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HIGHLY EFFICIENT ACTIVATION AND EXPANSION OF NATURAL KILLER CELLS FOR CLINICAL USE IN CANCER IMMUNOTHERAPY

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Content

Zusammenfassung

Natürliche Killer (NK) Zellen können Tumorzellen erkennen und zerstören und die Behandlung von Krebspatienten mit NK Zellen stellt eine mögliche Option der Krebstherapie dar. In diesem Zusammenhang wird die *ex vivo* Expansion genutzt, um große Mengen an aktivierten NK Zellen herzustellen, denn eine ausreichende Zahl dieser Effektorzellen ist essentiell für eine erfolgreiche NK Zell basierte adoptive Krebsimmuntherapie. Jedoch stellen die Entwicklung effizienter Protokolle für die NK Zell Expansion und der Transfer dieser Protokolle in klinisch anwendbare Methoden eine große Herausforderung dar. Daher war das Ziel meines Projekts die Entwicklung einer klinisch anwendbaren Methode, die große Mengen an hochfunktionellen NK Zellen hervorbringt.

Zuerst wurde ein vollautomatisierter technischer Prozess entwickelt für die Aktivierung und Expansion von NK Zellen mit Interleukin(IL)-2 und bestrahlten Feederzellen mit klinischer Qualität (EBV-LCL). Im Vergleich zur manuellen Prozedur lieferte der automatisierte Prozess ähnliche NK Zellen in Bezug auf die Zellzahlen, das Profil von Oberflächenmarkern, die Genexpression und die *in vitro* Effektorfunktion. Durch die Expansion hatten die NK Zellen funktionelle Oberflächenmoleküle hochreguliert, wie z.B. TRAIL, FasL, NKG2D und DNAM-1, sie erhöhten die Produktion von Interferon (IFN)-γ und Tumornekrosefaktor (TNF)- α und wurden zytotoxischer gegenüber Tumorzelllinien. Weil die NK Zell Expansion bei dem verwendeten Protokoll auf eine Dauer von 2-4 Wochen beschränkt war, wurde als nächstes ein effektiveres Protokoll für die Langzeitexpansion entwickelt. Die manuelle NK Zell Expansion mit EBV-LCL und IL-2 induzierte im Mittel nach einer Woche eine 22-fache NK Zell Expansion, welche durch die Zugabe von IL-21 deutlich auf eine 55-fache NK Zell Expansion erhöht wurde. Außerdem ermöglichte die wiederholte Stimulation mit EBV-LCL und IL-2 und die Zugabe von IL-21 zu Beginn der Kultur eine anhaltende NK Zell Proliferation mit 10^{11}-facher NK Expansion nach sechs Wochen, was eine einmalig hohe Expansionsrate darstellt, die durch andere Methoden bisher nicht erreicht wird. Am wichtigsten jedoch war, dass der adoptive Transfer von NK Zellen, die mit dem optimierten Protokoll expandiert wurden, zur Inhibierung des Tumorwachstums in einem Melanom Xenotransplantat Mausmodell führte, wodurch die therapeutische Wirksamkeit der *ex vivo* generierten NK Zellen nachgewiesen wurde. Dieser therapeutische Effekt war deutlicher ausgeprägt als bei konventionell mit IL-2 aktivierten NK Zellen und zeigt, dass die optimierte Methode für die NK Zellexpansion nicht nur die Quantität sondern auch die therapeutische Qualität der NK Zellen erhöht.

Zusammenfassend ist das Resultat dieses Projekts ein vollautomatisierter Prozess zur *ex vivo* Produktion von NK Zellen und ein optimiertes Protokoll für die NK Zellexpansion mit beispielloser Effektivität. Die expandierten NK Zellen besitzen Eigenschaften für eine wirksame Krebsbekämpfung und sie zeigten therapeutische Wirksamkeit in einem präklinischen Melanom Xenotransplantat Mausmodell. Damit dient das Projekt klinischen Anforderungen und macht es möglich hohe Dosen an funktionellen NK Zellen zu generieren für den Einsatz in der Krebsimmuntherapie.

Summary

Natural killer (NK) cells can detect and kill tumor cells and infusion of NK cells to cancer patients may be a promising option to treat cancer. In this context, *ex vivo* expansion is used to produce large quantities of activated NK cells, because sufficient numbers of these effector cells are essential for successful NK cell based adoptive cancer immunotherapy. The development of efficient NK cell expansion protocols and the transfer of these protocols to clinically applicable methods represent a major challenge. To overcome this issue, the aim of my project was to develop a clinically applicable method that yields large numbers of highly functional NK cells.

First, a fully automated technical process was developed to activate and expand NK cells with (interleukin) IL-2 and irradiated clinical-grade feeder cells (EBV-LCL). In comparison to the manual procedure, the automated process yielded similar NK cells in terms of cell numbers, surface marker profile, gene expression and *in vitro* effector functions. Upon expansion, NK cells up-regulated functional surface molecules, such as TRAIL, FasL, NKG2D and DNAM-1, they increased the production of interferon (IFN)-γ and tumor necrosis factor (TNF)-α and they became more cytotoxic against tumor cell lines. Next, because in the used protocol NK cell expansion was restricted to a period of 2-4 weeks, a more efficient protocol for long-term expansion was developed. Manual NK cell expansion with EBV-LCL and IL-2 induced a 22–fold mean NK cell expansion after one week that was significantly increased to 53–fold by addition of IL-21. Furthermore, repeated stimulation with irradiated EBV-LCL and IL-2 and addition of IL-21 at the initiation of the culture allowed sustained NK cell proliferation with 10^{11}–fold NK cell expansion after six weeks, which is an unprecedented high expansion rate not achieved by any other method so far. Most importantly, adoptive transfer of NK cells expanded with this optimized protocol led to significant inhibition of tumor growth in a melanoma xenograft mouse model, proofing the therapeutic efficacy of the *ex vivo* generated NK cells. This anti-tumor efficacy was superior over that from conventionally IL-2 activated NK cells, demonstrating that the improved NK cell expansion method enhanced not only the quantity but also the therapeutic quality of NK cells.

In conclusion, the outcome of this project is a fully automated process for *ex vivo* production of NK cells and an optimized protocol for NK cell expansion with unparalleled efficacy. The expanded NK cells possess potent anti-tumor features and showed therapeutic efficacy in a preclinical melanoma xenograft model. Thereby, the project serves clinical needs and makes it possible to generate high cell doses of functional NK cells for the use in cancer immunotherapy.

1. Introduction and Background

The herein described work deals with cells of the human immune system, so called natural killer (NK) cells, and their use in therapy of cancer. This introductory chapter starts with a brief description of cancer. Then, an overview of the immune system is given followed by background information about NK cells including their application in cancer therapy.

1.1 Cancer

1.1.1 A disease of the genome

When abnormal body cells start to grow and divide uncontrolled, this can cause a multitude of different and severe diseases, which are defined by a single term: cancer. Cancer is a leading cause of death in the world. “There were 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer (within 5 years of diagnosis) in 2012 worldwide.”[1] When Boveri, a German biologist who fundamentally coined the understanding of chromosomes and genetics, already proposed in 1914 that defects of the chromosomes lead to abnormal cell proliferation,[2] he postulated an important underlying mechanism of cancer development, because today we know that “cancer is a disease of the genome”.[3] Cancer can occur when specific mutations change the genome and give rise to cancer-causing genes, which can be subdivided in oncogenes and tumor-suppressor genes. Thus, mutagens that damage the genome such as ultraviolet light, certain viruses and chemicals like acryl amide increase the risk of cancer formation. However, most genome mutations are “passenger” mutations and don’t give rise to cancer-causing genes, while only a few “driver” mutations trigger cancerogenesis. Tumor suppressor genes, as the name says, keep tumor development under control, and loss of these genes can cause cancer. Whereas oncogenes are mutated versions of normal genes that favor cancerogenesis in their altered form. In general, cancer-causing genes modify cells in different ways during a multistep process, which starts from former normal cells and passes through pre-malignant stages until these cells finally become highly malignant tumor cells. Thereby, analogous to the Darwinian evolution, each modification results in an advantage for the cell to survive, such as increased cell growth for example.[4] Outgrowth of a whole population of malignant cells then results in tumor formation and has consequences beyond the cellular level. Over time, more knowledge about cancer development allowed better understanding of cancer characteristics and this finally made it possible to develop new strategies to treat cancer, such as immunotherapy, which is explained later.

1.1.2 The hallmarks of cancer

Cancer was introduced as the generic term for multiple diseases that arise from uncontrolled cell growth, but the nature of cancer is by far more complex and can be characterized by additional factors. Today eight major "hallmarks of cancer" plus two cancer "characteristics" are defined by Hanahan and Weinberg (Figure 1.1), including essential functional properties for cancer cell proliferation, survival and dissemination:[4,5]

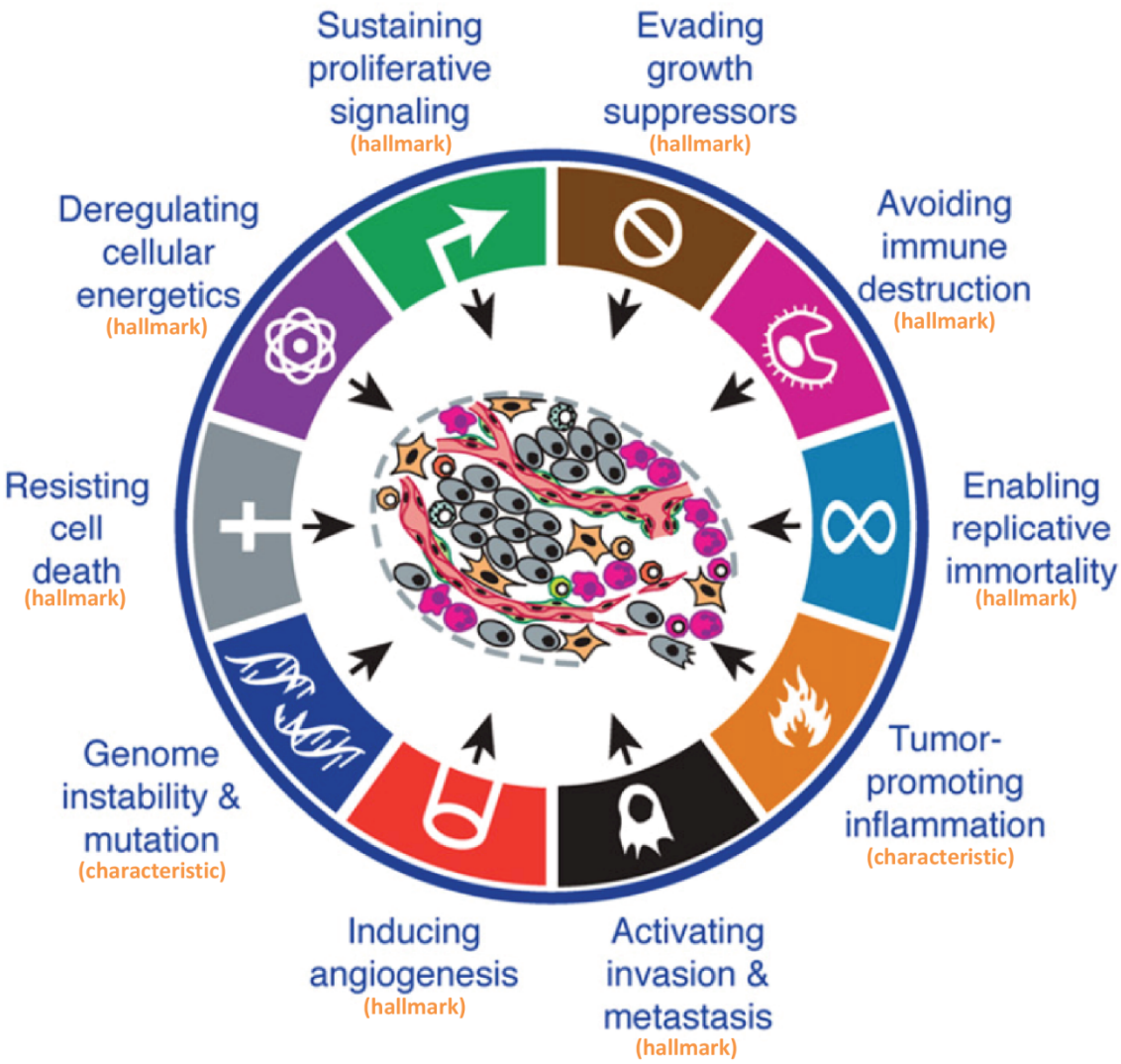


Figure 1.1 Scheme showing the hallmarks of cancer "that allow cancer cells to survive, proliferate, and disseminate; these functions are acquired in different tumor types via distinct mechanisms and at various times during the course of multistep tumorigenesis." Modified from Hanahan and Weinberg (2011)[5]

(1) Sustaining proliferative signaling is probably the most obvious cancer attribute. Cancer cells deregulate their cell cycle by producing growth factors themselves or by stimulating tumor-associated stroma cells for growth factor production.[6] Furthermore, cancer cells up-regulate receptors that are responsible for growth factor binding to reduce the threshold for growth factor signaling. Alternatively, a constitutive activation of the proliferation signaling is implemented by changing the structure of a growth factor receptor or by direct modification of a downstream pathway to maintain an activated proliferative signaling. An example for the latter would be the modification of the B-rapidly accelerated fibrosarcoma (B-Raf) protein that plays an important role for cell division and cell differentiation through the mercapturic acid pathway (MAP) and extracellular signal-regulated kinase (ERK) signaling pathway.[7,8]

(2) Evading growth suppressors is another strategy to bypass the regular cell cycle control, allowing cancer cells to become unresponsive to signals that normally dampen the cell growth. The frequency of mutations of such tumor suppressor genes is very high among different cancers, as shown exemplary by the tumor protein (TP)53.[9]

(3) Resisting cell death by evading the normal apoptosis machinery is an essential feature of cancer. The “programmed cell death” is controlled through a balance of pro- and anti-apoptotic regulators of the B cell lymphoma (Bcl)-2 family and the cell death is initiated in normal cells as a response to deoxyribonucleic acid (DNA) damage, energy stress, growth factor withdrawal or hypoxia.[10] In many cancers, the death program is circumvented by over-expression of anti-apoptotic Bcl-2 family members.[11]

(4) Enabling replicative immortality is required for cancer cells to overcome the normally limited capacity for cell division. Normal cells reach a senescence state after a certain amount of repeated cell divisions and this is regulated on the chromosomal level. The ends of the chromosomes, named telomeres, consist of hexanucleotide repeats and become shorter after each cell division. After a certain number of cell divisions the telomeres are completely destructed and the chromosomal DNA forms end-to-end fusions thereby initiating cell senescence followed by cell death.[12] However, certain cell types such as stem cells require an enhanced capacity for cell division that is achieved by longer telomeres and expression of telomerase, a specific enzyme capable of telomere synthesis and elongation.[13] Increased telomerase expression of tumor cells is needed for cancer progression.

(5) Inducing angiogenesis is required to ensure supply of nutrients, gas exchange and removal of metabolic end-products via the blood system. Angiogenesis is the spreading of new blood vessels and is controlled by angiogenic regulators, such as the pro-angiogenic vascular

endothelial growth (VEGF). In cancer, chronically activated angiogenesis is implemented for example by up-regulated VEGF expression directly by oncogenes or through hypoxia.[14]

(6) Activating invasion and metastasis facilitates cancer cells to disseminate. Adhesion molecules such as E-cadherin maintain the cohesion between cells within a tissue structure. During cancer, inactivation of E-cadherin allows single tumor cells to leave the tissue formation, to migrate to another location within the body and to rebuild a distant tumor.[15] Thereby, cancer cells make use of the epithelial-mesenchymal transition (EMT) process that is normally involved in the organ and tissue formation during development and tissue repair.[16]

(7) Deregulating cellular energetic or reprogramming the energy metabolism is necessary for cancer cells to ensure the supply of "fuel". The "Warburg effect" describes that cancer cells mainly produce energy through glycolysis even in the presence of oxygen, while normal cells mainly use oxidation of pyruvate in the mitochondria under aerobic conditions.[17] Surprisingly, this type of "aerobic glycolysis" is even less efficient in production of adenosine triphosphate (ATP). Probably the changed metabolism is a consequence of hypoxia within tumors and is used to bypass the mitochondria regulated apoptosis machinery of the cell. Furthermore, glycolysis provides intermediates for those biosynthetic pathways that lead to nucleosides and amino acids that are urgently required to generate new cells.

(8) Avoiding immune destruction is a protective strategy since the immune system normally detects and eliminates abnormal cells such as tumor cells. This topic is described in detail in chapter 1.2.3.

(A) Genome instability and mutation are fundamental for cancer development and cancer cells often actively increase the rate of occurring mutations. This is achieved by oncogenes that either increase the sensitivity to mutagenic agents or directly lead to DNA damage or inactivate the machinery for DNA maintenance and repair.[18–20]

(B) Tumor-promoting inflammation is a typical cancer associated condition characterized by infiltration of immune cells into the tumor tissue. These immune cells are actually supposed to protect the body and eradicate tumor cells, but instead, they generate a chronic inflammatory response that even enhances tumor cell growth and cancer progression. The mechanisms behind this misdirected immune attack are explained in chapter 1.2.3 after a short introduction about the immune system in the following section.

1.2 The immune system

The immune response is defined as protection of the body against potentially harmful pathogens such as viruses or bacteria. This protective response is mediated by a variety of molecules and effector cells that together form the immune system. This chapter gives a brief introduction into the very complex field of immunology based on current textbook knowledge,[21] and focuses on leukocytes, the white blood cells that build the cellular immunity. Leukocytes are derived from bone marrow hematopoietic stem cells that differentiate into myeloid and lymphoid progenitor cells and finally give rise to “innate” and “adaptive” immune cells.

1.2.1 The innate immune system

Cells of the innate immune system are called the first line of defense, since they play a major role during the first contact with pathogenic agents and they induce a fast immune response. The innate immune response relies on unspecific detection of general pathogen characteristics by means of a variety of immune cell receptors. The important class of Toll-like receptors (TLRs) for example recognizes a multitude of different parasites and leads to recruiting of immune cells, local control of pathogens and activation of adaptive immune cells. Innate immune cells are classified in granulocytes, monocytes, macrophages, dendritic cells (DCs) and NK cells.

Granulocytes destroy pathogens by release of toxic proteins and enzymes. An important feature of neutrophil granulocytes, macrophages, monocytes and DCs is the incorporation and neutralization of pathogens within the cell, called phagocytosis. Upon phagocytosis, soluble factors such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α are released that trigger other immune responses and induce an inflammatory condition. In addition, upon phagocytosis macrophages and DCs process the pathogenic proteins to smaller peptides and bind these protein fragments to major histocompatibility complex (MHC) structures. Later, the cells can act as antigen presenting cells (APCs) by presentation of the MHC-peptide complex on the cell surface to activate cells from the adaptive immune system. NK cells can be seen as the prototype of lymphocytes among innate immune cells and their biology and function is described in chapter 1.3.

1.2.2 The adaptive immune system

In contrast to the innate immune system, the adaptive immune system reacts delayed but more specific against a certain pathogen. An educated pool of cells generates a cellular memory, which accelerates the response when a specific pathogen is encountered again at later time

points. Adaptive immune cells are lymphocytes that can be subdivided in B cells and T cells and they highly efficiently detect specific structural substances defined as antigens. Every B cell or T cell bears an unique and highly specific receptor for a specific antigen. This high diversity of B cell receptors (BCRs) and T cell receptors (TCRs) is achieved during the development of B cells and T cells by randomized re-combination of segments of the receptor DNA sequences.

B cells get activated upon binding of the antigen to the BCR. The antigen gets internalized and is processed intracellular into smaller peptides that are finally presented on MHC class II on the B cell surface. Binding of certain T cells to the peptide-MHC II complex stimulates B cells to differentiate into memory B cells or into plasma cells. Plasma cells secrete antibodies, which are proteins that bind highly specifically to the encountered antigen. An antibody is also called immunoglobulin (Ig) and consists of a variable fragment for antigen-binding (Fab) and a constant fragment (Fc) that allows communication with other cells via Fc receptors. Fc receptor expressing immune cells can detect antibody coated cells, resulting in phagocytosis of the target cell or lysis of the target via antibody-dependent cellular cytotoxicity (ADCC). Furthermore, antibody-binding can directly lead to functional neutralization of the antigen, which is especially important in case the antigen is a toxin or a functional viral component.

The TCR enables T cells to recognize small peptides that are bound to MHC molecules. MHC class I can be found on virtually every cell within the body and its function is to present peptide structures of intracellular proteins on the cell surface. Thereby foreign structures such as pathogen derived peptides can be detected by cytotoxic T cells expressing the cluster of differentiation (CD)8 co-receptor that bind to the specific peptide-MHC class I complex and directly kill the target cell. Thereby CD8 T cells induce cell contact-mediated apoptosis or release cytotoxic granules. In addition, cytokines secreted by CD8 T cells, such as IFN-γ, TNF-α and lymphotoxin (LT)-α can contribute to target cell killing and stimulate other immune cells such as macrophages. Peptides presented on APCs are bound to MHC class II and can be recognized by T helper (Th) cells expressing the CD4 co-receptor or by regulatory T cells (Tregs). Naive Th cells differentiate into Th1 or Th2 cells after activation via antigen contact. Th1 cells are mainly involved in the activation of macrophages during infection and they interact with B cells to augment the production of antibodies. Th2 cells on the other hand activate naive B cells and carry an important function in the initiation of antibody production. Tregs are important for the regulation and attenuation of immune responses.

1.2.3 The role of immunology in cancer

A rough idea existed for long time that the immune system could not only eliminate pathogens but also combat cancer. Then, in the sixties Burnet and Thomas could show that the adaptive immune system indeed protects against cancer and they coined the term cancer immunosurveillance.[22,23] Today much more is known about the importance of the immune system in this context and the complex interplay between cancer and immune cells. The immune system prevents cancer by (1) protection against viruses and therefore virus induced cancers, (2) avoiding prolonged, cancer promoting inflammation by fast neutralization of pathogens that induce inflammation and (3) direct recognition and elimination of cancer cells.[24] However, cancer can also bypass or even utilize the immune system for cancer progression and the cancer immunoediting hypothesis describes the response of cancer cells to the immune system in three sequential phases: elimination, equilibrium, escape (Figure 1.2).[24–30]

Elimination is the first phase of cancer immunoediting and was mentioned already as a protective mechanism of the immune system. Recognition and subsequent elimination of cancer cells is possible, because cancer cells either up-regulate ligands for activation of the innate immune system or they express “cancer rejection antigens” that stimulate the adaptive immune system. In addition, danger signals such as type I interferons are released by tumor cells during cancer development and trigger adaptive immune responses. The stress ligands MHC class I polypeptide-related sequence (MIC)A and MICB are exemplary cancer associated ligands that are detected by NK cells and certain T cell subsets. Specific cancer rejection antigens can be derived from non-mutated highly over-expressed cellular antigens or from proteins that T cells normally don’t have access to, because the expression is restricted to germ line cells or specific tissues. The human epidermal growth factor receptor (HER)-2, the melanoma-associated antigen (MAGE) and the cancer-testis antigen NY-ESO-1 represent these types of tumor antigens for instance. On the other hand, cancer antigens can be neoantigens, meaning antigens that are normally absent from the human genome. In addition to cancer associated virus antigens, neoantigens are mainly generated by modified proteins as a result of tumor specific DNA mutations. The somatic mutation prevalence and therefore frequency of neoantigens that are immunogenic is cancer type dependent with the highest incidence for melanoma, lung and colorectal cancer.[31]

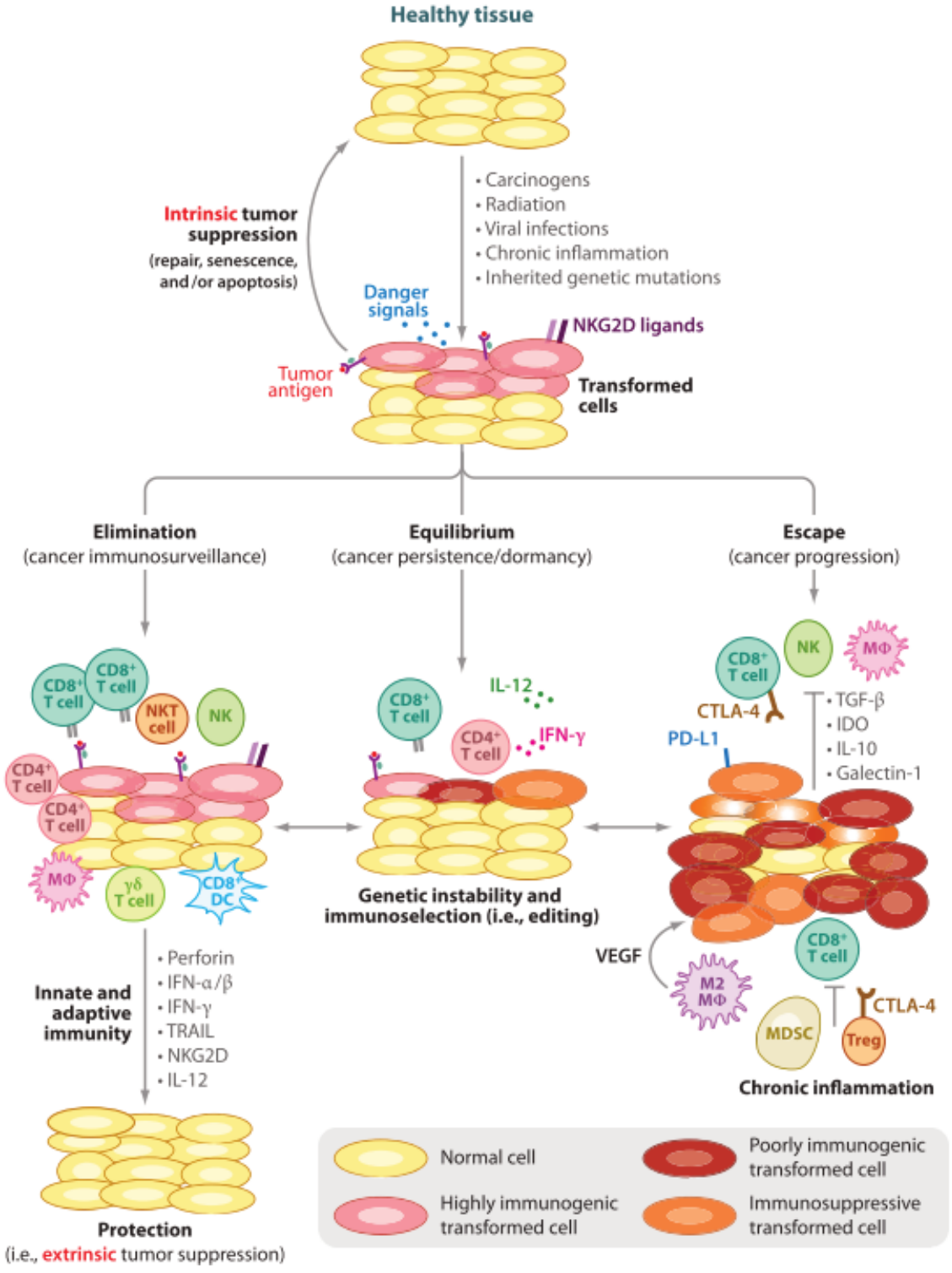


Figure 1.2 The three phases of cancer immunoediting: Elimination, Equilibrium, Escape. Schematic illustration of the complex interplay between tumor and immune cells. Modified from Vesely et al.[27]

Equilibrium is the next phase in case some tumor cells survive the elimination phase for any reason. The tumor cell outgrowth is still controlled by the immune system, but complete elimination is not achieved. This stable condition can persist for long time without cancer progression. Experiments in mice showed that IL-12, IFN-γ and T cells are mainly responsible for holding up the equilibrium phase.[32–34]

Escape occurs after tumor cells finally managed to circumvent the immune system and cancer progression is initiated. There are several reasons that can lead to tumor immune escape. Alterations of the tumor cells can cause the loss of cancer antigens or the tumor cells can acquire resistance to anti-apoptotic effector molecules. As a consequence of "natural selection", tumor cells with less susceptibility to immune responses grow out to visible tumors.[35] Furthermore local or systemic immunosuppressive mechanisms as well as chronic inflammation can cause immune escape.[36] This state can be actively induced by tumor cells through secretion of immunosuppressive factors such as transforming growth factor (TGF)-ß, Indoleamine-pyrrole 2,3-dioxygenase (IDO), IL-10, VEGF and galectin. Prolonged inflammation leads to presence of chronically activated leukocytes and accumulation of immunosuppressive Tregs, M2 Macrophages and myeloid-derived suppressor cells (MDSCs). All of these three, Tregs, M2 macrophages and MDCSs produce TGF-ß and IL-10. Tregs further inhibit T cells through the negative co-stimulatory checkpoint regulators cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1), causing T cell exhaustion and suppression of T cell activation. Tregs cells further consume IL-2 by expression of CD25, the α-chain of the high affinity IL-2 receptor, thereby depleting IL-2 that is required for maintenance of effector T cell functions. MDSCs can recruit Tregs via secretion of the cc-chemokine ligands (CCL)3, CCL4 and CCL5. MDSCs further act immunosuppressive by production of membrane-bound TGF-ß, and active inhibition of T cell functions by TCR nitrosylation and depletion of the amino acids arginine and tryptophan.[37–40]

Since the immune system is able to eliminate tumor cells and because cancer development is a consequence of cancer immune escape, it appears obvious to reinforce the immune system to treat cancer. In fact, cancer immunotherapy represents an emerging field and was acclaimed as "breakthrough of the year 2013" by the famous journal Science.[41] Immune checkpoint blockade targeting CTLA-4 and PD-1 is among the most promising approaches and show very encouraging clinical efficacies.[42,43] Another type of immunotherapy is to treat cancer patients by adoptive transfer of effector immune cells, such as DCs, T cells or NK cells.

1.3 Natural killer cell biology

NK cells were first described in 1975 and named natural killer cells due to their ability to kill tumor cells.[44,45] Since then, other important NK cell functions such as elimination of virus-infected cells became manifest and many researchers investigated these remarkable immune cells and their biology, leading to the knowledge that is available today about NK cell classification (chapter 1.3.1), NK cell receptors and NK cell activation (chapter 0) and NK cell effector functions (chapter 1.3.3).

1.3.1 NK cell classification – innate immune cells with adaptive features

NK cells are classically defined as innate immune cells, due to their ability to react against a certain target without prior sensitization. However, findings during the last years show that NK cells also feature typical characteristics of adaptive immune cells, indicating that the historical classification between innate and adaptive immune system might start to blur.[46–48]

First of all, adaptive immune cells and NK cells arise from the same lymphoid progenitor and share requirements for their development, such as common γ-chain-dependent cytokines.[49,50] Furthermore, similar to T cells, which undergo a selection process in the thymus to avoid unwanted responses against "self-antigens", NK cells pass through an education process to regulate their responsiveness and function.[51]

However, the most convincing adaptive attribute of NK cells is their ability for memory-like responses, characterized by an intensified response to a repeated stimulation. A first indication for NK cell memory has been observed in T and B cell deficient mice that showed an enhanced secondary immune response against chemical hapten antigens.[52] It was demonstrated that liver-resident NK cells are responsible for the effect dependent on the chemokine receptor CXCR6.[53,54] Another hint for mouse NK cell memory is given by a specific $Ly49H^+$ NK cell subset that is responsive against mouse cytomegalovirus (MCMV) glycoprotein m157 expressed on infected cells. Similar to T cells, these NK cells respond in three phases upon MCMV infection, starting with expansion of virus specific cells, followed by apoptosis of effector cells within a contraction phase and finally ending up in a long-lived stable pool of memory cells.[55] Upon MCMV re-challenge, these memory NK cells exhibit typical adaptive features as they undergo a secondary expansion phase and enabling a better control of the virus. Human CMV (HCMV) infection and reactivation is associated with specific expansion of a NK cell subset positive for CD57 and NK group 2 receptor (NKG2)C,[56] that is dependent on IL-12, monocytes and human leukocyte antigen (HLA)-E *in vitro*.[57] HCMV induced $CD57^+$ $NKG2C^+$

NK cells have a distinct epigenetic profile comparable to cytotoxic T lymphocytes,[58,59] but the relevance of this NK cell subset is still unknown.

Of note, treatment of NK cells with IL-12, IL-15 and IL-18 leads to a cytokine-induced NK cell memory, defined as long-term capacity to produce IFN-γ, so that several weeks after the initial activation the NK cells still mediate an enhanced cytokine response upon re-stimulation.[60–62] Thereby, cytokine-induced memory manifests as cell intrinsic effect that is passed on to offspring cells.

Besides the disputable historical classification of NK cells as innate immune cells, the former view of NK cells as the only lymphoid cell type among innate immune cells has changed after identification of a whole family of so called innate lymphoid cells (ILCs) with distinct characteristics and functions.[63] Thereby, striking similarities exist between the different ILCs and T-cell subsets regarding transcription factors and cytokine profiles, so that ILCs may resemble the T cell counterparts in the innate immune system.[64,65] NK cells are currently defined as killer ILCs due to the expression of IFN-γ, EOMES and TBET and their ability to directly kill target cells, similar to cytotoxic CD8 T cells, whereas the other ILC subsets, ILC1, ILC2, ILC3 and lymphoid tissue–inducer (LTi) cells are helper ILCs corresponding to the different types of Th cells. NK cells generally lack the expression of CD3 and human NK cells are classically subdivided in $CD56^{bright}$ and $CD56^{dim}$ NK cells depending on the CD56 expression level.[66] Of note, defining NK cells as $CD56^{+}/CD3^{-}$ NK cells spares $CD56^{-}$ NK cells, a subset that is rare in healthy individuals but that is predominantly found in patients infected with the human immunodeficiency virus (HIV) or hepatitis C patients.[67,68] Alternatively, the surface marker NKp46 is utilized to discriminate NK cells, but NKp46 is also expressed by subsets of NK-like T cells and LTi.[69,70] The $CD56^{dim}$ NK cell subset represents 90% of the NK cells in peripheral blood and is considered to be highly cytotoxic, whereas cytokine production is restricted to a short duration after activation.[71] On the other hand, $CD56^{bright}$ NK cells are predominant in lymph nodes and tonsils, have a low potential for natural cytotoxicity, but possess a high capacity to produce cytokines after stimulation. $CD56^{dim}$ NK cells express high levels of the Fc receptor CD16 and the chemokine receptors CXCR1 and CX3CR1, whereas $CD56^{bright}$ NK cells are CD16 low or negative and positive for CCR7, a chemokine receptor responsible for homing to secondary lymphoid organs.[72]

1.3.2 Regulation of NK cell activation

To mediate adequate protection, NK cells need to distinguish between normal cells on the one hand and tumor cells, virus infected cells or generally stressed cells on the other hand. Importantly, the essential NK cell ability to specifically recognize abnormal cells does not require prior sensitization for a specific target, because it's based on the interaction with target cells via a complex system of germ line coded activating and inhibitory receptors. Important receptors for human NK cells are described in the following.

Inhibitory receptors dampening NK cell activation

Normal autologous cells dampen NK cell activation by expression of MHC class I molecules, mainly by binding to inhibitory killer-immunoglobulin-like receptors (KIRs). Human MHC is classified as HLA and KIR genes are extremely diverse and differentially expressed, forming a heterogenic population of different NK cells with distinct KIRs and specificities for HLA alleles [73,74] Thereby, potentially auto-reactive NK cell clones expressing no inhibitory KIR for at least one self-HLA class I become anergic during NK cell development to ensure "self-tolerance".[75,76] This NK cell education process is similar to the selection process during development of adaptive immune cells, but the concrete mechanisms and involved cell types are still under investigation.[77] NKG2A is another inhibitory receptor on NK cells recognizing HLA-E, and same as KIRs, NKG2A signals through an immunoreceptor tyrosine-based inhibitory motif (ITIM).[78] The T-cell immunoreceptor with Ig and ITIM domains (TIGIT) is expressed by NK cells and it counteracts the NK cells activation. TIGIT binds to CD112 and CD113 and shares the ligand CD155 with another inhibitory receptor, CD96.[79] In addition, the killer-cell lectin like receptor G1 (KLRG-1) and the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) also inhibits NK cell activation.[80,81]

Activating receptors induce NK cell activation

The group of NK cell receptors containing an immunoreceptor tyrosine-based activating motif (ITAM) consists of activating KIRs, NKG2C, natural cytotoxicity receptors (NCRs) and CD16. Same as for their inhibitory counterparts, HLA class I molecules are the ligands for activating KIRs, and similar to NKG2A, NKG2C binds to HLA-E. The three NCRs discovered in the late 1990s, are NKp46 (NCR1; CD335), NKp44 (NCR2; CD336) and NKp30 (NCR3; CD337). NCRs recognize viral and bacterial structures but also tumor associated ligands.[82] NKp46 is the only NCR universally expressed by NK cells and it's conserved in humans and mice. Tumor relevant ligands for NKp46 are found for example on melanoma cells.[83] NKp44 is only expressed by activated NK cells and it recognizes ligands on tumor cells, such as the proliferating cell nuclear antigen (PCNA).[84] NKp30 is expressed on all mature NK cells and

binds to HLA-B-associated transcript 3 (BAT3) and B7 family member B7-H6, both produced by tumor cells.[85,86] CD16, also known as FcγRIIIa, belongs to a class of receptors that bind to IgG antibodies and enables NK cells to detect and eliminate antibody-coated cells, as described as ADCC before.[87] NK cell mediated ADCC represents an important mode of action of many therapeutic antibodies in cancer therapy such as trastuzumab, cetuximab or rituximab.[88] Of note, CD16 can act on its own to induce NK cell activation, whereas all other activating NK cell receptors require engagement of another co-receptor, making the activation process of NK cells even more complex.[89,90] Furthermore, non-ITAM bearing activating receptors comprise NKG2D or the DNAX accessory molecule-1 (DNAM-1) receptor. Human NKG2D signals though the DNAX activating protein (DAP)-10 and binds to its ligands MICA, MICB and UL16-binding proteins (ULBP)1–6.[91] Cancerogenesis and stress is linked to NKG2D ligand expression and NKG2D ligands are found on a variety of human cancer cell lines and primary tumors, such as glioma, leukemia, melanoma and colorectal cancer.[92–96] The activatory DNAM-1 competes with inhibitory TIGIT and CD96 for the ligands CD155 and CD112. DNAM-1 ligands are regulated by cellular stress, similar to ligands for NKG2D, and CD112 and CD155 are found over-expressed by many cancer types such as neuroblastoma and myeloma.[97,98] Interestingly DNAM-1 and other activating receptors synergize with 2B4 (CD244), the prototype of the signaling lymphocyte activation molecule (SLAM) family, that is involved in NK cell activation as well. CD2 is closely related to the SLAM family, binds to CD48 and works as co-receptor, for instance in NKp46 mediated NK cell effector functions.[89]

NK cell activation is regulated by the interplay of activating and inhibitory receptors

The multitude of activating and inhibitory receptors generates numerous signals during the NK cell-target cell interaction and integration of these signals determines whether the NK cell gets activated (Figure 1.3). Healthy cells express normal levels of inhibitory HLA class I and show only minor or no expression of NK cell activating ligands, so that NK cells don't get activated. In contrast, viral infected or tumor transformed cells tend to down-regulate their HLA class I expression as an immune escape mechanism to avoid the adoptive immune response by cytotoxic T cells.[99] The lack of HLA class I expression however increases the susceptibility to NK cells, since NK cells expressing specific KIRs for the missing HLA molecule perceive a "missing-self" signal and become responsive. The "missing self" hypothesis formulated more than 30 years ago was the first concept describing how NK cell activation is regulated.[100] Today it's known that also activating signals dictate the NK cell response and strong activating signal alone are sometimes efficient for activation despite inhibition in parallel.[101] As described before, abnormal cells or stressed cells up-regulate ligands for activating NK cell receptors and consequently the balance of incoming signals shifts towards activation.

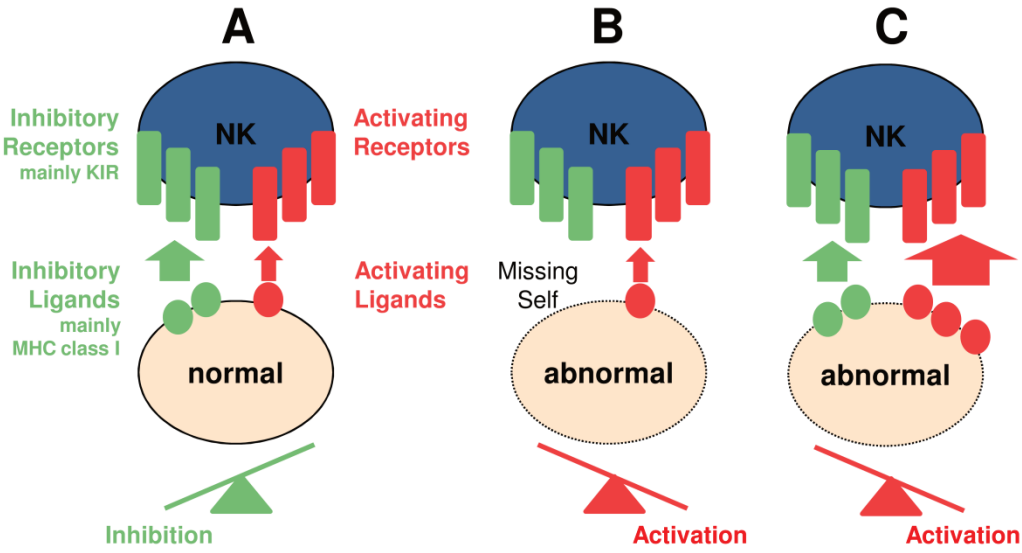


Figure 1.3 The integration of activating and inhibitory signals accounts for NK cell activation. During the interaction with normal cells, NK cell activation is prevented by dominating inhibitory signals (A), whereas abnormal cells trigger NK cell activation through missing inhibition (B) and/or intensive activating signals (C).

1.3.3 NK cell effector functions in cancer immunosurveillance

NK cell mediate cytotoxicity via the granule-exocytosis pathway

The direct elimination of target cells is a main function of NK cells and NK cells kill abnormal cells via different pathways.[102,103] After NK cells get activated by activating ligands or by antibodies that are recognized via CD16, NK cells mediate natural cytotoxicity or ADCC through the granule-exocytosis pathway. In the cytoplasm of NK cells several proteins such as granzymes and perforin are stored within cytotoxic granules.[104,105] NK cell activation triggers the release of these cytotoxic granules into the immunological synapse between target and NK cell and this degranulation process initiates the destruction of the encountered target.[106] Perforin disrupts the cell membrane and forms pores, allowing other cytotoxic substances such as granzymes to enter the cell. Furthermore, it cannot be excluded that perforin itself gets internalized and contributes to the cytotoxic effect by degradation of intracellular membranes and vesicles.[107] Granzymes are proteases that induce cell death in different ways.[108] Granzyme A cleaves molecules of the DNA repair system, while granzyme B induces apoptosis via the caspase cascade. The physiological significance of other granzymes (C, H, K and M) as well as their mode of action is unclear.

NK cells induce apoptosis via death receptor ligands

In addition to the release of cytotoxic granules and perforin-dependent killing of target cells, NK cells can express the TNF related apoptosis inducing ligand (TRAIL) and the FAS receptor

ligand (FasL), which bind to death receptors. Whereas DNA damage or energetic stress induces intrinsic, mitochondria-mediated apoptosis, death receptors cause cell death through an extrinsic apoptotic pathway after binding of a suitable ligand. TRAIL is normally not readily found on the surface of NK cells, but functional TRAIL can be induced on NK cells upon activation and enables efficient killing of tumor cells expressing receptors for TRAIL.[109] NK cells expressing FasL similarly eliminate Fas positive tumor cells.[110,111]

NK cell derived cytokines contribute to NK cell anti-tumor capacity

Besides other cytokines, such as IL-5, IL-10, IL-13 and the Granulocyte-macrophage colony-stimulating factor (GM-CSF), NK cells produce and release IFN-γ and TNF-α upon activation, which are two important factors for cancer immunosurveillance. NK cell derived IFN-γ carries several anti-tumor functions. IFN-γ inhibits the tumor angiogenesis and has anti-metastatic activity.[112,113] IFN-γ acts anti-proliferative and pro-apoptotic.[114] IFN-γ also triggers up-regulation of TRAIL on NK cells and thus enhances the sensitivity of TRAIL mediated target cell killing.[109] Furthermore, MHC class I up-regulation and enhanced antigen presentation is a common consequence of the treatment with IFN-γ and it consequently results in improved tumor clearance by the adaptive immunity.[115]

TNF-α is the soluble form of TNF and, same as its membrane-bound counterpart, it binds to the two receptors TNF-R1 and TNF-R2. However, soluble TNF-α preferentially binds to TNF-R2, whereas TNF has a higher affinity for TNF-R1, resulting in different signaling profiles for both factors.[116] Importantly, only TNF-R1 contains a death domain and directly mediates apoptosis,[117] while TNF-R2 signaling can also cause cell death by down-regulation of the anti-apoptotic factor Bcl-xL, as shown for T cells.[118]

Furthermore, IFN-γ and TNF-α complement one another in their anti-tumor capacity. Together, IFN-γ and TNF-α lead to growth arrest in various human cancers.[119] Both cytokines are required for efficient disruption of the tumor vasculature and clearance of established tumors.[120,121] Nevertheless, although IFN-γ and TNF-α together and by their own play an important role in cancer immunosurveillance, it should kept in mind that their pro-inflammatory character can be associated with chronic inflammation, potentially promoting immune escape mechanisms and tumor progression.[36,122]

Some NK cell anti-tumor functions arise from interactions with other immune cells

Importantly, NK cells not only combat cancer directly by themselves, but they also initiate anti-tumor activities arising from other parts of the immune system. The crosstalk of NK cells and T cells for instance has been shown to enable eradication of tumors that are resistant to NK cell

killing, because NK cell derived IFN-γ is crucial for the priming of tumor specific CD8 T cells that are then able to eliminate the cancer cells.[123] Similarly, it was observed that *in vivo* depletion of NK cells at the time of tumor challenge abolishes DC-mediated priming of T cells and abrogates the efficacy of vaccination immunotherapy with DCs.[124] In general, interaction between NK cells and DCs play a role in the activation and maturation of DC.[125] Furthermore, activated NK cells express MHC class II and can act as APCs.[126,127] Activated NK cells indeed trigger TCR induced function of CD4 T cells dependent on the expression of OX40 ligand and B7 by NK cells.[128] NK cells and T cells further interact directly with each other through 2B4 and CD48 on their surface, accelerating the proliferation of both cell subsets,[129] so that NK and T cell responses possibly amplify each other. In contrast, NK cells are able to directly dampen T cell responses, as they are able to lyse activated T cells.[130] Of note, CD48-CD2 interactions between B and NK cells carry a function in B cell antibody switch to IgG2a, representing the isotype that efficiently triggers NK cell-mediated ADCC.[131,132] CD48-2B4 interactions between B and NK cells trigger NK cells IL-13 production, representing a cytokine involved in the induction of Th2 immune responses.[133] The described examples demonstrate that cancer protection is based on a complex interplay between different immune cells and therefore NK cells should be seen as one part of the puzzle rather than separated effector cells.

1.4 NK cells in cancer therapy

Since they efficiently fight tumor cells, it seems obvious to utilize NK cells in cancer therapy and the progress of NK based therapy is frequently reviewed.[134–145] NK cells were applied during early therapies with lymphokine-activated killer (LAK) cells (chapter 1.4.1) and NK cells represent an essential factor for the outcome of stem cell transplantation (chapter 1.4.2). Furthermore, adoptive transfer of NK cells to cancer patients is a treatment option in early clinical evaluation with first promising results (chapter 1.4.3). However, NK cell therapy is also confronted with different challenges, still limiting its potential besides encouraging results in the past and reasonable strategies for the future (chapter 1.4.4).

1.4.1 Therapy with lymphokine-activated killer cells

The first use of NK cells in the clinics goes back to the infusion of so called LAK cells together with IL-2 into cancer patients starting in the 1980s.[146] The injection of *ex vivo* generated immune cells is originally defined as adoptive transfer.[147] LAK cells are derived from peripheral blood mononuclear cells (PBMCs) after *ex vivo* cultivation in IL-2 containing medium and consist of $CD3^-/CD56^+$ NK cells in addition to $CD3^+/CD56^+$ NKT-like cells and $CD3^+/CD56^-$ T cells. LAK therapy is mostly applied in an autologous setting, meaning that donor and recipient

of the cells is the same person. Transfer of LAK from foreign donors is critical, because incompatibility between donor T cells and recipient MHC molecules can result in lethal side effects, because alloreactive T cells from the donor attack the recipient tissue cells, which leads to the graft versus host disease (GvHD).[148] The cytotoxicity of NK cells is mainly responsible for the pronounced cytotoxicity of LAK cells against tumor cells *in vitro*.[149] Nevertheless, in first clinical trials the response did not exceed the efficacy of IL-2 monotherapy.[150] Since then, LAK therapy is considered inefficient, but later findings revealed possible reasons for the unfavorable clinical outcome. For instance, the administered high doses of IL-2 during early LAK therapies exhibit a toxic profile, it can cause activation induced cell death of NK cells *in vivo* and it drives the expansion of Tregs that inhibit the function of NK cells.[151,152] Another possible reasons for the failure of LAK therapy in the past is that the autologous NK cells exhibit a high level of self-tolerance due to a broad repertoire of inhibitory receptors that also depress the lysis of autologous leukemic cells.

Similar to LAK therapy, more recent approaches aim at the *ex vivo* expansion of NK cells from PBMCs using conditions favoring the specific outgrowth of NK cells such as cultivation of PBMCs together with anti-CD3 antibody in a cell culture medium known to support NK cell proliferation.[153] Surprisingly, although T cells are still the major cell type after three weeks of *ex vivo* cultivation under these conditions, adoptive transfer of the heterogeneous cell product to five cancer patients in an allogeneic setting did not cause side effects such as GvHD in a phase I safety study.[154] Indeed, experiments indicate that the T cell reactivity is lost *ex vivo*, when the cells are cultured longer than seven days using this protocol.[155] This simple approach for adoptive transfer of NK cells together with NKT-like cells and T cells represents a cost efficient concept among currently applied immunotherapies, but more clinical data are probably needed to definitely exclude safety risks associated with infusion of donor derived T cells in allogeneic settings.

1.4.2 The importance of NK cells for hematopoietic stem cell transplantation

Hematopoietic stem cell transplantation is an established cancer therapy

Clear importance of NK cells for therapy was first reported by Velardi and colleagues in 2002 in the context of allogeneic hematopoietic stem cell transplantation (HSCT) for the treatment of leukemia.[156] HSCT is used for long time and has become a standard therapy for the treatment of hematological malignancies.[157] First, it was thought that the treatment effect of HSCT was only mediated by the preceding irradiation or chemotherapy that is applied to eradicate the leukemia. But, the patient immune cells are eliminated as well and therefore donor derived allogeneic stem

cells were given to rebuild the missing immune system and counter this major side effect. Later, it became clear that the re-established donor derived alloreactive immune cells provide an important graft versus leukemia (GvL) effect that is essential to keep the patient in remission, meaning free of disease.[158] In addition, it was observed that allogeneic HSCT is useful for the treatment of solid tumors too, showing that the therapy provides a more general graft versus tumor (GvT) effect.[159] However, although HSCT fundamentally improved the treatment of leukemia, many of the treated patients still die, because re-growth of treatment-refractory cancer cells and reoccurrence of the disease.[160]

Alloreactive NK cells can play an important role for the outcome of HSCT

Impressively, Velardi and colleagues treated patients suffering from acute myeloid leukemia (AML) with hematopoietic stem cell grafts from allogeneic donors and revealed that a mismatch between donor and recipient KIRs, which determine the reactivity of NK cells, results in a significantly reduced relapse rates.[156] In detail, in a follow up of five years the relapse rate of 34 patients that were treated with a graft with KIR mismatch was 0% compared to 75% in the control group of 58 patients receiving a graft without KIR mismatch. The data were confirmed later in an enlarged patient cohort and Figure 1.4 shows the observed survival benefit for the patients.[161]

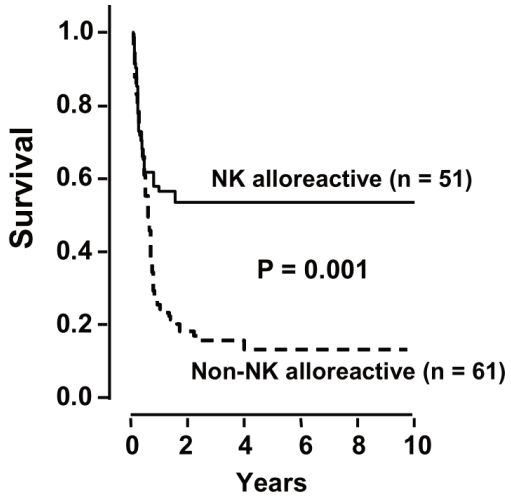


Figure 1.4 Alloreactive NK cells improve the survival of AML patients after allogeneic HSCT. AML patients received hematopoietic stem cell grafts from haploidentical donors and the survival is shown depending on the donor NK alloreactivity, estimated by KIR ligand mismatches. Adapted from Ruggeri et al. 2007.[161]

These data implied a functional relevance for the alloreactivity of transferred donor NK cells providing a strong GvL effect. In addition, whereas T cell alloreactivity is generally associated with rejection of the graft and a higher risk for GvHD, NK cell alloreactivity conversely correlated with improved engraftment and even protection from GvHD.[156] Velardi and colleagues showed in mouse models that NK cells not only kill leukemic cells, but they also lyse normal non-self hematopoietic cells, while other tissues are spared. The discrimination between hematopoietic and other non-self cells probably explains why NK cells don't mediate

GvHD, but the exact mechanisms remain an open question.[162] However, the NK cell alloreactivity during HSCT is thought to provide (i) eradication of the tumor by killing of leukemic cells, (ii) protection from graft rejection by killing of recipient T cells and (iii) protection from GvHD by killing of recipient T cells and DCs.[163] Therefore, analyzing the alloreactivity of donor NK cells prior to the treatment can improve the therapy of leukemia by a better donor selection. Furthermore, apart from the KIR repertoire of a given donor, the rate of NK cell reconstitution is directly linked to the clinical efficacy of HSCT. NK cells are the first lymphocytes that reappear after HSCT and faster reconstitution of NK cells correlates with a clearly reduced rate of relapse and improved survival of AML patients.[164–166]

Explanations for missing effects of alloreactive NK cells for HSCT in many studies

Some other groups confirmed the promising data of the Velardi group, but many subsequent studies did not reproduce the results associated with KIR mismatch in HSCT for the treatment of AML and these conflicting results may be explained by three critical factors, as reviewed recently by Wing Leung and described in the following.[143]

(1) Consideration of T cell alloreactivity is probably the most important factor for the outcome of HSCT, because HLA mismatch between donor and recipient can lead to severe GvHD mediated by the donor T cells. KIR ligands are HLA class I molecules and thus donor KIR mismatches often correspond to HLA mismatches with increased T cell alloreactivity. Therefore, KIR mismatch is even correlated with poor survival unless T cells are removed from the graft.[167,168] Importantly, the original data from the Velardi group and other studies with positive results were performed with T cell depleted grafts.

(2) Different models exist for the definition of ”KIR mismatch” plus another model defining a KIR haplotype. (a) The ligand-ligand model is defined as incompatibility between the donor KIR ligand and recipient KIR ligand repertoire. This model is based on the missing-self hypothesis arguing that it holds true for all KIR ligands that down-regulation or miss of a single KIR ligand results in activation of NK cells expressing the corresponding KIR. (b) The receptor-ligand model is based on incompatibility between the donor KIRs and recipient KIR ligands, taking into account that not all donors express all KIRs for every single recipient KIR ligand. Mismatches of KIR ligands in this model have no effect in case the corresponding donor KIR is absent, but a meaningful prediction requires donor KIR typing on the phenotype level. (c) The receptor-receptor model is defined as incompatibility between the donor KIRs and recipient KIRs. According to the KIR haplotype model, which is based on the receptor-receptor model, more activating donor KIRs correlate with a higher potential for alloreactivity. By

definition, the A KIR haplotype consist of only one activating KIR, whereas the B KIR haplotype comprises several activating KIRs.

(3) Besides the different definitions for KIR mismatch, the general comparison of different clinical studies is hindered by possible misclassification. Exact KIR ligand classification is not trivial, requires high resolution HLA typing and new ligand specificities for many KIRs are continuously discovered. In addition, the complex KIR allelic polymorphism causes misclassification. Similar to high resolution HLA typing, high resolution KIR typing is actually required to best possible select a suitable donor for HSCT.

In conclusion, the clinical relevance of NK cells for leukemia treatment using HSCT has been clearly shown. The alloreactivity of donor derived NK cells is essential for their therapeutic value, but factors such as T cell depletion and suitable determination of the NK cell alloreactivity are critical and have to be well-considered.

1.4.3 Adoptive NK cell therapy

The safety of adoptive NK cell therapy is shown by numerous pilot trials

Not least due to the relevance of reconstituted alloreactive NK cells during HSCT, adoptive transfer of NK cells for tumor therapy is tested in several early phase investigational studies as shown in Table 1.1. So far, numerous clinical studies started with the aim to investigate the feasibility and safety of NK cell adoptive transfer for the treatment of different types of leukemia and solid tumors. The vast majority of these studies utilize allogeneic NK cells, since donor derived alloreactive NK cells are expected to mediate a strong anti-tumor effect. First pioneering work showed that adoptively transferred allogeneic NK cells without T cells do not cause side effects such as GvHD.[169] Today, the safety of adoptive NK cell transfer is confirmed by numerous safety studies with heterogenic patient cohorts.[169–178]

Clinical efficacy of NK cell adoptive therapy is not yet clear due the early phase of clinical trials

Since the completed studies so far were designed with small patient numbers and without control groups, it’s not yet possible to adequately predict the therapeutic value of NK cell transfer. In addition, direct comparison of different studies is complicated because the applied treatment protocols are very different. The pre-conditioning varies among the studies and some of the clinical trials infused NK cells in combination with standard HSCT, whereas in other trials NK cell transfer was tested as independent therapy. The NK cells were purified in

different ways and several studies shortly pre-activated the NK cells with IL-2 before infusion or the NK cells were expanded *ex vivo* over long time with different expansion protocols.

Table 1.1 Clinical studies with adoptively infused NK cells in 2015. Adapted from Childs and Carlsten.[140]

Method	Patient population	Total number of clinical trials (number of active trials)	Comments
Non-expanded NK cells			
Autologous NK cells + IL-2	Melanoma, RCC, lung cancer and nasopharyngeal cancer	3 (1)	
Autologous NK cells + IL-15	Neuroblastoma, sarcoma, Wilms tumor and rhabdomyosarcoma	1 (1)	Intended to more specifically bolster NK cell anti-tumor activity than IL-2
Allogeneic NK cells + IL-2	AML, multiple myeloma, myelodysplastic syndromes, lymphoma, ovarian carcinoma, melanoma, neuroblastoma, Ewing sarcoma, breast cancer and Fallopian tube cancer	55 (29)	Most data published on adoptive NK cell therapy are from these studies
Allogeneic NK cells + IL-15	AML and myelodysplastic syndromes	2 (1)	Intended to more specifically bolster NK cell anti-tumor activity than IL-2
Expanded NK cells			
Autologous NK cells	CLL, RCC, lung cancer, multiple myeloma, sarcoma, colon cancer, melanoma, neuroblastoma, prostate cancer, ALL and pancreatic cancer	7 (6)	Various expansion methods used, including EBV-LCL and membrane-bound cytokine or 41BBL feeder cells; some studies use IL-2 post NK cell infusion
Allogeneic NK cells	AML, myelodysplastic syndromes, T cell lymphoma and multiple myeloma	11 (8)	Various expansion methods used, including EBV-LCL and membrane-bound cytokine or 41BBL feeder cells; some studies use IL-2 post NK cell infusion
Genetically modified NK cells			
CD19 CAR mRNA (expanded NK cells)	BCL	2 (2)	Designed to redirect tumor targeting. Haploidentical NK cells expanded with K562 membrane-bound IL-15 or 41BBL feeder cells; in Phase II clinical trials
NK cell lines			
NK-92	AML, multiple myeloma and lymphoma	2 (2)	Off-the-shelf NK cells; in dose-escalating Phase I clinical trials

41BBL, 41BB ligand; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BCL, B cell lymphoma; CAR, chimeric antigen receptor; CLL, chronic lymphocytic leukemia; LC, lung cancer; MDS, myelodysplastic syndromes; RCC, renal cell carcinoma. Data from ClinicalTrials.gov.

Adoptive transfer of autologous NK cells is reasonable but less effective

With the transfer of autologous NK cells one can definitely exclude the risk of donor related side effects arising from unintended co-transfer of alloreactive T cells, representing an advantage of this treatment concept. First patients were already treated with infusion of purified and *ex vivo* cultivated autologous NK cells more than 20 years ago.[179] However, autologous NK cells are less reactive, because inhibitory receptors generally dampen their response against autologous cells including tumor cells. In addition, NK cells from cancer patients often exhibit impaired responsibility due to weak expression of activating receptors. For instance, reduced expression of 2B4 by NK cells in multiple myeloma (MM) patients is considered a relevant factor in the immune escape of MM cells that express the 2B4 ligand CD48.[180] Importantly, autologous NK cells from MM patients up-regulate activating receptors including 2B4 after long-term *ex vivo* activation and show clear cytotoxicity against autologous MM cells.[181] Thus, the function of NK cells from cancer patients can be "rehabilitated" *ex vivo*, making them useful autologous effector cells for adoptive tumor therapy. On the other hand, the susceptibility of cancer cells to NK cell mediated killing can be enhanced for instance by bortezomib that triggers up-regulation of death receptors on tumor cells making them sensitive for TRAIL induced apoptosis.[182] Bortezomib treatment in combination with adoptive NK cell transfer is currently investigated in a Phase I clinical trial.[139] However, despite reasonable arguments for the use of autologous NK cells, their clinical value is limited at present. An example is given by a study from the Rosenberg group showing that adoptive transfer of autologous NK cells does not mediate tumor regression in patients suffering from metastatic melanoma and renal cell carcinoma (RCC), although high levels of circulating NK cells are found.[183]

Clinical benefit is achieved by adoptive transfer of allogeneic NK cells

In comparison to autologous NK cells, the transfer of allogeneic NK cells is more promising, because the mismatch of recipient MHC I molecules and donor NK cell inhibitory receptors increases the responsiveness of NK cells to tumor cells. Therefore, adoptive transfer of allogeneic NK cells in combination with HSCT is considered a beneficial strategy for cancer treatment, because the additionally transferred NK cells possess potent anti-tumor activity and may improve the stem cell engraftment and reduce the risk of infections and GvHD.[184] First published results for the combination of HSCT with adoptive NK cell transfer are indeed encouraging, but more advanced studies are required to identify the optimal dose and timing of the NK cell infusion.[170–176] Importantly, treatment of 27 patients with HSCT and subsequent transfer of allogeneic NK cells at relatively high median cell doses of $2x10^8$ cells/kg correlated with significant reduction in leukemia progression compared to 31 historical control patients treated under comparable conditions.[176] Of note, this high dose of NK cells was reached by *ex*

vivo expansion of the cells prior to the infusion. Thus, first data from patients show the safety of applying even high numbers of NK cells and these large NK cell doses are probably required to maximize the therapeutic effect. Furthermore, adoptive transfer can be advantageous for autologous HSCT. Autologous HSCT is often used when allogeneic HSCT is not applicable and adoptive transfer of donor derived allogeneic NK cells can be part of the pre-conditioning regimen to reduce the tumor mass before autologous HSCT.[185]

Unfortunately, HSCT itself is associated with transplant-related acute and late complications that cause disease relapse and reduce the chance for cure in case of AML.[186] Therefore, replacing HSCT by other suitable therapies is reasonable and applying chemotherapy combined with adoptive transfer of allogeneic NK cells is investigated by several studies for the treatment of patients with different types of cancer.[177,187–193] Data of clinical trials exploring this approach imply partial efficacy and one of the most promising results can be referred to a pilot study with 10 pediatric AML patients.[191] Remarkably, all children remained in remission and stayed free of disease in the follow up time of 2- 4.2 years. Based on this finding a comprehensive double-blinded study that involves multiple clinical centers was started and the results are expected soon.[194]

The use of NK cell lines as effector cells for adoptive immunotherapy represents a treatment with a special type of allogeneic NK cells that circumvent the need for a certain donor. Among the known NK cell lines the well characterized NK-92 is a considerable alternative for primary NK cells.[195] Nevertheless, NK cell lines have to be inactivated by irradiation prior to infusion into the patient to stop their uncontrolled cell proliferation that otherwise represents a major safety risk. The inactivation most likely goes along with an impairment of the therapeutic effect of NK-92 and is a major drawback compared to primary NK cells. Nevertheless, adoptive transfer of NK-92 to cancer patients with doses of up to $1x10^{10}$ cells/m^2 was proven to be safe.[196] So far, clinical studies with NK-92 indicate positive anti-tumor effects without off-target effects and very encouraging results were obtained in three patients with advanced chemotherapy-resistant lung cancer, which showed significant tumor responses including clearance of metastases in the lymph nodes and the lung.[197]

Ex vivo cultivation allows pre-activation of NK cells and administration of higher cell doses

Apart from the use of NK cell lines, adoptive NK cell therapy requires cells from a given donor, restricting the total NK cell dose to < $2x10^8$ primary NK cells that can be typically purified from one donor aphaeresis.[198] Thus, the achievable therapeutic effect is probably limited by a relatively low NK cell number that can be directly administered. To overcome this hurdle, cultivation and expansion of NK cell cells are utilized to maximize the NK cell dose prior to

infusion into the patient. Furthermore, the cultivation phase is helpful to pre-active the cells with cytokines and augment their anti-tumor functions before they are transferred to a patient. Of note, long-term activation of NK cells in cell culture medium containing high concentrations of IL-2 results only in a low proliferation and about five fold expansion of NK cells in two weeks, but it already yields NK cells with enhanced cytotoxicity.[171] In addition, the proliferation, cytotoxicity and migration of un-stimulated NK cells is strongly inhibited by the immunosuppressive drugs, such as mycophenolate mofetil that is often used during cellular therapy, but this effect is negligible for IL-2 stimulated NK cells.[199] Another good example for pre-activation of NK cells is the use of IL-12, IL-15 and IL-18. In combination these cytokines result in cytokine-induced memory-like NK cells with sustained high functionality.[61] Furthermore, these NK cells strongly up-regulate CD25, the α-chain of the high affinity IL-2 receptor, and become sensitive to very low levels of IL-2.[200] Therefore, a first clinical study was started with AML patients to test the safety of cytokine-induced memory-like NK cells together with low dose IL-2 therapy.[201] Thus, even without increasing the number of NK cells, short term cultivation using cytokines gives the opportunity to augment NK cells functionality.

In addition to cytokines, the co-culture of NK cells with certain feeder cell lines efficiently triggers NK cell expansion and provides higher NK cell doses for therapy (chapter 1.5.3). In an early report Escudier and colleagues utilized NK cells expanded with irradiated LAZ 388 cells in a combination therapy with IL-2 to treat patients with metastatic RCC.[179] Due to improved responses upon the applied treatment, they concluded that adoptive NK cell transfer might reduce the tumor burden of patients responsive to IL-2. Similarly, expansion of NK cells from PBMCs using the irradiated Wilms tumor cell line HFWT enabled up to three injections per patient with > 10^9 cells per injection in a pilot study showing the safety of this approach.[185] In recent years, engineered K562 feeder cells expressing 41BBL and membrane-bound IL-15 or IL-21 have proven its value as feeder cells for efficient expansion of NK cells and are currently assessed for clinical use.[202,203] In this context, acute GvHD surprisingly occurred in five of nine patients with solid tumors in a recent study evaluating the safety of adoptively transferred NK cells that were expanded with engineered K562 cells expressing membrane bound IL-15.[204] This was unexpected, because only a low dose of T cells (≤ $2x10^4$/kg) were co-transferred in the completely HLA-matched recipients and it raised some concerns about the general safety of adoptive NK cell therapy. However, the NK cells were also transferred at low doses (1-$10x10^5$/kg) following T cell depleted HSCT. The observed acute GvHD was associated with higher donor CD3 chimerism and more common with unrelated donor transplants, suggesting that alloreactive T cells were responsible for the acute GvHD. Nevertheless, it appeared that NK cells can at least indirectly contribute to acute GvHD and it's crucial to identify the underlying

mechansims.[205] Importantly, a different group utilized NK cells expanded with a similar K562 variant for the treatment of eight patients with MM and did not observe GvHD, although up to $1x10^8$ NK cells/kg were administered.[188] This event demonstrated that the exact treatment protocol including NK cell dose, type of NK cell activation, time point of NK cell injection and effects of other treatments and drugs that are applied in parallel still have to be carefully investigated in future trials to exclude potentially risks of NK cell transfer. Besides the use of engineered K562 feeder cells, NK cell expansion with clinical approved Epstein-Barr virus-transformed lymphoblastoid cell lines (EBV-LCLs) is established and yields large numbers of highly pure NK cells in clinical-grade quality.[206] Clinical-grade EBV-LCL-expanded autologous NK cells are currently tested for therapy of different cancers and because infusions of up to $2.5x10^8$ NK cells/kg were already well tolerated by 26 patients, further dose escalating studies with $1x10^9$ NK cells/kg are planned.[139]

1.4.4 Obstacles and perspectives for NK cell based cancer therapy

Tumor cells become unresponsive to NK cells as a result of immune escape

Although therapies with NK cells are promising, different issues challenge the success of these approaches. First of all, cancer can acquire resistance to NK cell mediated elimination due to immune escape mechanisms and NK cell immunoediting.[207] It was shown that cancer can lead to deficient expression of essential NK cell receptors, such as the NCR, NKG2D, DNAM-1 and 2B4, thereby suppressing the NK cell activation and function.[181,208–211] In addition, proteolytic cleavage of ligands for NK cell receptors from the tumor cell surface, known as ligand shedding, often circumvents recognition by NK cells as shown for the receptors NKp30 and NKG2D.[212–215] Besides proteolytic cleavage, tumor cells also inhibit NK cells by secreting ligands in exosomes as shown for the NKG2D ligands MICA and ULBP3.[216,217]. Of note, binding of NKG2D ligands in its soluble form to the corresponding receptors even blocks NK cell activation and leads to internalization of NKG2D.[212] Adoptive transfer of *ex vivo* activated and expanded NK cells expressing high levels of NKG2D is an suggested option to scavenge soluble NKG2D ligands from the patient serum and to at least transiently overcome the NKG2D-based NK cell resistance of the tumor cells.[218]

Antibodies can improve the NK cell anti cancer activity in different ways

The combination of NK cell therapy with therapeutic antibodies that trigger NK cell mediated ADCC could be another strategy to increase NK cells functionality and to overcome immune escape mechanisms. As an example, cetuximab, a therapeutic antibody targeting the epidermal growth factor receptor (EGFR), can restore the cytotoxic activity of soluble MICA-inhibited NK

cells as shown by *in vitro* experiments with tumor-like spheroids from primary cells of head and neck squamous cell carcinomas.[219] NK cells anti-tumor activity via ADCC is generally an attractive aspect for cancer immunotherapy.[88] Thereby, modifying the Fc-parts of used therapeutic antibodies can enhance the affinity for CD16-mediated binding by NK cells and it can boost the ability of NK cells for serial killing, meaning to lyse many targets one after another.[220–222] Further advancement is reached by antibody structures with multiple specificities. So called bi-specific or tri-specific killer cell engagers (BiKes or TriKes) are recombinant produced proteins, consisting of variable single chain fragments directed against one or two tumor antigens and CD16, which allow direct activation of NK cells through CD16 signaling upon binding and induce specific killing of cells bearing the targeted tumor antigens.[223] BiKes and TriKes against CD19 and CD22 clearly enhance the NK cell function against primary AML and chronic lymphocytic leukemia (CLL) cells, while a BiKe against CD133 successfully augments NK cell activity against colorectal cancer cells.[224,225] A BiKe against CD33, designed to target AML and myelodysplastic syndrome (MDS), allows potent killing and cytokine production by NK cells and overcomes the inhibition of NK cells by KIR.[226,227] Of note, antibodies against inhibitory KIR are applied to bypass the NK cell inhibition by MHC I molecules and strengthen NK cell responses against tumor cells. The antibody IPH2101 (lirilumab) is clinically tested alone or in combination with other treatments for several indications.[228–233] In conclusion, NK cell therapy and therapeutic antibodies can benefit from each other and open new treatment opportunities.

The use of IL-2 administration to support NK cell function after adoptive transfer is controversial

It's known for long time that injection of low dose IL-2 expands human NK cells *in vivo*.[234–236] Consequently, adoptive NK cell transfer is often combined with low dose IL-2 therapy with the aim to maintain an enhanced NK cell function *in vivo* and improve the therapeutic effect of the NK cells. Unfortunately, this IL-2 administration induces significant expansion of Tregs, which express CD25, the α-chain of the high affinity IL-2 receptor.[190,193,237] Tregs possibly impair the NK cell function via TGF-ß and reduce the availability of IL-2.[151,238] Specific elimination of Tregs could solve this issue and can be achieved by the IL-2-diphteria fusion protein that binds CD25 with high affinity.[239] In a clinical study with 57 AML patients investigating adoptive NK cell transfer, the IL-2-diphteria fusion protein resulted in significantly improved rates for complete remission and disease-free survival compared to the control group.[240] However, even with the depletion of Tregs only in 27% of the treated patients expansion of the donor NK cells was detectable 14 days after NK cell infusion. Thus, an alternative is needed to maintain the NK

cell function *in vivo*. IL-15 could be this alternative, because IL-15 does not induce Treg proliferation, while it expands NK cells in a similar way as IL-2 does.[241]

The function of NK cells can be improved by genetic engineering

With genetic modification of NK cells it's possible to arm NK cells with improved therapeutic features.[140,242] To achieve enhanced *in vivo* persistence, NK cells can be modified to produce IL-2 or IL-15 themselves, avoiding the need for exogenous cytokines as shown with NK cell lines.[243–245] With targeting the expression of IL-2 to the endoplasmic reticulum, secretion of the cytokine can be avoided and possible side effects on other cells are eliminated.[246] First preclinical evaluation of primary NK cells transduced with membrane-bound IL-15 yields positive results and supports clinical testing of this approach.[247]

Genetically engineered effector cells expressing chimeric antigen receptors (CARs) are among the most promising developments in immunotherapy in recent years. Although current approaches are typically realized with T cells, the interest in CARs with NK cells is emerging.[248–250] CARs are constructs consisting of an antibody-binding domain fused to a cellular signaling domain. After binding of the antibody domain to the corresponding antigen, the effector cell gets activated and responds to the encountered target. Therapy with CARs turned out to be extremely effective. For instance, the treatment of 30 pediatric acute lymphoblastic leukemia (ALL) patients with T cells expressing a CAR against CD19, resulted in complete remission in 90% of the children.[251] Therefore, the therapy was granted 'breakthrough therapy' by the United States Food and Drug Administration.[252] Consequently, many investigators develop CAR constructs for NK cells and preclinical evaluation of NK CARs cells is ongoing. At present, most of the reported work on NK CARs is done with the NK-92 cell line instead of primary NK cells (Table 1.2).

Table 1.2 Publications on CARs for use in NK cell cancer therapy

Used NK cells	**Targeted antigens of NK CARs with number of publications**									
	CD 19	CD 20	CD 138	CS1	EGFR	ErbB2	EpCAM	GD2	NKG2D ligands	**Total**
NK-92	**2**[253,254]	**4**[253–256]	**1**[257]	**1**[258]	**1**[259]	**4**[260–263]	**1**[264]	**1**[265]		**15**
Primary NK cells	**3**[266–268]				**1**[259]	**1**[269]		**1**[267]	**1**[270]	**7**

There are several reasons why NK-92 is preferably used for NK CAR approaches as described by Klingemann:[249] First, in contrast to primary NK cells, NK-92 doesn't require a donor apheresis and laborious cell purification steps prior to transduction. Second, NK-92 can be transduced relatively easy, while transduction efficacies for primary NK cells are low. Third, NK-92 is continuously growing, allowing unlimited cell doses in theory, whereas the number of primary NK cells is limited. The last point is an issue for adoptive NK cell therapy in general, but it's even more critical in the context of genetic engineering of NK cells, because due to the low transduction efficacy the number of obtained NK effector cells is even lower in the end. Therefore, methods to obtain more NK cells for adoptive NK cell therapy are urgently needed and efficient methods for *ex vivo* NK cell expansion are of great interest.

1.5 *Ex vivo* NK cell expansion

Ex vivo expansion of NK cells is an important strategy to produce NK cells for adoptive cell therapy and several review articles examine the progress in this discipline during the last years.[136,138,139,271–274] This section covers important factors for NK cell expansion and gives an overview about NK cell expansion protocols with focus on applications that reached clinical use.

1.5.1 The starting material and the importance of NK cell purity

NK cells from different sources are used for *ex vivo* expansion

First of all, NK cells for *ex vivo* expansion can be received from different sources. In 2015, most recruiting clinical trials utilized peripheral blood derived allogeneic (79%) or autologous (13%) NK cells, followed by other sources (8%) including the NK-92 cell line and umbilical cord blood.[275] This project focused on NK cells from peripheral blood as the commonly used starting material. However, it should be mentioned that differentiation and expansion of NK cells from cord blood CD34 cells represents an upcoming option to obtain NK cells with possible advantages over conventionally used peripheral blood, as pointed out by Anasetti et al and described in the following.[276] When a suitable cord blood unit for a distinct recipient is identified, it's rapidly available "off-the-shelf" from a cord blood bank, whereas obtaining cells from a peripheral blood donor is usually more time consuming. In general, it's less challenging to find an adequate cord blood donor, because the HLA matching of donor and recipient can be less stringent without increasing the risk for GvHD. The risk of infection transmission is minimal and a risk for the donor does not exist. Nevertheless, it has to be mentioned that the differentiation and expansion of NK cells from a rather limited starting cell number using cord blood takes about six weeks,[277] and the length of the process could hamper the cost efficient

translation to broad clinical use. Another promising future concept, which is still in early development, is the generation of NK cells for therapy from pluripotent stem cells.[278]

High NK cell purity of the NK cell graft is optimal for clinical requirements

The NK cell frequency in the starting material represents an important factor. Many protocols start with a small fraction of NK cells within a mixture of cells such as PBMCs and the NK cells grow out over time. This strategy is simple and practical for later use of the NK cells in autologous settings, but the approach appears critical for allogeneic applications since remaining non-NK cells in the final cellular product can trigger unwanted side effects. Alloreactive T cells cause GvHD and represent a severe risk factor. Same as T cells, alloreactive B cells should not be infused, because they can lead to B cell lymphoproliferative disorder upon Epstein-Barr virus (EBV) reactivation,[177,279] and they can result in the passenger lymphocyte syndrome.[280] Both are critical side effects for the patient. In general, a pure NK cell product is essential to clearly trace back positive and negative treatment results to NK cells and not to other cell subsets to accurately evaluate clinical efficacy and possible risks of NK cells for immunotherapy.[281] Therefore, NK cell purification is reasonable before *ex vivo* expansion or at least before adoptive transfer of the final cell product. Depletion of CD3 T cells by magnetic cell separation (MACS) is applied since more than 20 years.[282] During MACS, a conjugate consisting of a specific antibody and a magnetic particle binds specifically to a desired target, such as CD3 on T cells, allowing to retain and separate the target by a magnetic field.[283] After CD3 depletion, subsequent magnetic enrichment of CD56 cells can be performed to achieve highly purified NK cells. Automated NK cell purification in clinical scale is realized by CD3 depletion and CD56 enrichment using the CliniMACS system.[170,198,284–286] Good manufacturing practice (GMP)-compliant cell sorting represents an attractive option to start the clinical expansion of NK cells directly with a highly pure NK cell subpopulation of interest. A first proof of concept is shown by fluorescence-activated cell sorting (FACS) under GMP conditions to sort single KIR^+ NK cells, that are more cytotoxic against AML blasts than bulk NK cells.[287] Nevertheless, all antibodies for the intended sorting strategy are required in clinical-grade, hampering the general translation of this method to broad clinical use.

1.5.2 Cytokines for *ex vivo* NK cell expansion

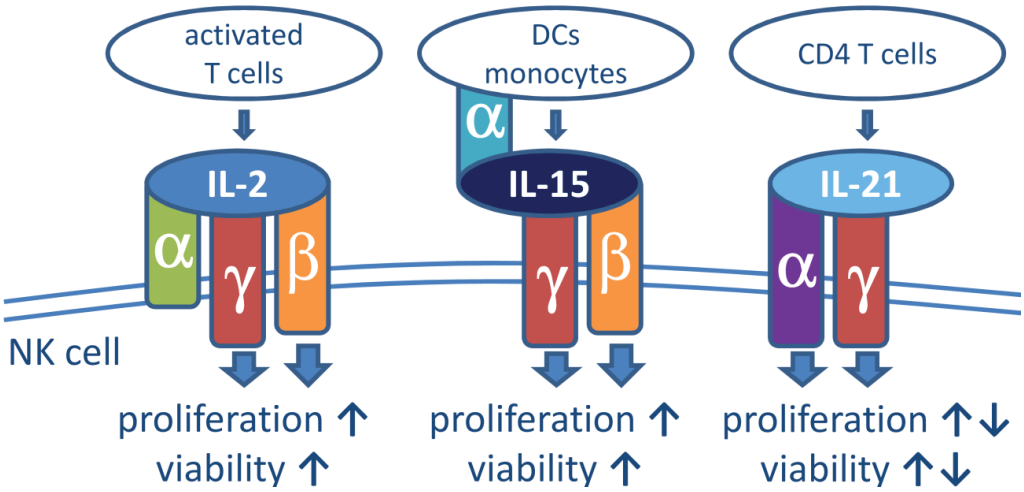


Figure 1.5 Structure of the receptors for IL-2, IL-15 and IL-21

IL-2 or IL-15 is essential for *ex vivo* culturing of NK cells

Activation and a basic expansion of NK cells are achieved by NK cell stimulating cytokines (Figure 1.5). IL-2 and IL-15 belong to the most essential components of NK cell expansion protocols, since it's known for long time that IL-2 and IL-15 are crucial for proliferation and survival of murine and human NK cells.[288–294] IL-2 and IL-15 belong to the family of cytokines that signal through the common γ-chain.[295] IL-2 is mainly secreted by activated T cells, while IL-15 is primarily produced by DCs and monocytes. IL-2 and IL-15 share the same receptor γ- and ß-chains, CD132 and CD122, which form the ßγ-heterodimer that is the primary subunit for the signal transduction. The common γ-chain is functional, but it binds cytokines only with extremely low affinity. The ßγ-heterodimer has a intermediate affinity, but it still requires nanomolar cytokine concentrations for activation, while the heterotrimer consisting of ßγ and additional α-chain strongly increases the affinity and allows signaling at picomolar cytokine concentrations.[296,297] Importantly, the α-chains of the IL-2 and IL-15 receptors alone don't mediate signal transduction. The expression of the IL-2 receptor α-chain (CD25) can be induced on NK cells after stimulation, e.g. with the combined cytokines IL-12, IL-15 and IL-18.[62,200] The receptor α-chain for IL-15 (CD215) is predominantly expressed on the surface of DCs and monocytes, it binds IL-15 with high affinity and IL-15 bound to CD215 can be trans-presented to NK cells, thereby enabling signaling through the αßγ-heterotrimer.[298] Recombinant IL-15 is currently investigated in clinical trials and recombinant IL-2 is utilized extensively since decades, so that both components are commonly available in clinical-grade.[201] The use of well defined and clinical-grade components is an important aspect for *ex vivo* NK cell cultivation to best possible meet regulatory requirements for clinical applications.

IL-21 is associated with NK cell proliferation, but its exact role is unclear

Besides IL-2 and IL-15, the cytokine IL-21 may play an important role for NK cell expansion, since feeder cells genetically engineered to express membrane-bound IL-21 were found to provide a long lasting *ex vivo* proliferation of NK cells.[203] However, for soluble IL-21 different or even contrary effects are published. As another IL-2 family cytokine, IL-21 signals though a heterodimer consisting of the common γ-chain and the IL-21 receptor α-chain. IL-21 is mainly produced by CD4 T cells and acts on various cell types including NK cells.[299] IL-21 has been originally discovered as cytokine that plays a role in the development of NK cells from bone marrow progenitors.[300] IL-21 can trigger apoptosis and shortens the lifespan of human and murine NK cells *in vitro*.[301,302] In mice, IL-21 acts inhibitory on the expansion of NK cells but induces functional NK cell maturation.[302,303] Although Wendt et al. published that human IL-21 appears to increase the proliferation of human CD56^{bright} NK cells[304], others did not observe an impact of IL-21 on the proliferation of NK cells from healthy human donors or HIV patients.[305] Taken together, IL-21 differentially affects the expansion of NK cells dependent on the experimental setup and this cytokine should be further investigated for its potential to enhance *ex vivo* NK cell expansion.

1.5.3 The role of accessory cells and feeder cells for NK cell expansion

Cytokines alone are not sufficient for stimulation of cell growth. Culturing purified NK cells in IL-2 containing medium for 2-4 weeks for instance results only in a minor 5-20 fold NK cell expansion.[171,273] Consequently, other stimuli are needed to enhance the proliferation and protocols for NK cell expansion often utilize effects of non-NK accessory cells or add irradiated autologous “feeder cells” to the culture (Table 1.3). The most efficient NK cell expansion however is achieved by culturing NK cells together with irradiated allogeneic feeder cells (Table 1.4).

Autologous accessory cells trigger NK cell expansion

Expansion of NK cells from the whole PBMC fraction is more effective than starting from purified NK cells, because non-NK cells within the cell mixture provide positive factors for the NK cell proliferation. CD14 cells within PBMCs are known for example to enhance the NK cell proliferation by soluble factors and via direct cell-to-cell contact with NK cells.[306] In this context, immature and mature DCs appear to be the main drivers of the NK cell stimulation, whereas monocytes have little or no effect.[307] DC derived exosomes were shown to induce NK cell expansion through NKG2D ligands and IL-15Rα.[308] T cells induce NK cell proliferation after they have been activated, for instance by concanavalin A.[309] Adding anti-CD3 antibody to

PBMCs lead to a profound NK cell proliferation,[153,154,181,310–313] probably as a result of activated T cells, but the exact mechanism is not reported. Clinical-grade NK cells can be grown out from PBMCs by use of OKT-3, a clinical approved anti-CD3 antibody.[153,154,181,311,312] This approach is suitable to expand autologous NK cells from different cancer patients as shown for B cell CLL and multiple myeloma (MM).[181,311] However, starting the *ex vivo* culture with PBMCs goes along with co-expansion of T cells and NKT-like cells that account for the majority of cells in the final product. This contamination with non-NK cells is problematic and undesirable, especially for the use in allogeneic settings, as described in chapter 1.5.1.

Irradiated autologous feeder cells can yield expanded NK cells with high purity

To make use of autologous non-NK cells for NK cell expansion but to avoid the outgrowth of these cells during culture, the non-NK cell fraction is often separated, then inactivated by irradiation and afterwards added again to NK cells as stimulating autologous feeder cells. Irradiation of bystander cells is not only helpful to stop them from growing, but it can also provoke up-regulation of surface ligands that activate NK cells, such as the NKG2D ligands ULBP1-3 for instance.[314] Nevertheless, co-culture of purified NK cells with irradiated autologous monocytes, B cells and T cells is a simple and GMP compliant method, but it's not very efficient without activation of these bystander cells (e.g. 16-fold NK cell expansion in two weeks).[315] In contrast, Sakamoto et al. showed recently that activated and afterwards irradiated autologous PBMCs as feeder cells allow for a median 4720-fold NK cell expansion after three weeks of cultivation with a final NK cell purity of 91% starting with PBMCs from digestive cancer patients.[310] Apart from OKT-3, Sakamoto and colleagues activated the autologous feeder cell fraction with two other clinical-grade components, OK-432 and FN-CH296, additionally explaining the good expansion performance.[310] Starting with already enriched NK cells using CD3-depleted PBMCs and cultivation for two weeks with OKT-3 and irradiated autologous PBMCs can yield a more pure cell population of 98% NK cells and ensures low numbers of unwanted T cells.[316] To maximize the purity even further, GMP-compliant cell sorting prior to cultivation is a smart strategy to expand distinct NK cell subpopulations. Siegler and colleagues demonstrated that sorted single KIR^+ NK cells without other contaminating cell populations can be expanded 160-390 fold in 19 days by OKT-3 and irradiated autologous PBMCs.[287]

Table 1.3 Clinical strategies for *ex vivo* NK cell expansion without allogeneic feeder cells

Stimulation	Year; Author	Starting Material	Cult. Syst.	Fold NK Expansion	Purity of final product
Without feeder cells					
IL-2	2004; Koehl[171]	CD3-depl CD56-enr PBMCs	?	5 (2-4 weeks)	99% NK <0.1%.
Autologous accessory cells					
IL-2; OKT-3	2010; Sultu[312]	PBMCs	bio-reactor; bags; flasks	77 bioreactor 530 bags 770 flasks (20 days)	38% bioreactor 31% bags 44% flasks
	2009; Barkholt[154]	PBMCs	?	1036 total cells (19 days)	~30% NK ~40% T cells
	2008; Alici[181]	Patient PBMC	flasks	1625 (20 days)	~65% NK ~22% T cells
	2001; Carlens[153]	PBMCs	plates	193 (21 days)	~55% NK ~22% T cells
IL-2; IL-21	2014; Choi[176]	CD3-depl PBMCs	?	3.7 $CD56^{+}CD122^{+}$ (13-20 days)	>90% $CD56^{+}CD122^{+}$ <3% NKT-like cells <0.3% T cells
Autologous feeder cells					
IL-2; IL-15; irr autologous PBMC	2015; Torelli[315]	CD3-depl CD56-enr PBMCs	flasks	16 (14 days)	97% NK 0.2% T
IL-2; OK432; FN-CH296 + OKT-3 activated irr autologous PBMC	2015; Sakamoto[310]	PBMCs	flasks and bags	4720 (21-22 days)	90.96% ~4% T cells
IL-2; OKT-3 irr autologous PBMC	2011; Parkhurst[183]	CD3-depl patient PBMCs	flasks and bags	278-1097 (21-26 days)	91-98% NK
	2013; Lim[316]	CD3-depl PBMC	bags	691 (14 days)	98.1% NK 0.06% T cells
	2013; Ahn[314]	CD3-depl CD56-enr PBMCs	plates and flasks	546 (14 days)	94.9% NK 2.2% T cells
IL-2 +/− IL-15; OKT-3; irr autologous PBMC	2010; Siegler[287]	CD3-depl 56-enr PBMCs	bags; plates	117/63 bags (+/−IL15) 993 plates (19 days)	bags 30%;NK 45% NK (+IL-15) 0.6% T cells
		+ GMP KIR sorted	bags	160–390 (+IL-15)	~100% NK > 0.01% T cells

depl, depleted; cult. syst., culture system; enr, enriched; irr, irradiated;

Allogeneic feeder cells are the most efficient stimuli for *ex vivo* NK cell expansion

Since NK cells are generally more responsive to allogeneic cells, it's not surprising that allogeneic PBMCs turned out to stimulate NK cell expansion better compared to autologous PBMCs. In a study directly comparing the outgrowth of NK cells from the PBMCs of patients with advanced lymphomas or terminal solid tumors, NK cells expanded 169-fold when irradiated PBMCs from the patient were added as additional feeder cells, whereas 300-fold expansion was achieved with irradiated PBMCs from healthy donors.[317] In addition, others already showed in the past that competent NK cell expansion is achieved when starting with purified NK cells and irradiated allogeneic PBMCs as feeder cells.[318]

Since allogeneic PBMCs have to be available from a donor, it's easier to use established cell lines as feeder cells for NK cell expansion. In fact, several cell lines are reported to induce NK cell expansion including HFWT, K562, RPMI 1866, Daudi, KL-1, MM-170 and EBV-LCL.[319–323] However, at least so far only a few cell lines are applied clinically for NK cell expansion. Presumably, this is because cell lines possess an unlimited capacity for proliferation, they are often tumorigenic and therefore represent a potential safety risk for the patient. Consequently, only cell lines with a proven safety profile are acceptable and it has to be ensured that the feeder cells are efficiently inactivated by irradiation when they are used to expand NK cells for adoptive NK cell therapy.

The Wilms tumor cell line HFWT selectively induces NK cell expansion from PBMCs and cord blood mononuclear cells,[324,325] while high NK cell numbers in co-culture with irradiated HFWT not only arise from mature $CD3^{-}CD56^{+}$ NK cells but also from $CD3^{-}CD14^{-}CD19^{-}CD56^{-}$ NK cell precursors expressing CD122.[326] Furthermore, after transfer of cord blood mononuclear cells together with HFWT cells into immunocompromised mice, the numbers of $CD56^{dim}CD16^{+}$ as well as $CD56^{-}CD16^{+}$ immature NK cells significantly increased *in vivo*. So far, NK cells expanded *ex vivo* with irradiated HFWT feeder cells were adoptively transferred to patients with recurrent malignant glioma, showing that the therapy is safe and partially effective.[185]

In recent years, expansion of NK cells from genetically engineered K562 cells has been shown to be highly effective and first encouraging results were obtained with K562 modified to express membrane-bound IL-15 and the ligand for 41BB (K562-mb15-41BBL). While unmodified K562 trigger NK cell proliferation from PBMCs only to some degree, allowing 2.5-fold NK cell expansion after seven days, K562-mb15-41BBL feeder cells yield around 20-and 1000-fold NK cell expansion after one or three weeks of culture.[266] Furthermore, stimulation of NK cells using K562-mb15-41BBL revealed for the first time that NK cells can undergo up to 30 population doublings *ex vivo*, allowing median 5.9 x 10^{4}-fold NK cell expansion in the long

run, and indicating the high proliferative potential of NK cells.[327] Compared to un-stimulated NK cells, NK cells expanded with K562-mb15-41BBL are more cytotoxic against several allogeneic and autologous tumor entities and efficiently mediate ADCC.[328] In addition, human NK cells expanded with K562-mb15-41BBL proved their therapeutic efficacy for the treatment of sarcoma and myeloma in mouse xenograft models. [329,330]

Denman and colleagues tested the expansion of NK cells with IL-15 or IL-21 as membrane-bound cytokines on modified K562 cells that were originally developed to expand antigen-specific T cells and therefore also expressed CD64, CD86, 41BBL and truncated CD19.[203] Surprisingly, after three weeks of cultivation with membrane-bound IL-21 an extensive increase in NK cells of around 48 x 10^3-fold was achieved, while membrane-bound IL-15 gave around 0.8 x $10x^3$-fold NK cell expansion, as seen with other IL-15 expressing K562 variants before. Moreover, weekly re-stimulation of NK cells with the K562 transfectants allowed a sustained level of NK cell expansion with K562 expressing membrane-bound IL-21, while the level of NK cell expansion declined over time with K562 expressing membrane-bound IL-15. Another group confirmed that K562 expressing membrane-bound IL-21 and 41BBL support sustained proliferation of NK cells and their experiments further suggested that activation of the STAT-3 signaling pathway is involved in the effect of membrane-bound IL-21 on NK cell expansion.[331] Using patient derived PBMCs or starting with PBMCs from healthy persons for NK cell expansion with K562 expressing membrane-bound IL-21 yields comparable high NK cell numbers and, most importantly, adoptive transfer of these expanded NK cells into mice bearing human neuroblastoma improved the survival of the animals, proofing the therapeutic efficacy of these NK cells.[332]

B cell lines derived by EBV transformation, which were already introduced as EBV-LCL, represent one of the first cell lines that were reported to stimulate NK cell expansion 30 years ago.[309,333,334] The EBV-LCL cell line LAZ 388 was already applied to expand NK cells for clinical use in 1994, but back then the efficacy of NK cell expansion was still relatively limited, only allowing around 43-fold NK cell expansion after 13-31 days of cultivation.[179] After the TM-LCL cell line was originally established to expand CD8 T cells,[335] this line was extensively characterized and qualified for use in clinical trials.[336] Later, the TM-LCL was applied to expand NK cells with clinical-grade quality. Co-culturing of purified NK cells with TM-LCL enables around 500-fold NK cell expansion after 2-3 weeks and results in a highly pure NK cell population for clinical applications.[206] The clinical grade SMI-LCL cell line was generated similar to TM-LCL and it was applied more recently to expand clinical-grade NK cells from cancer patients for adoptive NK cell therapy in an autologous setting.[139] Until 2013, 78 NK cell products for infusion were successfully generated with the SMI-LCL cell line that allows 3637-

fold NK cell expansion after 24-27 days of culture. Thus, co-culture of NK cells with the SMI-LCL line proved its ability to produce clinical-grade NK cells for adoptive immunotherapy in an efficient and reliable way and holds great promise for future NK cell applications.

Table 1.4 Clinical strategies for *ex vivo* NK cell expansion using allogeneic feeder cells

Stimulation	Year; Author	Starting Material	Cult. Syst.	Fold NK Expansion	Purity of product
Allogeneic feeder cells					
IL-2; IL-15; PHA, Iono irr ConA activated allogeneic PBMCs	2002; Luhm[318]	CD3-depl, CD4-depl, CD19-depl CD33-depl PBMCs	bags	80-200 (15 days)	Day 12 91% CD56 0.3% CD3
IL-2; OKT-3; irr autologous or allogeneic PBMCs	2013; Kim[317]	Patient PBMCs	plates	169/300 for auto/allo feeder; (14 days)	84%/94% NK for auto/allo feeder
IL-2; irr HFWT cells	2004; Ishikawa[185]	PBMCs	flasks	113 (2 weeks)	86% $CD56^+/CD16^+$
IL-2; irr K562 expressing membrane-bound IL-15 and 41BBL	2005; Imai[266]	PBMCs	plates	1089 (3 weeks)	"virtually pure"
	2009; Fujisaki[202]	PBMCs	bags	23, 152, 277 after 7, 14, 21 days	Day 21 96.8% NK 3.1% T cells
	2012; Lapteva[337]	PBMCs	G-Rex vessels; bags	442 G-Rex 227 bags (10 days)	70% NK 5-35% T cells
IL-15; irr K562 expressing membrane-bound IL-15 and 41BBL	2011; Zhang[338] 2014; Shah[204]	Untouched isolated NK (research kit);[338] CD3-depl/CD56-enr PBMCs [204]	?	~1000 (21 days)[338]	9-11 days[204] >90% NK ≤0.2% T cells
IL-2; irr K562 expressing membrane-bound IL-21 and 41BBL	2012; Denman[203]	PBMCs	flasks	$4{,}8 \times 10^4$ (21 days)	21.7% T cells
	2013; Liu[332]	Patient PBMCs	flasks	2363 (14 days)	83% NK 9.1% T cells
IL-2; PHA-P; irr allogeneic PBMCs; irr LAZ 388 cells	1994; Escudier[179]	PBMCs CD3-depl w/o monocytes	bags and plates	~43 (13-21 days)	90% NK < 5% T cells
IL-2; irr. EBV-LCL SMI-LCL[206] TM-LCL[139]	2009, Berg[206] 2013, Childs[139]	CD3-depl/CD56-enr PBMCs from patients or healthy persons	bags	TM-LCL 250-850 (2-3 weeks) SMI-LCL 198, 895, 3637 after 14-16,19-22, 24-27 days	98% NK (TM-LCL) 99.7% NK (SMI-LCL)

41BBL, 41BB ligand; conA, concanavalin A; cult. syst., culture system; depl, depleted; Iono, ionomycin, irr, irradiated; PHA, phytohemagglutinin

1.5.4 Challenges for NK cell expansion due to clinical requirements

Regulatory aspects can hamper NK cells on their way “from bench to bedside”

Since several preclinical studies revealed the efficacy of NK cell therapy, clinicians are interested in the translation of these findings into clinical applications and several investigational trials were already initiated, as described before. Nevertheless, the transfer of basic research results to clinical use is often hindered, because previously applied methods are not compliant with clinical requirements including up-scaling issue and the need for processes that are compliant with GMP. GMP is the umbrella term for official guidelines that cover regulatory aspects for the manufacturing and quality control of products with intended therapeutic use. Thereby, GMP assures an adequate high level of safety, quality and efficacy of these products. Importantly, GMP is not only critical for industrial manufacturers with focus on cellular products in the late phases of clinical evaluation, but also essential for academic investigators planning early phase clinical trials.[339] Furthermore, GMP guidelines differ between different regions of the world. The clinical use of expanded NK cells is complicated, because *ex vivo* processing of cells generally involves methods and components for cell isolation and cultivation that harbor the potential risk to be critical for GMP demands.

Standardized expansion of NK cells is challenging

Consistent clinical NK cell expansion depends on qualified and preferably well defined cell culture ingredients to be GMP-compliant. Whereas cytokines such as IL-2 and IL-15 fulfill these requirements, cellular components of expansion protocols such as feeder cells cannot be clearly defined and possibly vary in quality from batch to batch when they are maintained in culture over longer time. Using one large batch of previously produced and then cryopreserved and qualified feeder cells could circumvent this issue to some extent and the feasibility of this approach was demonstrated for K562 feeder cells expressing membrane-bound IL-15 and 41BBL.[340] Furthermore, for TM-LCL feeder cells it was shown using global gene expression profiling that the cells do not change in culture for at least three month, ensuring that TM-LCL cells at different culture periods yield expanded NK cells with a constant quality.[341] Still, feeder cells embody a factor for *ex vivo* NK cell expansion that is difficult to control. Thus, better understanding of the mechanisms for NK cell expansion is important to overcome the need for feeder cells in the future.

Automation of the cell processing is essential for the success of cellular therapy

Cellular therapies are generally associated with relatively high costs, due to the complex procedures that are applied and the often required expensive reagents. Sipuleucel-T, a cellular

therapy for prostate cancer based on *ex vivo* stimulation of DCs, successfully reached clinical use, but it's extremely expensive in relation to its relatively limited clinical efficacy, demonstrating the challenge for cellular therapies to compete with cheaper and more easily administered therapies.[342] Automation of the production process is cost-saving, it best possible assures constant product quality without the need for highly skilled experts, and it's therefore required to make cellular therapy more widely available beyond specialized academic centers.[343] For clinical purification of NK cells, NK cell enrichment from PBMCs is commonly applied by automated CD3 depletion and subsequent automated CD56 enrichment, using MACS with clinical-grade separation reagents.[136,274] Thus, a suitable solution for automated and GMP-compliant NK cell purification is established.

While GMP-conformity is not an issue for NK cell purification anymore, the cultivation of NK cells remains challenging. In some clinical trials, NK cells are still cultured in small scale tissue culture flaks (T flasks) by hand, so that it's required to handle for instance 51 T flasks for a single treated patient.[183] This high number of T flasks represents a workload that is hard to manage and it potentiates the risk of generating an unsterile product, because it's required to open the vessel from time to time to exchange culture medium. Compared to T flasks, cell culture bags allow to handle a larger culture volume at once over long time without opening the bag, but the expansion performance is reduced.[287,312] First investigators automated NK cell culture processes using a bioreactor system.[312,344,345] But, this bioreactor system still needs an initial manual cultivation phase, because relatively high culture volumes are needed to start the automated culture and this automated cultivation goes along with declined NK cell yields compared to manual cultivation in small scale. Consequently, better solutions have to be found for GMP-compliant NK cell cultivation to support the success of adoptive NK cell therapy in future.

To summarize this chapter, *ex vivo* expansion is a strategy to provide high numbers of activated NK cells that are originally derived from peripheral blood in most cases. Cytokines are essential but not sufficient to induce a high level of NK cell proliferation. Thus, NK cell expansion protocols take advantage of non-NK cell accessory cells and feeder cells and the highest possible expansion is achieved with allogeneic feeder cell lines. Although protocols for NK cell expansion progressively improved over time, proper translation of methods for *ex vivo* NK cell expansion to therapeutic use in clinical scale is still complicated because of GMP requirements.

2 Aim of the Study

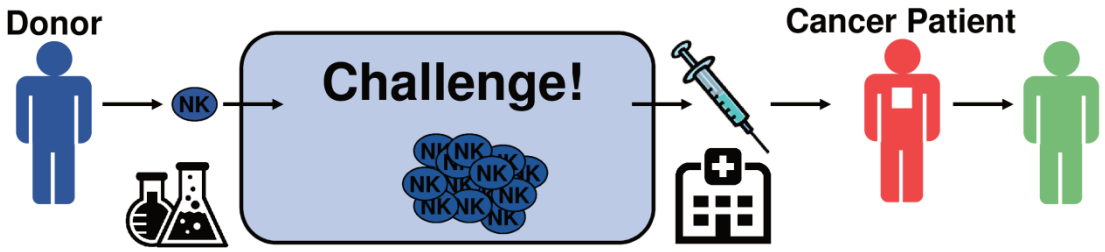


Figure 2.1 The project deals with the translation of NK cell therapy from "benchtop to bedside" and aims at the development of a method for clinical NK cell expansion, a big challenge for NK cell therapy.

Since NK cells are able to detect and kill tumor cells, adoptive NK cell transfer represents a therapeutic opportunity to combat cancer. However, the low number of NK cells that can be obtained from a donor possibly restricts the efficacy of adoptive NK cell therapy. Thus, protocols for *ex vivo* NK cell expansion and activation are needed to fulfill the requirement to generate high numbers of NK cell effector cells. In this context, NK cell therapy is hampered by the lack of suitable methods for clinical scale NK cell expansion that can be used routinely within a GMP regulated environment (Figure 2.1). Therefore, the aim of the project was to develop a highly efficient *ex vivo* activation and expansion of NK cells for clinical use in cancer immunotherapy. To reach this main goal, three basic questions were defined:

(I) What is a suitable technical method to translate protocols for NK cell expansion from early development in laboratory scale to large scale for clinical use?
(II) What is the most efficient way to induce NK cell proliferation *ex vivo* to serve clinical needs?
(III) How does *ex vivo* activation and expansion of NK cells affect the phenotype and function of the cells and what are the consequences for their anti-tumor efficacy?

These clinically relevant questions were addressed in this work. Thereby, the results of the project contribute to overcome a major challenge of NK cell adoptive immunotherapy, they gain a better understanding of NK cell biology and they hopefully result in an improved treatment of cancer.

3 Materials and Methods

Parts of the text in this chapter have been directly taken or slightly modified from Granzin et al. (2015)[346] and from a second manuscript that is currently submitted.[347] The text has been originally written by myself. This chapter describes all materials and methods used in experiments that were performed to meet the aims of the project.

3.1 Materials

3.1.1 Primary cells and cell lines

Primary NK cells were obtained from healthy donor buffy coats (Klinikum Dortmund) or leukapheresis products (Hannover Medical School, Hannover, Germany, or Institut für Klinische Transfusionsmedizin und Immungenetik Ulm Gemeinnützige GmbH, Ulm, Germany). The EBV-LCL (SMI-LCL) line was provided by Dr Richard W. Childs (National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD, USA). Human T cell leukemia cell line 1301 was obtained from Sigma-Aldrich, and K562, Raji and Daudi cell lines were purchased from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). UKRV-MEL-02, COLO-205, SK-MEL-28 and SK-MEL-28-luc were obtained from German Cancer Research Center (DKFZ, Heidelberg, Germany).

All cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine.

3.1.2 Mice

NOD-scid IL-2Rgamma^{null} (NSG) mice were bred at the DKFZ animal facility. Mice were housed under specific pathogen–free conditions and in accordance with all standards of animal care. All animal experiments were approved by the Regierungspräsidium Karlsruhe.

3.1.3 Cell culture media

Table 3.1 List of used cell culture media

Medium	Supplier
RPMI 1640	Biowest
RPMI 1640	Miltenyi
TexMACS research	Miltenyi
TexMACS GMP	Miltenyi

3.1.4 Laboratory accessories and cell culture products

Table 3.2 List of used laboratory accessories

Material	Supplier
Columns for MACS (LS, LD)	Miltenyi
Cell culture plates (6, 12, 24, 48, 96, wells; round and flat bottom)	BD Biosciences; Corning
Cell culture flasks (75, 150, 175 cm^2)	TPP; Greiner;
Cell strainer (40 µM, 70 µM)	BD Biosciences
Centrifugal filters 10K	Amicon
CliniMACS tubing set TS100	Miltenyi
CliniMACS tubing set TS 310	Miltenyi
CliniMACS tubing set TS 730	Miltenyi
Cryo-Vials	Thermo Scientific
Eppendorf cups	Eppendorf
FACS tubes	BD Biosciences; Corning
Falcon tubes (15, 50, 250 mL)	BD Biosciences

Material	Supplier
Filter Tips	Biozym
Lumaplate	Perkin Elmer
Mr Frosti Freezing Containers	Thermo Scientific
Needles Microlane 3 (30 G)	BD
Pipette combitips	Eppendorf
Pipette tips	Eppendorf
Pump MPC 101	ILMAC
Scalpels	Feather
Serological pipettes	Sarstedt; Costar
Surgery equipment	Dimeda
Syringes	
- with Lure-Lock	BD
- 5, 10, 50 mL	Discardit; Corning
- TBC 1 mL	mediware
Transfer bags	Terumo Transfusion
Transwell plates (0.4 µM)	Corning
Tubing interconnectors	Miltenyi
Quadro MACS separator	Miltenyi

3.1.5 Solutions

3.3 Lists of used solutions

PBS/EDTA/BSA (PEB) buffer *for use during flow cytometry staining*	
Sodium chloride	137 mM
Disodium chloride	8.1 mM
Potassium chloride	2.6 mM
Potassium dihydrogen phosphate	1.4 mM
EDTA	2 mM
BSA	0.5%

Buffer for CliniMACS processes *for automated GMP compliant cell processing*	
CliniMACS buffer	
Human serum albumin (HSA)	0.5%

Red blood cell lysis buffer *for preparation of cells from mouse blood*	
Ammonium chloride	8.3 g/L
Potassium bicarbonate	1 g/L
EDTA	37 mg/L

3.1.6 Antibodies

Antibodies for flow cytometry were conjugated with allophycocyanin (APC), phycoerythrin (PE), fluorescein isothiocyanate (FITC), VioBright FITC, APC-Vio770, PE-Vio770, VioBlue or Viogreen, Antibodies used for blocking during cell culture experiments were of functional grade or were concentrated with centrifugal filters and dissolved in CliniMACS buffer to ensure absence of sodium azide and endotoxins.

Table 3.4 List of used antibodies

Antigen / Target	Antibody Clone	Supplier
CX3CR1	2A9-1	Miltenyi
CD2	LT2	Miltenyi
CD3	BW264/56	Miltenyi
CD14	TÜK14	Miltenyi
CD15	VIMC6	Miltenyi
CD16	VEP13	Miltenyi
CD20	LT20	Miltenyi
CD20	rituximab	Roche
CD25	4E3	Miltenyi
CD25	REA570	Miltenyi
CD25	B-B10	eBioscience
CD40	HB14	Miltenyi
CD45	5B1	Miltenyi
CD48	REA426	Miltenyi
CD56	REA196	Miltenyi
CD57	TB03	Miltenyi
CD58	TS2/9	Miltenyi
CD62L	145/15	Miltenyi
CD94	REA113	Miltenyi
CD107a	H4A3	Miltenyi
CD137L	REA254	Miltenyi
CD158a	REA284	Miltenyi
CD155	PV404.19	Miltenyi
CD158b	DX27	Miltenyi
CD158e	DX9	Miltenyi

Antigen / Target	Antibody Clone	Supplier
CD159a	REA110	Miltenyi
CD159c	REA205	Miltenyi
CD178	NOK-1	Miltenyi
CD210	REA239	Miltenyi
CD226	DX11	Miltenyi
CD244	REA112	Miltenyi
CD253	RIK-2.1	Miltenyi
CD314	BAT221	Miltenyi
CD314	1D11	eBioscience
CD334	9E2	Miltenyi
CD336	2.29	Miltenyi
MICA/B	6D4	Miltenyi
mIgG1	P3.6.2.8.1	eBioscience
mIgG1	IS5-21F5	Miltenyi
mIgG2a	S43.10	Miltenyi
mIgG2b	IS6-11E5.11	Miltenyi
mIgM	IS5-20C 4	Miltenyi
IFNγ	LT27:295	Miltenyi
IL-10	JES3-9D7	Miltenyi
NKp80	4A4.D10	Miltenyi
TNF-α	cA2	Miltenyi
ULBP1	#170818	R&D Systems
ULBP3	#166510	R&D Systems
ULBP2/5/6	#165903	R&D Systems

3.1.7 Kits and reagents

Table 3.5 List of used kits and reagents

Material	Supplier
AB serum	Invitrogen, lifetechnologies
Agilent Gene Expression Hybridization Kit	Agilent Technologies
Agilent Low Input Quick Amp Labeling Kit	Agilent Technologies
Agilent RNA 6000 Nano Kit	Agilent Technologies
Agilent SurePrint G3 Human Gene Expression Microarrays 8 x 60K v2	Agilent Technologies
Ammonium chloride	Sigma Aldrich
Bovine Serum Albumin (BSA)	Bovogen Biologicals
CD3 MicroBeads	Miltenyi
CD56 MicroBeads	Miltenyi
Celltrace Violet Proliferation Dye	Life Technologies
Celltrace CFSE Proliferation Kit	Life Technologies
CliniMACS buffer	Miltenyi
CliniMACS anti-Biotin reagent	Miltenyi
CliniMACS CD19 reagent	Miltenyi
CliniMACS CD56 reagent	Miltenyi
CliniMACS TCRαβ-Biotin	Miltenyi
Dimethylsulfoxide (DMSO)	Sigma Aldrich
DNAse I	Sigma Aldrich
Fetal bovine serum	Biochrom
Ethylene-diamine-tetraacetic acid (EDTA)	Fluka; Biochrom
Fixable Aqua Dead Stain	Life Technologies
L-Glutamine	PAA

Material	Supplier
GolgiStop	BD Biosciences
Heparin-solution	Braun
Hyaluronidase Typ V	Sigma Aldrich
Human Serum Albumin	Grifols
IFNγ	Miltenyi
Ionomycin (Iono)	Sigma Aldrich
IL-2 (Proleukin)	Novartis
IL-12	Miltenyi
IL-15	Miltenyi
IL-21	Miltenyi
Isoflurane	Braun
D-Luciferin, Staybrite	Biovision
Lympholyte-M	Cedarlane
MACSplex Cytokine 12 kit,	Miltenyi
Monensin	eBioscience
NK cell isolation kit human	Miltenyi
NucleoSpin RNA kit	Machery-Nagel
PANCOLL	PAN Biotech
D-PBS	Sigma Aldrich
Propidium iodide	Miltenyi
Phorbol myristate acetate	Sigma Aldrich
Potassium bicarbonate	Sigma Aldrich
Potassium chloride	Merck
Potassium dihydrogen	Merck
RA1 buffer	Machery-Nagel
Sodium chloride	Merck
Telomere PNA Kit/FITC	Dako
TNF-α	Miltenyi
Triton x-100	Sigma Aldrich
Trypan blue	Sigma Aldrich

3.1.8 Devices and equipment

Table 3.6 List of used devices and equipment

Material	Type	Supplier
Cell processing device	CliniMACS Prodigy	Miltenyi
Centrifugues	Biofuge pico	Heraeus
	Multifuge 4KR	
	Multifuge X3R	
	Multifuge3SR	
	Varifuge 3 ORS	
Counting chamber	Neubauer chamber	Blau Brand
Flow cytometer	MACSQuant Analyzer 10	Miltenyi
Microarray Equipment	Agilent 2100 Bio-analyzer	Agilent Technologies
	Agilent's Microarray Scanner System G2505C	
	Hybridization chamber and oven	
	ND-1000	NanoDrop Technologies
Incubators	Heracell 240	Heraeus
	BBD6220	Heraeus
Irradiators	Animal irradiator OB 58/902-1	Buchler
	Gammacell 1000	Theratronics
	RS 2000 Biological Research Irradiator	Radsource
Imaging systems for *in vivo* luciferase activity	IVIS imaging system-100	Perkin Elmer
	IVIS lumina series III	
Laminar flow clean benches	Typ HS12	Heraeus
	Typ KS12	Heraeus
	Cell Gard, Lab Gard	Nuaire
Lumaplate reader	TopCount NXT	Perkin Elmer
Microscopes	DMIL	Leica
	Wilovert 30	Hund Wetzlar
Multichannel pipette	Transferpipette	Gilson
Multistepper	Multipipette plus	Eppendorf
Liquid nitrogen tanks	Cryostem 6000	MVE
	1500 series 190	MVE
Piepette boy	Cell Mate II	Matrix
Red light lamp	Heat Glo 75 W	ExoTerra
Sterile tubing welders	?	Terumo transfusion products
	Sterile tubing welder Hematron III	Baxter
Thermomixer	Compact	Eppendorf
Water bath	Heraeus Julabo TW20	Kendro
Vortexer	VortexGenie 2	VWR

3.2 Methods

3.2.1 Flow cytometry and cell counting

Cells were stained according to the product manual of the used staining antibody and analyzed by means of the MACSQuant Analyzer 10 and MACSQuantify 2.5 software. Dead cells were routinely excluded from the analysis by means of propidium iodide staining. Mouse IgG1, IgG2a, IgG2b, IgM or control REAfinity antibodies (REAs) conjugated with the respective dyes were used as isotype controls. Of note, the samples from the comparison between automated and manually expanded NK cells (chapter 4.1) were stored in liquid nitrogen first, so that they could be analyzed all together at a later time point. If not stated differently, determination of cell concentrations was always done by use of the MACSQuant Analyzer.

3.2.2 Cell counting with Neubauer chamber

For maintenance of cell lines, cells were counted using a Neubauer chamber in case no MACSQuant instrument was available. The cell suspension was diluted with an appropriate volume of trypan blue solution (0.05% w/v) for discrimination of dead cells. Viable cells were counted with the Neubauer counting chamber and the cell concentration was calculated using the following formula:

Cell concentration per mL = counted cells/counted squares x dilution factor x 10^4

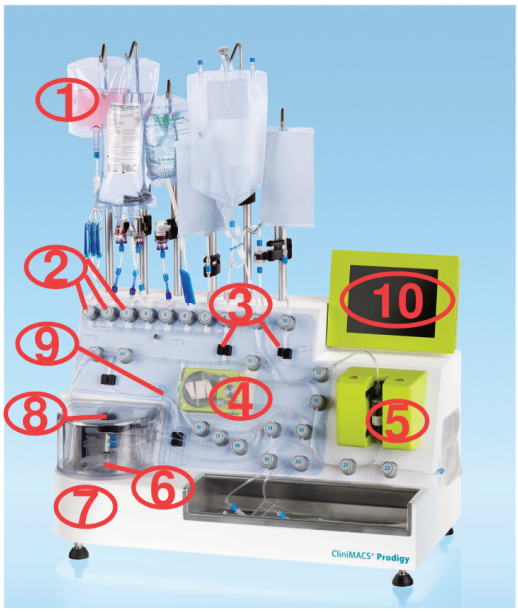
3.2.3 Freezing and thawing of cells

To freeze cells, the cells were suspended in RPMI medium with 20% fetal bovine serum and 10% dimethylsulfoxide (DMSO) at 1-10 x 10^7 cells/mL and transferred in cryo-vials. After initial storage in Mr Frosti Freezing Containers at -80°C for 24-72 hours, the frozen cells were transferred to liquid nitrogen. For thawing, cells were incubated at 37°C by use of a water bath until the sample is not frozen anymore and afterwards the cells were washed once before use (300 x g for 1 min).

3.2.4 The CliniMACS Prodigy system as tool for automated NK cell processing

The CliniMACS Prodigy system allows for automation of cell manufacturing processes for routine use in the clinic and it combines many different features as shown in Figure 3.1. Due to its suitable properties, the CliniMACS Prodigy was selected as a tool to develop a fully automated and GMP-compliant process for expansion of NK cells that is readily applicable for clinical use. For this purpose, the flexible programming suite (FPS) of the instrument was used

to design four different programs and the processing was performed with the CliniMACS Prodigy software version V.1.1.1 (build 2180). The programs were generated for use with Tubing Set 730 and they are described very briefly in the following. The program "NK_cell_setup_cultivation" allows the transfer of the NK cell suspension from a clinical reservoir bag to the CentriCult Unit (CCU) and mixing it with a selectable volume of cell culture medium from another reservoir bag. The program "NK_cell_culture" is used to ensure that the temperature and gas composition within the CCU is maintained at selectable parameters and spinning of the CCU at selectable intervals can be chosen to ensure mixing of cells. The program "NK_cell_media_feed" allows removing and adding selectable volumes of cell culture media from and to the CCU. The last program "NK_cell_take_sample" allows for shortly spinning of the cell suspension within the CCU and then 3 mL of the cell suspension are pumped in a sample pouch that can be welded off from the tubing for analysis. The detailed process steps during the automated NK cell cultivation using a clinical-grade NK cell expansion protocol are explained in chapter 3.2.6. Furthermore, the instrument was used for automated enrichment of NK cells from leukapheresis products as described in chapter 3.2.5.



CliniMACS Prodigy components

1. **Closed system consisting of sterile bags and tubing**
2. **Valves for liquid/gas transfer**
3. **Bubble sensors**
4. **Pump**
5. **CliniMACS separation magnet**
6. **CentriCult Unit + temperature control**
7. **Microscope**
8. **Camera**
9. **Gas supply**
10. **Touch screen**

Figure 3.1 The CliniMACS Prodigy system incorporates several features for complex processing of cells for clinical use within a closed system. (1) Clinical bags are connected to a closed tubing set and allow sterile conditions during the process. (2) Several valves are used for controlled transfer of liquids and gas to the different components of the instrument. (3) Sensors for air bubbles allow discrimination between liquid and air in defined sections of the tubing, ensuring proper process control for handling of liquids and gases. (4) A pump transports liquids and gases in a defined speed. (5) Magnetic cell separation in clinical scale can be performed by a large MACS column and a special clinical scale magnet for MACS. (6) The CentriCult Unit allows centrifugation and cultivation of the cells. (7) Cultured cells can be observed by an installed microscope. (8) A camera for layer detection can be used during centrifugation and allows density gradient centrifugation including isolation of cells from a detected layer. (9) Defined gas supply is provided during cell culture applications. (10) Operation of the system and in-process control is done by a touch screen.

3.2.5 Cell separation

PBMC preparation from buffy coats

Standard density gradient centrifugation was used to obtain PBMCs from buffy coats.[348] CliniMACS buffer with 0,5% human AB serum was added to 20-30 mL of buffy coat to a total volume of 35 mL and layered over 15 mL of Pancoll. Centrifugation was performed at 445 x g for 35 min with break 0 and the PBMC layer was isolated. After a first washing step at 300 x g for 15 min a second washing step was performed at 200 x g for 10 min to efficiently remove remaining platelets.

Magnetic cell separation (MACS)

Purification of NK cells was performed by MACS.

Manual untouched separation of NK cells

For untouched NK cell isolation from buffy coats, PBMC preparation was performed first. Then, the NK cell isolation kit human was applied for untouched enrichment of NK cells from PBMCs according to the user manual. This protocol resulted typically in >95% $CD56^+/CD3^-$ NK cells and was used routinely to obtain purified NK cells if not stated differently.

Manual separation of NK cells preceding automated NK cell expansion

To obtain NK cells for automated NK cell expansion (and manually performed NK cell expansion in comparison), NK cell separation was done by CD3 depletion and CD56 enrichment, representing the current standard for clinical NK cell enrichment. Briefly, NK cells were enriched from buffy coat derived PBMCs by means of CD3 depletion with the use of human CD3 MicroBeads and LD columns followed by CD56 enrichment with the use of human CD56 MicroBeads and LS columns, according to the user manuals.

Automated NK cell separation using the CliniMACS Prodigy system

For separation of NK cells from leukapheresis products, the CliniMACS Prodigy instrument and tubing set TS310 were used for automated TCR-α/β-CD19 depletion according to the available application sheet. Further automated CD56 enrichment with the use of the instrument was achieved by means of Program Enrichment 1, CliniMACS CD56 Reagent and tubing set TS100.

3.2.6 Expansion of NK cells

If not stated differently, purified NK cells were routinely cultivated in medium supplemented with 5% human serum type AB and 500 U/mL of IL-2, either together with 100 Gy irradiated EBV-LCL at a ratio of 1:20 and a starting concentration of 5.25 x 10^5 total cells/mL, or without feeder cells at a seeding density of 5 x 10^5 cells/mL. The AB serum was heat-inactivated for 20 min at 56°C. TexMACS GMP medium was used for automated NK cell culture and for manual NK cell expansion that was performed as a comparison to the automated cultivation. TexMACS Research medium was used for all other experiments. Irradiation of feeder cells was performed by x rays using the RS 2000 instrument or by gamma rays using the Gammacell 1000 device. .Under some conditions, human IL-21 was added to the medium, as indicated, with a concentration of 100 ng/mL if not stated differently. The NK cell density was checked during cultivation by staining and counting viable $CD3^-/CD56^+$ cells by flow cytometry.

Manual NK cell expansion

NK cell culture was started as described before using T flasks or cell culture plates. Seven days after start of cultivation fresh medium was added to double the culture volume and every second to fifth day thereafter fresh medium was added to dilute the cell density to 5-8 x 10^5 NK cells/mL. Expanded NK cells were re-stimulated at later time points in some experiments, as indicated, by co-culturing the expanded NK cells again with irradiated EBV-LCL at a ratio of 1:20 at a cell concentration of 5.25 x 10^5 total cells/mL. For practical reasons, only a fraction of the cells was kept in culture over longer time and the NK cell expansion fold was determined by dividing the theoretical NK cell number at the time point of interest by the NK cell number at start of cultivation.

Automated NK cell expansion using the CliniMACS Prodigy

Automated NK cell expansion was performed by means of the CliniMACS Prodigy instrument with the use of tubing set TS730 and programs that were generated with a process development platform provided with the instrument (see chapter 3.2.4). In short, a clinical bag containing the starting cell material was connected to the tubing set through sterile welding, and the cells were transferred automatically to the CCU of the instrument. A cultivation program for temperature control and repeated input of CO_2 maintained the cultivation conditions comparable to manual cultivation by means of an incubator at 37°C and 5% CO_2. The cultivation was initiated with 70 mL culture volume, and the volume was increased to 140 mL at day 7 and to 280 mL at day 9 by pumping in fresh medium from a reservoir bag. Medium (210 mL) was exchanged at day 12 while the cultivated cells were retained in the CCU by centrifugation. Until day 7, the cells were cultivated in a static culture, and, after day 7, short centrifugation intervals of 1 to 2 seconds

were used every 30 to 60 seconds to gently mix the cells, allowing high cell density cell culture. Transfer of small volumes of the cell suspension to sterile sample pouches was used at days 7, 9, 12 and 14 for sampling during the process.

3.2.7 Gene expression

RNA isolation, amplification and labeling

For total isolation of ribonucleic acid (RNA), 1 x 10^6 unexpanded and expanded NK cells per sample were lysed in RA1 buffer and stored at −20°C. To ensure high purity of the starting material, only CD3-depleted and CD56-enriched NK cells were considered for micro-array analysis. Human total RNA was isolated with the use of the NucleoSpin RNA kit. RNA quality and integrity were determined with the use of the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bio-analyzer, and RNA integrity numbers were confirmed to be between 8.1 and 10. According to published data, RNA integrity number >6 is of sufficient quality for gene expression profiling experiments.[349] RNA was quantified by measurement of A260 nm on the ND-1000 spectrophotometer. Total RNA from unexpanded and expanded NK cell samples (100 ng each) was used for the amplification and labeling step with the use of the Agilent Low Input Quick Amp Labeling Kit. Yields of complementary RNA measured with the ND-1000 Spectrophotometer were in all cases >5 mg, and dye incorporation rates were in all cases >15fmol/ng.

Hybridization of agilent whole human genome oligomicro-arrays

Hybridization was performed according to the Agilent 60-mer oligo-micro-array processing protocol with the use of the Agilent Gene Expression Hybridization Kit. Briefly, 600 ng of Cy3-labeled fragmented complementary RNA in hybridization buffer was hybridized overnight (17 h, 65°C) to Agilent SurePrint G3 Human Gene Expression Microarrays 8 x 60K v2 with the use of Agilent's recommended hybridization chamber and oven. Fluorescence signals of the hybridized Agilent Microarrays were detected with the use of Agilent's Microarray Scanner System. The Agilent Feature Extraction Software (FES 10.7.3.1) was used to read out and process the micro-array image files.

Pre-processing of micro-array data

Raw intensity data were extracted from Feature Extraction output files for Agilent Whole Human Genome Oligo Microarrays 8 x 60K v2 with the use of Rosetta Resolver software (Rosetta, Inpharmatics, LLC). All subsequent calculations were performed with the use of R/Bioconductor and software packages therein.[350,351] Background-corrected intensity values

were normalized between arrays by means of quantile normalization.[352] Reliable signal intensities were considered at $P \leq 0.01$, according to the Rosetta error model.[353] Log2-transformed normalized intensity values were used for subsequent statistical analysis.

The data set has been uploaded to the NCBI GEO public database: record No. GSE62654.

3.2.8 Cytotoxicity assays

Flow cytometry based assay for NK cell cytotoxicity and ADCC

Different target cells were labeled with CellTrace Violet according to the user manual (2 µM; 5 min). Labeled target cells (1 x 10^4) per well were seeded in 96-well round-bottom plates and cultivated alone, as a control, or with NK cells at different NK-to-target ratios, as indicated. To analyze antibody-dependent cytotoxicity, 1 mg/mL of rituximab was added directly to the co-culture of NK cells and target cells. After 4 h of incubation, plates were stored at 4°C for 0.5 to 2 h before the viable CellTrace Violet-positive target cells were quantified by use of the MACSQuant Analyzer 10. The difference between the number of viable target cells in samples with NK cells and in samples without NK cells was defined as killed target cells.

Killing assay based on chromium release

A standard ^{51}Cr- release assay was performed as follows. Target cells were suspended in 500 µL RPMI medium and incubated with 100 µCi ^{51}Cr for 60-90 min. Afterwards, the labeled target cells were seeded at 3x10^4 cells/mL in 96-well round-bottom plates and co-incubated for 4 hours with RPMI medium or with NK effector cells at different NK-to-target ratios or with RPMI medium containing 10% Triton x-100. The supernatants (100 µL) were transferred to a lumaplate and after drying over night the signal intensity was measured using a lumaplate reader. The percentage of specific lysis was calculated from counts for ^{51}Cr release as follows:

Specific lysis = (counts for NK cell sample – counts for medium)
/ (counts for Triton x-100 – counts for medium) x 100

3.2.9 Degranulation and production of IFN-γ and TNF-α

Cytokine production and degranulation were analyzed by means of flow cytometry. NK cells (2 x 10^4) per well were in seeded in RPMI medium using 96-well round-bottom plates. The NK cells were left untreated, as a control, or were stimulated with 1 x 10^4 K562 cells or with 50 ng/mL phorbol myristate acetate (PMA) and 0.5 µM ionomycin (Iono). During the stimulation, the cells were cultivated with CD107a-APC in addition to Monensin or GolgiStop according to the user manuals. For experiments using stimulation with K562, the cultivation was stopped

after 4 h, whereas stimulation with PMA/Iono was performed for 2 h. Then, labeling with Fixable Aqua Dead Stain was used in some cases to exclude dead cells during the analysis. Afterwards, the cells were fixed, permeabilized and stained for IFN-γ, TNF-α and CD56 (Inside Stain Kit, Miltenyi). The latter marker was used to discriminate NK cells from co-cultivated K562 target cells.

3.2.10 Cytokine detection assay using a multiplex bead-array assay

A multiplex bead-array assay was used to quantify cytokines in culture supernatants. The method is based on the binding of cytokines to different capture beads. The capture beads exhibit defined fluorescence properties and they can be analyzed by standard fluorescence cytometry. The MACSplex cytokine 12-kit, human was used according to the product manual to detect GM-CSF, IFN-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-17A, and TNF-α. The measurement was performed by means of the MACSQuant 10 and the data were automatically analyzed using the respective tool from the MACSQuantifiy software. To measure cytokines from EBV-LCL feeder cells and NK cells during expansion, NK cells and irradiated EBV-LCL were cultivated either alone or together as described in chapter 3.2.6. Cultures were terminated at different time points, as indicated, and the supernatants were taken and frozen at −20°C until the MACSplex measurement. To analyze the cytokine production of expanded NK cells upon stimulation, NK cells were expanded for 14 days using IL-2, EBV-LCL co-culture and adding IL-21 at start of cultivation. Then, these expanded NK cells ($1x10^6$/mL) were washed with RPMI medium and cultivated together with or without SK-MEL-28 cells ($4x10^5$/mL) for 24 h in RPMI medium. Afterwards, supernatants were taken and frozen at −20°C until MACSplex measurement.

3.2.11 Telomere length analysis

Telomere length was measured by means of flow cytometry with the use of a commercial Telomere PNA Kit/FITC (Dako) according to the user manual. Detection of the samples labeled with FITC-conjugated peptide nucleic acid was done with the use of the MACSQuant Analyzer X. As recommended, the cell line 1301 was used as internal control and relative telomere length (RTL) was calculated as follows:

RTL=

(MFI sample cells with probe – MFI sample cells without probe) x 2 x 100

/ (MFI control cells with probe – MFI control cells without probe

MFI = mean fluorescence intensity

3.2.12 Proliferation assay using Celltrace Violet

Celltrace Violet Proliferation Dye was used according to the product manual to perform a proliferation assay comparable to the commonly used carboxyfluorescein succinimidyl ester (CFSE) assay. A high concentration of the Celltrace Violet dye and a prolonged incubation time (10 µM and 30 min) was used to ensure a very bright and consistent staining.

3.2.13 Conjugate formation assay

To analyze the conjugate formation during the co-culture of NK cells and EBV-LCL, NK cells were labeled with Violet Dye (10 µM; 30 min) and EBV-LCL were labeled with CFSE (5 µM; 10min). Then, the cells were cultivated together as described in chapter 3.2.6. After 16h, the cells were analyzed by flow cytometry and the conjugate formation was determined by the frequency of Violet dye and CFSE double-positive events.

3.2.14 Transwell Assay

A transwell plate containing a membrane with 0.4 µM pores was used to culture NK cells and EBV-LCL together but without direct contact between the cells. NK cell cultures were set up as described in chapter 3.2.6 with NK cells (2.5 x 10^4/mL) cultivated either alone or together with 5 x 10^5 EBV-LCL/mL. As recommended, in the upper compartment (insert) 0.45 mL cell suspension was added, while the lower compartment contained 1.5 mL of the cell suspension. The upper chamber thereby contained medium or EBV-LCL or EBV-LCL together with NK cells. The lower chamber always contained NK cells, either alone or with EBV-LCL, and the number of these NK cells was quantified after 7 days.

3.2.15 Mouse xenograft models

Tumor cell injection and adoptive NK cell transfer

To evaluate for NK cell anti-tumor activity *in vivo*, tumors with human origin were engrafted into NSG mice to establish a xenograft mouse model. NSG mice (8 weeks old) were irradiated (3.5 Gy) to support optimal engraftment of xenogenic cells. Three to five hours after irradiation, the mice received 5x10^5 luciferase transfected SK-MEL-28 melanoma cells (SK-MEL-28-luc) intravenous (i.v.) through tail vein injection. For these injections, cells were always suspended in 200 µL PBS. NK cells (3-30 x 10^6 cells, as indicated, or PBS as a control) were injected via tail vein injection directly together with the tumor cells (co-injection model) or three days later, after successful engraftment of the human tumor cells (therapeutic model). In addition, during

the therapeutic model 10,000 units IL-2 per mice were injected intraperitoneal (i.p.) when NK cells or PBS were injected and 1, 2, 3, 5, 7 and 11 days thereafter.

Analysis of tumor burden

The tumor burden was estimated at different days during the treatment, as indicated. To measure the tumor burden, 4.5 mg StayBrite D-Luciferin dissolved in 150 µL sterile PBS was injected i.p. in the mice 10-15 min before the measurement. Then, the mice were anesthetized with 5% isoflurane followed by a second i.p. injection with 150 µL Luciferin solution. After arranging up to 5 mice together in the imaging chamber of an IVIS system, the *in vivo* luminescence was measured. Luminescence images were taken every 1-2 min with 60s exposure time, binning M, field of view 25, f1 until the maximum signal intensity was reached. Analysis of the pictures was performed using the software Living Image 2.5.

Re-isolation of injected human NK cells from mouse blood and tissues

To prepare the organs from treated mice, the mice were sacrificed by asphyxiation using CO_2 within a euthanasia chamber.

Preparation of NK cells from blood

The eyeball was removed and blood was collected from the retro-orbital vein. Heparin (25 µL) was added directly to 200-400 µL blood. Then, blood cells were removed by adding 5 mL red blood cell lysis buffer per 200 µL of heparinized blood and incubation for 10 min at room temperature. After two washing steps (300 x g for 10min), the cells that were prepared from the blood of one mouse were suspended in 250 µL PBS containing 0.5% AB serum and then they were stored at 4°C.

Preparation of NK cells from lungs (tumor) and spleen

Directly after the removal of blood, the mouse was dissected and the lung and spleen were removed and stored each at 4°C. The organs each were mechanically disrupted with a scalpel and transferred to a 50 mL falcon. Digestion buffer was added containing 5 mg hyaluronidase and 5 mg DNAse I dissolved in 10 mL PBS. After incubation for 30 min at 37°C, the digested tissue was plunged through a 70 µM cell strainer, washed (300 x g for 10 min at 4°C) and resuspended in 6 mL PBS. Lympholyte (5 mL) was added carefully and density gradient centrifugation was performed at 1500 x g for 26 min at room temperature. The leukocyte fraction was isolated and suspended in 250 µL PBS containing 0.5% AB serum and then it was stored at 4°C.

From the single cell suspensions from blood and tissues of each mouse, an aliquot of 50 µL was used directly to measure the cell count using the MACSQuant analyzer. The remaining volume was pooled with corresponding samples from other mice that were treated under the same experimental conditions and used for staining of surface markers and functional assays.

3.2.16 Statistics and data analysis

Apart from micro-array data analysis for gene expression, statistical comparisons were performed with the use of unpaired or paired Student's t-test, as indicated. In the micro-array data sets, significant expression differences were determined per reporter between the following sample groups: freshly isolated NK cells (day 0) and NK cells expanded for 14 days by the automated system CliniMACS Prodigy (P); manually with EBV-LCL feeder cells in T75 flasks (T) and manually without feeder cells only with the use of IL-2econtaining media (I). The analysis of variance test with repeated measurements design was applied by fitting a linear mixed-effects model (random effect: individual donors) on the normalized log2 intensity data. Correction for multiple testing occurred by use of the method of Benjamini & Hochberg (B-H). Further pairwise group comparisons were performed by means of Tukey's honestly significant differences post hoc test. The following selection criteria were applied: adjusted analysis of variance *P* values ≤0.05, Tukey *P* values ≤0.05 and median fold changes ≥2 or ≤−2. Reporters with a detection *P* value (flag) >0.01 for >3 of 6 samples per sample group were excluded because of insufficient signal reliability. The expression profiles of all reporters with differential gene expression in at least one of the pairwise comparisons was hierarchically clustered (Euclidean distance, complete linkage) and displayed in heat map images centered to the median value per reporter (TM4 suite, MeV_4_8_1).[354] Calculations were performed with the use of Excel (Microsoft Office Inc) or R/Bioconductor [R version 3.1.1 (2014e07e10)]. Because some of the reporters covered on the micro-array platform represent long non-coding RNAs or map to alternative transcripts of the same gene, functional grouping analysis was performed on the gene level with the use of QIAGEN's Ingenuity Pathway Analysis annotation tools (IPA, QIAGEN Redwood City, www.qiagen.com/ ingenuity). Significantly enriched functional groups were identified by use of default settings and a B-H multiple testing *P*-value cutoff of 0.025.

4 Results

Parts of the text in this chapter have been directly taken or slightly modified from Granzin et al. (2015)[346] and from a second manuscript that is currently submitted.[347] The text has been originally written by myself.

The chapter describes the results of experiments that were performed to meet the aims of the project as outlined in chapter 2. First, an automated method for clinical-grade NK cell expansion was developed and the *ex vivo* expanded NK cells were characterized in detail (chapter 4.1). Next, the protocol for clinical-grade NK cell expansion was further optimized to achieve higher numbers of functional NK cells and the mechanisms underlying NK cell expansion were investigated (chapter 4.2). Finally, *ex vivo* expanded human NK cells were evaluated for their therapeutic potential against melanoma in a xenograft mouse model (chapter 4.3).

4.1 Fully automated expansion and activation of clinical-grade NK cells for adoptive immunotherapy

The production of therapeutic effector cells in a standardized, GMP compliant and efficient way is challenging for several clinical applications, especially for the activation and expansion of NK cells. Therefore, a fully automated cell cultivation process was developed for clinical use by means of the CliniMACS Prodigy system as described in chapter 3.2.4. The automated process was applied to a clinical-grade NK cell expansion protocol that makes use of IL-2 and irradiated EBV-LCL (SMI-LCL) feeder cells to induce NK cell activation and proliferation (see chapter 1.5.3). The automation covered all steps that were needed within the cultivation time of 14 days including medium change, gentle cell mixing at high cell densities and sampling for analysis during the expansion process. To evaluate the automated expansion process in comparison to conventionally used manual expansion, NK cells were cultivated under three different conditions (Figure 4.1). The NK cell expansion was performed either automated using the CliniMACS Prodigy system or manual using T75 flasks. In addition, to further investigate the effect and necessity to use the irradiated EBV-LCL feeder cell line, NK cells were also cultivated manually in T75 flasks without irradiated EBV-LCL, representing a long-term NK cell activation with IL-2 containing medium alone, which can be seen as a commonly used standard and serves as additional control.

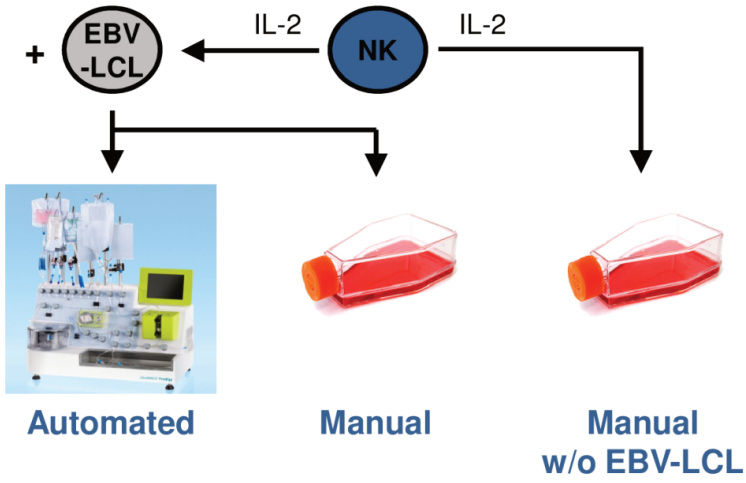


Figure 4.1 Experimental setup for evaluation of differentially expanded NK cells. NK cells were cultivated with IL-2, either in the presence of EBV-LCL feeder cells by means of the automated system (automated) or T-flasks (manual), or in the absence of EBV-LCL feeder cells by means of T flasks (manual w/o EBV-LCL).

4.1.1 Automated or manual NK cell cultivation results in comparable NK cell fold expansion

Purified NK cells from buffy coats of 10 donors were cultivated together with EBV-LCL feeder cells, either manually or by use of the automated system, resulting in comparable increase in NK cell numbers over time (Figure 4.2 A). In detail, automated or manual EBV-LCL feeder cell line-based expansion for two weeks led to 850 ± 509 or 1344 ± 1135 fold NK cell expansion respectively with high variability between different donors (as shown by standard deviations). Of note, without EBV-LCL only 14 ± 13 fold expansion was achieved, demonstrating the limitation of this approach and proving the strong proliferation-inducing effect of the EBV-LCL cell line on NK cells. On average, starting with only 1.5 x 10^6 NK cells, a number typically obtained from 20 ml of whole blood, a substantial number (mean 1.3 x 10^9) of NK cells could be generated within 14 days by a single run of the automated process. Both, the automated and manual expansion resulted in a highly pure NK cell product (>99% $CD3^-/CD56^+$) and no T or B cells could be detected.

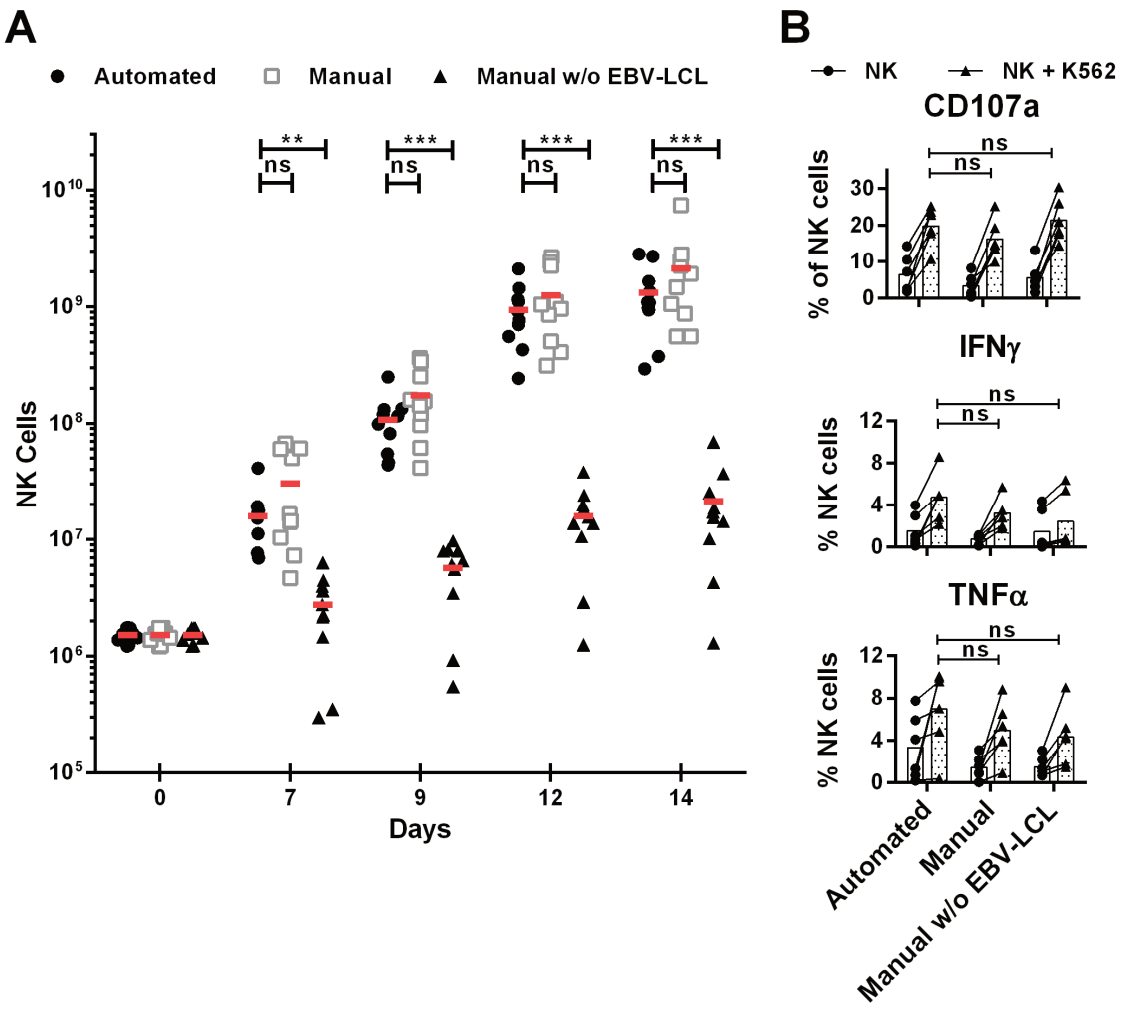


Figure 4.2 EBV-LCL co-culture induces a strong increase in NK cell numbers and automated and manual expansion of NK cells result in comparable expansion kinetics. Effector functions of expanded NK cells are enhanced. Modified from Granzin et al. (2015).[346] Automated EBV-LCL-based expansion of NK cells (circles) in comparison to manual NK cell expansion in T flasks with (squares) or without (triangles) irradiated EBV-LCL. NK cell numbers displayed for manual expansion are theoretical and were calculated by the NK cell fold expansion obtained in T flasks multiplied by the same starting NK cell number as in the automated approach. (B) Differentially expanded NK cells were tested for their reactivity by staining for CD107a, IFN-γ and TNF-α before (white bars) and after (gray bars) stimulation with K562 target cells. NK cells from ten (A) or six (B) donors were analyzed, and displayed are mean values and p values for paired Student t test with $p < .05$ considered as significant.

4.1.2 Automatically and manually produced NK cells have similar functionality

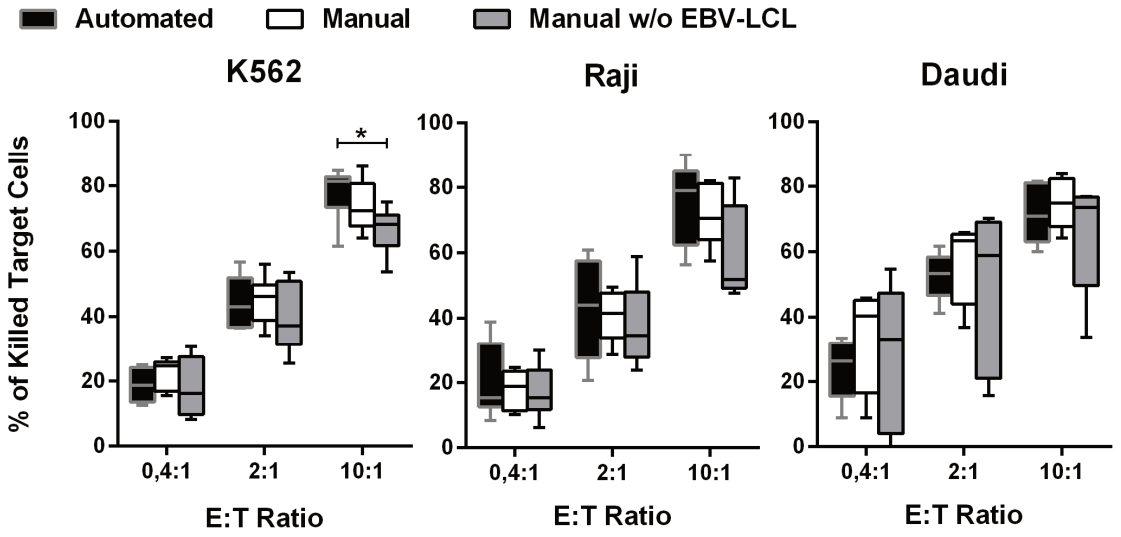


Figure 4.3 Differentially expanded NK cells show dose dependent cytotoxicity against tumor cell lines *in vitro*. Modified from Granzin et al. (2015).[346] NK cells were expanded for 14 days by the automated process (black bars) in comparison to manual NK cell expansion with (white bars) or without (gray bars) irradiated EBV-LCL and analyzed for cytotoxicity against K562, Raji and Daudi cell lines at different effector-to-target (E:T) rations. Statistical significance was determined by paired Student's t-test.

After testing the expansion performance, the effector functions of the expanded NK cells were analyzed. Stimulation of the differentially expanded NK cells with K562 target cells revealed no differences in the production of the pro-inflammatory cytokines IFN-γ and TNF-α and similar levels of degranulation as an indicator of NK cells cytotoxic function (Figure 4.2 B). NK cells were further evaluated for cytotoxicity against the human leukemic cell lines K562, Raji and Daudi (Figure 4.3). NK cells obtained by means of the automated or the manual approach showed comparable cytotoxicity against all three target cell lines in a dose-dependent manner, although significant differences in the cytotoxic intensity were observed between different donors. The level of killing tended to be higher compared with NK cells that had been expanded with IL-2 only and without EBV-LCL, but this trend only achieved statistical significance at a 10:1 effector-to-target ratio against K562 cells.

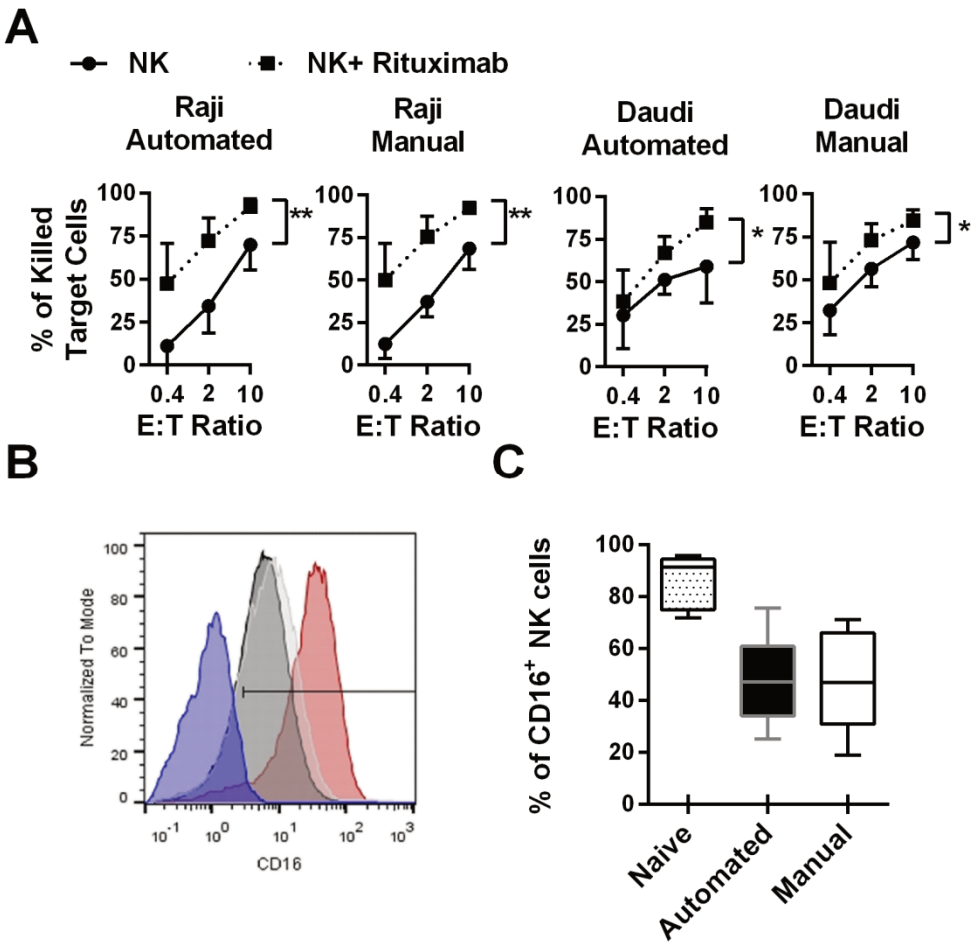


Figure 4.4 Expanded NK cells successfully perform antibody dependent cellular cytotoxicity against rituximab coated target cells despite low expression of CD16. Modified from Granzin et al. (2015).[346] (A) Cytotoxicity against CD20 positive Raji or Daudi cells that were untreated (circles) or treated (squares) with 1 µg/ml rituximab for 4 h during the assay. P values indicated are for paired Student t test with $p < .05$ considered as significant. Six or seven donors were analyzed and displayed are mean, Min to Max (whiskers in A) and SD. (B) Representative CD16 expression on NK cells from one donor is shown. Comparison of naive NK cells (red curve) and NK cells after 14 days of automated (black curve) or manual (white curve) expansion in the presence of irradiated EBV-LCL is displayed. CD16 negative PBMCs served as a control to determine the positive gate (blue curve). (C) An overview of the CD16 staining of all seven donors used in B is depicted and displayed are mean, min to max and SD

Because NK cells are able to induce antibody- dependent cellular cytotoxicity through the Fc receptor CD16, the killing of target cells could be enhanced by a monoclonal antibody. It's known that NK cell activation induces CD16 down-regulation,[355,356] and lower levels of CD16 were observed on the surface of the activated and expanded NK cells compared with freshly isolated cells (Figure 4.4 B,C). Still, rituximab significantly increased the NK cell mediated killing of CD20-positive Raji or Daudi cells, for both, automatically and manually obtained NK cells, proving that they had the capacity to mediate therapeutically relevant antibody-dependent cellular cytotoxicity (Figure 4.4 A). Of note, rituximab had no effect on the killing of CD20-negative K562 cells (Figure 4.5).

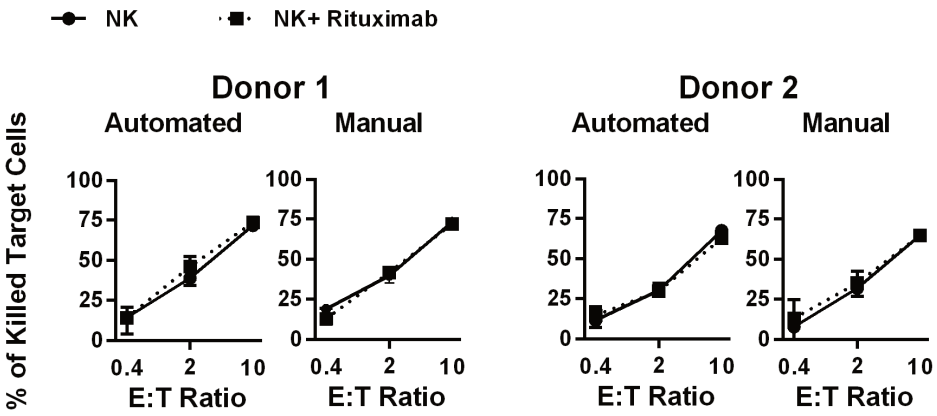


Figure 4.5 rituximab does not augment antibody dependent cellular cytotoxicity of NK cells against CD20-negative K562 cells. Modified from Granzin et al. (2015).[346] For two donors, NK cells were expanded for 14 days by co-culture with irradiated EBV-LCL using the automated process (left figures) or the manual approach (right figures) and analyzed for cytotoxicity against CD20-negative K562 at different effector-to-target (E:T) rations. The cells were untreated (circles) or treated (squares) with 1 µg/ml rituximab for 4h during the assay.

4.1.3 Expanded NK cells do not show a reduction in telomere length

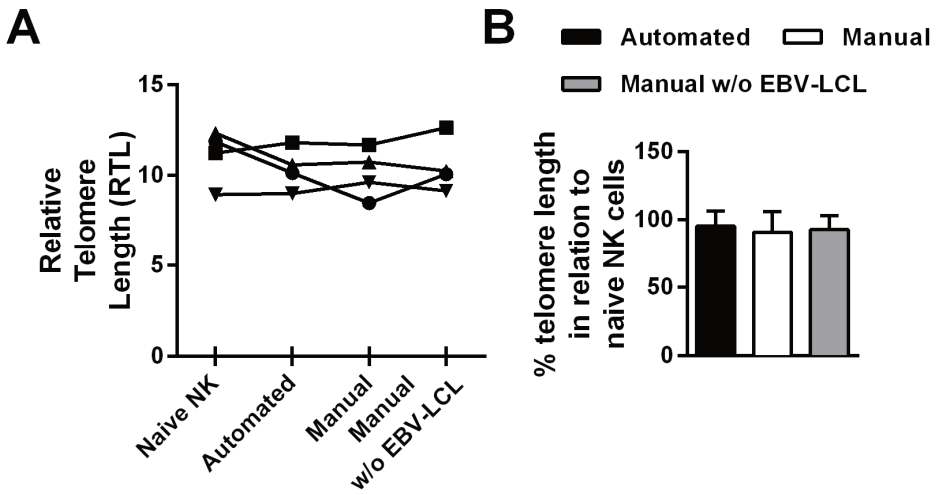


Figure 4.6 The telomere length of NK cells is not altered after two weeks of *ex vivo* expansion, independent of the expansion protocol. Modified from Granzin et al. (2015).[346] For four donors, naive NK cells and NK cells after automated or manual expansion with or without irradiated EBV-LCL were analyzed for telomere length using a commercial available assay based on flow cytometry. The tetraploid cell line 1301 with extremely long telomeres was used as an internal control and for each sample the telomere length was calculated in relation to this recommended control. Displayed are values for individual donors (A) or mean and SD for all donors (B).

Extensive expansion might result in telomere shortening, which would reduce the proliferative potential of NK cells for later *in vivo* applications. Therefore, the telomere length was investigated before and after expansion, because the potential of NK cells to proliferative *in vivo* after transfer to the patient is crucial for their therapeutic effect.[177] After 14 days of expansion, no noticeable difference in the telomere length was detected, independent of the expansion

method and expansion intensity, indicating that the proliferative potential of the NK cells was not reduced, even after extensive *ex vivo* expansion (Figure 4.6).

4.1.4 Flow cytometry profiles of automatically and manually expanded NK cells are similar, whereas they differ clearly from naive NK cells

Next, the phenotype of naive and expanded NK cells was compared by means of flow cytometry and stained for 18 selected markers (Figure 4.7). As previously described,[341] the pattern of many relevant NK cell markers changed on *ex vivo* expansion. Up-regulation of TRAIL and FasL as well as the activating NK cell receptors NKp30, NKp44, NKG2D and DNAM-1 indicated an activated state and correlated with the enhanced NK cell function after expansion, which was in line with the results of the preceding functional assays. Besides the strong phenotypic difference between naive and expanded NK cells in general, manually and automatically processed NK cells had a comparable marker profile. However, frequencies of NK cells expressing NKG2C, CX3CR1 and KIR2DL2/DL3 were slightly but still statistically significantly higher after automated expansion compared with manually expanded NK cells that showed slightly higher expression of NKG2A and NKp44.

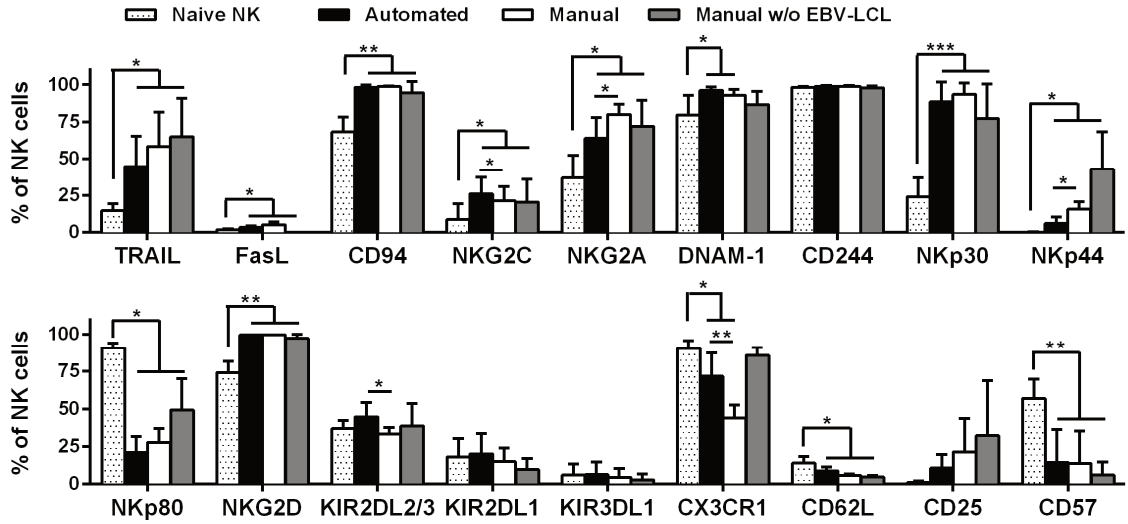


Figure 4.7 Differentially expanded NK cells show similar changes in surface marker expression after 14 days of *ex vivo* activation and expansion. Modified from Granzin et al. (2015).[346] NK cells were analyzed by flow cytometry for selected surface markers before (naive NK, dotted) or after automated EBV-LCL-based expansion (black) in comparison to manual NK cell expansion in T flasks with (white) or without (gray) EBV-LCL. Five donors were analyzed; mean values and standard deviations are shown. Statistical significance was determined by paired Student's t-test.

4.1.5 Gene expression analysis reveals only minor differences between NK cells after automated or manual NK cell expansion

The NK cells were further investigated at the gene expression level and a whole human genome micro-array with samples from six donors was performed. In total, 13,263 reporters corresponding to differentially expressed transcripts were identified in the comparisons among all sample groups (Figure 4.8 A). Of note, the most prominent expression differences were between freshly isolated and all other expanded NK cell samples. In contrast, and consistent with the flow cytometry analysis, gene expression after automated or manual expansion with EBV-LCL feeder cells was similar. Less than 2% of all differentially expressed reporters (247 reporters) varied significantly between both sample groups. However, to obtain further insight in associated functions for the small set of differentially expressed genes, functional grouping analysis was performed. The analysis revealed an association of the genes with hematological system development, cellular movement and immune cell trafficking. In particular, genes with known importance in movement of leukocytes were identified (Table 4.1), including a group of genes associated with NK cell migration (CMKLR1, CX3CR1, S1PR5, GNLY and CXCR1). The latter set of genes was expressed at slightly higher levels after automated compared with manual expansion.

Nevertheless, the expression profiles of NK cells after automated and manual expansion were strikingly similar and in strong contrast to the many differentially expressed genes between naive and expanded NK cells. Changes between NK cells after expansion with the automated system and naive NK cells before expansion were investigated in more detail and a list of the 100 most up-regulated and down-regulated genes is published in Granzin et al. (2015).[346] As expected for an expansion protocol, functional grouping analysis revealed the most significant functional association of the regulated genes (B-H *P* values $<1.5 \times 10^{-8}$) with cell cycle regulation, cell death and survival, DNA replication, DNA recombination and DNA repair, cellular growth and proliferation as well as cellular assembly and organization. Consistent with the results of the flow cytometry analyses, many NK cell relevant markers had a change in their expression levels after expansion (Figure 4.8 B). The most prominent effects were up-regulation of TRAIL, FasL, the inhibitory receptor TIGIT and the chemokine receptors CCR2, CCR5 and CXCR6. In addition, granzyme M was slightly down-regulated, but other effector molecules that play an important role in tumor killing, such as TNF-α, perforin and granzymes A, B and K, were up-regulated.

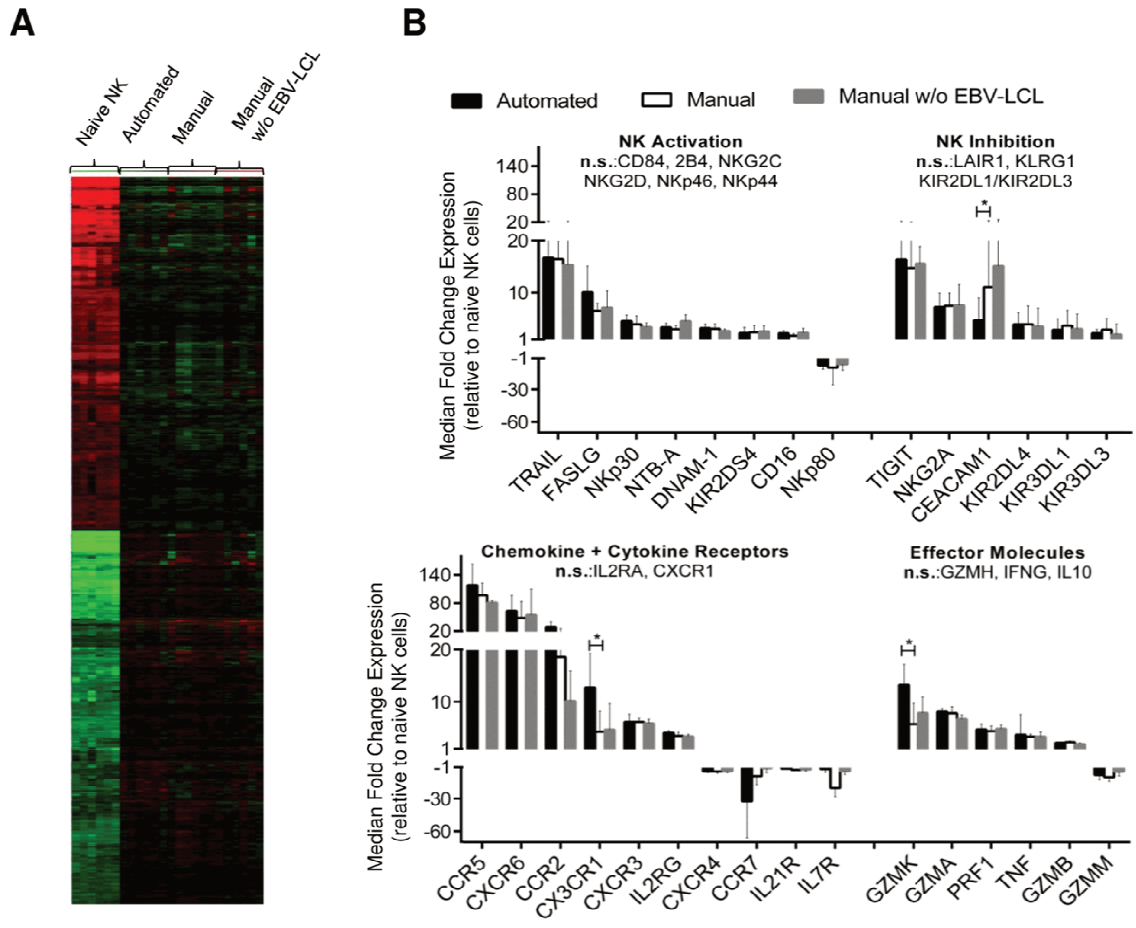


Figure 4.8 Many genes, including NK cell relevant markers, are regulated upon *ex vivo* expansion in the same way for differentially expanded NK cells. Modified from Granzin et al. (2015).[346] Samples from six donors were used for gene expression analysis of naive NK cells and NK cells after automated EBV-LCL-based expansion as well as NK cells after manual expansion in T flasks with or without irradiated EBV-LCL. (A) Differentially regulated reporters between the four sample groups were identified by filtering for statistical relevance and reliable signal intensities; median centered values for these reporters are shown in a heat map after hierarchical clustering analysis. Color saturation limits range from log2 intensities of –4 (green) to +4 (red). No changes relative to the reporter-wise median log2 intensity of all samples is displayed in black color. (B) Filtering for NK cell relevant genes among regulated reporters is displayed for NK cells obtained by the automated process (black bars), manual expansion with EBV-LCL feeders (white bars) or manual expansion without EBV-LCL feeders (gray bars) in relation to naive NK cells. Automated and EBV-LCL-based manual expansion was compared by means of Tukey's honestly significant differences post hoc test; genes with Tukey *P* value ≤0.05 and median fold change ≥2 or ≤–2 are indicated as significant (indicated by stars).

Table 4.1 Genes associated with movement of leukocytes for automatically expanded NK cells in relation to manually expanded NK cells. Both expansion methods utilized irradiated EBV-LCL feeder cells. Ingenuity functional grouping analysis with prediction for increased or decreased movement based on the number of published findings and the fold change of the gene of interest from Granzin et al. (2015).[346]

ID	Genes in dataset	Prediction (based on expression direction)	Fold Change	Findings
A_24_P766716	CMKLR1	Increased	4,036	Increases (7)
A_23_P131676	ACKR3	Increased	3,436	Increases (2)
A_33_P3298159	PTGDS	Increased	3,328	Increases (2)
A_23_P145024	ADRB2	Decreased	3,188	Decreases (1)
A_23_P407565	CX3CR1	Increased	2,872	Increases (6)
A_23_P129786	SREBF1	Increased	2,773	Increases (1)
A_23_P67932	CXCR1	Increased	2,588	Increases (15)
A_23_P107744	S1PR5	Increased	2,526	Increases (2)
A_23_P89249	ERBB2	Increased	2,477	Increases (4)
A_24_P941359	FAM65B	Decreased	2,445	Decreases (2)
A_33_P3353921	GNLY	Increased	2,232	Increases (11)
A_23_P156683	LTA	Decreased	-2,159	Increases (12)
A_24_P382319	CEACAM1	Decreased	-2,417	Increases (4)
A_23_P210210	EPAS1	Affected	-2,424	Affects (1)
A_33_P3354935	CSF1	Decreased	-3,211	Increases (24)
A_33_P3265739	PTGER3	Decreased	-3,603	Increases (1)

4.1.6 Automated NK cell expansion can be complemented by a preceding, automated NK cell separation, enabling a fully automated NK cell production process

Finally, it was shown that the complete cell processing needed for NK cell expansion, from the starting material to the final cell product inclusive of the NK cell enrichment process, can be achieved with the used system. Therefore, initial NK cell purification steps were also performed automated by means of the CliniMACS Prodigy system before the automated cultivation and expansion phase. In the experiments described so far, the NK cells were enriched manually from buffy coats as described in chapter 3.2.6.. For automated separation of NK cells from leukapheresis products in clinical scale, TCR-α/β-CD19 depletion was performed, to ensure efficient removal of potentially harmful contaminating TCR-α/β T and B cells, followed by CD56-positive selection in a second step to further enrich for purified NK cells. By use of this strategy, automated NK cell separations from leukapheresis products of three donors were performed and 4,1–4,4 and 2,7–3,1 log depletion of TCR-α/β and B cells was achieved. After subsequent CD56 enrichment, no TCR-α/β and B cells were detected, and NK cells with a purity of 71% to 92% could be obtained while remaining non-NK cells were mainly $CD14^+$ monocytes (12.4% ± 8.8%) and TCR-γδ $CD3^+CD56^+$ NK-like T cells (5.3% ± 4.7%). Automated expansion of automatically separated NK cells for 14 days resulted in 390-fold to 1185-fold expansion (Figure 4.9 A), within the same range of what was achieved by manually

separated NK cells before. The functionality of the fully automatically obtained NK cells was proven by the effective killing of K562, Raji and Daudi cells (Figure 4.9 B).

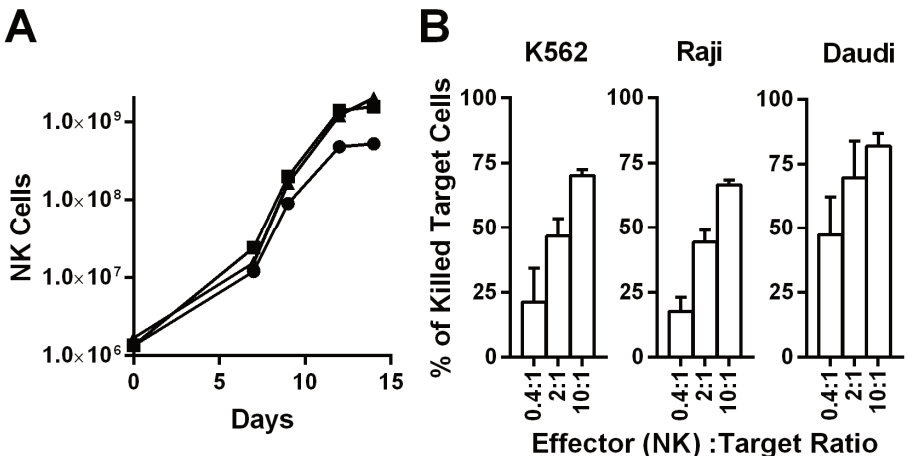


Figure 4.9 Fully automated NK cell separation and expansion is possible in clinical scale starting from leukapheresis. Modified from Granzin et al. (2015).[346] NK cells were separated from leukapheresis products by an automated system using TCRα/β-CD19 depletion followed by CD56 enrichment. Then NK cells were further used in the automated expansion process. (A) Increase in NK cell numbers over time during the expansion phase is displayed for three individual donors. (B) After 14 days of expansion, NK cell cytotoxicity against K562, Raji and Daudi cells was measured and mean values and SD of all donors is displayed.

In summary, substantial numbers of activated NK cells could be obtained with a completely automated system for *ex vivo* expansion. In comparison to manual NK cell expansion, the automated process yielded comparable high numbers of NK cells with similar phenotypic, transcriptional and functional profiles.

4.2 Development of an optimized protocol for NK cell expansion and activation

Production of NK cells in a way that is technically applicable in the clinics is a major issue for adoptive NK cell immunotherapy. This was addressed in the first part of the project by successful automation of a clinical-grade NK cell expansion, based on a protocol that utilizes irradiated EBV-LCL feeder cells with proven clinical applicability. However, efficient expansion of NK cells by simulation with EBV-LCL feeder cells is limited, because the proliferation of NK cells declines over time and efficient expansion is possible for two or maximum four weeks. Thus, the yield of achievable NK cells is still limited and this chapter deals with the development of an improved protocol for clinical-grade NK cell expansion to optimize the supply of effector cells for adoptive NK cell therapy. In addition, it's still unknown why EBV-LCL so efficiently stimulate NK cell proliferation and experiments in this section were performed to address responsible mechanisms.

4.2.1 EBV-LCL-based NK cell expansion is significantly increased by IL-21

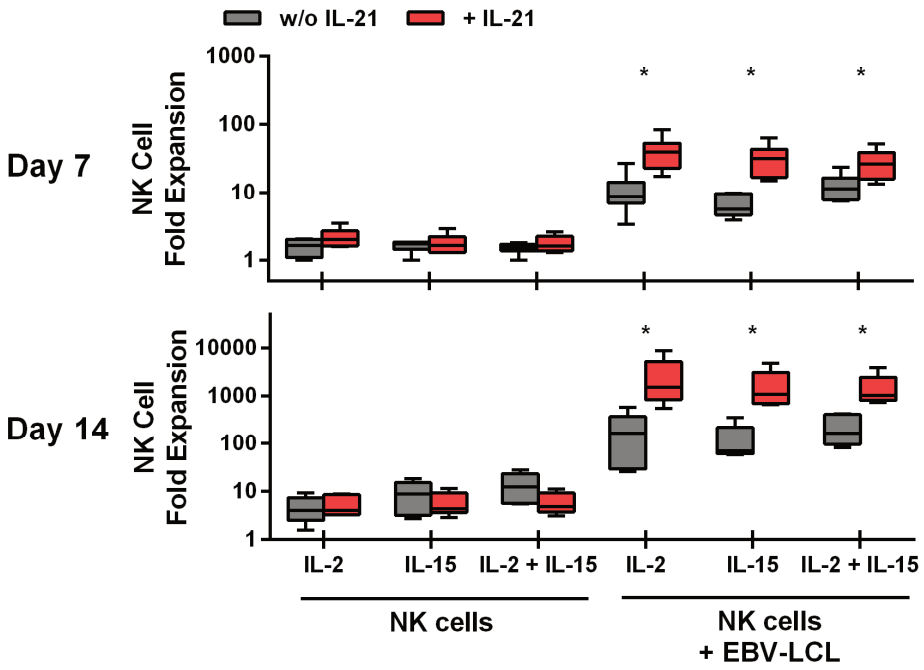


Figure 4.10 IL-21 enhances the EBV-LCL-mediated expansion of NK cells in the presence of IL-2 or IL-15. NK cells were expanded with different combinations of IL-2, IL-15 and IL-21 in the presence or absence of irradiated EBV-LCL feeder cells. The cultivation was performed for 7 days (top) or for 14 days (bottom), the NK cells were enumerated by flow cytometry and the NK cell fold expansion was calculated. NK cells from six donors were analyzed and mean, SD, min and max are shown. Statistical significance was determined by paired Student's t-test.

The standard protocol for clinical-grade EBV-LCL-mediated NK cell expansion utilize IL-2 and it was tried to enhance the yield of NK cells by IL-15 and IL-21, two cytokines that are known to affect NK cell proliferation (Figure 4.10). The use of IL-2, IL-15 or the combination of IL-2 and IL-15 resulted in comparable yields of NK cells, for both, the efficient EBV-LCL-mediated high NK cell expansion and the rather low expansion without irradiated EBV-LCL feeder cells. It was noticed that the presence of IL-2 or IL-15 was essential for efficient NK cell expansion *in vitro*. Use of IL-21 alone was insufficient to induce elevated NK cell expansion compared to IL-2 or IL-15 (data not shown). Strikingly, with IL-2 and/or IL-15 together, IL-21 strongly increased the EBV-LCL-mediated NK cell expansion, whereas IL-21 did not change the NK cell expansion when irradiated EBV-LCL were absent. IL-2 and IL-15 appeared interchangeable in this context and because IL-2 is routinely used in clinics for longer time and represents a better characterized clinical-grade reagent, IL-2 was considered for further experiments instead of IL-15.

The combination of IL-2, IL-21 and irradiated EBV-LCL co-culture was selected for in depth evaluation as an optimized protocol for NK cell expansion. First, the previous results were confirmed for NK cells from additional donors. Low NK cell expansion was observed in the absence of feeder cells despite the addition of IL-21 (Figure 4.11 A). Irradiated EBV-LCL co-culture and IL-2 induced a 22–fold mean NK cell expansion after one week that was further increased to 53–fold by adding IL-21 to the medium. To test whether addition of IL-21 directly affected the proliferation of NK cells, a proliferation assay was performed by monitoring CellTrace Violet dye labeled NK cells. Of note, NK cells did not start to proliferate until day 3 after initiation of culture (Figure 4.11 B). Thereafter, IL-21 enhanced the proliferation of NK cells in the presence of irradiated EBV-LCL, while IL-21 had no effect on the proliferation of NK cells by itself when feeder cells were absent. Furthermore, there was a pronounced positive correlation between the concentration of supplemented IL-21 and the increasing expansion of NK cells in co-culture with irradiated EBV-LCL feeder cells (Figure 4.11 C). Intriguingly, it was sufficient to add IL-21 only at the beginning of the culture to enhance the EBV-LCL-mediated NK cell expansion. Culturing in medium supplemented continuously with IL-21 did not enhance NK cell expansion compared to when IL-21 was added only at the beginning of the culture. Further, permanently adding IL-21 to the culture actually resulted in lower NK cell numbers after prolonged culture compared to an initial single exposure to IL-21 (Figure 4.11 D). Nevertheless, the rate of proliferation of NK cells declined after two weeks, limiting the *ex vivo* time period during which NK cell expansion occurred to 2-4 weeks, precluding further NK cell expansion past this time point (Figure 4.11 D). This limitation could be overcome by use of IL-21 at the start of culture combined with repeated addition of irradiated EBV-LCL feeder cells

every two weeks. This allowed for the sustained expansion of NK cells at very high levels for a longer time period, with a 2.7 x 10^{11}–fold mean increase in NK cell numbers being achievable after 46 days (Figure 4.11 E). Importantly, at later time points, NK cell expansion declined without repeated EBV-LCL stimulations, indicating that NK cells had not acquired an uncontrolled ability to proliferate, which would raise safety concerns for their clinical application.

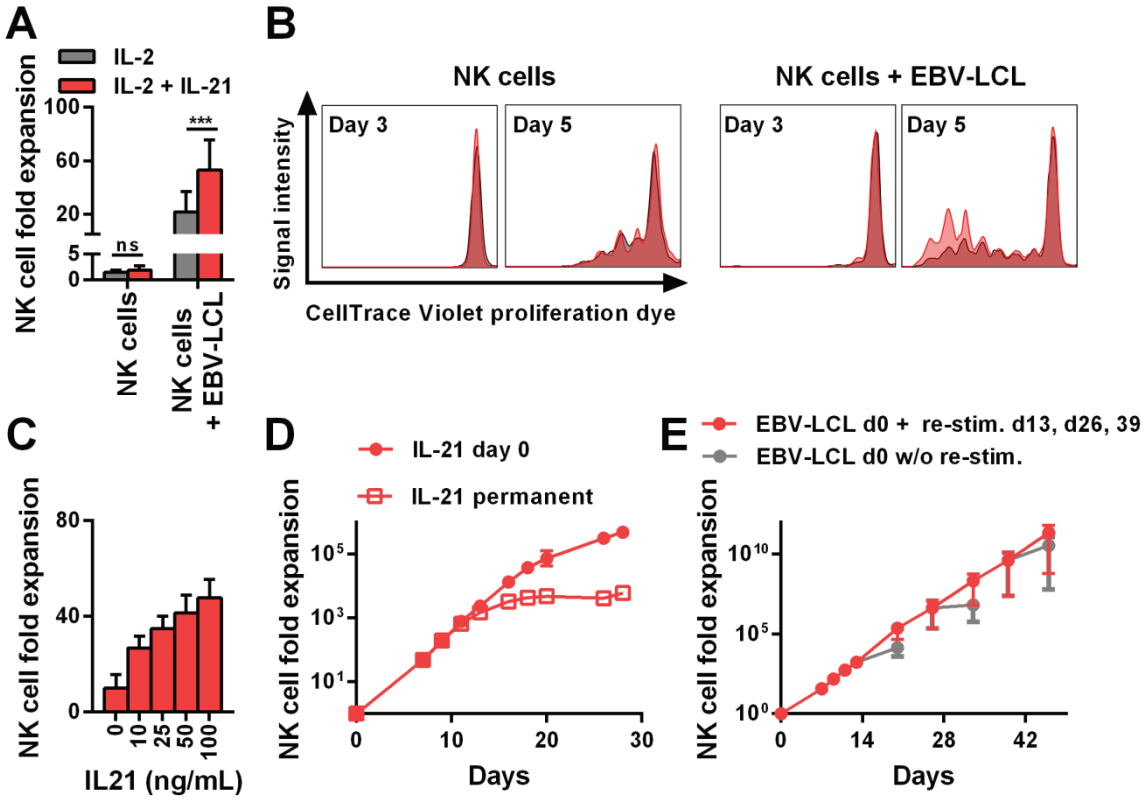


Figure 4.11 The combination of IL-21 supplementation at start of cultivation, IL-2 and irradiated EBV-LCL feeder cells enable a significant and long-lasting expansion of NK cells. (A) NK cells were cultivated with IL-2 (gray bars) or with IL-2 and 100 ng/mL IL-21 (red bars) in the presence or absence of irradiated EBV-LCL feeder cells. The expansion of NK cells was measured after 7 days. Displayed are mean values and standard deviation of eight NK cell donors and statistical significance was tested by paired Student's t-test. (B) NK cells were labeled with CellTrace Violet Proliferation Dye and cultivated with IL-2 (gray histograms) or IL-2 and 100 ng/mL IL-21 (red histograms) in the presence or absence of EBV-LCL feeder cells. The dilution of CellTrace Violet corresponding to cell proliferation was analyzed by flow cytometry at day 3 and day 5. A representative donor out of three donors is shown. (C) NK cells were co-cultured with irradiated EBV-LCL feeder cells and IL-2 at different concentrations of IL-21. Mean NK cell fold expansion and SD are displayed for three donors. (D) NK cells were co-cultured with EBV-LCL feeder cells and IL-2. IL-21 (100 ng/mL) was supplemented only at day 0 (red dots) or permanently (red open squares), meaning at day 0, 7, 9, 11, 13, 16, 18, 20 and 26 when fresh medium was added. Mean NK cell expansion fold and standard deviation of six donors are shown at different time points. (E) NK cells were cultured with IL-2 and 100 ng/mL IL-21 was added at day 0. NK cell expansion was initiated by EBV-LCL co-culture at day 0 and re-stimulation with irradiated EBV-LCL was performed at day 13, 26 and 39 (red line and dots). In addition, the expansion without EBV-LCL re-stimulation at day 13, 26 or 29 was tested and monitored for 7 days (gray line and dots). Shown are mean and range of the NK cell expansion folds of six donors at different time points.

Taken together, a highly efficient method for expansion of NK cells was developed using IL-21 supplementation only at the start of culture and repeated exposure of the NK cells to irradiated EBV-LCL feeder cells in the presence of IL-2. This protocol, which results in superior NK cell expansion using EBV-LCL and IL-2 alone, is further referred in the remainder of the manuscript as the "optimized expansion method".

4.2.2 IL-21 induces IL-10 production of EBV-LCL, but feeder cell derived IL-10 does not affect the expansion of NK cells

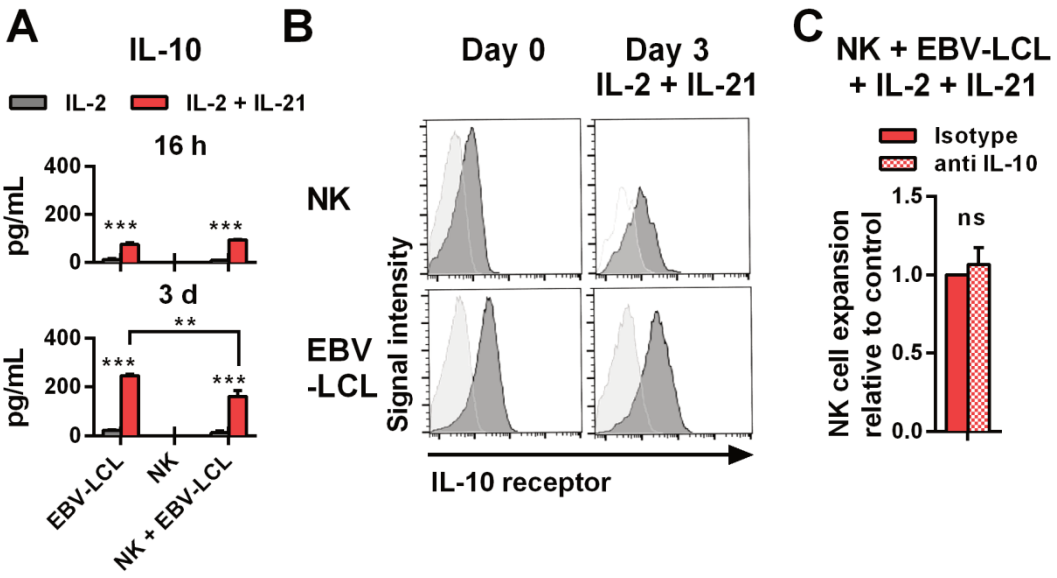


Figure 4.12 EBV-LCL produce elevated levels of IL-10 in presence of IL-21, but IL-10 is not responsible for enhanced NK cell expansion. (A) NK cells and EBV-LCL feeder cells were cultivated together or separated with IL-2 (gray bars) or with IL-2 and IL-21 (red bars). After 16 hours or 3 days the cultivation was terminated and the concentration of IL-10 in the supernatant was analyzed by a multiplex bead-array assay. Displayed are mean and SD for triplicate cultures with three different NK cell donors. (B) NK cells or irradiated EBV-LCL feeder cells were cultivated with IL-2 (gray bars) or with IL-2 and IL-21 (red bars). Representative histograms are displayed showing staining of the cells for human IL-10 receptor (dark gray) and isotype control (light gray) prior to cultivation and at day three of culture. (C) NK cells and irradiated EBV-LCL feeder cells were co-cultivated with IL-2 and IL-21, either together with an IL-10 neutralizing antibody (red-white checkered) or with an isotype control (red). The expansion of NK cells was measured after 7 days and for six donors the mean and SD of the NK cell expansion is shown relative to the isotype control. Statistical significance was determined by Student's t-test.

So far, little is known about the underlying mechanisms of NK cell proliferation and the exact role of EBV-LCL feeder cells as a trigger for NK cell expansion. Therefore, the EBV-LCL-based NK cell expansion and the observed effect of IL-21 in this context were investigated in more detail. First, it was checked whether cytokine release by irradiated EBV-LCL could explain the stimulating effect on NK cells. Supernatants of cultures containing irradiated EBV-LCL were tested for GM-CSF, IFN-α, TNF-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 and IL-17A. Independent of the presence of IL-21, none of these cytokines was found in a

noticeable amount within the first days of culture, with the exception of IL-10. Irradiated EBV-LCL produced significant levels of IL-10 in the presence of IL-21, whereas minor concentrations of EBV-LCL derived IL-10 was measured without IL-21 (Figure 4.12 A). Remarkably, the concentrations of IL-10 after three days of cultivation were significantly lower in cultures of EBV-LCL together with NK cells compared to EBV-LCL alone, indicating an uptake and consumption of IL-10 by NK cells. EBV-LCL and NK cells both express the IL-10 receptor as confirmed by flow cytometry (Figure 4.12 B). Nevertheless, adding an IL-10 neutralizing antibody had no consequence on the EBV-LCL-mediated expansion of NK cells in the presence of IL-21 (Figure 4.12 C). In conclusion, none of the tested cytokines played a role for the EBV-LCL-mediated NK cell expansion.

4.2.3 EBV-LCL-dependent NK cell expansion relies on direct cell-cell contact

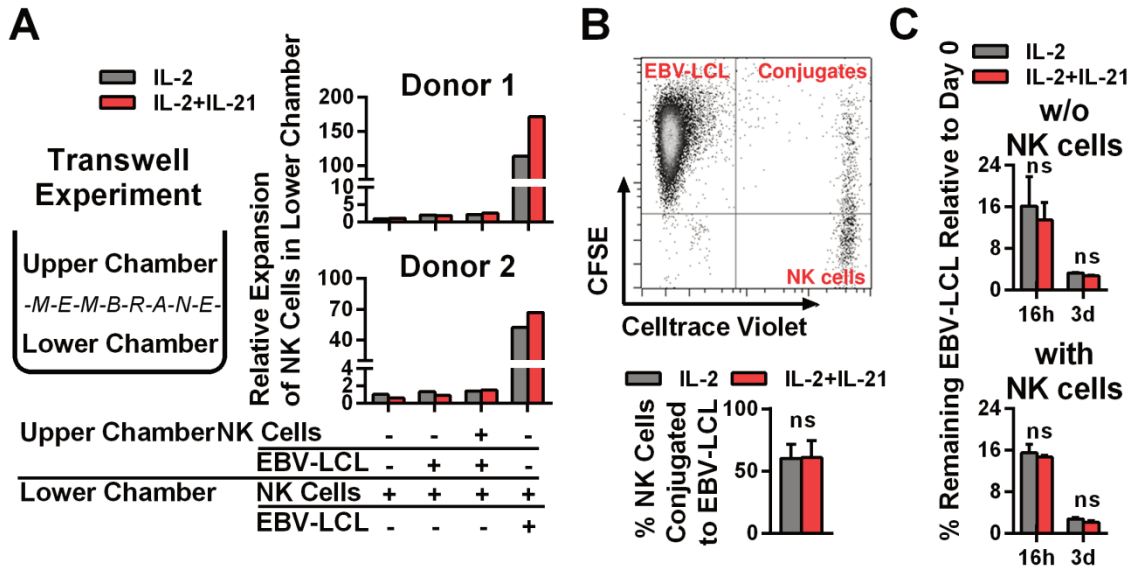


Figure 4.13 EBV-LCL-dependent NK cell expansion requires cell-cell contact and IL-21 neither influences the conjugate formation of NK cells and EBV-LCL nor does it affect the number of EBV-LCL during cultivation. (A) NK cells and irradiated EBV-LCL feeder cells were cultivated with IL-2 (gray) or with IL-2 and IL-21 (red) in the upper or lower chamber of transwells, as indicated. The NK cells in the lower chamber were quantified after 7 days and the displayed number is normalized to the condition without other cells added in the upper chamber. For two donors the mean of duplicate cultures each are shown. (B) Celltrace Violet dye labeled NK cells were cultivated together with irradiated and CFSE labeled EBV-LCL feeder cells at a ratio of 1:20 with IL-2 (gray) or with IL-2 and IL-21(red). The frequency of NK cells that are conjugated to EBV-LCL were analyzed after 16 hours by flow cytometry as displayed in a representative dot plot. Mean and SD are shown for three different NK cell donors. (C) Cells were cultivated as described in B and the number of EBV-LCL after 16 hours and 3 days are displayed in relation to the starting number. Mean and SD are shown for three different NK cell donors. Statistical significance was determined by Student's t-test.

Next, to investigate whether the EBV-LCL-dependent NK cell expansion requires cell-cell contact rather than soluble factors, NK cells and irradiated EBV-LCL were cultured in different compartments of a transwell plate, containing a filter membrane that allows exchange of soluble factor but no direct interactions of the cells (Figure 4.13 A). Because it's possible that soluble factors are not released until the feeder cells and NK cells are in contact, it was also tested to culture NK cells separated from irradiated EBV-LCL that were themselves in contact with another set of NK cells. However, the typical EBV-LCL-mediated high NK cell expansion was only achieved when NK cells and feeder cells were cultivated within the same compartment, allowing direct interactions between the two cell types. So, it was shown that direct cell-cell contact was essential for the EBV-LCL-dependent NK cell expansion, whereas EBV-LCL derived soluble factors appeared irrelevant. After clarifying the importance of cell-cell contact, it was tested whether IL-21 affects the conjugate formation between NK cells and irradiated EBV-LCL. But the frequency of formed conjugates between NK cells and EBV-LCL was comparable in the presence and absence of IL-21 (Figure 4.13 B). Next, it was tested whether IL-21 could enhance the viability of irradiated EBV-LCL, possibly ensuring that more EBV-LCL are available for interaction with NK cells. However, quantification of EBV-LCL during cultivation did not reveal any difference related to IL-21 (Figure 4.13 C). Probably because of the harsh irradiation, three days after start of cultivation the vast majority of EBV-LCL feeder cells have died already and disappeared independent of IL-21. Of note, the same number of EBV-LCL cells was found when EBV-LCL were cultured alone or together with NK cells, indicating that lysis of EBV-LCL by NK cells did not take place or was negligible.

Then, EBV-LCL feeder cells were investigated for surface ligands with potential importance for the NK-EBV-LCL interaction (Figure 4.14). It was also tested whether irradiation and culture of the EBV-LCL changed the marker expression. EBV-LCL did not express MICA, MICB or ULPBs, the ligands for the NK cell activating receptor NKG2D. CD155 was found on the surface of EBV-LCL and could possibly activate DNAM-1 expressing NK cells. Furthermore, the feeder cells were positive for CD137L, a ligand considered to trigger NK cell expansion and one of the factors expressed by engineered K562 feeder cells that commonly used for NK cell expansion. EBV-LCL expressed high levels of CD48, the ligand for 2B4 expressed by NK cells, which is known to play a role for the proliferation of murine NK cells in homotypic NK-to-NK cell interactions.[357]

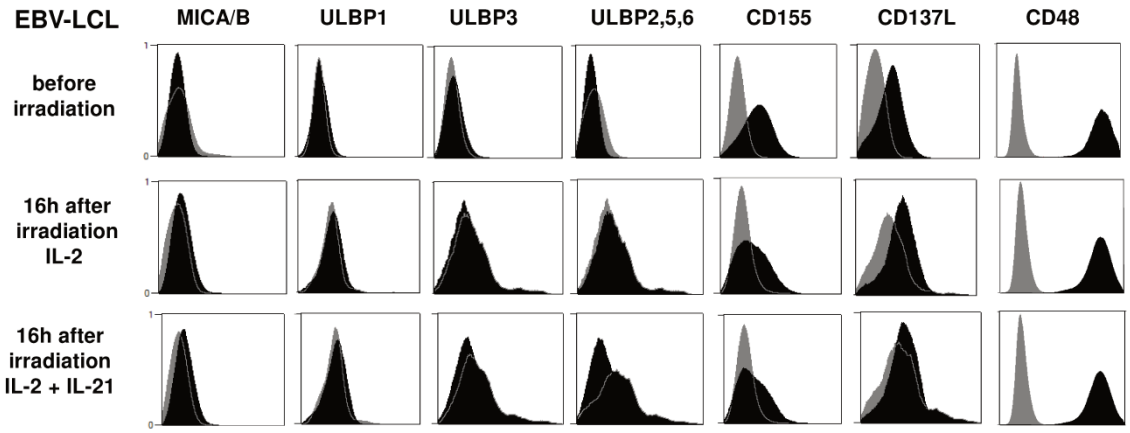


Figure 4.14 EBV-LCL express CD155, CD137L and CD48, but lack expression of NKG2D ligands. EBV-LCL were analyzed before and after irradiation for different surface markers, as indicated. After irradiation the cells were cultured for 16 h in medium containing IL-2 or IL-2 and IL-21, corresponding to the same conditions used for co-culture with NK cells.

4.2.1 NK cells exhibit significant expression of CD25 upon contact with EBV-LCL

Same as the expression of surface ligands by EBV-LCL, the marker repertoire of NK cells is of importance for the interplay between NK cells and EBV-LCL. NK cells change their marker expression during *ex vivo* expansion as described before (chapters 4.1.4, 4.1.5). Consequently, culture conditions, such as IL-21 supplementation, could specifically modify the marker profile of NK cells, possibly affecting relevant receptor-ligand interactions. Therefore, the surface marker expression of NK cells was analyzed by flow cytometry during the first days of culture when the proliferation is initiated. Staining of proliferating NK cells for several surface markers did not reveal significant differences specifically occurring upon EBV-LCL co-culture or upon IL-21 supplementation, with the exception of CD25 (Figure 4.15). Proliferating NK cells generally showed an enhanced expression of the activating receptors DNAM-1, NKG2D and TRAIL for example, independent of the presence of absence of irradiated EBV-LCL and IL-21. In contrast, proliferating NK cells in co-culture with irradiated EBV-LCL rapidly expressed high levels of CD25, whereas proliferating NK cells in the absence of irradiated EBV-LCL stayed mainly CD25 negative such as naive NK cells. Therefore, expression of CD25 possibly represents a relevant surface molecule for the pronounced EBV-LCL-mediated NK cell expansion

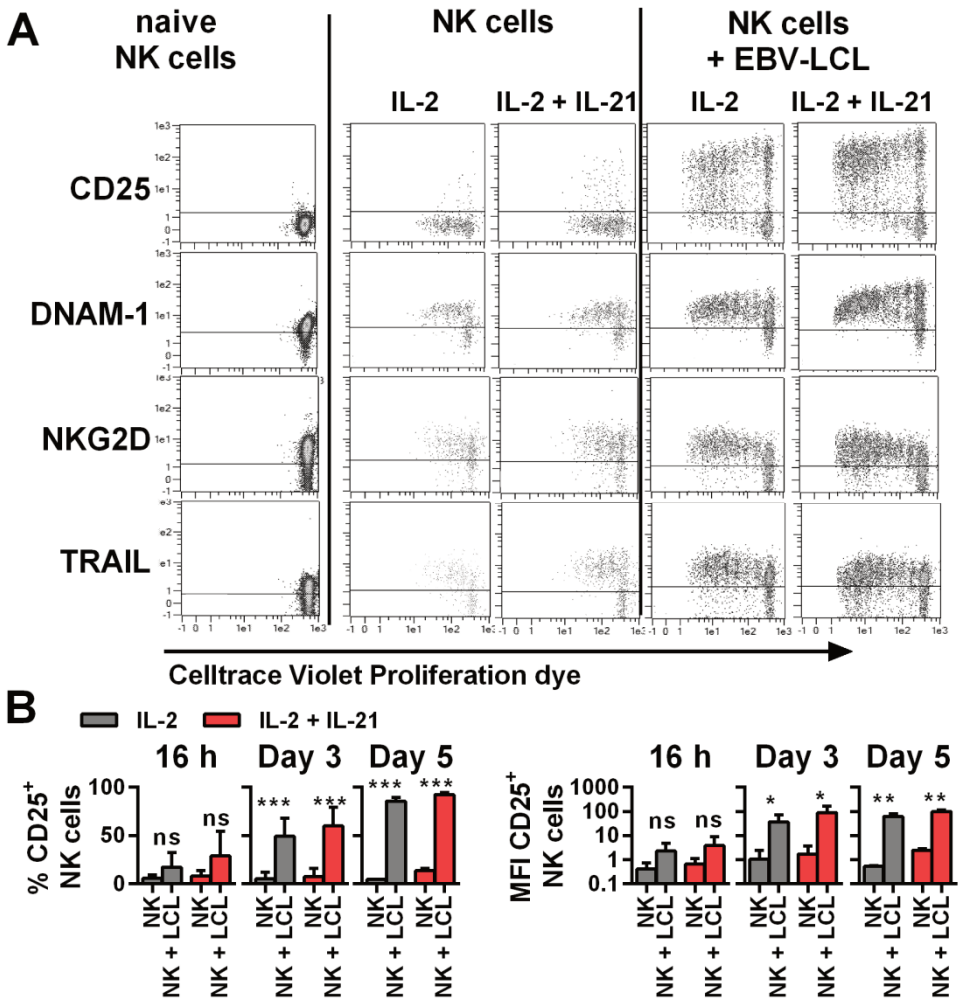


Figure 4.15 Proliferating NK cells in co-culture with EBV-LCL strongly up-regulate CD25, whereas regulation of other NK cell markers is independent of EBV-LCL. NK cells were labeled with Violet Proliferation dye and cultured with IL-2 (gray bars) and with or without IL-21 (red bars) in the presence or absence of EBV-LCL feeder cells. (A) The cells were stained for different surface markers before and 5 days after starting the cultivation and representative dot plots are shown. (B) NK cells were cultured as described and CD25 expression of NK cells was analyzed by flow cytometry after 16 hours, 3 days and 5 days. Displayed are mean and SD from three donors for the frequencies of CD25 expression (left) and the mean fluorescence intensities (MFI)(right). Differences between NK cell expansions obtained without EBV-LCL and NK cell expansions obtained with EBV-LCL were tested for statistical significance using the paired Student's t-test.

To further investigate factors with a potential relevance for the interaction between NK cells and irradiated EBV-LCL, the cells were cultured together with blocking antibodies against selected cell surface molecules (Figure 4.16 A). For most of the targeted surface markers no significant effect was observed, indicating that these receptor-ligand pairs were irrelevant.

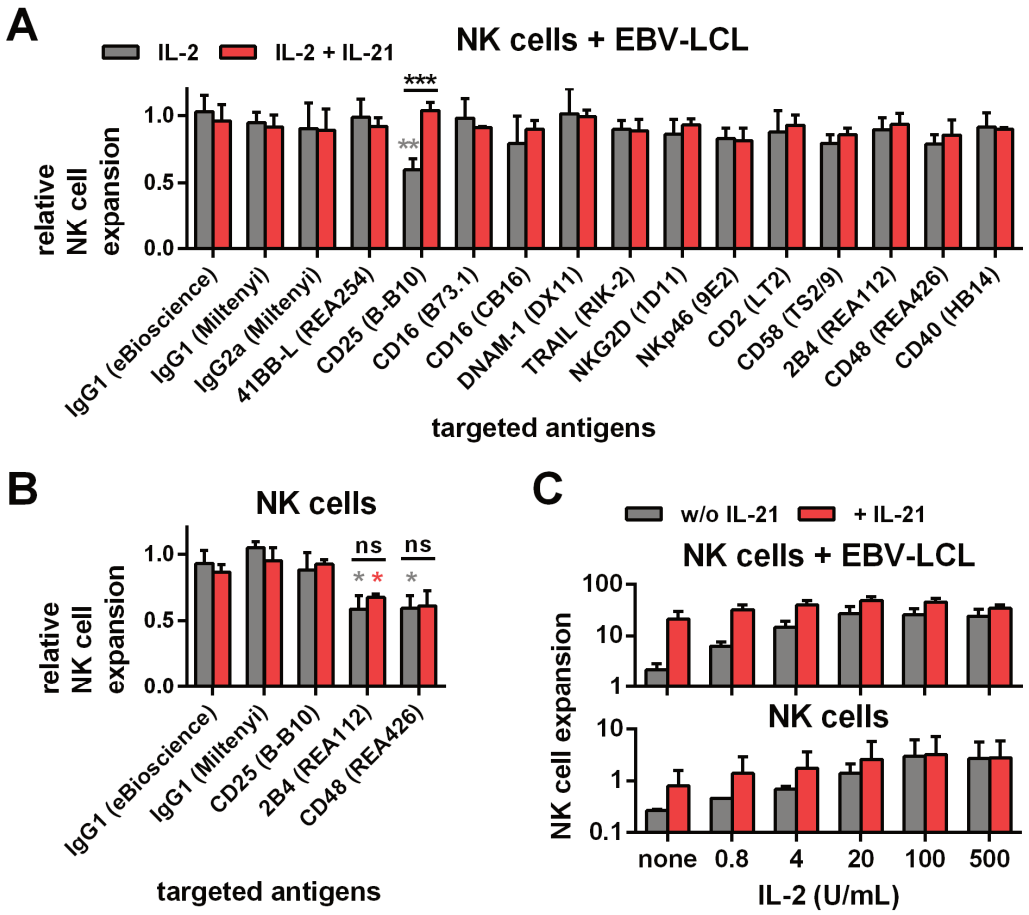


Figure 4.16 CD25 carries a functional role for NK cell expansion in co-culture with EBV-LCL, but CD25 becomes dispensable when IL-21 is present. NK cells were cultivated with 500 U/mL IL-2 (gray bars) or with IL-2 and IL-21 (red bars) in the presence or absence of EBV-LCL feeder cells. (A) Blocking antibodies (10 µg/mL) or medium as a control was added to the cultures with EBV-LCL and expansion of NK cells was measured after 7 days. To better compare different donors the expansion of the different cultures was calculated relative to the medium control for each culture. Mean and SD of the relative expansion fold from three to six donors are displayed and statistical significance was determined by paired Student's t-test. (B) Blocking experiments as described in A were performed for cultures with NK cells lacking EBV-LCL. Mean and SD of the relative expansion fold from three donors are displayed and statistical significance was determined by paired Student's t-test. (C) NK cells were cultured at different IL-2 concentrations, as indicated. The NK cell expansion was determined after 7 days and mean and SD of the NK cell expansion fold from three donors is shown.

Blocking CD25 clearly reduced the expansion of NK cells in culture with IL-2 and irradiated EBV-LCL, but surprisingly, this effect was completely abolished when IL-21 was present (Figure 4.16 A). Thus, CD25 expression indeed carried out a function during EBV-LCL-based NK cell expansion, but this function could be bypassed by IL-21, making CD25 expression dispensable. In the absence of irradiated EBV-LCL the blocking of CD25 had no effect on the expansion of NK cells, as expected due to the lack of CD25 expression under this condition (Figure 4.16 B). The results implied different IL-2 dependencies of the differentially expanded NK cells and the influence of the IL-2 concentration on NK cell expansion was tested (Figure 4.16 C). The EBV-LCL-mediated NK cell expansion profoundly decreased when the culture

was started with IL-2 concentrations lower than 20 U/mL and IL-21 was lacking. In contrast, the EBV-LCL-mediated NK cell expansion was nearly independent of IL-2 when IL-21 was present, showing that IL-21 definitely compensates for IL-2 signaling. Of note, IL-21 also compensated the lack of IL-2 when irradiated EBV-LCL feeder cells were not present, but then the effect was less pronounced. In general, concentrations of IL-2 higher than a specific threshold did not further increase the NK cell expansion. This seemed logical, because beyond a certain threshold the IL-2 concentration efficiently triggers IL-2 signaling through the intermediate affinity IL-2Rßγ receptor without the need for CD25 to form the high affinity IL-2Rαßγ receptor. Therefore, CD25 is considered redundant for efficient IL-2 signaling at higher IL-2 concentrations. However, standard NK cell cultivation as well as the blocking experiments were performed at a very high IL-2 concentration of 500 U/mL. Therefore, it could be demonstrated that CD25 is of functional importance for EBV-LCL-mediated NK cell expansion even at high concentrations of IL-2, because blocking of CD25 clearly reduced the expansion performance. The fact that IL-21 allows NK cells to expand more independently from IL-2 could explain why EBV-LCL-mediated NK cell expansion is generally increased by IL-21 and why blocking of CD25 by blocking monoclonal antibodies has no effect when IL-21 is present.

It's published that cell-cell interactions between murine NK cells via 2B4 and its ligand CD48 facilitate increased NK cell proliferation involving enhanced IL-2 signaling,[357,358] and it was further investigated whether this plays a role for the EBV-LCL-based NK cell expansion. Indeed, blocking of 2B4 or its ligand CD48 significantly decreased the expansion of NK cells in the absence of irradiated EBV-LCL (Figure 4.16 B), confirming the data from studies with mice. Nevertheless, 2B4 and CD48 seemed not necessary for the expansion of NK cells in co-culture with irradiated EBV-LCL, because blocking of these markers had no obvious effect (Figure 4.16 A). Thus, the exact factors that stimulate the NK cell proliferation upon cell-cell contact with irradiated EBV-LCL remained elusive. Nevertheless, the importance of CD25, which can be bypassed by IL-21, suggested an enhanced signaling through the common γ-chain receptor as one underlying mechanism.

4.2.2 EBV-LCL co-culture combined with IL-21 supplementation yields NK cells with potent anti-tumor functions *in vitro*

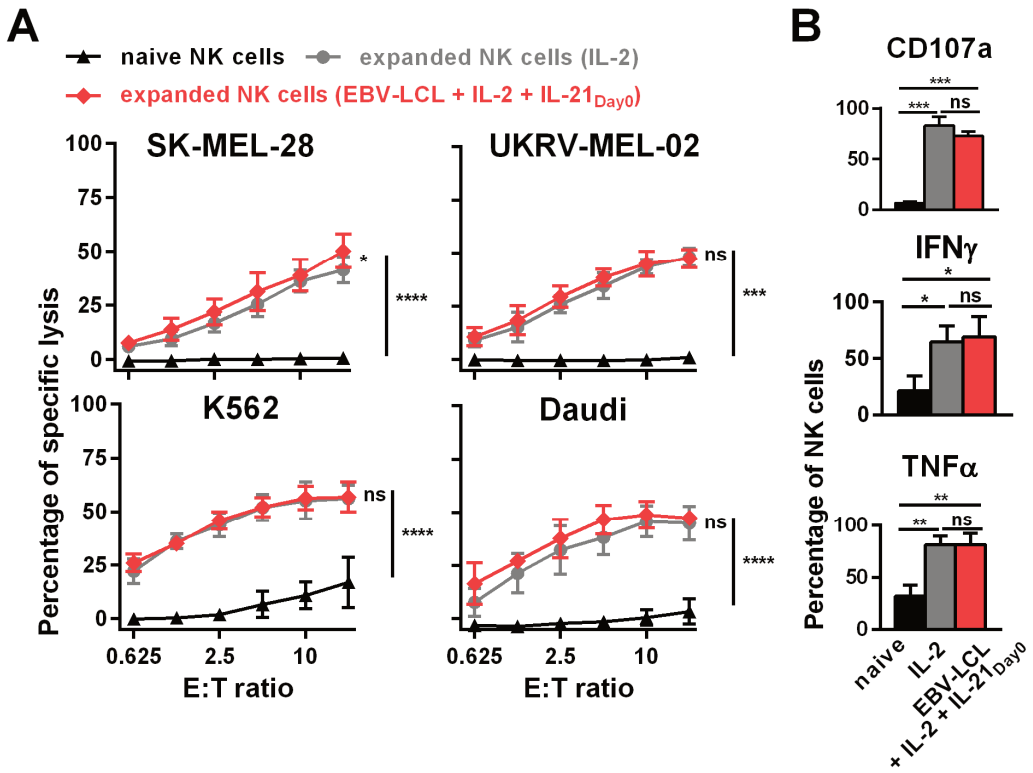


Figure 4.17 *Ex vivo* expanded NK cells were highly cytotoxic against different tumor cell lines and showed enhanced degranulation and production of IFN-γ and TNF-α. (A) Different NK cells were tested for cytotoxicity against four tumor cell lines using a standard chromium release assay. Specific lysis at different effector-to-target (E:T) ratios is shown for freshly isolated NK cells (black) and NK cells that have been expanded for 13 or 14 days, either with IL-2 (gray) or by use of IL-2, irradiated EBV-LCL and IL-21 supplemented at day 0 (red). Displayed are mean values and standard deviation of NK cells from 4-8 donors per target cell line and statistical significance was tested by Student's t-test. (B) NK cells were expanded as described in B and tested for degranulation and production of IFN-γ and TNF-α by flow cytometry upon stimulation with PMA/Iono. Displayed are mean values and SD of NK cells from five donors. Statistical significance was tested by paired Student's t-test.

To finally estimate the therapeutic value of NK cells that were obtained with the optimized expansion method, the functional competence of the cells was investigated *in vitro*. Before, it was verified that NK cells that have expanded *ex vivo* with irradiated EBV-LCL show similar cytotoxicity as long-term IL-2 activated NK cells without feeder cells (chapter 4.1.2). IL-2 activated NK cells have proven clinical applicability and can be seen as a standard. NK cells generated by the optimized expansion method showed similar cytolytic activity as IL-2 activated NK cells that were cultured without feeder cells (Figure 4.17 A). In contrast, freshly isolated, naive NK cells possessed only low cytotoxicity against leukemic K562 and Daudi cells and lacked cytotoxicity against SK-MEL-28 and UKRV-Mel-02 melanoma cells, confirming that *ex vivo* culturing led to NK cell activation and enhanced NK cell-mediated anti-tumor

activity. Compared to naive NK cells, *ex vivo* expanded NK cells displayed a significantly higher degranulation upon stimulation (Figure 4.17B). In addition, among *ex vivo* expanded NK cells the production of the immune stimulatory cytokines IFN-γ and TNF-α was significantly increased compared to naive NK cells. In conclusion, the optimized expansion protocol not only increased the numbers of NK cells but also enhanced their *in vitro* anti-tumor activity compared to freshly isolated NK cells from peripheral blood.

Taken together, it was shown that an improved and highly efficient *ex vivo* expansion of NK cells over long time is achieved by repeated stimulation with irradiated EBV-LCL feeder cells, IL-2 and initial supplementation of IL-21. It was demonstrated that the stimulating effect of EBV-LCL is not primarily dependent on soluble factors and requires direct cell contact. The developed NK cell expansion protocol makes it possible to produce high numbers of NK cells with potent effector function and therefore represents a highly relevant method for NK cell based adoptive therapy.

4.3 Evaluation of expanded human NK cells for therapeutic efficacy using a xenograft mouse model

After successful development of an optimized method for *ex vivo* NK cell expansion in the previous chapter, the *ex vivo* generated NK cells were tested for their anti-tumor efficacy *in vivo* using a xenograft mouse model, representing a meaningful preclinical evaluation.

4.3.1 High numbers of expanded human NK cells show anti-tumor effect in a xenograft "co-injection model"

First, the engraftment of human tumor cells in NSG mice was tested by i.v. injection of SK-MEL-28 melanoma cells. The mice were irradiated prior to the cell transfer, because irradiation is necessary for optimal engraftment of human melanoma cells into NSG mice as previously shown.[359] Furthermore, it was investigated whether tumor formation and growth can be prevented by simultaneous injection of *ex vivo* cultivated NK cells. This approach was previously established by Matthias Miller, a former PhD student in the lab, and named "co-injection model" due to the parallel injection of tumor cells and NK cells.[359]

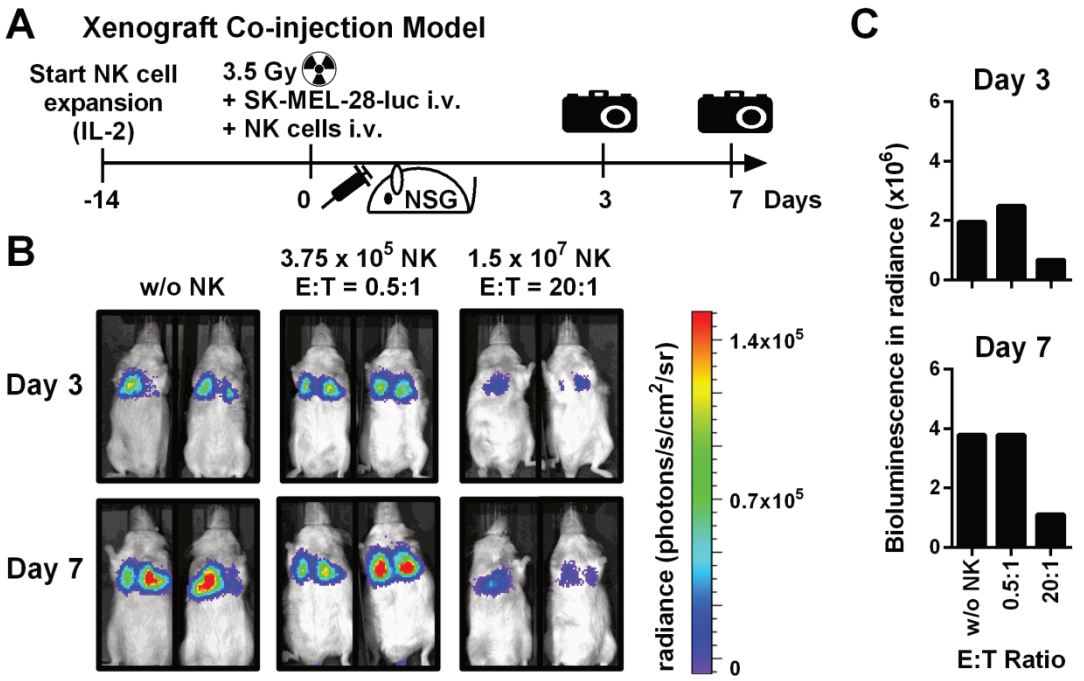


Figure 4.18 *Ex vivo* cultivated NK cells show anti-tumor activity in a xenograft "co-injection model" when injected at high cell doses. (A) Mice were irradiated and received human SK-MEL-28 melanoma cells expressing luciferase in addition to PBS, as a control, or NK cells by intravenous injection. Prior to injection, the NK cells were expanded by cultivation in IL-2 containing medium for 14 days. The tumor load was monitored by measuring the luciferase activity. The tumor loads at day 3 and day 7 were measured for mice that were treated with PBS ("w/o NK") or with NK cells at different effector-to-target ratios (2 mice per group). Bioluminescence pictures of the mice are shown in B and diagrams for the mean bioluminescence are shown in C.

Successful engraftment of SK-MEL-28 cells was observed in the lungs of the mice three days after transfer of the cells and an increased signal intensity seven days after injection indicated sustained growth of the tumors *in vivo* (Figure 4.18 B and C). As a first trial, NK cells that were cultivated with IL-2 for 14 days were evaluated. Transferring a high dose (1.5×10^7) of long-term IL-2 activated NK cells together with the tumor cells at an effector-to-target ratio of 20:1 resulted in a lower tumor burden compared to mice that did not receive NK cells (Figure 4.18 B and C). However, applying less NK cells at a lower E:T ratio of 0.5:1 had no obvious effect on the tumor load (Figure 4.18 B and C). This experiment demonstrated that tumors with human origin can be established in NSG mice and the mice tolerate injection of high NK cell numbers without unexpected side effects. The results further confirmed that transferred NK cells can mediate anti-tumor activity *in vivo* and it's possible to monitor the effect by means of this model.

4.3.2 High numbers of *ex vivo* generated NK cells control tumor growth in a therapeutic xenograft model

In a subsequent approach, tumors were established in the mice prior to the treatment with NK cells to better reflect the therapeutic situation. This xenograft mouse model was performed to extensively evaluate the efficacy of a therapeutic NK cell transfer (Figure 4.19 A). Human tumor cells successfully engrafted again three days after injection. Then, NK cells, which have been expanded by the optimized expansion method, were injected into tumor bearing mice. IL-2 was injected repeatedly to support the *in vivo* persistence of transferred NK cells. Single doses of up to 30×10^6 NK cells per mouse were tolerated without any noticeable side effects. With an average weight of about 30 g per mouse the used dose corresponds to 10^9 NK cells per kg, representing the upper limit of NK cell doses considered for human studies. In the control group without transfer of NK cells, the tumor load significantly increased within the two weeks follow-up period (Figure 4.19 B). In contrast, treatment with expanded NK cells significantly controlled the tumor growth, indicating a potent anti-tumor activity of the *ex vivo* generated NK cells *in vivo*. Furthermore, there appeared to be a dose response relationship in terms of the number of injected NK cells correlating with control of tumor growth, with the highest dose of 30×10^6 injected NK cells showing the best therapeutic effect (Figure 4.19 C). Injection of high human NK cell numbers also correlated with high numbers of NK cells that could be retrieved from blood and lungs 14 days after NK cell transfer (Figure 4.19 D). Thus, transferring higher numbers of NK cells resulted in increased numbers of NK cells *in vivo* post injection. Nevertheless, NK cell numbers continuously declined after the transfer (Figure 4.19 E), indicating that transferred NK cells were unable to sustain continuous expansion *in vivo*. In

conclusion, adoptive transfer of NK cells generated with the optimized protocol resulted in efficient control of tumor in a xenograft mouse model without noticeable side effects.

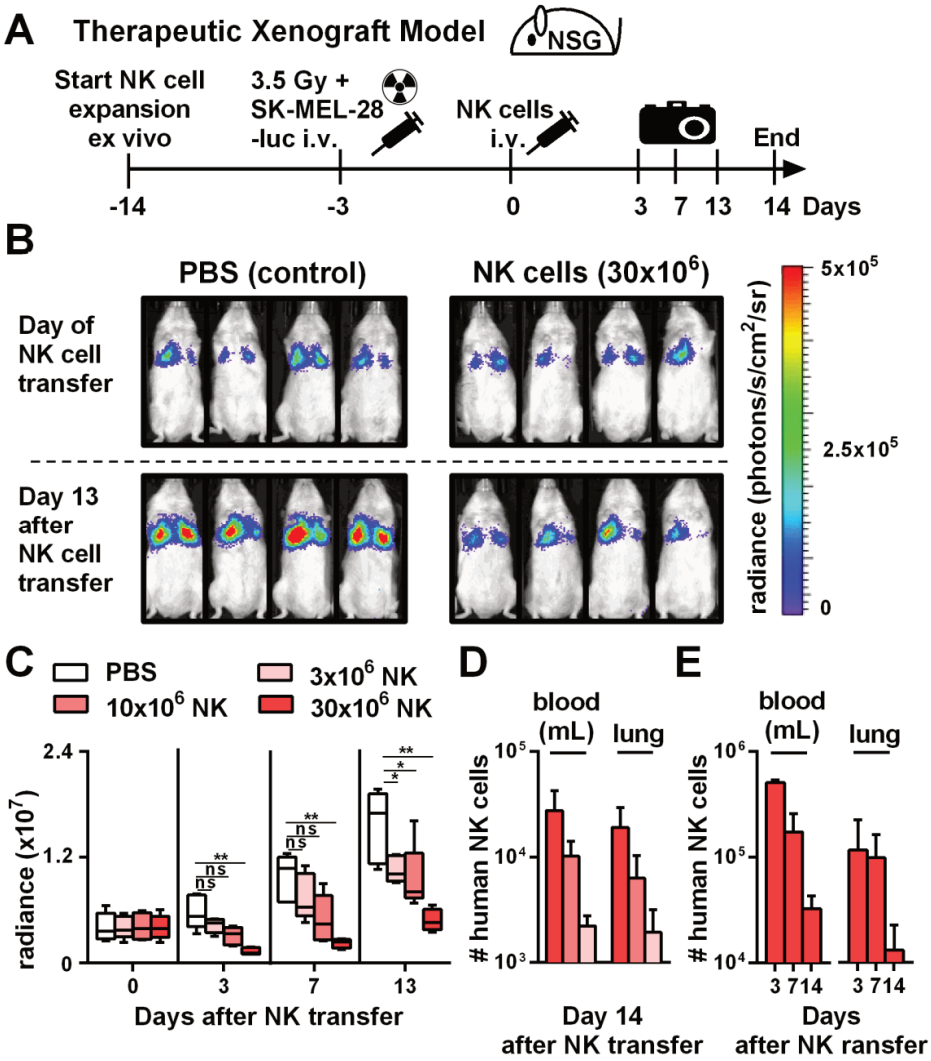


Figure 4.19 Adoptive transfer of NK cells expanded by the optimized protocol in tumor bearing mice resulted in pronounced tumor growth control. (A) Scheme is shown for evaluation of expanded NK cells *in vivo* using a xenograft model. Mice were irradiated and received human SK-MEL-28 melanoma cells expressing luciferase by intravenous (i.v.) injection. Three days later, after tumor engraftment, the mice were treated (i.v.) with human NK cells expanded with the optimized protocol and the tumor load was monitored by luciferase activity. IL-2 was repeatedly injected intraperitoneal. (B) Tumor bearing mice as described in A were treated with PBS, as a control, or with 30 x 10^6 NK cells that were expanded for 14 days using the optimized expansion protocol. The pictures display the bioluminescence (radiance) showing the *in vivo* luciferase activity of four representative mice of each group at the day of NK cell transfer (top) and 13 days thereafter (bottom). (C) Tumor bearing mice were treated with NK cells as described in B using different NK cell doses. Mean and range of the tumor burden, measured by bioluminescence, is shown at different time points for one representative experiment with 4-5 mice per group. (D) Mice were treated as described in C, and the transferred human NK cells were re-isolated from blood and lungs of the mice 14 days after NK cell injection and were enumerated using flow cytometry. Mean and standard deviation of NK cell numbers per lung and per mL of blood are shown for four mice per group. (E) Tumor bearing mice were treated with 30 x 10^6 expanded NK cells as described in B and at day 3, 7 and 14 the mice were sacrificed and human NK cells were re-isolated from blood and lungs. Mean and standard deviation of NK cell numbers per lung or mL of blood are shown for four mice per group. Statistical significance in all experiments was tested by Student's t-test.

4.3.3 *Ex vivo* generated NK cells change their phenotype and function *in vivo*

Next, changes in phenotype and function of NK cells *in vivo* were monitored and NK cells that were isolated from the lungs shortly after adoptive transfer were analyzed in comparison to naive NK cells and *ex vivo* expanded NK cells (Figure 4.20 A). Based on previous results showing that NK cells greatly change their surface marker profile upon *ex vivo* activation (chapter 4.1.4), TRAIL, DNAM-1 and NKG2D surface markers were selected to assess NK cell activation. As expected, *ex vivo* expanded and activated NK cells exhibited up-regulated TRAIL, DNAM-1 and NKG2D compared to naive NK cells (Figure 4.20 B, C). Intriguingly, three days after adoptive transfer of *ex vivo* expanded NK cells, the cells expressed again low levels of TRAIL, DNAM-1 and NKG2D similar to naive NK cells.

Next, the functional activity of NK cells was tested after their adoptive transfer. As expected from the low expression of TRAIL, DNAM-1 and NKG2D, NK cells retrieved from mice 3 days after adoptive transfer had low cytotoxicity against SK-MEL-28 and K562 target cells, compared to NK cells expanded from the same donors that were maintained in *ex vivo* cell culture (Figure 4.20 D). In line with the reduced cytotoxicity, re-isolated NK cells had a diminished potential for degranulation compared to expanded NK cells that were maintained in *ex vivo* cell culture. Importantly, the ability of *ex vivo* activated NK cells to produce IFN-γ and TNF-α was retained *in vivo* and remained at significantly higher levels than that observed with naive NK cells. In an additional experiment NK cells were re-isolated also at later time points after NK cell injection (Figure 4.21). The results indicated that the high productivity of IFN-γ and TNF-α was still retained after 7 and 14 days following NK cell injection, but the experiment was performed only once with NK cells from a single donor. Of note, similar data as obtained with NK cells isolated from lungs as shown in Figure 4.20 were obtained with NK cells from blood (data not shown). Taken together, although the increased potential for degranulation and direct killing of tumor cells was short lived *in vivo*, the data suggested that NK cells generated by the optimized protocol had a sustained competence for cytokine production *in vivo*.

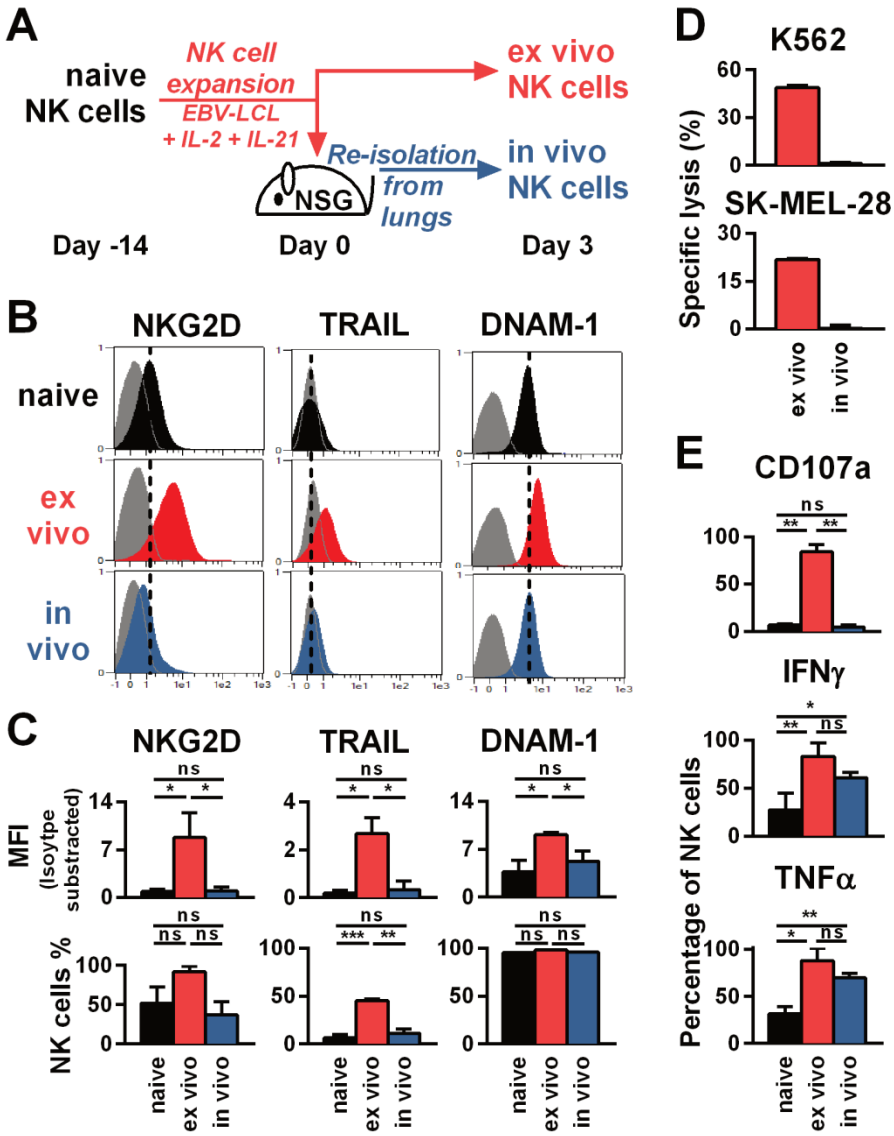


Figure 4.20 After transfer into NSG mice, *ex vivo* expanded NK cells rapidly changed their phenotype and lost their cytotoxicity and potential for degranulation, while they retained an enhanced ability to produce IFN-γ and TNF-α. (A) Scheme for characterization of three types of NK cells from the same donor. First, freshly isolated, naive NK cells were analyzed (black). Second, NK cells were expanded *ex vivo* for 17 days by use of short term IL-21 stimulation, IL-2 and irradiated EBV-LCL feeder cells (red). Third, NK cells were expanded in the same way for 14 days before they were transferred to tumor bearing mice (30 x 10^6 NK cells per mouse)(blue). Three days after the transfer, the transferred human NK cells were re-isolated from the mouse lungs and NK cells from 3-4 mice were pooled per donor (blue). (B) The differentially prepared NK cells as described in A were analyzed for the surface markers NKG2D, TRAIl and DNAM-1 by flow cytometry. Histograms for one representative NK cell donor are shown (back) together with isotype controls (gray). (C) The flow cytometric analysis as described in B is applied for three different donors. For each marker the mean and standard deviation of all donors are shown for the mean fluorescence intensity (MFI) corrected by isotype subtraction (top) and the frequency of NK cells expressing the marker (bottom). (D) The differentially prepared NK cells as described in A were analyzed for cytotoxicity against K562 and SK-MEL-28 target cell lines at a 3:1 effector-to-target ratio. Mean and standard deviation of one out of two representative experiments are shown using two different NK cell donors. (E) The differentially prepared NK cells as described in A were analyzed for degranulation and production of IFN-γ and TNF-α upon stimulation with PMA/Iono. Mean and standard deviation of three different NK cell donors are displayed. Statistical significance was tested by paired Student's t-test in all experiments.

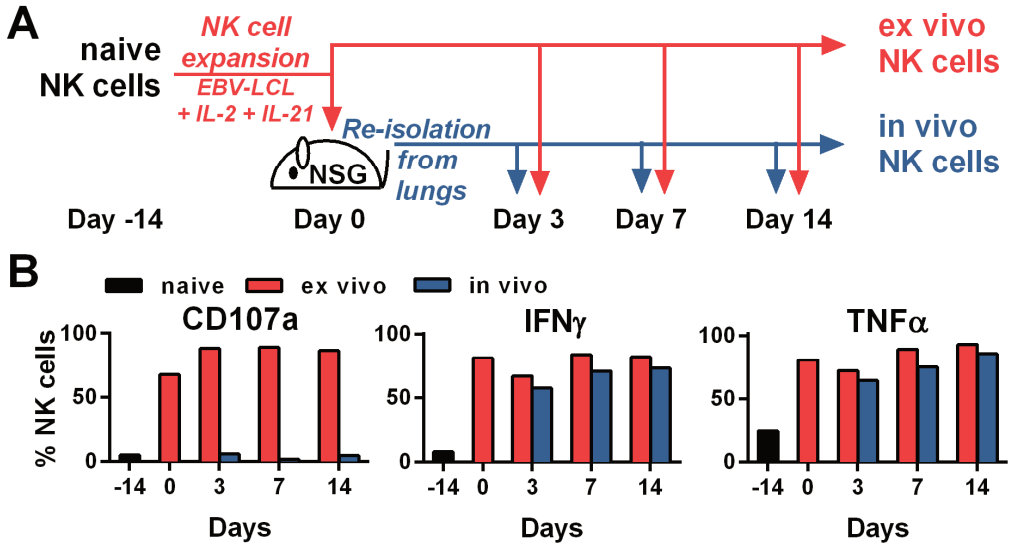


Figure 4.21 NK cells that have been expanded with the optimized protocol lose their high cytotoxic potential shortly after *in vivo* transfer, but they retain their high potential to produce IFN-γ and TNF-α over long time. (A) Scheme for characterization of three types of NK cells from the same donor. First, freshly isolated, naive NK cells were analyzed (black). Second, NK cells were expanded *ex vivo* for 17 days by use of short term IL-21 stimulation, IL-2 and irradiated EBV-LCL feeder cells (red). Third, NK cells were expanded in the same way for 14 days before they were transferred to tumor bearing mice (30 x 10^6 NK cells per mouse)(blue). After 3, 7 and 14 days following NK cell transfer, human NK cells were re-isolated from the mouse lungs and NK cells from four mice were pooled (blue). (B) The differentially prepared NK cells as described in A were analyzed for degranulation and production of IFN-γ and TNF-α upon stimulation with PMA/Iono. Displayed are results for NK cells from one donor.

Then, it was investigated whether long-term IL-2 activated NK cells show the same changes in phenotype and function upon *in vivo* transfer as NK cells obtained by the optimized protocol (Figure 4.22). Indeed, long-term IL-2 activated NK cells similarly reduced the expression of NKG2D, DNAM-1 and TRAIL and possessed a low potential for degranulation such as naive NK cells after *in vivo* transfer. However, in contrast to NK cells that have been generated by the optimized expansion method, long term IL-2 activated NK cells appeared also incapable of maintaining an enhanced productivity for IFN-γ and TNF-α *in vivo*. This implied a different functional quality of NK cells obtained by the optimized expansion method compared to conventionally used long-term IL-2 activated NK cells.

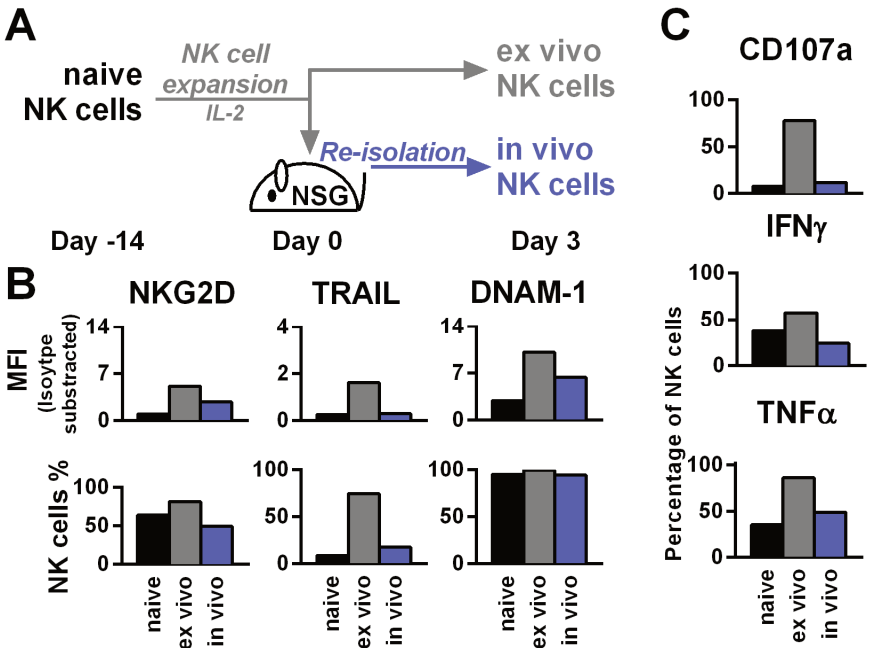


Figure 4.22 After transfer into NSG mice, NK cells that have been expanded with IL-2 and without irradiated EBV-LCL changed their phenotype and lost their *ex vivo* acquired functionality including enhanced production of IFN-γ and TNF-α. (A) Scheme for characterization of three types of NK cells from the same donor. First, freshly isolated, naive NK cells were analyzed (black). Second, NK cells were expanded *ex vivo* for 17 days in IL-2 containing medium.(gray). Third, NK cells were expanded in the same way for 14 days before they were transferred to tumor bearing mice (30 x 10^6 NK cells per mouse)(blue). Three days after the transfer, the transferred human NK cells were re-isolated from the mouse lungs and NK cells from two mice were pooled per donor (blue). (B) The differentially prepared NK cells as described in A were analyzed for the surface markers NKG2D, TRAIl and DNAM-1 by flow cytometry. For each marker the mean value of two analyzed donors is shown for the mean fluorescence intensity (MFI) corrected by isotype subtraction (top) and the frequency of NK cells expressing the marker (bottom). (C) The differentially prepared NK cells as described in A were analyzed for degranulation and production of IFN-γ and TNF-α upon stimulation with PMA/Iono. Mean values of two analyzed donors are displayed.

4.3.4 IFN-γ and TNF-α inhibit the growth of SK-MEL-28 cells *in vitro*

Because NK cells in the xenograft model exhibited a sustained potential to produce IFN-γ and TNF-α, it was tested whether these factors can directly contribute to the NK cell mediated anti-tumor efficacy. Since it was published recently that COLO-205 are highly sensitive and K562 are less susceptible for growth inhibition by IFN-γ and TNF-α,[119] the effect of IFN-γ and TNF-α on SK-MEL-28 melanoma target cells was analyzed in comparison to K562 and COLO-205 cells (Figure 4.23 A). As expected, IFN-γ and TNF-α strongly reduced the cell growth of COLO-205 cells to 28%, while the effect was less pronounced in case of K562 cells (71%). Similar to COLO-205 cells, the growth of SK-MEL-28 dropped to 20% in the presence of IFN-γ and TNF-α, suggesting a high sensitivity of SK-MEL-28 for IFN-γ and TNF-α. The growth inhibition was dose dependent and low concentrations of IFN-γ and TNF-α were less efficient but still sufficient to mediate a detectable effect *in vitro* (Figure 4.23 B).

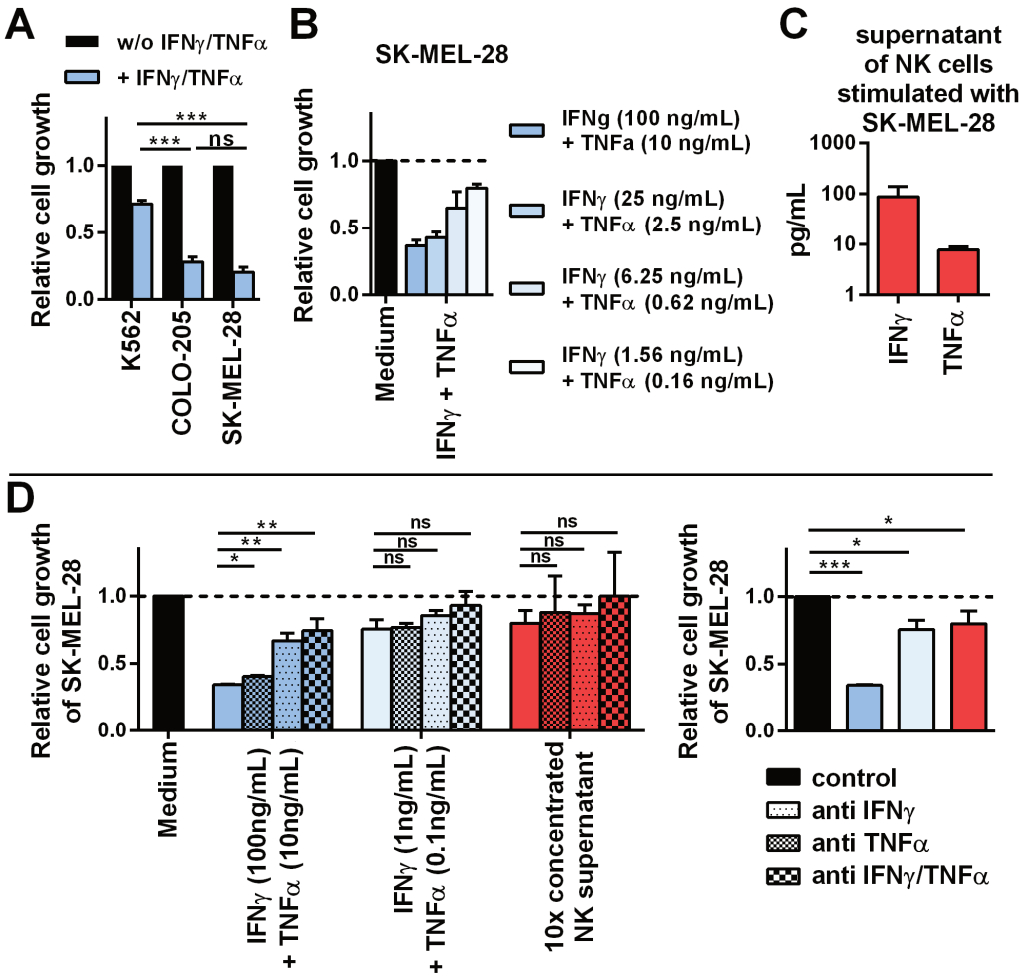


Figure 4.23 The growth of SK-MEL-28 melanoma target cells is inhibited by IFN-γ and TNF-α *in vitro*. (A) Different target cell lines were cultivated with or without IFN-γ (100 ng/mL) and TNF-α (10 ng/mL) for 4 days. The cell growth normalized to cultivation without IFN-γ and TNF-α is depicted for triplicate cultures. (B) Expanded NK cells were stimulated with SK-MEL-28 cells for 24 h and the culture supernatants were analyzed for IFN-γ and TNF-α using a multiplex bead-array assay. The NK cells were expanded before for 14 days by means of the optimized expansion protocol and mean and SD of three donors is shown. (C) SK-MEL-28 cells were cultured as described in A at different concentrations of IFN-γ and TNF-α. (D) SK-MEL-28 cells were cultured as described in C. Adding supernatants of stimulated NK cells as described in B were investigated in addition to IFN-γ and TNF-α. NK cell supernatants were 10-fold concentrated using centrifugal filters. Adding blocking antibodies (10 µg/mL) against IFN-γ or TNF-α at start of cultivation was tested. Mean and SD is shown for triplicate cultures and three different NK cell donors. Statistical significance was tested by Student's t-test.

Cultures of expanded NK cells that were stimulated with SK-MEL-28 cells contained concentrations of IFN-γ and TNF-α of about 100 pg/mL and 10 pg/mL, respectively (Figure 4.23 C). To evaluate NK cell derived IFN-γ and TNF-α for growth inhibition of SK-MEL-28, the supernatants were concentrated by factor 10 to reach the concentration range of IFN-γ and TNF-α that is required to detect a possible effect *in vitro*. Indeed, NK cell derived supernatants reduced the growth of SK-MEL-28 in the same way as recombinant IFN-γ and TNF-α did at low concentrations (Figure 4.23 D). Blocking antibodies against IFN-γ and TNF-α clearly

abrogate the inhibitory effect of recombinant IFN-γ and TNF-α at high concentrations of the cytokines. But, at lower concentrations of IFN-γ and TNF-α the effect of the blocking antibodies did not reach statistical significance due to the worse "signal-to-noise ratio", so that it was not possible to proof that IFN-γ and TNF-α were responsible for the inhibitory effect of the NK cell supernatants. In conclusion, it was shown that IFN-γ and TNF-α clearly inhibit the growth of SK-MEL-28. Soluble factors derived from stimulated NK cells reduced the growth of SK-MEL-28 cells, but it could not be confirmed that this was dependent on IFN-γ and TNF-α *in vitro*.

4.3.5 NK cells obtained by the optimized protocol show better anti-tumor efficacy and *in vivo* persistence compared to conventionally IL-2 activated NK cells

Finally, the *in vivo* anti-tumor activity of NK cells generated with the optimized expansion protocol were compared to NK cells activated long-term with IL-2 alone without feeder cells. A comprehensive evaluation was performed using expanded NK cells from three different NK cell donors during different experiments. For both expansion protocols, a high number of 30 x 10^6 NK cells per mouse were applied. Both protocols were suitable to obtain this high NK cell dose for use in this mouse model, even though expansion with IL-2 without feeder cells resulted in a minor NK cell expansion of 2- to 10-fold, whereas the optimized expansion protocol yielded a mean 2900 fold NK cell expansion after two weeks. However, it's important to clarify that only the optimized expansion method would be able to provide this dose of NK cells for humans receiving this type of therapy. As shown before, the optimized expansion protocol led to NK cells that were able to control tumor growth also at later time points. In contrast, even though conventionally IL-2 activated NK cells showed a similar anti-tumor efficacy shortly after the transfer, they were less effective at later time points (Figure 4.24 A). Importantly, despite infusing identical numbers of IL-2 activated and optimally expanded NK cells, there was a striking difference in the numbers of NK cells that could be isolated from the mice two weeks after the transfer. On day 14 after injection, the numbers of NK cells in blood, lungs and spleen were around ten times lower for IL-2 activated NK cells compared to NK cells that have been expanded with the optimized expansion method (Figure 4.24 B). These data indicate that NK cells that were generated with the optimized protocol exhibited enhanced *in vivo* persistence and significantly controlled tumor growth at later time points.

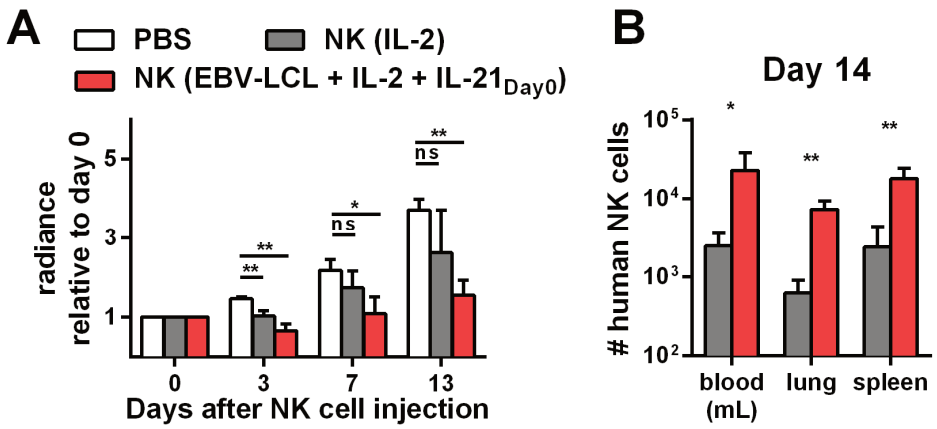


Figure 4.24 Expansion of NK cells using the optimized protocol results in NK cells with better *in vivo* persistence and anti-tumor activity compared to IL-2 expanded NK cells. (A) Mice bearing SK-MEL-28 cells expressing luciferase received either intravenous (i.v.) PBS (white bars) or human NK cells. The injected NK cells were previously expanded *ex vivo* for 14 days, either with IL-2 (gray bars) or with IL-2, irradiated EBV-LCL and IL-21 supplemented at day 0 (red bars). In total, three independent NK cell donors were used in different experiments and the bioluminescence (radiance) of each mouse at day 3, 7 and 13 was measured and analyzed relative to the bioluminescence at day 0. For each donor two to eight mice were used per group and mean and SD of all donors are shown for different time points. (B) Tumor bearing mice were treated as described in A and human NK cells were re-isolated from blood, lungs and spleen of the mice 14 days after NK cell injection. NK cells were enumerated using flow cytometry and mean and SD of the NK cell numbers are shown for one representative donor using four mice per group. Statistical significance was tested by Student's t-test in all experiments.

In summary, adoptive transfer of *ex vivo* expanded NK cells has a therapeutic effect against melanoma derived human tumors in a xenograft mouse model and a high dose of transferred NK cells is crucial for the outcome of the treatment. Furthermore, the optimized method for *ex vivo* NK cell expansion, which was developed in this project, enables the generation of NK cells that feature enhanced *in vivo* persistence after adoptive transfer and the ability for sustained productivity of IFN-γ and TNF-α in response to re-stimulation. Most importantly, the therapeutic efficacy of these generated NK cells is superior over conventionally IL-2 expanded NK cells.

5 Discussion

Parts of the text in this chapter have been directly taken or slightly modified from Granzin et al. (2015)[346] and from a second manuscript that is currently submitted.[347] The text has been originally written by myself.

In this chapter the conclusions of the project results are discussed in the context of existing knowledge. The chapter first deals with aspects that arise from the development of the fully automated NK cell expansion (chapter 5.1). Then, findings of the development of the optimized NK expansion protocol are discussed (chapter 5.2), followed by conclusions that can be drawn from the therapeutic xenograft mouse model for adoptive NK cell transfer (chapter 5.3). Finally, the results of the entire project are reviewed in the context of the aims (chapter 5.4).

5.1 Fully automated expansion and activation of clinical-grade NK cells for adoptive immunotherapy

The developed expansion process is a valuable tool to bring NK cells "from bench to bedside"

An automated cell expansion process was successfully developed by the use of an automated system and results were reported for the production of activated NK cells for their use in clinical cell therapy applications. For large-scale expansion of clinical-grade cells, NK cell cultures normally are maintained for 14 to 28 days and typically require frequent interventions such as media changes to refresh cytokines and other growth factors as well as to ensure that NK cells are maintained at a concentration that optimizes their growth and viability.[139] This procedure was efficiently automated allowing for clinical-grade production of high NK cell numbers that showed the same *in vitro* functionality and similar phenotype and gene expression as manually expanded NK cells. The automation requires financial investment for the instrument but enables significantly reduced running costs for an actively used clean-room, representing a major expense factor for the cellular product. Therefore, the automated process will be cost-saving in production scale in case of numerous performed processes per year. But, most importantly, the automation within a closed system substantially facilitates the expansion procedure by saving not only time but also minimizing the risk of culture contamination while introducing consistency in the production process. Thus, the process allows efficient GMP-compliant expansion of NK cells to best possible meet clinical needs and thereby it supports the translation of NK cell therapy to therapeutic use.

Ex vivo expansion changes the NK cell phenotype with possible consequences for their use in therapy

In line with previous findings,[341] many NK cell relevant markers and apoptosis-inducing molecules were up-regulated upon *ex vivo* expansion (chapters 4.1.4, 4.1.5). This change in phenotype might contribute to an increase of the therapeutic potential of the *ex vivo* generated NK cells. In particular, up-regulation of TRAIL by expanded NK cells can be utilized to efficiently treat tumors that express TRAIL death receptors and/or are sensitized to TRAIL by drugs such as bortezomib or doxorubicin.[182,360,361] Furthermore, the up-regulated expression of DNAM-1 and NKG2D by expanded NK cells increases their responsiveness and lead to enhanced NK cell mediated natural killing of tumor cells expressing ligands for these receptors, which is important for improved elimination of leukemia and solid tumors (chapter 0). Here, similar to TRAIL, the susceptibility of cancer cells can be further increased by different chemotherapeutic agents or hyperthermia, which induce the expression of ligands for DNAM-1 and NKG2D on tumor cells.[362,363] Importantly, NK cells from cancer patients often show diminished cytotoxic response due to impaired expression of activating NK cell receptors.[364] Therefore, *ex vivo* activation and expansion of patient derived autologous NK cells can be helpful to restore the repertoire of activating receptors on autologous NK cells, pointing out the importance of *ex vivo* expansion in particular for adoptive NK cell therapy in autologous settings.

Similar to a bioreactor, the presented system for NK cell expansion is very convenient, but it does not require manual pre-cultivation and allows lower starting cell numbers

Although some methods for effective NK cell expansion were developed in the past and have proven their applicability in large-scale by use of manual cultivation systems, such as cell culture bags or G-Rex containers,[154,206,287,318,337,365] there has been virtually no progress in the development of a fully automated and controlled process for clinical-scale NK cell expansion. An early report in 1996 showed the feasibility of automated NK cell cultivation by means of a stirred bioreactor,[366] but no further applications of this approach have been published. Sutlu et al. and Lapteva et al., independent of each other, applied an automated Wave Bioreactor system for clinical-grade NK cell expansion from PBMCs,[312,344] and Spanholtz et al. used the same system to generate clinical-grade NK cells expanded from cord blood hematopoietic progenitor cells.[277] Sutlu et al. concluded that automation of the cultivation is more practical and generated more activated NK cells compared with manual approaches. The results presented here confirm the practicability of an automated system, whereas, in this project, NK cells neither differed significantly in phenotype nor in function, whether they have been cultivated manually or

automatically (chapters 4.1.2, 4.1.3, 4.1.4, 4.1.5), similar to observations from Lapteva et al. and Spanholtz et al.. In comparison, the Wave Bioreactor system requires a high cell number to initiate the culture, which inevitably implicates a manual pre-cultivation until enough cells are generated to start the automated process. In the system presented here, low starting cell numbers were not a limitation, because the automation covered the whole cultivation phase including an early static cultivation phase with very low cell numbers, with 10^6 NK cells being sufficient to initiate the current process.

The combination of automated cell separation and NK cell cultivation within a single system is unique and allows full-automation of the whole NK cell expansion procedure

In contrast to other expansion approaches, this project shows that the entire cell cultivation phase, including preceding cell separation steps, can be done fully automated by a single instrument, enabling complete cell processing from the starting material to the final "ready-to-use" cell product (chapter 4.1.6). Starting the NK cell production process with a cell separation step to enrich for NK cells is beneficial, because it results in high-purity expanded NK cells without contaminating T cells in the final cell product.[206] The purity of the produced NK cells is important to avoid T cell mediated toxicities such as GvHD, especially in allogeneic settings, but also to directly trace back any treatment effects, positive and negative ones, to NK cells and allow a proper clinical evaluation of NK cells as therapeutic effector cells (chapter 1.5.1).

Similar to a bioreactor, the presented system for NK cell expansion yields sufficient NK cells for cell doses typically used in clinical trials, but considering strategies to increase the achievable number are needed since future applications probably require more NK cells

On average, with one instrument 1.3 x 10^9 activated NK cells could be generated within two weeks, enough to treat a typical 70- to 100-kg patient with 1 to 2 x 10^7 NK cells/kg. This would fall within the range of NK cells typically used in most investigational trials of adoptive NK cell immunotherapy.[367] Nevertheless, the optimal dose for NK cell injections has not yet been determined, and, because no dose dependent side effects have been observed, NK cell injections of 10^8 to 10^9/kg are imaginable in future.[139] Whereas bioreactor systems provide a volume of up to 3 L for cultivation and can yield 2 x 10^9 NK cells derived from umbilical cord blood hematopoietic stem cells,[277] or 9.8 x 10^9 NK cells from expansion of PBMCs,[312,344] the current system presented here is equipped with a medium scale culture volume of only 300 mL, which allowed a maximum NK cell number of 2.7 x 10^9. Thus, if higher doses of NK cells are needed, several process runs or multiple devices might be necessary for one application. In case that NK cell therapy turns out to be an efficient treatment option, it's even imaginable that production of NK cells could be realized in highly standardized bioreactor systems equipped with volumes up

to 10,000 liters similar to large cell cultures for production of therapeutic antibodies. Of note, the optimized protocol for NK cell expansion, which is discussed later, makes it possible to reach extremely high NK cell numbers, which could indeed require the capacity of such large culture systems. Then, the developed automated NK cell expansion would be ideal for initial NK cell processing, which includes NK cell separation and early cultivation steps, to provide clinical-grade NK cells as inoculum for subsequent expansion using a suitable bioreactor system.

Multiple NK cell infusions over time, which could be achieved by continuous production of NK cells, could be an alternative to infusion of a single large NK cell dose

Another strategy is the continuous production of NK cells that can be infused during multiple courses, allowing a high dose of applied NK cells in the end. With the current instrumentation, it's already possible to perform a continuously running process that maintains the NK cells in the expansion phase with repeated harvesting of cells whenever the maximum cell density is reached. Of note, with the optimized expansion protocol, a continuously running process could be maintained over weeks and very high NK cell doses could be achieved from a single blood donation. Repeated administration of *ex vivo* expanded NK cells at low numbers could be already sufficient for inducing a long-lasting anti-tumor response, since it has been shown in mice that adoptively transferred NK cells are able to trigger tumor specific endogenous memory T cell responses as antigen presenting cells or through cross-talk with DCs.[368]

The expanded NK cells keep their proliferative potential during *ex vivo* expansion, so that NK cells could further expand *in vivo* after adoptive transfer

The number of NK cells that is required for the transfer to the patient may be lower, if better *in vivo* persistence and expansion of the transferred cells could be achieved through post infusion strategies, such as cytokine administration. Clinical trials showed that it's possible to induce *in vivo* NK cell expansion in humans by means of IL-2 injection or endogenous production of IL-15 that can be stimulated by preparative chemotherapy with high dose cyclophosphamide and fludarabine.[177,190] Importantly, NK cells produced with the use of the automated method showed no noticeable telomere length shortening after expansion (chapter 4.1.3), indicating that the cells do not become senescent, and the regular proliferative potential is conserved, potentially allowing *ex vivo* expanded NK cells to further expand *in vivo*.

Specific expansion of therapeutically relevant NK cells could reduce the required NK cell dose

Another aspect to consider is that NK cells are heterogeneous in phenotype and function, with only a fraction of NK cell subsets driving their major cytotoxic effects.[369,370] As a consequence, effective NK cell based immunotherapy may not necessarily require the transfer of a high

number of bulk NK cells but the transfer of sufficient cells of therapeutically relevant NK cell subsets. For example, expanded single KIR^+ NK cells are proven to be advantageous over bulk NK cells in an AML xenograft model.[287] But, detailed characterization of the optimal NK cell subset for therapy is still pending. Nevertheless, because the automated expansion system combines the feature of cell separation and the possibility to use a very low starting NK cell number, it would be possible to isolate only a rare, therapeutically relevant NK cell subset and expand these cells to clinically needed numbers within a single process, representing a promising future strategy.

The applied clinical NK cell enrichment using TCR-α/β-CD19 depletion followed by CD56 enrichment could be better than conventional CD3 depletion and CD56 enrichment

In the presented work, the used system was automated through the use of TCR-α/β-CD19 depletion followed by CD56 enrichment as a novel strategy to enrich for bulk NK cells in clinical scale from leukapheresis products (chapter 4.1.6). This strategy may be promising, because TCR-α/β depletion has been shown to be superior over conventional CD3/CD19 depletion in stem cell transplantation settings due to a more efficient removal of TCR-α/β T cells,[371,372] which are responsible for GvHD. Furthermore, remaining TCR-γ/δ T lymphocytes rather possess positive features as they exhibit direct anti-tumor activity,[373,374] augment NK cell activity through CD137 engagement,[375] and eliminate recipient DCs and T cells, thereby preventing GvHD.[162] Indeed, a highly efficient depletion of TCR-α/β cells was achieved in the presented project and the obtained NK cells had the same potential to proliferate and showed the same functionality after expansion as NK cells, which were obtained by CD3 depletion and CD56 enrichment. The latter method has been used as a standard strategy for clinical scale NK cell separation in therapeutic settings so far.[170,198,284,285]

As the automated NK cell expansion process allows centralized and de-centralized production, it facilitates different strategies for large scale manufacturing of NK cells for therapeutic use

In view of the need to develop standardized methods to expand NK cells for clinical use, this automated process enables easy up-scaling for cost-efficient, centralized manufacturing of the therapeutic cell product. On the other hand, the closed system allows scale-out strategies and decentralized cell processing directly at the location of use,[376] avoiding the need for cell shipping, which often represents a logistic challenge, and, if done incorrectly, can compromise the NK cell product quality.[344] In general, both models, centralized and de-centralized manufacturing, have advantages and disadvantages and the choice for the best production model may rely mainly on the product stability and the necessity for a fast delivery to the patient, which is more critical for autologous than for allogeneic cellular therapies in most cases.[377]

Importantly, NK cells are applied in autologous but also in allogeneic therapeutic settings and the presented system supports the optimal NK cell production model for both settings.

5.2 Development of an optimized protocol for expansion and activation of human NK cells

In the first part of the project, a fully automated process for NK cell expansion was developed based on a clinical protocol that makes use of IL-2 and irradiated, clinical-grade EBV-LCL feeder cells to stimulate NK cell. Although this is an attractive strategy for clinical NK cell expansion, it's restricted to a relatively limited period of 2-4 weeks when expansion occurs and the achievable yield of NK cells remains limited. Here, it was demonstrated for the first time that the expansion of NK cells from peripheral blood by use of clinical-grade irradiated EBV-LCL feeder cells can be greatly increased by one single initial addition of IL-21 into the culture medium. This resulted in a long-lasting highly efficient proliferation of NK cells with potent *in vitro* and *in vivo* anti-tumor activity (chapters 4.2, 4.3).

The efficacy of the developed method for NK cell expansion is unprecedentedly high

Repeated stimulation with K562 feeder cells bearing membrane-bound IL-21 has been reported to facilitate a long-term expansion of NK cells allowing around 10^8–fold NK cell expansion after six weeks.[203] Here, a much higher 10^{11}–fold NK cell expansion was reached after six weeks by combining repeated stimulation with irradiated EBV-LCL feeder cells with IL-21 addition at the initiation of culture (chapter 4.2.1). It was sufficient to supplement soluble IL-21 only at start of cultivation to achieve high expansion of NK cells using stimulation with irradiated EBV-LCL. These data are in concordance with recent data showing exposure time of soluble IL-21 is critical for the yield of NK cells during co-culture with K562 feeder cells expressing membrane-bound IL-15.[378] Importantly, IL-21 has been shown to cause apoptosis of NK cells which is enhanced when this cytokine is membrane-bound compared to when it's in its soluble state.[301] This implies that stimulation of NK cells using feeder cells expressing membrane-bound IL-21 may have disadvantages compared to the approach utilized here, where IL-21 was incorporated only briefly into the media at the start of cell culture. The combination of repeated stimulation with clinical-grade irradiated EBV-LCL feeder cells and the short term presence of soluble IL-21 in the medium results in unparalleled efficacy in expanding NK cells.

The optimized expansion method would allow to generate an off-the-shelf NK cell product

Unlike other effector cells, NK cells can be applied not only in an autologous but also allogeneic setting and receptor-ligand mismatches of donor and recipient may support a better NK cell versus tumor effect without inducing unwanted toxicities.[379] Thus, at some stage NK

cell products may become universally applicable "off-the-shelf" with the optimized expansion method enabling the production of activated NK cells to an industrial scale. Until now, this was only possible by using continuously growing NK cell lines like NK-92 that proved its applicability in mouse studies and in pilot trials with cancer patients.[380,381] However, due to safety concerns, NK cell lines require proper inactivation by irradiation prior to infusion in the patient, preventing their ability to proliferate *in vivo* which could potentially compromise their therapeutic potential. To avoid this major drawback of NK cell lines, it would be advantageous to use primary NK cells and expand them to very high cell numbers using the presented optimized method, to manufacture off-the-shelf NK cell products that are suitable for use in protocols treating a variety of different cancer patients. Compared to individually generated therapeutic NK cell units, off-the-shelf NK cell products would not only ensure a more constant product quality and efficacy, but also the production process could be designed in a centralized and more cost-efficient way. Apart from the therapeutic efficacy, these aspects are essential for the success of a cellular therapy in the long run.

EBV-LCL-mediated NK cell expansion is not primarily dependent on soluble factors

Although EBV transformed B cell lines are used for NK cell expansion for long time, the expansion inducing mechanisms are still unclear. Interestingly, IL-12 is known to induce NK cell activation and IFN-γ production and IL-12 was originally purified from the supernatant of the EBV-LCL cell line RPMI 8866.[323,382] However, the EBV-LCL used in this project did not secrete noticeable amounts of IL-12 (chapter 4.2.2). This can be explained by the heterogeneity of EBV-LCL cell lines. In a study analyzing the cytokine secretion of different EBV-LCL cell lines only 19 out of 39 tested EBV-LCL cell lines produced IL-12.[383] Furthermore, early reports already claimed that EBV-LCL-mediated NK cell expansion is not dependent on soluble factors,[309] which is in agreement with the here presented data that imply an important role for the direct cell-cell contact between EBV-LCL and NK cells (chapter 4.2.3).

EBV-LCL-mediated NK cell expansion relies on cell-cell contact, but the relevant factors for this interaction remain elusive

It's known that expansion of NK cells in co-cultures with different other cells is often based on heterotypic cell-cell contact, as shown for autologous PBMCs[384] and $CD14^+$ cells[306] or allogeneic feeder cells including K562[385], HFWT[324,325], KL-1[321] and MM-170.[322] However, in most cases, the essential factors for this interaction are unknown. Therefore, different surface marker were targeted with blocking antibodies to reveal important factor for the interaction between the used EBV-LCL and NK cells, (chapter 4.2.1). In general, allogeneic feeder cells can support NK cell expansion *in vitro* due to the mismatch of expressed KIR ligands and NK

cell KIRs.[386] Interactions with CD16 on NK cells were required for KL-1–mediated expansion of NK cells from PBMCs, and, interestingly, depletion of B cells from the PBMCs abrogated NK cell expansion, while addition of an EBV-LCL cell line further increased the expansion.[321] However, in my experiments CD16 was irrelevant for the interaction between the EBV-LCL and NK cells, since two different blocking antibodies directed against CD16 did not reveal any effect on the EBV-LCL-mediated NK cell expansion. 41BBL is an important ligand on engineered K562 triggering NK cell proliferation,[266] but blocking of this ligand also had no consequence on the expansion of NK cells in co-culture with EBV-LCL. Because EBV infection can induce CD40 ligand expression it was suggested that CD40/CD40L signaling could play a role,[387] but blocking CD40 in the NK-EBV-LCL co-culture did not make a difference. CD48 and 2B4 are critical for the proliferation of NK cells in response to IL-2 during homotypic interactions between different NK cells, and they play a role for interactions between $CD48^+$ T cells and $2B4^+$ NK cells.[129,357,358] Indeed, it was observed that blocking of 2B4 or CD48 reduced the expansion of NK cells in cultures with NK cells alone, but these blockings had no significant effect when EBV-LCL were present, implying that the character of the NK-EBV-LCL interaction is independent of CD48 and 2B4. Unfortunately, none of the targeted receptors appeared to be important for the EBV-LCL NK cell expansion with the exception of the high affinity IL-2 receptor CD25, pointing to the relevance of IL-2 signaling, which is addressed in the next section.

The γ-chain cytokines IL-2 and IL-21 seem critical for EBV-LCL-mediated NK cell expansion

To understand the relevant factors of EBV-LCL-mediated NK cell expansion, it would be necessary to understand the general mechanisms of NK cell expansion. For T cell expansion, it's an accepted model that engagement of the TCR followed by a second co-stimulatory signal, such as CD28 triggering, enables full T cell activation and induces up-regulation of cytokine receptors, which then allow T cell proliferation in response to different cytokines.[388] Although the relevant parameters for NK cell expansion are less clear, one could imagine a similar model for NK cells, as NK cell expansion during formation of memory NK cells seems to rely on comparable mechanisms as for memory T cells. For NK cells in mice, MCMV lead to engagement of the Ly49H receptor and co-stimulation through DNAM.-1, provoking a clonal expansion of $Ly49H^+$ NK cells in response to pro-inflammatory cytokines such as IL-12.[48] In humans, HCMV causes the specific expansion of $NKG2C^+$ NK cells and the exact ligand that act as first signal and drives this expansion is unknown, but, similar to mice, IL-12 plays a critical role, because it induces CD25 expression and drives expansion of $NKG2C^+$ NK cells in response to CMV *in vitro*[57]. Intriguingly, induction of high CD25 expression was observed by NK cells in co-culture with EBV-LCL (chapter 4.2.1). This may indicate that EBV-LCL

provide the required signals for an initial NK cell activation, which then lead to up-regulation of cytokine receptors, such as CD25, which in turn would allow cytokine-driven expansion of NK cells, for instance by IL-2. In fact, even at high IL-2 concentrations blocking of CD25 significantly reduced the EBV-LCL-mediated NK cell expansion, showing the relevance of CD25 for EBV-LCL-mediated NK cell expansion. Importantly, blocking of CD25 had no effect on the expansion of NK cells in response to EBV-LCL when IL-21 was present, indicating that IL-2 signaling can be replaced by IL-21 that as well can signal through the common γ-chain. This hypothesis was confirmed by the fact that a relatively robust NK cell expansion was still possible without IL-2 when IL-21 was present, whereas the expansion was dramatically reduced when both were missing. The fact that IL-21 compensates the need for IL-2 could at least partially explain the good performance of the optimized expansion method, using EBV-LCL and adding of IL-21 at start of the culture. However, total replacement of IL-2 by IL-21 seems not to be an option for NK cell expansion protocols, because IL-21 counteracted the NK cell expansion when it was present in culture over longer time (chapter 4.2.1). This emphasizes the actual need to dissect the effects of the different cytokines during NK cell expansion in more detail. In addition, the signals that are provided by EBV-LCL represent an important topic to better understand the mechanisms behind the effective NK cell expansion.

Better understanding of the mechanisms behind the EBV-LCL-mediated NK cell expansion would help to develop NK cell expansion protocols without the need for feeder cells

Identification of the signals provided by EBV-LCL for NK cell expansion would not only help to understand the biology of NK cell proliferation, but it could also help to design NK cell expansion approaches without feeder cells. The avoidance of feeder cells would further improve the standardization of the NK cell expansion procedure, since it can be assumed that feeder cells are undefined cell culture components with varying quality. A recent study showed that it can be sufficient to utilize cell membrane particles of feeder cells for NK cell expansion rather than intact feeder cells,[389] which could already be an improvement from a regulatory standpoint. An even more advanced strategy could be the use of GMP-grade artificial particles carrying the relevant factors for NK cell expansion, similar to T cell expansion that can be achieved by non-biological particles loaded with anti-CD3/anti-CD28 antibodies.[390] A recent approach using bead-bound IL-21 and 41BBL demonstrated the general feasibility of this strategy for NK cell expansion, but the achieved performance is far behind that achievable with feeder cells (140-fold NK cell expansion in 3 weeks), showing that it's necessary to better understand the relevant factors for NK cell proliferation first.[391] Since it was demonstrated that the EBV-LCL-dependent NK cell expansion relies on cell-contact, the use of membrane particles of EBV-LCL

for NK cell expansion should be feasible and could simplify the identification of relevant membrane components of these feeder cells in a next step.

5.3 Evaluation of expanded human NK cells for therapeutic efficacy using a xenograft mouse model

With the optimized expansion method it's possible to produce high NK cell doses for adoptive transfer that are required to mediate a noticeable anti-tumor effect

NK cells expanded with the optimized method mediated pronounced anti-tumor activity in a mouse model and they showed superior persistence *in vivo* compared to conventional IL-2 activated NK cells (chapters 4.3.2, 4.3.5). To optimize this anti-tumor effect, injection of up to 30 x 10^6 NK cells per mouse is needed, corresponding to 10^9 NK cells/kg, which is in the upper range of considered doses for studies in humans.[139] Thus, the results of the here presented xenograft mouse model indeed support the rationale for transferring preferably high NK cell doses to maximize a therapeutic anti-tumor effect. This is in agreement with a published xenograft model with NSG mice bearing human myeloma derived tumors, where transfer of 140 x 10^6 *ex vivo* expanded K cells were required to achieve NK cell mediated tumor growth control, while injection of 40 x 10^6 NK cells was not sufficient for tumor control.[330] Importantly, the optimized NK cell expansion method is capable of providing these high numbers of clinical-grade NK cells for treatment of humans, whereas conventional NK cell expansion protocols may not reach these NK cell doses required for clinical use.

IL-2 injections did not maintain NK cell activation and persistence in the experimental system *in vivo*

To maintain sustained activation and expansion of NK cells *in vivo*, injection of low dose IL-2 has frequently been utilized in clinical studies. However, despite treatment with low dose IL-2 in the animal model, neither noticeable *in vivo* expansion was detected, nor did adoptively transferred NK cells maintain the phenotypic profile of activation that was acquired during *ex vivo* culture (chapters 4.3.2, 4.3.3). Possibly, the great quantity of transferred NK cells caused a very high demand for IL-2 that could not be obtained by the low dose of administered IL-2. Of note, it was published recently that, in contrast to IL-15, IL-2 was inefficient to promote noticeable *in vivo* NK cell expansion in a similar xenograft mouse model.[392] Therefore, IL-15 may be a better cytokine to improve NK cell persistence, which also has the added benefit of avoiding unfavorable proliferation of regulatory T cells which occurs commonly with IL-2.[190,193,237] An additional option would be to utilize an IL-2 "superkine", which is an engineered

IL-2 that binds with high affinity to the IL-2Rß and eliminates the functional requirement for CD25.[393]

Limited *in vivo* persistence of NK cells could also be of advantage for their use in therapy

Although an extending lifespan of adoptively transferred effector cells seems desirable,[394] the limited *in vivo* persistence and the short lifespan of transferred NK cells could have benefits that would make sustained *in vivo* NK cell expansion needless. Adoptive therapy using T cells expressing CARs currently represents a breakthrough for cancer immunotherapy, but the longevity of T cells may cause severe and potentially life-long side effects, such as B cell aplasia upon infusion of anti-CD20 CAR T cells.[395] These unwanted side effects of CAR T cells could be avoided by the use of short-lived CAR expressing NK cells, potentially making them better controlled, potentially superior "car drivers".[249]

Similar to cytokine-induced memory-like NK cells, NK cells obtained with the optimized NK cell expansion method maintain an increased potential for production of IFN-γ and TNF-α

Although activated NK cells had a rapid decline in their ability kill tumor target cells after adoptive transfer, they retained their enhanced potential to produce IFN-γ and TNF-α in response to stimulation (chapter 4.3.3). Similarly, pre-activation of murine or human NK cells with IL-12, IL-15 and IL-18 leads to cytokine-induced memory-like NK cells that maintain the ability to respond to stimulation with higher production of IFN-γ.[60,61] Furthermore, murine and human cytokine induced memory-like NK cells possess improved anti-tumor activity *in vivo*, as shown by a mouse lymphoma model and a leukemia xenograft model.[62,396] Thus, the results presented here indicate that expansion of NK cells with the optimized expansion method may lead to a type of NK cells similar memory-like NK cells with sustained production of IFN-γ and TNF-α upon stimulation. Importantly, up-regulation of CD25 is another important characteristic of cytokine-induced memory-like NK cells, allowing them to respond to picomolar concentrations of IL-2.[62,200] As the optimized expansion also results in NK cells with significantly up-regulated CD25 (chapter 4.2.1), this could be another hint that NK cells obtained with the optimized expansion protocol share features with the type of memory-like NK cells generated with IL-12, IL-15 and IL-18.

NK cell derived IFN-γ and TNF-α could contribute to the anti-tumor effect *in vivo*

IFN-γ and TNF-α could play a direct role in the anti-tumor effect that was observed in the xenograft mouse model. In combination, IFN-γ and TNF-α can induce permanent growth arrest in numerous human cancers and both cytokines together are essential for destroying established tumors in mice by eradication of tumor associated stroma cells.[119,120] Importantly, it could be

shown that SK-MEL-28 melanoma cells, which were engrafted in the mouse xenograft model, were sensitive for growth arrest by IFN-γ and TNF-α *in vitro* (chapter 4.3.4). Unfortunately, at least *in vitro* it was not possible to proof that IFN-γ and TNF-α derived from expanded NK cells are sufficient to mediate direct growth inhibition of SK-MEL-28. Nevertheless, IFN-γ also can polarize T cells into type-1 effector cells and upregulates MHC class I on target cells and DCs that could potentially facilitate subsequent T cell mediated anti-tumor responses. Accordingly, in a therapeutic mouse model of RMA-S lymphoma, the production of IFN-γ by transferred NK cells was essential for their tumor growth control.[62] Thus, it would be reasonable to further investigate whether IFN-γ and TNF-α produced by adoptively transferred NK cells contribute to the NK cell-mediated anti-tumor activity. While current NK cell based therapies focus on the direct cytotoxic effect of NK cells, the possible importance of NK cell derived cytokines could be a relevant aspect for NK cells therapeutic function.

NK cell derived anti-tumor responses that engage other immune cells cannot be determined in the utilized xenograft mouse model

As already discussed before, some effects from the transferred NK cells could involve other immune cells to induce effective anti-tumor immunity (see also chapter 1.3.3). Of note, some of these effects, such as IFN-γ secretion that triggers MHC class I up-regulation on target cells and thereby makes them more vulnerable for T cell responses could in turn also dampen NK cells by inhibition through KIR signaling.[115] Effects that inhibit the function of transferred NK cells would affect the tumor control in the xenograft mouse model. However, effects from the adaptive immune system are not reflected by the used xenograft mouse model, because the mice are immunocompromised and they lack T cells and B cells to allow engraftment of human cells without rejection. This is a possible drawback of the used model that could be overcome by utilizing mice with a humanized immune system. These humanized mice can be generated by injection of human primary hematopoietic cells that give rise to the different human immune cells.[397,398] This model then allows to engraft human derived tumors without rejection of the graft as shown for breast cancer for example, and, in addition, the established human immune system enables better investigation of the complex anti-tumor immunity.[399] To further improve the informative value of the model, engraftment of different primary human tumors instead of tumor cell lines would be more close to the situation in the clinic, but this approach could be complicated by the poor availability of these tumor materials.[400] Nevertheless, although the here used xenograft mouse model lacks other immune cells than the transferred NK cells, it could be demonstrated that adoptive NK cell transfer clearly controlled the tumor growth. Thus, the model was suitable to proof the therapeutic efficacy of expanded NK cells *in vivo*. Nevertheless,

with view on the possible importance of other immune cells, which could be triggered by the transferred NK cells, the observed anti-tumor effect in the xenograft model could be underestimated or at least different to the expected effect with an immune competent individual.

The optimized expansion of clinical-grade NK cells and the established xenograft model are useful tools for the implementation and testing of improved NK cell therapy concepts in future

Adoptive transfer of expanded NK cells in combination with other anti-cancer therapies could open new perspectives for cancer treatment in future. Considering the described recent developments in the field and taking the results of the presented project into account, future scenarios of cancer immunotherapy with NK cell transfer could combine several aspects. An exemplary scenario in the near future could be the adoptive transfer of clinical-grade *ex vivo* expanded NK cells at very high cell doses during multiple courses together with multi-specific antibodies directed against tumor antigens. An outlook to the more distant future could be for instance the therapy with genetically modified off-the-shelf NK cell products, which express their own IL-2 and CARs against tumor antigens, and which could be applied together with antibodies targeting inhibitory KIRs. Many different treatment settings that include NK cell transfer are possible and these approaches could be evaluated using the established preclinical xenograft model. Furthermore, the here developed method for clinical-grade NK cell expansion is essential to overcome the normally limited numbers of available primary NK cells. These limited NK cell numbers could be particular relevant for intended approaches involving genetic modification of NK cells, such as generation of CAR expressing NK cells. Because genetic engineering of NK cells require further processing steps that are associated with potential loss of NK cells, further reducing the amount of NK cells that is available for the intended therapy.

5.4 Conclusion and Perspectives

The first aim of the project was to identify a suitable technical method to translate protocols for NK cell expansion from early development in laboratory scale to large scale for clinical use. This was worked out by the automation of an entire NK cell expansion process with the use of a single instrument, allowing for the efficient production of clinical-grade NK effector cells. Because all processing steps are done automated in a closed system, this provides the highest standards for GMP conformity and it best possible meets clinical requests. Importantly, apart from the clinical-grade quality, the automated procedure yields sufficient quantities of activated NK cells for most of the current clinical NK cell applications. Strategies to obtain higher NK cell doses that are possibly required in future could be the combination of the current processing system with a bioreactor, or increasing the capacity of the available culture volume of the current system, or producing NK cells in a continuously running expansion process that is already possible with the current system.

A second aim of the project was the development of a highly efficient method to expand NK cells *ex vivo* to best possible serve clinical needs. Therefore, an optimized protocol was established for *ex vivo* expansion of primary human NK cells with outstanding cell yields. This was achieved by stimulation of NK cells with irradiated EBV-LCL feeder cells, IL-2 and adding IL-21 at the start of the culture. NK cells expanded and activated under this condition possess potent anti-tumor activity. Thus, the method is suitable to provide very high doses of functional NK cells for clinical use, which is at least in terms of quantity not possible by other protocols reported so far. It's imaginable that this method can pave the way for off-the-shelf primary NK cell products, opening new perspectives for cellular therapy with NK cells. Furthermore, identification and understanding of the EBV-LCL-derived signals for NK cell expansion would be a reasonable next step to allow the development of NK cell expansion methods without feeder cells, further improving NK cell expansion from a regulatory standpoint.

The third aim of the project was to investigate how *ex vivo* expansion and activation of NK cells affect their anti-tumor properties. Characterization of *ex vivo* activated and expanded NK cells revealed that expanded NK cells exhibit alterations in gene expression, surface marker profiles and function. Upon *ex vivo* expansion, NK cells up-regulate activating receptors and effector molecules, they become more cytotoxic against several tumor cell lines, and they exhibit enhanced production of IFN-γ and TNF-α upon stimulation *in vitro*. Furthermore, adoptive transfer of NK cells expanded with the optimized expansion method led to significant inhibition of tumor growth in a melanoma xenograft mouse model *in vivo*. This anti-tumor efficacy was

superior over that from conventionally IL-2 activated NK cells, demonstrating that the developed NK cell expansion method enhances not only the quantity but also the therapeutic quality of NK cells. Intriguingly, NK cells expanded with the optimized method maintained their enhanced potential to produce IFN-γ and TNF-α after adoptive transfer, although these NK cells again became similar to naive NK cells in terms of their surface marker profile and a low potential to kill tumor cells. Thus, subsequent work could focus on the role of IFN-γ and TNF-α for NK cells anti-tumor function. Further, it could be investigated why the cytotoxicity of expanded NK rapidly declines after adoptive transfer and whether it's possible to anticipate this loss of cytotoxic function. In addition, the established xenograft model could be used for the testing of strategies to further improve the NK cell anti-tumor effect. Such strategies could include repeated injection of high NK cell doses instead of a single NK cell injection. The injection of IL-15 or other suitable agents to enhance the NK cell persistence and function *in vivo* could be tested. Additional promising treatment options are the combination of NK cell transfer with therapeutic antibodies or the use of genetically modified NK cells, such as CAR-expressing NK cells.

In summary, the project yields a novel technical procedure for automated *ex vivo* expansion of clinical-grade NK cells and an optimized method for NK cell expansion with unparalleled efficacy, allowing to generate large numbers of NK cells with pre-clinically approved therapeutic function. Thereby, the outcome of the project meets a critical clinical need as it allows for the production of functional NK cells that can be applied in the clinics for adoptive NK cell therapy at high cell doses. Furthermore, the outcome of the project creates a basis to develop and improve future strategies for cancer therapy with NK cells.

6 Abbreviations

1301	T cell leukemia cell line
ADCC	Antibody-dependent cellular cytotoxicity
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC	Antigen presenting cell
ATP	Adenosine triphosphate; allophycocyanin
BAT3	HLA-B-associated transcript 3
BCR	B cell receptor
BiKe	Bi-specific killer cell engagers
B-RAF	B-rapidly accelerated fibrosarcoma
C	Celsius
CAR	Chimeric antigen receptor
CCL	CC-chemokine ligand
CCU	CentriCult Unit
CD	Cluster of differentiation
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1
CFSE	Carboxyfluorescein succinimidyl ester
CLL	Chronic lymphocytic leukemia
CO_2	Carbon dioxide
COLO-205	Human colorectal adenocarcinoma cell line
CTLA-4	T-lymphocyte-associated protein 4
Daudi	Burkitt's lymphoma cell line
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNAM-1	DNAX accessory Molecule-1
EBV	Epstein-Barr virus
EBV-LCL	Epstein-Barr virus-transformed lymphoblastoid cell line
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
Fab	Fragment for antigen-binding
FasL	FAS receptor ligand
Fc	Constant fragment
FITC	Fluorescein Isothiocyanate
FPS	Flexible programming suite
g	Gram; gravity acceleration
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GvHD	Graft versus host disease
GvL	Graft versus leukemia
GvT	Graft versus tumor
Gy	Gray
h	Hour(s)
HCMV	Human cytomegalovirus
HER	Human epidermal growth factor receptor
HFWT	Wilms tumor cell line
HSCT	Hematopoietic stem cell transplantation
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFN	Interferon
IG	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
Iono	Ionomycin
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
k	Kilo

K562	Human myelogenous leukemia cell line
KIR	Killer-immunoglobulin-like receptor
KLRG1	Killer-cell lectin like receptor G1
L	Liter
LAK	Lymphokine-activated killer
LAZ 388	Specific EBV-LCL cell line
LN	Lymphotoxin
LTi	Lymphoid tissue–inducer
m	Milli; meter
µ	Mirco
M	Molar
MACS	Magnetic cell separation
MAGE	Melanoma-associated antigen
MAP	Mercapturic acid pathway
MCMV	Mouse cytomegalovirus
MDS	Myelodysplastic syndrome
MDSC	Myeloid-derived suppressor cells
MFI	Mean fluorescence intensity
MHC	Histocompatibility complex
MIC	MHC class I polypeptide-related sequence
min	Minute(s)
MM	Multiple myeloma
n	Nano
NCR	Natural cytotoxicity receptor
NK	Natural killer
NKG2	NK group 2
NSG	NOD-scid IL-2Rgamma^{null}
O_2	Oxygen
PBMC	Peripheral blood mononuclear cell
PCNA	Proliferating cell nuclear antigen
PD-1	Programmed cell death protein 1
PE	Phycoerythrin
PEB	PBS/EDTA/BSA buffer
PMA	Phorbol myristate acetate
Raji	Burkitt's lymphoma cell line
RCC	Renal cell carcinoma
REA	REAfinity antibody
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RPMI-8866	ß-lymphoid cell line from chronic myelogenous leukemia patient
RTL	Relative telomere length
s	Second(s)
SK-MEL-28	Human melanoma cell line
SLAM	Signaling lymphocyte activation molecule
TCR	T cell receptor
T-flask	Tissue culture flask
TGF	Transforming growth factor
Th	T helper
TIGIT	T-cell immunoreceptor with Ig and ITIM domains
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TP	Tumor protein
TRAIL	TNF related apoptosis inducing ligand
TRIKE	Tri-specific killer cell engagers
U	Units
UKRV -MEL-02	Human melanoma cell line
ULBP	UL16-binding protein
VEGF-A	Vascular endothelial growth factor
w/o	Without

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