Establishment of a good manufacturing practice-compliant procedure for expansion of therapeutic doses of genetically modified, CAR expressing NK-92 cells for the treatment of ErbB2-positive malignancies

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1 Introduction

1.1 Natural killer cells

1.1.1 Function and distribution

Natural killer cells are an important component of the innate immune system; as such, they are responsible for the elimination of virally infected, malignant or otherwise stressed cells. They do not require prior stimulation to efficiently lyse target cell in MHC-independent manner. In addition, they provide a link between innate and adaptive immunity by robust secretion of immunomodulatory cytokines (e.g. IFN-γ and TNF-α) and by interacting with dendritic cells. Natural killer cells are large granular lymphocytes derived, like all hematopoietic cells, from lineage-undetermined CD34+ hematopoietic progenitor cells. They constitute 10 % of peripheral blood lymphocytes and can be phenotypically separated from T and B lymphocytes by the absence of T- and B-lineage determining surface antigens, such as CD3 and surface Ig and B cell receptor expression, respectively. In humans, two major NK cell subsets can be distinguished based on the surface markers expression, namely $CD56^{dim}CD16+$ and $CD56^{bright}CD16-$ [1]. The first constitutes the majority of circulating NK cells (> 90 %) and possesses robust cytolytic properties, whereas the $CD56^{bright}$ subtype secretes abundant amounts of cytokines and is significantly enriched in uterus, liver and lymphoid tissue (lymph nodes, spleen, bone marrow, tonsil) where it acquires a tissue-specific phenotype and function (tissue-resident $CD56^{bright}$ population) [2] (Figure 1.1).

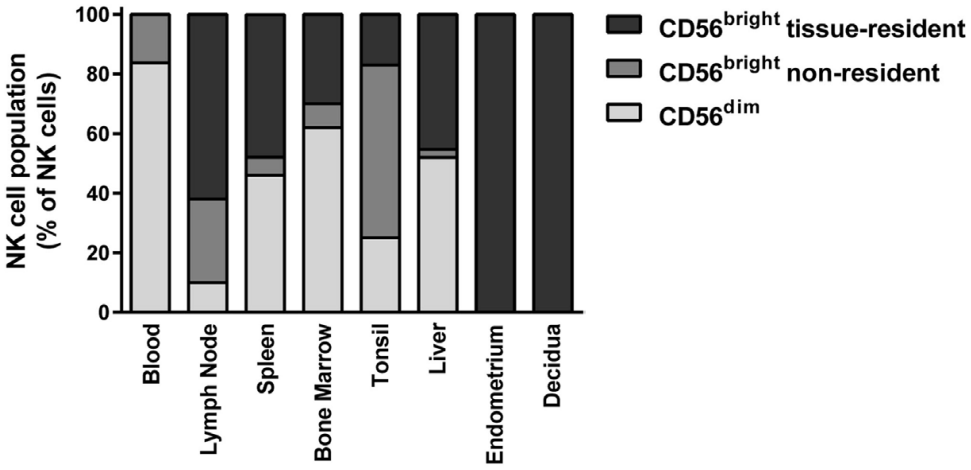

Figure 1.1 Distribution of NK cell populations. Adapted from Melsen et al. [2]

1.1.2 Maturation of NK cells

Natural killer cells are part of the hematopoietic system and are produced in bone marrow; however, the maturation of NK cells is complex and does not occur completely in this organ. It has been hypothesized that the $CD56^{bright}$ population is less mature than the $CD56^{dim}$ and is highly enriched in secondary lymphoid tissue (SLT) (lymph nodes and tonsils). Activation of the SLT-derived $CD56^{bright}$ subsets induced expression of receptors characteristic to $CD56^{dim}$ NK cells [3]. Furthermore, a population of pre-NK cells described as 34+CD45RA+ was found to be selectively enriched in SLT when compared to peripheral blood and bone marrow. High frequencies of these two immature NK cell populations, together with IL-15 expression on antigen presenting cells (APC), suggest SLT as a site of NK-cell development [4]. Based on recent findings the maturation of NK cells may be divided into 5 phases: pro-NK cells from bone marrow circulate in the peripheral blood (1) and via high endothelial venules enter the lymph node (2) where in the parafollicular space they become activated by APC and mature through pro-, pre and iNK cell intermediate stages followed by $CD56^{bright}$ and $CD56^{dim}$ (3). Next, mature $CD56^{dim}$ population returns to the periphery via the efferent lymph (4) providing the pool of circulating NK cells, whereas the majority of $CD56^{bright}$ cells support the tissue-resident population (5) [1] (Figure 1.2).

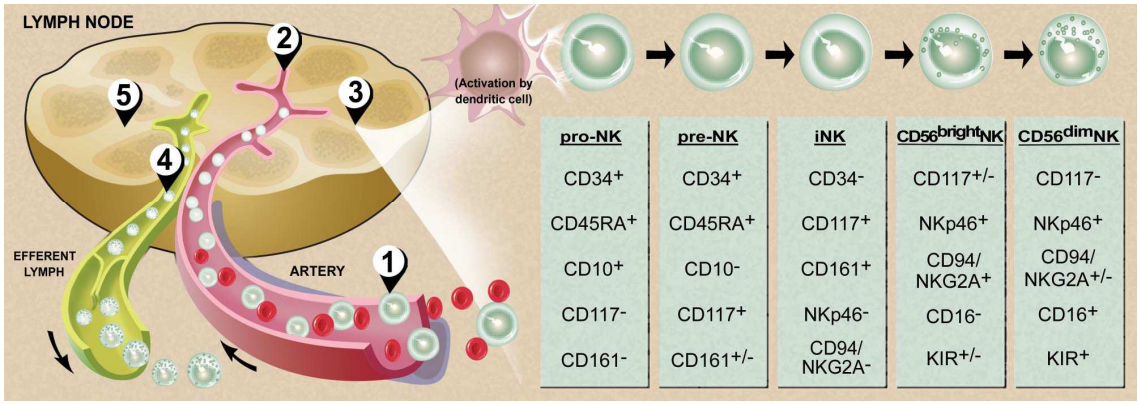

Figure 1.2 Model of human NK cell maturation [1]

1.1.3 Target recognition and mechanisms of killing

Natural killer cells express a wide spectrum of inhibitory and activating receptors in a germline configuration that enable them to distinguish stressed cells (malignant, infected or otherwise injured cells) from normal cells. The leading mechanism preventing the elimination of healthy cells is MHC class I induced self-tolerance, where inhibitory killer immunoglobulin like receptors (KIRs) recognize and bind classical MHC class I molecules (HLA-A, B and C) expressed on every cell in the body except RBCs. Upon ligand binding, the KIR sends a strong inhibitory signal that overcomes activating ones and prevents NK cell from killing [5]. Upon malignant transformation, MHC class I molecules are often downregulated leading to the lack of KIR-mediated NK cell inhibition and hence, target cell lysis, in a mechanism called missing-self recognition. However, downregulation of MHC class I molecules on the target cell in the absence of an activating signal is not sufficient to activate NK cell. Conversely, if activating signals derived from stress ligands are strong enough to overcome KIR-induced inhibition, the presence of MHC class I molecules on malignant cells is not sufficient to inhibit NK cell activation (Figure 1.3), in other words, the suppressive action of KIRs is relative [6]. In addition to KIRs, NK cells bear other receptors with inhibitory function, namely C-type lectin-like receptors: heterodimeric CD94/NKG2A which binds non-classical MHC class Ib ligand (HLA-E) and NKR-P1A (CD161) which recognizes non-MHC ligand lectin-like transcript-1. Moreover, a lack of inhibition is not sufficient to activate NK cell, implying that the predominant signal derived from activating receptors is indispensable for NK cell mediated target cell lysis [7,8]. There are numerous NK-cell activating receptors described to date including

NKG2D, the group of NCR (NKp30, NKp44, NKp46), NKp80 and DNAM1 [9-12]. NKG2D is the best characterized activating receptor and plays a crucial role in cancer immunosurveillance via a mechanism called stress-induced self-recognition [13,14]. NKG2D recognizes self-proteins selectively upregulated by cells which have undergone various forms of stress, e.g. infection with intracellular pathogens, transformation or DNA damage. These stress-ligands include MHC class I chain-related proteins A and B (MICA and MICB) and a family of UL16-binding proteins (ULBP1-6) both of which are not detectable on healthy cells [15,16]. Ligands for natural cytotoxicity receptors (NCR) are still poorly characterized, with the exception of B7-H6 which was identified as a tumor-specific ligand interacting with NKp30 [17]. In addition to aforementioned activating receptors, the mature population of $CD56^{dim}$ NK cells bears FcγRIII (CD16), the low affinity receptor binding the Fc portion of IgG bound to opsonized target cells. The FcγRIII-IgG interaction initiates a process called antibody-dependent cell-mediated cytotoxicity (ADCC), where the NK cell becomes activated and kills the target cell via the cytolytic granule-dependent mechanism [7]. Exocytosis of cytolytic granules containing perforin and granzymes is the major killing mechanism of NK cells. Upon NK cell activation and degranulation, perforin acts as a membrane-disrupting protein and forms holes in the membrane of a target cell enabling diffusion of granzyme B. Granzyme B is a serine protease, which induces apoptosis via the activation of cellular caspases or by direct DNA fragmentation. An alternative mechanism of NK cell mediated killing is through surface expression of apoptosis-inducing ligands (FasL, TRAIL, TNF-α) for death receptors expressed on the target cell. Death ligand-receptor interaction leads to the activation of the caspase-dependent extrinsic apoptosis pathway [18].

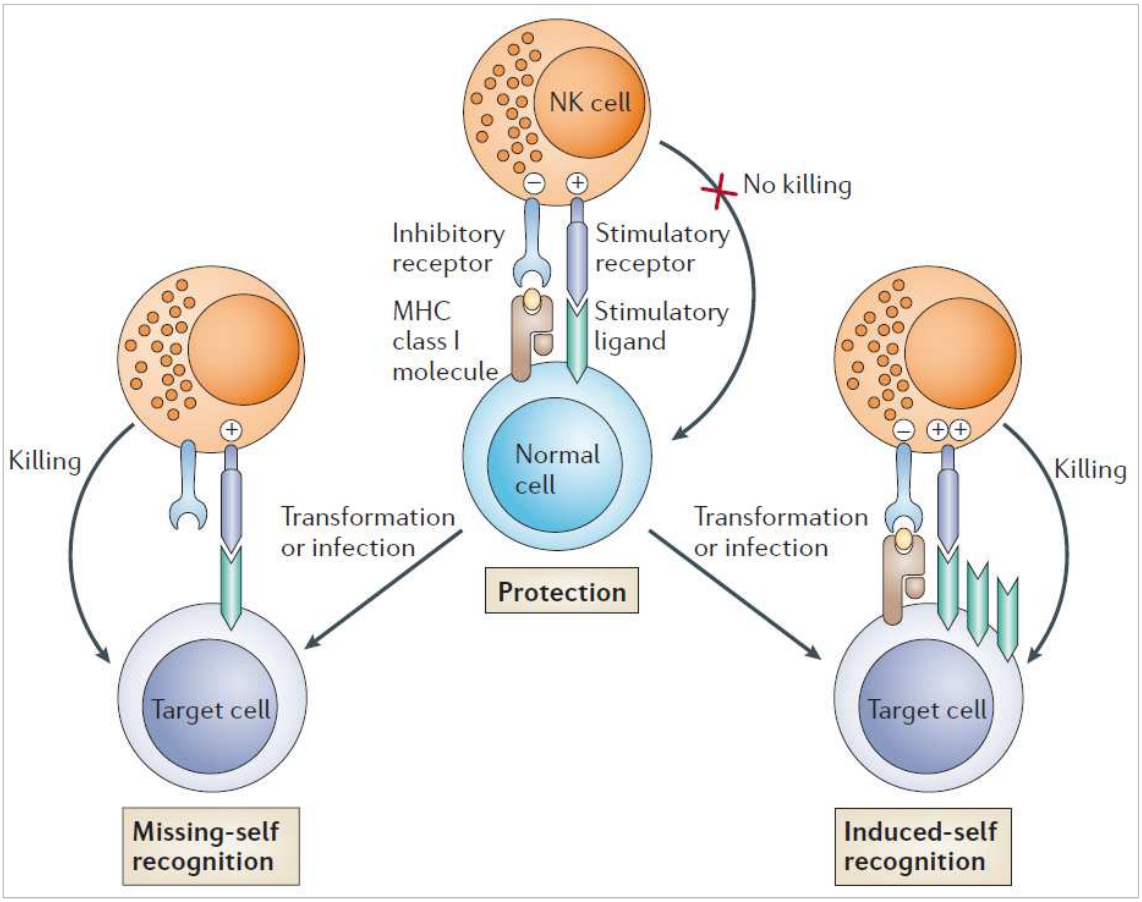

Figure 1.3 Balance between activating and inhibitory signals regulates the interaction between NK cell and target cell. Adapted from Raulet et al [6].

1.1.4 NK cells in cancer immunosurveillance

Natural killer cells demonstrate natural innate cytotoxicity against malignant cells without the need for prior stimulation or immunization [19,20]. NK cells can eradicate tumor cells directly upon stimulatory receptor-mediated activation (discussed in the previous chapter) via several mechanisms including: (1) release of perforin/granzyme containing granules, (2) expression of ligands for death-receptors, (3) FcγRIII associated ADCC and (4) release of effector molecules (e.g. IFN-γ) [21]. In addition, abundant production of cytokines, chemokines and growth factors by activated NK cells contributes to the development of antitumor adoptive immunity by the stimulation of T, B and dendritic cells [7,22]. NK-derived IFN-γ promotes tumor-specific antibody production by B cells, activates CD8+ T lymphocytes to develop into cytotoxic T cells (CTLs) and stimulates CD4+ T cells to secrete Th1 cytokines which further supports CTL differentiation and B cell induction [23,24].

Moreover, NK induced tumor cell lysis leads to the release of tumor antigens, which are taken up by dendritic cells leading to their maturation and antigen presentation to CTL (Figure 1.4) [25].

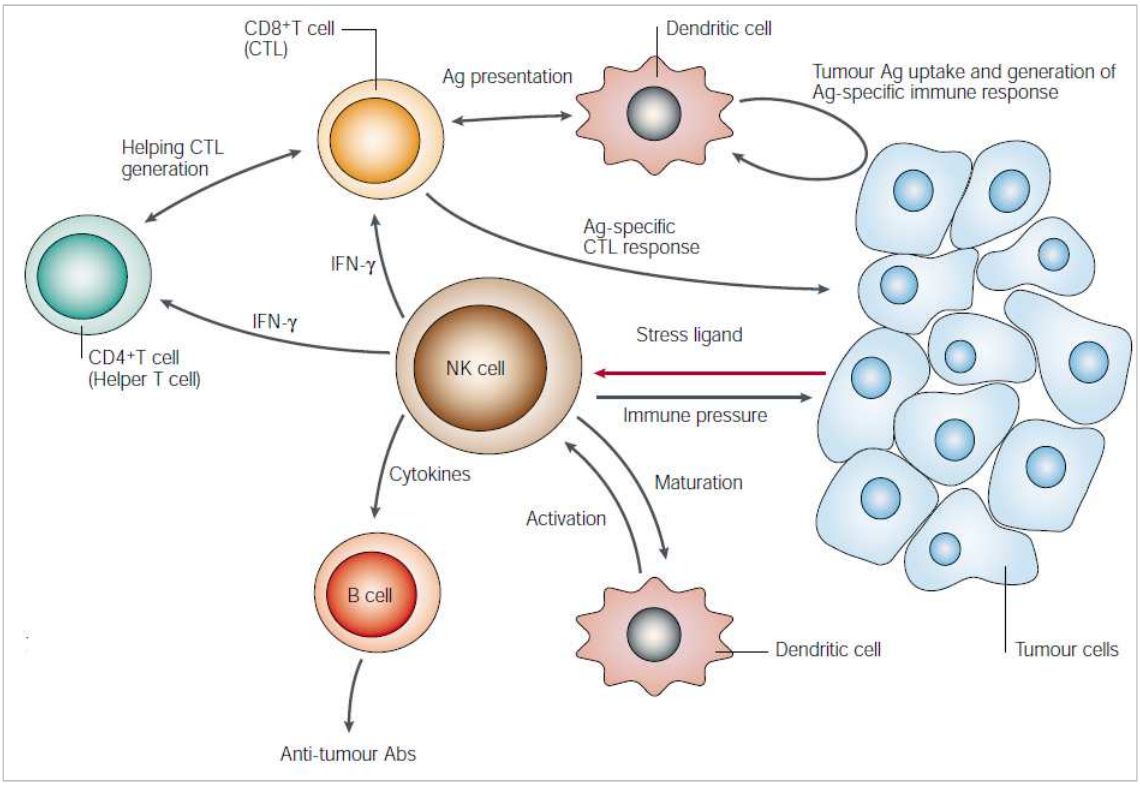

Figure 1.4 Role of NK cells in antitumor immunity. Adapted from Smyth et al. [13]

1.1.5 NK cell lines

There are seven human natural killer cell lines established hitherto: HANK-1, KHYG-1, NK-92, NK-YS, NKL, SNK-6, YT, all of which are derived from patients suffering from NK/NKT neoplasms (Table 1.1). The cells have a large granular lymphocyte (LGL) morphology with pronounced azurophilic granules. Characterization of their immunophenotype revealed the immaturity of tested cell lines, defined as CD16- CD56+. Only KHYG-1 and NK-92 show significant and reproducible NK activity against the MHC-I-negative K562 target cell line. This discrepancy might be due to the fact that the other NK-lines have yet reached the maturity stage at which they acquire the typical NK cytotoxicity. Five of seven NK cell lines were found to be positive for EBV in PCR testing, excluding KHYG-1 and NKL [26]. This could be explained by the recent finding showing that, while primary NK cells are an early target

for EBV, [27] they may not be necessarily associated with the pathogenesis of the malignant disease.

Table 1.1 NK cell lines

NK-cell line	Original disease	NK activity against K562
HANK-1	Nasal-type NK/T-cell lymphoma	–
KHYG-1	Aggressive NK-cell leukemia	+
NK-92	NHL with LGL cells	++
NK-YS	Nasal NK-cell lymphoma	–
NKL	NK-LGL leukemia	–
SNK-6	Nasal NK/T-cell lymphoma	–
YT	ALL + thymoma	–

Adapted from Matsuo et al. [26] ALL, acute lymphoblastic leukemia; LGL, large granular lymphocytes; NHL, non-Hodgkin's lymphoma; NK, natural killer-cell; T, T-cell; (−) < 10 % lysis; (+) 10–50 % lysis; (++) > 50 % lysis.

1.2 NK-92

1.2.1 Establishment of the NK-92 cell line

NK-92 is a continuously growing, IL-2 dependent cell line with the characteristics of activated natural killer cells. It was established and further characterized in 1992 by the Dr. H. Klingemann's laboratory. The cells were isolated from a 50-year-old male patient suffering from an aggressive non-Hodgkin's NK cell lymphoma. The cells have an LGL morphology and the following immunophenotype: CD2+, CD7+, CD11a+, CD28+, CD45+, CD54+, CD56++ and CD1-, CD3-, CD4-, CD5-, CD8-, CD10-, CD14, CD16-, CD19-, CD20-, CD23-, CD34-, HLA-DR-. The expression of CD25 (the α subunit of IL-2 receptor) correlates inversely with the IL-2 concentration in the culture medium [28]. NK-92 is highly cytotoxic against a broad range of established solid (neuroblastoma, ovarian and prostate cancer cell-lines) and hematologic tumor cell lines. NK-92 is also cytotoxic against freshly isolated malignant cells of the B- and T-lineage [29-31]. High cytolytic activity of NK-92 cells is due to their unique repertoire of expressed receptors (Figure 1.5). They express only a few inhibitory

receptors (NKG2A/B and ILT-2) and lack almost all of the killer-cell immunoglobulin-like receptors (KIRs) except KIR2DL4, which, unlike other KIR-DL-receptors, has activating potential and according to recent findings plays a role in the remodeling of the maternal uterine vasculature in the early phase of pregnancy [32,33]. Conversely, NK-92 cells express a whole range of activating and co-stimulatory receptors (NKp30, NKp46, 2B4, NKG2D/E, CD28) and high levels of perforin and granzyme, as well as effector molecules of the TNF-superfamily (FasL, TRAIL, TWEAK and TNF-α). However, the lack of CD16 (FcγRIII) expression deprives them of the capacity for ADCC (antibody-dependent cell-mediated cytotoxicity), which is one of the major NK-cell killing mechanisms. In addition, the absence of CD16 which is normally expressed by mature peripheral blood NK cells together with lack of KIRs, confirms the relative immaturity of NK-92 cells [32].

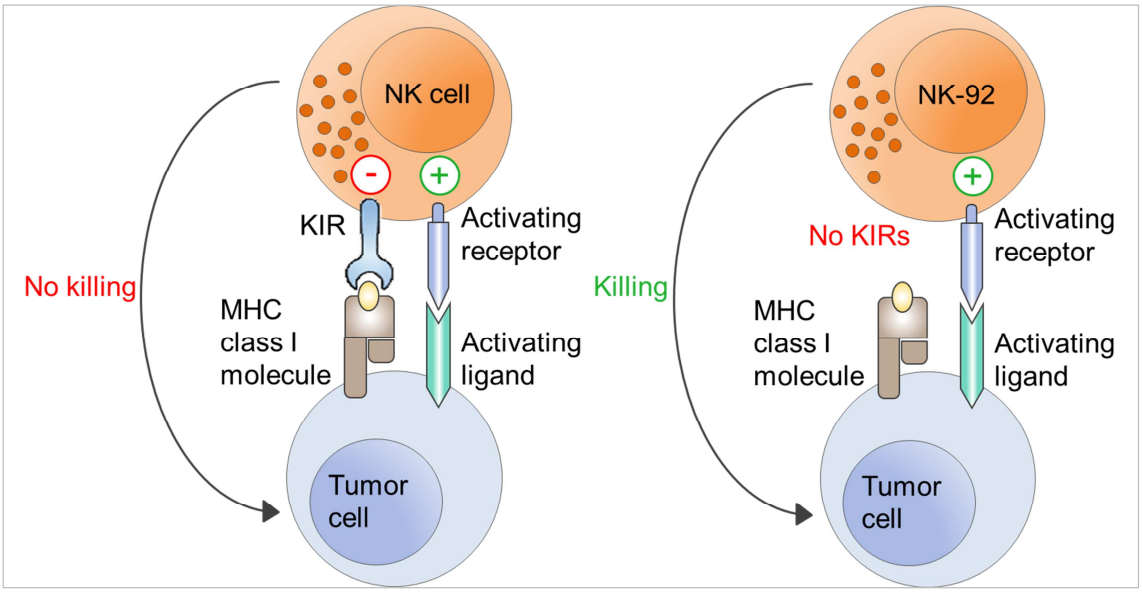

Figure 1.5 Lack of KIR expression increases the cytolytic activity of NK-92 cells. Based on Raulet et al. [6] and Maki et al. [32]

1.2.2 Immunotherapy with parental NK-92 cells

NK-92 is the only natural killer cell line ever to enter clinical trials for in-vivo anti-cancer therapy. Two phase I studies with intravenous infusions of up to 1 x 10^{10}/m² of body surface area of gamma-irradiated NK-92 cells in patients with advanced malignancies have been completed to date. One clinical trial, conducted in Chicago (USA) by Arai et al., included 11 patients with renal cell carcinoma (RCC) and 1 patient

with melanoma [34]. Another trial, performed in Frankfurt, Germany by Tonn et al., recruited a heterogeneous group comprising 15 patients suffering from end-stage solid tumors and hematologic malignancies (medulloblastoma, PNET, SCLC, NSCLC, colorectal cancer, sarcoma, rhabdomyosarcoma, osteosarcoma, adrenal carcinoma, CLL and B-NHL) [35,36]. None of the 27 patients treated in the above mentioned clinical trials showed severe toxicity and there was also no evidence of prolonged persistence of NK-92 cells. Both initial studies confirmed the feasibility of clinical scale expansion and the safety of administrating high numbers of parental NK-92 cells (up to 10^{10} cells/m² BSA). Due to the early phase of the trials, definitive conclusions regarding the effectiveness of NK-92 treatment are limited. The impression of some beneficial effect was gained in a group of patients, with a partial tumor regression reported in 1 patient, stable disease in 5 patients, and a mixed response in 3 more patients. Despite the allogenic character of NK-92 cells, antibody formation against NK-92 HLA was observed only in a small group of patients; however, this can be explained by their immunocompromised status. These are encouraging results considering the presumably advanced stages of the targeted diseases and the extensive pretreatment of enrolled individuals where NK-92 cell therapy might be considered. However, further strategies improving the efficacy of parental NK-92 are necessary.

1.2.3 Genetically engineered NK-92 variants

Unlike primary NK cells, NK-92 can be sufficiently engineered, not only with viral vectors, but also with physical methods like transfection with mRNA. Post-modification cultivation and serial cell sorting allows obtaining highly purified population of genetically modified NK-92 cells with the expression of desired transgene [37]. To date, NK-92 cells were engineered in a number of ways in order to make them more attractive and feasible for cancer immunotherapy. As mentioned previously, NK-92 cells lack expression of FcγRIII (CD16). Therefore, they are not able to kill via ADCC. This issue was solved by Campbell et al. who engineered NK-92 cells by means of retroviral transduction to express a high affinity CD16 Fc receptor (NK-92.26.5 cells) [38]. Taking into account the limited therapeutic efficacy of monoclonal antibodies in the majority of patients with intermediate or low affinity variants of FcγRIII receptor [39], therapy combining high affinity NK-92Fc with therapeutic antibodies

(e.g. Rituximab and Ofatumumab) might be a promising strategy to fight the cancer [40,41]. Currently, the Fc expressing NK-92 variant is successfully being used as an effector in a commercialized bioassay panel for screening of monoclonal antibodies to determine their contribution to ADCC. Another interesting approach of NK-92 engineering was presented by Tam et al. and Sahm et al. [42,43]. Both groups introduced the ectopic expression of cytokines essential for NK-92 cell proliferation and function, namely IL-2 and IL-15 respectively, which resulted in the generation of NK-92 variants independent from the exogenous supply of these cytokines.

1.2.4 CAR expressing NK -92 cells for cancer immunotherapy

While NK-92 cells seemed to be well tolerated after systemic infusion in early clinical trials, the activity of parental NK-92 cells appears to be restricted to certain tumor entities and their potency limited. NK-92 cells showed efficacy *in vitro* and in animal models against NK cell sensitive hematologic malignancies, such as AML, B-ALL and T-ALL. However, most solid tumors are resistant to NK cell mediated killing [44]. It has prompted further development of NK-92 with grafted specificity to tumor associated antigens by virtue of expressing chimeric antigen receptors (CAR). CAR expressing NK-92 cells specific against tumor antigens, like CD38, CD19, CD20, epithelial cell adhesion molecule (EPCAM), GD2, EGFRvIII and ErbB2 (HER2), have been generated and proved to be specific and highly cytotoxic against otherwise resistant tumor cells [41,45-48].

1.3 Chimeric antigen receptors (CARs)

1.3.1 Design of chimeric antigen receptors

Chimeric antigen receptors are recombinant molecules which were initially designed to redirect and improve the specificity and potency of T lymphocytes against virally infected or malignant cells. The first functional chimeric receptor was developed by Eshhar et al. in 1989. It consisted of TCR constant domains of α and β chains recombined with variable region heavy (VH) and light (VL) chains of anti-TNP (2,4,6-trinitrophenyl) antibody (Sp6). Expression of chimeric TCR (cTCR) in cytotoxic T cell hybridoma cells resulted in specific antigen recognition in an MHC-independent manner and in efficient T-cell activation [49]. Over the past 3 decades, chimeric receptors have gained great attention from researchers and a huge amount of effort has been invested to make them more attractive for immunotherapy. Currently, there are two leading strategies for antigen specific activation of immune effector cells: recombinant T-cell receptors (TCRs) and chimeric antigen receptors (CARs). The first, however, is associated with serious limitations like patient specific HLA-restriction and a risk of mispairing with the endogenous TCRs and thus severe autoimmune reactions [50]. In contrast, CARs recognize the specific antigen directly. A downside of CARs is their inability to target intracellular targets. Historically, the development of CAR has gone through three phases, or generations, thus far, determined by the number of signaling molecules included in the receptor construct. First generation CARs comprised of an antigen **recognition/binding domain**, **hinge region (spacer)** and CD3-ζ or FcRγ as a **signaling domain**. Second generation CARs included an intracellular **costimulatory domain** (e.g. CD28, CD134 or CD137), while chimeric antigen receptors of the third generation are equipped with two costimulatory molecules in order to further enhance the stimulatory potential (Figure 1.6).

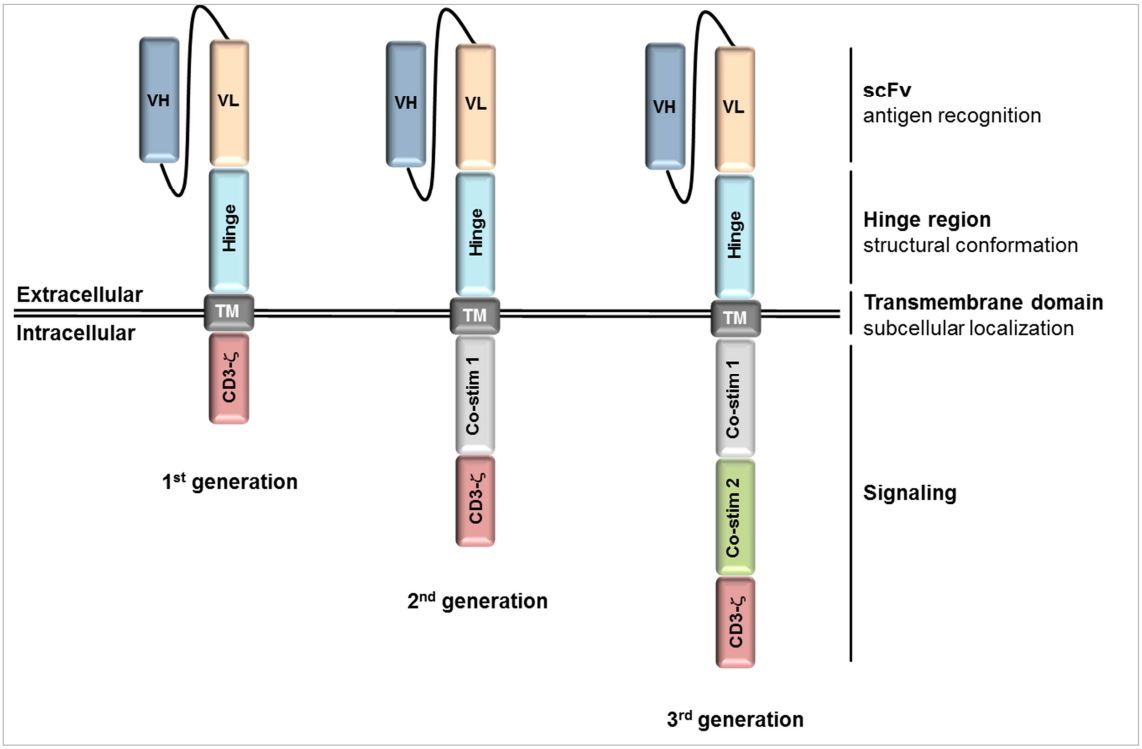

Figure 1.6 Generations of chimeric antigen receptors. Modified from Abate-Daga et al. [51]

Binding domains

The most commonly used antigen-binding domain is a scFv (single chain variable fragment) molecule. It is generated by joining the light (VL) and heavy chain (VH) variable regions of a monoclonal antibody with a linker (Figure 1.7). The major role of the antigen binding domain is that it determines CAR specificity and affinity, which is consistent with the specificity and affinity of the original monoclonal antibody from which the light and heavy chain have been derived. Moreover, positioning of a binding epitope within the antigen and its distance from the cell membrane may have an impact on the potency of CAR-equipped effector cells. It was observed that CAR-T cells recognizing membrane-proximal epitopes of CD22 on malignant B-cells had superior anti-leukemic potency than those binding to membrane-distal epitopes [52]. The affinity of the scFv binding domain may determine the functional properties of a CAR in the context of safety. CARs equipped with low affinity scFv mediate killing of tumor cells exhibiting target antigen overexpression, while healthy human tissues expressing low or intermediate levels of the same antigen remain intact [53].

Hinge region (Spacer)

The hinge region can be derived from IgG Fc (CH2-CH3) (Figure 1.7), CD8α or CD28 and together with transmembrane domains anchors the CAR in the cell membrane and provides the link between the antigen-binding domain and intracellular signaling domains. Length and flexibility of the hinge region may play an important role in spatial orientation of CAR during antigen binding. However, finding an optimal spacer is difficult, as the optimal length and structure are unpredictable and can only be approached experimentally. To a significant degree, which spacer will work is determined by the position of the binding epitope and its proximity to the cell membrane which may assist spacer design, provided the two are known [54].

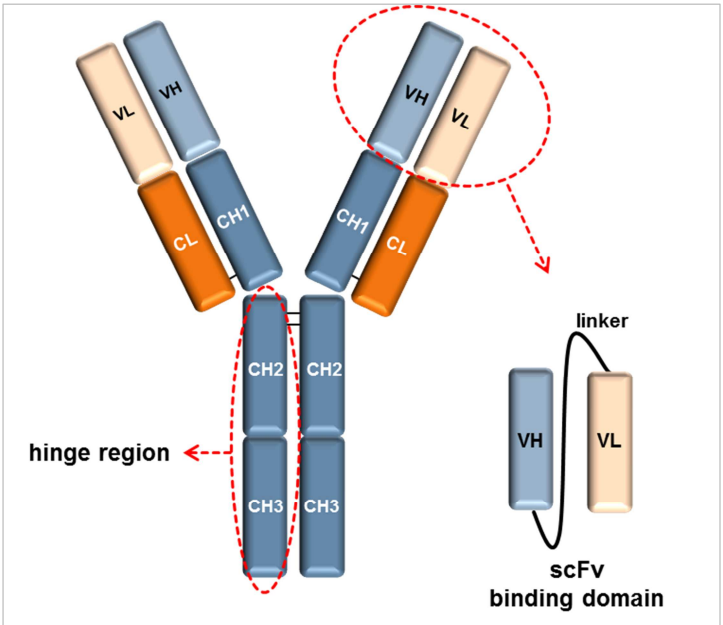

Figure 1.7 Immunoglobulin-derived regions utilized in CAR construct

Signaling domains

ITAMs (immunoreceptor tyrosine based activation motifs)-containing cytoplasmic portions of CD3-ζ or FcRγ are typically used as signaling domains and have been shown to be sufficient (first generation CAR) to trigger T and NK cell cytotoxicity and cytokine secretion upon antigen binding to the scFv region [48]. What is more, *in vivo* animal models showed improved survival and tumor elimination in mice treated with scFv- CD3-ζ CAR T cells [55]. However, the therapeutic potency of 1st generation

CARs was disappointing, with limited clinical efficacy, in vivo expansion and persistence, fueling further CAR development.

Costimulatory domains

To improve the potency of 1st generation CARs, additional costimulatory domains were introduced resulting in the development of the 2nd (one costimulatory domain) and 3rd (two costimulatory domain) generations. Molecules typically used to provide costimulatory signals play a role in primary T cell activation. The most commonly utilized and clinically explored are CD28 and CD137 (4-1BB) derived domains. CD134 (OX 40) and ICOS have also been extensively investigated. A secondary signal provided from costimulatory molecules significantly improves cytotoxicity, cytokine secretion, persistence and proliferation capacity of effector cells. *In vivo* studies showed superior efficacy of ErbB2-specific 2nd generation chimeric receptors comprising CD28 when compared to the first generation CARs with the same specificity [56]. In a clinical trial performed by Savoldo et al. CD19-redirected T cells bearing CD28-comprising CAR showed a higher proliferation rate and longer persistence *in vivo*, when compared to their first generation counterparts [57]. Furthermore, CAR constructs containing CD28 moieties induced higher proliferation and cytokine secretion (IFN-γ, TNF-α, IL-2 and IL-10), when compared to CARs containing other costimulatory domains [58]. In contrast, introduction of CD137 resulted in a higher percentage of specific target cell lysis and reduced effector cell exhaustion [59]. The 3rd generation CARs, combining early costimulatory molecule CD28 and late costimulatory molecule OX40 (CD28/OX40) or 4-1BB (CD28/4-1BB) exhibited some improvement with regard to *in vivo* anti-tumor efficacy and effector cell persistence. However, disappointing *in vivo* data, showing only little benefit over 2nd generation CARs has thus far limited clinical application of 3rd generation variants [60].

1.3.2 Clinical experience with CAR-modified immune cells

To date, numerous clinical trials have been conducted with genetically modified immune effector cells equipped with chimeric antigen receptors mainly for cancer treatment using second generation CARs comprising CD28 or 4-1BB as costimulatory

molecules. The largest studies reported hitherto focused on targeting the CD19 antigen for the treatment of B-cell hematologic malignancies: adult and pediatric B-ALL (B-cell acute lymphoblastic leukemia), CLL (chronic lymphatic leukemia), B-NHL (B-cell non-Hodgkin lymphoma). These studies provided encouraging results with a high percentage of patients in CR (complete remission) [61]. However, disease relapse is still a problem. CD19 targeted therapies seem to promote the outgrowth of CD19-negative leukemic variants, which is probably the primary cause for disease recurrence [62]. Immunotherapy employing highly activated effector cells is associated with the risk of side effects. Indeed, all aforementioned studies reported similar treatment related complications such as: cytokine release syndrome (CRS), neurotoxicity and B-cell aplasia. The last complication was expected, considering CD19 expression by normal B cells. Thus CD19-CAR therapies require lifelong intravenous plasma donor-derived immunoglobulin replacement [63]. Cytokine release syndrome was the most serious toxicity manifested at different grades from mild to life-threatening, which has been strongly correlated with tumor burden [64]. CRS is caused by the secretion of great amounts of pro-inflammatory cytokines (IL-6, IL-2, IFN-γ and TNF-α) after stimulation of CAR-T cells with malignant targets and may occur several hours (severe CRS) to several weeks (mild CRS) post CAR-T cell injection. In most cases, CRS is fully reversible with corticosteroids and/or anti-cytokine therapy with tocilizumab (IL-6 receptor inhibitor) [65]. Although the main focus of CAR-based therapies has been on hematologic malignancies due to the availability of a sufficiently specific molecular target, i.e. CD19, there are some early phase clinical trials targeting solid tumors-associated antigens. To date, 11 clinical studies on solid tumors have been completed and published including: HER2-positive sarcoma, neuroblastoma, mesothelioma, colon cancer, ovarian cancer and metastatic renal cell carcinoma (RCC) [66]. There was some substantial antitumor efficacy observed in the clinical trials including neuroblastoma patients where CAR-T cells targeting either CD171 or GD2 antigens were employed without any serious treatment-related toxicity [67,68]. Conversely, in a small study, including three RCC patients subjected to CAR-T cell therapy targeting CAIX tumor associated antigens, all individuals experienced hepatotoxicity [69]. Taken together, the above discussed clinical experiences employing CAR-based strategies revealed the great potential of this

therapeutic approach. However, there are still many issues to be solved concerning safety and long-term efficacy.

1.4 ErbB2

1.4.1 ErbB2 in tumorigenesis

ErbB2, also called HER2 (human epidermal growth factor receptor 2), is a 185 kDa protein and transmembrane receptor with tyrosine kinase activity [70]. Together with ErbB1 (EGFR), ErbB3 and ErbB4 belong to the epidermal growth factor receptor family. All four receptors (ErbB1-4) consist of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular signaling domain. Despite intensive research, the natural external ligand for ErbB2 is unknown. ErbB2 exhibits both ligand-dependent and ligand-independent dimerization activity. It can heterodimerize with ligand-bound ErbB1, ErbB3 and ErbB4. In addition, it can form signaling-active homodimers in a ligand-independent manner; suggesting its constitutively active conformation, when overexpressed at the plasma membrane [71]. Receptor dimerization and subsequent phosphorylation of tyrosine residues in the receptor cytoplasmic tails activate mitogenic and survival signaling pathways such as the MAP kinase and the PI3 kinase pathways. Overexpression of ErbB2 disturbs homeostatic receptor interactions and preferentially induces the formation of ErbB2 complexes containing hetero- and homo-dimers. As a consequence, inappropriate activation of downstream signaling can lead to dysregulated cell growth, inhibition of apoptosis and in turn to oncogenic transformation [72,73]. The best described mechanism of ErbB2 mediated tumorigenesis is deregulation of G1/S control by degradation of p27 (CDKN1B; cyclin dependent kinase inhibitor 1B) and upregulation of c-Myc and D-cyclins involved in sequestration of p27. Binding of p27 to cyclin E-CDK2 or cyclin D-CDK4 complexes prevents their activation and thus controls the cell cycle progression during the G1 phase. Reduction of the free p27 leads to activation of the mentioned complexes, and as a consequence to uncontrolled cell proliferation [74].

1.4.2 ErbB2 overexpressing malignancies

ErbB2 is overexpressed by many tumors of epithelial origin including breast, ovarian, stomach, colorectal and aggressive forms of uterine cancer (Table 1.2) In a number of malignancies such as osteosarcoma and glioblastoma, ErbB2 overexpression is associated with increased disease recurrence and poor patient prognosis [75]. A large retrospective study, including 149 samples of glioblastoma patients, was performed to assess the prognostic value of ErbB2 in this disease. This study showed that the median survival of the patients with ErbB2 overexpressing tumors was shorter by half compared to the patients without overexpression (4 months versus 8 months) [76]. *ERBB2* gene amplification is the most common mechanism leading to protein overexpression. The most pronounced example of gene amplification-dependent ErbB2 overexpression is breast cancer, where concordance rates between these two parameters measured by FISH and IHC respectively were between 73 %-98 %. The second example is gastric cancer showing concordance between *ERBB2* gene amplification by FISH and protein overexpression in IHC between 58.5 %-88 % [77]. However, analysis conducted in early 90's on gastric cancer patients by Kameda et al. [78] showed, in some cases, high ErbB2 protein expression without gene amplification. Furthermore, in the study investigating human glioblastoma cases, expression of the ErbB2 protein was present in 54 % (20/37) of analyzed tumor specimens. However, no gene amplification was detected [79]. Above findings suggest that ErbB2 overexpression is not always gene amplification-dependent and may result from different mechanisms like high polysomy of chromosome 17, where the ErbB2 encoding gene is located, epigenetic changes or dysregulation of transcriptional or post-transcriptional processes [80].

Table 1.2 ErbB2 overexpression in different types of tumors

Tumor type	**Frequency of ErbB2 overexpression (%)**
Medulloblastoma	86.5 [81]
Endometrial carcinoma	14-80 [82]
Wilms' tumor	68.5 [83]
Glioblastoma	15.4-54 [76,79]
Inflammatory breast cancer	50 [84]

Tumor type	Frequency of ErbB2 overexpression (%)
Osteosarcoma	42.6 [75]
Gastric cancer	8.2-41.3 [85,86]
Ovarian cancer	9-32 [87]
Breast cancer	18 [84]
Lung cancer	7 [84]
Pancreatic cancer	4 [84]
Colon cancer	3 [84]

1.4.3 ErbB2 as a therapeutic target

The most important feature of the therapeutic target is its selective expression on tumors, but not on normal healthy cells, thereby avoiding on-target, off-tumor toxicities. ErbB2 is overexpressed by a variety of malignancies, but its presence on normal tissues is limited to few tissues across the body (e.g. cardiomyocytes, respiratory epithelial cells) where ErbB2 is expressed at low levels [88]. Moreover, the role of ErbB2 in tumorigenesis and tumor progression was widely demonstrated. All these observations make ErbB2 an attractive target for cancer immunotherapies. Indeed, a few ErbB2 targeted treatments have been already approved and investigated in early and advanced phase clinical studies with promising results. ErbB2-specific therapeutic monoclonal antibodies play the leading role in this field, where trastuzumab (marked as Herceptin) is the best investigated one. Mechanism of action of trastuzumab includes downmodulation of signaling pathways (PI3K/AKT and ras-Raf-MAPK) and inhibition of cell cycle progression by induction of the p27 protein which leads to Cdk2 inactivation and G1 arrest. Moreover, trastuzumab prevents ErbB2 ectodomain (ECD) cleavage, a phenomenon observed in some breast cancers, which is associated with constitutive activation of remaining receptor domains resulting in increased signal transduction. *In vivo*, trastuzumab induces host immune responses by recruiting immune effector cells carrying Fc receptors (NK cells, monocytes), which leads to ADCC and killing of trastuzumab coated tumor cells [89]. Trastuzumab has been approved by the Food and Drug Administration for the treatment of ErbB2-overexpressing, early stage and metastatic breast cancer and metastatic gastric/gastroesophageal (GE) junction

cancers with ErbB2 overexpression or gene amplification. It has been applied as a single-agent therapy or in combination with standard chemotherapy, showing significant improvement of overall survival and disease-free survival when compared with chemotherapy alone [90]. However, except common side effects like flu-like symptoms, nausea and diarrhea, cardiac dysfunction was reported in 3 %-7 % of the patients receiving trastuzumab as a single-agent [91]. In a large phase 3, international ToGA (Trastuzumab for Gastric Cancer) trial, including ErbB2-positive gastric/GE junction cancer patients from 24 countries, trastuzumab was investigated as a first-line therapy in combination with chemotherapy. Trastuzumab-containing regimens significantly improved median overall survival (mOS=13.8 months) and progression free survival (PFS=6.7 months) of the patients, when compared to the group treated with chemotherapy alone (mOS=11.1 months; PFS=5.5 months). As expected, analysis of ErbB2 status suggested that trastuzumab-containing treatment was more efficient in patients with high expression of this protein (mOS=16 months). Importantly, the incidence rates of cardiac adverse events or grade 3/4 adverse events did not differ between the trastuzumab plus chemotherapy and chemotherapy alone treated group [92].

There are more approved therapies under evaluation, specifically targeting ErbB2 protein, including the monoclonal antibody pertuzumab (trade name Perjeta), the small molecule tyrosine kinase inhibitor lapatinib and a novel antibody–drug conjugate trastuzumab emtansine (T-DM1; marked as Kadcyla). Pertuzumab binds an extracellular dimerization domain of ErbB2 (domain II), a moiety different from that of trastuzumab binding (domain IV), by which it prevents dimerization of ErbB receptors and inhibits downstream signaling (Figure 1.8A). Like trastuzumab, pertuzumab causes ADCC mediated killing of antibody coated tumor cells. Lapatinib is a dual tyrosine kinase inhibitor binding to ATP-binding sites of ErbB1 (EGFR) and ErbB2 tyrosine kinase in the cytoplasmic domain, preventing phosphorylation and subsequent activation of signaling pathways (Figure 1.8B). T-DM1 is a combination of trastuzumab (T) with the cytotoxic agent emtansine (DM-1), which is specifically derived to target cells by means of trastuzumab to ErbB2 overexpressing cells and subsequent internalization. DM1, once entering the tumor cell, prevents microtubules polymerization and thereby inhibits tumor cell division (Figure 1.8C).

Currently, efficacy and safety of all mentioned therapeutic agents in combination with each other and with standard chemotherapy are under evaluation in numerous clinical trials mainly in breast cancer and gastric cancer patients [93].

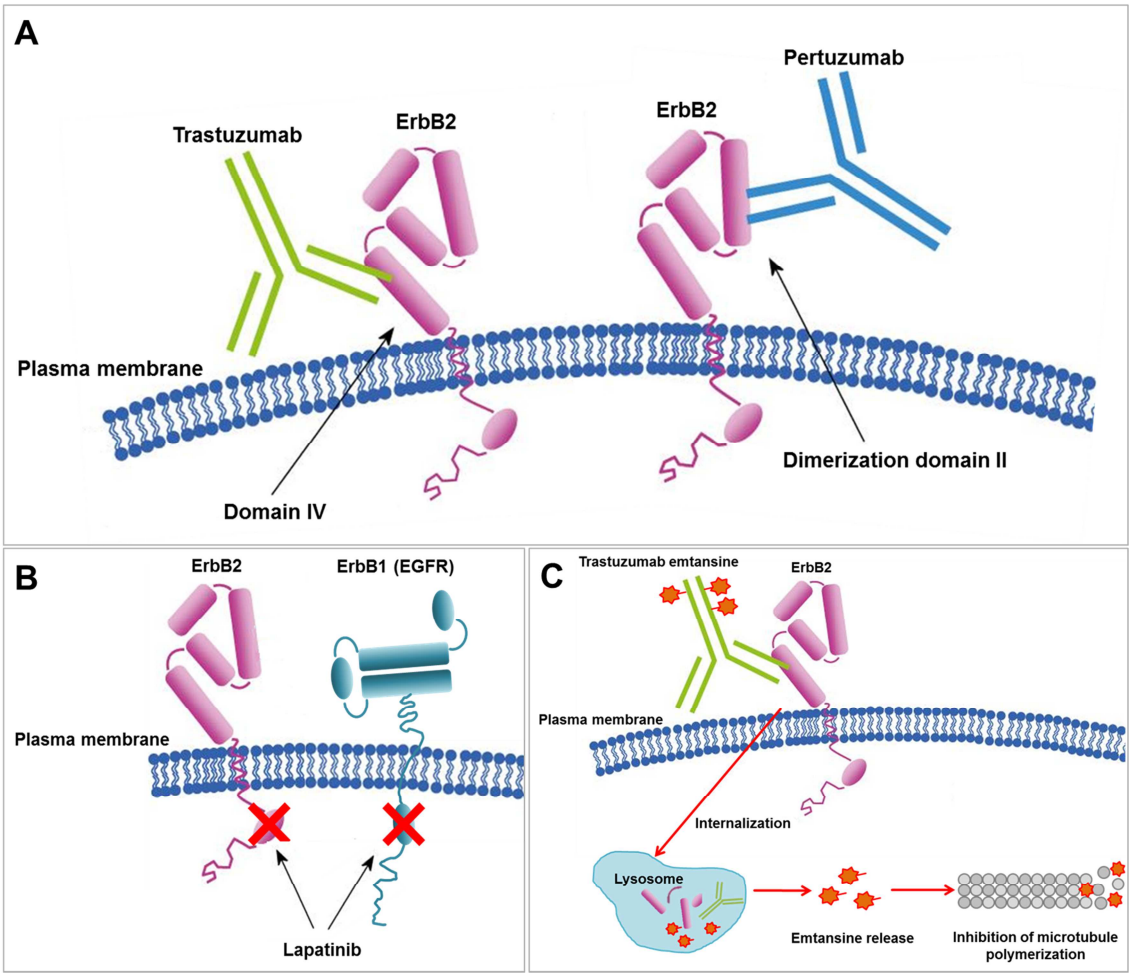

Figure 1.8 Mechanism of action of ErbB2 targeting therapeutic agents. Modified from Park et al. [93]

1.5 ErbB2-CAR expressing NK-92 cell line: NK-92/5.28.z

While T-cells are certainly attractive vehicles to carry CARs in the therapy of malignancies, NK cells do offer certain advantages over T cells. The most prominent is that NK cells do not cause GvHD and can be used in an allogeneic fashion [94]. In the case of the immortalized NK-92 cell line, this feature will enable further development of “off-the-shelf” based cell therapies significantly simplifying a complex cell therapy process as genetic engineering. The first CAR expressing NK-92 cells were generated almost 15 years ago. These cells were highly effective against ErbB2 positive

breast and ovarian cancer cells *in vitro* and *in vivo* [41]. Based on these promising results, ErbB2-CAR expressing NK-92 cells were regenerated, following GMP-compliant procedures, in order to make them suitable for clinical evaluation. Parental NK-92 cells from a FDA-licensed master cell bank were transduced with lentiviral vector encoding a humanized, second generation CAR construct, comprising an ErbB2-specific single chain variable fragment (scFv) derived from the FRP5 monoclonal antibody, CD28 as a costimulatory molecule and the CD3-ζ signaling domain (5.28.z) (Figure 1.9) [95]. The thusly generated NK-92/5.28.z cell line exhibited high specific cytotoxicity toward ErbB2-expressing tumor targets which were resistant to parental NK-92 cells. *In vivo* antitumor efficacy was extensively tested in several established mouse models of orthotopic human GBM, renal cell carcinoma and breast carcinoma xenografts, showing specific homing of NK-92/5.28.z cells to tumor sites, significant reduction of metastases and, in some cases, complete tumor clearance [96].

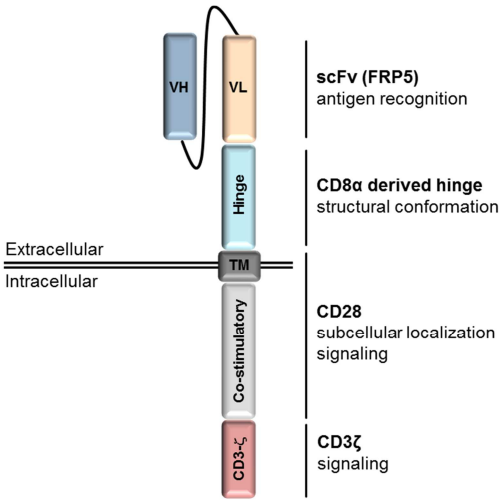

Figure 1.9 ErbB2 specific 2nd generation CAR expressed by NK-92/5.28.z [95]

1.6 Aim of the study

Treatment of cancer patients with innovative cellular products is preceded by the tedious translation of laboratory scale experiments and upgrading research-grade procedures to clinically applicable, robust and reproducible processes compliant with GMP guidelines and feasible for clinical-scale production. Generation of the cellular product of NK-92/5.28.z planned in this study is based on concept applied by Tonn et al. for parental NK-92 cells [35], where a qualified GMP-compliant master cell bank serves as a platform for further expansion and generation of individual therapeutic doses. While NK-92/5.28.z cells were generated under GMP compliant conditions, by means of lentiviral transduction, any further development for clinical application will involve the establishment and qualification of a GMP-compliant master cell bank. One major hurdle in NK-92 based therapies is the general sensitivity of NK-92 cells and their derivative to cryopreservation and thawing. Other obstacles are the demand of this cell line for complex cell culture media and its IL-2-dependent proliferation and potency. Therefore, the overall aim of this thesis was to develop a process which will allow the generation of a safe and efficient cellular therapy product of NK-92/5.28.z cells following standardized procedures in order to be able to support a subsequent phase I/II clinical trials in ErbB2-overexpressing malignancies. The first task of this study was the establishment of GMP-grade culture and of cryopreservation protocols optimal for efficient growth and satisfactory post-thaw recovery of NK-92/5.28.z cells in order to generate a GMP-compliant master cell bank. In addition, it was necessary to develop adequate methods to enable determination and monitoring of the identity (CD56 expression, lack of CD16 and CAR expression) and functional properties (retargeted killing of ErbB2+ targets and natural killing ability against K562 cells) of the NK-92/5.28.z cell line to control the quality of the cells during manufacturing processes and as a final product. Since the clinical application of NK-92/5.28.z cells will most probably include γ-irradiation to prevent *in vivo* expansion, different irradiation doses were analyzed in order to find a dose which will inhibit proliferation but retain cell potency. The establishment of a robust manufacturing process yielding high numbers (up to 5×10^9) of potent NK-92/5.28.z cells was of major interest. In order to achieve this goal, the growth kinetic of CAR-expressing NK-92 cells had to be investigated to determine an expansion schedule and optimal harvest point of

individual patient doses. Other issues associated with clinical application, including final product formulation: the maximal cell density at which effector cells retain their viability and potency and optimal shipment/injection solution, were addressed in this study in order to obtain a stable cellular therapy product with clinically relevant shelf-life. Considering the fact that a patient's premedication may attenuate natural killer cell cytotoxicity [97], it was reasonable to test the impact of corticosteroids used in cancer patients on the potency of NK-92/5.28.z cells. Since CAR-T cell therapies were associated with cytokine release syndromes [98], it was of importance to determine the profile of soluble factors secreted by target stimulated NK-92/5.28.z cells. To further explore the safety aspect, the influence of NK-92/5.28.z cells on normal human hematopoietic stem cells was tested with colony forming assay.

2 Materials and Methods

2.1 Chemicals

Table 2.1: List of chemicals

Chemicals	Company
7-AAD (7-Amino-Actinomycin D)	BD Pharmingen™, San Jose, CA, USA
Calcein violet AM	Molecular Probes Invitrogen, Karlsruhe, Germany
DMSO	WAK Chemie Medical, Steinbach, Germany
Lymphocyte separation medium	BioWhittaker, Lonza, Basel, Switzerland
HEPES	Carl Roth, Karlsruhe, Germany
Probenecid	Alfa Aesar, Karlsruhe, Germany
Recombinant Human ErbB2/Her2 Fc Chimera	R&D systems, Minneapolis, USA
Hydrogen peroxide	Allied Signal, Morristown, USA

2.2 Cell culture reagents

Table 2.2: List of cell culture reagents

Reagents	Company
Accutase	GE Healthcare, Pasching, Austria
CellGro SCGM (Stem Cell Growth Medium)	CellGenix, Freiburg, Germany
CryoStor CS10 medium	BioLife Solutions, STEMCELL Technologies, Vancouver, Canada
DMEM-GlutaMax™ (Dulbecco's Modified Eagle Medium (High Glucose)	Gibco Life Technologies, Carlsbad, USA
FBS (Fetal Bovine Serum)	Biochrom, Berlin, Germany
Human plasma	German Red Cross, Blood donation service, Frankfurt am Main, Germany
Human serum albumin 200g/L	Baxter, Deerfield, Illinois, USA
PLTMax (hPL 1)	Mill Creek Life Sciences, Rochester, USA

Reagents	Company
Human platelet lysate 2 (hPL 2)	German Red Cross, Blood donation service, Frankfurt am Main, Germany
IL-2 (Proleukin)	Novartis Pharma, Nürnberg, Germany
PBS (Dulbecco's Phosphate Buffered Saline without Ca^{2+} and Mg^{2+})	Gibco Life Technologies, Carlsbad, USA
Penicillin-Streptomycin	Gibco Life Technologies, Carlsbad, USA
RPMI-1640-GlutaMax™ (Roswell Park Memorial Institute Medium)	Gibco Life Technologies, Carlsbad, USA
StemMACS HSC-CFU lite with Epo	MiltenyiBiotec, Bergisch Gladbach, Germany
Trypan blue	Sigma-Aldrich, St. Louis, USA
X-Vivo 10 without Phenol red and Gentamicin	Lonza, Basel, Switzerland
X-Vivo 10 without Phenol red and Gentamicin contains recombinant transferrin	Lonza, Basel, Switzerland

2.3 Buffers

Table 2.3: List of buffers

Buffers	Composition
FACS buffer	0.5 % FBS in 1x PBS
EuTDA assay buffer	1 % Probenecid 2 % HEPES in 1x PBS
HEPES	Gibco Life Technologies, Carlsbad, USA
PAGGS-M based transport buffer	1 % HSA in 1 x PAGGS-M
SAG-M based transport buffer	1 % HAS

	in 1x SAG-M
PBS/EDTA based transport buffer	1 % HSA in 1x PBS/EDTA
SSP based transport buffer	1 % HSA in 1x SSP

2.4 Antibodies and kits

Table 2.4: List of antibodies

Antibody	Company	Clone	Dilution factor
FACS (fluorescence-activated cell sorting)			
FITC-labeled mouse anti-human CD16	BD Biosciences	NKP15	1:50
PE-labeled mouse anti-human CD56	BD Biosciences	NCAM16.2	1:100
APC-conjugated mouse anti-human CD56	BD Biosciences	B159	1:30
V450-labeled mouse anti-human CD3	BD Horizon	UCHT1	1:20
APC-conjugated goat anti-human IgG $F(ab')_2$	Jackson ImmunoResearch	Polyclonal	1:200
PE-labeled mouse anti-human ErbB2	R&D Systems	191924	1:20
Isotype controls			
Mouse IgG_{2B} isotype control-PE	R&D Systems	133303	1:20

Table 2.5: List of kits

Kit	Company
QuantiBRITE™PE*	Becton Dickinson
EasySep™ Negative Human NK cell Enrichment kit	STEMCELL Technologies
BD Cytometric Bead Array (CBA) Human Soluble Protein Master Buffer Kit	BD Biosciences
BD CBA Flex Set (human soluble proteins) ▪ CD178 (Fas ligand) Flex Set ▪ G-CSF Flex Set ▪ GM-CSF Flex Set ▪ TNF Flex Set ▪ IFN-γ Flex Set	BD Biosciences

Kit	Company
▪ IL-2 Flex Set ▪ IL-6 Flex Set ▪ IL-8 Flex Set ▪ IL-10 Flex Set ▪ MIP-1α Flex Set	
Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent	eBioscience
DELFIA EuTDA Cytotoxicity Reagents kit	Perkin Elmer

2.5 Cell culture methods

2.5.1 Cell lines and culture conditions

Human MDA-MB-453, MDA-MB-468, (DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen), MDA-MB-361, HepG2 and G55T2 (ATCC) were maintained in DMEM, high glucose, containing GlutaMAX (Life Technologies), the erythroleukemic K562 cells (DSMZ) were cultured in RPMI 1640 medium containing GlutaMAX (Life Technologies), both media were supplemented with 10 % of heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies). For routine maintenance, NK-92 cells as well as NK-92/5.28.z were cultured in X-Vivo 10 medium containing recombinant transferrin (Lonza, Basel, Switzerland) supplemented with 500 U/ml of IL-2 (Proleukin; Novartis Pharma, Nürnberg, Germany) and 5 % of heat-inactivated human plasma (German Red Cross, Blood Donation Service, Baden-Württemberg–Hessen, Frankfurt, Germany) unless otherwise specified in the respective experiment. NK cells were split twice a week and kept in culture at a concentration ranging from 5 x 10^4 cells/ml, defined as seeding concentration, to 5 x 10^5 cells/ml.

2.5.2 Culture media and supplements for expansion of NK-92 and NK-92/5.28.z

To establish optimal culture protocols enabling efficient expansion of NK-92/5.28.z cells, the following GMP-grade culture media were tested:

- X-Vivo 10 w/o phenol red and Gentamycin containing human holo-Transferrin (Lonza #BE04-743Q)
- X-Vivo 10 w/o phenol red and Gentamycin containing recombinant Transferrin (Lonza #BE02-055Q)
- CellGro (SCGM) (CellGenix)

In addition, diverse culture conditions (serum-free culture, human serum substitutes, and different IL-2 concentrations) were assessed for their capability to optimally support proliferation of NK-92/5.28.z cells. To test the above mentioned variables, cells were seeded at an initial concentration of 5 x 10^4/ml in triplicates in vented T75 cell culture flasks (Greiner BioOne) in a combination of media supplements as indicated in the respective experiment. Large-scale expansion was conducted in gas-permeable VueLife750-C1 culture bags (CellGenix). Cell number and viability were measured using either Trypan blue exclusion assay or NucleoCounter NC-100 (Chemometec, Denmark) according to manufacturer's protocol.

2.5.3 Cryopreservation solutions

Human serum albumin (HSA) (200g/L; Baxter), as well as combination of HSA with commercially available CryoStor CS10 medium at 1:1 ratio (BioLife Solutions, STEMCELL Technologies) were used as a base cryopreservation solution to freeze parental NK-92 and NK-92/5.28.z. Human serum albumin was supplemented with various amounts of DMSO to yield final concentrations of 5-15 % DMSO. NK-92 and NK-92/5.28.z cells from exponentially growing culture were harvested by centrifugation in conical, 50 ml Falcon tubes (Greiner Bio-One, Kremsmünster, Austria) at 1800 rpm, for 10 min at RT. Subsequently, supernatant was discarded and the cell concentration was adjusted to 1 x 10^7 cells/ml in respective base medium (HSA, CryoStor, HSA + CryoStor). 500 µl of cell suspension (5 x 10^6 cells) was transferred to 1 ml cryovial (Greiner Bio-One, Kremsmünster, Austria) and filled up to 1 ml with respective cryopreservation base medium supplemented with DMSO. Samples were performed in triplicates. Cryovials were transferred into isopropanol-based freezing boxes and stored overnight at -80 °C. Next day cells were placed in a liquid nitrogen tank and stored in the vapor phase at < -145 °C.

After 3 weeks, cells were thawed rapidly in a water bath pre-warmed to 37 °C. Next, the content of each cryovial was gently resuspended in 10 ml of heat inactivated human plasma pre-cooled to 4 °C and centrifuged at 2000 rpm, for 10 min at RT. Supernatant was discarded and cell pellet was resuspended in 20 ml of pre-warmed, complete culture medium and transferred to a T75 culture flask (Greiner Bio-One, Kremsmünster, Austria). Subsequently, cell concentration and viability were determined using trypan blue exclusion assay according to manufacturer's protocol. Measurement was repeated after 24 hours. Additionally, the influence of cryopreservation solution supplemented with 20 % of conditioned medium on post-thaw recovery and cell growth of NK-92/5.28.z was investigated. NK-92/5.28.z cells were seeded at a concentration of 7 x 10^4 cells/ml in complete X-Vivo 10 rTF medium and cultured for 72 hours. After this time, the cell suspension was transferred to a 50 ml falcon tubes and centrifuged at 4000 rpm for 15 min at RT. Supernatant was harvested, supplemented with IL-2 to a final concentration of 500 U/ml and used as conditioned medium in subsequent experiments. NK-92/5.28.z cells from exponentially growing culture were harvested and frozen in triplicates at a concentration of 5 x 10^6 cells/ml in 1 ml cryovials. Cryopreservation medium consisted of human serum albumin supplemented with 20% of conditioned medium and 7.5 % of DMSO. NK-92/5.28.z cells were frozen using the ThermoForma computer controlled freezing device with a temperature drop rate of 1 Kelvin/min down to -100 °C and subsequently placed in a cryostorage tank. After 7 days, cells were thawed and viability and cell concertation were analyzed as described above.

2.5.4 Gamma irradiation

Cells were exposed to various irradiation doses ranging from 0-30 Gy (0 Gy, 2 Gy, 5 Gy, 10 Gy and 30 Gy) using a cesium source with a dose rate of 3.75 Gy/min (IBL 437C blood irradiator). After exposure, cells were centrifuged (1800 rpm, 10 min, RT) and washed in 20 ml of complete X-Vivo 10 rTF medium. After centrifugation, supernatant was discarded and cells were seeded in 20 ml of complete X-Vivo 10 rTF medium at an initial concentration of 1 x 10^5/ml and cultured for 120 hours in a cell culture incubator. Cell proliferation, viability, specific cytotoxicity and natural killing

ability of parental and genetically modified NK-92 cells were tested at indicated time points post-irradiation.

2.5.5 Shipment/injection solutions

Stability of NK-92/5.28.z was investigated in 6 buffered solutions. Testing included 4 solutions routinely used for storage/preparation of blood products: saline-adenine-glucose-mannitol hypertonic solution (SAG-M; 376 mOsm/l; Fresenius Kabi, Bad Homburg, Germany) and phosphate-adenine-glucose-guanosin-saline-mannitol isotonic solution (PAGGS-M; 285 mOsm/l; Fresenius Kabi, Bad Homburg, Germany) both erythrocyte storage buffers, SSP (Macopharma, Langen, Germany) a thrombocyte additive solution and PBS/EDTA (MiltenyiBiotec, Bergisch Gladbach, Germany) used for preparation of stem cell products, all buffers were supplemented with 1 % of human serum albumin. In addition, pure HSA (200 g/1L) and X-Vivo 10 rTF medium as pure or complete (supplemented with 5% heat inactivated human plasma and 500 U/ml IL-2) formulation were included in the analysis. Cells were expanded in VueLife 750-C1 culture bags prefilled with complete culture medium, harvested by centrifugation and irradiated with 10 Gy. After irradiation, the cell suspension was centrifuged (2000 rpm, 10 min, RT) and washed with 20 ml of pure X-Vivo 10 rTF medium. After centrifugation, supernatant was discarded and cell concentration was adjusted to 5 x 10^6 cells/ml in respective buffer. Non-irradiated cells stored in complete X-Vivo 10 rTF were included as controls. Cell suspension was transferred to CryoMACS bags and stored under ambient conditions (temperature range: 22-22.5 °C) for 48 hours. Viability and pH measurements were performed in duplicates over the storage period using the NucleoCounter NC-100 (Chemometec) device and Microprocessor pH Meter pH 211 (Hanna instruments), respectively. Samples were analyzed in duplicates in 3 independent experiments. Subsequently, the analysis of stability was extended to cell potency. Specific cytotoxicity of NK-92/5.28.z cells in suspensions prepared as described above in pure X-Vivo 10, pure HSA and PAGGS-M + 1 % HSA was analyzed in quintuplicates using EuTDA assay (described in chapter 2.7.3) after 6-hour storage under ambient conditions.

2.5.6 Clinical scale expansion of NK-92/5.28.z

Clinical scale expansion was conducted in gas-permeable VueLife 750-C1 culture bags (CellGenix), which proved to be optimal (data not shown). NK-92/5.28.z cells were inoculated into culture bags prefilled with 1000-2000 ml of X-Vivo 10 medium containing recombinant transferrin, supplemented with 5 % of heat inactivated human plasma and 500 U/ml of IL-2 at a concentration of 5 x 10^4 cells/ml. After 5 days, cells were harvested at a concentration of 5-6 x 10^5/ml by centrifugation (2000 rpm, 20 min RT, R9 acceleration/R3 breaks) in 400-ml transfer bags (Fenwal), γ-irradiated with 10 Gy, washed with fresh X-Vivo 10 rTF + 100 U/ml IL-2 medium, concentrated by centrifugation and transferred to CryoMACS bag (MiltenyiBiotec, Bergisch Gladbach, Germany) serving as a final bag. After cell counting performed with Sysmex XT-1800i (Sysmex, Kobe, Japan), cell concentration in the final product was adjusted to 5 x 10^7/ml in X-Vivo 10 containing recombinant transferrin, supplemented with 100 U/ml of IL-2. Three independent final products were generated and tested in terms of cell viability, CAR expression and potency. Additionally, sterility testing for aerobic and anaerobic bacteria was performed using the BacT/ALERT system, presence of mycoplasma was tested using the PCR-based Intego Mycoplasma Detection Kit and endotoxin level was measured by Endosafe PTS, all used for routine testing of blood products.

2.6 Establishment of a GMP-compliant master cell bank of NK-92/5.28.z

2.6.1 Cell expansion and cryopreservation

Generation of master cell bank (MCB) was performed in a class 100 cleanroom in the GMP facility of the Institute for Transfusion Medicine in Frankfurt, with qualified starting materials only. The manufacturing process consisted of three main phases: (i) thawing and recovery, (ii) expansion in cell culture bags, (iii) harvesting and freezing. Genetically modified NK-92 cells were thawed and after recovery and a pre-expansion phase seeded at a concentration of 1-3 x 10^4 cells/ml in 10 VueLife 750-C1 culture bags prefilled with 1.5 L of X-Vivo 10 containing recombinant transferrin supplemented with 5 % of heat inactivated human plasma and 500 U/ml of

IL-2. Sterility testing for aerobic and anaerobic bacteria was performed using the BacT/ALERT system. After 50 days NK-92/5.28.z cells were harvested with the concentrations of 7-13 x 10^5 cells /ml considered as optimal and confluent cell density. 10 VueLife 750-C1 bags were connected one by one with 4 transfer bags (Fresenius Kabi, Bad Homburg, Germany) using a sterile tubing welder (TSCD II; Terumo, Tokio, Japan) and 15 liters of cell suspension were transferred by means of a peristaltic pump (ISMATEC). Transfer bags containing cell suspension were centrifuged (1800 rpm, 20 min RT, R9 acceleration/R9 breaks) and supernatant was pressed out using a manual plasma extractor (Baxter laboratories) into an empty transfer bag. One bag with supernatant was kept to serve as a conditioned medium in the end of procedure. Transfer bags were connected using a sterile tubing welder (Terumo TSCD II) and cell pellets were pooled into 1 transfer bag. Cells were resuspended in 100 ml of PBS/EDTA solution and incubated in the refrigerator for 15 min in order to avoid clumping. Subsequently, the cell suspension was centrifuged (1800 rpm, 20 min RT, R9 acceleration/R9 breaks) and resuspended in conditioned medium. 500 µl of cell suspension was transferred into 1 ml cryovial (Greiner Bio-One) to which were added 500 µl of HSA supplemented with 15% of DMSO. 200 vials containing in total 5 x 10^9 cells (approximately 2.5 x 10^7cells/vial) were frozen in cryopreservation solution consisting of 50 % HSA, 50% of conditioned medium and DMSO at a final concentration of 7.5 %. Freezing was performed using a ThermoForma computer controlled freezing device with a temperature drop rate of 1 Kelvin/min down to -100 °C (Figure 2.1). Subsequently, the vials were placed into the vapor phase of liquid nitrogen at < -145 °C.

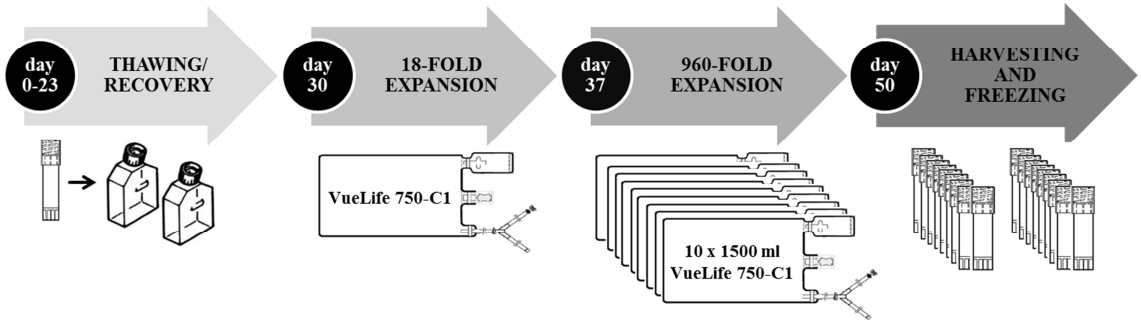

Figure 2.1 Generation of a master cell bank

2.7 Potency assays

2.7.1 Verification of identity and stability of chimeric antigen receptor expression

Identity and CAR expression stability of NK-92/5.28.z cells, which were kept in maintenance culture for up to 12 months, were monitored monthly using a two-step FACS staining. Parental NK-92 cells were included as a control. 1 x 10^5 cells/sample from an exponentially growing culture were transferred to FACS tubes and centrifuged (1500 rpm, 5 min, RT). Supernatant was discarded and 2 ml of PBS + 0.5 % FCS were added to each tube and centrifuged again. After discarding the supernatant 1 µg/sample of rhErbB2/Fc fusion protein (R&D Systems) was added and samples were incubated for 30 min on ice. Afterwards, cells were washed twice with 2 ml of FACS wash buffer (PBS + 0.5 % FCS) and 5 µl/sample of goat anti-human IgG F(ab')$_2$-APC (Jackson ImmunoResearch) was added forming FACS-detectable complexes with fusion protein bound to CAR expressed by NK-92/5.28.z cells (Figure 2.2). To confirm an immunophenotype characteristic for NK-92 cell line (CD16^{neg}, CD56^{bright}) and to estimate cell viability, 1µl/sample of anti-human CD56-PE (BD Biosciences), 2 µl/sample of anti-human CD16-FITC (BD Biosciences) and 5 µl/sample of 7-AAD (BD Pharmingen) were included into the staining. Samples were incubated for 15 min on ice and washed twice with 2 ml per tube of FACS wash buffer. After the last centrifugation, supernatant was discarded and the pellet was resuspended in 250 µl of Cytofix solution (BD Bioscience). Samples were incubated for 15 min at 4 °C protected from light and washed with 2 ml of FACS wash buffer. After centrifugation and decantation of supernatant, the cell pellet was resuspended in 400 µl of FACS wash buffer. Analysis was performed in triplicates. Measurement and data analysis were performed using FACSCanto II flow cytometer equipped with FACSDiva software.

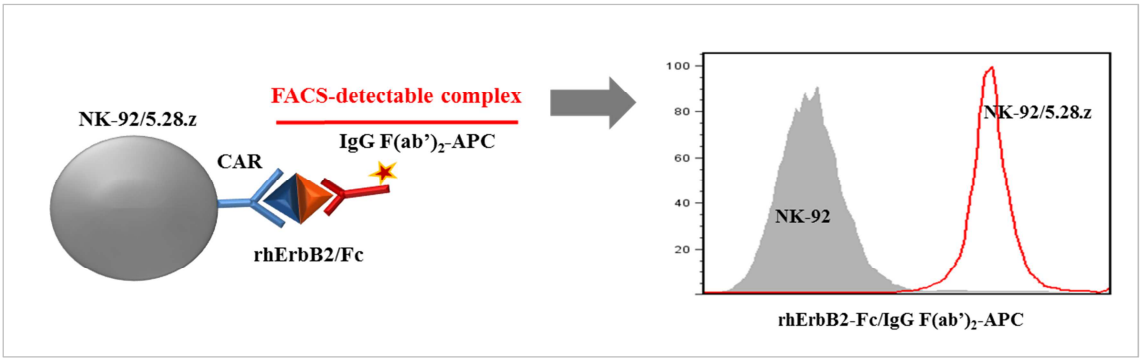

Figure 2.2 Principle of CAR staining

2.7.2 FACS-based cytotoxicity assay

Cytotoxicity of NK-92/5.28.z and parental NK-92 cells toward tumor targets (MDA-MB-453, MDA-MB-468 and K562) was analyzed using a FACS-based killing assay. Adherent target cells were harvested using Accutase solution (GE Healthcare, Pasching, Austria) and washed in complete DMEM culture medium (centrifugation 1400 rpm, 5min, RT). The supernatant was discarded and the concentration of target cells was adjusted to 1 x 10^6 cells/ml for subsequent staining with calcein violet AM (Molecular Probes, Invitrogen, Karlsruhe, Germany). Briefly, calcein violet AM powder was resuspended in DMSO and 1.5 µl of calcein violet solution per 1 ml of target cell suspension was added. Target cells were incubated for 30 min on ice, centrifuged (1400 rpm, 5min, RT) and washed twice with 20 ml of complete DMEM medium. After the last centrifugation, supernatant was discarded and the concentration of target cells adjusted in complete X-Vivo 10 rTF to 5 x 10^5 cells/ml. NK-92/5.28.z and parental NK-92 were harvested by centrifugation (1600 rpm, 5 min, RT) and resuspended in complete X-Vivo 10 rTF. Effector cells were counted and concentration was adjusted to 5 x 10^6 cells/ml in complete X-Vivo 10 rTF. Co-incubation of target and effector cells was performed in triplicates at a 10:1 effector to target (E:T) ratio in 96-well Ultra Low Attachment plates (Corning, New York, USA). 100 µl/well of target cell suspension (5 x 10^4 cells) and 100 µl/well of effector cells (5 x 10^5 cells) were pipetted into wells and incubated for 2 hours in a cell culture incubator. To determine spontaneous lysis, target cells were incubated in assay medium without effector cells. After incubation time the content of each well was transferred to an appropriately labeled FACS tube. Cells were labeled with 7-AAD (BD Pharmingen)

viability dye at 0.5 µg/test and 3 µl/sample of anti-human CD56-APC (BD Biosciences) and incubated for 15 minutes on ice. Samples were measured with the FACSCanto II flow cytometer and data were analyzed using FACSDiva software version 6.1.3. Percentage of 7-AAD and calcein violet AM double positive cells measured in the test tube were considered as sample lysis and used for calculation of specific cytotoxicity according to the equation:

% Specific cytotoxicity = [(sample lysis - spontaneous lysis)/(100 - spontaneous lysis)] x 100

2.7.3 Europium TDA (EuTDA) cytotoxicity assay

In addition to a FACS-based killing assay, europium release cytotoxicity assay was established. Adherent target cells were harvested and washed as described in chapter 2.7.2. Target cells were counted and concentration was adjusted to 1 x 10^6 cells/ml. Subsequently, loading of target cells with the acetoxymethyl ester of the fluorescence enhancing ligand (BATDA) (Perkin Elmer) was performed. A volume of 1µl of BATDA reagent per 1 x 10^6 of target cells was added. Target cells were incubated for 30 min at 37 °C in cell culture incubator, thereafter cells were pelleted by centrifugation (1100 rpm, 4 min, RT), washed twice with 20 ml of washing buffer (PBS + 2.5 mM Probenecid + 20 mM HEPES) and counted using NucleoCounter NC-100 (Chemometec) device. Target cell concentration was adjusted to 1 x 10^5 in assay medium (X-Vivo 10 rTF + 5 % of human plasma + 500 U/ml of IL-2 + 2.5 mM Probenecid). NK-92/5.28.z and parental NK-92 were harvested by centrifugation (1700 rpm, 5 min, RT), supernatant was discarded and the cell pellet was resuspended in assay medium. Effector cells were counted and concentration was adjusted to 1 x 10^6 cells/ml in assay medium. Co-incubation of target and effector cells was performed in triplicates at 10:1 effector to target (E:T) ratio in 96-well V-bottom plate (Greiner Bio-One, Kremsmünster, Austria): 50 µl/well of target cell suspension (5 x 10^3 cells) and 50 µl/well of effector cells (5 x 10^4 cells) were pipetted into wells and incubated for 2 hours in a cell culture incubator. To determine spontaneous lysis, 50 µl/well of target cells were incubated with 50 µl/well of assay medium without effector cells. To determine the background derived from BATDA reagent, target cell

suspension was centrifuged and 50 µl/well of supernatant was mixed with 50 µl/well of assay medium. Maximal lysis was assessed by treatment of target cells with 20 µl/well of Lysis buffer (Perkin Elmer) mixed with 30 µl/well of assay medium. After incubation time, 20 µl of supernatant was transferred from each well to a 96-well Flat bottom Optic Plate. Next, 200 µl of europium solution was added to each well and the plate was incubated for 15 min on a rocker-shaker protected from light. After this time, measurement of the fluorescent signal using Victor X4 (Perkin Elmer) set on Delfia Europium Protocol was performed. Specific lysis was calculated according to the following formula:

% Specific release = [(Sample release - Spontaneous release)/(Maximal release - Spontaneous release)] x 100

Spontaneous release was calculated as follows:

% Spontaneous release = [(Spontaneous release - Background)/(Maximal release - Background)] x 100

2.7.4 Cytometric bead array

Release of soluble factors by NK-92/5.28.z and parental NK-92 upon stimulation with target cells was analyzed using the FACS-based Cytometric Bead Array (BD Biosciences). 5 x 10^5 effector cells per sample were co-incubated with tumor cells at 10:1 effector to target (E/T) ratio for 2 hours at 37 °C. Unstimulated effector and target cells, as well as cells stimulated with PMA (50 ng/ml)/Ionomycin (500 ng/ml), were included for determination of basal release and maximal release respectively. After incubation, the assay plate was centrifuged and 50 µl of supernatant was collected from each assay well and further analyzed for the presence and concentration of soluble factors according to the manufacturer's protocol. Briefly, samples were incubated with capture beads specific to a defined set of analytes (Flex Set). Next, a master mix of PE-conjugated antibodies (detection reagent) was added forming FACS-detectable complexes (capture bead-analyte-detection reagent). Qualitative and quantitative analysis of the sample was based on the fluorescence signal characteristic for bead and

detector, respectively (Figure 2.3). Data were collected using BD LSRFortessa flow cytometer and analyzed with FCAP Array software version 3.0.

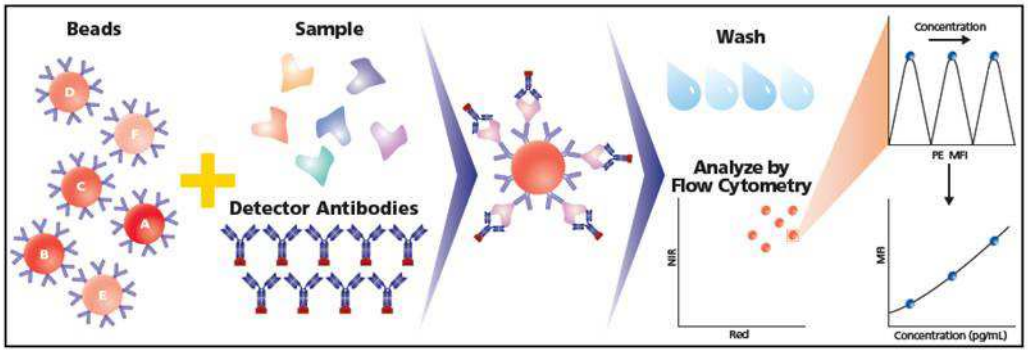

Figure 2.3 Cytometric bead array assay. Modified from bdbiosciences.com

2.7.5 Isolation of human primary NK cells

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of 3 random healthy donors by centrifugation over a density gradient lymphocyte separation medium (BioWhittaker, Lonza). Subsequently, purification of human primary NK cells from the PBMC fraction was performed using the EasySep^{TM} Negative Human NK cell Enrichment kit (STEMCELL Technologies) according to the manufacturer's protocol. Briefly, 2 ml of PBMC resuspended in PBS/EDTA (CliniMACS, MiltenyiBiotec) at a concentration of 5×10^7 cells/ml were placed in a polystyrene tube (1×10^8 cells/tube). 50 µl/ml of EasySep^{TM} Human NK cell Enrichment Cocktail containing bispecific antibody complexes targeted against non-NK cell surface antigens and against dextran were added. Samples were mixed by vortexing and incubated for 10 min at RT. After incubation, 100 µl/ml of dextran-coated EasySep^{TM} D magnetic particles were added and samples were incubated for a further 5 min. Afterwards, tubes were placed into an EasySep^{TM} magnet. After 2.5 min, the desired fraction of unbound NK cells was poured off into a new tube. To increase the purity, the selection step was repeated. After isolation, positive fraction of NK cells was centrifuged (1400 rpm, 5 min, RT) resuspended in complete X-Vivo 10 medium and conditioned in a cell culture incubator for 60 min. To assess the purity and viability of the isolated NK cell population, FACS staining for the detection of CD3 and CD56 was performed. Cells were stained with 3 µl/sample of anti-human CD56-APC

(BD Biosciences), 5 µl/sample CD3-V450 (BD Horizon) and 5 µl/sample 7-AAD (BD Pharmingen) and incubated for 15 min at RT protected from light. Afterwards, samples were washed with 2 ml of FACS wash buffer and measured using a BD LSRFortessa flow cytometer.

2.7.6 Human stem cell colony formation assay after NK-cell treatment

Peripheral blood stem cells (PBSCs) from G-CSF mobilized donors (n=3) were resuspended in complete X-Vivo 10 rTF medium. Based on the analysis of apheresis product provided by Cell Separation Department of Blood Transfusion Centre, Frankfurt, the concentration of CD34+ cells was adjusted to 3000/ml. NK-92 and NK-92/5.28.z were harvested by centrifugation (1600 rpm, 5 min, RT) and the concentration of the cells was adjusted in fresh complete X-Vivo 10 rTF to the concentration resulting in a 10:1 ratio between effector cells and PBSCs. Conditioned medium of NK cells was performed as follows. NK-92 and NK-92/5.28.z cells were seeded at a concentration of 1 x 10^5 cells/ml in complete X-Vivo 10 rTF medium and cultured for 48 hours. After this time, cell suspensions were transferred to 50 ml falcon tubes and centrifuged at 2000 rpm for 10 min at RT. Supernatant was harvested and used as conditioned medium for treatment of PBSCs. Co-incubation of PBSCs and effector cells/conditioned medium was performed in triplicates in 96-well Ultra Low Attachment plates (Corning, New York, USA): 100 µl/well of PBSCs suspension (300 CD34+ cells/well) and 200 µl/well of effector cells or conditioned medium were pipetted into wells and incubated for 15 hours in a cell culture incubator at 37 °C. After incubation, the content of each well was seeded into a tube containing 3 ml of methylcellulose medium (StemMACS HSC-CFU lite with Epo, MiltenyiBiotec). Samples were vortexed and 1.1 ml of methylcellulose containing approximately 100 CD34+ cells was transferred into one 35-mm culture plate in duplicates (Greiner Bio-One, Kremsmünster, Austria). Controls included NK-92, NK-92/5.28.z and PBSC incubated alone in assay medium (X-Vivo 10 containing recombinant transferrin, supplemented with 500 U/ml of IL-2 and 5 % of heat inactivated human plasma). After 14 days, colonies of myeloid and erythroid linages were enumerated (colony definition ≥ 30 cells) (Figure 2.4).

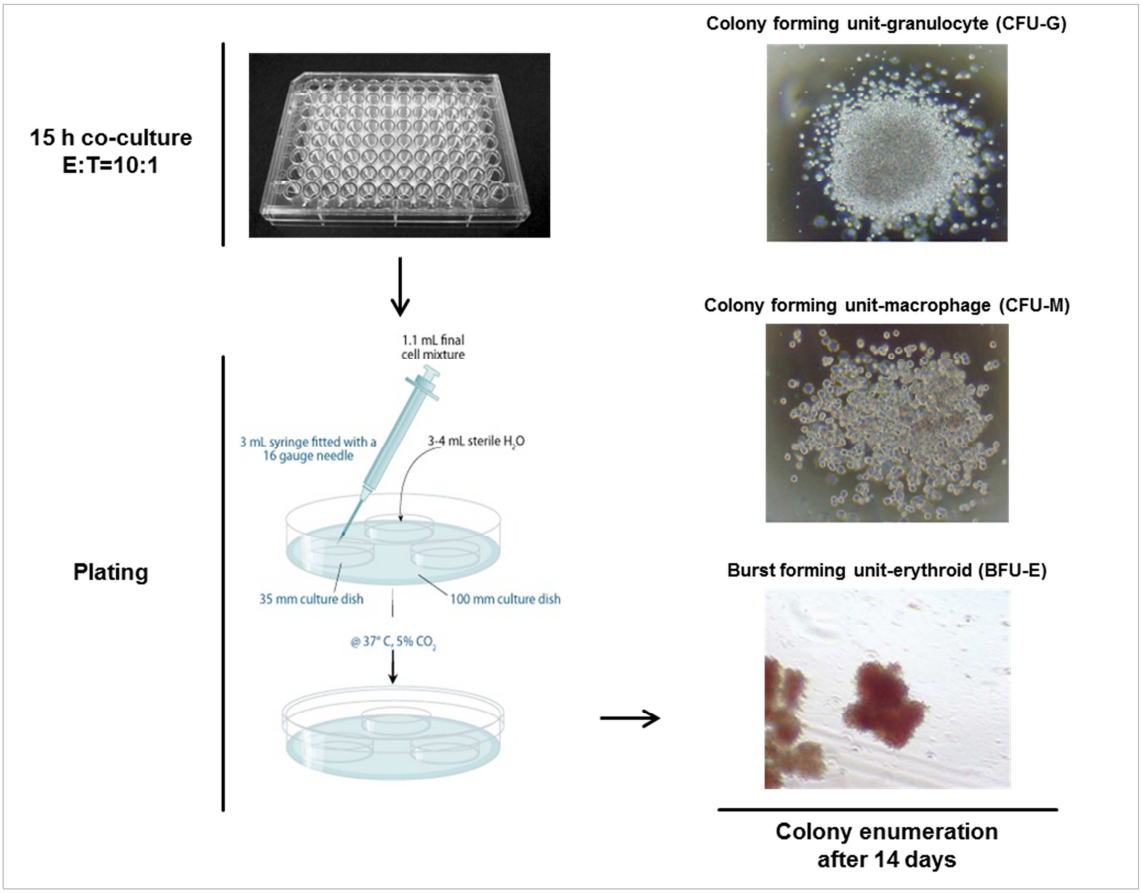

Figure 2.4 Scheme of the CFU-C assay. Modified from rndsystems.com

2.7.7 Enumeration of ErbB2 molecules on target cells

The average number of ErbB2 molecules per target cell was determined using QuantiBRITE-PE beads (BD Biosciences). 1 x 10^5 target cells/tube were stained with 5 µl of anti-human ErbB2-PE antibody (R&D Systems) and incubated for 30 min at room temperature in dark. After incubation, samples were washed twice with 2 ml of FACS wash buffer (PBS + 0.5% FCS). After the last centrifugation, supernatant was discarded and the cell pellet resuspended in 300 µl of FACS wash buffer. A ready-to-use tubes containing four populations of QuantiBRITE-PE beads with various, defined MFI of PE signal served as a standard sample and was run at the same instrument setting as tested samples. Samples were measured using BD LSRFortessa flow cytometer. Data derived from the standard sample were used for generation of the standard curve and further calculation. The MFI signal, derived from PE conjugated antibodies bound to the tested population, was converted into the average number of

ErbB2 molecules per cell. Isotype control was included to eliminate background derived from unspecific binding. Calculations were performed according to the manufacturer's instructions.

2.7.8 Effect of corticosteroids on cytotoxicity

NK-92/5.28.z were seeded in complete X-Vivo 10 rTF medium at a concentration of 2 x 10^5/ml in triplicates and treated with 4 different doses of Natrium (prednisolone-21-succinat) (Solu-Decortin H1000mg, *Merck* Serono GmbH) ranging from 0-200 µg/ml (0 µg/ml, 0.2 µg/ml, 2 µg/ml, 20 µg/ml,200 µg/ml) of culture medium. Prednisolone powder was dissolved in culture medium and 1:10 serial dilution was performed. 100 µl of diluted stock solutions were pipetted into respective culture flask. Cells were cultured for 24 hours in a cell culture incubator. Subsequently, specific cytotoxicity toward ErbB2-positive MDA-MB-453 cells and natural killing ability toward a HLA-negative K562 cell line were tested using EuTDA cytotoxicity assay (described in chapter 2.7.3).

2.8 Statistical analysis

Statistical analysis of data was performed using GraphPad Prism software version 5.02. Results are presented as mean ± SEM. Two-tailed Student's t-test was used for comparison of two groups. For multiple comparisons one way analysis of variance (ANOVA) was carried out. Statistical differences were considered significant at a $P < 0.05$.

3 Results

3.1 Development of a GMP-compliant manufacturing processes

3.1.1 GMP-grade culture media

NK-92 cell line was adapted to GMP-grade X-Vivo 10 culture medium in order to make it suitable for clinical application [35]. Due to its high human serum albumin (HSA) content (approximately 30 %), X-vivo 10 medium is intended to promote cell growth without the need for further human plasma substitution. Nevertheless, NK-92 cells cultured in X-Vivo 10 medium still require human plasma supplementation for efficient cell expansion. Therefore, commercially available GMP grade cell culture media: 2 variants of X-Vivo 10 (Lonza, Basel, Switzerland) and CellGro (CellGenix, Freiburg, Germany) were compared with regard to their ability to promote growth of NK-92.5.28.z cells as plasma-free and plasma-supplemented formulation. The first assessment was made between X-Vivo 10 containing human holo-transferrin and X-Vivo 10 containing recombinant transferrin (X-Vivo 10 rTF). Both media were supplemented with 500 U/ml of IL-2 and 5 % of heat inactivated human plasma. Although up to day six a difference in cell proliferation was not detected, the presence of recombinant transferrin seemed to improve cell growth in long-term culture. The most pronounced difference was observed at day 7 with fold expansion of 20.73 ± 0.33 and 15 ± 0.6 for X-Vivo 10 rTF and X-Vivo 10 containing human holo-transferrin (*P<0.0011*), respectively. These data suggest that X-Vivo 10 rTF gives the opportunity to reach higher cell concentrations in batch culture, without medium exchange or further supplementation (Figure 3.1A) when compared to X-Vivo 10 containing human-derived transferrin with maximal cell concentrations/ml of 12.49 x 10^5 ± 0.32 x 10^5 and 7.5 x 10^5 ± 0.3 x 10^5, respectively. Subsequently, X-Vivo 10 rTF was compared with CellGro (SCGM) giving equivalent results in terms of supporting cell proliferation as plasma-supplemented composition with fold expansion of 21.08 ± 0.1 (X-Vivo 10 rTF) and 20.07 ± 0.37 (CellGro) at day six. However, CellGro was not able to promote cell expansion in plasma-free conditions (max. conc./ml in batch culture 1.52 x 10^5 ± 0.1 x 10^5), whereas

X-Vivo 10 containing rTF maintained gradual cell growth (max. conc./ml in batch culture 2.65 x 10^5 ± 0.09 x 10^5) (Figure 3.1B), thus facilitating acclimation to plasma-free culture conditions. Summary of the cell growth rates obtained in tested conditions are presented in Table 3.1.

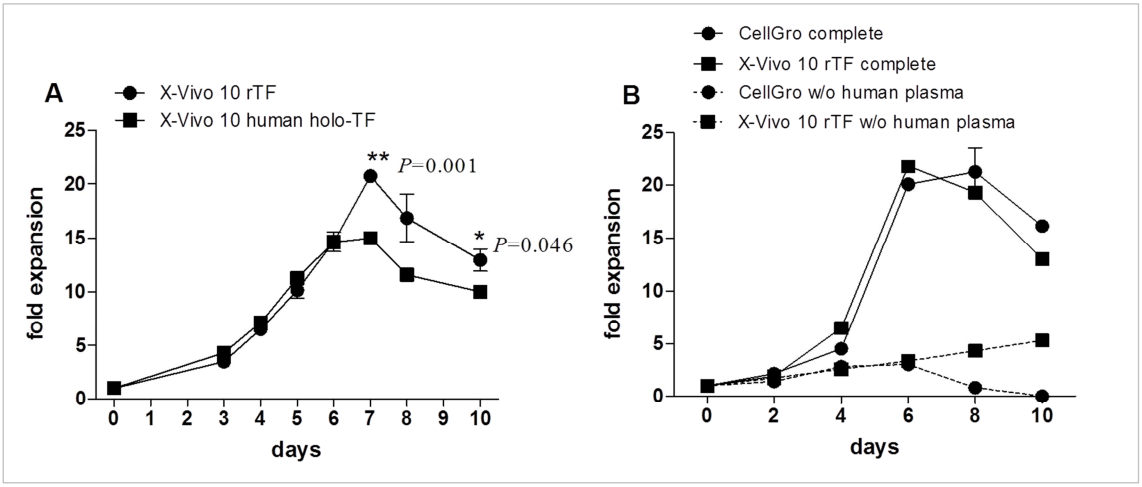

Figure 3.1 Comparison of NK-92/5.28.z cell growth in GMP-compliant serum-free culture media. (A) Comparison of two variants of X-Vivo 10 media containing transferrin from two different sources. (B) Growth of NK-92/5.28.z cells in X-Vivo 10 rTF and CellGro. Solid lines represent plasma-supplemented culture. Dotted lines represent plasma-free cultures.

3.1.2 Serum/plasma-free culture

To eliminate undefined, donor-derived supplements, NK-92/5.28.z cells were subjected to the 32-day process of acclimation to serum/plasma-free conditions. An applied feeding regimen enabled maintenance of cell viability over ≥ 80 % during the whole acclimation course and maximal concentration in batch culture of 10.2 x 10^5 ± 0.85 x 10^5 cells/ml (Mean ± SEM) at day 30 (Figure 3.2A). At the end of the acclimation procedure, the quality of the cells was evaluated with the following results for tested parameters: CAR expression > 99 %, identity (CD56+ CD16-) > 99 % and specific cytotoxicity against ErbB2(+) targets with the mean value of 94.4 %. NK-92/5.28.z cells cultured in plasma-supplemented medium were included for comparison (Figure 3.2B). Established plasma-free culture was further maintained and analyzed providing favorable results in terms of cell proliferation when compared to not acclimated control cells cultured in plasma-free medium with doubling time of 47.37 h ± 2.75 h and 83.11 h ± 3.08 h ($P < 0.001$), respectively. Cell growth parameters obtained in all tested culture conditions were summarized in Table 3.1.

Significantly shorter doubling time of the cells subjected to acclimation demonstrated the importance of the performed procedure. CAR expression and phenotype of NK-92/5.28.z were further investigated and remained stable over the 28-week analysis period with values > 99 % (Figure 3.2C).

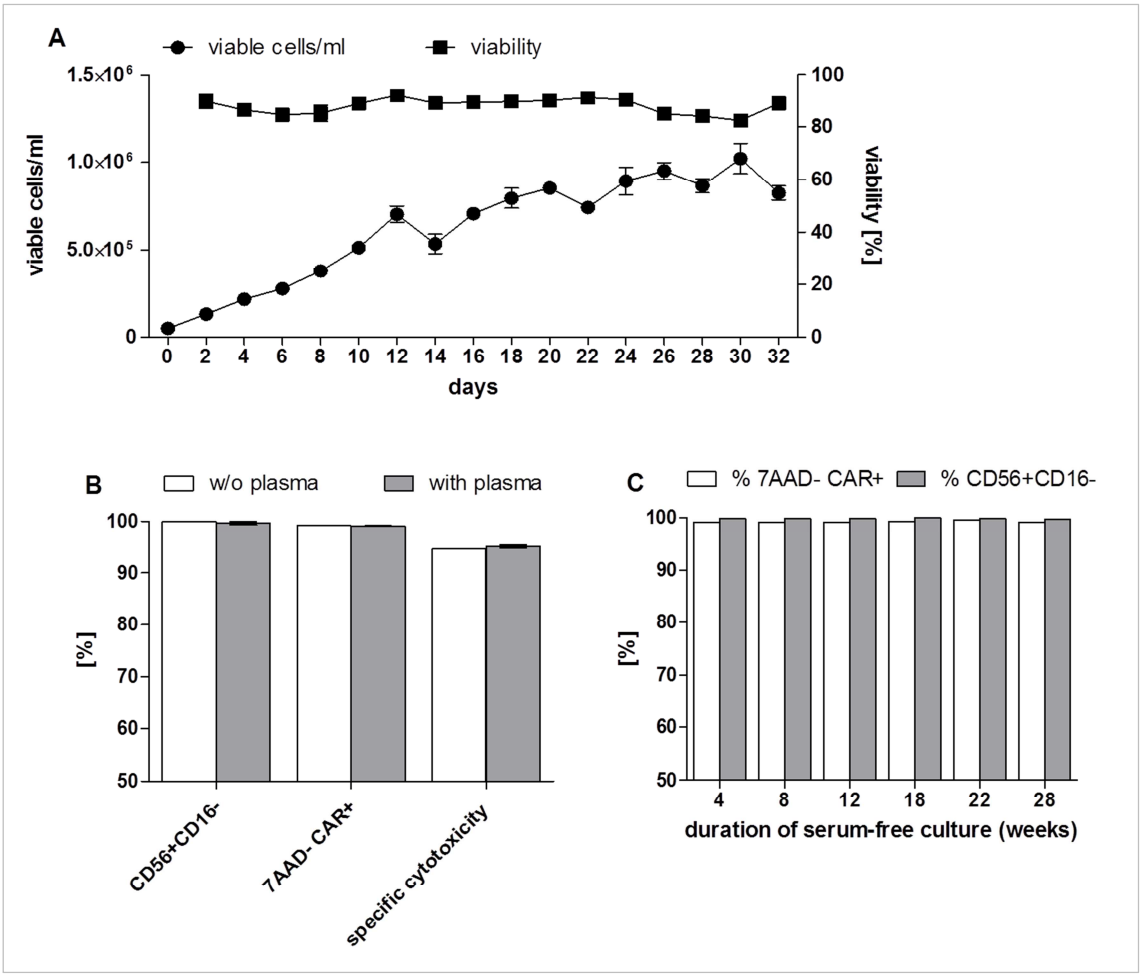

Figure 3.2 Acclimation of NK-92/5.28.z cells to serum/plasma-free culture. (A) Cells were seeded at an initial concentration of 5 x 10^4/ml in X-Vivo 10 rTF supplemented with 500 U/ml of IL-2 without addition of plasma/serum. Every second day, half the culture supernatant was removed and culture was replenished with fresh plasma-free medium. Subsequently, cells were resuspended and cell number and viability were determined. Cells were kept in batch culture for 32 days until reaching confluency defined as 1 x 10^6/ml. (B) At the end of the batch culture, identity (CD56+CD16-), CAR expression (7AAD-CAR+) and specific cytotoxicity against ErbB2(+) targets were tested and compared with the results obtained with cells cultured in plasma-supplemented medium (with plasma). (C) CAR expression and identity were further tested up to 28 weeks. Experiments were performed in triplicates. Data are presented as Mean ± SEM

3.1.3 Impact of IL-2 concentration on cell proliferation and functionality

Natural killer cells can reach optimal potency and expansion capacity only in an optimized cytokine milieu where IL-2 plays a crucial role and, in the case of NK-92, seems to be sufficient to reach satisfactory levels of cell activation. Therefore, finding an optimal concentration of this cytokine for *ex vivo* cell expansion was an important issue investigated in this study. Cell proliferation of NK-92 and NK-92/5.28.z cells was tested at five different concentrations of IL-2: 0 U/ml, 10 U/ml, 100 U/ml, 500 U/ml and 1000 U/ml. Obtained results show that IL-2 is essential for proliferation of NK-92 and NK-92/5.28.z cells. Low concentration of this cytokine (10 U/ml) is able to maintain cell proliferation with maximal fold expansion in batch culture of 8.85 ± 0.53 and 10.53 ± 1.64 (Mean ± SEM) for NK-92 and NK-92/5.28.z cells, respectively, but it is not sufficient to promote robust cell expansion. Increase of IL-2 concentration in culture medium leads to more efficient cell expansion, however, there were no significant differences in fold expansion of NK-92 and NK-92/5.28.z cells when cultured in 100 U/ml, 500 U/ml, 1000 U/ml. 500 U/ml was indicated as the optimal IL-2 concentration giving slightly better results than 100 U/ml. In addition, doubling this concentration (1000 U/ml) did not promote further cell expansion (Figure 3.3A). Interestingly, CAR-mediated specific cytotoxicity seems not to be strictly dependent on the IL-2 concentration in culture media. All three tested concentrations (50 U/ml, 100 U/ml, 500 U/ml) resulted in specific cytotoxicity of NK-92/5.28.z against ErbB2-positive targets of more than 90 % with values of 97.26 % ± 0.19 % for 50 U/ml, 96.84 % ± 0.14 % for 100 U/ml and 91.98 % ± 0.83 % for 500 U/ml. As expected, increasing levels of IL-2 supported natural killing when tested against K562 cells, of both genetically modified and parental NK-92 cells (Figure 3.3B). Furthermore, stability of cell potency in IL-2-free culture was tested over time, up to 72 hours. An instantaneous, significant decrease in natural killing of both NK-92 and NK-92/5.28.z amounting 17.59 % ± 2.5 % and 19.27 % ± 4.57 % after 24 hours, respectively, was observed. Conversely, retargeted killing of NK-92/5.28.z toward ErbB2-positive targets was decreasing gradually with cytotoxicity values of more than 50 % up to 48 h (67.77 % ± 3.16 % at 24 h; 59.02 % ± 5.16 % at 48 h) and 45.50 % ± 5.99 % after 72 hours (Figure 3.3C).

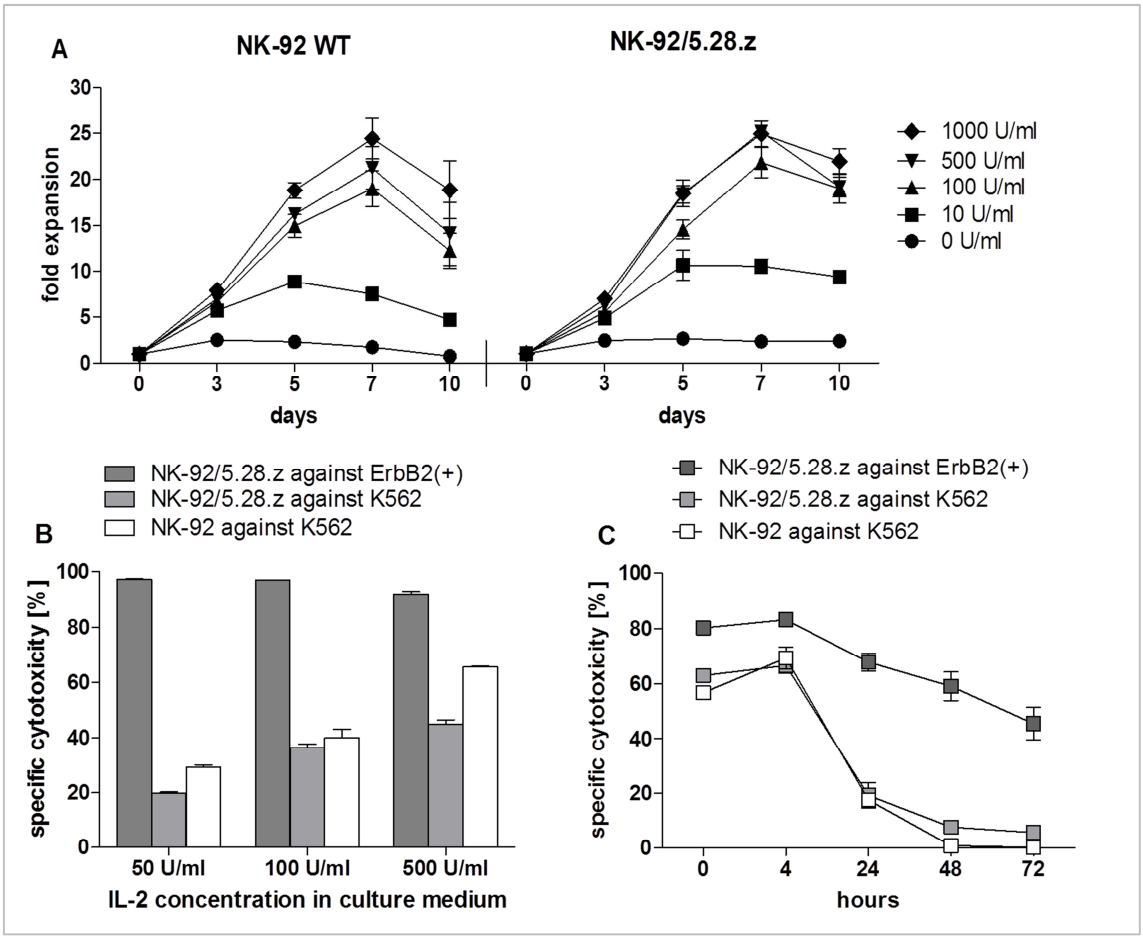

Figure 3.3 Expansion and functionality of NK-92 and NK-92/5.28.z cultured in medium supplemented with various IL-2 concentrations. (A) Proliferation of parental (NK-92 WT) and genetically modified NK-92 cells (NK-92/5.28.z) in X-Vivo 10 rTF supplemented with 5 % of human plasma and various concentrations of IL-2 up to 10 days. (B) Natural killing of NK-92 (white bars) and NK-92/5.28.z (light grey bars) and retargeted killing of NK-92/5.28.z (dark grey bars) cultured for 5 days in X-Vivo 10 rTF supplemented with 5 % of human plasma and 50 U/ml, 100 U/ml, 500 U/ml of IL-2. (C) Stability of natural killing of NK-92 (white symbol) and NK-92/5.28.z (light grey symbol) and retargeted killing of NK-92/5.28.z (dark grey symbol) in IL-2-free culture was tested up to 72 hours.

3.1.4 Influence of human platelet lysate on NK-92/5.28.z proliferation and functionality

Promising data on expansion of other cell types (e.g. MSCs, osteoblasts) cultured in media supplemented with platelet lysate encouraged us to test the proliferation of NK-92/5.28.z in a similar setting and to replace the serum substitution by platelet lysate. Therefore, platelet lysate from two different companies/sources (hPL1 and hPL2) in comparison with human plasma was assessed for its ability to promote cell expansion and cytotoxicity. However, there was no advantageous influence of cultivation in medium supplemented with 5 % of hPL in terms of natural killing (human

plasma: 47.97 % ± 4.12 %; hPL1: 62.44 % ± 5.5 %; hPL2: 54.6 % ± 4.24 %) and specific killing of ErbB2-positive (MDA-MB-453) targets (human plasma: 95.1 % ± 3.08 %; hPL1: 91.42 % ± 4.62 %; hPL2: 92.65 % ± 4.7 %) (Figure 3.4A). What is more, cells cultured in medium supplemented with 5 % of human plasma proliferated significantly better ($P < 0.05$) when compared to hPL2 with fold expansion of 23.87 ± 0.22 and 17.37 ± 0.67 (Mean ± SEM), respectively at day 9 (Figure 3.4B).

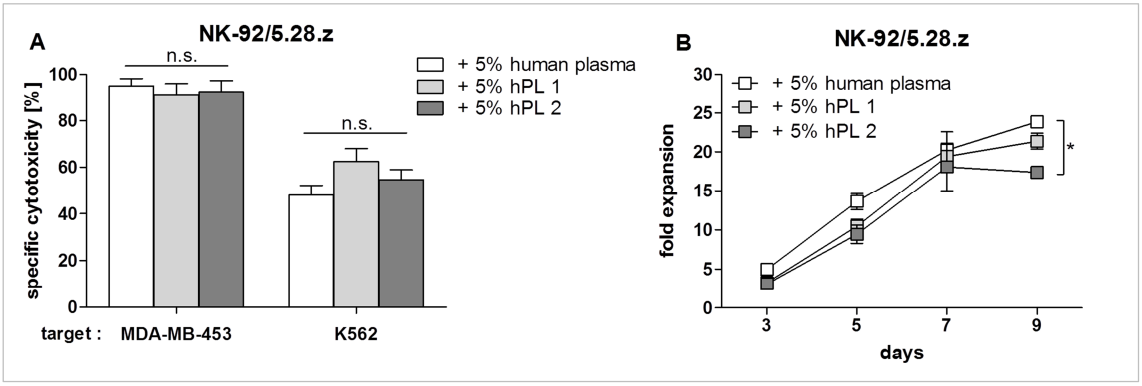

Figure 3.4 Comparison of functionality and proliferation of NK-92/5.28.z cells cultured in human plasma and platelet lysate (hPL) supplemented medium. (A) NK-92/5.28.z cells were cultured in X-Vivo 10 rTF supplemented with 500 U/ml of IL-2 and 5 % of either human plasma (white bars) or hPL1 (grey bars) or hPL2 (dark grey bars). After 72 hours natural killing against K562 and CAR-mediated killing against ErbB2-overexpressing MDA-MB-453 cell line were tested in EuTDA killing assay. (B) Proliferation of NK-92/5.28.z cultured in X-Vivo 10 rTF supplemented with 500 U/ml of IL-2 and 5 % of either human plasma (white symbols) or hPL1 (grey symbols) or hPL2 (dark grey symbols) was measured up to 9 days.

Table 3.1 Impact of different serum substitutes in GMP-compliant, albumin containing culture media supplemented with 500 U/ml of IL-2 on NK-92/5.28.z cells proliferation

Culture medium	Human serum substitute	Doubling time [h]	Maximal fold expansion	Maximal concentration/ml in batch culture [x10^5]
X-Vivo 10 w/o Phenol red and Gentamycin containing recombinant Transferrin (Lonza, #BE02-055Q)	5 % of heat inactivated human plasma	28.84 ± 0.5	24.97 ± 0.65	12.49 ± 0.32
	5 % of human platelet lysate 1	35.6 ± 1.38	21.37 ± 1.01	10.7 ± 0.52
	5 % of human platelet lysate 2	37.48 ± 1.89	18.07 ± 3.04	9.01 ± 1.52
	serum-free culture w/o acclimation	83.11 ± 3.08	5.3 ± 0.18	2.65 ± 0.09
	serum-free culture post-acclimation	47.37 ± 2.75	20.4 ± 1.71	10.2 ± 0.85
X-Vivo 10 w/o Phenol red and Gentamycin (Lonza, #BE04-743Q)	5 % of heat inactivated human plasma	34.39 ± 0.63	15 ± 0.6	7.5 ± 0.3
CellGro SCGM (CellGenix)	5 % of heat inactivated human plasma	33.29 ± 0.2	21.23 ± 2.34	10.62 ± 1.17
	serum-free culture	90.97 ± 5.47	3.03 ± 0.2	1.52 ± 0.1

Grey field indicates optimal formulation and respective growth parameters. Results shown as Mean ± SEM

3.1.5 Establishment of cryopreservation protocols

The freeze/thaw cycle is the first, and most stressful, step for the cells in the whole course of expansion, due to it impacting the quality and quantity of the starting cell population, thereby determining efficiency of the expansion procedure. In order to evaluate the best cryopreservation and thawing protocol for NK-92/.528.z cells, the recovery of parental and retargeted NK-92 cells was analyzed directly and 24 hours after thawing following freezing in HSA, commercially available CryoStor CS 10 or a combination of both containing various concentrations of DMSO (5 %, 7.5 %, 10 %, 12.5 %, 15 %). Obtained results demonstrate that HSA supplemented with 7.5 % of DMSO gave the most beneficial results for NK-92/5.28.z with recovery of 68.84 % ± 5.07 % and 23.91 % ± 9.05 % directly post-thaw and 24 hours after thawing, respectively. In the case of parental NK-92 cells, recovery values at 0 h and 24 h were not sufficient to determine the optimal cryopreservation solution (Figure 3.5A). Therefore, an additional measurement of cell concentration was performed 7 days post-thaw (Figure 3.5B) showing the best results for the cells frozen in HSA + 7.5 % of DMSO ($5.47 \times 10^5 \pm 0.44 \times 10^5$ cells/ml) confirming the optimal character of this combination to cryopreserve parental and genetically modified NK-92 cells. Hoping to further improve the quality of NK-92/5.28.z cells after thawing, HSA + 7.5 % DMSO was supplemented with 20 % of NK-92/5.28.z conditioned medium (CM) from 72-hour culture. There was no significant difference detected: neither directly post-thaw with recovery values of 73.06 % ± 2.66 % for cryopreservation solution without CM (w/o CM) and 61.11 % ± 5.79 % for cryopreservation solution supplemented with CM (+20% CM), nor in recovery measured after 24 hours (w/o CM: 48.06 % ± 6.16 %; +20% CM: 53.61 % ± 4.19 %) (Figure 3.6A). Further analysis of cell growth also did not show any differences between solution without or with CM (day 6 w/o CM: $10.73 \times 10^5 \pm 0.57 \times 10^5$; day 6 +20% CM: $11.86 \times 10^5 \pm 0.57 \times 10^5$) (Figure 3.6B).

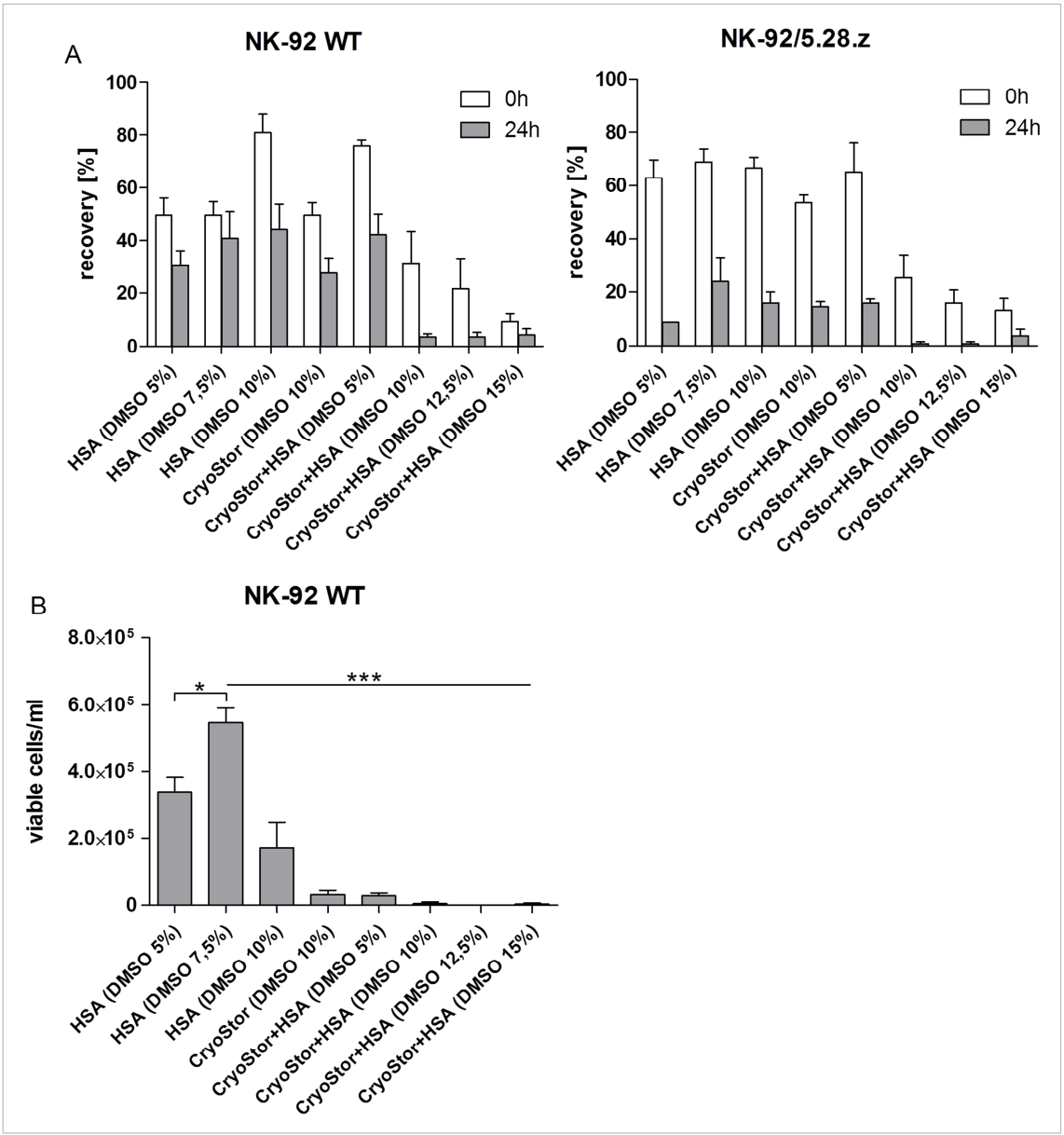

Figure 3.5 Influence of cryopreservation solutions on NK-92 and NK-92/5.28.z cells post-thaw recovery. (A) Recovery of parental NK-92 and NK-92/5.28.z was measured directly (0 h; white bars) and 24 h (grey bars) after thawing. (B) An additional measurement of concentration of viable NK-92 cells after 7 days in culture was performed.

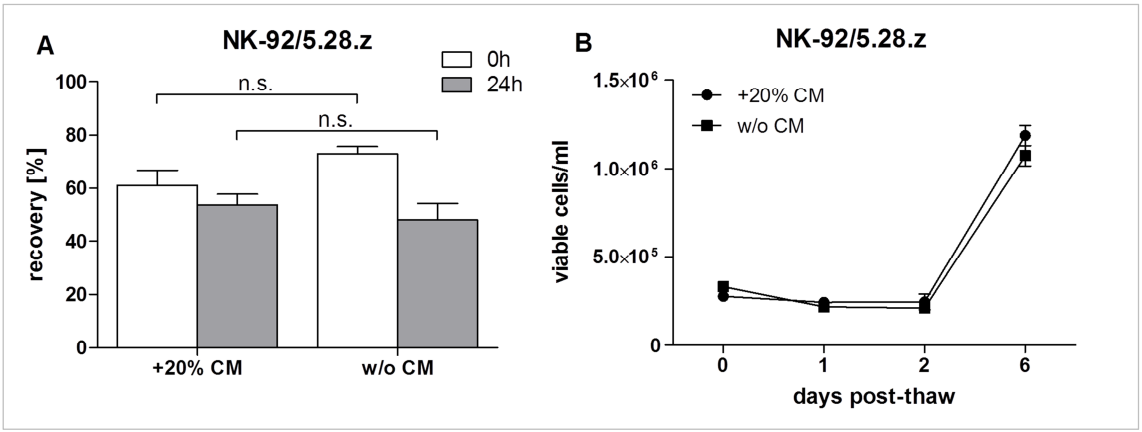

Figure 3.6 Influence of the presence of NK-92/5.28.z.-derived conditioned medium (CM) in cryopreservation solution on (A) post-thaw recovery and (B) cell growth of NK-92/5.28.z. Graphs show the data obtained from the analysis of 4 independent vials. Results shown as Mean ± SEM

Table 3.2 Summary of tested cryopreservation solutions for freezing of NK-92/5.28.z

Cryopreservation solution	**DMSO concentration**	**Direct post-thaw recovery [%]**	**Recovery after 24h [%]**
Human Serum Albumin (HSA)	5 %	63.05 ± 6.64	8.7 ± 0.0
	7.5 %	68.84 ± 5.07	23.91 ± 9.05
	10 %	66.67 ± 4.03	15.94 ± 4.03
CryoStor CS10	10 %	53.62 ± 2.90	14.49 ± 1.92
Human Serum Albumin (HSA) + CryoStor CS10	5 %	65.22 ± 10.94	15.94 ± 1.45
	10 %	25.36 ± 8.54	0.72 ± 0.72
	12.5 %	15.94 ± 4.75	0.72 ± 0.72
	15 %	13.04 ± 4.53	3.62 ± 2.62

Grey field indicates the optimal combination. Results shown as Mean ± SEM

3.1.6 Impact of gamma irradiation on proliferation and potency of NK-92/5.28.z

In all clinical trials performed hitherto, NK-92 cells were subjected to γ-irradiation exposure to prevent permanent engraftment in patient. In this study, proliferation and viability of NK-92 and NK-92/5.28.z following γ-irradiation with 5 doses (0 Gy, 2 Gy, 5 Gy, 10 Gy, 30 Gy) every 24 h up to 5 days were analyzed. Figure 3.7A shows that 30 Gy, 10 Gy and 5 Gy, but not 2 Gy, caused complete inhibition of cell proliferation when compared to the non-irradiated control. It resulted in a significant decline in viability that was first observed 48 h post-irradiation of parental NK-92 cells with the dose of 30 Gy with a viability value of 59.33 % ± 7.27 % versus 98.79 % ± 0.3 % for non-irradiated cells. Treatment with 2 Gy significantly retarded cell growth but did not affect cell viability which exceeded 80% for both cell lines over the entire analysis period. Moreover, genetically modified NK-92 were markedly less radiosensitive; an effect that first became visible 48 h after irradiation with 30 Gy (92.4 % ± 0.1 %) when compared to parental cells (59.33 % ± 7.27 %) (Figure 3.7 B). Subsequently, potency of NK-92 and genetically modified NK-92 was examined 2 hours post-exposure where no significant decrease, in either specific cytotoxicity against ErbB2-positive targets of NK-92/5.28.z cells (0 Gy: 97.7 % ± 0.1 %; 10 Gy: 96.7 % ± 0.36 %; 30 Gy: 94.8 % ± 1.39 %; Mean ± SEM) or natural killing ability of both cell lines, was detected (Figure 3.7C). In addition, differences in the secretion of soluble factors between non-irradiated and irradiated NK-92/5.28.z cells upon stimulation with three cell lines routinely used as targets in previously performed experiments were investigated. GZMB, IFN-γ, IL-10, TNF, and MIP-1α were produced at similar levels by irradiated and non-irradiated NK-92/5.28.z cells while IL-6 was not detected. There was a significant drop in sFasL production upon stimulation with MDA-MB-453 following irradiation with 30 Gy from 385.02 pg/ml ± 4.04 pg/ml (0 Gy) to 202.69 pg/ml ± 9.95 pg/ml (30 Gy) (Mean ± SEM) and a significant increase in IL-8 secretion following irradiation with 10 Gy from 479.38 pg/ml ± 112.77 pg/ml (0 Gy) to 1433.01 pg/ml ± 135.12 pg/ml (10 Gy) (Mean ± SEM) but not with 30 Gy (768.58 pg/ml ± 105.09 pg/ml) compared to the non-irradiated control (Figure 3.8). Taken together, the above results suggest that 10 Gy is the optimal irradiation dose for NK-92/5.28.z, inhibiting cell proliferation with satisfactory viability values of 87.6 ± 4.61 % versus 99.86 ± 0.14 % for non-irradiated cells at the 72 hour time point.

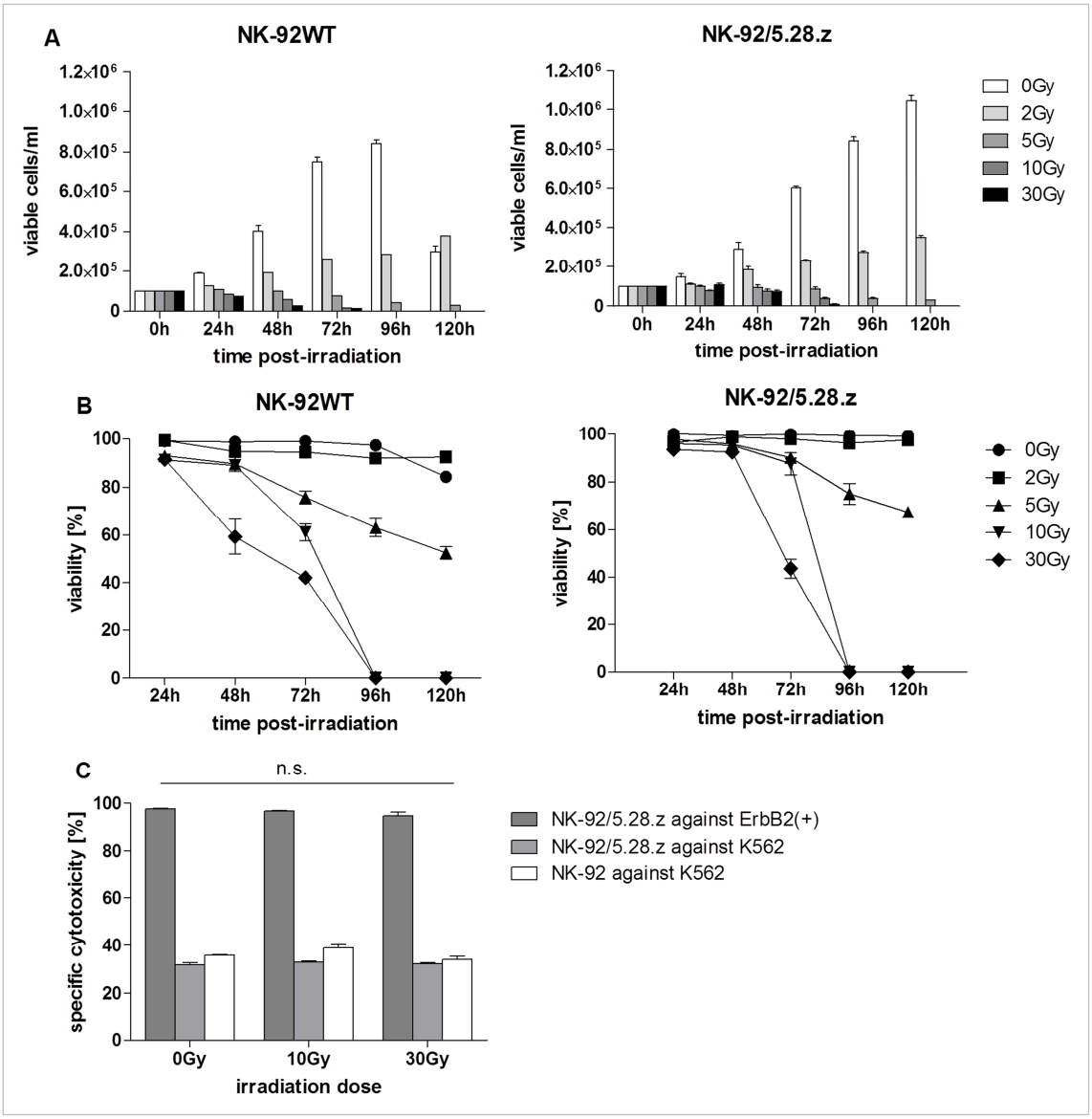

Figure 3.7 Impact of γ-irradiation on the proliferation, viability and potency of NK-92 and NK-92/5.28.z. Cells were exposed to various irradiation doses (0 Gy, 2 Gy, 5 Gy, 10 Gy, 30 Gy) washed and seeded at an initial concentration of 1 x 10^5/ml. (A) Cell concentration and (B) viability were tested every 24 hours up to 5 days. (C) Specific cytotoxicity and natural killing ability of parental and genetically modified NK-92 cells were measured 2 hours post-irradiation using an EuTDA killing assay. Results are presented as Mean ± SEM; n=3

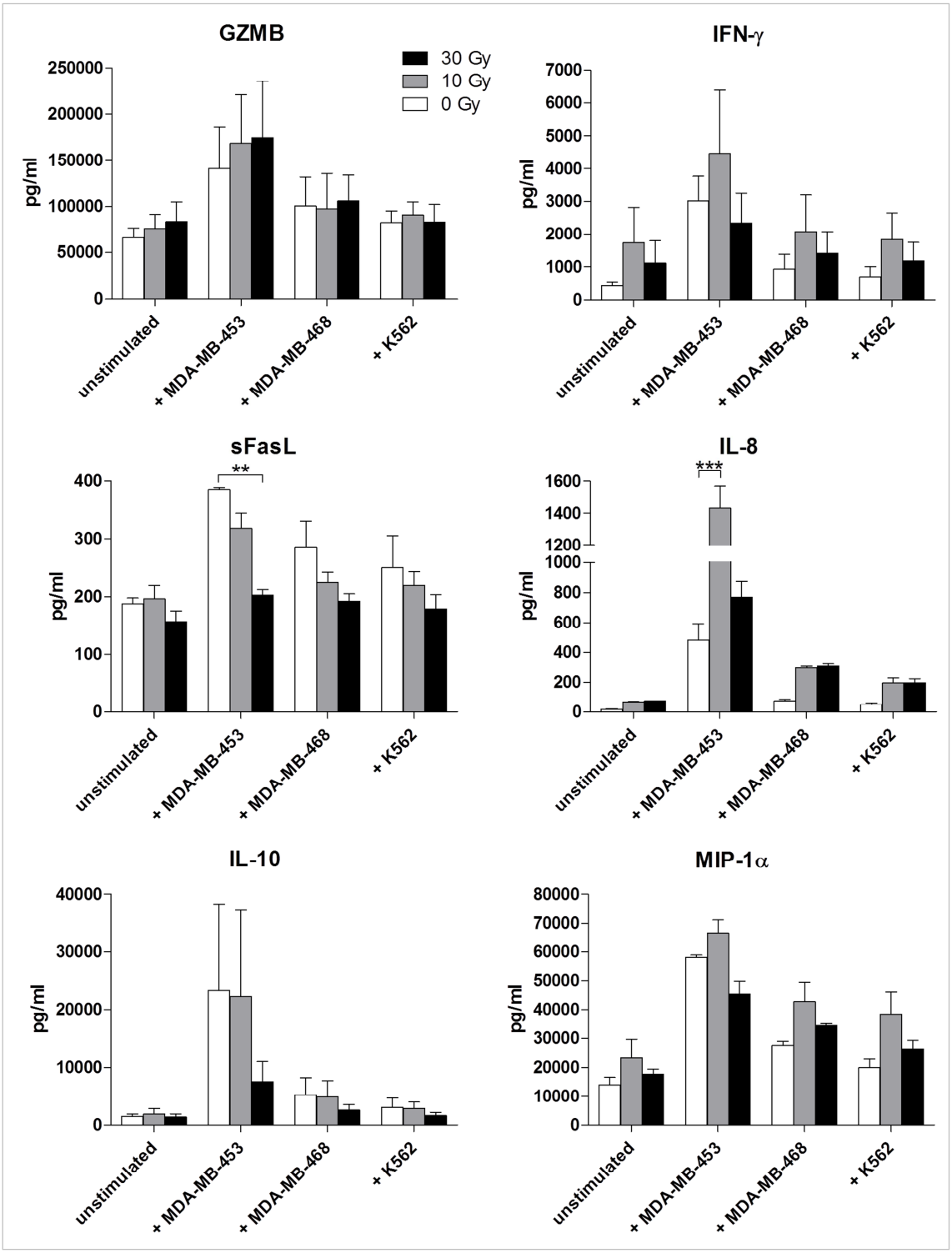

Figure 3.8 Influence of irradiation dose on soluble factors release by stimulated NK-92/5.28.z. NK-92/5.28.z were irradiated with 10 Gy (grey bars) and 30 Gy (black bars) and co-cultured with target cells at E/T ratio of 10:1. NK-92/5.28.z cells cultured in the absence of target cells, as well non-irradiated NK-92/5.28.z (0 Gy, white bars) were included as controls. Concentrations of soluble proteins in test supernatants were measured using a cytometric bead array.

3.2 Qualification of a GMP-compliant master cell bank of NK-92/5.28.z

3.2.1 Testing of manufacturing related parameters

A representative sample (5%) of the cryopreserved master cell bank was allocated to qualification and testing. Results of the analysis of safety parameters (e.g. sterility testing by direct inoculation method, LAL test for endotoxin, presence of viral contaminants, presence of mycoplasma and DNA fingerprinting) performed in accordance with the principles of good manufacturing practice by the contract research organization (CRO) (BioReliance Inc., Glasgow, UK) confirmed the identity and biological safety of the MCB. Additionally, according to established methods (method section), manufacturing related parameters like purity (% of CD56+ CD16-), stability of CAR expression, potency and the post-thaw recovery were tested. Results of the cell recovery from 6 independent vials enable determination of actual vial content, which is crucial information for the master cell bank qualification (Figure 3.9A). A vial, in average, contained a total number of $2.06 \times 10^7 \pm 0.14 \times 10^7$ cells (Mean ± SEM) (range: $1.51\text{-}2.53 \times 10^7$/vial) with a mean viability post-thaw of 65.65 % ± 3.08 % (range: 54.27 %-74.28 %). Cells thawed from MCB demonstrated a satisfactory post-thaw recovery with the mean value of 57.25 % ± 7.06 (Mean ± SEM), with maximal and minimal recovery of 84.52 % and 39 %, respectively (Figure 3.9B). Moreover, cells expanded from 3 (n=3) independent vials exhibited reproducible post-thaw growth in VueLife 750-C1 culture bags prefilled with 1 L of culture medium, with an evident recovery phase between day 0 and 7 and exponential growth phase from day 7 to 12 (Figure 3.9C). What is more, the freeze/thaw procedure did not attenuate stability of the cells defined as CAR expression level, phenotype and functionality with the values obtained 1-3 weeks post-thaw of 99.11 % ± 0.18 % (percentage of CAR+ 7AAD- cells analyzed 1 week post-thaw), 99.42 % ± 0.12 % (percentage of CD56+ CD16- cells analyzed 1 week post-thaw), 88.7 % ± 1.65 % (specific cytotoxicity against ErbB2-positive targets analyzed 3 weeks post-thaw), respectively, when comparted to results, obtained shortly before freezing (CAR+ 7AAD-: 99.17 % ± 0.15 %; CD56+ CD16-: 99.6 % ± 0.06 %; specific cytotoxicity: 63.59 ± 1.15 %). The analysis period was extended to 12 months showing

no decrease in values of stability parameters (CAR+ 7AAD-: 99.77 ± 0.03 %; CD56+ CD16-: 100 %; specific cytotoxicity: 89.75 ± 3.77 %) (Figure 3.9D).

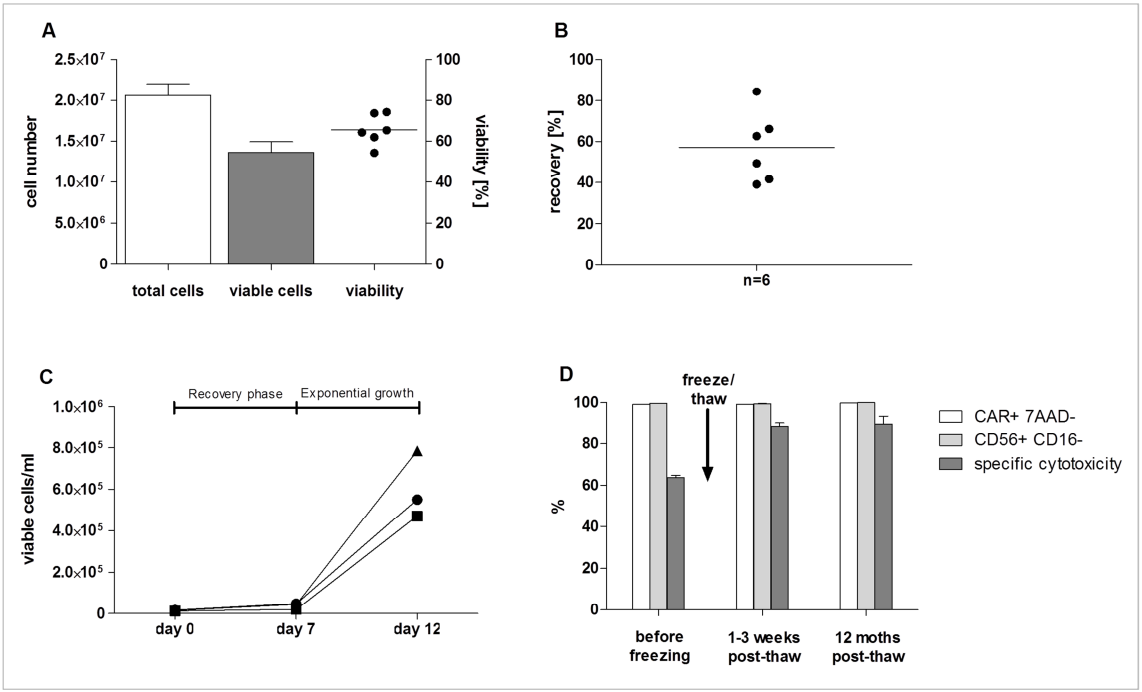

Figure 3.9 Master cell bank qualification with regard to manufacturing parameters. (A) Mean vial content analyzed directly post-thaw (n=6). (B) Post-thaw recovery of NK-92/5.28.z cells derived from 6 independent vials (each dot represents 1 vial). (C) Post-thaw growth of NK-92/5.28.z cells thawed from 3 independent vials inoculated directly into VueLife 750-C1 culture bags prefilled with 1 L of X-Vivo 10 rTF supplemented with 5 % of human plasma and 500 U/ml of IL-2. (D) Analysis of stability parameters before freezing of MCB and after thawing (n=3). Results shown as Mean ± SEM.

3.3 Manufacturing and release testing of therapeutic patient doses, expanded from the master cell bank

3.3.1 Establishment of shipment/injection solution (excipient)

To find the optimal excipient for shipment and subsequent infusion, which will preserve cell viability and cytotoxicity, 6 different buffered solutions, including 4 routinely used for storage and/or the application of blood products, were evaluated. Testing included: saline-adenine-glucose-mannitol hypertonic solution (SAG-M; 376 mOsm/l; Fresenius Kabi, Bad Homburg, Germany), phosphate-adenine-glucose-guanosin-saline-mannitol isotonic solution (PAGGS-M; 285 mOsm/l; Fresenius Kabi, Bad Homburg, Germany), SSP (Macopharma, Langen, Germany) and PBS/EDTA (MiltenyiBiotec, Bergisch Gladbach, Germany). All buffers were supplemented with 1 % of human serum albumin. In addition, pure HSA (200 g/1L) and X-Vivo 10 rTF medium as pure or complete (supplemented with 5% heat inactivated human plasma and 500 U/ml IL-2) formulation were tested. X-Vivo 10 rTF, HSA, PBS/EDTA + 1 % HSA and SSP + 1 % HSA maintained a stable pH between 7.0 and 8.0 over the 48-hour analysis period, whereas SAG-M and PAGGS-M showed an acidic pH (<6.5) characteristic for erythrocyte storage buffers. All tested storage solutions except for SSP + 1 % HSA and PBS/EDTA + 1 % HSA, were able to maintain cell viability at satisfactory levels (> 85 %) for up to 48 hours. Viability of the cells stored in SSP + 1 % HSA and PBS/EDTA + 1 % HSA declined significantly (65.12 % ± 1.26 % and 47.3 % ± 1.38 %, respectively) despite having a stable, near-physiological pH of these buffers (SSP min. and max pH: 7.00 ± 0.11 and 7.46 ± 0.02; PBS/EDTA min. and max pH: 7.07 ± 0.04 and 7.15 ± 0.01). Interestingly, three solutions with different pH: slightly basic pure X-Vivo 10 rTF (min. pH: 7.33 ± 0.01 and max. pH: 8.14 ± 0.02), neutral pure HSA (min. pH: 7.3 ± 0.03 and max pH: 7.63 ± 0.03) and acidic PAGGS-M + 1 % HSA (min. pH: 5.89 ± 0.01 and max pH: 6.39 ± 0.04) gave comparable results and were indicated as optimal for cell viability measured after 48-hour storage under ambient conditions (pure X-Vivo 10 rTF: 90.27 % ± 0.18 %; pure HSA: 86.24 ± 0.33 %; PAGGS-M + 1 % HSA: 86.0 ± 0.16 %), suggesting a marginal importance of pH values for cell viability in this experiment. There was no difference in cell viability between supplemented X-Vivo 10 rTF and pure X-Vivo 10 rTF with viability values at

the 48-hour time point of 91.49 % ± 0.62 % and 90.27 % ± 0.18 %, respectively. Furthermore, irradiation of the cells stored in X-Vivo 10 rTF with 10 Gy did not impair cell viability in the analyzed time range when compared to a non-irradiated control with viability values of 91.49 % ± 0.62 % and 93.24 % ± 0.07 %, respectively (Figure 3.10A). Subsequently, three selected solutions (pure X-Vivo 10, pure HSA and PAGGS-M + 1 % HSA) were tested for their ability to preserve cell cytotoxicity during 6 hours of storage under ambient conditions. Pure X-Vivo 10 rTF and PAGGS-M + 1 % HSA provided comparable results in the specific cytotoxicity of NK-92/5.28.z against ErbB2-positive targets, amounting to 86.18 % ± 2.58 % and 81.31 % ± 1.07 % (Mean ± SEM), respectively. Storage in HSA significantly ($P<0.0001$) diminished the potency of NK-92/5.28.z cells, when compared to X-Vivo 10 rTF and PAGGS-M + 1 % HSA, with a specific cytotoxicity value of 63.38 % ± 0.99 % (Figure 3.10B). Obtained data suggest that pure X-Vivo 10 rTF culture medium with its slightly basic pH and ability to preserve cell viability and potency at satisfactory levels may be used for final formulation of the cellular product and thereby serve as storage/injection solution.

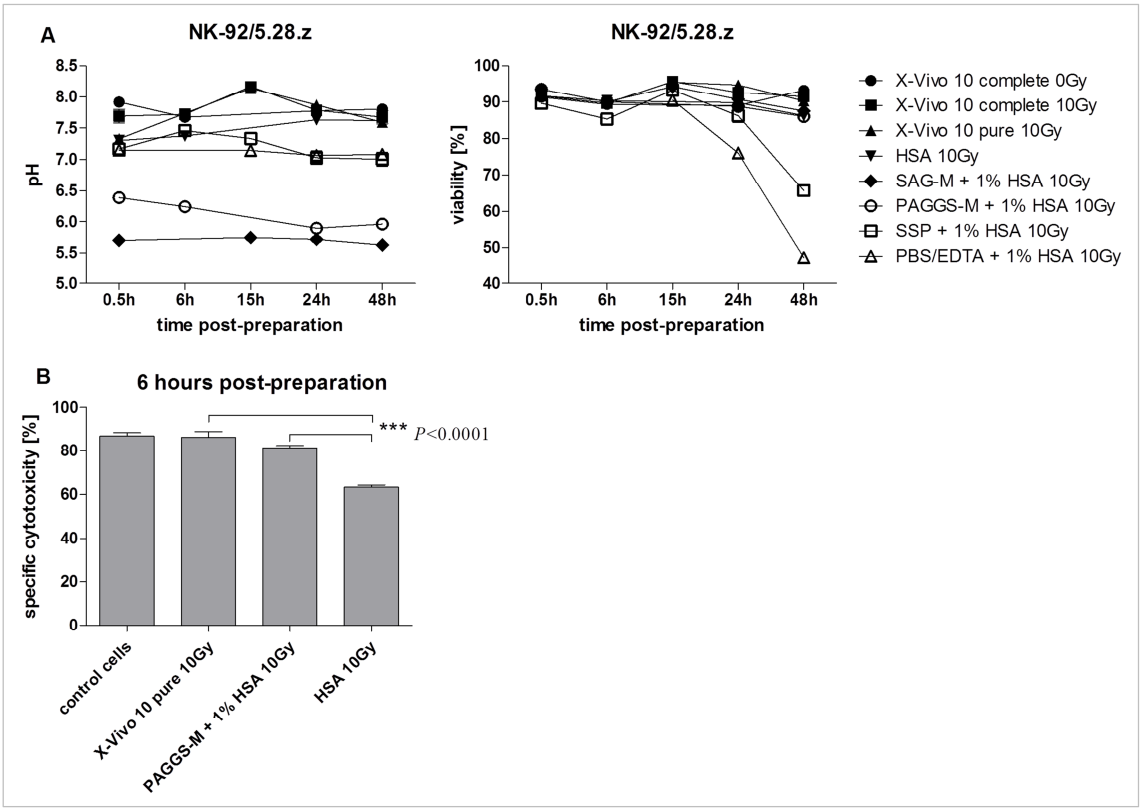

Figure 3.10 Impact of storage buffers on the stability of NK-92/5.28.z. (A) pH and viability values of cell suspensions prepared in 6 tested buffers measured over 48-hour storage under ambient conditions. (B) Specific cytotoxicity against ErbB2(+) targets of NK-92/5.28.z cells stored in 3 preselected buffered solutions under ambient conditions, analyzed 6 hours post-preparation. NK-92/5.28.z. cells stored in complete X-Vivo 10 rTF in cell culture incubator were included as a control (control cells).

3.3.2 Stability of NK-92/5.28.z at high cell densities

The intended route of administration affects the final formulation of the cellular product. Thus local intratumoral or peritumoral injection limits the injection volume and consequently demands high cell densities in the final product. To evaluate the maximally feasible cell density in a potential patient dose, viability and potency of irradiated (10 Gy) NK-92/5.28.z in high density cell suspensions (2 x 10^7/ml; 4 x 10^7/ml; 6 x 10^7/ml), stored under ambient conditions, were tested to model patient dose potency and stability. The data demonstrated high stability of the irradiated cells with a viability of > 90 % for 24 hours (96.41 % ± 0.36 % for 2 x 10^7/ml; 94.84 % ± 0.22 % for 4 x 10^7/ml; 92.83 % ± 0.63 % for 6 x 10^7/ml) and specific cytotoxicity against ErbB2(+) targets of > 50 % up to 18 hours for all three tested concentrations. However, cytotoxicity of NK-92/5.28.z at the density of 6 x 10^7/ml after 18 hours was significantly lower (51.48 % ± 1.5 %) when compared to 4 x 10^7/ml

(67.01 % ± 1.89 %; $P<0.0001$) and 2 x 10^7/ml (75.36 % ± 2.44 %; $P<0.0001$) (Figure 3.11), implying cell concentration as a factor potentially limiting the stability of patient doses in the clinically relevant period of time. Based on the obtained results, the concentration of 5 x 10^7/ml was indicated as feasible and used as a concentration of the final product in 3 subsequent validation runs (chapter 3.3.3).

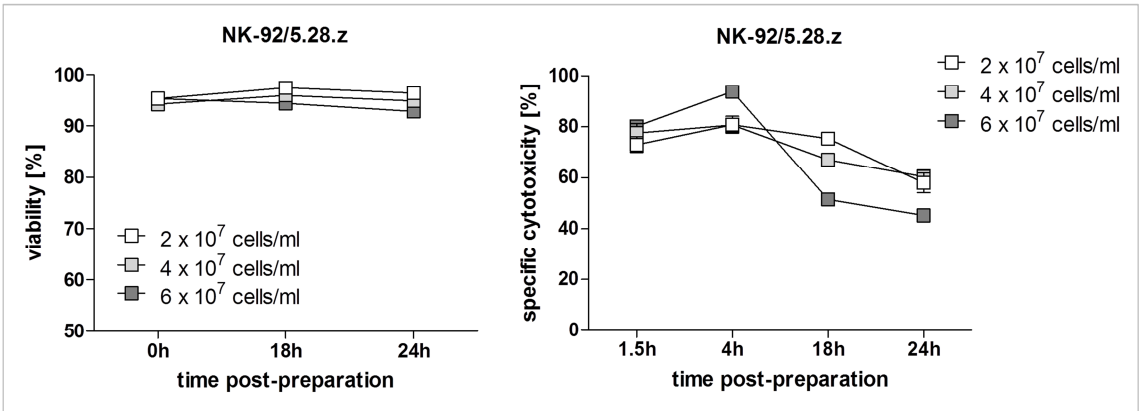

Figure 3.11 Stability of γ-irradiated NK-92/5.28.z cells in high density cell suspensions during storage under ambient conditions in X-Vivo 10 + 100 U/ml of IL-2. Viability (left panel) and specific cytotoxicity against ErbB2(+) targets (right panel) were analyzed at the indicated time points using EuTDA cytotoxicity assay; n=3.

3.3.3 Generation of a therapeutic doses of NK-92/5.28.z

To confirm the feasibility of established clinical scale manufacturing protocol covering the following issues: efficient cell expansion, optimal irradiation dose, cell density in the final product and optimal transport solution; three independent validation runs of patient dose generation were performed under full GMP conditions with subsequent testing of final product. Three independent batches of NK-92/5.28.z cells seeded at the concentration of 5 x 10^4 cells/ml in VueLife 750-C1 culture bags prefilled with complete X-Vivo 10 rTF showed stable and reproducible growth with the average doubling time of 32.76 h ± 0.32 h over a 5-day culturing period (Figure 3.12A). Cells were harvested at a concentration of approximately 6 x 10^5 cells/ml (batch 1: 6.5 x 10^5 cells/ml; batch 2: 6.6 x 10^5 cells/ml; batch 3: 5.94 x 10^5 cells/ml). All three patient doses fulfilled predefined specification in terms of cell viability (specification > 80 %; result: 92.16 % ± 0.74 %; Mean ± SEM of 3 independent products), CAR expression (specification > 95 %; result: 97.67 % ± 0.4 %; Mean ± SEM of 3 independent products) and specific cytotoxicity

(specification > 50 %; result: 83.43 % ± 2.48 %; Mean ± SEM of 3 independent products) post-preparation. Moreover, the stability of generated products in terms of viability and potency was further tested up to 24 hours showing high viability values of > 85 % for three products over the whole analysis period (viability values 24 hours post-preparation: product 1: 87.4 %; product 2: 87.7 %; product 3: 87.35%) and potency of more than 70 % at 6 hours post-preparation (specific cytotoxicity: product 1: 86.13 % ± 5.08 %; product 2: 84.78 % ± 0.39 %; product 3: 73.67 % ± 4.55 %; Mean ± SEM of triplicates) (Figure 3.12B). Importantly, the generated cellular products were negative for culturable microbes (BacT/Alert) and free of mycoplasma and endotoxin and thus within specification for these outcomes as well.

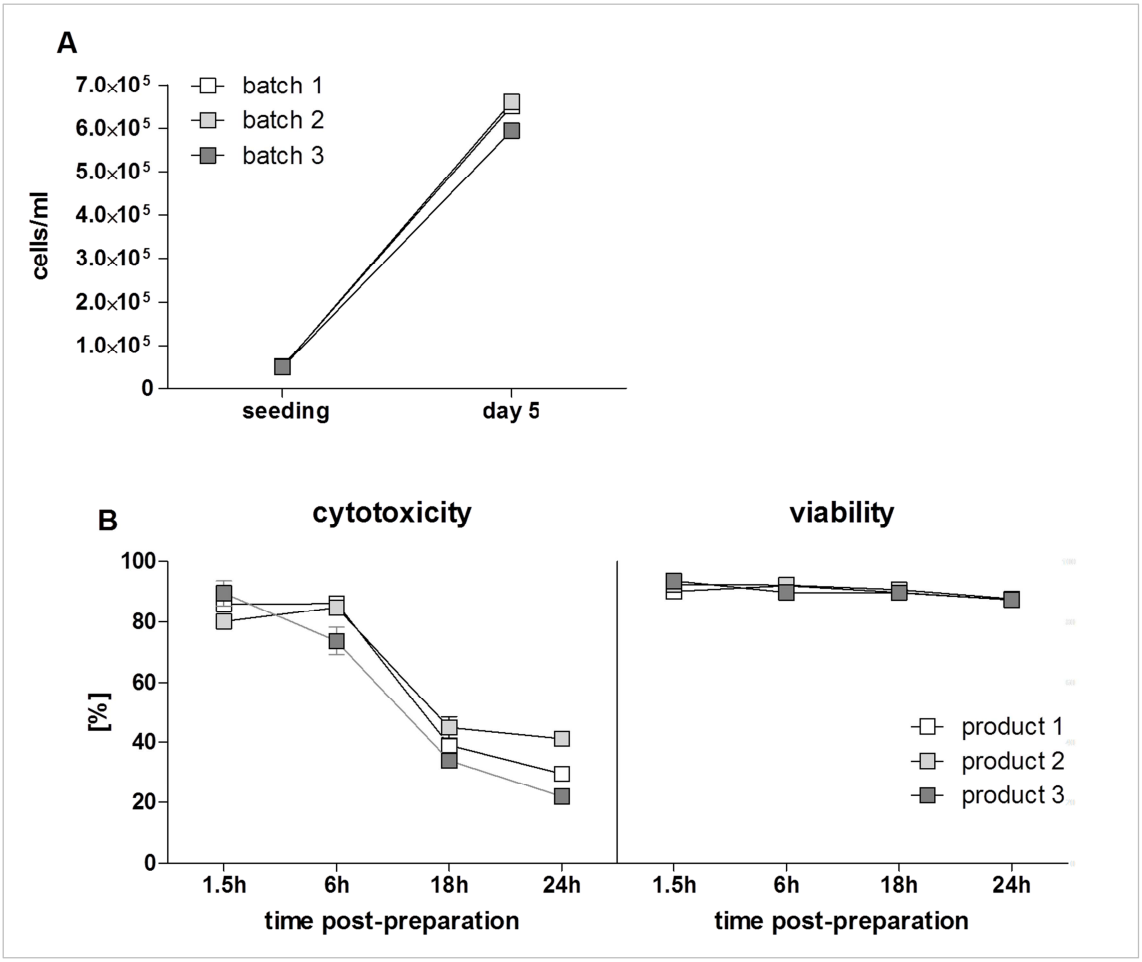

Figure 3.12 Expansion of individual patient dose and testing of final product stability. (A) 3 independent batches of NK-92/5.28.z cells were expanded to a concentration > 5 x 10^5 cells/ml in VueLife 750-C1 culture bags. Subsequently, cells were harvested, irradiated and, after serial centrifugation and washing steps, 3 final products were formulated. (B) Specific cytotoxicity against ErbB2(+) targets and viability of the cells in final formulation were tested for up to 24 hours.

3.4 Patient related issues in the context of clinical application

3.4.1 Impact of corticosteroid treatment on NK-92/5.28.z cytotoxicity

Corticosteroids are potent immunosuppressive agents used in cancer patients to mitigate the side effects caused by first line anticancer therapies [99]. The mechanisms of action include, *inter alia*, inhibition of natural killer cell cytotoxicity. For that reason, the impact of 4 clinically relevant concentrations (0.2 µg/ml, 2 µg/ml, 20 µg/ml, 200 µg/ml) of corticosteroids on the potency of NK-92/5.28.z. was assessed. The dose of 200 µg per milliliter of culture medium corresponds with the highest clinical dose of prednisolone applied intravenously (1000 mg of prednisolone) in the case of pulmonary edema. 24-hour prednisolone treatment did not significantly attenuate CAR-dependent cytotoxicity of genetically modified NK-92 cells against ErbB2-overexpressing targets with the following results: 0.2 µg/ml: 95.56 % ± 1.1 %; 2 µg/ml: 93.05 % ± 2.87 %; 20 µg/ml: 91.43 % ± 2.62 %; 200 µg/ml: 86.86 % ± 3.48 %, when compared to non-treated control (0 µg/ml): 90.07 % ± 2.78 %. Natural killing ability was significantly reduced in a dose-dependent manner with twofold difference between the untreated control (57.84 % ± 2.8 %) and cells treated with the highest tested prednisolone dose (29.76 % ± 1.37 %; Mean ± SEM) (Figure 3.13).

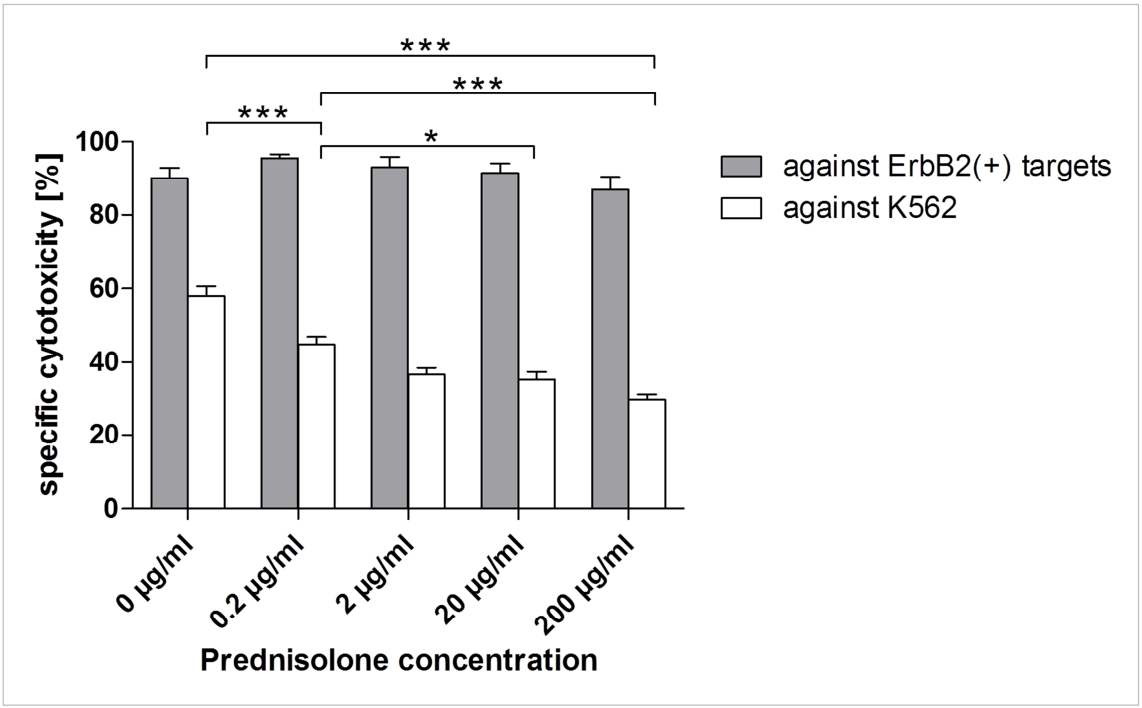

Figure 3.13 Impact of 24-hour prednisolone treatment on CAR-mediated cytotoxicity against ErbB2(+) cells (grey bars) and natural killing against K562 cells (white bars) of NK-92/5.28.z. Results presented as Mean ± SEM of triplicates from 3 independent experiments.

3.4.2 Analysis of soluble factors secreted by target stimulated NK-92 cells, NK-92/5.28.z and primary NK cells

The profile of soluble factors, including effector molecules, secreted upon short-term stimulation was determined in order to test cell potency as well as to predict potential (systemic) side effects of the cell therapy. Release of GZMB, IFN-γ, sFasL, TNF, IL-2, IL-6, IL-8, IL-10, G-CSF and GM-CSF by parental NK-92 and NK-92/5.28.z was measured after 2-hour stimulation with target cells. Adequate controls of unstimulated and PMA/Ionomycin stimulated effector cells as well as PMA/Ionomycin stimulated target cells were included (Table 3.3). Secretion of GZMB, IFN-γ, sFasL, IL-8 and IL-10 by NK-92/5.28.z was distinctly higher compared to parental cells, not only when stimulated with K562, but also in unstimulated controls. However, in the case of soluble Fas ligand the differences did not reach statistical significance (P=0.1692 and P=0.2110 for unstimulated and K562 stimulated cells, respectively). These data demonstrate a specific and significant increase in GZMB (2-fold; P<0.05), IFN-γ (4-fold; P<0.001), IL-8 (24-fold; P<0.01) and IL-10 (5-fold; P<0.01) production by NK-92/5.28.z,

co-cultured with ErbB2-overexpressing targets (MDA-MB-453), when compared to baseline secretion (unstimulated NK-92/5.28.z). This phenomenon was not observed in the case of parental NK-92 cells, indicating resistance of our ErbB2(+) target cell line to natural killing mechanisms, which can be overcome by specific CAR-mediated activation (Figure 3.14). TNF and GM-CSF were detected only upon strong stimulation with PMA/Ionomycin, but not in test samples. Secretion of IL-6, G-CSF and IL-2 by target stimulated effector cells was not detected (data not shown).
In addition, the profile of soluble factors secreted by primary NK (pNK) cells was tested, where the experimental setting was comparable to the one applied to NK cell lines. Primary NK cells stimulated with K562 target cells secreted higher amounts of effector molecules like GZMB (1.8-fold), IFN-γ (31.8-fold) and sFasL (2.7-fold), when compared to unstimulated controls. Increased concentrations of effector molecule secreted by pNK cells stimulated with MDA-MB-453 and MDA-MB-468 were not detected, indicating resistance of these target cell lines to the pNK cell-mediated lysis (Figure 3.15). As expected, unstimulated peripheral blood primary NK cells secreted significantly lower amounts of effector molecules (GZMB, IFN-γ, sFasL), when compared to unstimulated parental NK-92 (GZMB: 61-fold more, IFN-γ: 67.4-fold more, sFasL: 12-fold more) and NK-92/5.28.z cells (GZMB: 277-fold more, IFN-γ: 934.6-fold more, sFasL: 21.6-fold more). Table 3.4 shows the concentrations of soluble factors secreted by unstimulated and K562 stimulated primary NK cells in comparison to NK-92 and NK-92/5.28.z. IL-10 was not detected in the supernatants of primary NK cells, whereas abundant amounts of this cytokine were secreted by NK cell lines.

Table 3.3 Cytokine profile of PMA (50ng/ml)/Ionomycin (500ng/ml) stimulated target cells.

Target cell line	Soluble factor	Concentration [pg/ml]
MDA-MB-453	IL-8	9.93 ± 1.2
MDA-MB-468	IL-6	247 ± 30.09
	IL-8	635 ± 27.53
	TNF	6.64 ± 2.72
K562	GM-CSF	286.61 ± 76.55
	IL-6	235.26 ± 64.34
	IL-8	524.61 (n=1)
	TNF	311.92 ± 47.92

Results shown as Mean ± SEM

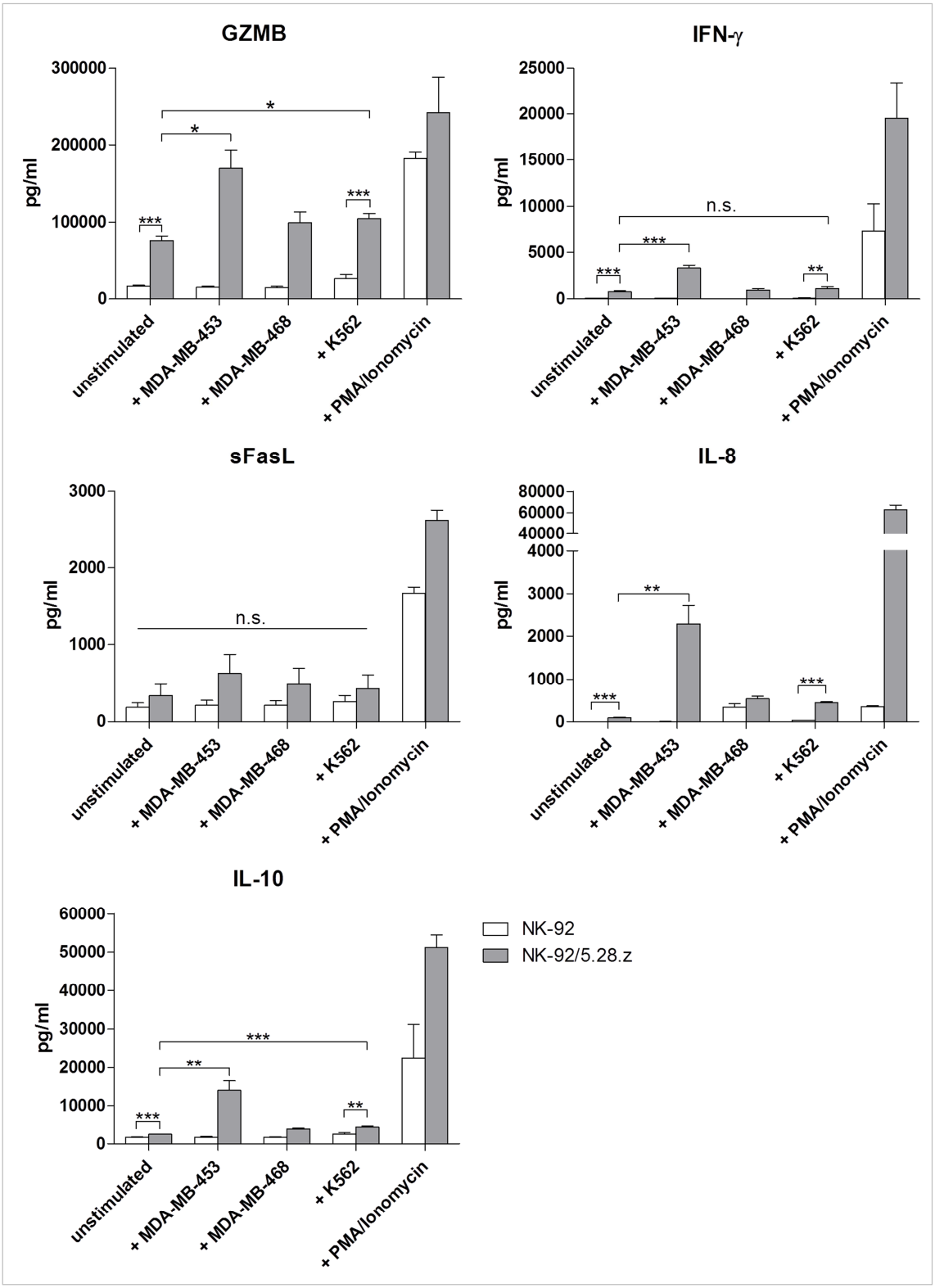

Figure 3.14 Influence of second generation ErbB2-CAR expression on the release of soluble factors by stimulated NK-92 cells. NK-92 (white bars) or NK-92/5.28.z (grey bars) were incubated for 2 hours with ErbB2(+) MDA-MB-453, ErbB2(-) MDA-MB-468 or K562 cells at 10:1 E/T ratio. Effector cells incubated without target cells (unstimulated), as well as stimulated with PMA/Ionomycin served as negative and positive controls, respectively.

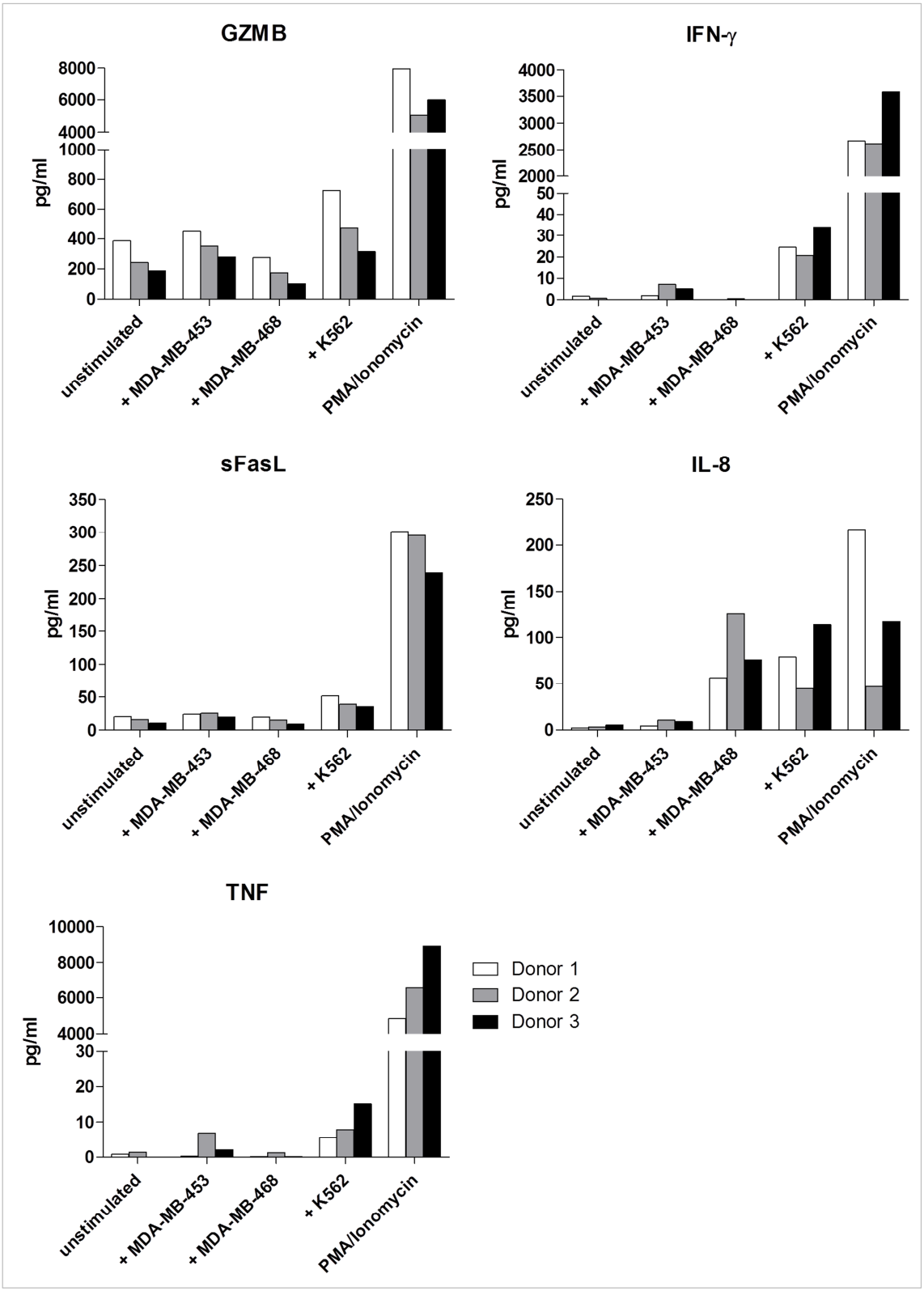

Figure 3.15 Release of soluble factors by target stimulated primary NK cells isolated from buffy coats of normal peripheral blood from 3 independent donors. NK cells were incubated for 2 hours with ErbB2(+) MDA-MB-453, ErbB2(-) MDA-MB-468 or K562 cells at 10:1 E/T ratio. NK cells incubated without target cells (unstimulated), as well as stimulated with PMA/Ionomycin served as negative and positive controls, respectively.

Table 3.4 Secretion of soluble factors by unstimulated and K562 stimulated primary NK cells (pNK) and NK cell lines.

Soluble factor	unstimulated			+K562		
	pNK	NK-92	NK-92/5.28.z	pNK	NK-92	NK-92/5.28.z
GZMB	272.07 ± 59.07	16584 ± 990.3	75331 ± 5963	504.29 ± 119.65	26718 ± 5066	104881 ± 6329
IFN-γ	0.83 ± 0.49	55.97 ± 19.52	775.7 ± 107	26.4 ± 3.96	82.97 ± 31.36	1146 ± 177.3
sFasL	15.65 ± 2.73	187.6 ± 35.32	338.2 ± 95.29	42 ± 4.9	261.3 ± 50.22	429.9 ± 115.7
TNF	BDL	BDL	BDL	9.59 ± 2.87	BDL	BDL
IL-10	BDL	1735 ± 46.51	2671 ± 58.15	BDL	2659 ± 406.2	4587 ± 206.5
IL-8	3.65 ± 0.94	BDL	93.25 ± 11.39	79.12 ± 20.05	27.63 ± 4.41	442.2 ± 23.63

BDL, below detection limit. Results shown as Mean ± SEM. Concentrations of soluble factors secreted by primary NK cells are presented as mean values of 3 independent donors.

3.4.3 NK-92/5.28.z activation depends on ErbB2 expression on target cells.

To investigate the specificity of NK-92/5.28.z. and evaluate a threshold level of ErbB2 on the target cell surface required for effector cell activation, three breast cancer cell lines (MDA-MB-361, MDA-MB-453 and MDA-MB-468), human hepatocellular carcinoma cell line (HepG2) and a glioblastoma cell line (G55T2) were tested for their ErbB2 status, showing expression ranging from negative (< 300 ErbB2 molecules/cell for MDA-MB-468 and G55T2) to highly positive (> 1 x 10^5 ErbB2 molecules/cell for MDA-MB-453). A subsequently conducted EuTDA cytotoxicity assay, showed that all target cells expressing ErbB2 protein (MDA-MB-453: 1.06 x 10^5 ± 0.03 x 10^5 molecules/cell, MDA-MB-361: 6.99 x 10^4 ± 0.23 x 10^4 molecules/cell, HepG2: 11.59 x 10^3 ± 0.29 x 10^3 molecules/cell; Mean ± SEM) were efficiently and selectively killed by NK-92/5.28.z (MDA-MB-453: 89.94 % ± 1.95 %, MDA-MB-361: 77.04 % ± 1.34 %, HepG2: 71.7 % ± 3.97 %) confirming the dependency of NK-92/5.28.z cell activation on an ErbB2 presence on target cell surface (Figure 3.16). However, even in the case of ErbB2-negative targets a certain amount of specific lysis mediated by CAR-expressing NK-92 cells was observed (MDA-MB-468: 13.34 % ± 3.05 %, G55T2: 17.72 % ± 1.55 %).

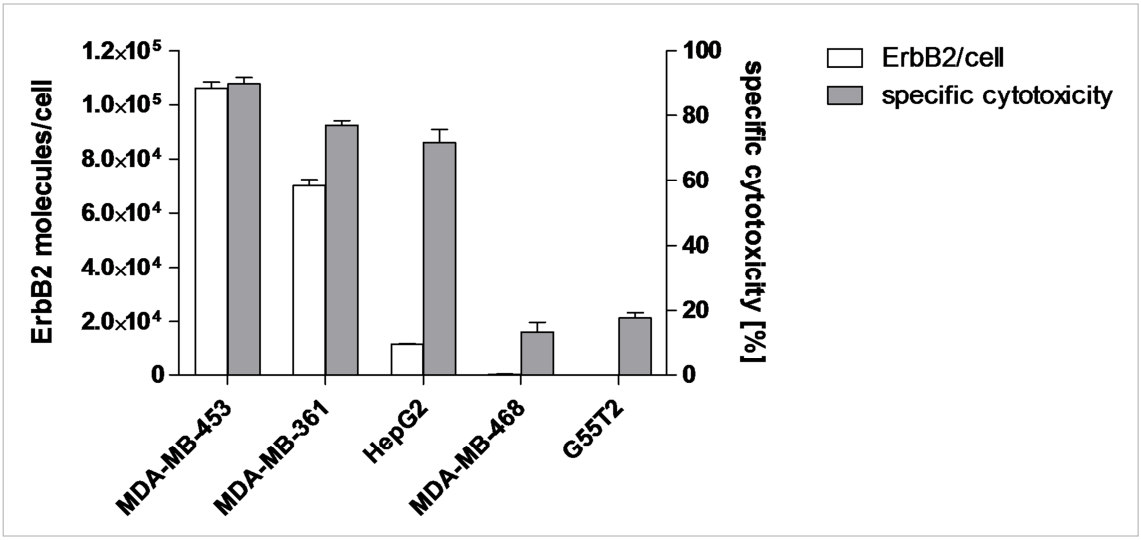

Figure 3.16 Specific cytotoxicity of NK-92/5.28.z depends on ErbB2 expression on target cells. Number of ErbB2 molecules per target cell (white) was analyzed using QuantiBRITE-PE beads. Specific cytotoxicity of NK-92/5.28.z toward target cells was measured in EuTDA killing assay.

3.4.4 Impact of NK-92/NK-92/5.28.z on colony forming capacity of peripheral blood stem cells

The possibility of off-tumor effects of the CAR-modified NK-92 cells, either through NK-receptor mediated mechanisms or through CAR-mediated on- or off-target effects must be considered, although previous work has not suggested such. Therefore, to test the impact of NK-cell treatment on normal hematopoietic cells, the colony forming capacity of peripheral blood stem cells (PBSCs) after co-incubation with NK-92, NK-92/5.28.z and conditioned medium (CM) derived from both NK cell lines was analyzed. There was no difference in myeloid colony number in any of the analyzed treatment combinations (+NK-92: 107.89 ± 5.73, +NK-92/5.28.z: 102.67 ± 2.66, +CM-NK-92: 95 ± 3.79, +CM-NK-92/5.28.z: 93.33 ± 5.47) when compared to an untreated control (PBSC: 97.63 ± 4.15). Interestingly, a slight but reproducible increase in the number of erythroid colonies post-treatment with conditioned medium of NK-92 (+CM-NK-92: 87.33 ± 3.28) and NK-92/5.28.z (+CM-NK-92/5.28.z: 74.67 ± 2.06) from 48-hour culture was found, when compared to untreated control (PBSC: 64.88 ± 2.14) giving statistically significant differences of $P<0.0001$ and $P=0.005$ for CM-NK-92 and CM-NK-92/5.28.z, respectively. However, an increase in the frequency of erythroid colonies was not observed when PBSCs were treated with NK-92 (+NK-92: 72.44 ± 4.89) or NK-92/5.28.z cells (+NK-92/5.28.z: 64.56 ± 4.65), implicating soluble factors rather than direct cell-cell interaction in the observed phenomenon (Figure 3.17).

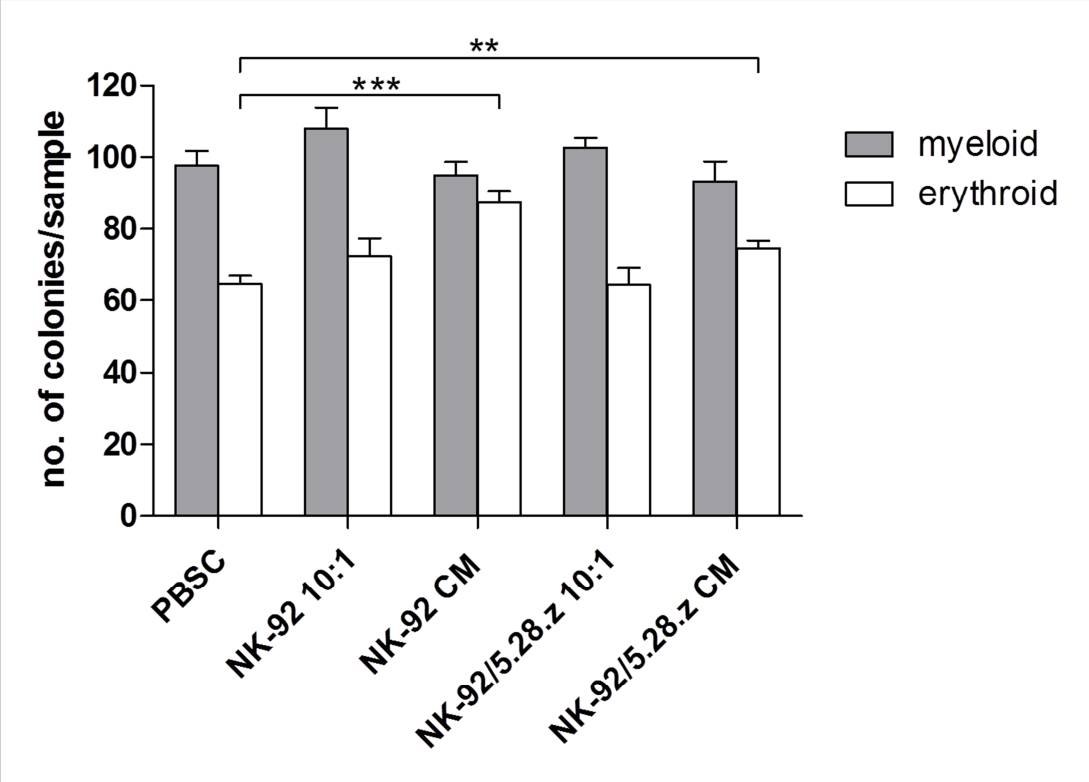

Figure 3.17 Colony forming capacity of peripheral blood stem cells post-treatment with NK-92 (NK-92 10:1) and NK-92/5.28.z (NK-92/5.28.z 10:1) cells, as well as with conditioned medium derived from both NK cell lines (NK-92 CM and NK-92/5.28.z CM). Myeloid (grey bars) and erythroid (white bars) derived colonies were enumerated. Peripheral blood stem cells incubated in assay medium without effector cells were included as a control (PBSC).

4 Discussion

To meet the regulatory requirements for clinical-grade cell expansion, reagents and protocols have to be well-defined so as to enable manufacturing of a cellular product in a reliable, safe and reproducible way. Elimination of components causing undue variability in cell quality or cell growth parameters is highly expected [100]. Three GMP-grade culture media were assessed for their ability to support cell proliferation in plasma-free and plasma-supplemented cultures. X-Vivo 10 containing recombinant transferrin (X-Vivo rTF) became our gold standard for the cultivation of parental and retargeted NK-92 cells, even as a plasma-free formulation, maintaining cell growth, phenotype and functionality at satisfactory levels. However, significantly longer doubling time of the cells cultivated in plasma-free (DT post-acclimation: 47.37 h ± 2.75 h, Mean ± SEM) when compared to plasma-supplemented conditions (DT: 28.84 h ± 0.5 h, Mean ± SEM), stresses the need for further supplementation with human plasma in order to reach the required cell dose in a time-effective manner. CAR expressing NK-92, same as the parental cell line, is dependent on exogenous IL-2 for cell survival, proliferation and potency. Although, other cytokines like IL-7, IL-12 and IL-18 were tested as alternatives, only IL-2 was able to efficiently maintain the long-term growth and functionality of NK-92 cells [101,102]. In the current study, the optimal saturating concentration of this cytokine (500 U/ml of culture medium), efficiently supporting cell proliferation and cytotoxicity was successfully established. However, it is worth mentioning that genetically modified variants of NK-92 with ectopic expression of IL-2 (NK-92MI and NK-92 CI) and IL-15 (NK-92/IL-15-EGFP) have been generated and proved to be independent from exogenous cytokine support [42,43]. Although, the aforementioned cell lines are available only in their research-grade formats, the strategy seems to be promising and may simplify and economize established GMP-compliant protocols for the large-scale expansion of NK-92 cells. A growing body of literature stresses the beneficial effect of human platelet lysate (hPL) on the growth and function of various cell types, albeit mostly for primary, adherent cells [103,104]. We demonstrated a decreased proliferation rate of NK-92/5.28.z cells cultured in medium supplemented with platelet lysate when compared to cells cultured in human plasma supplemented medium. Moreover, an

inhibitory effect of TGF-beta, which is one of the main components of platelet alpha granules, on the functionality of primary NK cells and NK-92 cell line by downmodulation of NKp30 and NKG2D was reported [105,106]. Adverse effects on cytotoxicity were not observed in our studies, probably due to relatively low concentration (5 %) of platelet lysate (and hence, TGF-beta) in the culture medium and the short cultivation time (72 h). Indeed, the possibility that long-term culturing of NK-92/5.28.z cells in hPL supplemented medium could lead to loss of cytotoxicity should be considered. Based on the data obtained by the other researchers on TGF-beta concentration in human platelet lysate [107], we calculated that concentration of TGF-beta in our culture medium supplemented with 5 % of hPL (approximately 7 ng/ml) might, be sufficient to attenuate the natural killing of NK-92 and NK-92/5.28.z if culturing period was extended.

Long-term culturing and cell passaging may lead to the accumulation of genetic and epigenetic changes and as a consequence to alterations in phenotype and/or functional properties [108]. It also carries a potential risk for microbial or viral contaminations, as well as cross-contamination by other cell lines. To prevent these incidents, cryogenic preservation of valuable cell lines and generation of frozen stocks is widely used. However, in this approach, poor viability of NK cells following thawing may be an important factor, potentially limiting the clinical application by prolongation of the recovery phase, and thus the cell expansion period [109-111]. Therefore, finding optimal cryopreservation conditions appears to be substantial for efficiency of patient dose generation of NK-92/5.28.z cells. In the freezing/thawing cycle, the cryoprotective agent (CPA) plays an important role. The most extensively used CPA is DMSO, probably due to its ability to efficiently permeate most cell types and hence provide better recovery values, when compared to other CPAs. Moreover, concerning the clinical purpose of NK-92/5.28.z cells cryopreserved as master cell bank, it is of importance to use CPAs available at pharmacopeia-grade. DMSO from certified suppliers meets these standards. DMSO at suboptimal, low concentrations is not able to prevent ice crystal formation during the freezing process which, as a consequence, leads to rapid membrane rupture and cell death, whereas too high of a DMSO concentration might be cytotoxic [112]. The second important task in developing a cryopreservation process is selection of a base media used for CPA preparation. At the research scale,

animal sera were widely used with a great success due to their high content of lipids and proteins. Obviously, upgrading a cell line to the clinical grade-product renders the usage of animal derived components obsolete because of regulatory reasons, stressing the need of investigation of human derived (or, better yet, synthetic) alternatives [113]. Therefore, in this study, several combinations of GMP-compliant cryopreservation solutions with different DMSO content and human serum albumin (HSA) as a base medium were tested. After systematic testing, human serum albumin (HSA) supplemented with 7.5% of DMSO was identified as the optimal cryopreservation solution for recovery of CAR-expressing and parental NK-92 cells, after a freeze/thaw cycle, with post-thaw recovery values of 68.84 % ± 5.07 % and 49.51 % ± 5.12 %, respectively. Furthermore, results obtained in this study showed a significant decrease in recovery values of NK-92 (40.69 % ± 10.2 %) and NK-92/5.28.z (23.91 % ± 9.5 %) cells 24 hours after thawing when compared to measurements performed directly post-thaw. One possible reason could be freezing-induced damage of metabolic organelles, which resulted in cell death within a couple of hours post-thaw. This finding suggests that testing cell recovery only directly post-thaw may be insufficient for the establishment of optimal cryopreservation conditions. Extension of recovery analysis and comparisons of growth parameters of the cells frozen in different cryopreservation solutions, within the first few days post-thaw, may be necessary. Establishment of optimal expansion and cryopreservation protocols enabled the generation of a GMP-compliant master cell bank (MCB) of NK-92/5.28.z cells. Post-thaw analysis of manufacturing parameters confirmed the suitability of the procedure, as it resulted in a high quality of thawed NK-92/5.28.z cells with predictable cell growth, which ensures reproducible expansion of individual patient dose. The generation of a GMP-compliant MCB was an important part of the current study, providing an "on demand" well characterized platform for the expansion of therapeutic doses.

Although NK-92 cells never engrafted in immunocompromised SCID mice, the few clinical trials that were done so far, always included γ-irradiation of the cell product prior to transfusion as a definitive safeguard against proliferation and engraftment in the patient. In clinical trials, employing parental NK-92 cells irradiated with 10 Gy, rapid clearance was reported, where NK-92 cells were detected in the patient’s blood up to

48 hours post-infusion [35,36]. For that reason, radiation sensitivity and maintenance of functionality after irradiation were investigated over time with the main focus on high density cell suspensions imitating therapeutic doses of NK-92/5.28.z. Our data confirmed irradiation dose of 10 Gy as the optimal treatment of genetically modified NK-92 cells intended for clinical application, offering a convenient balance between effector cell stability/efficacy (cell viability > 90 % for 24 hours; specific cytotoxicity > 50 % up to 18 hours) and definitive proliferation arrest. Additionally, the release of soluble factors by effector cells as a stress response to γ-irradiation was tested. As described for other cell types, we expected elevated levels of pro-inflammatory cytokines: IL-8 and IL-6 after exposure [114]. However, there was significant increase only in IL-8 production, whereas the release of IL-6 was not induced upon irradiation. This confirms the results of other experiments where IL-6 was not detected even after stimulation with ErbB2-positive targets.

Formulation of the cellular product depends on the clinical application, where local injection into the vital body organs seems the most challenging. A good example of such an approach is an imminent clinical trial employing NK-92/5.28.z cells for the treatment of patients with ErbB2-overexpressing GBM, where intracranial injection of cell suspension will be performed through a Rickham reservoir implanted during resection surgery. This route of administration is associated with several limitations, for instance: the restricted injection volume which necessitates high cell densities in the final product in order to achieve clinically relevant doses. Another issue is the clinically applicable injection medium (excipient) optimal to preserve cell quality [115,116]. In this study, the concentration of 5 x 10^7 cells/ml was determined as a maximal stable cell concentration in the final product. Performed experiments showed that, too high of a concentration of the cells could be a limiting factor especially in the case of NK-92 cells, which are particularly sensitive to overgrowth and hence rapidly lose their cytotoxic properties when stored in high densities. Moreover, prolonged storage in a closed system leads to nutrient and cytokine exhaustion and an accumulation of toxic metabolites like lactate, which may additionally impair the quality of the cells as well as cause a pH acidic shift of the storage/shipment solution. Therefore, to ensure clinical efficacy of NK-92/5.28.z, several buffers were tested to serve as an excipient for shipment and injection. X-Vivo 10 containing recombinant transferrin gave the most

promising results in terms of cell viability and potency over 6-hour storage (91.49 % ± 0.62 % and 86.18 % ± 2.58 %, respectively), indicating that the combination of supplements contained in this medium is optimal not only for NK-cell culturing but also for cell storage/shipment. The manufacturing of cellular products is associated with numerous limitations specific to the particular cell type. The aim of this study, therefore, was to solve the issues of reproducible and efficient cell expansion, optimal irradiation dose and maximal cell concentration in final product, which resulted in the establishment of a clinical grade manufacturing procedures enabling generation of sufficient numbers of functional cells to form a stable patient dose. Three independent cellular therapy product processing of NK-92/5.28.z cells were successfully completed. Post-preparation analysis of viability and cell potency revealed that the stability of generated patient doses was satisfactory in the context of clinical application, proving the optimal character of established conditions.

In some ErbB2-overexpressing tumors potentially eligible for NK-92/5.28.z treatment, corticosteroids are part of the standard of care, used to reduce tumor-related symptoms or side effects of first line antitumor therapies (e.g. vasogenic edema in patients with malignant brain tumors) [117]. Considering the numerous studies reporting inhibitory effects of corticosteroids on NK-cell cytotoxicity [118,119], analysis of a potential impact of prednisolone treatment on CAR-mediated and natural killing by genetically modified NK-92 cells was of importance. As expected based on published reports, the natural killing ability against HLA-negative targets was strongly reduced in a dose-dependent manner. The effect is probably due to a downregulation of natural cytotoxicity receptors (NCR) as reported by other researchers [97]. Remarkably, retargeted cytotoxicity of NK-92/5.28.z was not affected, showing high potency in killing ErbB2-positive targets even after prednisolone treatment with the highest tested dose of 200 µg/ml of culture medium, which corresponds with the highest clinical dose applied intravenously (1000 mg). This interesting finding indicates the feasibility of NK-92/5.28.z as a combined or second line therapy and confirmed the independence of CAR-mediated cell activation from natural cytotoxicity mechanisms. Consequently, it may suggest the resistance of NK-92/5.28.z to common tumor-escape mechanisms utilizing the downregulation/exhaustion of natural cytotoxicity receptors by tumor cells (e.g. downregulation of NKp30 by soluble variants of its ligand B7-H6 in

ovarian carcinoma patients [120] or down-modulation of NKG2D by soluble MIC ligands [121-123]).

Cytokine release syndrome (CRS) is the most common and most severe complication observed in patients subjected to immune-based therapies (e.g. CAR-engineered T cells or therapeutic antibodies). In most cases CRS is fully reversible and manageable with a combination of supportive care and the IL-6 receptor-blocking antibody, tocilizumab. This indicates IL-6 is a major player at least in the early, acute phase of the syndrome. Moreover, IL-6 and TNF-α were the first inflammatory cytokines highly elevated in the serum of pediatric ALL patients suffering from grade 3 CRS after CD19-CAR T cells infusion [124,125]. In performed experiments, with CAR-armed NK-92 cells IL-6 was not produced by effector cells and TNF was secreted at very low levels, even upon strong stimulation. This suggests the superiority of engineered NK cells over T cells in this pivotal safety aspect. Therapies employing NK and T cells as effectors are diametrically opposed strategies where manipulated T cells are intended to persist in the patient's circulation as memory cells and after tumor clearance serve as cancer immunosurveillance, while NK-cell based therapies preconceive effector cell degradation after rapid tumor elimination (with rather classical pharmacokinetics similar to conventional drugs), thus reducing the risk of long-term side effects [126]. On the other hand, intensified secretion of pro-inflammatory cytokines (IFN-γ, MIP-1α) by NK-92/5.28.z cells upon stimulation with ErbB2-positive targets, may contribute to the enhancement of endogenous antitumor immunity by stimulation of the host immune system, as recently explored by Zhang et al in mouse models of GMB [96]. Possible mechanisms of this phenomenon could be natural killer-dendritic cell (DC) interaction resulting in the conversion of DC into type-1 polarized DCs (DC1); demonstrating resistance to tumor-related suppressive mechanisms and endowed with the ability to induce Th1 and CTL antitumor responses [127,128]. Moreover, IFN-γ, secreted by tumor stimulated NK-92/5.28.z, may promote tumor-specific antibody production by B cells and activation of CD4+ T cells to secrete Th1 cytokines which lead to further CTL and B cell induction.

The obtained results confirmed that ErbB2 expression on target cells is essential for robust activation of retargeted killing mediated by genetically modified NK-92 cells. However, it should be noted that all targets utilized in our experiments were of tumor

origin, expressing stress molecules and other factors inducing the activation of NK-92/5.28.z cells via natural killer receptors. Therefore, even in the case of ErbB2-negative targets (MDA-MB-468, G55T2) a certain amount of cell lysis was observed. The fact, that even low expression of the target molecule is sufficient to trigger potent and rapid activation of CAR-expressing NK-92 cells should be considered in terms of potential on-target/off-tumor effects and associated side effects. ErbB2 is not only overexpressed by cancer cells but also expressed at low levels by normal human tissues of epithelial origin [88]. In this safety aspect, the ErbB2-specific original monoclonal antibody from which the antigen binding domain is derived plays a critical role. A phase I clinical study utilizing immunotoxin (erb-38) consisting of the Fv portion of ErbB2-specific monoclonal antibody e23 fused to a truncated form of Pseudomonas exotoxin A, demonstrated hepatotoxicity in all treated patients [129]. Whereas in a similar approach with the recombinant antibody toxin scFv(FRP5)-ETA (binding domain derived from FRP5 mAb), administered at therapeutic doses, no induction of toxicity was reported [130]. Trastuzumab is an ErbB2-specific monoclonal antibody showing great potential as a therapy for the ErbB2-overexpressing breast cancers either alone or in combination with chemotherapy regimens; however, it is associated with a risk of cardiac dysfunction, due to a recognition of low levels of ErbB2 protein on adult human cardiomyocytes [91]. Conversely, the safety of the FRP5-based binding domain used for generation of the CAR applied in NK-92/5.28.z was recently confirmed. In that study, which included a group of recurrent/refractory sarcoma patients who were treated with ErbB2-specific CAR-T cells equipped with targeting domain, derived from FRP5, severe toxicities were not observed [131]. The few available clinical reports suggest, that the position of the binding epitope relative to the target cell membrane plays a crucial role in the safety issue. The FRP5 antibody recognizes the membrane distal domain of ErbB2 (epitope on subdomain I) distant from the trastuzumab-binding domain (epitope on subdomain IV) [130,132]. Furthermore, no cytotoxicity above background values was observed in *in vitro* killing assays against normal human cardiomyocytes, lung epithelial cells and PBMCs, as reported by Schonfeld et al [95]. In this work, analysis of potential adverse reactions caused by NK-treatment was extended to the investigation of colony-forming capacity of peripheral blood progenitor cells (apheresis material from G-CSF mobilized

donors) treated *in vitro* with NK-92, NK-92/5.28.z or CM of both, where no reduction in clonogenic potential was observed. Interestingly, there was a slight but significant increase in the number of erythroid colonies suggesting the presence of growth factors in NK-conditioned medium [133] which in combination with the erythropoietin present in the cytokine-replete methylcellulose medium used for our studies could induce modest erythroid skewing [134].

In this study, GMP-compliant procedures enabling the production of therapeutic doses of NK-92/5.28.z were successfully established. Moreover, crucial factors concerning safety and efficacy in the context of imminent clinical trials were discussed.
In summary, it was shown that NK-92 is not only a very good candidate to become a potent, standard therapy for treatment of tumors, but it is also a perfect platform for further modification. Its CAR-expressing derivative NK-92/5.28.z. retains main features of parental cells (phenotype, doubling time, the same optimal culture and cryopreservation conditions) while exhibiting superior, selective potency against ErbB2-positive targets normally resistant to NK-cell cytotoxicity.

5 Summary

5.1 Abstract

The NK-92/5.28.z cell line is an ErbB2 (HER2) specific, CAR-expressing continuously growing derivative of the clinically applicable NK-92 line, and exhibits profound and highly specific cytotoxicity against ErbB2-expressing tumor targets which are resistant to parental NK-92 cells. This study aimed to establish good manufacturing practice procedures enabling the generation of therapeutic doses of ErbB2-CAR expressing NK-92 cells for upcoming phase I/II clinical trials in ErbB2-overexpressing malignancies. The concept of patient dose manufacturing presented here encompasses the generation of a GMP-compliant master cell bank, which serves as a well-defined source of NK-92/5.28.z cells for further expansion of individual patient doses. To reach sufficient numbers of genetically modified NK-92 cells, expansion protocols were developed by systematic testing of culture conditions including GMP-grade culture media (X-Vivo 10 with human holo-transferrin, X-Vivo 10 with recombinant transferrin, CellGro) and human serum substitutes (human serum albumin, platelet lysate, heat inactivated human plasma). Since growth and potency of NK-92/5.28.z cells are IL-2 dependent, identification of a suitable concentration of this cytokine was an important issue addressed in this work. As a result of performed analyses, X-Vivo 10 containing recombinant transferrin supplemented with 5 % of heat inactivated human plasma and 500 U/ml IL-2 was selected as the culture medium for clinical expansions. This combination supported reproducible cell growth with doubling time of 28.84 h ± 0.5 h ±, maximal -fold expansion in batch culture of 24.97 ± 0.65 (max. conc.: 12.49 x 10^5 ± 0.32 x 10^5 cells/ml) stable CAR expression and potency of the cells, even in long-term culture (CAR expression: 99.77 % ± 0.03 %; specific cytotoxicity: 89.75 % ± 3.77 % after 12 months of culturing). Considering the sensitivity of NK-92 and its CAR-expressing derivative to cryopreservation procedures, a part of this thesis focused on selection of cryopreservation solutions providing satisfactory post-thaw recovery of frozen cells, where human serum albumin as a base medium supplemented with 7.5 % DMSO gave the best results (post-thaw recovery of

NK-92/5.28.z cells: 68.84 % ± 5.07 %). Following established GMP-compliant culture and cryopreservation procedures, a master cell bank comprising 200 vials of NK-92/5.28.z cells was generated. By means of concurrently developed quality control methods, 6 representative vials from the master cell bank were tested in terms of manufacturing related parameters (mean cryovial content, post-thaw recovery and cell growth, cell identity, specific cytotoxicity) confirming the high quality of the cells and thereby the validity of the performed process. Multiparameter analysis revealed that therapeutic doses of 5 x 10^9 NK-92/5.28.z cells can be expanded from a master cell bank within 5 days. Application of X-Vivo 10 rTF medium supplemented with 100 U/ml of IL-2 as an excipient, supported 6-hour shelf life of irradiated (10 Gy) NK-92/5.28.z cells formulated as a final product at the density of 5 x 10^7/ml. This concentration translates to the final dosage form of 1 x 10^8 cells/dose in 2 ml, a volume which is suitable for intracranial injection in the upcoming clinical trial in glioblastoma. The systemic (intravenous) route of administration planned in an upcoming breast cancer trial will allow the infusion of even higher volumes of up to 100 ml, and thereby higher cell numbers up to 5 x 10^9 cells/dose. In addition, investigation of factors impacting the efficacy and safety of clinical application was another important part of this thesis. Analysis of soluble factors released by target stimulated NK-92/5.28.z cells showed selective and significant increase in the secretion of GZMB (2-fold), IFN-γ (4-fold), IL-8 (24-fold) and IL-10 (5-fold) NK-92/5.28.z upon stimulation with ErbB2(+) targets. This phenomenon was not observed in the case of parental NK-92 cells and primary NK cells, indicating resistance of ErbB2(+) target cell line to natural killing mechanism, which can be overcome by specific CAR-mediated activation. Abundant amounts of immunomodulatory cytokines secreted by tumor activated NK-92/5.28.z may stimulate the host immune system by interaction with dendritic cells, induction of CTL and tumor-specific antibody production by B cells. Of possible relevance for clinical tolerability, IL-6 which plays the leading role in cytokine release syndrome was not detected. Treatment with clinically relevant doses of corticosteroids (maximal dose of 200 µg/ml corresponds with the highest dose of prednisolone applied clinically) did not affect retargeted killing of ErbB2(+) targets by NK-92/5.28.z cells, while natural killing against K562 cells was attenuated in a dose-dependent manner, as expected. Colony forming assays with mobilized

peripheral blood progenitor cells pretreated with NK-92 or NK-92/5.28.z showed no evidence of NK cell impact on the colony forming capacity of PBSCs. In this study, GMP-compliant procedure enabling production of therapeutic doses of NK-92/5.28.z was successfully established. The data identify genetically modified, CAR-expressing NK-92 cells as a powerful, clinically feasible candidate for efficient and safe adoptive immunotherapy of ErbB2-overexpressing malignancies like breast cancer or glioblastoma.

5.2 Zusammenfassung

Die NK-92/5.28.z-Zelllinie ist unter optimalen Zellkulturbedingungen eine kontinuierlich wachsende, ErbB2 (Her2) Tumorantigen spezifische CAR-exprimierende Variante der klinisch einsetzbaren NK-92-Zellen. Sie zeichnet sich durch ihre hohe Zytotoxizität gegenüber ErbB2-positiven Tumorzellen aus, die gegen parentale NK-92-Zellen resistent sind. Das Ziel dieser Arbeit war die Etablierung von GMP-konformen Protokollen zur Herstellung von therapeutischen Dosen ErbB2-CAR-exprimierender NK-92/5.28.z-Zellen für bevorstehende klinische Phase I/II Studien zur Therapie ErbB2-überexprimierender Tumore. Das in dieser Arbeit vorgestellte Konzept zur Herstellung therapeutischer Dosen schließt die Erstellung einer GMP-konformen Masterzellbank ein, die als qualifizierte Ausgangsbasis für die Herstellung von individuellen Patientendosen der NK-92/5.28.z-Zelllinie dienen soll.
Um eine ausreichende Anzahl genetisch modifizierter NK-92/5.28.z-Zellen zu erhalten, wurden optimale Expansionsprotokolle entwickelt, indem systematisch die Kulturbedingungen getestet wurden. Insbesondere wurde die Eignung verschiedener Zellkulturmedien (X-Vivo 10 mit humanem holo-Transferrin, X-Vivo 10 mit rekombinantem Transferrin (rTF), CellGro) sowie humaner Serumsubstitute (humanes Serumalbumin, humanes Plättchenlysat, hitzeinaktiviertes humanes Plasma) hinsichtlich einer GMP-konformen Zellexpansion untersucht. Da Zellproliferation und Funktionalität von NK-92/5.28.z-Zellen IL-2-abhängig sind, war die Findung einer optimalen Konzentration dieses Zytokins ein wichtiger Aspekt dieser Arbeit. Es zeigte sich, dass das Zellkulturmedium X-Vivo 10 mit rTF, supplementiert mit 5% hitzeinaktiviertem humanem Plasma und 500 IU/ml rekombinantem IL-2, ein

reproduzierbares Zellwachstum und gleichbleibende Funktionalität mit stabilen Zelldopplungszeiten (28,84 h ± 0,50 h), einer 24,97 ± 0,65 –fachen maximalen Expansion (max. Zellkonzentration: 12,49 x 10^5 ± 0,32 x 10^5 Zellen/ml) sowie einer stabilen CAR-Expression und funktionellen Aktivität (CAR-Expression: 99.77 % ± 0.03 %; spezifische Zytotoxizität: 89.75 % ± 3.77 %) auch in einer Langzeitkultur bis 12 Monate garantiert. In Anbetracht der Empfindlichkeit von NK-92-Zellen und der davon abgeleiteten genetisch modifizierten Variante wurde ein besonderer Schwerpunkt auf die Optimierung der Kryokonservierungsmedien hinsichtlich der erzielbaren Zellausbeuten nach dem Auftauprozess gelegt. Hier zeigte sich 7,5% DMSO in humanem Serumalbumin als am besten geeignet (Rate vitaler NK-92/5.28.z-Zellen nach dem Auftauen: 68.84 % ± 5.07 %). Auf Basis dieser GMP-konformen Zellkultur und Kryokonservierung wurde eine Masterzellbank von NK-92/5.28.z-Zellen mit 200 Ampullen angelegt. Entsprechend der entwickelten Qualitätskontrollprotokolle konnte bei allen 6 repräsentativ untersuchten Ampullen der Masterzellbank die Einhaltung von herstellungsrelevanten Aspekten, wie Gesamtzellzahl, Zellkonzentration, Zellausbeute nach dem Auftauen, Zellwachstum, Zellidentität und spezifische Zytotoxizität bestätigt werden. Anschließend wurden Faktoren untersucht, die hinsichtlich der Herstellung einer Patientendosis essentiell sind. Dies umfasste Transportbedingungen, Injektionsmedium, Bestrahlungsdosis und die maximale Zellkonzentration des finalen Produktes. Therapeutische Patientendosen von 5 x 10^9 NK-92/5.28.z-Zellen konnten innerhalb von 5 Tagen aus der Masterzellbank generiert werden. Die Verwendung von X-Vivo 10 rTF, supplementiert mit 100 IU/ml rekombinantem IL-2 als Exzipient erlaubte für die Formulierung des Endproduktes eine Zelldichte von 5 x 10^7/ml bestrahlter (10Gy) NK-92/5.28.z-Zellen und ermöglichte eine Haltbarkeit des Produktes von 6 Stunden. Zur intrakranialen Applikation der NK-92/5.28.z-Zellen in der geplanten Glioblastom-Studie können maximal 2 ml Zelldispersion verwendet werden, so dass als maximale finale Dosis 1 x 10^8 Zellen injiziert werden können. Die systemische Gabe in der geplanten klinischen Studie für Brustkrebspatienten ermöglicht die Injektion von bis zu 100 ml Zelldispersion, wodurch bei diesen Patienten bis zu 5 x 10^9 NK-92/5.28.z-Zellen pro Injektion appliziert werden können. Zusätzlich wurden weitere Faktoren untersucht, die wesentliche Auswirkungen auf die Effizienz und Sicherheit einer klinischen

Anwendung von NK-92/5.28.z-Zellen haben. So konnte nach Zellkontakt mit Tumorantigen-exprimierenden Zielzellen mit NK-92/5.28.z-Zellen ein signifikanter Anstieg der Sekretion von Granzyme B (2-fach), IFN-γ (4-fach), IL-8 (24-fach) und IL-10 (5-fach) durch NK-92/5.28.z-Zellen bestimmt werden. Diese Sekretion war spezifisch für NK-92/5.28.z-Zellen und konnte sowohl für parentale NK-92-Zellen als auch für primäre NK-Zellen nicht beobachtet werden, was die Resistenz ErbB2(+)-Zielzellen gegenüber der natürlichen Zytotoxizität nahe legt. Große Mengen immunmodulatorischer Zytokine, die durch aktivierte NK-92/5.28.z-Zellen sekretiert werden, können durch Interaktionen mit dendritischen Zellen, Induktion von CTL und tumor-spezifischen Antikörper produzierenden B-Zellen Einfluss auf das Wirtsimmunsystem nehmen. IL-6, ein pro-inflammatorisches Zytokin, dem eine entscheidende Rolle beim sogenannten „c*ytokine-release syndrom*“ zukommt, konnte nicht nachgewiesen werden. Weiterhin konnte gezeigt werden, dass klinisch relevante Dosen von Kortikosteroiden (die maximale eingesetzte Dosis von 200 µg/ml entspricht der höchsten verabreichten Prednisolonmenge, die klinisch appliziert wird) keinen Einfluss auf die spezifische Zytotoxizität von NK-92/5.28.z-Zellen haben, während die natürliche Zytotxizität gegenüber K562-Zellen dosisabhängig inhibiert wurde. Schließlich konnte auch kein Einfluss der parentalen NK-92-Zellen sowie der NK-92/5.28.z-Zellen auf die Koloniebildungsfähigkeit von humanen peripheren Blutstammzellen in sogenannten CFU- (*colony forming units*) Assays nachgewiesen werden.

In dieser Arbeit wurde ein GMP-konformer Prozess zur Herstellung therapeutischer Dosen von NK-92/5.28.z-Zellen erfolgreich etabliert und validiert. Die Ergebnisse demonstrieren, dass gegen das Tumorantigen ErbB2 gerichtete CAR-exprimierende NK-92-Zellen ein wirkungsvolles, klinisch anwendbares Therapeutikum für die Behandlung ErbB2-überexprimierender Tumore, wie Brustkrebs und Glioblastom, darstellt.

6 Addendum

6.1 Abbreviations

° C	Degree Celsius
ADCC	Antibody-dependent cell-mediated cytotoxicity
ALL	Acute lymphoblastic leukemia
APC	Allophycocyanin
CAIX	Carbonic anhydrase IX
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CLL	Chronic lymphatic leukemia
CM	Conditioned medium
Conc.	Concentration
CR	Complete remission
CRS	Cytokine release syndrome
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DT	Doubling time
E/T	Effector to target ratio
EBV	Epstein–Barr virus
EGFR	Epidermal growth factor receptor
EpCAM	Epithelial cell adhesion molecule
Eu	Europium
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
FBS	Fetal bovine serum
FDA	Food and drug administration
FFP	Fresh frozen plasma
FITC	Fluorescein isothiocyanate
G-CSF	Granulocyte-colony stimulating factor

GD2	Disialoganglioside
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Good manufacturing practice
GvHD	Graft versus host disease
Gy	Gray
GZMB	Granzyme B
h	Hour
HER2	Human epidermal growth factor receptor 2
HER2taNK	HER2 specific target activated natural killer cells
HI	Heat inactivated
HLA	Human leukocyte antigen
hPL	Human platelet lysate
HSA	Human serum albumin
IFN	Interferon
IL	Interleukin
kDa	Kilodalton
L	Liter
LGL	Large granular lymphocyte
Max.	Maximal
min	Minute
Min.	Minimal
ml	Milliliter
mM	Millimolar
mOS	Median overall survival
mOsm	Milliosmole
NCR	Natural cytotoxicity receptor
NHL	Non-Hodgkin lymphoma
NK	Natural killer cells
PBSC	Peripheral blood stem cells
PE	Phycoerythrin

PFS	Progression free survival
PMA	Phorbol 12-myristate 13-acetate
pNK	Primary natural killer cell
R	Receptor
r	Recombinant
RCC	Renal cell carcinoma
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
scFv	Single chain variable fragment
SCGM	Stem cell growth medium
SEM	Standard error of the mean
TF	Transferrin
TNF	Tumor necrosis factor
TRAIL	TNF–related apoptosis-inducing ligand
TRF	Time-resolved fluorometry
TWEAK	TNF-like weak inducer of apoptosis
WT	Wild-type cells

6.2 List of figures

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7 References

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9 Word of honor

I assure herewith on my word of honor, that I wrote this thesis by myself. All quotes, whether word by word, or in my own words, have been put in quotation marks or otherwise identified as such. The thesis has not been published anywhere else or has been presented to any other examination board.

Darmstadt, ……..

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(signature)

10 Curriculum Vitae

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11 Publications and congress contributions

11.1 Publications

Nowakowska P, Romanski A, Miller N, Odendahl M, Bonig H, Zhang C, Seifried E, Wels WS, Tonn T. Clinical grade manufacturing of genetically modified, CAR-expressing NK-92 cells for the treatment of ErbB2-positive malignancies. Cancer Immunol.Immunother. (submitted)

Becker PS, Suck G, **Nowakowska P**, Ullrich E, Seifried E, Bader P, Tonn T, Seidl C. Selection and expansion of natural killer cells for NK cell-based immunotherapy. Cancer Immunol.Immunother.2016; DOI: 10.1007/s00262-016-1792-y

Suck G, Odendahl M, **Nowakowska P**, Seidl C, Wels WS, Klingemann HG, Tonn T. NK-92: an 'off-the-shelf therapeutic' for adoptive natural killer cell-based cancer immunotherapy. Cancer Immunol.Immunother.2016; DOI: 10.1007/s00262-015-1761-x.

Schonfeld K, Sahm C, Zhang C, Naundorf S, Brendel C, Odendahl M, **Nowakowska P**, Bonig H, Kohl U, Kloess S, Kohler S, Holtgreve-Grez H, Jauch A, Schmidt M, Schubert R, Kuhlcke K, Seifried E, Klingemann HG, Rieger MA, Tonn T, Grez M, Wels WS. Selective inhibition of tumor growth by clonal NK cells expressing an ErbB2/HER2-specific chimeric antigen receptor. Mol.Ther.2015; DOI: 10.1038/mt.2014.219.

11.2 Congress contributions

11.2.1 Oral presentation

Nowakowska P, Odendahl M, Schonfeld K, Zhang C, Bonig H, Becker P, Grez M, Naundorf S, Seifried E, Wels WS, Tonn T. 2015. GMP-Process optimization to allow the clinical scale expansion of genetically modified, ErbB2-specific CAR expressing "off the shelf" natural killer (NK-92) cells. 48th Annual Meeting of Deutsche Gesellschaft für Transfusionsmedizin und Immunhämatologie (DGTI), Basel, Switzerland

11.2.2 Poster

Nowakowska P, Odendahl M, Bonig H, Schonfeld K, Zhang C, Naundorf S, Becker P, Seifried E, Grez M, Wels WS, Tonn T. 2015. Optimization and validation of processes related to the GMP compliant manufacture of genetically modified CAR expressing NK-92 cells. 21st Annual Meeting of International Society for Cellular Therapy, Las Vegas, United States of America

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