chiron, München, Germany) and 10ng/ml of IL-15 (CellGenix GmbH, Freiburg, Germany), while the other fraction was incubated without cytokines.

Cell line

The A431 (epidermoid carcinoma) cell line was obtained from ATCC and cultured in Dulbecco's modified medium (DMEM; Invitrogen, Carlsbad CA, USA) containing 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum (FCS; Integro, Zaandam, The Netherlands). Cells were passaged every 5 days and early-passage cells (passage numbers between 20-25) were used for the experiments. Cells were maintained in a 37°C, 95% humidity, 5% CO_2 atmosphere.

Anti - EGFR monoclonal antibody

The anti-EGFR monoclonal antibody cetuximab (Erbitux®, Merck, Darmstadt, Germany) was purchased from the VU University Medical Center pharmacy for NK cell ADCC experiments.

Flow cytometers and instrument settings

Experiments were performed with three different flow cytometers: BD LSRFortessa™ X-20 (Becton Dickinson (BD) B.V, Breda, The Netherlands), BD FACSCanto™ II (Becton Dickinson B.V, Breda, The Netherlands) and MACSQuant® Analyzer X (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). All devices were equipped with 3 solid state lasers (Violet, Blue and Red). Comparable band pass and long pass filters enabled the use of same fluorochrome-conjugated antibodies between the three machines (Table 1). The chosen fluorochromes were distributed accordingly; two light scatter parameters and 8 fluorescence detectors: Violet laser excited VioGreen and VioBlue fluorochromes, Blue laser for forward and side scatter, besides detecting signals from FITC, VioBrightFITC, PE, PerCP-Cy5.5, PerCP-Vio700 and PE-Vio770 channels and red laser for APC and APC-Vio770. Daily maintenance and initial photomultiplier tube (PMT) voltage determination were performed using recommended cytometer tracking and set-up beads (CS&T) (BD biosciences, Breda, The Netherlands) for BD instruments, and MACSQuant® calibration beads (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) for MACSQuant® Analyzer X.

Antibodies, staining protocol and compensation settings

The antibody clones and fluorochrome combinations were selected based on lab user experience and antibody sensitivity and with the aim of achieving minimal spill over between detectors. Moreover, the antibody - fluorochrome conjugates were chosen in order to attain high sensitivity for measuring dimly expressed antigens besides overcoming

sterical hindrance. For staining, the PBMC concentration was adjusted in washing buffer (PBS containing 0.5% bovine serum albumin) to 1 x 10^6/ml and stained in 96 well U bottom plates (Corning, Amsterdam, The Netherlands). PBMC were incubated with relevant antibody mixes in a final premix volume of 30µl for 10 minutes at 4°C in the dark. After incubation cells were washed once with 170 µl of washing buffer per well and centrifuged at 300xg for 5min, supernatants were discarded, and cell pellets suspended in 200µl of washing buffer until measurement. Samples were measured within 1 hour.

The antibody mix for the NK phenotype panel consisted of CD45 VioGreen, CD3 VioBlue, TCRγδ VioBlue, CD14 VioBlue, CD19 VioBlue and SYTOX® Blue for live NK cell discrimination and NK cell phenotypic antigens PanKIR2D FITC, NKG2A PE, NKG2C PE-Vio770 and NKG2D PerCP-Cy5.5. Similarly, the antibody mix for the NK function panel consisted of CD45 VioGreen, CD14 VioBlue, CD19 VioBlue and SYTOX® Blue, together with CD3 PerCP-Vio700 and TCRγδ PerCP-Vio700 to identify NK cells, with inclusion of NK function antigens CD25 VioBrightFITC, CD107a PE, NKp44 PE-Vio770. Most of the antibodies were supplied by Miltenyi Biotec except NKG2D PerCP-Cy5.5 (Biolegend, Fell, Germany) and dead cell stain SYTOX® Blue (Thermo Fisher Scientific, Berlin, Germany). Manufacturer recommended dilutions of the antibodies were first checked and finally used. Details on antibody clones and dilutions used in the NK phenotyping and NK function panel are listed in tables 2 and 3.

Due to the absence or low expression of some antigens (CD25, NKp44 or CD107a) on NK cells in unstimulated PBMC, we used Ig-capture beads (MACS® Comp Bead anti-mouse Igκ and MACS® Comp Bead anti-human Igκ, Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) to establish initial fluorescence compensation matrices using the automatic compensation feature of each instrument. Further, unstained PBMC and single staining of all antibodies was acquired to assess the compensation matrices obtained with the Ig-capture beads. In addition to this, FMO controls were performed for drop in antigens (CD25 VioBrightFITC, NKp44 PE-Vio770 and CD107a PE) in the NK function panel (Supplementary figure 1). Compensation matrices were calculated using automated settings from BD FACS DIVA™ software for BD FACSCanto™ II and BD LSRFortessa™, and MACSQuantify™ 2.8 software for MACSQuant® Analyzer X. In situations where instrument compensation was not optimal, adjustments were made based on the single staining controls from PBMC using KALUZA® data analysis software (Beckman Coulter, California, US). Compensation matrices of the NK phenotype and function panels for the three flow cytometers are shown in supplementary tables S1, S2, and S3 online.

NK cell cytotoxicity assays

Activated and non-activated PBMC (containing effector cells) were stimulated with A431 cells (targets) in the presence or absence of 5µg/ml of cetuximab (CET) in a total volume of 100 µl in 96 well U bottom plates. PBMC counts were adjusted so that NK cell numbers

matched with the number of target cells (5 x 10^4 target and 5 x 10^4 NK cells from PBMC) at a 1:1 E: T ratio. PBMC without targets and PBMC + CET without targets were included as controls. All treatments and corresponding conditions were performed in triplicate. After 4h incubation at 37°C, 95% humidity and 5% CO_2 atmosphere, cells were pelleted and stained with the NK phenotype (Table 2) or NK function antibody (Table 3) mixes. To assess degranulation by NK cells, anti-CD107a PE (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) was added at the beginning of the assay.

Acquisition and data analysis for harmonisation between different flow cytometers

Individual acquisition protocols following standardized instrument settings were set up in three centers for three different flow cytometers using the same reference samples (n=6) and the same staining antibody mixtures. In the first step, to compare variability between flow cytometers, reference PBMC samples were isolated and stored as described above for testing. Single-stain controls were acquired for antibody - fluorochrome conjugates in the NK phenotype and NK function panel. Further, to detect expression of NK cell receptors and NK cell function-associated antigens and effector molecules, PBMC were exposed to target cells (A431). FCS files acquired from three flow cytometers were analysed in KALUZA®. For comparison of different PBMC conditions (fresh versus cryopreserved, no cytokine versus cytokine activated and no CET versus CET stimulated), data acquisition was done using two flow cytometers BD LSRFortessa™ and MACSQuant® Analyzer X. In this experimental part, the FCS files obtained were analysed by individual laboratories, with KALUZA® software for analysis of data generated from BD LSRFortessa™ and MACSQuantify™ 2.8 software for data generated from MACSQuant® Analyzer X.

Gating strategy

Two different gating strategies were defined, one for the NK phenotype and another for the NK function panel following a modified ISHAGE gating strategy[51]. For the NK phenotype panel, cell doublets were excluded by plotting forward side scatter area and height parameters. Next, CD45 was plotted against CD3/TCRγδ/CD14/CD19 (lineage) and SYTOX® Blue to gate on all live and lineage- CD45+ cells. Thus, selected cells were further plotted against CD56, identifying only viable NK cells. This NK cell gate was used to assess the expression levels of CD16, NKG2A, NKG2C, NKG2D and KIR2D antigens (Supplementary Fig. S2 online). Of note, the gating strategy for the NK cell function panel was essentially the same with one exception, i.e. the inclusion of CD3 and TCRγδ as drop in antigens, excluding them from the backbone markers. With this panel, we differentiated between three cell populations: NK cells (CD45+CD3-/TCRγδ-CD56+) T cells (CD45+CD3/TCRγδ+CD56-) and non-conventional T cell subsets (CD45+CD3+/TCRγδ+CD56+). The gate on the NK cell

population (CD45+CD3-/TCRγδ- CD56+) was used to analyse the expression of CD107a, CD25 and NKp44 (Supplementary Fig.S3 online).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Differences between centers were determined using Kruskal Wallis test and difference between fresh and cryopreserved were determined using non-parametric Wilcoxon test. P value of < 0.05 was considered statistically significant.

Ethics Statement

The research presented in this paper, all methods and experimental protocols were approved and carried out in accordance with relevant guidelines and regulations of the Committee for Scientific Research of the VU University Medical Center, Cancer Center Amsterdam.

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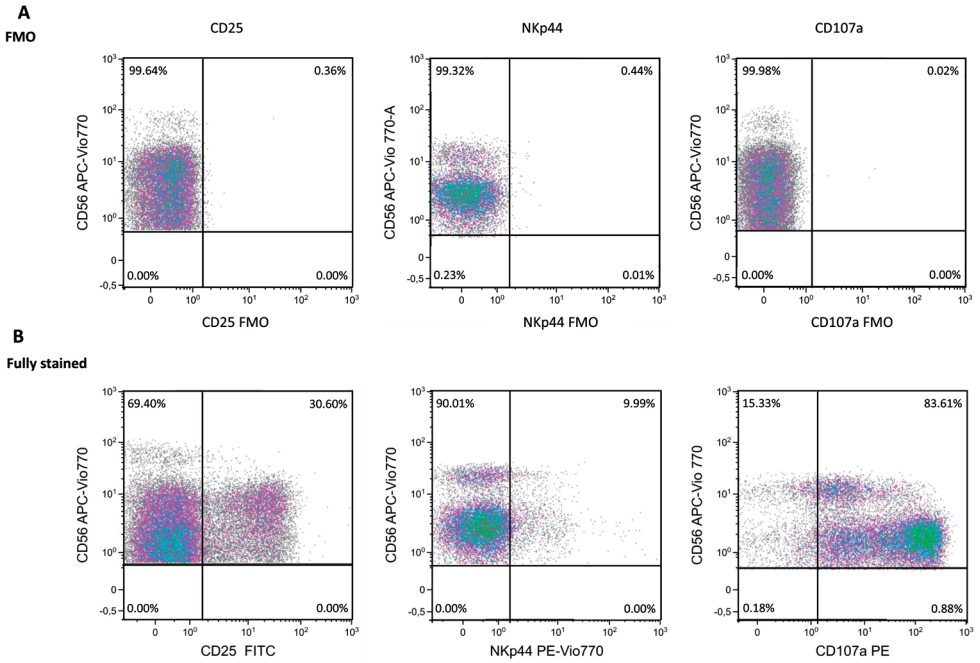
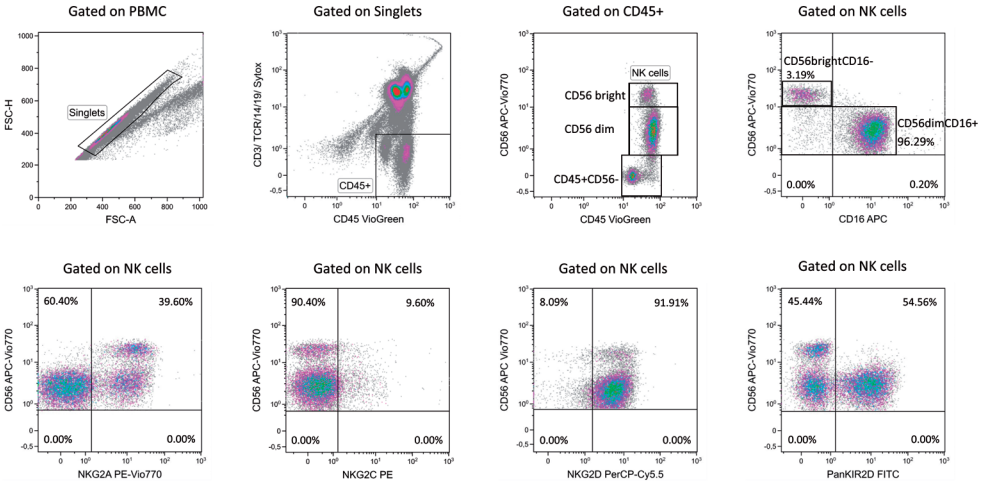


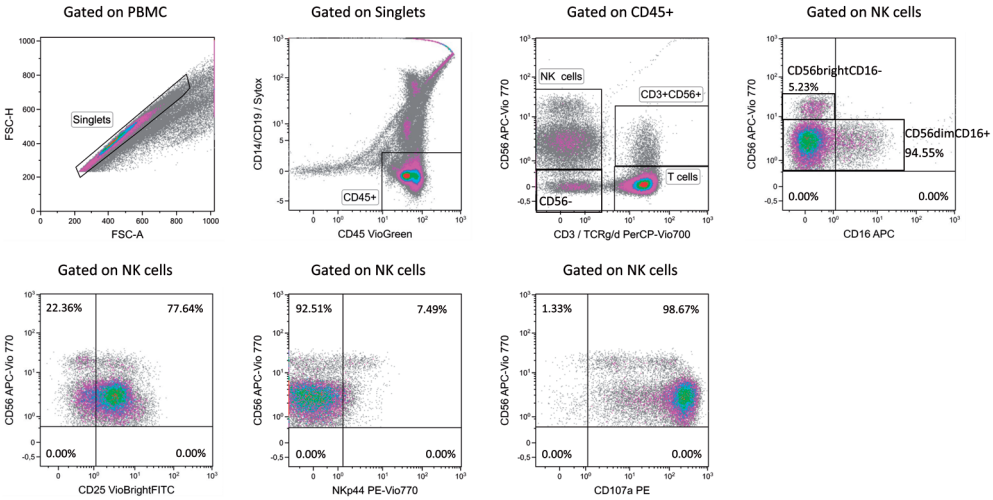
SUPPLEMENTARY FIGURE 1: Fluorescence minus one (FMO) controls for gating NK function panel receptors

In case of NK cell function panel experiments, a Fluorescence Minus One (FMO) control was devised to optimize the identification of NK cell activation antigens CD25, NKp44 and CD107a under different stimulated conditions. FMO stains and a fully stained sample of NK cell stimulated with cetuximab coated A431 tumor target cells is shown in figure A and B. The FMO gate clearly indicates the negative control position.



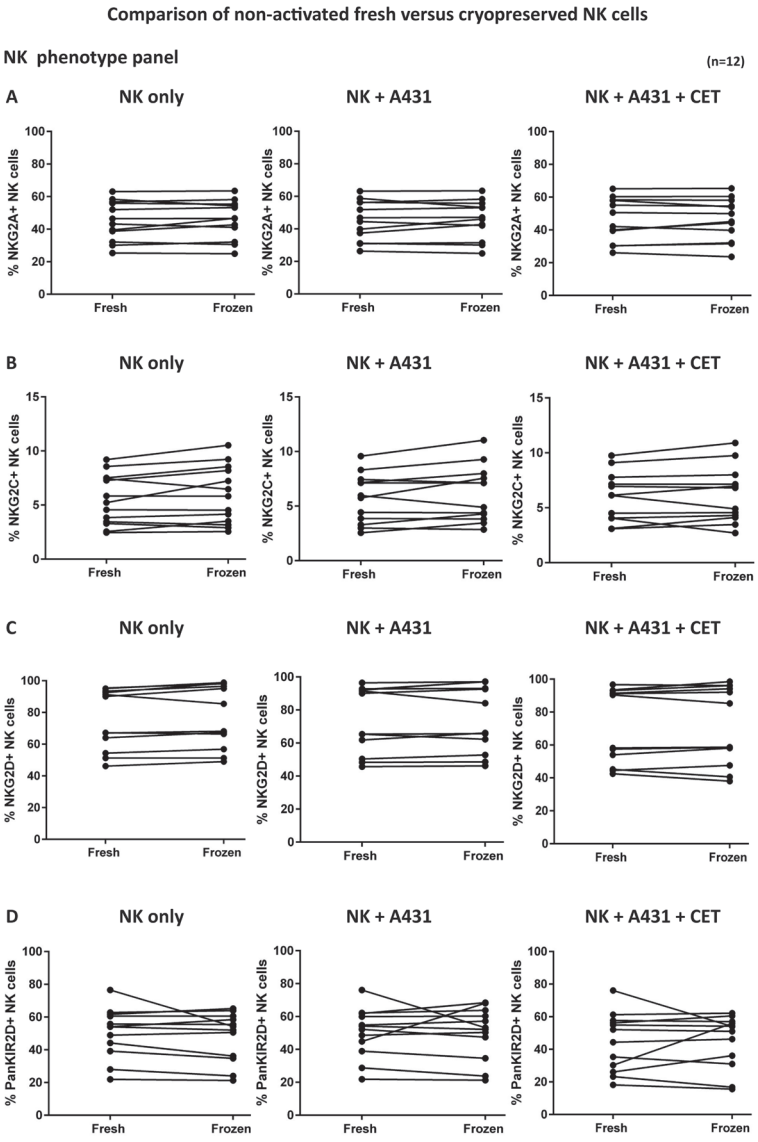
SUPPLEMENTARY FIGURE 2: Gating strategy for the NK cell phenotype panel

The gating procedure to analyse NK cell phenotypes from PBMC population is shown. Cells acquired were gated FSC-H/FSC-A to eliminate doublets, followed by gating on the exclusion channel (CD3/ TCRγδ/CD14/CD19/SYTOX® Blue) against CD45. Lymphocytes negative for exclusion channel antigens and positive for CD45 were then plotted for CD45+CD56+ cells. CD56+ NK cells were then gated to define single receptor positive cells CD56+CD16+, CD56+NKG2A+, CD56+NKG2C+, CD56+NKG2D+, CD56+KIR2D+ plots from CD56+NK cells. Further to facilitate NK subset analysis, NK cells were sub-divided into NK bright and NK dim populations based on CD56 expression and CD16 levels. CD45+CD56- cells can also be studied using this gating strategy.



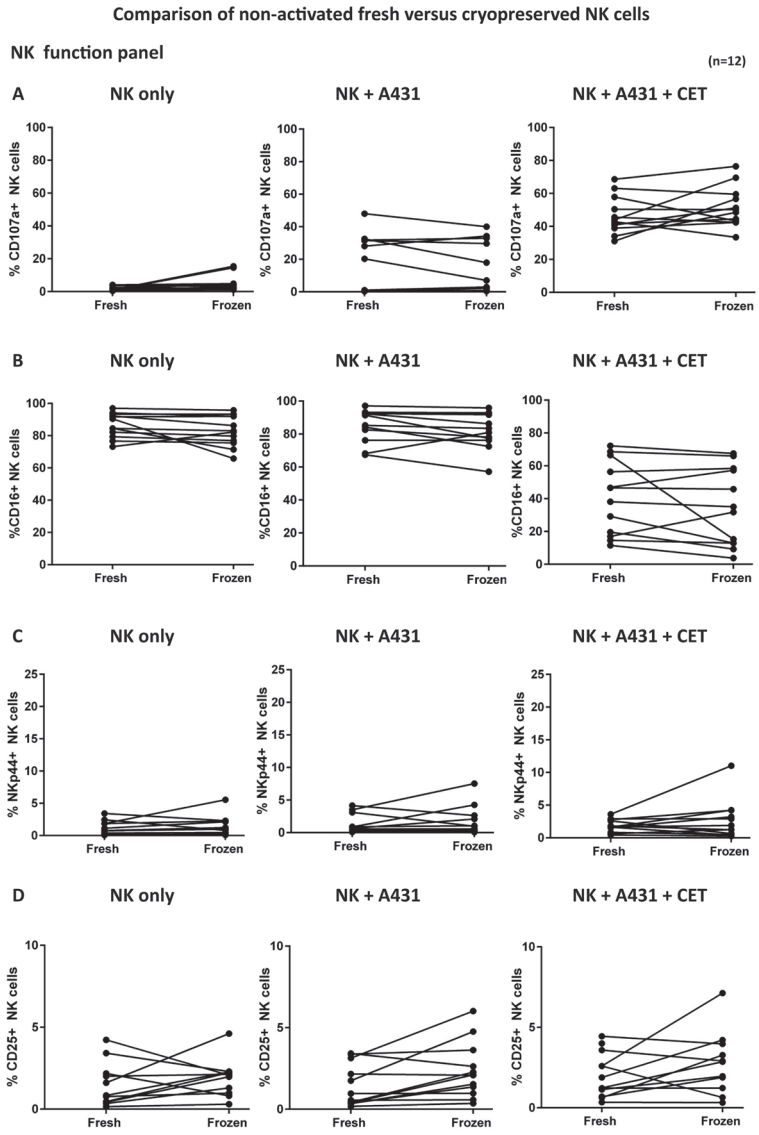
SUPPLEMENTARY FIGURE 3: Gating strategy for the NK cell function panel

The gating procedure to analyse NK cell functions from PBMC population is shown. Singlets were selected gating PBMC against FCS-A versus FSC-H. CD45+ cells negative for CD14, CD19 and SYTOX® Blue were further gated against CD3/TCRγδ and CD56 to differentiate NK cells, T cells and CD3+CD56+ cells. CD45+CD56+ NK cells were further analysed for CD16, CD107a, NKp44 and CD25 expression. NK cells shown in this figure were stimulated with A431 + CET to show higher CD107a and lower CD16 levels. Further to facilitate NK subset analysis, NK cells were sub-divided into NK bright and NK dim populations based on CD56 expression and CD16 levels. CD45+CD56- cells can also be studied using this gating strategy.



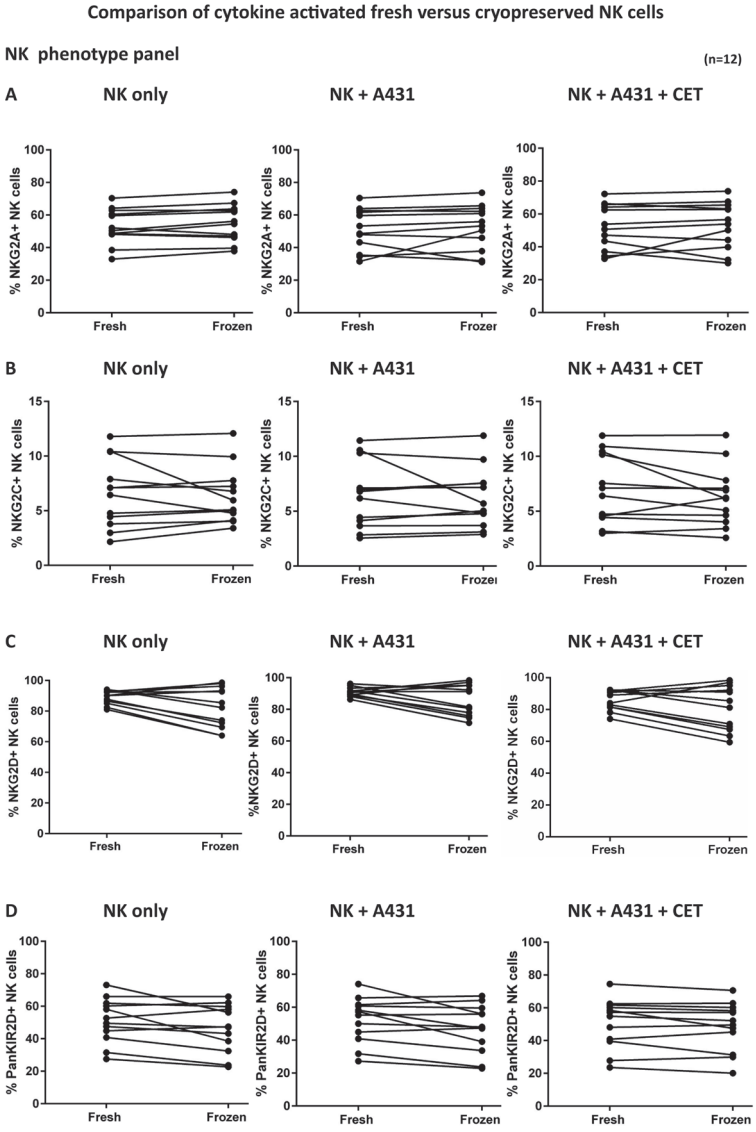
SUPPLEMENTARY FIGURE 4: Comparison of NK phenotype panel marker differences between fresh and cryopreserved non-activated NK cells

Non-activated NK cells were stimulated with target cells (A431) alone or targets coated with cetuximab (CET). Expression levels of NKG2A (A), NKG2C (B), NKG2D (C), KIR2D (D) were compared between fresh and cryopreserved NK cells for the following conditions: i) NK only ii) NK + A431 and iii) NK + A431 + CET. Data points represent the mean of triplicate values from independent experiments from 12 PBMC donors (6 donors: BD LSRFortessa + 6 donors: MACSQuant). Statistical analysis was done using Wilcoxon test and no statistically significant differences were observed between non-activated fresh and cryopreserved NK cells.



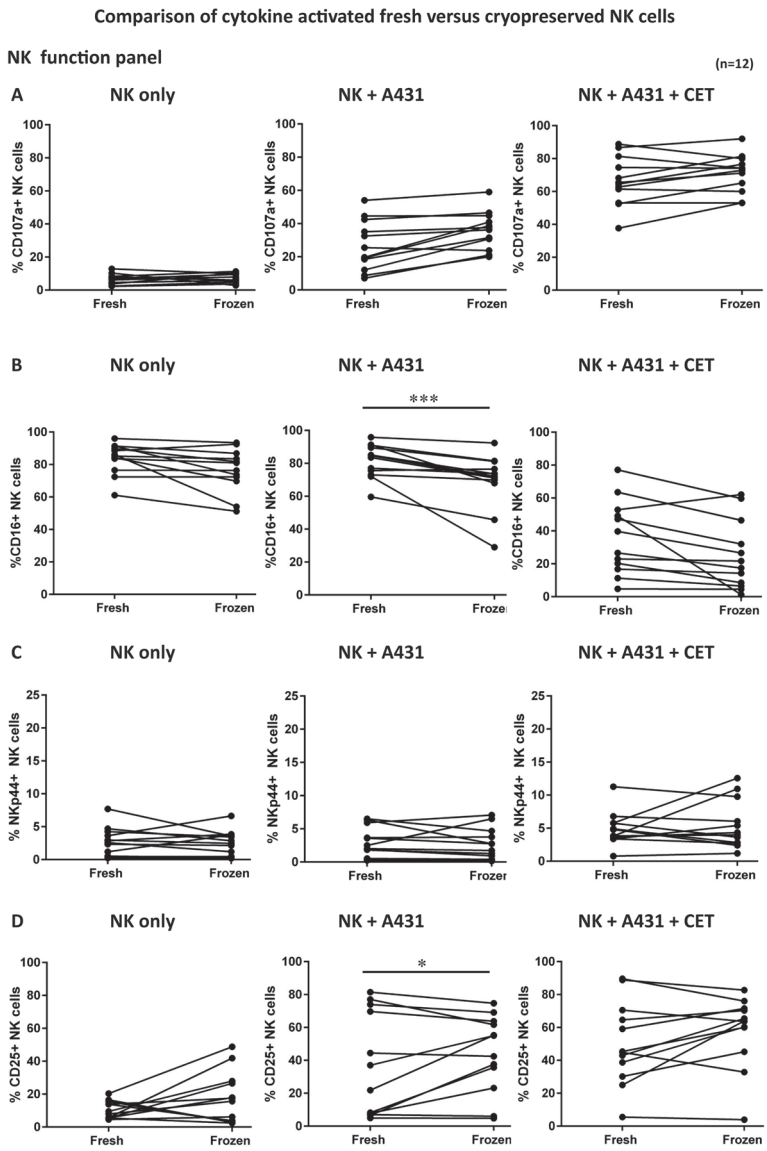
SUPPLEMENTARY FIGURE 5: Comparison of NK function panel marker differences between fresh and cryopreserved non-activated NK cells

The capacity of NK cells to perform natural cytotoxicity and ADCC under different NK stimulated conditions were analysed on 12 healthy donors using the NK function panel antibody mix. Expression levels of CD107a (A), CD16 (B) NKp44 (C) and CD25 (D) were compared between fresh and cryopreserved non-activated NK cells for the following conditions: i) NK only ii) NK + A431 and iii) NK + A431 + CET. Data points represent the mean of triplicate values from independent experiments from 12 PBMC donors (6 donors: BD LSRFortessa + 6 donors: MACSQuant). Statistical analysis was done using Wilcoxon test and no statistically significant differences were observed between fresh and cryopreserved non-activated NK cells.



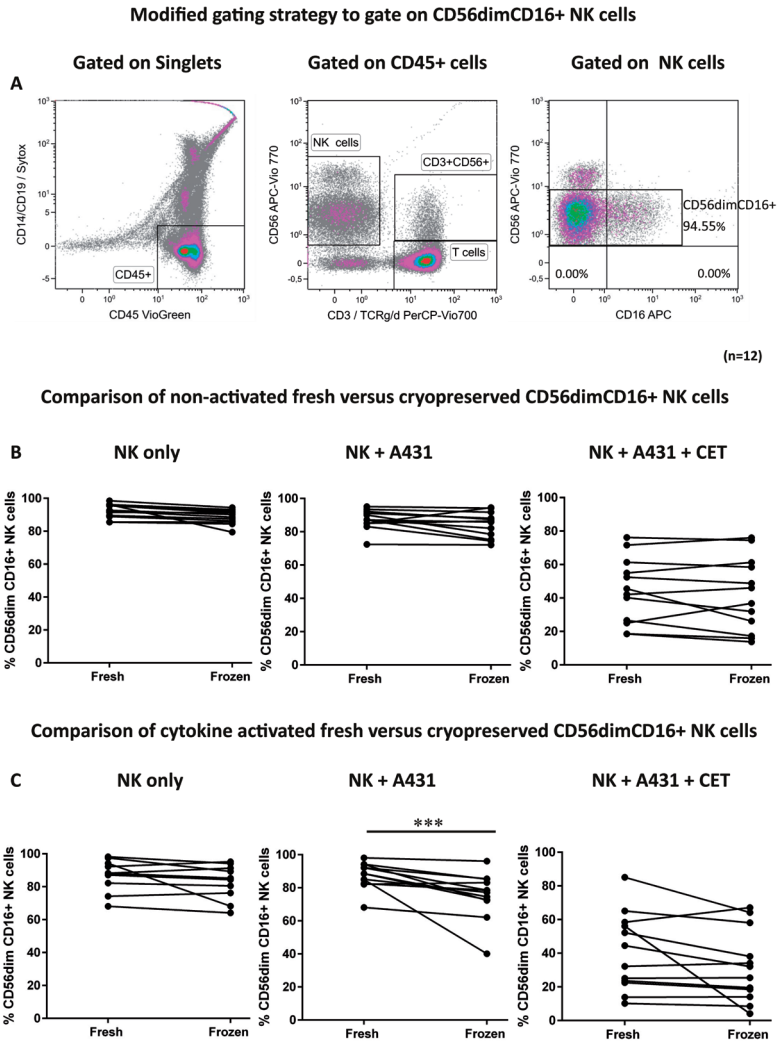
SUPPLEMENTARY FIGURE 6: Comparison of NK phenotype panel marker differences between fresh and cryopreserved cytokine activated NK cells

NK cells were activated overnight with IL-2 and IL-15 and stimulated with target cells (A431) alone or targets coated with cetuximab (CET). Expression levels of NKG2A (A), NKG2C (B), NKG2D (C), KIR2D (D) were compared between fresh and cryopreserved NK cells for the following conditions: i) NK only ii) NK + A431 and iii) NK + A431 + CET. Data points represent the mean of triplicate values from independent experiments from 12 PBMC donors (6 donors: BD LSRFortessa + 6 donors: MACSQuant). Statistical analysis was done using Wilcoxon test and no statistically significant differences were observed between fresh and cryopreserved cytokine activated NK cells.



SUPPLEMENTARY FIGURE 7: Comparison of NK function panel marker differences between fresh and cryopreserved cytokine activated NK cells

*The capacity of NK cells to perform natural cytotoxicity and ADCC under different NK stimulated conditions were analysed on 12 healthy donors using the NK function panel antibody mix. Expression levels of CD107a (A), CD16 (B), NKp44 and CD25 (D) were compared between fresh and cryopreserved cytokine activated NK cells for the following conditions: i) NK only ii) NK + A431 and iii) NK + A431 + CET . Data points represent the mean of triplicate values from independent experiments from 12 PBMC donors (6 donors: BD LSRFortessa + 6 donors: MACSQuant). Statistical analysis was done using Wilcoxon test and only statistically significant differences are mentioned (*p<0.05, ***p<0.001).*



SUPPLEMENTARY FIGURE 8: Paired comparison analysis of CD56dim CD16+ subset using modifed NK cell gating strategy

*(A) The gating procedure to analyse NK cell phenotypes from PBMC population is shown. Lymphocytes negative for exclusion channel antigens and positive for CD45 were plotted for CD45+CD56+ cells. CD56+ NK cells were sub-divided into NK bright and NK dim populations based on CD56 expression and further the CD56dimCD16+ NK cells (NK effectors) were defined by gating CD45+CD3-CD56+ NK cells against CD16. (B) CD16 levels were compared between non activated fresh and cryopreserved NK cells (C) CD16 levels were compared between cytokine activated fresh and cryopreserved NK cells. In, B and C the following conditions: i) NK only ii) NK + A431 and iii) NK + A431 + CET were compared between fresh and cryopreserved NK cells for changes in CD56dim CD16+ marker expression. Data points represent the mean of triplicate values from independent experiments from 12 PBMC donors (6 donors: BD LSRFortessa + 6 donors: MACSQuant). Statistical analysis was done using Wilcoxon test and only statistically significant differences are mentioned (***p<0.001).*

CHAPTER 3

HLA independent killing of cervical tumors by UCB-NK cells

Published as
"High-efficiency lysis of cervical cancer by allogeneic NK cells derived from umbilical cord progenitors is independent of HLA status"

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ABSTRACT

Down-regulation of HLA in tumor cells, low numbers and dysfunctionality of NK cells are commonly observed in patients with end-stage cervical cancer. Adoptive transfer of high numbers of cytotoxic NK cells might be a promising treatment approach in this setting. Here, we explored the cytotoxic efficacy on ten cervical cancer cell lines of activated allogeneic NK cells from two sources, i.e. peripheral blood (PBNK) -with and without cetuximab (CET), a tumor-specific monoclonal antibody directed against EGFR, or derived from umbilical cord blood (UCB-NK). Whereas CET monotherapy was ineffective against the panel of cervical cancer cell lines, irrespective of their EGFR expression levels and despite their *RAS*^{wt} status, it significantly enhanced the *in vitro* cytotoxic efficacy of activated PBNK ($P=0.002$). Equally superior cytotoxicity over activated PBNK alone was achieved by UCB-NK ($P<0.001$). Both PBNK and UCB-NK-mediated cytotoxic activity was dependent on the NK-activating receptors NKG2D (natural killer group 2, member D receptor) and DNAM-1 (DNAX Accessory Molecule-1) ($P<0.05$) and unrelated to expression levels of the inhibitory receptors HLA-E and/or HLA-G. Most strikingly, whereas the PBNK's cytotoxic activity was inversely correlated with HLA-ABC levels ($P=0.036$), PBNK + CET and UCB-NK cytotoxicity were entirely independent of HLA-ABC expression. In conclusion, this study provides a rationale to initiate a clinical trial for cervical cancer with adoptively transferred allogeneic NK cells, employing either UCB-NK, or PBNK + CET for EGFR-expressing tumors. Adoptive transfer of UCB-NK might serve as a generally applicable treatment for cervical cancer, enabled by HLA-, histology- and HPV-independent killing mechanisms.

INTRODUCTION

Persistent infection of the cervical epithelium by high-risk HPV can lead to cervical intraepithelial neoplasia which may progress to invasive cervical cancer, such as squamous cell carcinoma, adenosquamous cell carcinoma or adenocarcinoma[1-3].

Treatment for cervical cancer includes conventional surgery, chemotherapy and/or radiation. In addition, in advanced (metastatic) disease, targeted therapies are widely explored. Unfortunately, targeted intervention strategies using small molecules, angiogenesis inhibitors and monoclonal antibodies directed against specific tumor antigens and proliferation pathways have had limited success in restricting cervical tumor growth so far[4,5]. In cervical cancer, EGFR is variably expressed in 80% of the tumor tissues[6]. Overexpression of EGFR has been associated with poor prognosis in cervical cancer, making EGFR an obvious candidate for therapeutic targeting[7,8]. However, treatment with cetuximab (CET) (chimeric IgG_1, anti-EGFR mAb) as monotherapy or CET in combination with chemotherapy was ineffective in patients with cervical cancer, in spite of the apparent absence of activating mutations in the EGFR pathway[9,10].

Immunotherapy of cervical cancer has been clinically explored with limited success. Efforts so far have mostly focused on vaccination approaches against HPV-derived oncogenes (E6 and E7) to trigger an efficacious antitumor T-cell response[11]. Failure to improve clinical outcome may at least in part be due to extensive HLA down-regulation commonly observed in cervical cancer[12-14]. In these cases, NK cell-based therapies may prove more effective than T-cell-based approaches. Indeed, the role of the innate immune response in host defense and viral clearance during (early) infection is well recognized[15]. NK cells are potent in exerting rapid cytotoxicity by releasing cytotoxic granzyme B and perforin in order to lyse virus-infected cells and tumor cell targets. Functional activity of NK cells is regulated by an equilibrium between inhibitory (e.g. CD94/NKG2A) and activating (e.g. CD16, DNAM-1, CD94/NKG2C, CD94/NKG2D) receptors[16,17].

Infiltrating NK cells are observed in low-grade and high-grade cervical intraepithelial neoplasia lesions and to a lesser extent in cervical carcinoma[13,18-21]. *In vitro* studies have shown that peripheral blood NK cells (PBNK) are able to kill HPV-infected cell lines[19,21,22]. However, NK cells are often dysfunctional and low in number in cervical cancer patients, and thereby unable to mount efficient cytotoxicity against tumors[23,24]. NK cytotoxic function is also counteracted by several cervical tumor escape mechanisms, including low expression of activating NK cell receptor ligands (e.g. MICA/B, ULBPs, Nectin, PVR) and aberrant expression of suppressive non-classical HLA molecules (e.g. HLA-E and -G) by tumor cells [14,19,25-27]. *Ex vivo* expanded autologous NK cells, adoptively transferred for the treatment of solid tumors, in most studies have yielded disappointing results, underscoring the dire need for the development of more powerful therapeutic approaches to overcome tumor-

associated NK cell dysfunctionality and the inherent resistance to cytolysis of cancer cells. Clinical studies exploring the use of *ex vivo* expanded allogeneic PBNK from healthy donors also yielded low antitumor efficacy[28,29], which may have been due to their limitations in terms of cell yield, purity, ability to expand *in vivo*, and cytotoxic capacity[30.]

An attractive alternative approach would be the use of umbilical cord blood $CD34^+$ stem cell derived NK cells (UCB-NK), which are feeder cell-free cultures that can be differentiated and efficiently expanded up to 10.000 fold, resulting in a highly pure product with a high cytolytic capacity[31]. Yet another alternative might be to enhance PBNK cell-mediated cytolysis of cervical tumor cells by the tumor-targeted IgG1 monoclonal antibody CET, to invoke antibody dependent cell mediated cytotoxicity (ADCC)[32].

In this comparative study we explored the anti-tumor efficacy of two clinically applicable therapeutic strategies, i.e. UCB-NK vs. allogeneic PBNK+CET, for cervical cancer. Of note, the combination with CET is not a viable option for UCB-NK as *in vitro* they do not express sufficient levels of the required Fc receptor CD16 to obtain functional benefit[33-34]. A series of *in vitro* NK cytotoxicity assays was conducted to compare anti-tumor potency of PBNK from healthy volunteers, with or without co-incubation with CET with that of umbilical cord blood derived NK cell (UCB-NK) monotherapy against various cervical cancer cell lines. These cell lines (n=10) were stratified based on infection with different HPV types, histological origins, and differential expression levels of NK activating- and inhibitory ligands. The findings from this pre-clinical study strongly support the use of allogeneic UCB-NK derived from umbilical cord precursor cells and outline the advantages of their use as monotherapy in the treatment of cervical cancer.

RESULTS

Comparative analysis of NK cell cytotoxic activity against cervical cancer cell lines

Initially, we compared the anti-tumor potency of healthy activated PBNK in the presence or absence of CET. Ten cervical cancer cell lines (EGFR-expressing except for C33A, and all *RAS*^{wt}; Table 1) were subjected to PBNK only, CET only, or to a combination of PBNK with CET in order to examine ADCC effects. In line with previous studies, CET as monotherapy did not induce cell death in any of the cell lines tested (data not shown). However, cervical cancer cell lines were sensitive in varying degrees to PBNK-induced cell lysis (Figure 1a), independent of their EGFR expression levels (Figure 1b), with consistently and significantly higher cytotoxicity rates when coated with CET ($P = 0.002$) (Figure 1c). C33A (EGFR- cell line) was the only cell line that did not display a higher cytotoxicity rate when PBNK were combined with CET (Figure 1a-c).

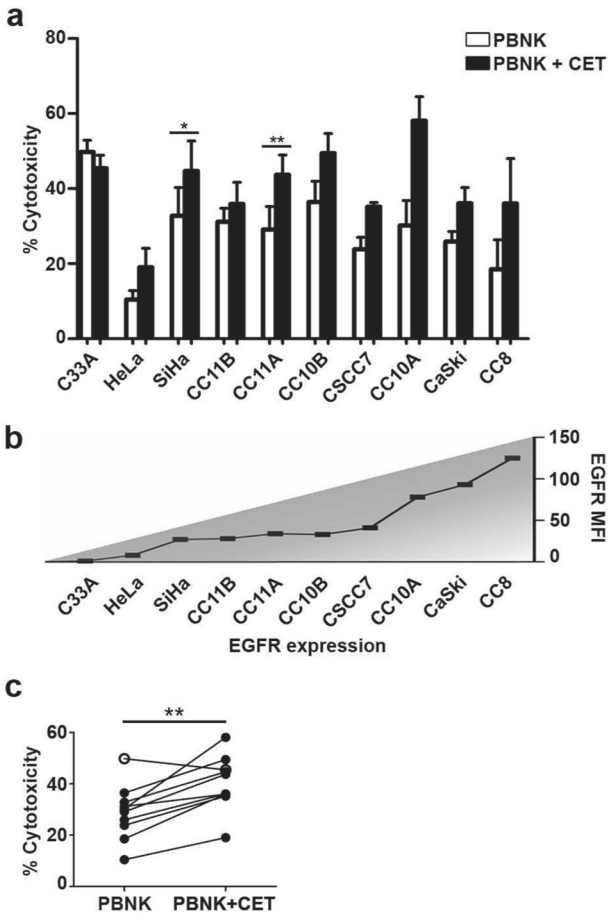

FIGURE 1: PBNK cytotoxicity against cervical cancer cells alone and in combination with CET

(a) *Cytotoxicity levels (Δ7AAD) of activated PBNK (open bars) and PBNK + cetuximab (CET) (closed bars) against ten cervical cancer cell lines,* **(b)** *arranged in order of EGFR expression level. Bars are means of triplicate values from four experiments with four different donors for C33A, HeLa, SiHa, CC11B, CC11A, CC10B, CC10A, CaSki and two experiments with two different donors for CSCC7 and CC8. Bars represent mean ± SEM.* **(c)** *Significantly higher cytotoxicity levels (Δ7AAD) were observed in all cell lines after co-culture with PBNK + CET compared to PBNK, except for C33A (open circle). * P <0.05 and **P < 0.01 calculated with paired t test.*

Next, activated PBNK were compared with UCB-NK for their ability to induce target cell death. UCB-NK were significantly more cytotoxic than PBNK, consistently inducing higher rates of tumor cell death in all tested cell lines ($P < 0.001$) (Figure 2a, b). Note that the PBNK cytotoxicity data presented in Figure 2a are the same as those in Figure 1a. Indeed, the cytotoxicity levels were similar for UCB-NK and PBNK + CET (Figure 1a, 2a). This was

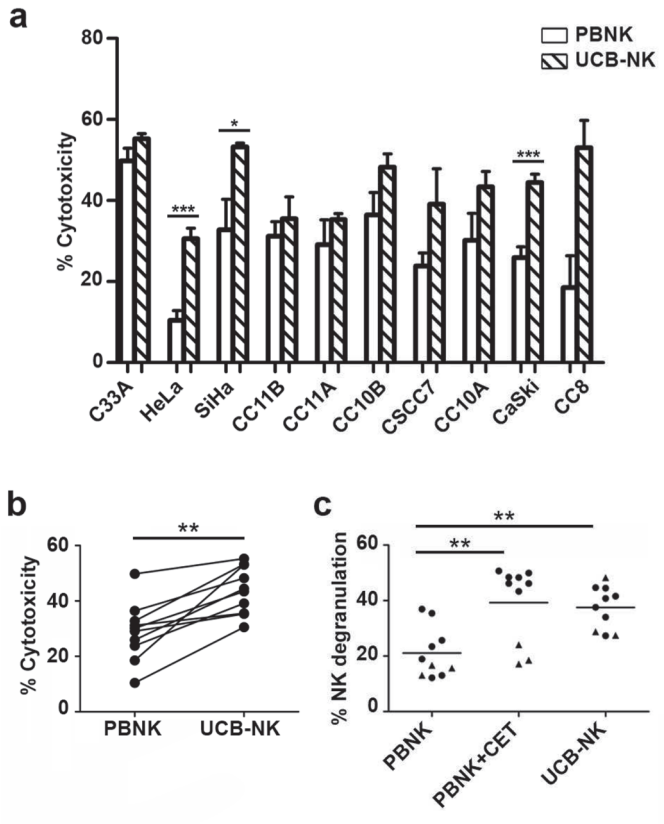

FIGURE 2: PBNK and UCB-NK cytotoxicity against cervical cancer cells
(a) *Cytotoxicity levels (Δ7AAD) of PBNK (open bars) and UCB-NK (hatched bars) against ten cervical cancer cell lines. Bars are means of triplicate values from four experiments with four different donors for C33A, HeLa, SiHa, CC11B, CC11A, CC10B, CC10A, CaSki and two experiments with two different donors for CSCC7 and CC8 using PBNK and five experiments for UCB-NK using five different donors for all cell lines; Bars represent mean ± SEM. PBNK data used to compare with UCB-NK in Figure a are the same dataset as Figure 1a.* **(b)** *Significantly higher cytotoxicity levels (Δ7AAD) were observed in all cell lines after co-culture with UCB-NK compared to PBNK.* **(c)** *Significantly higher levels of NK degranulation (ΔCD107a) in PBNK + cetuximab (CET) and UCB-NK conditions compared to PBNK only condition. Triangles denote cell lines with low EGFR levels, i.e. C33A, HeLa, and SiHa. *P <0.05, **P < 0.01 and ***P < 0.001 calculated in A and B with unpaired t test, in C with one-way ANOVA, Bonferroni's multiple comparison test.*

further borne out by observed degranulation levels of NK cells in response to exposure to the cervical cancer cell lines, as measured by CD107a surface expression. These were comparably and significantly elevated in the PBNK + CET and UCB-NK conditions over PBNK alone (Figure 2c, Supplementary Figure 2). UCB-NK were not tested in combination with CET due to their low surface expression of CD16a, which is essential for ADCC in combination with therapeutic mAbs (data not shown). Interestingly, PBNK degranulation levels were low

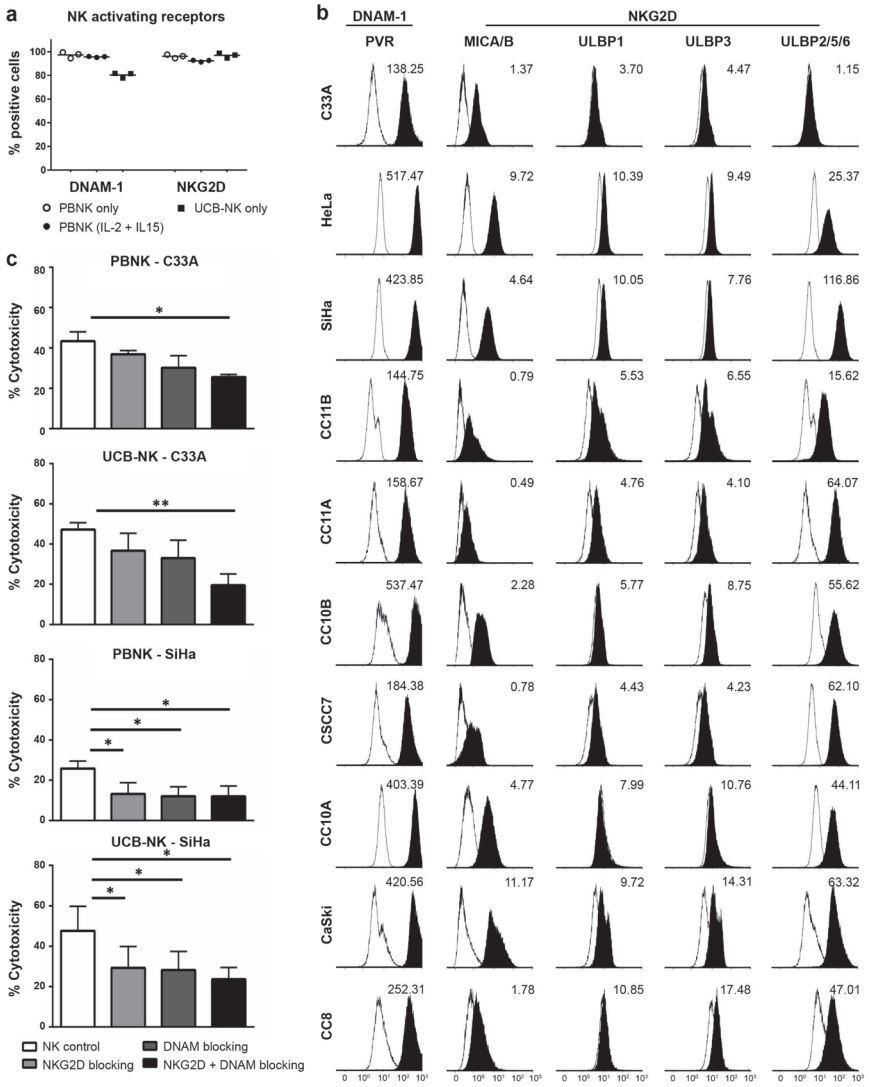

FIGURE 3: NK-activating receptors in PBNK and UCB-NK and their ligand expression in cervical cancer cell lines influencing NK cytotoxicity

(a) *Percentage of positive cells within the NK cell population for NK activating receptors DNAM-1 and NKG2D for PBNK only, PBNK stimulated with cytokines (IL-2+IL-15) and UCB-NK only were determined by flow cytometry. The data presented is from three representative donors for PBNK and UCB-NK. PBNK only are denoted by open circles, PBNK (IL-2+IL-15) are denoted by closed circles and UCB-NK only by closed squares.* **(b)** *Representative example of histograms showing geometric mean fluorescence intensity (MFI) for NK activating ligands PVR (ligand of DNAM-1 receptor), MICA/B, and ULBP1, -3 and -2/5/6 (ligands of NKG2D receptor).* **(c)** *PBNK and UCB-NK were coated with NKG2D and/or DNAM-1 blocking antibodies and incubated with C33A and SiHa cells. Cytotoxicity levels (Δ7AAD) were measured from 7AAD⁺ C33A and SiHa cells at the end of a 4h assay. Data presented are means of triplicate values from three different PBNK and three different UCB-NK donors; Bars represent mean ± SEM. * P <0.05 and ** P < 0.01 calculated with paired, two-way ANOVA multiple comparisons of column means.*

in combination with CET upon exposure to cervical cancer cell lines expressing low levels of EGFR (C33a, HeLa and SiHa: denoted in Figure 2c by triangles). In contrast, degranulation levels in UCB-NK were generally high. PBNK, PBNK + CET and UCB-NK cytotoxicity levels per histological subtype and HPV type of cervical cancer cell lines are shown in Supplementary Figure 3. It shows that irrespective of HPV or histological tumor type, highest cytotoxicity was consistently achieved by UCB-NK.

Expression of NK activating receptors and their ligands and their contribution to mediating cytotoxicity

To investigate the involvement of activating receptors in mediating the cytotoxic activity of PBNK and UCB-NK, the expression of the two major NK activating receptors DNAM-1 and NKG2D on the NK cells described to be involved in the recognition of cervical cancer cells, and their respective ligands, i.e. PVR and MICA/B, ULBPs, on the tested cervical cancer cell lines, were assessed. Similarly, high levels of DNAM-1 and NKG2D were observed on both PBNK and UCB-NK (Figure 3a). The cell lines showed differential expression of the NK activating ligands, but all were positive for PVR, the DNAM-1 ligand, and at least one of the NKG2D ligands (Figure 3b). From the panel of cell lines, SiHa (with highest expression levels of PVR and ULBP-2/5/6) and C33A (with lowest expression levels of PVR and ULBP-2/5/6) were selected as target cells in functional blocking studies. The relatively low ligand expression levels on C33A required combined blocking of DNAM-1 and NKG2D to achieve a significant reduction in either PBNK- or UCB-NK-mediated cytotoxicity (Figure 3b). In contrast, blocking either DNAM-1 or NKG2D already led to significant reductions of cytotoxicity in SiHa cells (Figure 3c). These data show dependence (at least in part) of both PBNK and UCB-NK on DNAM-1 and NKG2D for their cytotoxic potency.

Differential expression of NK inhibitory receptors and their ligands in relation to level of cytolysis

To investigate the effect of NK inhibitory receptors on the observed cytotoxic efficacy, the expression levels of KIR2D and NKG2A on the NK cells, and of their respective ligands, i.e. HLA-ABC/-G and HLA-E[39], on the cervical cancer cell lines, were assessed (Figure 4a, b). Irrespective of overnight activation with IL-2/IL-15, PBNK expressed high levels of both KIR2D and NKG2A, whereas UCB-NK only expressed equivalent levels of NKG2A, but no KIR2D. All classical and non-classical HLA molecules were expressed on all ten cervical cancer cell lines, but in widely varying degrees (Figure 4b). Correlation analyses showed a relationship only between HLA-ABC expression levels and levels of cytotoxicity achieved by PBNK, with lower HLA-ABC levels allowing for higher levels of cytotoxicity ($P = 0.036$, Figure 4c). In contrast, PBNK + CET (Figure 4d) and UCB-NK cytotoxicity was totally independent of HLA-ABC expression levels (Figure 4e). No other correlations were found between cytotoxicity levels and HLA-E or HLA-G expression levels on cervical cancer cell lines (data not shown).

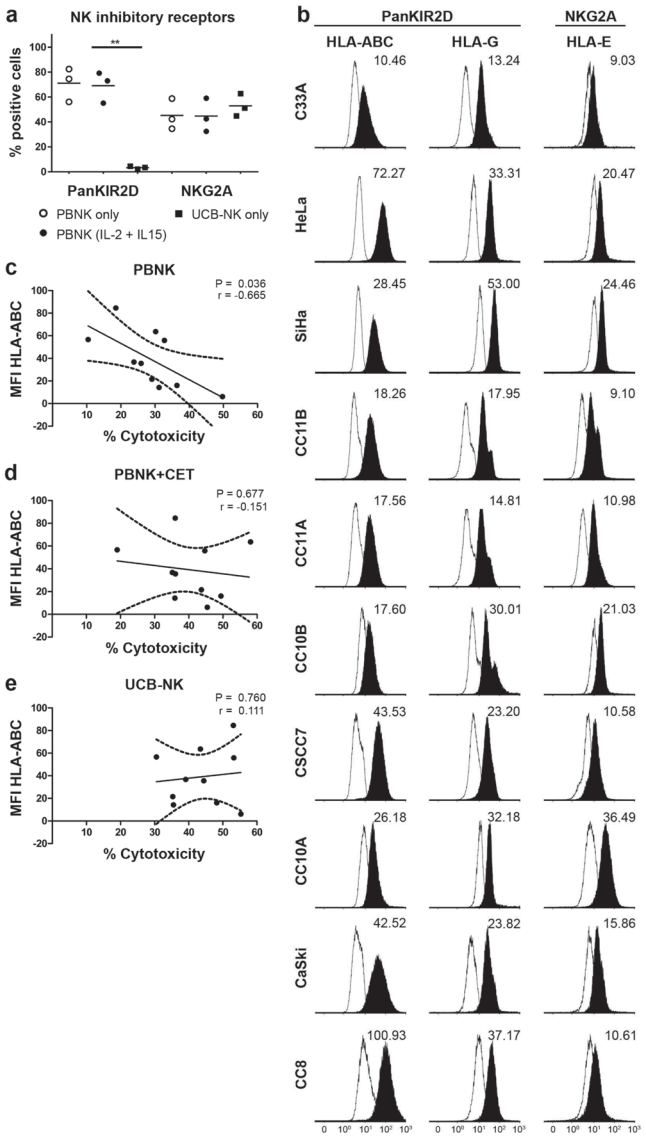

FIGURE 4: Effects of NK inhibitory ligands on NK cytotoxicity against cervical cancer cells
(a) *Percentage of positive cells within the NK cell population for NK inhibitory receptors KIR2D and CD94/NKG2A for PBNK only, PBNK stimulated with cytokines (IL-2+IL-15) and UCB-NK only were determined by flow cytometry. The data presented is from three representative donors for PBNK and UCB-NK. PBNK only are denoted by open circles, PBNK (IL-2+IL-15) are denoted by closed circles and UCB-NK by closed squares.* **(b)** *Representative histogram plots showing geometric mean fluorescence intensity (MFI) of NK inhibitory ligands HLA-ABC, HLA-E and HLA-G on cervical cancer cells; representative plots of 2-3 separate analyses are shown. Correlation analysis of MFI of HLA-ABC with % cytotoxicity (Δ7AAD) by* **(c)** *PBNK,* **(d)** *PBNK + cetuximab (CET), and* **(e)** *UCB-NK. Dotted lines represent 95% confidence interval of the regression line. Four experiments with four different PBNK donors for C33A, HeLa, SiHa, CC11B, CC11A, CC10B, CC10A, CaSki, two experiments with two different PBNK donors for CSCC7 and CC8, and five experiments with five different UCB-NK donors were used for this experiment. P-value was calculated with Pearson analysis.*

DISCUSSION

Cervical cancer is the fourth most common malignancy in women worldwide. Survival is severely reduced in case of lymph node metastasis, with no curative treatment options available. In cervical cancer, ACT involving T cell or NK cell based therapies have not yet been widely explored, but they have been successfully applied in the treatment of various other cancer types[40-42]. In one clinical trial adoptive transfer of tumor infiltrating T-cells in metastatic cervical cancer resulted in tumor responses in 3/9 patients with complete remission in 2/9 patients[43]. These findings suggest that ACT could be a viable treatment option for some patients with cervical cancer. However, most cervical tumors have HLA class I alterations and will therefore not respond completely to T-cell-based therapies[13,14,44]. NK cell-based therapies present a viable alternative in that case, but in advanced cervical cancer these effector cells are often impaired in their functionality[24,25]. In this study, we therefore explored the possible therapeutic efficacy of allogeneic NK cells. Clinically applicable NK cells may be derived from two sources, i.e. NK cells derived from peripheral blood and NK cells cultured and expanded from umbilical cord blood stem cells. We tested their cytotoxic efficacy (with and without CET for PBNK) on ten cervical cancer cell lines representing different histological subtypes, HPV types, and expressing differential levels of NK activating and inhibitory ligands.

Initially, we investigated the effect of PBNK alone and a combination of PBNK with CET on the cervical cancer cell lines. From literature it is known that cervical tumors often present with variable levels of EGFR[6,8]. In colorectal cancers, mutant KRAS is associated with resistance to CET[45]. Although most of the cervical cancer cell lines were EGFR-positive and all were *RAS*^{wt}, their EGFR expression levels were relatively low, and, in keeping with clinical observations for cervical cancer, they did not respond to CET as a single agent[9,10,46]. Our observation of increased PBNK cytotoxicity upon combination with CET is in line with a report by Meira and colleagues who showed that one of the antitumor effector mechanisms upon combined CET and chemoradiation actually was ADCC[47].

Next, we compared the functionality of PBNK with that of *ex vivo* generated UCB-NK derived from cord blood stem cells, and showed that UCB-NK were significantly more cytotoxic than PBNK (Figure 2). NK cytotoxicity and NK degranulation levels were equivalent for UCB-NK and PBNK + CET. Further study of the NK killing mechanism, revealed that the cytotoxic activity of both PBNK and UCB-NK was at least in part dependent on DNAM-1 and NKG2D receptors, as also previously reported for an NK cell line (NKL) and cytotoxicity it induced in the CaSki and SiHa cell lines[19]. This was in keeping with high expression levels of both NKG2D and DNAM-1 observed on both PBNK and UCB-NK. As complete abrogation of tumor cell killing was not achieved by combined blocking of DNAM-1 and NKG2D on activated PBNK and UCB-NK, other NK killing mechanisms such as NKp44/NKp44L, TRAIL

(Tumor necrosis factor-related apoptosis-inducing ligand), and FAS (Fas-Ligand interactions) also might contribute to the observed target cell lysis[48,49]. Indeed, NKp44 has been previously reported as highly expressed on expanded UCB-NK, in sharp contrast to PB-NK cells, which in the steady state don't express NKp44[33]. The known ligands for NKp44 have mostly been associated with microbial responses, whereas the identity of cancer associated ligands until recently has remained mostly obscure. A ligand for NKp44 has now been identified on tumor cells, designated NKp44L, which opens the way for further exploration of the relative importance of this activating receptor axis in NK-mediated tumor cytolysis[50].

Interestingly, in the present study we have shown the predominant effect of HLA class I expression on the functionality of PBNK. In contrast to PBNK, UCB-NK have the ability to overcome resistance to cytolysis due to HLA-ABC expression as demonstrated by the correlative studies with all ten cell lines which revealed efficient UCB-NK-mediated cytolysis of both HLA-ABC high and low expressing cell lines (figure 4c). A lack of expression of inhibitory KIRs on UCB-NK may provide a mechanistic explanation for their ability for HLA class I independent cytotoxicity. Indeed, whereas PBNK and UCB-NK expressed similar levels of NKG2A, inhibitory KIRs, as measured by a panKIR2D antibody, were completely lacking from the UCB-NK cell surface. In keeping with this observation, we previously published the profiling of UCB-NK using an expanded panel of antibodies to inhibitory KIR, which revealed low expression levels of KIR2DL1/DS1, KIR2DL2/DL3/DS2, and KIR3DL1/DS1 as compared to PBNK[33]. Cervical tumors have been shown to also have aberrant non-classical HLA class I expression which might help them to escape from NK cell killing[14]. Remarkably, in our hands NK cytotoxicity was not impaired by higher levels of HLA-E or HLA-G expression. The apparent ability of UCB-NK to overcome the possible resistance related to expression of both inhibitory classical and non-classical HLA molecules may offer an excellent treatment modality for cervical cancer.

NK cells are often dysfunctional and low in number in cervical cancer patients[19,23,24]. In order to achieve a more potent and effective cytotoxic effect of NK cells in patients with cervical cancer it is therefore critical to have adequate numbers of functional effector NK cells. In regard to generating large numbers of NK cells for therapeutic purposes, NK cells expanded from PBMC and other sources have limited expansion capacity as compared to cord blood derived NK cells[51]. Adoptive transfer of large numbers of cytotoxic UCB-NK could be a viable treatment option, because UCB-NK have a highly activated phenotype with more than 75% stable expression rates of NKG2D, DNAM-1, NKp30, NKp44 and NKp46 in all mature UCB-NK and lack inhibitory KIRs resulting in HLA independent cytolytic efficacy; additional advantages of UCB-NK over PBNK are fewer impurities (such as T and B cells) detected upon full NK maturation, thereby reducing chances of GVHD upon adoptive transfer[31,38]. In this study, UCB-NK were not tested in combination with CET due to their low surface expression of CD16a *in vitro*, however, UCB-NK further mature upon adoptive

transfer *in vivo* which is accompanied by an increase in CD16a expression[52], and this feature could be exploited to enhance tumor killing even more via ADCC using CET and other IgG_1 therapeutic antibodies. To facilitate clinical application, a GMP based NK cell expansion and differentiation protocol has already been established, approved by regulatory authorities and applied in a Phase-I clinical trial for elderly Acute Myeloid Leukemia patients and numbers of over $30 \cdot 10^6$/kg body weight cytotoxic UCB-NK (oNKord®) can easily be achieved for therapeutic purposes (CCMO no NL31699 & Dutch trial register no 2818). Therefore, it is now entirely feasible to develop clinical protocols to explore, for the first time, adoptive transfer of UCB-NK in patients with solid tumors like cervical cancer.

In conclusion, our data provide a clear rationale for the use of UCB-NK to treat cervical tumors and also the possibility of using PBNK in combination with CET for EGFR-expressing tumors, with both significantly higher cytotoxicity and degranulation levels than in PBNK only conditions. Notably, treatment with UCB-NK might serve as a generally applicable treatment for cervical cancer enabled by HLA-, histology- and HPV-independent killing mechanisms.

MATERIAL & METHODS

Cell lines

Cervical cancer cell lines CSCC7, CC8, CC10A, CC10B, CC11A, and CC11B were generated in the department of Pathology of Leiden University Medical Center (The Netherlands) from primary tumors as described previously[35]. These patient-derived cell lines as well as commercially obtained cervical cancer-derived cell lines, HeLa, SiHa, CaSki and C33A (American Type Culture Collection) were maintained in DMEM (Lonza) medium containing 4.5 g/L glucose, 10% FCS (Hyclone), 10 µg/mL gentamicin and 0.25 µg/ml amphotericin B (Gibco), 100 Units Penicillin/100 Units Streptomycin/ 0.3 mg/mL Glutamine (Thermo Fisher Scientific). Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. See Table 1 for cell line characteristics.

Phenotyping of cervical cancer cell lines

To phenotype cervical cancer cell lines, cell suspensions in PBS supplemented with 0.1% BSA and 0.02% NaN_3 (FACS buffer) were stained for 30 min at 4°C using antibodies to HLA-ABC (clone w6/32, Immunotools) (labeled with FITC), HLA-E (clone 3D12HLA-E, eBioscience), HLA-G (clone 87G, Biolegend), EGFR (clone EGFR.1, BD Biosciences), PVR (clone SK11.4, Biolegend), MICA/B (clone 6D4, Biolegend), ULBP2/5/6 (clone #165903, R&D systems), ULBP1 (clone #170818, R&D systems) and ULBP3 (clone #166510, R&D systems) (all labeled with PE). IgG_1, IgG_{2a}, and IgG_{2b} isotype antibodies were used as negative controls. After incubation, the cells were washed with FACS buffer and analyzed using a

flow cytometer LSR Fortessa (BD Biosciences). Phenotypic analyses were obtained from at least two independent experiments performed on each cell line. Data were analyzed using Kaluza software (Beckman coulter) and calculated as specific (geometric) mean fluorescence intensity (MFI) (MFI; geometric mean fluorescence of marker - geometric mean fluorescence of isotype).

RAS typing

RAS status was obtained from Rational molecular Assessments and Innovative Drugs selection (RAIDs) project data (http://www.raids-fp7.eu/project-overview.html) and www.lgcstandards-atcc.org for cell lines HeLa, SiHa, CaSki, C33A, CSCC7, CC10A and CC10B. In addition, full *RAS* typing (i.e., *BRAF* exon 15, *KRAS* exon 2-3-4 and *NRAS* exon 2-3-4) was performed for cell lines CC8, CC11A and CC11B at the molecular pathology lab of the Department of Pathology of the VU University medical center (Amsterdam, The Netherlands) using high resolution melting assay followed by Sanger sequencing of using high resolution melting-PCR products with an aberrant melt curve, essentially as described previously[36,37].

PBMC isolation & NK cell isolation

Whole blood samples from four healthy volunteers were collected. PBMC were isolated using Lymphoprep™ (STEMCELL Technologies, The Netherlands) density gradient centrifugation. $CD56^+$ NK cells were isolated from PBMC using a MACS® Human NK cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The cell number and purity of the isolated PBNK was analyzed by flow cytometry. Isolated NK cells were activated overnight with 1000 U/ml IL-2 (Proleukin®; Chiron, München, Germany) and 10 ng/ml IL-15 (CellGenix) before use in cytotoxicity assays. NK cell purity and viability were checked by flow cytometry using the following antibodies: 7-Aminoactinomycin D (7AAD; Sigma Aldrich), CD3 (labeled with VioBlue), CD56 (labeled with APC-Vio770), and CD16 (labeled with APC) (all from Miltenyi Biotech). Purity of NK cells obtained from NK donors was 87± 6%. For cytotoxicity assays, only PBNK with CD16 expression rates exceeding 80% were used.

UCB-NK isolation and cultures

Allogeneic NK cells were generated from cryopreserved umbilical cord blood hematopoietic stem cells as previously described[38]. $CD34^+$ UCB cells (3×10^5 per ml) were plated into 12-well tissue culture plates (Corning Incorporated, Corning, NY) in Glycostem Basal Growth Medium (GBGM®) (Clear Cell Technologies, Beernem, Belgium) supplemented with 10% human serum (Sanquin Bloodbank, The Netherlands), 25 ng/mL of SCF, Flt-3L, TPO, and IL-7 (CellGenix, Germany). In the expansion phase II, from day 9 to 14, TPO was replaced

with 20 ng/mL IL-15 (CellGenix). During the first 14 days of culture, low molecular weight heparin (LMWH) (Clivarin®; Abbott, Wiesbaden, Germany) in a final concentration of 20 µg/ml and a low-dose cytokine cocktail consisting of 10 pg/ml GM-CSF (Neupogen), 250 pg/ml G-CSF and 50 pg/ml IL-6 (CellGenix) were added to the expansion cultures. Cells were refreshed with new medium twice a week and maintained at 37°C, 5% CO_2. On day 14, the NK cell differentiation process was initiated by addition of NK cell differentiation medium consisting of the same basal medium with 2% human serum but with high-dose cytokine cocktail consisting of 20 ng/ml of IL-7, SCF, IL-15 (CellGenix) and 1000 U/ml IL-2 (Proleukin®; Chiron, München, Germany). Cultures were refreshed every 2-3 days and maintained till day 35. For cytotoxicity assays, UCB-NK was used with $CD56^+$ cells >85% purity.

In vitro NK cytotoxicity assays

Cervical cancer cell lines (target cells) were labelled with 5 µM pacific blue succimidyl ester (PBSE; Molecular Probes Europe, Leiden, The Netherlands) in a concentration of $1x10^7$ cells/mL for 15 min at 37°C. After incubation, cells were washed and resuspended in DMEM culture medium to a final concentration of $1x10^6$/mL. PBNK and UCB-NK were washed with PBS and also resuspended in GBGM medium to a final concentration of $1x10^6$/mL. Target cells were co-cultured in triplicate with effector cells (PBNK or UCB-NK), with or without 5 µg/ml CET at an E:T ratio of 1:1 in a total volume of 100 µl in FACs tubes (5×10^4 targets in 50 µl of DMEM culture medium incubated with 5×10^4 effectors in 50 µl of GBGM medium). PBNK, UCB-NK and target cells alone were cultured in triplicate as controls. To measure degranulation by PBNK and UCB-NK, anti-CD107a PE (Miltenyi Biotech, Germany) was added at the beginning of the assay. After incubation for 4h at 37°C, cells were harvested and stained with 7AAD, CD56 (labeled with APC-Vio770) and CD16 (labeled with APC) (all from Miltenyi Biotech, Germany). For NK flow cytometry and blocking experiments NKG2D PE (clone ON72, Beckman Coulter) and DNAM-1 FITC (clone DX11, BD Pharmingen™) were used at 10 µg/ml. Further, Killer-cell immunoglobulin-like receptor 2D (PanKIR2D) FITC (clone NKVFS1) and CD94/NKG2A PE-Vio770 (Clone REA110) (both from Miltenyi Biotec) were used to screen inhibitory receptor expression on PBNK and UCB-NK. BD LSR Fortessa™ was used for read-out of the cytotoxicity assays. Data were analyzed using Kaluza software (Beckman coulter). Percentages of specific NK degranulation were calculated as $\Delta CD107a^+$ NK cells (i.e. [target cells + NK cells] minus [NK cells only]) and percentages of cytotoxicity as $\Delta 7AAD^+$ target cells (i.e. [target cells + NK cells] minus [target cells only]). See Supplementary Figure 1 for a representative gating example.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism software. Statistical significance of differences between conditions were determined using a parametric paired t test, unpaired

t test or a one-way ANOVA with Bonferroni's multiple comparison test, and a two-way ANOVA with multiple comparisons between column means. Correlation analyses between percentages NK degranulation, cytotoxicity and MFI were performed using Pearson analysis. A P value of < 0.05 was considered statistically significant.

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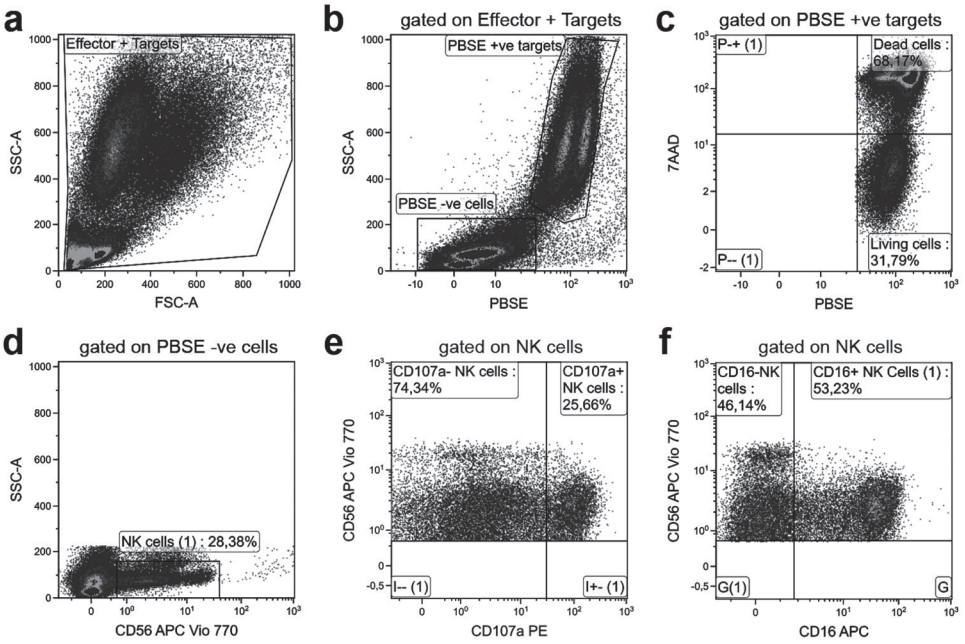
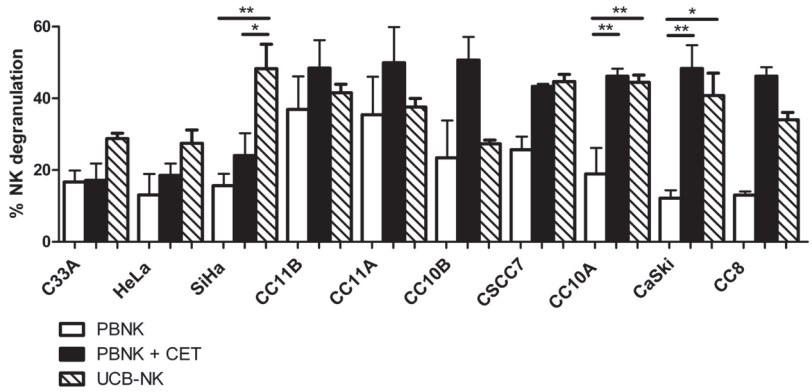


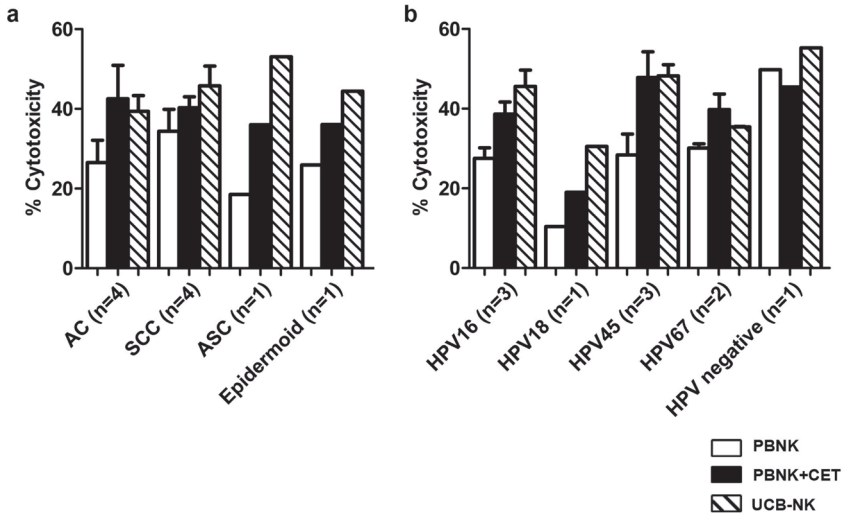
SUPPLEMENTARY FIGURE 1: Representative example of flowcytometric analysis

A representative example of flowcytometric analysis of Natural Killer (NK) cytotoxicity assay with cell line CC10A. (a) Identification of effectors and targets using SSC and FSC gating. (b) Target cells stained with PBSE were gated out from effector cells and cell debris. (c) PBSE-positive target cells were then gated against 7AAD to determine the percentage of cell death induced by NK cells. (d) Cells staining negative for PBSE and low on SSC were gated against CD56 marker to identify NK cells, further on NK cells, the levels of (e) NK degranulation (CD107a) and (f) NK CD16^{+} cells percentages were calculated and compared with respective target only controls.



SUPPLEMENTARY FIGURE 2: NK degranulation levels per cell line for PBNK, PBNK + CET, and UCB-NK

*Significantly higher levels of NK degranulation (ΔCD107a) in PBNK + cetuximab (CET) and UCB-NK conditions compared to PBNK only condition for SiHa, CC10A and CaSki. Bars are means of triplicate values from four experiments with four different donors for C33A, HeLa, SiHa, CC11 B, CC11A CC10B, CC10A, CaSki and two experiments with two different donors for CSCC7 and CC8 using PBNK and five experiments for UCB-NK using five different donors for all cell lines. *P <0.05 and **P 0.01 calculated with one-way ANOVA, Bonferroni's multiple comparison test.*



SUPPLEMENTARY FIGURE 3: Cytotoxicity levels per histological subtype and HPV type

PBNK (open bars), PBNK + cetuximab (CET) (closed bars), and UCB-NK (hatched bars) cytotoxicity levels per (a) histological subtype and (b) HPV type of cervical cancer cell lines. Bars represent mean ± SEM. Four experiments with four different PBNK donors for C33A, HeLa, SiHa, CC11B, CC11A, CC10B, CC10A, CaSki, two experiments with two different PBNK donors for CSCC7 and CC8, and five experiments with five different UCB-NK donors were used for this experiment. AC: adenocarcinoma; SCC: squamous cell carcinoma; ASC: adenosquamous cell carcinoma.

CHAPTER 4

NK cell ADCC enhances treatment efficacy in colorectal cancer

Published as
"Combination of NK cells and cetuximab to enhance anti-tumor responses in RAS mutant metastatic colorectal cancer"

Veluchamy JP, Spanholtz J, Tordoir M, Thijssen VL, Heideman DA, Verheul HM, de Gruijl TD, van der Vliet HJ.

PLOS ONE, 2016

ABSTRACT

The ability of Natural Killer (NK) cells to kill tumor targets has been extensively studied in various hematological malignancies. However, NK cell therapy directed against solid tumors is still in early development. Epidermal Growth Factor Receptor (EGFR) targeted therapies using monoclonal antibodies (mAbs) such as cetuximab and panitumumab are widely used for the treatment of metastatic colorectal cancer (mCRC). Still, the clinical efficacy of this treatment is hampered by mutations in RAS gene, allowing tumors to escape from anti-EGFR mAb therapy. It is well established that NK cells kill tumor cells by natural cytotoxicity and can in addition be activated upon binding of IgG_1 mAbs through Fc receptors (CD16/FcγRIIIa) on their surface, thereby mediating antibody dependent cellular cytotoxicity (ADCC). In the current study, activated Peripheral Blood NK cells (PBNK) were combined with anti-EGFR mAbs to study their effect on the killing of $EGFR^{+/-}$ cancer cell lines, including those with RAS mutations. *In vitro* cytotoxicity experiments using colon cancer primary tumors and cell lines COLO320, Caco-2, SW620, SW480 and HT-29, demonstrated that PBNK cells are cytotoxic for a range of tumor cells, regardless of EGFR, RAS or BRAF status and at low E:T ratios. Cetuximab enhanced the cytotoxic activity of NK cells on $EGFR^{+}$ tumor cells (either RAS^{wt}, RAS^{mut} or $BRAF^{mut}$) in a CD16 dependent manner, whereas it could not increase the killing of $EGFR^{-}$ COLO320. Our study provides a rationale to strengthen NK cell immunotherapy through a combination with cetuximab for RAS and BRAF mutant mCRC patients.

INTRODUCTION

Epidermal Growth Factor Receptor (EGFR) is expressed on cell surfaces in normal tissues and binding to its ligands activates two important pathways, the RAS-RAF-MAPK and PI3K-PTEN-AKT pathway, which both control cell proliferation, survival and motility[1]. Dysregulation of the EGFR signaling cascade can result in rapid cell division ultimately supporting tumor growth. Several solid tumors show elevated EGFR expression levels, which were shown to be related to poor prognosis[2]. Cetuximab (IgG_1 chimeric) and panitumumab (IgG_2 fully humanized) are clinically approved anti-EGFR mAbs that bind to the extracellular domain of EGFR thereby blocking EGFR dimerization, resulting in apoptosis and preventing tumor growth[3].

Regrettably, mutations in the EGFR downstream signaling pathway (e.g. RAS mutations), can lead to constitutive RAS signaling, resulting in unresponsiveness to anti-EGFR therapy[4-6]. The fact that in about 40% of patients with metastatic colorectal cancer (mCRC) mutations in the RAS gene can be observed, means that anti-EGFR therapy is applicable in only half of the mCRC patients[7]. Therefore, several approaches have been proposed and are currently tested to increase the efficacy of anti-EGFR mAb therapy by overcoming the inhibitory effect of RAS mutation, e.g. by immune effector cell-mediated antibody dependent cell-mediated cytotoxicity (ADCC)[8,9].

Several immune effector cells in the body have the ability to recognize target molecules on the tumor cell surface, like EGFR on CRC cells, through their FcR-mediated binding of antibodies directed against these targets, leading to potent antitumor immunity. However, due to cytotoxic treatment regimens in solid tumor patients, the immune system can be temporarily dysfunctional, signified by a decrease in immune effector cell subsets[10,11]. This limitation may be overcome by cellular immunotherapy, such as the adoptive transfer of activated cytolytic Natural Killer (NK) cells. NK cells are part of the innate immune defense, with the ability to kill tumor cells. NK cells comprise of two subsets, from which the majority (about 90%) are phenotypically $CD56^{dim}$ $CD16^{bright}$ and exert mainly cytolytic functions, whereas the other subset of $CD56^{bright}$ $CD16^{dim}$ NK cells primarily exert immune regulatory functions[12]. CD16a (FcγRIIIa), a low affinity Fc receptor, preferably binds to IgG_1 antibodies and can actively mediate ADCC[13,14].

This study aims to prove that NK cells are able to induce strong ADCC responses in combination with therapeutic EGFR-targeting mAbs and can thereby overcome the potential limitations of stand-alone anti-EGFR therapy. Therefore, activated PBNK cells were combined with cetuximab or panitumumab to test their ADCC efficacy on $EGFR^{+/-}$, $RAS^{wt/mut}$, $BRAF^{mut}$ cell lines and primary tumor cells from patients with CRC.

RESULTS

More potent NK effector cell activation and ADCC effected by cetuximab than by panitumumab

To establish which of the anti-EGFR mAbs, cetuximab or panitumumab, exerted higher functionality with respect to EGFR recognition and cytotoxicity, both were tested on strongly EGFR positive ($EGFR^{+++}$) A431 cells. Flowcytometric detection of EGFR using biotinylated cetuximab (**Δ**MFI=217) was nearly two-fold intense than observed with biotinylated panitumumab (**Δ**MFI=123), as shown in Figure1A and B. Next, A431 cells were treated with cetuximab or panitumumab to calculate the concentration required to induce 50% of maximal cytotoxicity. At higher concentrations than 1000µg/ml both mAbs were equally cytotoxic (61 ± 2%). Titrating down, the concentrations required to induce 50% of maximal cytotoxicity (EC_{50} value) of $EGFR^{+++}$targets were found to be 5µg/ml for cetuximab (31 ± 2%) and 100µg/ml for panitumumab (36 ± 1%) respectively (Figure 1C). Based on these findings concentrations of 5µg/ml of cetuximab and 100µg/ml of panitumumab were used in all subsequent experiments to assess their ADCC efficacy when combined with NK cells. To this end A431 cells were coated with either cetuximab or panitumumab and co-incubated with activated NK cells. A significant increase in A431 cell death was observed over NK cells only, when NK cells were combined with cetuximab, but not with panitumumab coated targets (Figure 2A). This is consistent with the IgG_2 isotype of panitumumab, which precludes high-affinity binding to NK CD16a. In line with these ADCC data, degranulation of NK cells, as assessed by CD107a expression, was significantly increased when tumor target cells were coated with cetuximab (Figure 2B and supplementary figure 2B). Similarly, to the observed ADCC and CD107a levels, IFNγ production was increased in NK cell and cetuximab co-cultures (Figure 2C). To formally demonstrate that the cetuximab-related increase in target cell killing was due to ADCC, NK-FcR receptors were blocked using a FcR blocking reagent (Miltenyi Biotec) and then incubated with cetuximab coated target cells. As shown in Figure 2D and supplementary figure 2C, FcR blocking resulted in a considerable and statistically significant reduction in degranulation of the $CD16a^{+}$ NK cell compartment. Together, these data provide a clear rationale to combine NK cells and cetuximab to increase the killing of $EGFR^{+++}$ targets by ADCC.

NK CD16a (FcγRIIIa) polymorphism does not significantly influence cetuximab induced ADCC *ex vivo*

Several clinical studies reported that NK FcγRIIIa receptor polymorphisms affected the clinical efficacy of cetuximab, due to a variable binding affinity to NK cells, thereby directly affecting the potency of ADCC[15,16]. In order to test if V158V (V/V) and V158F (V/F) polymorphic versions of CD16 translated to differences in ADCC upon engagement with

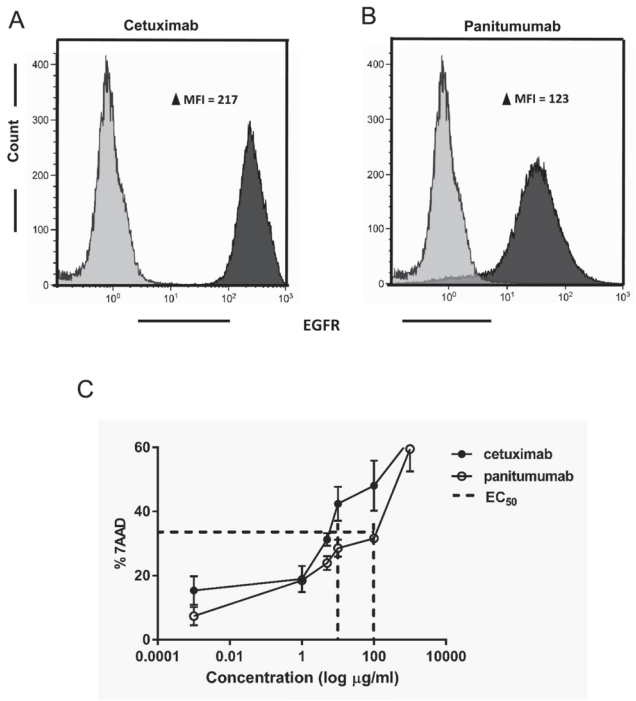


FIGURE 1: Cetuximab and panitumumab binding affinities and their cytotoxicity towards EGFR^{+++} tumor targets

EGFR overexpressing A431 cells were used to test anti-EGFR mAbs. A and B, Histograms showing binding of 100μg/ml of biotinylated cetuximab (A) and panitumumab (B) to A431 cells. Grey shades represents the streptavidin APC control, black shades represent binding of the biotinylated mAbs. (C) Dose response curve to measure EC_{50} concentration for cetuximab and panitumumab. A431 cells treated with cetuximab and panitumumab at concentrations of 1ng, 1μg, 5μg, 10μg, 100μg and 1000μg per ml for 4 hrs were analyzed for target cell death.

cetuximab, cytotoxicity assays were performed with NK cells from 6 donors, 3 with the V/V polymorphism and 3 with the V/F polymorphism. Cytotoxicity against A431 and degranulation of the CD16^{+} NK cell fraction was assessed after a 4hr co-culture of cetuximab coated A431 cells and NK cells. No significant differences in target cell death (Figure 3A) or degranulation (Figure 3B) were observed between NK cell donors with V/V and V/F CD16/FcγRIIIa polymorphism.

NK cells efficiently lyse EGFR$^{+/-}$ and RAS$^{wt/mut}$ colon cancer cell lines

Having established the anti-tumor efficacy of the cetuximab/NK cell combination with the EGFR^{+++} cell line A431, our next aim was to extend these findings to colon cancer cell lines COLO320 (EGFR^{-}, RAS^{wt}), SW480 (EGFR^{+} RAS^{mut}; KRAS exon 2 c.35>T; p.G12V), SW620 (EGFR^{+}

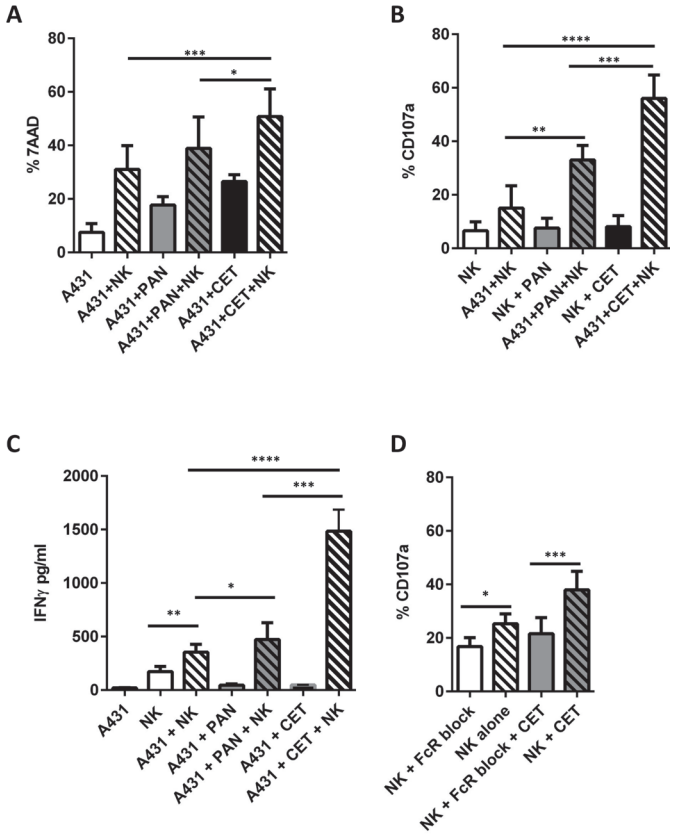


FIGURE 2: Anti-EGFR mAb cytotoxicity in combination with NK cells
*The effect of IL-2 and IL-15 activated PBNK cells, cetuximab and panitumumab on lysis of A431 tumor cells was assessed. The percentage of ADCC was calculated based on percentage of PBSE labelled A431 cells staining positive for 7AAD in different co-culture conditions (A). Effector cells from the assay were stained with CD107a to measure NK cell degranulation by flow cytometry (B). Cell free culture supernatants were collected at the end of a 4hr co-culture and analyzed for IFNγ release by ELISA (C). The contribution of FcR mediated effector functions of NK cells (i.e. ADCC) on NK cell mediated A431 tumor cell lysis was tested by blocking the FcR receptor on NK cells (D). Data presented is from six individual PBNK donors. Columns are mean of triplicate values; with bars showing SD. Mean ± SD for each significant condition are represented as p = <0.05 *, <0.01 **, <0.005 ***, <0.001 ****.*

RAS^{mut}; KRAS exon 2 c.35>T; p.G12V) and HT-29 (EGFR^{+}, RAS^{wt} , BRAF^{mut}). Binding ability towards biotinylated cetuximab was negative for COLO320 EGFR (ΔMFI=1), with a relatively low EGFR expression detected on SW480 cells (ΔMFI=17) as shown in supplementary figure 1A and B. The EGFR expression level (ΔMFI) on the other colon cancer cell lines was 23 for CaCo-2, 7 for HT-29, and 3 for SW620 (data not shown). In addition, cetuximab as a single agent could not induce cytotoxicity, even at increasing concentrations of up to 1000µg/ml (Supplementary figure 1C and D). In the next step, to test NK killing effects alone and in combination with cetuximab, all five colon cancer cell lines were coated with 5µg/ml

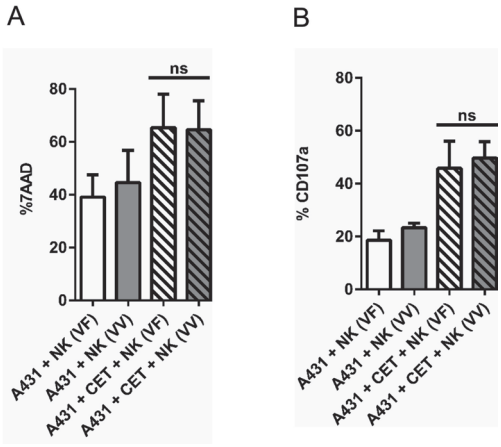


FIGURE 3: Evaluation of NK cell CD16a polymorphism on cetuximab induced ADCC
*Differences in cetuximab induced ADCC between NK cells expressing V/V and V/F polymorphic versions of CD16 were analyzed. Natural cytotoxicity and NK cell CD16a mediated degranulation in the absence and presence of cetuximab coated A431 cells are shown in figure A and B. Columns are mean of triplicate values; with bars showing SD. Data are from independent experiments performed in triplicates from 3 V/V and 3 V/F donors. Mean ± SD for each significant condition are represented as p = <0.05 *, <0.01 **, <0.005 ***, <0.001 ****.*

cetuximab to see if sensitizing target cells with cetuximab could contribute to improved NK cell killing through ADCC. The results showed that COLO320, Caco-2 and SW620 cells are more sensitive for NK killing compared to SW480 and HT-29 cells (Figure 4A). However, while co-incubation with cetuximab did not result in a significant increase in COLO320 tumor cell death, a significant increase in killing was seen in four $EGFR^{+}$ cell lines independent of RAS and BRAF status. With respect to Caco-2, SW480, SW620 and HT-29, the cytotoxic effect could be increased by cetuximab (Figure 4A), this enhancement of the cytolytic NK cell response was also evident from an increased rate of degranulation in the condition where NK cells and cetuximab were added in combination to $EGFR^{+}$ colon cancer cell lines (Figure 4B). Interestingly, we also assessed tumor cell expression of HLA-E and NK cell expression of NKG2A as HLA-E is a known ligand for the NK cell inhibitory receptor NKG2A. As can be seen in the supplementary figure 3A and B, while NKG2A levels were comparable on NK cells in all donors that were tested, HLA-E expression levels were strikingly different and correlated with the overall susceptibility of tumor cells to NK cell lysis as shown in figure 4, where cell lines with low HLA-E levels (COLO320, Caco-2, SW620) were more sensitive to NK cell killing compared to higher HLA-E expressing cell lines (SW480 and HT-29).These data suggest that NK cells have the potential to improve anti-EGFR mAb therapy efficacy even in situations where tumors carry RAS^{mut}, $BRAF^{mut}$ or are $EGFR^{-}$.

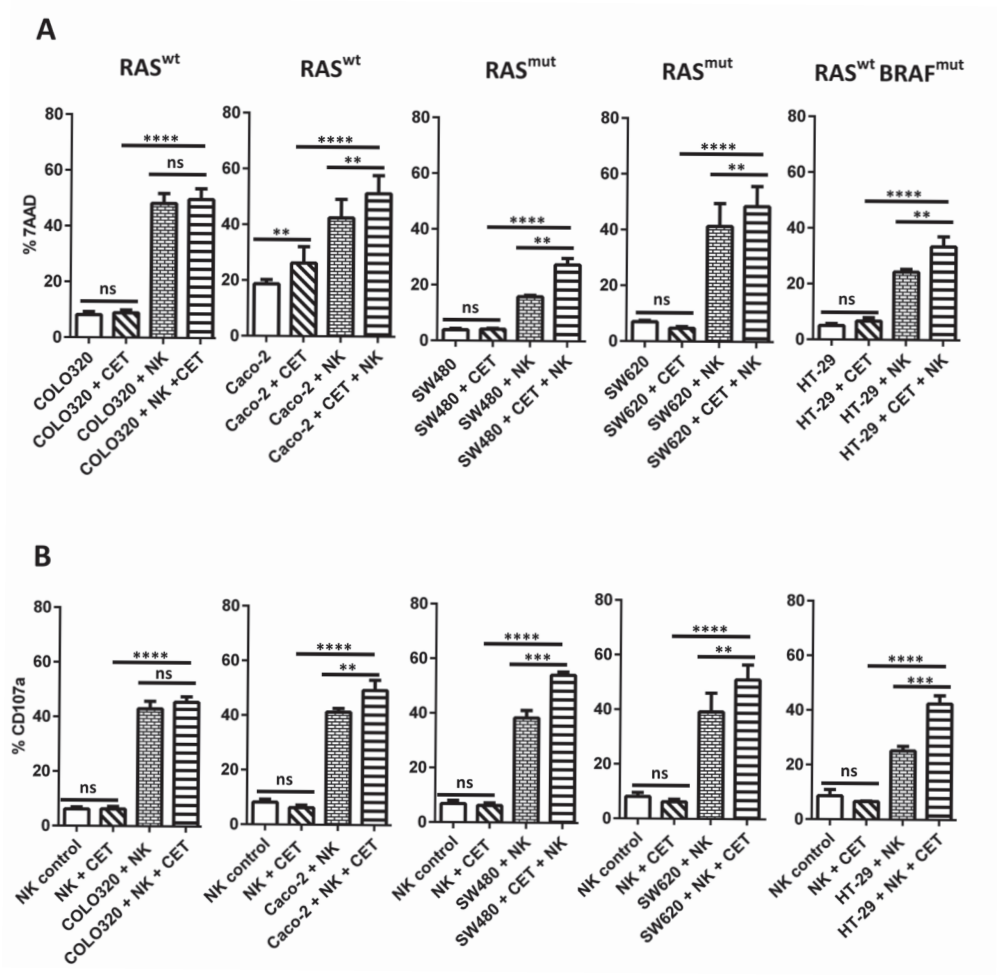


FIGURE 4: Combinatorial effect of NK cells and cetuximab on EGFR$^{+/-}$, RAS$^{wt\,/\,mut}$ and BRAF^{mut} cells

*Cytotoxicity effected by cetuximab and NK cells either alone or in combination against COLO320 (EGFR^{-}, RAS^{wt}), Caco-2 (EGFR^{+}, RAS^{wt}), SW480 (EGFR^{+}, RAS^{mut}), SW620 (EGFR^{+}, RAS^{mut}) and HT-29 (EGFR^{+}, RAS^{wt,} BRAF^{mut}), was compared between these cell lines measuring target cell death (A). Functional differences between NK cells that were co-cultured with tumor cell lines in the presence or absence of cetuximab were evaluated by studying NK cell degranulation (B). Data presented is from five individual PBNK donors. Columns are mean of triplicate values from five experiments; bars represent SD. Mean ± SD for each significant condition are represented as p = <0.05 *, <0.01 **, <0.005 ***, <0.001 ****.*

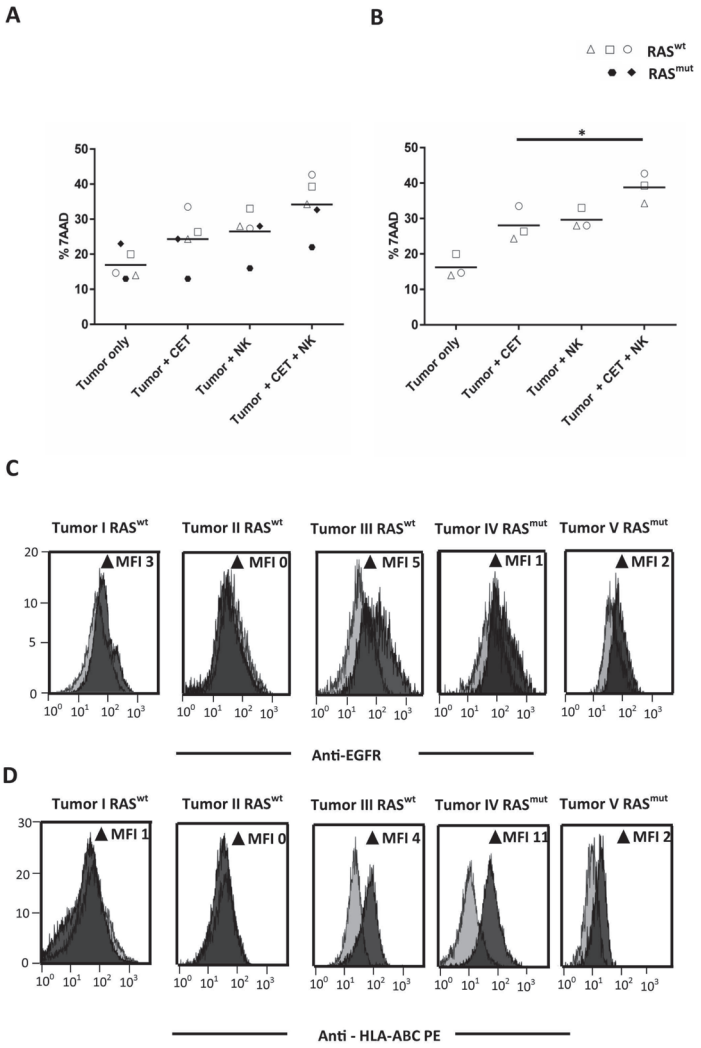


FIGURE 5: Cetuximab enhances NK cell killing of primary colon tumors through ADCC

*Primary colon tumor cells were pretreated with cetuximab and incubated with activated NK cells to monitor ADCC effects. NK cells and cetuximab alone controls were included to document the effects of monotherapy. Percentages of tumor cell death from five CRC patients are shown in figure A and each symbol represents one specific patient. RAS^{wt} tumors I, II and III are indicated by open squares, triangle and circles, and RAS^{mut} tumors IV and V are shown as closed rhombus and hexagon. Figure B shows only the cytotoxicity data of RAS^{wt} tumors. All five primary tumors used in the cytotoxicity assay were tested for EGFR and HLA-ABC expression as shown in figures C and D. Grey histograms represent the isotype controls, black shades represent EGFR and HLA-ABC expression for tumor samples I-V. Data points from figures A and B are mean of triplicate values from five patients; each significant condition are represented as p = <0.05 *, <0.01 **, <0.005 ***, <0.001 ****.*

NK cells efficiently target and kill primary colon tumor cells

As an indication that our observations regarding combined cetuximab/NK cell antitumor efficacy could be extrapolated to the clinical situation, primary tumor material from five patients with CRC was subjected to NK cell killing in the presence or absence of cetuximab in an experimental set-up that was essentially the same as used for the cytotoxicity experiments with colon cancer cell lines described above. In all 5 patients, tumor cells were effectively lysed by NK cells, but only 3 out of 5 tumors responded to cetuximab monotherapy. Importantly, and as shown in Figure 5A and B, cetuximab increased NK cell tumor cell lysis regardless of whether tumors were susceptible to cetuximab monotherapy. The differences in response to cetuximab monotherapy were related to the RAS mutation status of the tumors as the 2 tumors not responding to cetuximab monotherapy had a mutation (KRAS exon 2, c.35>T; p.G12V) in the RAS gene. Figure 5C and D show tumor cell EGFR and HLA-ABC expression levels respectively; all tumors tested had low levels of EGFR and HLA-ABC. Together, these experiments have demonstrated that NK cells have the ability to kill primary tumor cells, and that this can be further increased via cetuximab-mediated ADCC in both RAS^{wt} and RAS^{mut} tumors.

DISCUSSION

Anti-EGFR therapies currently in practice are not adequate to prevent mCRC. The main aim of this study was to determine whether NK cells and cetuximab could be combined to improve their anti-tumor efficacy and widen their applicability in mCRC independent of EGFR and RAS status. NK cells are the immune effectors of choice for ADCC induced by cetuximab[17,18]. Higher frequencies of NK cells in the peripheral blood and increased NK tumor infiltration have both been associated with a favorable prognosis in CRC[19]. The ability of NK cells to target cancer stem cells has also been reported[20]. Thus, the presence of an adequate number of cytolytic NK cells has the potential to drive potent ADCC in combination with cetuximab.

EGFR is overexpressed in various solid tumors, making it an attractive biomarker for anti-EGFR therapy[21]. Cetuximab, when administered following chemotherapy or in chemo-refractory RAS^{wt} mCRC patients improves their progression-free survival[22]. However, although the ensuing anti-tumor response is reliant on the presence of a non-mutated RAS gene, the overall response rate of cetuximab is still only around 23%, even among patients with RAS^{wt} tumors [23-25]. We hypothesized that NK cells in combination with cetuximab could enhance the cytotoxic effects of cetuximab in $EGFR^{+}$ $RAS^{wt/mut}$ $BRAF^{mut}$ CRCs, besides NK cell natural cytotoxicity can mediate anti-tumor activity on $EGFR^{-}$ CRCs that do not respond to anti-EGFR therapy.

In our experiments, a group of solid tumor cell lines was selected based on EGFR expression, RAS and BRAF status. Titrating cetuximab in cultures of A431 cells ($EGFR^{+++}$,

RAS^{wt}), SW480 cells (EGFR^{+}, RAS^{mut}) and COLO320 cells (EGFR^{-}, RAS^{wt}) demonstrated that cetuximab alone induced cytotoxicity only in EGFR^{+} RAS^{wt} tumor cells (Figure 2A and supplementary figure 1C and D). In a next set of experiments, NK cells were combined with cetuximab and this was shown to enhance the lysis of EGFR^{+++} RAS^{wt} tumor cells. Using NK-FcR blocking assays this enhancement was shown to be mediated through ADCC (Figure 2D and supplementary figure 2A-C). Investigating the role of NK cell CD16a (FcγRIIIa) polymorphisms in relation to cetuximab efficacy, we did not see a statistically significant difference between V/F and V/V donors with respect to the induction of NK cell degranulation or tumor target cell lysis. These data are in line with the recent clinical study observations published by Mellor et al, concluding that FcγRIIIa genotype differences were not predicting significant therapeutic benefits for cetuximab[26].

Of interest, our data demonstrated that NK cells were capable of also killing EGFR^{-} RAS^{wt} cells, EGFR^{+} RAS^{wt}, EGFR^{+} RAS^{mut} and EGFR^{+} BRAF^{mut} cells. Furthermore, though cetuximab was completely ineffective as monotherapy against SW480 (EGFR^{+} RAS^{mut}), SW620 (EGFR^{+} RAS^{mut}) and HT-29 (EGFR^{+} RAS^{wt} BRAF^{mut}) cells, cetuximab could increase the target cell killing of NK cells in this setting. The ability of NK cells to lyse tumor targets independent of EGFR, RAS and BRAF status combined with the observation that cetuximab can enhance this cytotoxic effect in EGFR expressing tumors regardless of RAS and BRAF mutational status is an added advantage as it can result in effective target cell lysis of tumors responsive or non-responsive to cetuximab monotherapy. In the clinics, the eligibility criteria for anti-EGFR therapy is based on RAS^{wt} status, whereas the CRC patients are not evaluated for EGFR expression levels; the fact that NK cells can effectively target EGFR^{-} tumor cells could provide an ideal platform to treat metastatic CRC patients having variable levels of EGFR expression.

We observed that although NK cells can efficiently kill EGFR$^{+/-}$ RAS$^{wt\ /\ mut}$ cells (A431, COLO320, Caco-2 and SW620), its efficacy was relatively low on EGFR^{+} RAS^{mut} SW480 and EGFR^{+} RAS^{wt} BRAF^{mut} HT-29 cells. This difference could be related to the differential expression of the inhibitory non-classical HLA-E; i.e. RAS^{mut} SW480 and BRAF^{mut} HT-29 cells were found to have high expression of HLA-E, an inhibitory ligand for the NK cell inhibitory receptor NKG2A (supplementary figure 3), making them less susceptible to NK cell killing[27-29]. Of interest, though NK cell induced tumor cell lysis was still lowest in SW480 and HT-29, cetuximab appeared to at least in part bypass the inhibitory effect mediated through NKG2A/HLA-E interactions, probably by tipping the balance of activating and inhibitory signals more towards NK cell activation through binding of cetuximab to the activating FcγRIIIa. Furthermore, blocking HLA-E on SW480 and HT-29 cells could pave the way for more effective NK cell killing and could hence translate into superior cell death when combined with cetuximab in this setting[30,31].

From the data obtained using primary colon cancer cells as target cells, it was evident that NK cells could kill primary tumor cells and that while cetuximab monotherapy was effective only in RAS^{wt} tumor samples, NK cell cytotoxicity could be increased by cetuximab irrespective of RAS mutational status, making these primary tumor cells ideal targets for observing the potentiating effect of cetuximab induced ADCC on NK cell natural cytotoxicity against $EGFR^{low}$ primary tumors (Figure 5A and B)[32]. Menon et al, showed that HLA Class-I was down-regulated in 72% of patients with CRC[33], thereby making the majority of CRC cells highly susceptible to NK cell mediated killing. The clinical relevance of FcR polymorphisms and NK ADCC influencing the treatment of RAS mutated mCRC patients have been studied by Bibeau and his team reporting disease stabilization in 10 out of 27 patients with RAS mutant tumors following cetuximab monotherapy[34]. In accordance with their findings, our in vitro data support the notion that NK cell ADCC could exert relevant cytotoxicity in this setting. Of note, clinical studies point out that most of the cancer patients present with an imbalance in their immune subsets, which tilts the balance towards tumor progression. Additionally, adoptive transfer of ex vivo activated autologous NK cells has failed to demonstrate clinical responses in mCRC patients[35-37]. Combining all these factors, allogeneic NK cell transplantation might be the preferred choice to treat solid tumors and hence support the immune system of the patient with sufficient cytolytic NK cells to mount a strong anti-tumor attack. Furthermore, adoptive transfer of large number of allogeneic cytolytic NK cells could be an option to strengthen tumor eradication in combination with cetuximab on RAS mutated tumors via ADCC. Allogeneic NK cell therapies are widely explored over recent years due to their cytotoxic nature providing a window to induce a HLA-mismatch setting to efficiently target tumor cells[38]. Different sources of allogeneic NK cell products are currently in use for clinical applications from adult PBNK cells[39], umbilical cord blood stem cell derived NK cells[40,41] and engineered NK cell lines[42]. Adoptive transfer of large number of allogeneic NK cells could repopulate the immune system, providing sufficient numbers of cytolytic NK cells to support ADCC with cetuximab. An increased number of NK cells in the blood stream could also target circulating tumor cells preventing metastasis and with a further role in decreasing or removing residual tumor load[43]. Further clinical confirmation of NK cell adoptive transfer advantages in combination with cetuximab on RAS^{mut} or $BRAF^{mut}$ mCRC tumors is warranted and calls for clinical trials.

In conclusion, the ability of functional NK cells to overcome the limitations of anti-EGFR therapy has been clearly demonstrated. The availability of allogeneic NK cells, combined with cetuximab could pave the way to demonstrate the therapeutic efficacy of this approach in patients with RAS^{mut}, $BRAF^{mut}$ and $EGFR^{low/-}$ CRC.

MATERIALS AND METHODS

Cell lines

Cell lines A431, COLO320, SW480, Caco-2, SW620, HT-29 were obtained from ATCC and cultured in Dulbecco's modified medium (DMEM; Invitrogen, Carlsbad CA, USA) containing 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum (FCS; Integro, Zaandam, The Netherlands) Cell cultures were passaged every 5 days. Cultures were maintained in a 37°C, 95% humidity, 5% CO_2 incubator.

Peripheral blood NK cell isolation and activation from whole blood specimens

Mononuclear cells (MNCs) were isolated from peripheral blood using Lymphoprep™ (STEMCELL Technologies, The Netherlands) density gradient centrifugation from buffy coats obtained from anonymous healthy blood donors (Sanquin Blood Supply, Amsterdam) with written informed consent for research use, in accordance with the ''Code for Proper Use of Human Tissues'' as formulated by the Dutch Federation of Medical Scientific Organizations (www.fmwv.nl)[44]. $CD56^+$ NK cells were isolated from MNCs using a MACS Human NK cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The cell number and purity of the isolated NK cell fraction were analyzed by flow cytometry. Isolated NK cells were activated overnight with 1000U/ml IL-2 (Proleukin®; Chiron, München, Germany) and 10ng/ml IL-15 (CellGenix) for use in cytotoxicity assays. NK cell purity and viability were checked using CD3 PE, 7AAD (BD Biosciences), CD56 APC Vio 770, and CD16 APC (Miltenyi Biotech). The parameters compared before and after activation were NK purity ($CD56^+$%, 83 ± 9 % vs. 82 ± 9%), NK CD16% 88 ± 10 % vs 85 ± 11%) and NK viability (91 ± 3 % vs 86 ± 2%) respectively.

Anti- EGFR monoclonal antibodies used for the cytotoxicity assay

The anti-EGFR mAbs cetuximab (Erbitux®) and panitumumab (Vectibix®) were purchased through the VU University medical center pharmacy.

Biotinylation of anti-EGFR mAbs

To assess the binding of cetuximab and panitumumab to EGFR expressing target cells, anti-EGFR mAbs were concentrated using Amicon ultra centrifuge 0.5ml 30K tubes (EMD Millipore, Netherlands). Concentrations of the mAbs were adjusted to 20mg/ml and then biotinylated using Biotin-N-hydroxysuccinimide ester (Sigma Aldrich, St Louis, USA) according to the manufacturer's instructions. The biotinylated anti-EGFR mAbs were incubated with A431 ($1x10^6$) cells for 1hr, washed twice in ice cold PBS and stained with

streptavidin APC (BD biosciences, Netherlands). A nonspecific IgG_1 and IgG_2 specific APC labeled antibody was used as a negative control.

DNA extraction and FCGR3A V158V and V158F (V/V and V/F) polymorphism genotyping

Genomic DNA (gDNA) was isolated from MNCs using QIA amp DNA kit (QIAGEN, Westburg, The Netherlands). Purified DNA was eluted in a volume of 100µl. Purity and Quantity of gDNA was measured using the nanodrop method (NANODROP 1000, Thermo Scientific). About 40-200ng of gDNA was used for FcγRIIIa polymorphism assays. FcγRIIIa primers ID: C__42463377_10 were purchased from Life Technologies, The Netherlands. 40ng of gDNA, 6.25µl of PCR master mix and 12.5µl mix of forward and reverse primers with VIC and FAM labeled probes for V/V and V/F polymorphism respectively were added. Readings were interpreted using v2.0.1 software (Bio Rad, Netherlands). Known controls for VV and VF genotypes were included in the experiment.

Flow cytometry

Flow cytometry analysis was done on a BD LSRFORTESSA X-20 (BD Biosciences). Cell numbers and expression of cell-surface markers were determined by flow cytometry. The cell numbers and the population of live cells was determined by gating on $CD45^+$ cells based on forward scatter (FSC) and side scatter (SSC). For analysis of phenotype, the cells were gated only on FSC/SSC and further analyzed for the specific antigen of interest. Cells were incubated with the appropriate concentration of antibodies for 30 min at 4°C. After washing, cells were suspended in FACS buffer.

Flow cytometry-based cytotoxicity and degranulation studies

Flow cytometry was used for the read-out of cytotoxicity assays. Target cells were labelled with 5µM pacific blue succinimidyl ester (PBSE; Molecular Probes Europe, Leiden, The Netherlands) in a concentration of $1x10^7$ cells per ml for 10 min at 37°C. The reaction was terminated by adding an equal volume of FCS, followed by incubation at room temperature for 2 min after which stained cells were washed twice with 5 ml DMEM/10% FCS. After washing, cells were suspended in DMEM/10% FCS to a final concentration of 5×10^5/ml. $CD56^+$ NK cells were washed with PBS and suspended in Glycostem Basal Growth Medium (GBGM) + 2% FCS to a final concentration of 5×10^5/ml. Target cells were co-cultured with effector cells at an E:T ratio of 1:1 in a total volume of 250 µl in 96-wells flat-bottom plates (5×10^4 targets in 100 µl of DMEM + 10% FCS incubated with 5×10^4 effectors in 100 µl of GBGM + 2% FCS, further supplemented with 25 µl of GBGM + 2% FCS and DMEM + 10% FCS medium). NK cells and target cells alone were plated out in triplicate as controls. Target cells were coated with anti-EGFR mAbs for 1h at 4°C. Cells (A431, COLO320, Caco-2,

SW80, SW620 and HT-29) were washed and co cultured with activated NK cells. To measure degranulation by NK cells, anti-CD107a PE (Miltenyi Biotech, Germany) was added in 1:20 dilution to the wells. After incubation for 4h at 37°C, 75 µl supernatant was collected and stored at -20°C for analysis of cytokine production. Cells in the remaining volume were harvested and stained with 7AAD (1:20). Degranulation of NK cells was measured by detecting cell surface expression of CD107a. After 4 hrs of incubation at 37°C, CD56 APC Vio 770 (1:25) and CD16 APC (1:25) (Miltenyi Biotech, Germany) were added to the co-cultures and NK CD107a degranulation was measured for $CD56^+$ NK, $CD56^+CD16^+$ NK and $CD56^+CD16^-$ NK cells.

IFNγ production assay

Production of IFNγ by target cell-stimulated NK cells was measured in the supernatant of the co-cultures by ELISA (Sanquin, Amsterdam, The Netherlands). Absorbance was measured at 450 nm with a Multiscan MCC/340 ELISA reader (Titertek, Huntsville, Alabama, USA).

Primary colon tissue dissociation and storage

Colon cancer tissues from patients participating in trials conducted at the VU University medical center in Amsterdam, The Netherlands were collected and processed as described[45] after written informed consent and used for this study according to protocols approved by the VUmc IRB (IRB00002991; IORG number 0002436)[46]. Tumor material was further confirmed histologically and were screened for HLA class I (Clone W6/32), and EGFR expression. Tumor tissues were washed thrice, scraped, cut into small fragments and digested mechanically using collagenase A (Roche Diagnostics, The Netherlands) based growth medium. Digestion was carried out in a sterile glass flask with continuous stirring for 45 mins. This step was repeated twice, and the single cell suspension was collected through a 45µm sterile filter. Cells were counted and then frozen under controlled conditions in liquid nitrogen. Primary tumor cells were thawed, ficoll based density grade separation was done to remove dead cells and finally resuspended in DMEM +10% FCS medium for the cytotoxicity assays.

RAS typing

The mutational status of *KRAS* exon 2/3/4, *NRAS* exon 2/3/4 and *BRAF* exon 15 was assessed by high resolution melting (HRM) assay followed by Sanger sequencing of HRM-PCR products with an aberrant melt curve, essentially as described previously[47,48].

Statistical analysis

Statistical analysis was performed using Graph Pad Prism software. Differences between conditions were determined using two-way anova with multiple comparisons between

column means. Results from cytotoxicity experiments are described as mean ± standard deviation of the mean (SD). A p-value of <0.05 was considered statistically significant.

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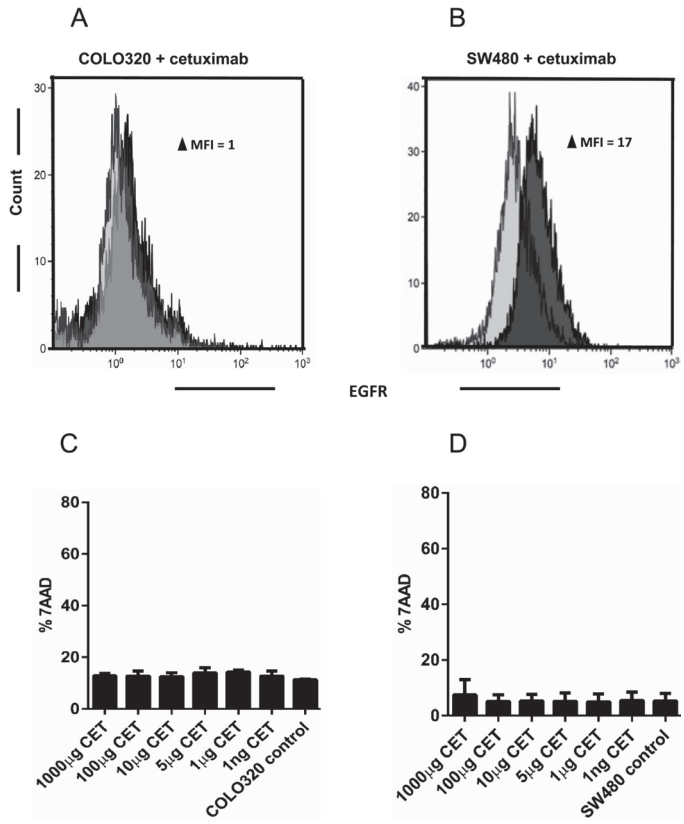
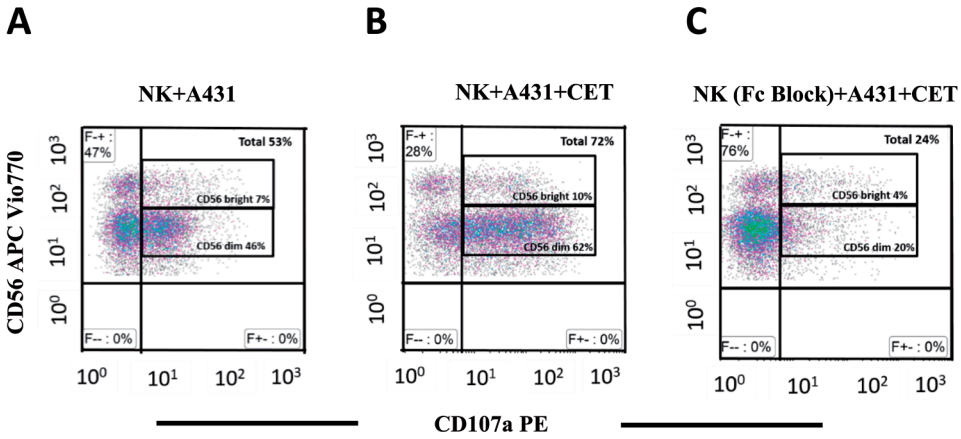


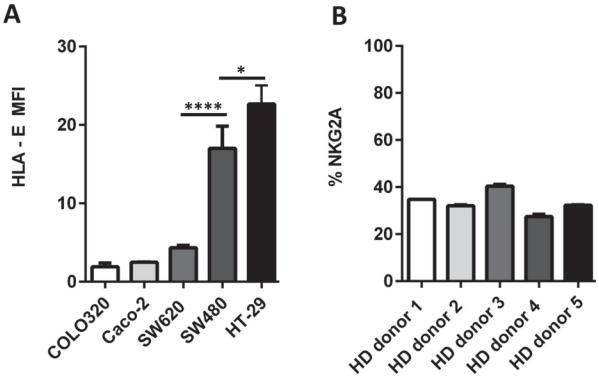
SUPPLEMENTARY FIGURE 1: Cetuximab cytotoxic activity against EGFR^{+} RAS^{mut} and EGFR^{-} RAS^{wt} colon cancer cells

SW480 and COLO320 cells were incubated with 100µg/ml of biotinylated cetuximab for 1hr at 4°C and analyzed for their EGFR recognition by cetuximab. A and B, Histograms showing binding of cetuximab to (A) COLO320 and (B) SW480 cells. Grey shades represent the streptavidin APC control, black shades represent binding of cetuximab. Further, SW480 and COLO320 cells were stained with PBSE and exposed to increasing concentrations of cetuximab for 1hr at 4°C after which unbound antibodies were removed and cells were cultured for an additional 4hrs at 37°C. Target cell death was determined by assessing the percentage of 7AAD positive COLO320 (C) and SW480 (D) cells at the end of incubation.



SUPPLEMENTARY FIGURE 2: Degranulation of NK cell CD56^{bright} and CD56^{dim} subsets in response to cetuximab coated tumor target cells

A representative example of PBNK degranulation pattern upon target exposure to A431 tumor targets in the presence or absence of cetuximab. CD56^{bright} and CD56^{dim} degranulate upon target cell recognition (Figure A), with an increase in degranulation in CD56^{dim} CD16^{+} subset of NK cells when target cells are coated with cetuximab (Figure B). The degranulation can be reduced by blocking Fc receptors (Figure C) and this also decreases the degranulation in the CD56^{bright} subset of NK cells.



SUPPLEMENTARY FIGURE 3: Expression of HLA-E on tumor cell lines and NKG2A on NK cells used for cytotoxicity experiments

*Surface expression of HLA-E on COLO320, Caco-2, SW620, SW480 and HT-29 and NK cell NKG2A expression levels of five healthy donors used for the cytotoxicity assays were determined by flow cytometry as shown in figure A and B. Columns are mean of triplicate values from two independent experiments, bars represent SD. Mean ± SD for each significant condition are represented as p = <0.05 *, <0.01 **, <0.005 ***, <0.001 ****.*

CHAPTER 5

Towards UCB-NK cells treatment in colorectal cancer

Published as
"In vivo efficacy of umbilical cord blood stem cell-derived NK cells in the treatment of metastatic colorectal cancer"

Veluchamy JP, Lopez-Lastra S, Spanholtz J, Bohme F, Kok N, Heideman DA, Verheul HM, Di Santo JP, de Gruijl TD, van der Vliet HJ.

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ABSTRACT

Therapeutic monoclonal antibodies (mAbs) against the epidermal growth factor receptor (EGFR) act by inhibiting EGFR downstream signaling and by eliciting an NK cell-mediated anti-tumor response. The IgG_1 mAb cetuximab has been used for treatment of RAS^{wt} metastatic colorectal cancer (mCRC) patients, showing limited efficacy. In the present study, we address the potential of adoptive NK cell therapy to overcome these limitations investigating two allogeneic NK cell products, i.e. activated peripheral blood NK cells (A-PBNK) and umbilical cord blood stem cell derived NK cells (UCB-NK). While cetuximab monotherapy was not effective against $EGFR^-$ RAS^{wt}, $EGFR^+$ RAS^{mut} and $EGFR^+$ $BRAF^{mut}$ cells, A-PBNK were able to initiate lysis of $EGFR^+$ colon cancer cells irrespective of RAS or BRAF status. Cytotoxic effects of A-PBNK (but not UCB-NK) were further potentiated significantly by coating $EGFR^+$ colon cancer cells with cetuximab. Of note, a significantly higher cytotoxicity was induced by UCB-NK in $EGFR^-RAS^{wt}$ (42 ± 8% versus 67 ± 7%), $EGFR^+$ RAS^{mut} (20 ± 2% versus 37 ± 6%) and $EGFR^+$ $BRAF^{mut}$ (23 ± 3% versus 43 ± 7%) colon cancer cells compared to A-PBNK and equaled the cytotoxic efficacy of the combination of A-PBNK and cetuximab. The anti-tumor efficacy of UCB-NK cells against cetuximab resistant human $EGFR^+$ RAS^{mut} colon cancer cells was further confirmed in an *in vivo* preclinical mouse model where UCB-NK showed enhanced anti-tumor cytotoxicity against colon cancer independent of EGFR and RAS status. As UCB-NK have been proven safe in a recently conducted phase I clinical trial in acute myeloid leukemia, a fast translation into clinical proof of concept for mCRC could be considered.

INTRODUCTION

Colorectal cancer (CRC) is the fourth leading cause of cancer related deaths in the world[1]. Despite substantial advances in the treatment of metastatic CRC (mCRC) over the last decades that have contributed to better survival rates[2,3], the disease is still frequently fatal. Monoclonal antibodies targeting the Epidermal Growth Factor Receptor (EGFR) pathway, such as panitumumab and cetuximab are approved for the treatment of patients with advanced CRC either in combination with chemotherapy or, as monotherapy, in chemo-refractory conditions[4]. Cetuximab (CET) and panitumumab block the interaction between EGFR and its ligands, thus inhibiting the downstream RAS-signalling cascade and tyrosine kinase activation[5]. However, mutations in tumour suppressor genes and proto-oncogenes in EGFR signalling pathways, such as in RAS, BRAF and PIK3CA are common in patients with CRC. These mutations represent a poor prognostic marker and render anti-EGFR mAbs ineffective, leaving 42% of the chemo-refractory mCRC population without standard treatment option[6, 7].

Besides the blockade of the EGFR-ligand interaction on tumor cells, therapeutic mAbs can also interact with Natural Killer (NK) cells triggering antibody-dependent cell-mediated cytotoxicity (ADCC)[8-10], and this can translate into superior anti-tumor effects[11]. Two NK cell subsets can be identified based on the expression of CD16, the low affinity FcγRIIIa receptor. The majority of NK cells are $CD56^{dim}CD16^{+}$, and play an active role in NK cell cytotoxicity and are capable of performing ADCC upon IgG_1 engagement via CD16, whereas $CD56^{bright}CD16^{-}$ NK cells are mainly immune regulatory in function, secreting cytokines, and are less cytotoxic than $CD56^{dim}$ cells[12]. NK cell functions are tightly regulated by a delicate balance between activating receptors (like the natural cytotoxicity receptors NKp46, NKp30 and NKp44, or C-type lectin-like receptor NKG2D)[13] and Major Histocompatibility Complex (MHC) class I binding inhibitory receptors, including Killer-cell immunoglobulin-like receptors (KIRs), LIR1/ILT2 and NKG2A/CD94[14]. The importance of NK cells in controlling tumors has been extensively demonstrated since their identification 40 years ago[15-17].

Several studies have shown a dysfunctional phenotype and poor infiltration of NK cells in the CRC tissue from early stages on, together with an immunosuppressive tumor microenvironment[18,19]. Hence, various strategies e.g. using cytokines or therapeutic ADCC enhancing mAbs, have been explored to increase NK cell numbers and function and to enhance their trafficking to tumor sites[20]. Another approach entails the adoptive transfer of *in vitro* manipulated and expanded autologous or allogeneic NK cells. Autologous NK cells so far have failed to demonstrate significant therapeutic benefits in solid tumors[21-23]. Therefore, the focus has shifted to the development of allogeneic NK cells as a potential adoptive cell therapy for treatment in solid tumors. Previously, we demonstrated that the combination of allogeneic activated PBNK (A-PBNK) cells and CET can effectively target RAS mutant (RAS^{mut}) CRC tumors[24].

Here, we compared two feeder cell free allogeneic NK cell products, i.e. activated peripheral blood NK cells (A-PBNK) and cord blood stem cell derived NK cells (UCB-NK), alone or in combination with cetuximab for anti-tumor effects against RAS^{mut} CRC.

RESULTS

Highly dysfunctional NK cells in CRC patients

Flow cytometry was used to determine the frequency, phenotype and functionality of NK cells in PBMC of healthy volunteers (n=10, age range 56-64, 6 males/4 females) and patients with metastatic CRC (n=10, age range 66-74, 8 males/2 females) before and after the first cycle of first line palliative chemotherapy consisting of oral capecitabine (1000 mg/m^2, bid, day 1-14), i.v. oxaliplatin (130 mg/m^2, day 1) and i.v. bevacizumab (7.5 mg/kg, day 1, in 4/10 mCRC patients). As illustrated in figure 1A, mCRC patients harbored on average a 20% lower percentage of $CD3^-CD56^+$NK cells in the total $CD45^+$ lymphocyte population as compared to healthy controls ($p<0.05$). These lower NK rates, which are in line with a previous report in colorectal cancer[28], further declined after the first cycle of chemotherapy ($p<0.01$).

We next evaluated whether this quantitative NK cell defect was also accompanied by functional defects in the NK cell population. For this purpose, the ability of NK cells from healthy volunteers and mCRC patients to induce both natural cytotoxicity and mediate ADCC of the epidermoid carcinoma cell line A431 (MHC-I^{low}, $EGFR^{high}$, $KRAS^{wt}$) was assessed. For ADCC tumor target cells were coated with cetuximab before the addition of NK cells. It was evident that the cytotoxic potential of NK cells from mCRC patients, as reflected by degranulation (i.e. CD107a surface expression), was highly impaired both before chemotherapy and after the first cycle of chemotherapy. Though NK cells of mCRC patients were capable of ADCC, as evidenced by significant increases in degranulation when target cells were coated with cetuximab ($p<0.05$), levels were still low compared to those observed in healthy volunteers. (Figure 1B). Of note, although the NK cells of healthy volunteers and mCRC patients expressed similar levels of CD16 (Figure 1C), this did not translate into comparable levels of ADCC. NKp44 expression, known to reflect the activation status of NK cells, was similar between the HD and mCRC groups used in NK cytotoxicity experiments (Figure 1D). Furthermore, no significant differences were observed in expression levels of NK activating (NKG2D, NKG2C) and NK inhibiting (NKG2A, KIR2D) receptors between healthy controls and CRC patients (Supplementary figure 1).

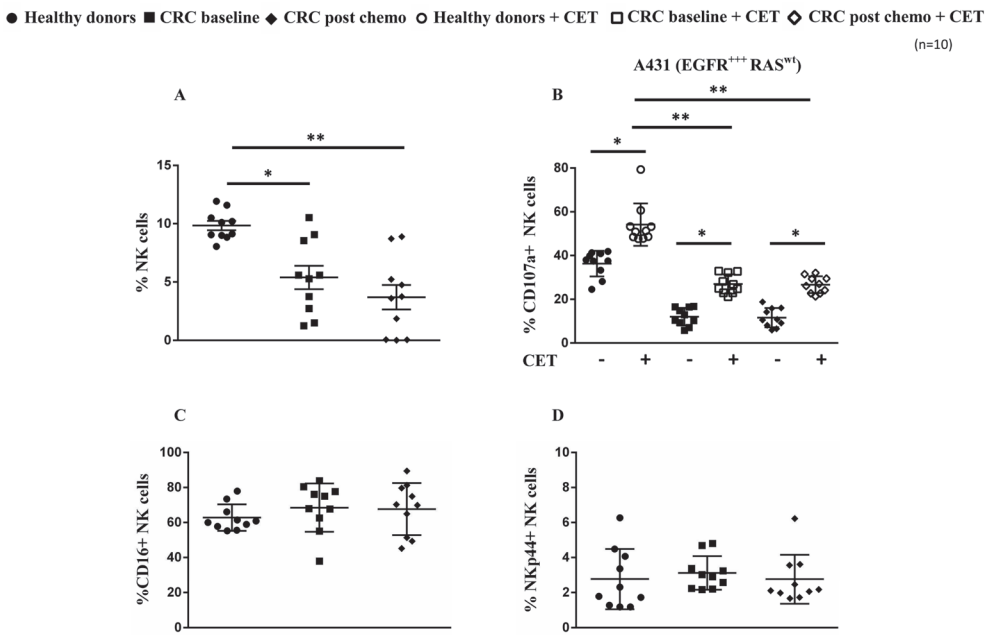


FIGURE 1: Low prevalence and functionally impaired NK cells in CRC patients
*(A) Frequency of NK cells within PBMC from healthy controls and from mCRC patients at baseline and after the first cycle of chemotherapy. (B) NK cell degranulation in healthy controls and mCRC patients after a 4 hr. co-culture of resting NK cells with A431 cells in the presence (open symbols) or absence (closed symbols) of cetuximab at an E:T ratio of 1:1. (C) Expression levels of resting NK cell CD16 and (D) NKp44 in healthy controls and in mCRC patients before and after 1 cycle of chemotherapy. Data represent mean ± SEM from 10 mCRC patients and 10 age and sex matched healthy controls. *P < 0.05, **P < 0.01, ***P < 0.005, calculated with one-way ANOVA, multiple comparison between column means.*

Enhanced *in vitro* cytotoxicity of colon cancer cells mediated by UCB-NK cells

In order to explore novel therapies to replace dysfunctional NK cells in patients with advanced CRC, we tested two different sources of allogeneic NK cell products (A-PBNK and UCB-NK), that could eventually be used for adoptive transfer strategies. We next compared the activity of A-PBNK cells (age range 22-37 years) and UCB-NK cells using a flow-based NK cell cytotoxicity assay based on detection of 7-AAD accumulation in tumor cells. Three different cell lines of colon cancer origin were compared, i.e. COLO320 ($EGFR^-$ RAS^{wt}), SW480 ($EGFR^+$ RAS^{mut}) and HT-29 ($EGFR^+$ RAS^{wt}, $BRAF^{mut}$). As expected, addition of cetuximab to $EGFR^-$ RAS^{wt} COLO320 cells did not result in increased killing. Of interest, lysis was consistently and significantly higher ($p<0.01$) using UCB-NK compared to A-PBNK. As reported previously, the combination of cetuximab and A-PBNK resulted in increased killing

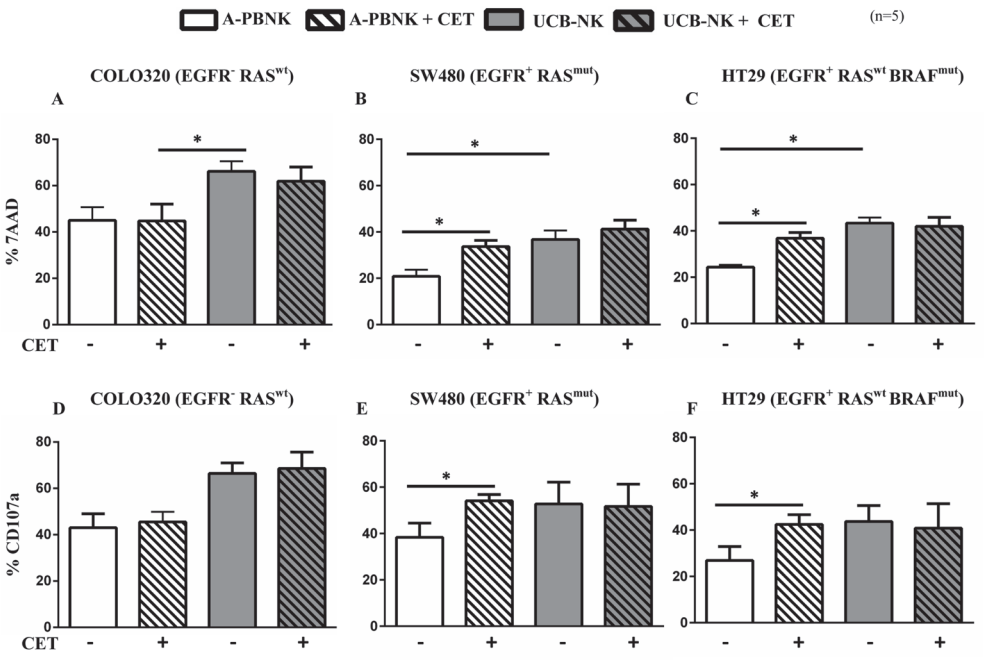


FIGURE 2: *Ex vivo* cytotoxic efficacy of A-PBNK and UCB-NK cells against CRC cells
*(A) CRC cell lines COLO320 (EGFR$^-$, RAS^{wt}), SW480 (EGFR$^+$, RAS^{mut}) and HT-29 (EGFR$^+$, RAS^{wt}, BRAF^{mut}) were subjected to NK killing using two allogeneic NK cell products, i.e. A-PBNK and UCB-NK cells. 7AAD (A) and CD107a (B) were measured after a 4 hr. co-culture of A-PBNK and UCB-NK cells with CRC targets in the presence or absence of cetuximab at an E:T ratio of 1:1. Experiments were carried out in triplicate. Bars represent mean ± SEM, n=5. *P < 0.05 and **P < 0.01, calculated with two-way ANOVA, multiple comparison between column means.*

of EGFR$^+$RAS^{mut} SW480 and EGFR$^+$ BRAF^{mut} HT-29 via ADCC[24]. CD16 was expressed by 88 ± 8% (n=5) of A-PBNK after overnight stimulation with cytokines and by 7 ± 2% (n=5) of UCB-NK cells at the end of the 35 day culture period. No added effect of cetuximab was observed when using UCB-NK cells, which is possibly related to their lower *in vitro* CD16 levels[29]. Of note, tumor cell lysis induced by UCB-NK cells was comparable to that observed with the combination of A-PBNK and cetuximab (Figure 2A). Measurements of NK cell degranulation reflected equivalent trends observed for tumor cell lysis (Figure 2B). These results show that UCB-NK cells have superior cytotoxic efficacy over A-PBNK cells against cetuximab resistant colon cancer cells *in vitro.*

UCB-NK cells inhibit *in vivo* tumor growth and increase survival

To address whether UCB-NK cells exhibit similar anti-tumor efficacy *in vivo,* we transferred Gluc transduced SW480 cells to immunodeficient mice (BRGS; see methods). SW480 cells

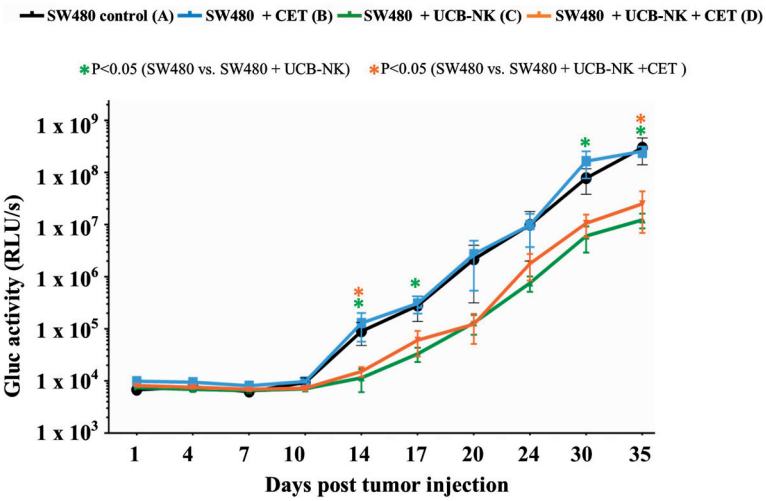


FIGURE 3: Significant anti-tumor effects of UCB-NK cells *in vivo*
*Real time monitoring of tumor progression and treatment response was performed measuring Gluc levels from mice blood twice a week. Baseline Gluc values were obtained from all mice a day before tumor injection (day-1), and further monitoring continued until day 35. Blood Gluc levels were compared between control SW480 only (A) group and treatment groups SW480 + cetuximab (B), SW480 + UCB-NK (C) and SW480 + UCB-NK + cetuximab (D) for statistical significance. Data presented is from 6 mice per group (n=6). Scatter plots represent mean ± SEM. *P < 0.05, calculated with unpaired-t test.*

are $EGFR^{+}RAS^{mut}$ and cetuximab monotherapy resistant. Mice were divided into 4 groups of 6 mice per group: SW480 only (group A), SW480 + cetuximab (group B), SW480 + UCB-NK (group C) and SW480 + UCB-NK + cetuximab (group D). Gaussia luciferase activity in whole blood was measured every three days to monitor the tumor burden (Supplementary figure 2). These data confirmed our *in vitro* observations that SW480 cells were resistant to cetuximab mediated growth inhibition (blue line). Of note, while treatment with UCB-NK cells alone significantly decreased the tumor load (green line), this effect was not increased by combining UCB-NK cells with cetuximab and thereby further confirmed both the inefficacy of cetuximab in treating RAS mutated tumors as well as the inability of cetuximab to induce ADCC of UCB-NK cells *in vivo* (orange line) (Figure 3). CD16 expression levels on UCB-NK cells were monitored in two mice upon adoptive transfer and increased from 6.0% before transfer to 14.0% (mouse 1) and 19.1% (mouse 2) at day 5 post UCB-NK cell infusion (data not shown).

While the blood Gluc assay measurements provided evidence of a reduction in the total tumor burden after UCB-NK treatment, we wanted to explore the impact of the therapy on the localization and size of the metastases. For that purpose, BLI was performed at day

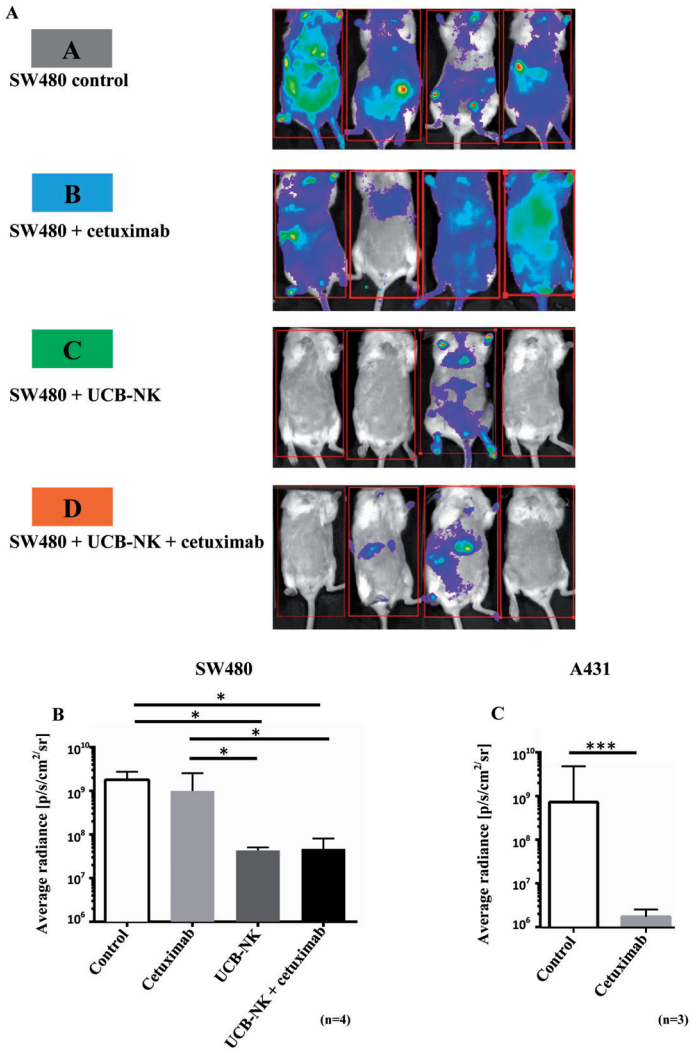
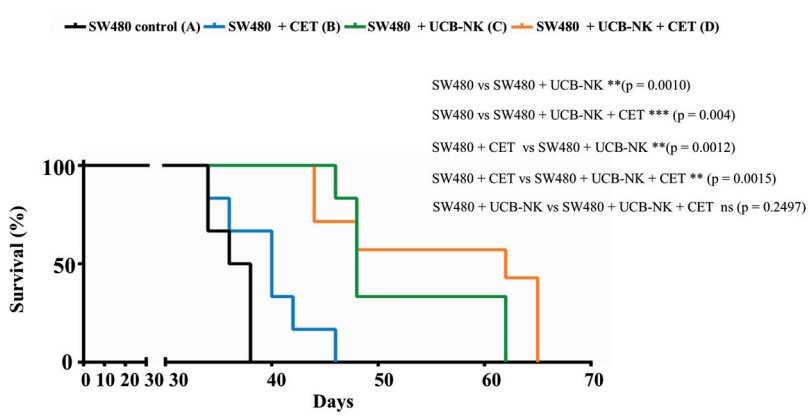


FIGURE 4: Successful tumor elimination by UCB-NK cells as revealed by bioluminescence imaging *in vivo*

*(A) Four mice from control and treatment groups were imaged at day 35 for tumor load and distribution. Mice were injected retro-orbitally with Gluc substrate coelenterazine and images were acquired for 5 min. In SW480 control and SW480+cetuximab groups, tumor growth was extensive and highly disseminated, spreading to most parts of the body. However, in UCB-NK and UCB-NK + cetuximab groups there was a significantly lower tumor load, which was further verified by calculating the average radiance between groups as shown in figure B (n=4 mice per group). (C) Cetuximab functionality against EGFR^{+++} RAS^{wt} A431 cells was tested in parallel to SW480 studies in BRGS mice (n=3 mice per group). For figures B and C, bars represent mean ± SEM. *P< 0.05 for figure B and figure C was calculated with unpaired t test.*



Median survival (days)			
SW480 control	SW480 + CET	SW480 + UCB-NK	SW480 + UCB-NK + CET
37	40	48	62

FIGURE 5: Significant survival benefit in cetuximab resistant RAS mutant tumor bearing mice treated with UCB-NK cells

Kaplan -Meier survival curves were plotted for the total experimental study period from day 0 until day 65. Survival rates of SW480 ($EGFR^{+}$, RAS^{mut}) tumor bearing mice (n=6 per group) following treatment with PBS only (black line), cetuximab only (blue line), UCB-NK only (green line) and UCB-NK + cetuximab (orange line) were plotted over time to monitor treatment outcome. Statistical differences between groups were calculated using log rank (Mantel-Cox) test and indicated in the figure.

35 after tumor inoculation. Figure 4A depicts four representative BLI images from each group at day 35 post-tumor injection and average radiance from range of interest (ROI) measurements are shown in Figure 4B. It is clear that mice from groups A and B showed a higher and more diffuse tumor load compared to mice treated with UCB-NK alone or in combination with cetuximab. In order to demonstrate the possibility of antitumor efficacy of cetuximab in the BRGS mouse model we performed a similar tumor challenge using the cetuximab sensitive A431 cell line, which bears wild-type RAS and overexpresses EGFR. A significant decrease in tumor load was observed when A431 tumors were treated with the same concentration of cetuximab as in the SW480 study (Figure 4C), confirming the *in vivo* functionality of cetuximab. We next assessed whether treatment of SW480 bearing mice with UCB-NK cells alone or in combination with cetuximab translated into a survival advantage (Figure 5). Indeed, treatment of mice with UCB-NK cells alone resulted in a significant prolongation in their life span (p=0.01), whereas combinatorial therapy did not add significantly to this. Treatment with cetuximab alone did not translate into a significant survival advantage, consistent with the observed effects on tumor growth.

DISCUSSION

In order to test the cytotoxic potential of NK cells for treating advanced colorectal cancer patients, we compared their functional status before and after chemotherapy. We observed that peripheral blood NK cell numbers were reduced in mCRC patients and that residual NK cells were dysfunctional and unable to mount a strong effector response when stimulated with an NK cell sensitive tumor target. Though an increase in NK cell cytotoxicity was observed when tumor target cells were coated with the anti-EGFR mAb cetuximab, reflecting a capacity for ADCC, cytotoxicity was still significantly lower (both before and after chemotherapy) than that observed in healthy controls. These data indicate a decreased functional state of NK cells in patients with mCRC, which is in line with studies in mice where the cytokine production and anti-tumor activity of adoptively transferred NK cells were highly affected following long-term exposure to tumors[30]. Through recognition of MHC class I molecules KIRs prevent NK cells from targeting healthy cells while allowing them to detect tumor or infected cells with low or downregulated expression of MHC class I in a process known as "missing self"[31]. Severely diminished or aberrant expression of MHC class I has been reported in the majority of colorectal adenocarcinomas[32,33], which makes them an ideal target for NK cell-mediated killing. Although NK cells are infrequent in colorectal tissues[18], several independent studies investigated the clinical impact of NK cell infiltration on the prognosis of CRC, as well as in other types of carcinoma. These clinical studies, including a recent tissue microarray of 1414 CRC biopsies, led to the conclusion that NK cell infiltration in tumors correlated with better overall response rates and progression-free survival in CRC patients[34-37], suggesting that therapies aimed at boosting NK cell functions could be beneficial in mCRC and possibly also in other types of cancer.

We evaluated and compared the cytotoxic efficacy of two different sources of feeder cell free allogeneic NK cells, i.e. A-PBNK cells and *in vitro* expanded and differentiated UCB-NK cells. *In vitro* NK cell cytotoxicity experiments revealed that the cytotoxic activity of UCB-NK cells against CRC cells was significantly higher than that of A-PBNK cells and in addition demonstrated that, while an increase in cytotoxicity through ADCC was not evident with UCB-NK cells, their cytotoxic potential was still comparable to that observed with A-PBNK potentiated by cetuximab mediated ADCC. It is possible that the stronger cytotoxic effects of UCB-NK cells result from a more intense stimulation with cytokines in comparison to A-PBNK cells. The failure to observe ADCC-enhanced cytotoxicity with UCB-NK cells *in vitro* can be explained by their low expression levels of CD16[29]. As we previously observed *in vivo* up-regulation of CD16 on UCB-NK cells upon their transfer to NOD/SCID/IL2Rgnull (NSG) mice[38], we decided to also test the efficacy of cetuximab treatment in combination with UCB-NK cells in an *in vivo* model. Treatment of SW480 RAS^{mut} tumors in BRGS mice with UCB-NK cells, resulted in control of disease progression and translated into a significantly longer survival. As expected, cetuximab monotherapy did not result in a decreased SW480

tumor load or improvement in survival, recapitulating the clinical data from patients bearing RAS^{mut} CRC tumors. Unexpectedly, we failed to demonstrate superior *in vivo* anti-tumor effects or survival when we combined the transfer of UCB-NK cells with cetuximab infusions. The underlying causes for this latter finding remain obscure but may be related to sub-optimal *in vivo* upregulation of CD16 in the used mouse model or CD16 polymorphisms in the employed batch of UCB-NK cells, both of which could have hampered efficient ADCC.

Taken together, UCB-NK cells displayed significant anti-tumor efficacy, suggesting a potential beneficial role for UCB-NK cells in the treatment of RAS and BRAF mutant CRC. As an important present limitation in treating mCRC patients is related to resistance to anti-EGFR mAbs, adoptive transfer of cytolytic UCB-NK cells could thus constitute a viable treatment option. Our *in vitro* and *in vivo* data demonstrating that adoptive transfer of UCB-NK cells alone was as effective as the combination of A-PBNK and cetuximab raises the possibility that UCB-NK administration could obviate the use of cetuximab in RAS^{wt} mCRC. Furthermore, UCB-NK can also lyse RAS^{mut} CRC cells at levels higher than those observed with A-PBNK. Importantly, allogeneic NK cells have demonstrated their safety in clinical trials in several solid tumors[39, 40], and more specifically, the UCB-NK cell product used in our experiments was found to be safe in a clinical trial in Acute Myeloid Leukemia (AML) patients[41].

Several features make UCB-NK attractive for further clinical development. For example, our GMP based expansion and differentiation protocol reproducibly resulted in a more than 10,000-fold expansion of cytotoxic UCB-NK cells from single donors. Furthermore, UCB-NK cells can be supplied as an “off the shelf” product, stored in large aliquots facilitating multiple infusions. Also, the low immunogenicity by UCB grafts prevents adverse reactions that are prevalent after repeated PBNK transfusions[42]. In this respect, it is relevant to mention that while NK cells in general are often inhibited by recognition of MHC class I molecules on the surface of tumor cells, UCB-NK display relatively low levels of Killer cell - immunoglobulin like receptors (KIRs) supporting their ability to effectively lyse MHC class I expressing tumor cells[29]. Finally, the ability of UCB-NK cells to proliferate and home to liver, lungs, spleen and bone-marrow after adoptive transfer has been previously demonstrated in NSG mice[38], though additional studies are required to determine whether UCB-NK cells have a similar migratory pattern upon adoptive transfer in solid tumor patients. Together, these features and observations provide UCB-NK cells with several unique advantages for further development as a universal NK cell platform.

Considering the size and heterogeneity of the tumor mass in advanced stages of CRC and other types of cancer, UCB-NK may not provide a sufficient therapeutic effect as a single agent. However, rational combinations of UCB-NK cells with existing drugs or drugs that are in clinical development can be envisioned to further increase their efficacy.

Previous studies have pointed out that the proteasome inhibitor (bortezomib)[43] and the immunomodulatory drug (lenalidomide)[44] sensitize tumor cells to NK mediated killing. In addition, UCB-NK cell application together with bispecific or trispecific antibodies that bind to tumor and UCB-NK cell activating receptors can also increase NK cell tumor specificity[45]. Though we did not specifically assess ADCC induced by other mAbs, it is very likely that the failure of UCB-NK to mediate ADCC is a more general phenomenon as this depends on binding to CD16/FcγRIII, which was found to be expressed at only low levels in the UCB-NK cell product. However, recent data from a clinical phase 1 study with the same UCB-NK cell product in patients with AML revealed significant upregulation of CD16 on UCB-NK cells post transfusion suggesting that the UCB-NK cell product may acquire the capacity to mediate ADCC in patients following adoptive transfer[46]. Further, this phenomenon may also provide a strong rationale for combining UCB-NK cells with bispecific or trispecific killer cell engagers[47]. Taken together, these approaches can substantially increase UCB-NK cell responses to advanced solid tumors, including mCRC.

In conclusion, in this study we have demonstrated the *in vitro* efficacy of UCB-NK cells against multiple colorectal cancer cell lines independent of EGFR expression and EGFR downstream signaling mutations, and in addition have demonstrated the *in vivo* antitumor efficacy of adoptively transferred UCB-NK cells against $EGFR^{+}RAS^{mut}$ tumors. As the adoptive transfer of UCB-NK cells (oNKord®) has been shown to be safe in patients with AML (CCMO nr NL31699 & Dutch trial register no 2818), our data provide a rationale for the clinical exploration of UCB-NK cells in the treatment of mCRC.

MATERIALS AND METHODS

Cell lines

Cell lines A431 (epidermoid carcinoma), COLO320, SW480 and HT-29 (colon carcinoma) were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified medium (DMEM; Invitrogen, Carlsbad CA, USA) containing 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum (FCS; Integro, Zaandam, The Netherlands). Cell cultures were passaged every 5 days and maintained in a 37°C, 95% humidity, 5% CO2 incubator.

PBNK isolation and activation

Peripheral blood mononuclear cells (PBMC) were isolated from the heparinized blood of healthy donors (6 males, 4 females, age range = 56-64 and CRC patients (8 males, 2 females, age range = 66-74) after written informed consent and according to protocols approved by the institutional review board of VU University Medical Center, Amsterdam (NCT01792934). Blood samples were collected at baseline and after the first cycle of first-

line palliative chemotherapy consisting of oral capecitabine (1000 mg/m^2, bid, day 1-14), i.v. oxaliplatin (130 mg/m^2, day 1) and i.v. bevacizumab (7.5 mg/kg, day 1, in 4/10 mCRC patients). PBMC were isolated using Lymphoprep™ (STEMCELL Technologies, Cologne, Germany) density gradient centrifugation. CD56$^+$ NK cells were isolated from PBMC using a MACS Human NK cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. PBNK cells purity and viability were checked using CD3 VioBlue, CD56 APC Vio 770, and CD16 APC (Miltenyi Biotech) and 7AAD (Sigma Aldrich, Zwijndrecht, The Netherlands). Isolated PBNK cells were activated overnight with 1000U/ml IL-2 (Proleukin®; Chiron, München, Germany) and 10ng/ml IL-15 (CellGenix) for use in cytotoxicity assays. The parameters compared before and after stimulation with cytokines were NK purity (87 ± 5 % versus 84 ± 2%), NK CD16$^+$, 92 ± 12 % versus 88 ± 8%) and NK viability (89 ± 5 % versus 84 ± 8%), respectively.

Flow cytometry

The antibody staining mix for the assessment of NK cell functionality consisted of CD45 VioGreen, CD14 VioBlue, CD19 VioBlue and SYTOX® Blue, together with CD3 PerCP-Vio 700 and TCRγδ PerCP-Vio700 to exclude dead cells, debris and non-NK populations from PBMC. NK cells were identified by the expression of CD45$^+$CD3$^-$CD56$^+$ cells, and further characterized for NK functionality by plotting against CD16 APC, CD25 VioBrightFITC, CD107a PE, and NKp44 PE-Vio770 and for NK cell phenotype by plotting against NKG2A PE-Vio770, NKG2C PE, NKG2D PerCP-Cy5.5 and PanKIR2D FITC. All antibodies were supplied by Miltenyi Biotec except SYTOX® Blue (Thermo Fisher Scientific, Berlin, Germany).

UCB-NK cultures

Allogeneic NK cells (UCB-NK) were generated from cryopreserved umbilical cord blood (UCB) hematopoietic stem cells as previously described[25]. CD34$^+$ UCB cells from six UCB-donors were plated (4 x 10^5/ml) into 12-well tissue culture plates (Corning Incorporated, Corning, NY, USA) in Glycostem Basal Growth Medium (GBGM®) (Clear Cell Technologies, Beernem, Belgium) supplemented with 10% human serum (HS; Sanquin Bloodbank, Amsterdam, The Netherlands), 25ng/mL of SCF, Flt-3L, TPO, and IL-7 (CellGenix, Freiburg, Germany). In the expansion phase II, from day 9 to 14, TPO was replaced with 20ng/mL IL-15 (CellGenix). During the first 14 days of culture, low molecular weight heparin (LMWH) (Clivarin®; Abbott, Wiesbaden, Germany) in a final concentration of 20µg/ml and a low-dose cytokine cocktail consisting of 10pg/ml GM-CSF (Neupogen), 250 pg/ml G-CSF and 50 pg/ml IL-6 (CellGenix) were added to the expansion cultures. Cells were refreshed with new medium twice a week and maintained at 37°C, 5% CO_2. On day 14, the NK cell differentiation process was initiated by addition of NK cell differentiation medium consisting of the same basal medium with 2% HS but with high-dose cytokine cocktail consisting of 20ng/ml of IL-7, SCF, IL-15 (CellGenix)

and 1000 U/ml IL-2 (Proleukin®; Chiron, München, Germany). Cultures were refreshed every 2-3 days and maintained till day 42. Five UCB-NK cultures were used for cytotoxicity assays and one UCB-NK culture for *in vivo* studies (both with a $CD56^+$ cell purity of >95%). UCB-NK CD16 levels in matured UCB-NK cells were monitored using an antibody mix of human CD45VioGreen (1:11), CD56 APC-Vio770 (1:11) and CD16 APC (1:11). Similarly, UCB-NK CD16 expression in BRGS mice was monitored using an antibody mix of BV650 anti-mouse CD45 (clone 30-F11), Alexa Fluor® 700 anti-human CD45 (clone HI30), PE-CF594 anti-human CD56 (clone B159), all from BD, and APC-Vio770 anti-human CD56 (clone REA196) and APC CD16 (clone REA423) both from Miltenyi Biotec.

NK cell cytotoxicity assays

Flow cytometry was used for the read-out of cytotoxicity assays. Target cells (COLO320, SW480 and HT-29 were labelled with 5µM pacific blue succinimidyl ester (PBSE; Molecular Probes Europe, Leiden, The Netherlands) at a concentration of $1x10^7$ cells per ml for 10 min at 37°C. The reaction was terminated by adding an equal volume of FCS, followed by incubation at room temperature for 5 min after which stained cells were washed twice and suspended in DMEM + 10% FCS to a final concentration of 5 x 10^5/ml. Overnight activated PBNK cells and UCB-NK cells were washed with PBS and suspended in Glycostem Basal Growth Medium (GBGM) + 2% FCS to a final concentration of 5 x 10^5/ml. Target cells were co-cultured with effector cells at an E:T ratio of 1:1 in a total volume of 250 µl in 96-well flat-bottom plates (5 x 10^4 targets in 100 µl of DMEM + 10% FCS incubated with 5 x 10^4 effectors in 100 µl of GBGM + 2% FCS, further supplemented with 25 µl of GBGM + 2% FCS and DMEM + 10% FCS medium). NK cells and target cells alone were plated out in triplicate as negative controls. Target cells were coated with 5µg/ml cetuximab (Merck, Darmstadt, Germany) for 1h at 4°C. To measure degranulation of NK cells, anti-CD107a PE (Miltenyi Biotech) was added in 1:20 dilution at the beginning of the assay. After incubation for 4hr at 37°C, cells were harvested and stained with CD56 APC Vio 770 (1:25) and CD16 APC (1:25) (Miltenyi Biotech) and 7AAD (1:500) (Sigma Aldrich). Degranulation of NK cells was measured by detecting cell surface expression of CD107a.

In vivo studies

The $EGFR^+RAS^{mut}$ SW480 cell line and $EGFR^{+++}RAS^{wt}$ A431 cell line were stably transduced with Gaussia Luciferase (Gluc) for *in vivo* studies. Lentiviral (LV) supernatant of Cerulean Fluorescent Protein (CFP) positive Gluc virus (LV-CFP-Gluc) was kindly provided by Dr. Tom Würdinger[26]. SW480 and A431 cells with Gluc expression of 95% were used for mouse studies.

Immunodeficient BRGS mice (BALB/c *Rag2*^{tm1Fwa} *Il2rg*^{tm1Cgn} *Sirpa*^{NOD}) were used in this study. Twenty-four adult mice (male, 8 weeks old) received an intravenous (i.v) tail vein injection

with 0.5 x 106 SW480 Gluc cells at day 0 and were randomized into 4 groups. Group A only received SW480 cells, group B received SW480 in combination with cetuximab intraperitoneally (i.p., 0.5 mg, days 1, 4, 7), group C received SW480 in combination with UCB-NK i.v. (1 x 107, days 1, 4, 7), and group D received SW480 cells in combination with UCB-NK i.v. (1 x 10^7, days 1, 4, 7) and cetuximab i.p. (0.5 mg, days 1, 4, 7). Groups C and D received i.p. 0.5µg IL-15 + 7.5µg IL-15Rα every 2-3 days from day 0 till day 14. Further, three adult mice received i.v tail vein injection of 0.5 x 10^6 A431 Gluc cells at day 0 and were treated with 0.5mg cetuximab (i.p., 0.5mg days 1, 4, 7), was used as a cetuximab efficacy control. Treatment effects were monitored using blood Gluc levels and bioluminescence imaging (BLI). All manipulations of BRGS mice were performed under laminar flow conditions.

Blood Gluc quantification *in vitro*

Secreted Gluc was measured according to a protocol described previously[27]. Ten µl of blood was collected by capillarity into EDTA containing Microvette® CB tubes. Blood samples were distributed in 96 well black plates then mixed with 100µl of 100mM Gluc substrate native coelenterazine in PBS (P.J.K. GmbH; Kleinblittersdorf, Germany) and 5 minutes later light emission was quantified. Blood that was withdrawn before tumor inoculation served to determine a baseline value. Measurements were done twice a week until day 35. Gluc activity was measured using IVIS spectrum luminescence detector (PerkinElmer, Villebon-sur-Yvette, France). Data obtained were quantified using Living Image 4.0 software (PerkinElmer, Villebon-sur-Yvette, France).

Bioluminescence imaging *in vivo*

Mice were anesthetized using isofluorane gas in an induction chamber at a gas flow of 2.5 pm. Retro orbital injection of coelenterazine (4mg/kg body weight) was administered and mice were placed in the anaesthesia manifold inside the imaging chamber and imaged within 5 mins following substrate injection. Mice were placed into the light chamber and overlay images were collected for a period of 15min using IVIS spectrum *in vivo* imaging system (PerkinElmer, Villebon-sur-Yvette, France). Images were then analysed using Living Image 4.0 software (PerkinElmer, Villebon-sur-Yvette, France).

Ethics statement

Animals were housed in isolators under pathogen-free conditions with humane care and anaesthesia was performed using inhalational isoflurane anaesthesia to minimize suffering. Experiments were approved by the Institut Pasteur's ethical committee for animal use in research, Comité d'étique en expérimentation animale (CETEA) #89, protocol reference # 2007–006 and validated by the French Ministry of Education and Research (Reference # 02162.01).

Statistical analysis

Data were analyzed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). Differences between conditions were determined using one-way ANOVA or two-way ANOVA with multiple comparisons between column means, unpaired-t-test and log rank (Mantel-cox) test as deemed appropriate. A p-value of <0.05 was considered statistically significant.

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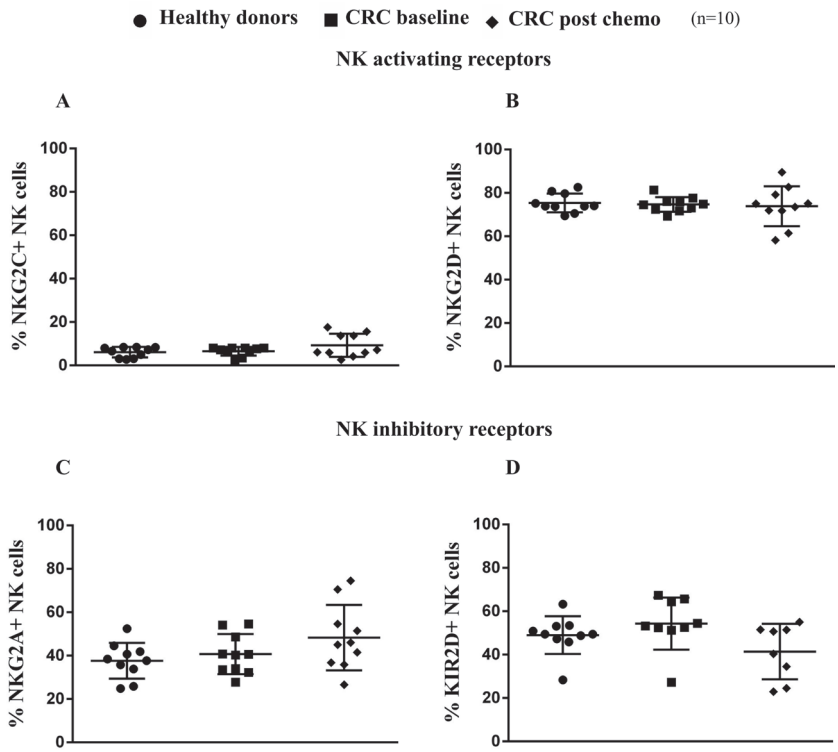
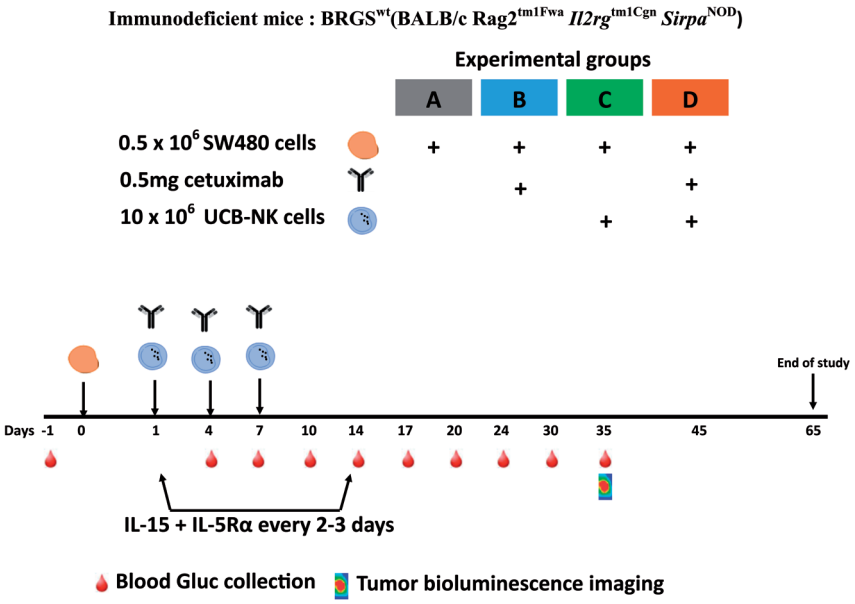


SUPPLEMENTARY FIGURE 1: Expression profiles of NK cell receptors in CRC patients

Resting NK cells within PBMC populations from healthy controls and from mCRC patients at baseline and after 1 cycle of chemotherapy were monitored for the NK activating receptors NKG2D (A), NKG2C (B) and NK inhibitory receptors NKG2A (C) and KIR2D (D). Data represent mean ± SEM from 10 mCRC patients and 10 healthy controls. Statistical difference was calculated with one-way ANOVA, multiple comparison between column means.



SUPPLEMENTARY FIGURE 2: Schedule of *in vivo* BRGS^{wt} mouse experiments.
BRGS^{wt} mice were divided in 4 groups of 6 mice each. SW480 (A) is the control group, followed by treatment groups SW480 + cetuximab (B), SW480 + UCB-NK (C) and SW480 + UCB-NK + cetuximab (D). 0.5 x 106 Gluc transduced SW480 cells per mouse were administered i.v. to all groups at day 0. On day 1 (dose I) post tumor injection, Groups B and D mice were treated with 0.5mg cetuximab i.p. and Groups C and D were infused i.v. with 10 x 106 UCB-NK cells. Same doses of cetuximab and UCB-NK cells were again administered on day 4 (dose II) and day 7 (dose III) to the respective groups. 0.5µg IL-15 was mixed with 7.5 µg IL-15Rα and administered to the UCB-NK-treated groups on days 1, 4, 7, 10 and 14. Treatment effects were monitored using blood Gluc levels and BLI imaging studies, 10µl of blood was withdrawn twice a week and tumors were imaged 4 weeks after end of treatment.

CHAPTER 6

General discussion and Future Prospects

Adapted in part from the publication
"The rise of allogeneic Natural Killer cells as a platform for cancer immunotherapy: Recent innovations and future developments"

Veluchamy JP, Kok N, van der Vliet HJ, Verheul HMW, de Gruijl TD, Spanholtz J.

Frontiers in Immunology, 2017

Allogeneic NK cells from human umbilical cord blood cells

NK cells represent a promising immunotherapeutic treatment option for cancer. Stem cell progenitors from cord blood offer a unique clinically applicable platform for the expansion and differentiation of cytotoxic NK cells. The low immunogenicity of cord blood cells strongly reduces the risk of relapse and GvHD after transplantation[1]. Considering the advantages of using cord blood, Glycostem Therapeutics, a clinical stage biotech company, which in the last decade has developed a flexible platform technology to expand and differentiate NK cells from CD34+ cells[2], upgraded this into a large scale GMP UCB-NK platform for clinical implementation (oNKord®)[3]. UCB-NK cells were infused at up to 30 x 10^6 cells/kg/bodyweight in elderly AML patients, resulting in excellent safety and early signs of efficacy in a phase I trial. Infused oNKord® cells showed active migration to the marrow and further matured in the absence of any exogenous cytokine injections. This confirms previous findings from a preclinical model, showing migration to the bone marrow and upregulation of KIRs and CD16a *in vivo* as well as antileukemic activity[4]. oNKord® is well characterized and was found to have a similar functionality and gene expression profile as PBNK cells[5]. Furthermore, as demonstrated in this thesis, oNKord® is highly cytotoxic against solid tumor targets such as cervical cancer cells, in which killing was independent of HLA expression levels, tumor histology and HPV types[6], or colorectal cancer cells, in which killing was independent of tumor EGFR levels, and RAS and RAF mutations[7], thus paving the way for oNKord® as immunotherapy for advanced solid tumors. It has become clear from the *in vitro* and *in vivo* experiments described in this thesis, using cervical, colon and epidermoid carcinoma cells, that activated allogeneic NK cells can be used to efficiently target different solid tumor types, with superior toxicity exhibited by UCB-NK cells. However, not all tumor targets are sensitive to NK cell killing and these underlying differences need to be addressed to improve the clinical efficacy of NK cell-based cancer immunotherapy. Several strategies are being explored that aim to improve the anti-tumor potency of NK cells, which may also be applied to the UCB-NK platform. A summary of currently applied and newly developed NK cell potentiating lead products of biotech companies and potential gene modification approaches are discussed below.

Approaches to augment NK cell functions as pursued by biotech industries

As reviewed in chapter 1, various clinical trials have been published, mainly initiated by academia, proposing allogeneic NK cells as an effective therapeutic option. As a result of these studies, interest in NK cell-based immunotherapy strategies has been engendered in an increasing number of biotech companies. Clinical trials conducted in academia are often restricted to phase I or II, as progression of experimental therapies to Phase III clinical trials and further on to commercialization and marketing requires a level of funding that surpasses the capacity of academic institutions. The financing of market enabling studies

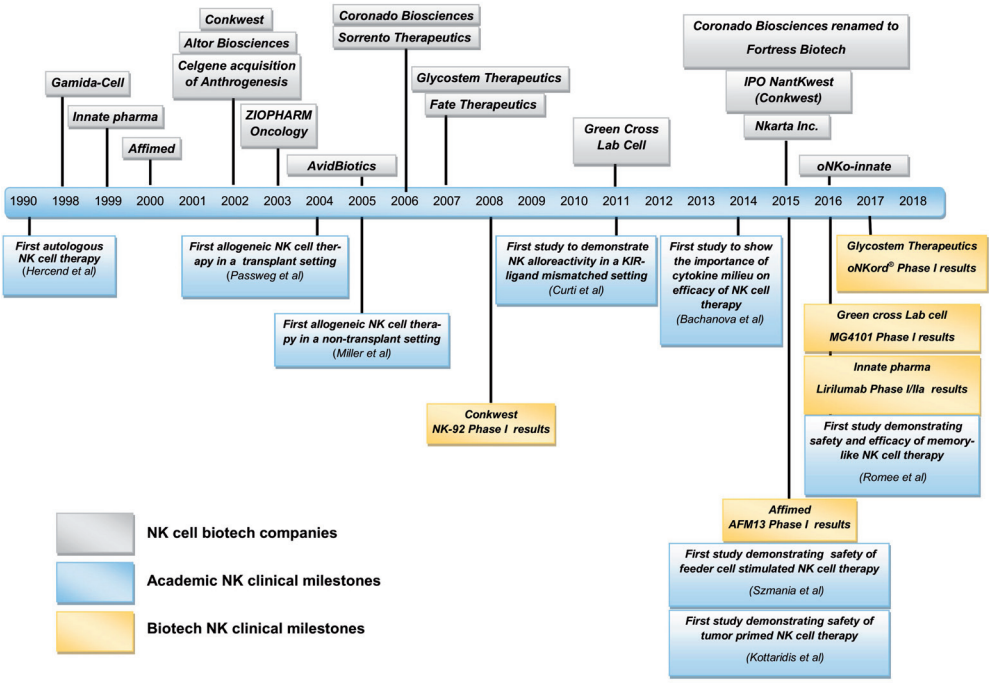


FIGURE 1: Summary of NK cell biotech companies and landmark clinical trials from academia and biotech industries.

is coming mainly from industry. Although NK cells can be effective in some types of cancer as a monotherapy, considering their heterogeneity, complex networking and the inherent adaptability of several tumors to evade killing by immune cells, it is likely necessary to improve on the efficacy of currently available NK cell products. In this respect, it is worthwhile to consider combinatorial approaches of different treatment strategies involving NK cells. A summary of biotech companies involved in NK cell research is listed in Table 1 and Figure 1. Here, we review for a selected group of NK cell companies, which develop NK cell specific treatments, the underlying scientific principles and findings of their product pipelines, revealing highly innovative concepts that herald future clinical applications.

Cytokines to enhance NK cell functions

To improve the anti-tumor activity of autologous NK cells, systemic administration of clinical grade recombinant IL-2 (rIL-2) and single chain IL-15 (scIL-15) have been used in high doses and this has resulted in severe grade 3/4 toxicities[8-10]. Since then their safety and efficacy have been tested in low doses following NK cell adoptive transfer in cancer patients[11-13]. However, IL-2 resulted in expansion and mobilization of inhibitory T-regs, severely limiting

NK cell cytotoxicity[14]. This shifted the focus to the use of IL-15 for clinical trials involving NK cells. Currently more potent and advanced heterodimeric IL-15, which has a longer half-life than scIL-15, is being tested in several studies[15]. IL-15 is known to be more effective in membrane-bound form (i.e. bound to its receptor), engaging target immune cells in a cell contact dependent manner. Campana and his team (from Nkarta Therapeutics) addressed this by stably transducing the membrane bound IL-15 (mbIL-15) gene into proliferating PBNK cells which were stimulated with K562-mb15-41BBL. mbIL-15 resulted in increased survival, proliferation and enhanced cytotoxic functions of NK cells[16]. Further, Cyto-Sen Therapeutics compared mbIL-15 to K562-based artificial APCs with mbIL-21. From their findings, it was evident that mbIL-21 NK cells have a significantly higher expansion and proliferation ability compared to mbIL-15 NK cells[17]. Cyto-Sen also developed plasma membrane particles (PM21) engineered from K562-mb21-41BBL cells and found that these PM21 particles stimulated efficient NK cell expansion in PBMC samples from AML patients[18].

Altor BioSciences came up with an alternative approach to overcome the limitations associated with the short-half-life of recombinant IL-15. It developed an IL-15 super agonist known as ALT-803. It consists of a human IL-15 mutant N72D variant which is stably complexed with a soluble human IL-15Rα sushi-Fc dimer protein. Enhanced biological activity of ALT-803 was reported in several pre-clinical studies showing durable anti-tumor activity in various solid and haematological malignancies[19-22]. Furthermore, ALT-803 facilitated expansion of effector and migratory NK cell subsets and significantly decreased the metastatic activity of tumor cells in a murine colon cancer pulmonary metastasis model[23]. ALT-803 stimulated primary human NK cells to exhibit increased degranulation, IFNγ production and ADCC when exposed to B cell lymphoma cell lines coated with IgG_1 therapeutic anti-CD20 mAbs[24]. Several clinical trials are currently ongoing with ALT-803 as monotherapy in patients with advanced solid tumors, haematological malignancies, and AIDS as summarized in Table 1.

Priming NK cells to enhance tumor killing

Mark Lowdell and his team, proposed that for an NK cell to be able to kill tumor cells, it requires a priming and triggering signal. NK cells failing to kill tumor cells, though they are exposed to the triggering signal, would remain inactive due to the absence of a priming ligand. To address this, Fortress Biotech (previously known as Coronado Biosciences) developed a technology to increase NK cell tumor killing using cell lysates from the leukemia cell line CTV-1, known as CNDO-109, to prime NK cells. A phase I/II clinical trial of activated PBNK cells from haploidentical donors co-incubated with CNDO-109, infused at doses of up to 3 x 10^6 kg/recipient/body weight was tolerable without any adverse reactions. Out of 7 evaluable patients, 4 remained disease relapse free for more than 1 year [25].

Another NK cell activating product is ENKASTIM-ev, developed by Multimmune GmbH, which mimics the functions of heat shock protein 70 (Hsp70). ENKASTIM-ev resulted in NK specific activation and actively targeted Hsp70 expressing tumors. Safety of Hsp70 activated autologous NK cells has been documented in a phase I study in patients with metastatic colorectal and non-small cell lung cancer[26].

Enhancing NK cell homing functions

Gamida-Cell developed a feeder cell free NK cell culture and expansion system containing nicotinamide (NAM) to generate NK cells from PBMC apheresis products. Nicotinamide, a derivative of vitamin B3, serves as a potent inhibitor of NAD dependent enzymes. Results from *in vivo* studies in mice showed that PBNK cells expanded with NAM in feeder free cultures exhibited increased homing potential towards lymphoid organs, with a significant increase in the expression of CD62L (L-selectin) compared to cultures without NAM[27].

Genetic modification of NK cells

In addition to successful expansion, differentiation and demonstrable anti-tumor effects of NK cells, NK cell tumor targeting can be made more specific by employing chimeric antigen receptors (CARs) as demonstrated for T cell adoptive transfer strategies[28]. CARs are recombinant Ab-based molecules that upon expression in immune effector cells bind antigens of interest on target cells, resulting in immune activation and enhanced immune effector cell survival through specific intracellular signalling motifs fused to the antigen binding domain (usually a single-chain Fv fragment [scFv]). PBNK-CARs against breast cancer (HER-2), neuroblastoma (CD244) and CD19+ B-cell precursor cell ALL (CD19)[29] have demonstrated efficacy in preclinical studies, while two clinical trials are ongoing using modified haplo-identical PBNK cells with anti-CD19 CARs in B cell malignancies (NCT00995137 and NCT01974479)[28]. NantKwest, is actively involved in enhancing the functions of its lead product, parental NK-92 cells (activated NK cells, aNK), through gene modifications employing CARs to make them target specific. NK-92 CARs (taNK) are developed against tumor markers in neuroblastoma (GD2), melanoma (GPA7)[30], breast cancer (EpCAM, HER-2, EGFR)[31,32], multiple myeloma (CS1[33], CD138[34]) and leukemias (CD19, CD20)[35] and have shown efficacy in preclinical studies. In an alternative approach NK-92 cells have also been modified to express CD16a (high affinity NK cells, haNK) to promote ADCC[36]. NantKwest has also partnered with Sorrento Therapeutics to develop NK-92 CARs targeting programmed death-ligand1 (PD-L1)[37] and receptor tyrosine kinase like orphan receptor 1 (ROR-1)[38].

Besides specific targeting of tumor antigens and strategies to promote ADCC, Nkarta therapeutics developed NKG2D CARs (NKG2D- CD3ζ - DAP10) using NK-92 cells and PBNK cells, which exhibited enhanced cytotoxicity against osteosarcoma and hepatocellular

carcinoma when compared to activated and expanded PBNK cells[39,40]. mRNA based genetic engineering has been used to enhance migration of NK cells to tumors.

Apart from gene modification, gene editing is also widely used to overexpress or knock out genes of interest to augment NK cell function. Expression of HLA-A on allogeneic NK cells leads to rejection of allogeneic NK cells by the recipient's T and NK cells. Cooper and colleagues from Ziopharm Oncology, used zinc finger nuclease (ZFN) technology to remove HLA-A sequences from allogeneic NK cells, thus enabling these immune effector cells to escape rejection from recipient T cells. However, in that case there is yet a high probability of being attacked by endogenous NK cells targeting HLA-A negative allogeneic cells. This was further addressed by retaining HLA-B and HLA-C genes in donor NK cells[41-43]. To increase NK cell persistence *in vivo,* scientists at oNKo-innate identified a group of proteins called suppressor of cytokine signalling (CIS, SOCS 1-7), which negatively regulate cytokine signalling pathways. SOCS1 and SOCS3 bind to JAK1, JAK2 and TYK2 molecules and inhibit JAK activity. Similarly, CIS protein binds to JAK1, and suppresses IL-15 signalling in NK cells. It became evident from *in vivo* studies in mice with *Cish*$^{-}$/$^{-}$ knockout NK cells that loss of CIS led to prolonged IL-15 signalling, resulting in an increased proliferation, survival and functionality of NK cells[44].

Fc optimized monoclonal antibodies

The potential of NK cells to mediate ADCC with therapeutic mAbs has been well described over the years[45]. However, concerns have been voiced based on results from certain clinical trials, showing that polymorphisms in NK CD16 (V158V, V158F and F158F) could influence the efficacy of mAb treatment and ADCC[46]. To address this issue and limit the variations between different CD16 sequences, Fc glyco-engineered (defucosylated) mAbs with enhanced binding affinities to NK CD16a were developed. The Fc optimized anti-CCR4 mAb mogamulizumab[47] (Kyowa Hakko Kirin) has entered Phase III clinical testing in patients with adult T cell leukemia, emerging as the lead NK cell ADCC product to reach the market soon. Fc optimized anti-CD20 mAbs obinutuzumab (Genentech)[48] and ocaratuzumab (Mentrik Biotech, LLC)[49] are currently tested in patients with chronic lymphocytic leukemia and follicular lymphoma. Similarly, the Fc optimized anti-EGFR mAb imgatuzumab (Roche Glycart) is tested in phase I/II clinical trials for head and neck cancer and in KRAS mutant colorectal cancer[50,51]. Although Fc engineered mAbs address NK-mAb binding issues, reports of serious side effects, like from the imgatuzumab study[52], have made the scientists rethink this strategy and call for the careful study of the advantages and disadvantages of this approach.

Bispecific antibodies

In the last decade, several bispecific and trispecific Ab platforms, simultaneously targeting immune cells and tumor cells, have been developed in the field of cancer immunotherapy[53]. To date, the majority of bispecific Abs that has been developed targets T cells, whereas only a limited number of bispecific approaches targets NK cells[54]. Affimed is a clinical stage pharmaceutical company developing bifunctional antibodies that recruit immune cells such as T and NK cells to tumor sites. These bispecifics (TandAbs) are tetravalent in nature, thus offering four binding sites, two aimed at tumor antigens and two aimed at immune cells. Currently, Affimed's AFM13 that targets CD30 on cancer cells and CD16a on NK cells is in clinical phase II testing in patients with Hodgkin's lymphoma. In phase I studies AFM13 was found to be safe and well tolerated, and resulted in an overall response rate of 23%. Furthermore, AFM13 treatment resulted in an increase in NK cell activation and a decrease in soluble CD30 levels in peripheral blood (NCT01221571)[55]. Further, two other bispecific CD16a based tumor targeting antibodies are in preclinical phase development, i.e. AFM22 and AFM24 that bind to EGFRvIII expressed by several solid tumors, including glioblastoma (GBM), and wild-type EGFR respectively. Another promising NK cell focused bispecific platform is developed by AvidBiotics to target tumors that evade NK killing via downregulation or shedding of the NKG2D ligand MICA, which is a major limiting step in NK mediated tumor targeting. To overcome this, AvidBiotics designed MicAbody proteins that bind to the NK cell NKG2D receptor with high affinity. Further, this MicAbody was engineered with an additional binding site to target tumor antigens of interest, thus enabling recruitment of NK cells to tumors[56].

NK cell checkpoint inhibitors

Another strategy to increase NK cell functionality is the disruption or blocking of NK inhibitory signals. Innate Pharma is a clinical stage pharmaceutical company currently focused on developing NK cell checkpoint inhibitors. Lirilumab (IPH2102/ BMS 986015) is a fully humanized IgG_4 anti-KIR mAb against the inhibitory KIRs KIR2DL1, L2 and L3, which are expressed predominantly on NK cells and on some T cells. Lirilumab induced significant anti-tumor activity of NK cells against HLA-C expressing tumor cells, contributing to increased survival in lirilumab treated mice[57]. Similar to KIRs, the NK cell inhibitory receptor NKG2A binds to its ligand HLA-E on tumor cells resulting in an inhibition of NK cell function. HLA-E is overexpressed in colon, cervical and ovarian cancers, thus serving as an escape mechanism for NK killing in these tumors[58,59]. The anti-NKG2A mAb monalizumab, was developed to block the interaction between NKG2A and HLA-E and is currently under clinical investigation. IPH4102, which targets KIR3DL2, is under phase I clinical investigation in cutaneous T cell lymphoma (CTCL). Clinical trials testing lirilumab, monalizumab and IPH4102 are listed in Table 1.

Tumor disruptive technology aiding NK tumor recognition

NOXXON Pharma is targeting the chemokine receptor CXCL12, with the aim of increasing the sensitivity of tumor cells to drugs and immune cells. Their product NOX-A12 functions as a CXCL12 inhibitor and enables the release of CXCL12 from the surface of tumor stromal cells and blocks its interaction with cell surface receptors CXCR4 and CXCR7. This mechanism facilitated the mobilization of CXCR4 expressing tumor cells from their tissue niches to areas, where they become more easily accessible by NK cells or T cells[60,61]. Using tumor spheroids, increased mobilization of T and NK cells towards tumor cells in the tumor microenvironment was demonstrated. NOX-A12 also enhanced NK killing of obinutuzumab coated Raji cells *in vitro,* mediated by ADCC[62].

NK cells from iPSCs

Generally applicable NK cell platforms, like UCB-NK, are very attractive for clinical therapeutic purposes. In recent years, NK cells generated from induced pluripotent stem cells (iPSC-NK) and human embryonic stem cells (hESC-NK) have been gaining more interest as an NK cell therapeutic product. Fate Therapeutics developed a platform technology to generate NK cells from iPSC. hESC/iPSC were made into aggregates by centrifugation to form so-called embryoid bodies (spin EBs)[63], giving rise to hematopoietic progenitor cells expressing CD34 and CD45, which were then differentiated into mature NK cells using a specific cytokine cocktail. iPSC/hESC derived NK cells were shown to express common NK cell markers, such as KIRs, CD16, NKp44, NKp46, NKG2D and TRAIL and were cytotoxic against several haematological and solid tumor cells in vitro[64,65]. In the next stage, iPSC/hESC derived NK cells were successfully expanded using IL-2 and K562-based aAPCs with membrane-bound IL-21 to generate sufficiently high numbers for clinical applications[66].

Conclusions

From the information obtained from NK cell clinical trials, we conclude that adoptive transfer of allogeneic NK cells in a non-transplant setting is safe and shows early signs of clinical efficacy against haematological and certain solid tumors. Current data are mostly based on phase I clinical trials and hence it is still too early to get an overall picture of NK cell alloreactivity in different kinds of cancer. Most of the clinical studies conducted so far have used primary NK cells but with limited efficacy, pointing to the need to improve the functionality of these NK cells after their transfer to patients. The growing opportunities to augment NK cell functions have attracted several biotech companies to invest in NK cell research, spearheading NK therapy development with different innovative approaches. This also stresses the need for combining adoptive transfer of allogeneic NK cells with NK function-augmenting products to achieve a maximum anti-tumor effect. As NK cells are safe to infuse, the use of CAR-NK cells may be instrumental in providing a much safer but

still very effective platform, to bring CAR-based therapies to broader clinical applications. It may also facilitate effective tumor targeting of NK cells. oNKord® and iPSC derived NK cells could serve as alternative allogeneic platforms to develop CAR-NK products, beside NK cell lines. In a solid tumor setting, NK cells are challenged by several factors that affect their homing and penetration into the tumor tissues. Moreover, they should achieve and maintain an activated effector state, even in the face of immune suppressive conditions, that are prevalent in patients with cancer. To overcome these bottlenecks in NK therapy of solid tumors, a plethora of creative solutions are being pursued by numerous research labs as well as by biotech companies in clinical or close-to-clinical phase. Strategies to enhance NK cell functions from leading NK cell products are summarized in Figure 2. With all these exciting developments, NK cells are set to make a considerable impact on the future treatment of patients with haematological as well as with solid tumors.

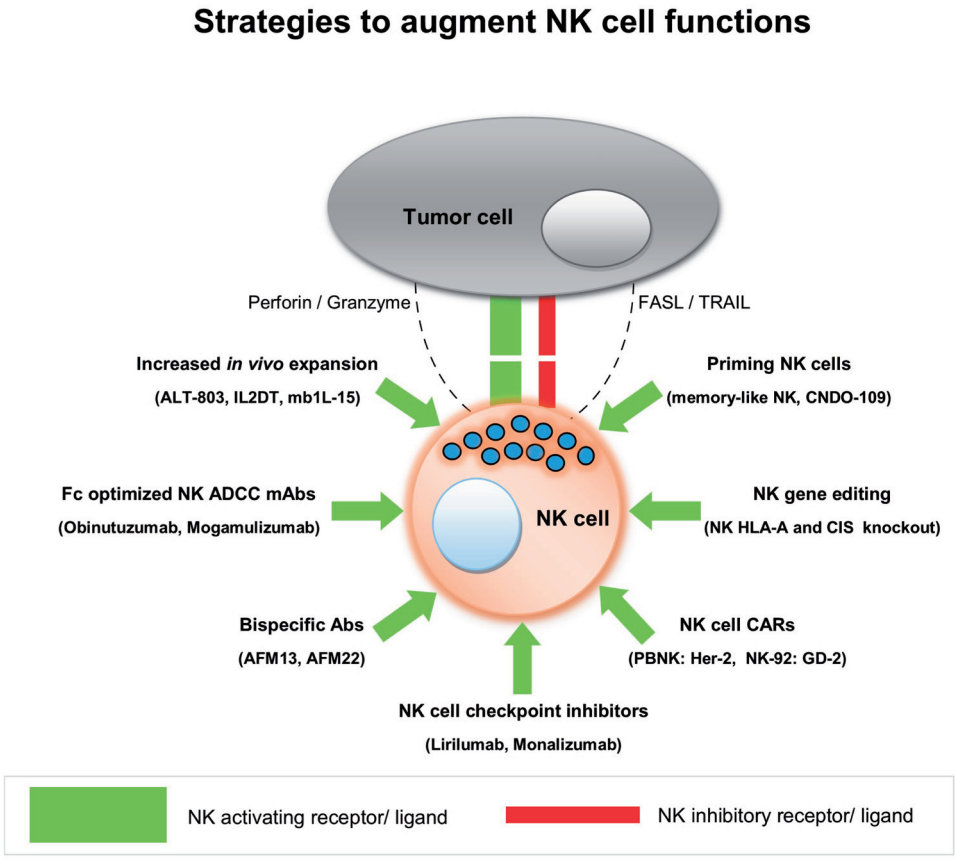


FIGURE 2: A Summary of various strategies to augment NK cell functions.

TABLE 1: List of biotech NK cellular therapies and NK cell function enhancing compounds

Company	NK cell product	Product characteristics
Fortress Biotech Inc.	CNDO-109	Tumor primed NK cells
Multimmune GmbH	ENKASTIM-ev	a synthetic peptide which mimics Hsp70 and activates NK cells *ex vivo*
Glycostem Therapeutics	oNKord®	NK cells derived from umbilical cord blood (UCB) progenitor cells
NantKwest Inc.	Activated NK-92 cells (aNK cell)	IL-2 dependent tumor cell derived NK cell line
	High affinity NK cells (haNK)	aNK cells genetically modified to express CD16 for ADCC with therapeutic mAbs
	Target - activated NK cells (taNK)	aNK cells genetically modified to express CARs
Green Cross Lab Cell	MG4101	Ex vivo expanded NK cells derived from CD3 depleted unrelated donors
Gamida Cell	NAM-NK cells	Nicotinamide based PBNK cell culture system
Celgene Cellular Therapeutics	NK cells	NK cells derived from UCB and placenta.
Fate Therapeutics Inc.	iNK cells	NK cells derived from induced pluripotent stem cells
Sorrento Therapeutics Inc	CARs to enhance tumor homing of NK-92 cells	NK-92 cells CAR targeting programming death ligand-1 and NK-92 CAR targeting receptor tyrosine kinase like orphan receptor to increase NK-92 tumor homing.
Nkarta Therapeutics	NKG2D CARs	NKG2D CARs developed with NK-92 and PBNK to enhance the functions of NKG2D receptor in NK cells
Ziopharm Oncology Inc.	HLA gene editing	Zinc finger nuclease technology to delete HLA-A sequences from allogeneic NK cells, allowing them to evade recipient T cell killing.
Kyowa Hakko Kirin	Mogamulizumab	Fc optimized anti CCR4 CD20 mAb
Genentech	Obinutuzumab	Fc optimized anti CD20 mAb
Mentrik Biotech, LLC	Ocaratuzumab	Fc optimized anti CD20 mAb
Roche Glycart	Imgatuzumab	Fc optimized anti EGFR mAb
Affimed N.V.	AFM13	Bispecific antibody binding to CD16a on NK cells and CD30 on tumor cells
	AFM22	Bispecific antibody binding to CD16a on NK cells and epidermal growth factor receptor (EGFR) vIII on tumor cells
	AFM24	Bispecific antibody binding to CD16a on NK cells and wild type EGFR on tumor cells

Disease target	Product stage
AML	Phase I/II
Metastatic colon and non-small cell lung cancer	Phase II
AML and Solid tumors	Phase I (AML)
Solid tumors and haematological malignancies	Phase I
Ideally in combination with IgG_1 therapeutic mAbs in solid tumors (ex: cetuximab) and haematological malignancies (ex: rituximab)	Preclinical
NK-92 CARs are developed targeting tumor antigens in neuroblastoma, melanoma, breast cancer, multiple myeloma and leukemias	Preclinical
Solid tumors and lymphoma (NCT01212341)	Phase I
Solid tumors and haematological malignancies	Preclinical
Solid tumors and haematological malignancies	Preclinical
Solid tumors and haematological malignancies	Preclinical
Solid tumors and haematological malignancies	Preclinical
Osteosarcoma and hepatocellular carcinoma	Preclinical
Solid tumors and haematological malignancies	Preclinical
Cutaneous T cell lymphoma	Phase III
Chronic Lymphocytic leukemia	Phase II
Chronic lymphocytic leukemia	Phase II
Head and neck and KRAS mutant colorectal cancer	Phase I / II
Hodgkin's lymphoma and lymphomas	Phase II
Head and neck and solid tumors	Preclinical
EGFR expressing solid tumors	Preclinical

Company	NK cell product	Product characteristics
Innate Pharma S. A.	Lirilumab	mAb to block NK cell inhibitory signalling from KIRs (KIR2DL1 -3).
	Monalizumab	mAb to block NK cell inhibitory receptor NKG2A
	IPH4102	mAb to block NK cell inhibitory receptor KIR3DL2
	IPH4301	mAb to target NKG2D ligands MICA/MICB and it also mediates ADCC with NK cells
Altor Biosciences corporation	ALT-803	IL-15 super agonist reported to stably express IL-15. Increases NK cell proliferation *in vivo*, also enhances expansion of migratory NK subsets.
NOXXON Pharma	NOX-A12	Functions as chemokine receptor CXCL12 inhibitor, enables the release of CXCL12 from the surface of tumor stromal cells, thus facilitating migration of tumor cells towards NK cells.
AvidBiotics	MicAbody proteins	Dual role: binds to NKG2D receptor in NK cells and to target antigens of interest simultaneously

Abbreviations: Killer cell immunoglobulin-like receptors (KIRs), Antibody dependent cell mediated cytotoxicity (ADCC), aNK-activated NK cells, haNK-high affinity NK cells, taNK-target activated NK cells, monoclonal antibodies (mAbs), Nicotinamide (NAM), Chimeric antigen receptors (CARs), Epithelial cell adhesion molecule (EpCAM), Acute Myeloid Leukemia (AML), Killer cell immunoglobulin like receptor two domains long cytoplasmic tail 1-3 (KIR2DL1-3), Killer cell immunoglobulin like receptor three domains long cytoplasmic tail 2 (KIR3DL2). MHC class-I-chain related protein A and B (MICA/MICB), Human immunodeficiency virus (HIV), non -small-cell lung cancer (NSCLC), Bacillus Calmette Guerin (BCG).

Disease target	Product stage
As monotherapy (Phase II, NCT02399917), with nivolumab (Phase I, NCT01592370), with ipililumab (Phase I, NCT01750580), 5-azacytidine (Phase I, NCT02399917), with nivolumab + 5-azacytidine (Phase II, NCT02599649), with elotuzumab (NCT02252263) and with rituximab (Phase I, NCT02481297).	Phase I/ II
As monotherapy (Phase I/II, NCT02459301, NCT02331875 with cetuximab (NCT02643550), with ibrutinib (NCT02557516) and with durvalumab (NCT02671435).	Phase I/II
As monotherapy in cutaneous T cell lymphoma (NCT02593045)	Phase I
Solid tumors and haematological malignancies	Preclinical
Advanced solid tumors (NCT01946789), multiple myeloma (NCT02099539), HIV patients (NCT02191098), with nivolumab in NSCLC (NCT02523469), with rituximab (NCT02384954) in B cell Non-Hodgkin Lymphoma (NHL) (NCT02384954), with (BCG) in Non-Muscle Invasive Bladder Cancer (NCT02138734), with chemotherapy drugs gemcitabine and Nab-paclitaxel in advanced pancreatic cancer (NCT02559674).	Phase I/II
Solid tumors and Multiple Myeloma	Preclinical
Solid tumors and haematological malignancies	Preclinical

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CHAPTER 7

Summary

SUMMARY

The active role of NK cells in controlling cancer has been intensively studied. From the clinical trial summary provided in Table 2 from Chapter 1, it is evident that adoptive transfer of NK cells in a non-transplant setting for hematological malignancies is safe and has been shown to mediate a Graft versus Tumor (GvT) effect without causing Graft versus Host Disease (GvHD).

It is well known that NK cell functions are tightly regulated by the balance between arrays of NK cell activating and inhibitory receptors. In several cancer types, NK cell activating receptors and are often down-regulated, thus limiting NK cell target killing. Hence it is essential to have a deeper understanding of the expression levels of relevant NK cell receptors and the functional status of NK cells in tumor conditions. This led us to the develop two eight-color NK cell flowcytometry (FACS) panels, one to study the NK cell phenotype and the other to study the NK cell function in PBMC (or single-cell tumor) samples from multiple centers as described in Chapter 2. The NK cell FACS panels were designed, optimized and tested across three different centers using three different flow cytometers with comparable configuration, thus providing a unique platform to generate comparable and reproducible data for multicenter clinical trials. Furthermore, this study also emphasized that cryopreserved NK cells are suitable for studying NK cell phenotypes and functions including NK cell mediated ADCC.

In Chapter 3, to treat anti-EGFR/cetuximab and immunotherapy-resistant cervical cancer cells, allogeneic NK cell-based therapy was explored. The cytotoxic effects of UCB-NK cells and activated PBNK cells were compared either as monotherapy or in combination with cetuximab in an in-vitro set-up. A panel of ten cervical cancer cell lines with different histology and different HPV types were subjected to NK cell killing. All these cell lines expressed low to moderate levels of EGFR (except C33A, which was EGFR negative) and were wild type for the RAS gene, but failed to respond to cetuximab monotherapy. Upon performing NK cell cytotoxicity assays, it was evident that all cervical cancer cell lines were sensitive to NK-mediated killing, independent of tumor histology and HPV type. Interestingly, UCB-NK-mediated cytolysis rates were significantly higher than those achieved with PBNK alone and equalled those of PBNK + cetuximab. The superior cytotoxicity with UCB-NK cells correlated to their low expression levels of inhibitory KIRs, in keeping with the observed lack of inhibition by HLA-ABC expressed on the cervical tumor cells. These data point to the potential application of UCB-NK cells in the treatment of cervical cancer.

In Chapter 4, we addressed a major hurdle in the treatment of colorectal cancer, i.e. resistance against cetuximab therapy. RAS mutations in the EGFR signalling pathway have left nearly half of the metastatic colorectal cancer (mCRC) population, ineligible for anti-EGFR treatment. In our set-up, using allogeneic and highly activated PBNK cells, we could

induce effective killing of colon cancer cells irrespective of EGFR expression levels and RAS status. PBNK induced killing of $EGFR^+$ cells was significantly higher when colon cancer cells were coated with cetuximab, mediating ADCC. Importantly, PBNK cells were also highly cytotoxic to $EGFR^-$ colon cancer cells, which will obviously not respond to cetuximab therapy. The superior cytolysis observed for both cell lines and primary colon tumors, with variations in EGFR expression levels and RAS mutation status, indicates the potential of combined PBNK and cetuximab application in the treatment of cetuximab-resistant colon cancer.

In Chapter 5, we monitored the frequency and function of NK cells in mCRC patients before and after the first cycle of chemotherapy, and found that mCRC patients not only had a 20% lower frequency of NK cells in peripheral blood before the initiation of chemotherapy, but that this percentage further declined during chemotherapy. In addition to this quantitative defect in NK cells of mCRC patients, their cytotoxic capacity was also impaired. Of interest, though the cytolytic activity of NK cells of mCRC patients could be increased by cetuximab through ADCC, the level of cytotoxicity was still markedly reduced compared with that mediated by NK cells from healthy adult volunteers. These data suggest that adoptive transfer of fully functional NK cells might be of benefit to restore the NK effector cell pool in mCRC patients. For this purpose, the cytotoxic effects of two allogeneic NK cell products, i.e. activated PBNK and UCB-NK cells, were tested and compared *in vitro* against a panel of colon cancer cell lines. UCB-NK cells were found to exert superior cytotoxicity compared to PBNK cells, their cytotoxicity being comparable to that achieved when PBNK were additionally stimulated using cetuximab. These superior cytotoxic effects of UCB-NK cells were verified *in vivo,* where treatment with UCB-NK cells alone significantly reduced the tumor load in mice inoculated with $EGFR^+$ RAS^{mut} colon cancer cells. Of interest, this effect was not increased by the addition of cetuximab *in vivo*, which could be due to a sub-optimal up-regulation of CD16 cell surface expression levels on the adoptively transferred UCB-NK in the immunodeficient mice that was used for these studies. Importantly, as a clinical study in AML patients revealed that CD16a expression levels steadily increased on UCB-NK cells post-infusion, synergy between both approaches may be expected when applied clinically.

To summarize, through the studies described in this thesis we have demonstrated that UCB-NK cells have superior anti-tumor efficacy against epidermoid, colon and cervical cancers as compared to activated PBNK cells, and are equally cytotoxic as the combination of PBNK and cetuximab, demonstrating significant anti-tumor benefit against $EGFR^+$ RAS mutant and $EGFR^+$ BRAF mutant tumors. This novel UCB-NK expansion and differentiation technique from Glycostem allows the generation of large numbers of cytolytic UCB-NK cells that may overcome the limitations of current NK cell based adoptive transfer strategies and supplement the immune system with sufficient numbers of NK cells to mount an effective

anti-tumor immune response in immunosuppressed cancer patients. Soon, UCB-NK cell functions, and thereby antitumor efficacy, may be further improved by genetic modification or combination therapy approaches encompassing novel immune modulatory genes or agents as outlined in chapter 6.

NEDERLANDSE SAMENVATTING

NK cellen of “Natural Killer” cellen bieden een aangeboren bescherming tegen ziekteverwekkende indringers zoals virussen maar ook tegen kankercellen. In patiënten met kanker functioneren NK cellen vaak slecht door afweer onderdrukkende stoffen die door tumoren worden uitgescheiden. Eén manier om deze sabotage van de antikanker afweer te omzeilen is het intraveneus toedienen van grote aantallen NK cellen bij patiënten met kanker (adoptieve toediening). Een haalbare manier om aan voldoende grote aantallen NK cellen te komen voor de behandeling bestaat uit de expansie en differentiatie van NK cellen uit bloedvormende stamcellen uit navelstrengbloed (UCB-NK). In een eerste klinische trial in patiënten met acute myeloïde leukemie is toediening van uit navelstrengbloed afkomstige NK cellen veilig gebleken met eerste aanwijzingen van antikanker effectiviteit. In de studies beschreven in dit proefschrift werd onderzocht hoe adoptieve toediening van uit navelstreng verkregen NK cellen ook effectief ingezet zou kunnen worden bij patiënten met solide tumoren.

De rol van NK cellen in de bestrijding van kanker is intensief bestudeerd zoals samengevat in Hoofdstuk 1. Klinische studies hebben aangetoond dat adoptieve toediening van NK cellen buiten de transplantatie setting voor hematologische maligniteiten veilig is. Bovendien is er aangetoond dat infusie van NK cellen een Graft versus Tumor (GvT) effect initieert zonder de ernstige gevolgen van een omgekeerde afstotingsziekte (van transplantaat tegen de tumor, de zogenaamde Graft versus Host disease, GvHD).

Het is algemeen bekend dat NK cel functies strak gereguleerd zijn door een evenwicht tussen activerende en remmende NK cel receptoren. Verschillende soorten kanker kunnen verminderde expressie van activerende NK cel receptoren veroorzaken, wat leidt tot een verminderd vermogen van de NK cellen om tumorcellen te doden. Daarom is het van groot belang om een beter begrip te krijgen van de expressie niveaus van relevante NK cel receptoren en de functionaliteit van NK cellen in tumoren. Dit heeft ons ertoe gebracht om twee acht-marker NK cel panels voor flowcytometrie (FACS) te ontwerpen en operationeel te krijgen (beschreven in hoofdstuk 2): één om het NK cel fenotype te bestuderen en een andere om de NK cel functionaliteit te bepalen, zowel in bloed als in gedissocieerde tumor monsters. De NK cel FACS panels werden geoptimaliseerd en getest in drie verschillende centra, gebruikmakend van drie verschillende flowcytometers met vergelijkbare configuratie, Zo werd er een uniek platform gecreëerd voor het genereren van vergelijkbare en reproduceerbare gegevens afkomstig uit multicentrische klinische studies. Bovendien benadrukt deze studie ook dat levend ingevroren cel suspensies geschikt zijn om het NK cel fenotype en functionaliteit te bestuderen, zoals bijvoorbeeld NK cel gemedieerde antilichaam afhankelijke cellulaire toxiciteit (antibody dependent cellular cytotoxicity, ADCC).

In hoofdstuk 3 is allogene NK cel therapie bestudeerd als combinatie behandeling met anti-EGFR antistoffen (b.v cetuximab) voor tumoren van de baarmoederhals. De cytotoxische effecten van UCB-NK cellen en geactiveerde, uit perifeer bloed verkregen, PBNK cellen werden *in vitro* vergeleken als monotherapie of in combinatie met cetuximab. De NK cel therapie werd getest op tien baarmoederhalskanker cellijnen met verschillende histologische kenmerken en verschillende HPV-types. Deze cellijnen brachten in verschillende mate EGFR tot expressie (van geen tot hoge expressie) en hadden geen RAS mutatie. Niettemin waren alle resistent voor cetuximab als monotherapie. *In vitro* experimenten toonden aan dat alle baarmoederhalskanker cellijnen gevoelig waren voor NK-gemedieerde celdood, onafhankelijk van de histologie en het HPV type van de tumor. Een interessante uitkomst was dat de UCB-NK gemedieerde celdood van baarmoederhalskanker cellijnen aanzienlijk hoger was dan de celdood veroorzaakt door PBNK cellen en vergelijkbaar was met de mate van celdood ten gevolge van PBNK + cetuximab. De superieure cytotoxiciteit veroorzaakt door UCB-NK cellen was geassocieerd met lage expressie niveaus van NK-remmende receptoren, de zogenaamde Kiiling Inhibitory Receptors (KIRs), wat ook overeen kwam met de bevinding dat er geen remming door HLA-ABC op de baarmoederhals tumorcellijnen plaats vond. Deze resultaten wijzen op de mogelijkheid om UCB-NK cellen toe te passen in de behandeling van baarmoederhalskanker.

In hoofdstuk 4 hebben we een grote barrière in de behandeling van dikke darmkanker aan de kaak gesteld, namelijk resistentie tegen anti-EGFR/cetuximab therapie. Doordat bijna de helft van de patiënten met uitgezaaide darmkanker RAS mutaties heeft in de EGFR signalering, komen zij niet in aanmerking voor een anti-EGFR behandeling. Echter, door anti-EGFR te combineren met allogene en geactiveerde PBNK cellen, konden we een effectieve celdood van dikke darm kanker cellen induceren, ongeacht het niveau van EGFR expressie en de aan- aof afwezigheid van RAS mutaties. Het percentage van dode $EGFR^+$ tumorcellen na blootstelling aan PBNK cellen was aanzienlijk hoger wanneer dikke darm kankercellen tevens met cetuximab waren behandeld, leidend tot effectieve ADCC. Nog een belangrijke bevinding was dat PBNK cellen ook zeer cytotoxisch waren voor $EGFR^-$ dikke darm kankercellen, die niet op cetuximab therapie reageerden. Deze resultaten wijzen op de mogelijke potentie van PBNK cellen, al dan niet gecombineerd met cetuximab, in de behandeling van cetuximab-resistente dikke darmkanker.

In hoofdstuk 5 hebben we de frequentie en de functionaliteit van NK cellen in patiënten met uitgezaaide darmkanker (“metastatic colorectal cancer, mCRC”) voor en na de eerste behandeling van chemotherapie bepaald. We vonden dat patiënten met mCRC niet alleen een verlaagde NK cel frequentie hadden in het perifere bloed vóór de start van chemotherapie, maar dat de frequentie verder was afgenomen na chemotherapie. Naast deze kwantitatieve vermindering in NK cellen van de patiënten was de cytotoxische capaciteit ook verslechterd. Hoewel de cytolytische activiteit van de NK cellen middels

ADCC kon worden verhoogd na toevoeging van cetuximab, was het niveau van cytotoxiciteit nog steeds sterk verminderd in vergelijking met de cytotoxische capaciteit van NK cellen van gezonde volwassenen. Deze resultaten lijken aan te geven dat adoptieve toediening van volledig functionele NK cellen wellicht noodzakelijk zal zijn voor het herstel van de NK effector populatie bij patiënten met mCRC. Om dit te testen werden de cytotoxische effecten van twee allogene NK cel producten, namelijk geactiveerde PBNK en UCB-NK cellen vergeleken *in vitro* tegen een panel van dikke darm kanker cellijnen. UCB-NK cellen bleken in deze studie een superieure cytotoxiciteit te vertonen. In overeenstemming met onze bevindingen voor baarmoederhalskanker (Hoofdstuk 3) bleek de cytotoxische capaciteit van UCB-NK cellen vergelijkbaar met de capaciteit van PBNK-cellen in combinatie met cetuximab. Deze superieure cytotoxische effecten van UCB-NK cellen werden bevestigd door een *in vivo* studie, waarin de behandeling met UCB-NK cellen resulteerde in een aanzienlijk verlaagd tumor volume in immuun deficiënte muizen die eerder geïnjecteerd waren met humane $EGFR^{+}RAS^{mut}$ dikke darm kankercellen. Het is opvallend dat dit effect niet verhoogd werd door de toevoeging van cetuximab *in vivo*. Dit zou te wijten kunnen zijn aan een suboptimale inductie van de Fc receptor CD16 (een antilichaam bindende receptor) op de adoptief toegediende UCB-NK cellen. Omdat een studie met AML patiënten inmiddels heeft aangetoond dat CD16 expressie levels hoger worden op de UCB-NK cellen na infusie, kan er een synergie verwacht worden door toevoeging van cetuximab in een klinische setting.

In de studies die zijn beschreven in dit proefschrift hebben we aangetoond dat allogene UCB-NK-cellen een superieur anti-tumor effect hebben tegen in dikke darm en baarmoederhalskanker in vergelijking met geactiveerde allogene PBNK cellen. Bovendien bleek de cytotoxische capaciteit van UCB-NK cellen equivalent aan die van PBNK cellen wanneer die werden gecombineerd met cetuximab. Gebaseerd op onze bevindingen concluderen we dat UCB-NK cellen een veelbelovend therapeutisch platform bieden voor de behandeling van solide tumoren, onafhankelijk van hun EGFR of RAS status. De unieke expansie en differentiatie techniek ontwikkeld door Glycostem maakt het mogelijk om grote aantallen allogene en cytotoxisch actieve UCB-NK cellen te genereren. Zo worden een aantal beperkingen van de huidige adoptieve NK cel strategieën omzeild en wordt het mogelijk tegen de immuun suppressieve druk van tumoren in de *in vivo* NK cel effector pool aan te vullen en de aangeboren (natuurlijke) afweer tegen tumoren in patiënten met kanker te versterken. Zoals tenslotte beschreven in hoofdstuk 6, zal in de nabije toekomst de functionaliteit van UCB-NK cellen verder verbeterd worden door gebruik te maken van genetische modificatie of combinatie therapieën, leidend tot een nog effectievere anti-tumor afweer respons.

CURRICULUM VITAE

John Pradeep Veluchamy was born November 17, 1984 in Oddanchatram, Tamil Nadu, India. He completed his secondary school education in 2002, and joined Christian Medical College, Vellore for his bachelor's, and graduated with honours in Biomedical Laboratory Sciences in 2006. Next one year, he underwent rotatory internships in the departments of Clinical Biochemistry, Microbiology, Pathology and Virology labs at Christian Medical College, Vellore. In 2007, he did a bridge course in "Advanced Biomedical Laboratory science methods" followed by a report entitled "A comparison study on the soluble expression of CXCL16 in macrophage cultures" at Örebro University in Sweden. In 2008, John returned to diagnostics, where he worked as a laboratory technologist in a Clinical laboratory and Blood bank at Christian Fellowship Hospital in Oddanchatram, India. In September 2010, John moved to London to pursue his master's in "Biomedical and Molecular Sciences Research" at King's college London. The master's thesis project entitled ""Assessment of T cell, B cell and NK cell recovery after haematopoietic stem cell transplantation using lymphocyte-depleting antibodies to prevent graft versus host disease" motivated him to dive deep into Immunology. In 2011, he had an excellent opportunity to work in a research project focussed on NK cells and ADCC mechanism entitled "An exploratory open label multicentre study to investigate pharmacodynamics of a human monoclonal antibody antagonist of EGFR, in head and neck cancer patients in the Department of Haematological medicine at King's College London, UK. In March 2013, he enrolled into the PhD program sponsored by Marie Curie FP7 NATURIMMUN consortium, a joint project between VU university Amsterdam and Glycostem, where his focus for the last 4 years, was on research into application of allogeneic NK cells towards treatment in solid tumors. John has authored 7 peer reviewed articles so far. John continues to work in the field of allogeneic NK cell immunotherapy at Glycostem Therapeutics in Oss, Netherlands.

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