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Presented by:

Dipl-biochem. Matthias Miller

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TOWARDS THE GENERATION OF NOVEL NATURAL KILLER CELL-BASED IMMUNOTHERAPIES AGAINST MELANOMA

REFEREES:

1st referee: Prof. Dr. Viktor Umansky

2nd referee: PD Dr. Adelheid Cerwenka

Declaration

I hereby declare, that I wrote this thesis on my own and that I did not use any other sources or materials than the explicitly mentioned ones. The work described in this thesis was performed from May 2010 to November 2013 in the 'Innate Immunity' research group at the German Cancer Research Center (DKFZ), Heidelberg, Germany under the supervision of PD Dr. Adelheid Cerwenka. I further declare that this thesis has not been submitted at another university for dissertation.

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1 ZUSAMMENFASSUNG

Seit der Entdeckung, dass verschiedene Immunzellen über die Fähigkeit verfügen, Tumorzellen zu erkennen und zu zerstören, wurden unterschiedliche Ansätze getestet, um unter anderem Natürliche Killerzellen (NK-Zellen) zur Therapie maligner Erkrankungen einzusetzen. So wurden mit NK-Zell-basierten Therapien wichtige Durchbrüche bei der Behandlung hämatologischer Tumore, doch nur geringe Erfolge in Patienten mit soliden Tumoren erzielt. Dass die Anzahl Tumor-infiltrierender NK-Zellen *in vivo* zu einer Verbesserung der Prognose führen kann, wurde jedoch für mehrere Krebsarten gezeigt. Wir vermuteten daher, dass es für das Ausbleiben relevanter therapeutischer Erfolge bei Patienten mit soliden Tumoren zwei Gründe geben könnte: Zunächst könnten die infundierten NK-Zellen evtl. nicht effizient in das Tumorgewebe eingewandert sein oder aber sie haben ihre Aktivität nach der Infusion in die Patienten rasch verloren. Das Ziel dieser Studie war es daher, die anti-Tumor-Aktivität adoptiv transferierter NK-Zellen zu verbessern, indem (1) die Anlockung von NK-Zellen in Tumore durch die Expression von chemotaktischen Zytokinen (Chemokinen) in Tumorzellen verbessert wird und (2) ein neuartiges Aktivierungsprotokoll etabliert wird, welches NK-Zellen mit lang anhaltender Aktivität generiert. Hierfür wurde zunächst ein Tiermodell entwickelt, um die *in vivo*-Relevanz unserer Studien zu belegen. Das Modell ermöglichte das Anwachsen humaner Luciferase-transduzierter Melanom-Zellen und NK-Zellen in immunsupprimierten Mäusen, in denen die Tumorgrößen durch Lumineszenzmessungen bestimmt werden konnten. Für unser erstes Ziel konnten wir weiterhin CX3CL1 als geeignetes Chemokin für die spezifische Anlockung zytotoxischer NK-Zellen identifizieren. CX3CL1-überexprimierende Melanom-Zelllinien lockten im Vergleich zu den jeweiligen Kontrollzellen NK-Zellen *in vitro* und *in vivo* im Xenograftmodell stärker an. Von großer Bedeutung ist, dass eine stärkere Kontrolle des Wachstums CX3CL1-positiver Tumore durch NK-Zellen *in vivo* gezeigt werden konnte. Für unser zweites Ziel testeten wir ein neuartiges Stimulierungsprotokoll. Cooper *et al.* haben in einer früheren Studie gezeigt, dass murine NK-Zellen, die mit Interleukin (IL)-12, IL-15 und IL-18 (IL-12/15/18) stimuliert worden waren, über lang anhaltende Reaktivität verfügten, weswegen wir dieses Aktivierungsprotokoll für humane NK-Zellen testeten. Als Referenz wurden IL-15-stimulierte NK-Zellen verwendet. Unsere Untersuchungen zeigten, dass IL-12/15/18-stimulierte NK-Zellen durch die verstärkte Expression der α-Kette des IL-2-Rezeptors sensitiver gegenüber IL-2 wurden. Demgemäß teilten sich die NK Zellen *in vitro* und *in vivo* in den Versuchstieren unter IL-2-Gabe schneller als die Kontrollzellen und lieferten größere Zellzahlen. Weiterhin reagierten IL-12/15/18-stimulierte NK-Zellen nach längerer *in vitro*-Kultivierung oder nach Re-Isolation aus Versuchstieren bei Restimulierung mit einer verstärkten Produktion von Interferon-γ (IFN-γ). Von großer Bedeutung ist weiterhin, dass die IL-12/15/18-Stimulierung eine stark verminderte Expression inhibitorischer Killer cell Immunoglobulin-like Receptors (KIR)-Rezeptoren bewirkte, deren niedrigere Expression auf NK-Zellen nach Stimulierung mit einer Zunahme der Reaktivität gegenüber Tumorzellen einherging, welche die zugehörigen inhibitorischen Liganden exprimierten. Zudem, verglichen mit IL-15-stimulierten Kontrollzellen, zerstörten IL-12/15/18-aktivierte NK-Zellen Tumorzellen *in vitro* stärker und verminderten das Tumorwachstum *in vivo*.

Zusammenfassend wird in dieser Arbeit beschrieben, dass sowohl die Anlockung von NK-Zellen durch die gezielte Expression von CX3CL1 in Tumorzellen, als auch die Aktivierung von NK-Zellen durch IL-12, IL-15 und IL-18 über ein großes therapeutisches Potential verfügen. In unserem prä-klinischen Mausmodell konnten wir eine verstärkte Anlockung von NK-Zellen in CX3CL1-exprimierende Tumore zeigen und für beide Ansätze eine verbesserte Tumorabstoßung durch NK-Zellen. Diese Daten verfügen damit über erhebliche klinische Relevanz und sollten in das Design klinischer Studien zur NK-Zell-basierten Tumorimmuntherapie einbezogen werden.

2 SUMMARY

Since the initial observation that various immune effector cells can detect and lyse tumor cells, different approaches have been developed in order to harness for instance natural killer cells (NK cells) for the treatment of malignant diseases. Despite important breakthroughs in the therapy of hematological malignancies, only small successes could be achieved in the NK cell-based treatment of patients with solid tumors. Nevertheless, it was reported for several cancer entities that the amount of tumor-infiltrating NK cells correlated with a better prognosis. Thus, we were assuming that at least two reasons exist why in patients suffering from solid malignancies following NK cell infusion no relevant clinical responses could be observed: first, the transferred NK cells could not have properly infiltrated into the tumor tissue or second, they could have rapidly lost their responsiveness. Accordingly, the aim of this study was to improve the anti-tumor responses of adoptively transferred NK cells (1) by improving NK cell infiltration into tumors by over-expressing chemoattracting cytokines (chemokines) in tumor cells and (2) by establishing a novel activation protocol in order to generate effector NK cells with sustained effector functions. First, to explore the *in vivo* relevance of our studies, a xenograft mouse model was established. This model allowed for the engraftment of human luciferase-transduced melanoma cells and NK cells in immunocompromised mice and for the subsequent quantitative detection of tumor cells using optical imaging. Regarding the first aim, CX3CL1 was identified as a suitable chemokine that specifically attracted cytotoxic NK cells. Accordingly, we have found that CX3CL1-expressing melanoma cells attracted NK cells *in vitro* in transwell migration assays and *in vivo* in the xenograft model more pronouncedly compared to the respective control cells. Importantly, we also showed that the growth of CX3CL1-overexpressing cell lines compared to control lines was more potently controlled by NK cells *in vivo*. With regards to the second aim, we tested a new NK cell stimulation regimen in our model. Recently, Cooper *et al.* reported that murine NK cells stimulated with Interleukin (IL)-12, IL-15 and IL-18 (IL-12/15/18) exhibited more potent and sustained reactivity. Based on these findings, we investigated the effect of this triple cytokine stimulation on human NK cells. As a control, NK cells were stimulated with IL-15 alone. Our study showed that IL-12/15/18-stimulated NK cells expressed higher levels of the IL-2 receptor α-chain, rendering the NK cells more sensitive towards IL-2. Accordingly, IL-12/15/18-stimulated NK cells proliferated faster both *in vitro* and *in vivo* upon IL-2-supplementation and yielded higher cell recovery. Furthermore, IL-12/15/18-stimulated NK cells produced higher levels of interferon-γ (IFN-γ) after prolonged culture periods *in vitro* or after recovery from immunodeficient mice. Most importantly, IL-12/15/18-stimulation led to a decreased expression of several inhibitory NK cell receptors, the so-called killer cell immunoglobulin-like receptors (KIRs) resulting in increased reactivity against targets that expressed the cognate ligands. Moreover, compared to IL-15-stimulated cells, IL-12/15/18-stimulated NK cells exerted increased cytotoxicity *in vitro* and, most remarkably, also exhibited more potent anti-tumor effector functions *in vivo* leading to a decreased tumor load.

In summary, this work suggests that both the attraction of NK cells through CX3CL1-expression and the activation of NK cells with IL-12, IL-15 and IL-18 harbor great therapeutic potential for novel NK cell-based immunotherapies against melanoma and human malignancies in general. In our pre-clinical mouse model we could demonstrate a more pronounced NK cell recruitment to CX3CL1-expressing tumors and for both approaches an improvement in NK cell-mediated tumor control. These data might be clinically relevant and thus we propose that these findings should be implemented into the design of clinical trials for NK cell-based tumor immunotherapy.

3 INTRODUCTION

3.1 Cancer biology

3.1.1 Oncogenesis

Malignant transformation which leads to the development of cancer is a complex process that can be initiated by a variety of events, all ultimately being mutagenic such as ultraviolet (UV)- or γ-irradiation or DNA intercalating agents. Typically, cancer arises after a multi-step acquisition of various genetic or epigenetic mutations that ultimately lead to a fatal deregulation of cellular processes involved in DNA repair, cell survival, proliferation and motility. As a result, a healthy cell acquires the capability to accumulate mutations and to proliferate in an uncontrolled way, ultimately forming a tumor mass from which single cells disseminate to form metastasis at distant sites in the body. This spread of tumor disease is often fatal and leads to the death of the respective cancer patient.

The required mutations are typically sub-classified into driver and passenger mutations with the first ones being crucial to drive tumorigenesis[1]. Driver genes typically regulate cell fate, cell survival and genome maintenance processes that upon deregulation cause loss of growth control. Compared to driver mutations passenger mutations do not *per se* confer growth advantage. Whole genome sequencing of the DNA of cancer patients led to the estimate that a typical tumor contains 2-8 driver mutations which are typically accompanied by approximately 30-60 passenger mutations indicating the complexity of malignant transformation. Melanoma, which is studied in this thesis, is an outlier from this general rule harboring typically approximately 200 mutations per tumor, indicating the power of UV irradiation as a mutagen[2]. This is a clinically relevant feature, as these data prove that probably every tumor will be unique in terms of acquired mutations and most likely also immunogenicity, which will be discussed later on.

Many of these mutations are located in so-called oncogenes and tumor-suppressor genes[3,4]. Oncogenes are genes that upon chronic activation, for example due to a mutational event, confer a growth advantage of the mutation harboring cell whereas tumor-suppressor genes are genes that require de-activation to allow for uncontrolled cell growth. Prototypical oncogenes are myc, human epidermal growth factor receptor 2 (Her2/neu), Ras and the Abl gene in the Philadelphia chromosome whereas typical tumor-suppressor genes are the retinoblastoma protein (pRb), p53 or the phosphatase and tensin homolog (PTEN).

Importantly, many proteins can harbor mutations at various sites indicating their importance for tumorigenesis in general and many tumor entities share similar mutation patterns providing them with similar features which led to the classification of the hallmarks of cancer by Hanahan and Weinberg that will be discussed under 3.1.2[5].

3.1.2 General hallmarks of cancer

As introduced previously, the genetic instability that neoplastic cells acquire can cause a tremendous amount of genetic and epigenetic mutations that ultimately grant tumor cells their abnormal growth and later on disseminating behavior[1]. Hanahan and Weiberg in 2000 have proposed an organizing principle showing that mutations during oncogenesis serve basically one of six purposes[6]. Due to the emerging importance of additional hallmarks in 2011 a new version featuring two new emerging hallmarks and two enabling characteristics was released (**figure 3.1**)[5].

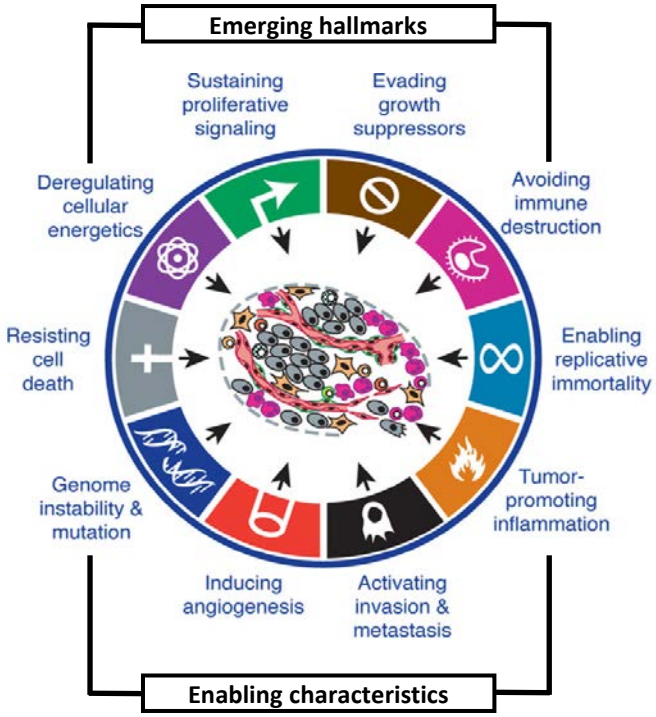


Figure 3.1 – The next generation of hallmarks of cancer. Schematic representation of key features of tumor cells together with the underlying mechanisms allowing for a rapid evolution of cancer cells. The represented abilities can be observed in virtually any patient and tumor entity and are ultimately causative for cancer-related deaths (Adopted from Hanahan and Weinberg, *Cell*, 2011; 5, 646-74)[5].

Due to the importance of those hallmarks for cancer biology they will be briefly introduced below:

a) Enabling replicative immortality

Tumor cells acquire a state of immortality. Typically, after many rounds of cell division, the telomeres of the chromosomes get overly truncated and aggregate ultimately leading to cell death. As tumor cells unlike non-immortalized cells express a functional telomerase this chromosomal crisis is avoided as the protein constantly adds nucleotide repeats to the end of the chromosomes in tumor cells.

b) Activating invasion and metastases

Primary tumors often initiate a sequence of certain steps, the invasion-metastasis cascade, leading to the dissemination of single tumor cells. These are transported via lymphatic or blood vessels to distant organs which they colonize. This complex process features the de-regulation of adhesion molecules like E-cadherin but also tumor-infiltrating bone-marrow derived cells such as macrophages that can supply matrix-degrading enzymes and thus facilitate metastasis.

c) Inducing angiogenesis

As cancer cells are metabolically highly active, a tumor permanently needs proper nurturing with various metabolites and in addition requires a well-established gas exchange, both processes which are mediated by the bloodstream. Thus, for sustained growth tumors require to establish an extensive blood vessel system, the generation of which is termed angiogenesis or neo-angiogenesis. The vascular endothelial growth factor (VEGF), in this context, is a prototypical pro-angiogenic molecule which participates in triggering the angiogenic switch.

d) Resisting cell death

As sustained proliferative signaling leads to induction of cell death, for example via Bcl-2 homology 3 (BH3)-only proteins, anti-apoptotic factors are required to keep malignant cells alive. This is often achieved via loss of p53, overexpression of anti-apoptotic factors such as B-cell leukaemia/lymphoma 2 (Bcl-2) or down-regulation of pro-apoptotic factors such as Bax or Bim.

e) Sustaining proliferative signaling

On their way to oncogenic transformation, normal cells constantly need to receive growth and/or survival signals. This is achieved, for instance, via autocrine stimulation with growth factors or constant, i.e. ligand-independent, signaling of growth factor receptors. One example would for instance be a B-Raf mutation yielding a constitutively active version of the protein that can be detected in around 40% of melanoma samples[7].

f) Evading growth suppressors

In line with the sustained proliferative signaling, tumor cells also need to evade growth suppressing signaling. One very famous example for a tumor-suppressor gene would be p53, the so-called ‘guardian of the genome’. As genetic mutations are accumulating, control proteins which can drive cells into senescence or apoptosis when DNA damage is overwhelming need to be kept in check. Accordingly, p53 is one of the most frequently mutated control proteins in cancer showing point mutations in about 50% of all sequenced tumors.

The acquisition of these six important hallmarks is crucial for cancer cells, yet they may occur in varying orders and with different timings in individual cancers. All of the introduced six hallmarks can be acquired because of two so-called enabling characteristics that will be introduced briefly in the next section:

a) Genome instability and mutation

As introduced earlier, genetic instability enables cells or cancer cells to acquire mutations in a random way that later on are selected for conferring growth advantage which leads to the outgrowth of the most successful or tumorigenic subclones. This instability needs to be achieved by either increasing the sensitivity towards mutagenic agents or by circumventing the cellular DNA repair machinery to prevent repair of introduced mutations. Important alterations include variations in gene copy numbers, mutations in coding parts of DNA but also mutations in non-protein coding DNA, for instance in regulatory elements.

b) Tumor-promoting inflammation

Infiltrates of various immune cells can be found in any given tumor. Despite the fact that many of the cell types found can control tumor growth, tumor-associated inflammation can severely contribute to assist tumor cells in advancing to states of elevated malignancy. Inflammatory environments can hence lead to the modification of the extracellular matrix (ECM), to neo-vascularization and even effector molecules like reactive oxygen species (ROS) have been shown to be able to foster genetic instability and thus drive the evolution of cancer cells.

Due to immense research efforts that have been put into the understanding of tumor development and progression, two new emerging hallmarks were identified and added to the original list. These will be briefly discussed in the next section:

a) Deregulating cellular energetics

Tumor cells require many nutrients to fuel their excessive growth. On the other hand, the tumor microenvironment is often hypoxic disabling the usage of the citric acid cycle that critically depends on normal oxygenation. This fact has been realized in the 1930s by Otto Warburg. Since then it became increasingly clear that hypoxia and, for instance, Ras signaling can independently increase the hypoxia-inducible factor 1 alpha (HIF1α) which subsequently up-regulates glycolysis. Together with the overexpression of glucose transporters like the glucose transporter-1 (GLUT-1) these phenomema provide enough energy to drive the uncontrolled proliferation of tumor cells in hypoxic tissues.

b) Avoiding immune destruction

It has been shown successfully in models of adoptive transfer and/or depletion that amongst others cytotoxic T lymphocytes (CTL) and NK cells can control tumor growth *in vivo*. In line with this it has been shown that immuno-suppressed individuals, for example organ recipients, also display a higher risk for developing cancer. Also, several studies reported that usually the level of infiltration of tumor tissue with T and/or NK cells correlated with better prognosis (these findings will be discussed later). These date led to the addition of the hallmark of immune evasion to the existing list.

3.1.3 Melanoma

Malignant melanoma is a subtype of cancer involving the melanocytes in the basal layer of the epidermis (stratum basale). It is overall the third most common malignancy and is notably a very aggressive neoplasia that after metastatic spread is characterized by a 5 year-survival rate of less than 5%[8]. Further, although the lifetime risk of developing cutaneous malignant melanoma with around 1-2% is comparably low the incidence rate increases faster than for any other malignancy[9,10].

As already introduced, melanoma cells harbor usually more mutations than other cancer entities. This is due to the fact that melanocytes are frequently exposed to mutating UV irradiation which is part of the spectrum of sunlight. Of note, prominent mutations in melanoma samples are directly in line with issues that were discussed under 3.1.2. Like mentioned the proto-oncogene B-Raf is mutated in about 40% of melanoma samples but also N-Ras, Bcl-2, PTEN or nuclear factor-κB (NF-κB)

frequently exhibit mutations providing progenitors of melanoma cells with growth advantages leading to oncogenic transformation[7,11].

One of the reasons why despite increasing incidence the melanoma-related death rate is virtually stable is that it is more often detected in its early stages where it can be removed with surgery. However, as melanocytes are derived from highly motile neural-crest progenitors they are very prone of initiating metastatic spread. Also, melanoma cells are often refractory to most existing treatment strategies and can exhibit a pronounced apoptosis-resistance *in vitro* and *in vivo*[12]. Thus, novel strategies targeting melanoma are desperately needed.

3.2 The immune system

The human body is exposed to many different pathogens, such as viruses, bacteria or fungi. Yet, it evolved an entire system of organs and cellular and soluble components that is able to potently fight off virtually any infectious threat. The most important organs being part of the immune system are the bone marrow, the spleen, the thymus, lymph nodes, tonsils and the mucosa- and gut-associated lymphoid tissues. Importantly, the cells of the immune system are all created by specialized hematopoietic stem cells (HSC) in the bone marrow from where they either migrate towards secondary lymphoid tissue like the thymus for development or circulate in the blood or lymph to detect pathogenic or malignant threats to the body. In order to do so, all immune cells rely on a distinct set of receptors with which they discriminate self/healthy from non-self/diseased tissues to ensure that their effector potential is only released in danger situations. In vertebrates, the cells of the immune system are classically assigned to either the innate or the adaptive immune system. This is done according to the type of recognition receptors used and the timing of the subsequent immune reaction but also by the ability of the cell to exert immunological memory.

3.2.1 The innate immune system

The innate immune system is characterized by the usage of receptors that are germline-encoded, non-clonally distributed and that recognize a broad array of pathogens. Upon recognition of for example a virus an immune reaction is instantly initiated without the need for prior sensitization. Therefore, innate immunity serves as a first line of defense against invaders. It consists of various cell types as well as soluble factors. The most important cell types of this branch of the immune system are monocytes, macrophages, dendritic cells (DCs), granulocytes and NK cells. The soluble factors belonging to the innate immune system are comprised of the acute phase proteins (pentraxins like the C-reactive protein (CRP) and the mannose binding lectin (MBL)) that are secreted by hepatocytes upon challenge with macrophage-derived IL-1, IL-6, tumor necrosis factor-α (TNF-α) and the complement proteins. The latter comprise a set of plasma proteins that get activated in a triggered-enzyme-cascade and subsequently assist in opsonizing pathogens for phagocytes, lyse them directly or produce inflammatory peptides[13].

Upon penetration of a pathogen through epithelium or mucosa the cellular components of the innate immune system can recognize structures which are shared by many different pathogens (pathogen-associated molecular patterns (PAMPs)) with their cognate pattern recognition receptors (PRRs). Important membrane-bound PRRs, for instance, would be toll-like receptors (TLRs), C type lectin receptors or scavenger receptors. Notably, besides these membrane-bound receptors there are also soluble and intracellular PRRs. The entire PRRs bind to PAMPs, promiscuously expressed on various pathogens, such as bacterial lipopolysaccharide (LPS, recognized by TLR4), flagellin (recognized by TLR5), double-stranded RNA (recognized by TLR3) or unmethylated CpG (recognized by TLR9) conferring self-non-self discrimination capabilities. Of note, these receptors do not reach the extent of specificity that receptors of the adaptive immune systems confer.

When a phagocytic cell (neutrophil granulocytes, monocytes, macrophages, DCs), for instance a tissue-resident DC, recognizes PAMPs with its PRRs it will phagocytose the PAMP bearing structure[14]. This triggers the secretion of inflammatory cytokines and chemokines but also allows macrophages

and DCs to break incorporated proteins down into peptides that subsequently get presented on major histocompatibility complex (MHC) molecules[15]. After full maturation, which includes the up-regulation of the co-stimulatory CD80 and CD86, and homing to the draining lymph node these peptides in a MHC context are presented to naïve T cells which thereby get activated[14]. This cross-talk between cells of the innate and adaptive immune system is absolutely crucial for mounting adaptive immune responses. In particular DCs, the most professional antigen presenting cells (APC) play a crucial role in the cross-talk between innate and adaptive immunity.

Of note, PRRs are not exclusively expressed on cells of the innate immune system. It has been shown for instance that PRRs are also expressed on cells of the vascular endothelium. These endothelial cells (EC) upon PRR signaling produce chemotactic proteins and increase the expression of certain adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) and E-selectin. This process, being termed endothelium activation, efficiently assists effector cells in extravasating into infected tissue (refer to section 3.2.4).

Another important cell type of the innate immune system is the NK cell. As they are the central cell type for this thesis they will be discussed comprehensively in chapter 3.3.

3.2.2 The adaptive immune system

The innate immune system, as introduced in 3.2.1, harbors PRR-expressing cells that respond quickly towards various invading pathogens, keeping the infection under control until several days later the adaptive immune system is fully activated. The reason for this delayed type of response is the structure of the respective receptors on adaptive effector cells. They require somatic gene re-arrangement and are clonally expressed on cells. During development, B and T cells randomly recombine their receptor genes which are located in various segments, a process called V(D)J recombination. Key to this process are the recombination activation genes (RAG) 1 and 2[16]. The entire process leading to the expression of one functional receptor can create up to 10^{14}-10^{18} specificities that subsequently get depleted of self-reactive receptors. This selection and depletion of autoreactive specificities is called clonal deletion. Due to the random assembly of receptors this process is an important pre-requisite to maintain tolerance to self.

The cells of the adaptive immune system are B and T cells, both of which are generated in the bone marrow. Whereas B cells remain in the bone marrow for maturation, T cells egress into the thymus where their final maturation takes place. Due to the stochastic expression of receptors with a distinct specificity, only few B or T cells directly after an infection express the correct specificity for a given pathogen-derived epitope. Thus, a process named clonal expansion is triggered. This process generates antigen-specific cells out of very few B or T cells expressing a receptor detecting pathogenic molecules. This required expansion explains the delay until full effector functionality can be provided. For T cells, this activation is achieved by recognition of cognate peptides in the context of MHC molecules on APCs, like for example DCs, with their T cell receptor (TCR). T cells expressing the CD4 co-receptor hereby bind to peptides in the context of MHC class II presentation that is expressed on APCs whereas cells expressing the CD8 antigen bind to peptides presented on MHC class I which is expressed on virtually all nucleated cells. T cells mature in the thymus and later patrol secondary lymphoid tissue in search for APCs presenting specifically their cognate peptide:MHC complex that can be detected by their TCR. After activation, $CD8^+$ T cells develop into CTLs. These

cells mediate direct cytotoxicity against cells presenting their cognate peptide on MHC class I. $CD4^+$ T cells can differentiate into Th1 or Th2 cells depending on the cytokine milieu during activation. Th1 cells are generated in the presence of IL-12 and/or IL-18. They activate macrophages to kill intracellular pathogens more efficiently and stimulate B cells to produce opsonizing antibodies. Th2 cells on the other hand differentiate in the presence of IL-4 and assist in the activation of antigen-specific B cells to produce neutralizing antibodies.

B cells prior to activation express a membrane bound immunoglobulin as B cell receptor (BCR). For activation, they require binding to their specific antigen. This antigen subsequently gets internalized and peptides of it are presented on MHC class II on the B cell surface. Certain T cell subsets are required for B cell activation by binding to the peptide: MHC II complex stimulating the B cell. Thus, the TCR recognizes a peptide of the antigen the respective BCR reacted against. Upon activation, B cells differentiate either into memory cells or into antibody-secreting plasma cells that are a crucial part of the defense against extracellular pathogens or toxins. Different classes of secreted antibodies hereby mediate various functions such as antibody-dependent cytotoxicity (ADCC), opsonization of pathogens for phagocytes or complement activation.

Of note, other cells like NKT cells or γδ T cells exert bridging functions or show features of either of the two branches. γδ T cells for instance use TCR rearrangement, yet their TCR is working in a PRR-like fashion[17]. NKT cells on the other hand exert a bridging function between the two arms of immunity in a way that they get strongly activated by signaling of CD1d in the presence of DC derived IL-12 and subsequently activate $CD4^+$ Th1 cells and CTLs[18]. Notably, there are more cell-types for example Th17 or regulator T cells (T_{reg}) that are not introduced in this section.

3.2.3 Tumor immunology

Besides fighting off pathogenic invaders, the immune system also has developed the ability to recognize altered self as target structures and mount an immune reaction against these changed, i.e. transformed cells. However, the effector cells and molecules are not always exerting beneficial effects, rendering tumor immunity a double-edged sword[19].

Of note, more than 100 years ago in 1909, Paul Ehrlich introduced the concept of immune cells controlling cancer growth. After 50 years and many discoveries made in the field of immunology, Burnet and Thomas proved that Paul Ehrlich's assumptions were correct and introduced the renaissance or real starting point of modern tumor immunology featuring the concept of tumor immune-surveillance. Yet, the similarity of tumor cells to healthy cells were discussed controversially, since danger signals like bacterial LPS for instance were clearly missing in a tumor context. Also data were differing in several groups. The very first clear cut result was achieved in the 1990s with IFN-$\gamma^{-/-}$ or IFN-γ receptor (IFN-γR)$^{-/-}$ mice that showed defective tumor rejection compared to wild type (wt) mice upon carcinogen challenge or injection of tumor cell lines. Several important findings are comprehensively reviewed by Vesely *et al.* and ultimately led to the general appreciation of tumor immune-surveillance[20].

A way of summarizing all of the findings in a comprehensive way was introduced by Schreiber and his colleagues who were arranging the cancer-immune cell cross-talk throughout several stages and thus creating the immunoediting scheme, explaining how immune cells can either be beneficial or

detrimental for tumor bearing patients (**figure 3.2**)[21,22]. Most importantly, the immune system, in addition to the amount of tumor cells, also impacts the quality of the remaining cancer cells by actively selecting for non-immunogenic clones, thus explaining the word editing:

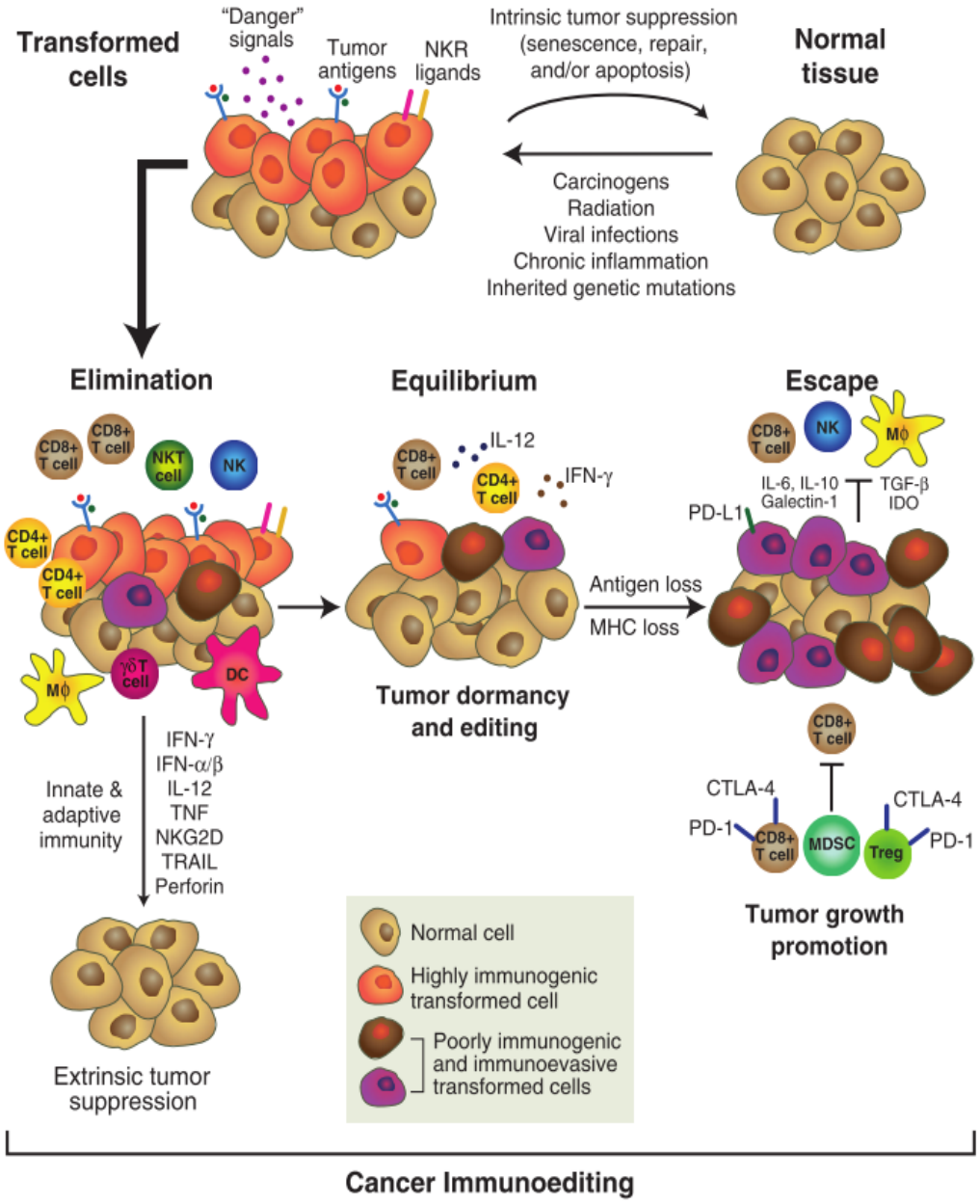


Figure 3.2 – The three E's of cancer immunoediting. The scheme displays the interplay between tumor cells at various stages with multiple immune cells, leading ultimately either to tumor destruction (elimination) or outgrowth (escape). CTLA-4, Cytotoxic T-Lymphocyte Antigen 4; IDO, indoleamine 2,3-dioxygenase; MΦ, macrophage; NKR, NK cell receptor; PD-1, Programmed cell death protein 1; PD-L1, programmed cell death-1 ligand; TGF-β, transforming growth factor- β; TRAIL, TNF-related apoptosis-inducing ligand, (Schreiber *et al. Science* 2011; 6024: 1565-70)[19].

As pointed out in section 3.1 mutations can cause malignant disease, if they allow for a growth advantage, by conferring the previously introduced hallmarks of cancer. However, several modes of intrinsic tumor suppression exist, for instance p53 mediated apoptosis induction due to excessive DNA damage or induction of senescence due to oncogene activation[23]. Upon circumventing these

regulatory mechanisms the transformed cell grows out and eventually is detected by the immune system due to the expression of markers of non-self. These markers can for instance be activating NK cell ligands (see section 3.3.2), some of which are induced upon malignant transformation or tumor-associated T cell epitopes[24].

Important signals by the developing tumor that alert the immune system are featuring type I IFNs, IL-1, TNF-α, high mobility group box 1 (HMGB1) or hyaluronan fragments from damaged tissue[25]. Importantly, HMGB1 can associate with other molecules and trigger TLR or the receptor for advanced glycation end products (RAGE) signaling, inducing inflammation therefore being assigned to the class of damage-associated molecular patterns (DAMPs). Notably, perturbances in the tissue integrity by neo-vascularization or stromal remodeling due to invasive tumor growth were proposed to be the first danger signal for the immune system as they could produce inflammatory cytokines, chemokines and molecules such as heat shock proteins (hsps) that activate tissue resident DCs[26,27]. These in turn attract and subsequently activate NK and T cells.

Elimination

The first phase of immunoediting represents the elimination phase, the stadium in which immune cells are fighting to prevent tumor outgrowth. Ideally, this phase from early on involves cells of the innate as well as cell of the adaptive immune system. NK cells can detect activating transformation induced ligands, like ligands for their activating receptor NKG2D, whereas T and NKT cells can detect tumor antigens in the context of MHC or CD1[24]. The activation of these cells leads to the triggering of cytotoxic effector mechanisms but also the secretion of inflammatory cytokines such as IFN-γ. Likewise, IFN-γ stimulates macrophages to produce IL-12 that in turn stimulates IFN-γ secretion by NK cells resulting in a positive feedback loop[28,29]. Activated macrophages can then kill tumor cells via reactive oxygen species, whereas for NK cells TNF-related apoptosis-inducing ligand (TRAIL) and perforin dependent mechanisms have been described[30,31]. This first wave of anti-tumor effector mechanisms, as pointed out, is mainly driven by innate effector cells. Of note, high levels of IFN-γ also exert direct anti-proliferative, anti-angiogenic and pro-apoptotic functions on tumor cells[32–34].

As already mentioned, tissue resident sentinel DCs will get activated in this milieu either by innate cytokines such as IFN-γ or by tumor-infiltrating NK cells themselves, leading to their activation[35]. Also, the micromilieu in the meantime is enriched for hsp and apoptotic cell fragments that harbor excessive amounts of tumor antigens[36,37]. Subsequently, tumor-antigen loaded DCs migrate to the draining lymph node to mount an adaptive immune response against these antigens and induce a specific anti-tumor T cell response. This response ideally involves a $CD4^+$ Th cell induction that subsequently supports the activation of cytotoxic $CD8^+$ CTLs via cross-presentation[38]. Notably, the contribution of $CD4^+$ Th cells to tumor immunity has not been realized for a while. Now, data clearly point to an important role of $CD4^+$ T cells[39]. Accordingly, $CD8^+$ CTLs after their clonal expansion will ideally infiltrate back into the tumor tissue, detect specifically antigen-expressing tumor cells and respond with cytotoxicity and/or IFN-γ secretion that in turn can activate macrophages, DCs, NK cells or exert anti-tumoricidal effects as discussed, possibly leading to the elimination of the tumor.

Equilibrium

If during the elimination phase not the entire tumor burden gets cleared, a population of tumor cells co-exists with the effector cells of the immune system in a state of dormancy. This state is called equilibrium and is believed to be the longest phase in the process of immunoediting. It thus has been estimated that it can take 20 years from the first contact with a carcinogen to clinically manifest malignant disease during which most of the time the tumor exists in an occult state in equilibrium phase[40].

Animal experiments were proving the existence of this dormant state. Fully immunocompetent C57BL/6 mice were treated with low dose 3'-methylcholanthrene (MCA) and due to the low dosing did not develop any apparent tumor. Later, upon antibody-mediated depletion of IFN-γ and T cells, roughly 50% of the animals quickly developed tumors at the site of MCA treatment[41]. Of note, NK cells were not found to contribute to this state of dormancy as administration of anti-NK1.1 or anti-NKG2D did not lead to tumor outgrowth. Similar data that support this hypothesis of the existence of an equilibrium phase were also gathered in various mouse models[42]. Thus, the adaptive immune system prevents the outgrowth of resident tumor cells and sculpts their immunogenicity by selecting for less immunogenic tumor cells. This renders the equilibrium phase distinct from elimination phase that requires both innate and adaptive immunity.

Despite the equilibrium phase keeping a growing tumor under control, this phase is crucial for the creation of an evolutionary force driving the tumor cells to develop escape variants as it keeps high immunological pressure on the cancer cells. These variants are more aggressive compared to the original tumor cells as they are less immunogenic and eventually cannot be killed by immune cells anymore and thus grow out leading to malignant disease.

Escape

The last step in the immunoediting process is the escape phase. As discussed, during the equilibrium phase immune cells shape tumor cells leading to the accumulation of immune-evasive mutations. These mutations, or intrinsic mechanisms can be a) loss of immunodominant antigens b) loss of MHC class I and/or II antigens or c) defects in the antigen processing machinery[43]. According to Darwin's survival of the fittest theory, these cloaked, malignant altered-self cells progressively grow leading to clinically manifest disease. The described cancer-inherent mechanisms are only one possibility to prevent destruction by the immune cells. As shown in **figure 3.2**, cancer-induced immunosuppression of effector cells is another way of evading cytotoxic immune cells and is termed the cancer-extrinsic way. To render the microenvironment immunosuppressive several possibilities exist, all of which are exploited by tumor cells. First, the secretion of immunosuppressive cytokines directly by tumor cells exerts strong tumor-protective functions. The most prominent molecules are IL-10, VEGF[44], TGF-β[45] and indoleamine 2,3-dioxygenase (IDO)[46]. A second possibility is the specific recruitment of immunosuppressive immune cells such as T_{reg} or myeloid derived suppressor cells (MDSC). T_{reg} upon stimulation with their specific antigenic peptide produce immunosuppressive cytokines such as TGF-β or IL-10. Further, via their high expression of CD25 they sequester IL-2, an important molecule to promote NK cell and CTL activation and survival. Furthermore they express inhibitory molecules such as CTLA-4 or programmed death-1 (PD-1)[47]. MDSC are a heterogeneous population of immature myeloid cells and similarly to T_{reg}, exhibit various functions that suppress the functions of various

immune cells. They secrete TGF-β, deplete arginine and tryptophan and are able to nitrate the TCR or chemokine receptors of tumor-specific T cells. Interestingly, tumor-derived factors trigger a distorted differentiation of immature DCs and myeloid cells thus driving *in situ* MDSC differentiation. Important factors were described to be constitutive signal transducer and activator of transcription 3 (STAT3) and defective NF-κB signaling[48]. Notably, previous work from our group showed in a mouse model of lymphoma, that tumor-infiltrating MDSC are able to actively recruit T_{reg} via the secretion of the chemokines CCL3, CCL4 and CCL5 thus establishing a link between the accumulation of these immune-suppressive populations[49]. Lastly, also the secretion of soluble ligands which block activating receptors of immune effector cells has been described[50].

3.2.4 Leukocyte migration and the chemokine system

3.2.4.1 Transendothelial migration

After their maturation leukocytes are transported in blood or lymphatic vessels through the body searching for inflamed tissue. To help and clear an infection, lymphocytes for instance from the blood stream need to specifically detect where they are needed and pass through a dense layer of EC to migrate to the site of the infection. Etravasation is a multi-step process which is introduced in the following section (**figure 3.3**)[51].

First, the EC in proximity to the site of infection needs to display an activated phenotype. Notably, PRR-activated, tissue-resident macrophages secrete for instance TNF-α. This cytokine can trigger the secretion of Weibel-Palade-bodies in proximal EC, leading to the elevated expression of E-selectin[52]. Also TLR-stimulation of EC, for instance TLR4 stimulation via LPS can directly induce P-selectin.

The first step of transmigration requires a loose attachment of leukocytes to the EC, a process termed capturing that leads to rolling of the respective cell on the EC layer. This interaction is mostly dependent on the selectin family of adhesion proteins, the L-, P- and E-selectins with L-selectin being expressed on most leukocytes and E- and P-selectin being expressed by inflamed EC[52]. The P-selectin glycoprotein ligand 1 (PSGL1) has a role as ligand for all three selectins and can be expressed on EC and almost all leukocytes. Triggering of leukocyte selectin can subsequently lead to integrin activation via g protein-coupled receptor (GPCR) and MAPK signalling[53]. The likewise activated integrins after binding to their ligands slow down rolling. Depending on the cell type one of various interactions is possible, for instance the interaction between leukocyte functional antigen 1 (LFA1) and ICAM1 or the very late antigen 4 (VLA4) and the vascular cell adhesion molecule 1 (VCAM1)[54]. Of note, the VLA4-ICAM1 axis for instance mostly recruits monocytes and T cells. Thus, on the pattern of adhesion molecule expression specific effector cells can be recruited. For the firm arrest of slowly rolling leukocytes in addition chemokines are required. This is a family of chemoattractant cytokines that orchestrate the tissue distribution of leukocytes[55]. They will be introduced in detail later in this section. The origin of the chemokines can be either the EC itself, as they produce chemokines upon activation by inflammatory cytokines, cells in the blood stream for instance platelets, that deposit CCL5 on the luminal side or effector cells on the abluminal side or immune cells in the tissues[56,57]. Notably, the chemokines are mostly deposited on glycosaminoglycans (GAG) on the EC surface. It was shown that this interaction is crucial for efficient recruitment and that GAG binding might increase the half-life time of chemokines[58]. Binding to a cognate chemokine instantly increases the affinity and valency of the integrins to their respective EC-bound ligands leading ultimately to a firm

arrest of the receptor bearing lymphocyte on the EC[59]. The signaling underlying this mechanism - characteristically for heterotrimeric G proteins - involves Ca^{2+}- flux induced by phospholipase C signaling which produces inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). Ca^{2+} and DAG together activate various GTPases such as RAP1 or RHOA that modulate the actin-binding protein talin-1 to activate the integrins[60–63].

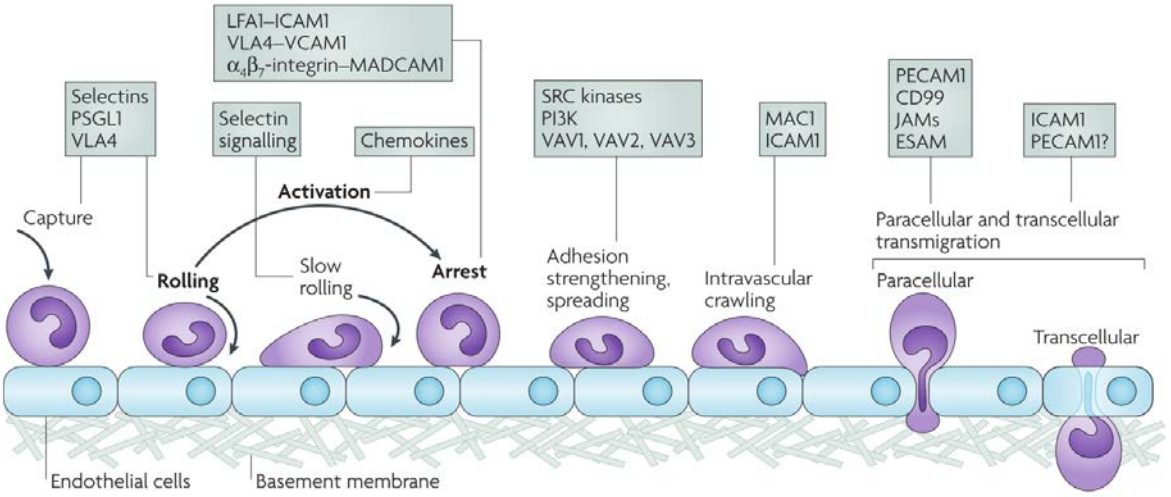


Figure 3.3 – The updated leukocyte adhesion cascade. Shown are the three traditional steps in bold and recently added steps leading to leukocyte transmigration through EC. The most important adhesion molecules involved in each step are indicated in boxes. ESAM, endothelial cell specific adhesion molecule; ICAM1, intracellular cell adhesion molecule 1; LFA1, leukocyte functional antigen 1; JAM, junction adhesion molecule; MAC1, CD11b/CD18; MAdCAM1, mucosal vascular addressin cell adhesion molecule 1; PECAM1, platelet endothelial cell adhesion molecule; PI3K, phosphatidylinositide 3-kinase; PSGL1, P-selectin glycoprotein ligand 1; VCAM1, vascular cell adhesion molecule 1; VLA4, very late antigen 4, (Ley *et al.* Nature Reviews Immunology 2007; 9: 678-89)[51].

Finally, the firmly attached cell needs to transmigrate through the layer of inflamed EC it is fixed to. Some cell types such as neutrophils or monocytes can crawl the surrounding vessel parts in order to find the best suited spot for transmigration. This crawling is mostly CD11b/CD18 (MAC1) and ICAM1 dependent[64]. To migrate past the EC layer to the basement membrane, two possibilities exist for migrating leukocytes. The first option is the paracellular migration. Here it has been shown that inflammatory stimuli and integrin signaling in EC re-distribute junction molecules facilitating transmigration of leukocytes through less tightly attached ECs. Here, various stimuli activate different junctional molecules. Due to the complexity, the details will not be discussed in this thesis[51]. The second possibility to migrate past ECs is transcellularly[65,66]. This mode requires vesiculo-vacuolar organelles (VVOs). These specialized membraneous paths through EC seem to require neutrophil adhesion to EC, cytokine activation of the respective EC and high ICAM1 levels. Leukocytes thus can inefficiently but quickly migrate through these channels in the cells[65,67,68]. Having passed the EC layer extravasating leukocytes need to pass the basement membrane and eventually the pericyte sheath. Notable, whereas the EC layer can be passed usually in 2-5 minutes, the basement-membrane can require more than 15 minutes. Interestingly, similar to GAG, heparin-sulphate in the basement-membrane can bind chemokines and thus provide freshly extravasated leukocytes with further migratory stimuli. Having invaded the inflamed tissue, effector leukocytes next follow the chemokine gradient to reach the site of inflammation.

3.2.4.2 The chemokine system

Chemokines are a chemotactic family of cytokines of 8-10 kDa of size that orchestrates the complex distribution of leukocytes (circulation, homing and retention)[69]. They are the only cytokines that signal via G-protein coupled receptors and share a very similar folding, due to a distinct motif containing 4 cysteine residues. **Figure 3.4** gives an overview over most of the so far identified chemokines and their cognate receptors.

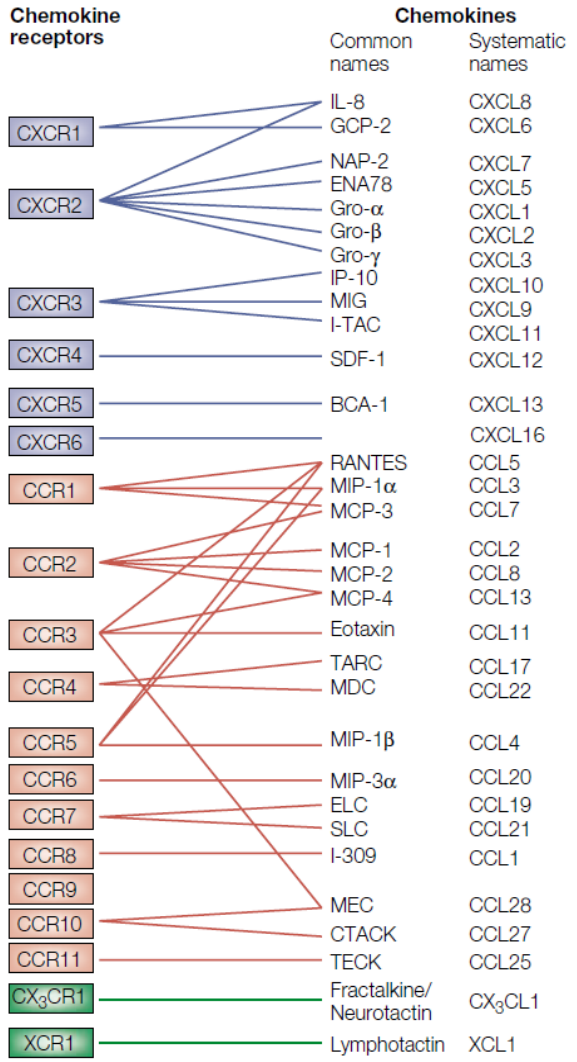


Figure 3.4 – Chemokine receptors and their ligands. Color codes indicate the respective chemokine group. Lines indicate respective specific receptor-ligand pairs. The system today contains more than 50 chemokines and 20 chemokine receptors. BCA-1, B-cell-attracting chemokine 1; CTACK, cutaneous T-cell-attracting chemokine; ELC, Epstein–Barr-virus-induced gene 1 ligand chemokine; ENA78, epithelial-cell-derived neutrophil-activating peptide 78; GCP-2, granulocyte chemotactic protein 2; Gro, growth- regulated oncogene; IL-8, interleukin 8; IP-10, interferon-inducible protein 10; I-TAC, interferon-inducible T-cell α chemoattractant; MCP, monocyte chemoattractant protein; MDC, macrophage-derived chemokine; MEC, mucosae-associated epithelial chemokine; MIG, monokine induced by interferon γ; MIP, macrophage inflammatory protein; NAP-2, neutrophil-activating peptide 2; RANTES, regulated on activation, normal T-cell expressed and secreted; SDF-1, stromal-cell-derived factor 1; SLC, secondary lymphoid-tissue chemokine; TARC, thymus and activation-regulated chemokine; TECK, thymus-expressed chemokine (Proudfoot *Nature Reviews Immunology* 2002; 2: 106-15)[91].

The segregation of chemokines into families is done by analyzing the cyteine-containing N-terminal motif. If the first two cysteines are separated by one amino acid, the chemokine belongs to the CXC-family of chemokines. This family is also called the α-chemokine family. If no amino acid separates the first two chemokines it is assigned to the CC-family which is also called the β-chemokine family. Some identified chemokines do not fit into this classification for instance XCL1 or lymphotactin, harboring only 2 instead of 4 cysteines in this motif, CX3CL1 or fractalkine that harbors three amino acids between the first two cysteines and SLC the 6Ckine with a total of 6 cysteines in that motif. Notably, the system evolved to be highly redundant, as usually several chemokines bind to one receptor and one receptor to various chemokines.

The chemokines were primarily identified as chemotactic proteins. They are secreted by various cell types, amongst others by platelets, EC, microglia, monocytes, macrophages, fibroblasts, T cells and

many more[70]. After secretion they exert two functions. First, they are crucial mediators of efficient leukocyte extravasation and tissue invasion and second they guide effector cells along the chemokine gradient from the vessel to the chemokine producing cell. Their cognate receptors are all serpentin receptors that span biological membranes 7 times and signal as described under 3.2.4.1. Of note, this signaling is highly sensitive to pertussistoxin as this compound efficiently ADP-ribosylates the G_i unit of the heterotrimeric complex. Also, chemokine receptors frequently get internalized after they bound their cognate ligand[71].

As discussed under 3.2.4.1 lymphocytes eventually leave the blood or lymph through inflamed or activated EC. After passing the basement membrane they need to migrate in the interstitium towards the source of the chemokine to reach the inflamed tissue. This chemotaxis is crucial for immune cell infiltration of inflamed tissue[70,72]. Signaling mainly involves PI3K with the chemokine gradient inducing a guiding system in the leukocytes that translates into polarization of the cell. This polarization ultimately leads to directed migration through tissue[73,74]. Chemokines are therefore presented or anchored at the ECM via GAG or heparin-sulphates. These molecules were also reported to be relevant for chemokine transport and stability[75,76]. Migrating leukocytes thus integrate signals from various chemokines that are either free or presented by the ECM and polarize accordingly, a process that was reported to depend on LFA1, the phosphatidylinositide 3-kinase (PI3K), Syk, Bruton's tyrosin kinase (Btk) and Rho GTPase[74,76–80]. Importantly, infiltrating leukocytes express a variety of proteolytic enzymes enabling them to aggressively invade different tissues by digesting their way through ECM[81,82].

In addition to the classical chemokine receptors, most of which are displayed in figure 3.2.2, there are 5 atypical chemokine receptors. They are called CXCR7, Chemokine-binding protein 2 (CCBP2) also called D6, C-C chemokine receptor type 11 (CCRL1), C-C chemokine receptor-like 2 (CCRL2) and Duffy antigen/chemokine receptor (DARC) also called Duffy. Importantly, due to mutations that abolish efficient binding of small, heterotrimeric G proteins to the receptor they do not signal properly and thus serve as scavenger receptors or interceptors of chemokine signaling[83]. DARC likewise binds to many inflammatory chemokines. Its expression on erythrocytes is important as it serves as a chemokine sink. Also by presenting chemokines on venular endothelial cells it assists leukocyte emigration at the sites of inflammation[84]. Similarly it was shown that D6 is expressed on EC of lymphatic vessels, depriving activating chemokines from the lymph. Accordingly, $D6^{-/-}$ mice exhibited higher levels of inflammatory cytokines as excess activating chemokines were not sequestered[85].

In the last years, it was increasingly recognized that chemokines exert more functions than just chemoattraction. Various effects on leukocytes and correlations to various diseases have been reported. The CCL25-CCR9 axis for instance was shown to be important for thymic T cell development, whereas several chemokines have been shown to increase NK cell proliferation and /or cytotoxicity[86–88]. However, de-regulation or overexpression of chemokines can lead to various diseases for instance asthma via the CCL11-CCR3 axis that attracts eosinophils or the CXCL10-CXCR3 axis that attracts Th1 cells to promote inflammation in lesions of patients with multiple sclerosis[89,90]. Not surprisingly, various modalities have been tested to block chemokine receptor signaling to decrease the infiltration of overly inflamed tissues[91].

As chemokine receptor signaling also triggers survival pathways, tumor cells are exploiting that pathway in that they express chemokine receptors and produce the respective chemokines to create

an autocrine growth stimulation loop. Also, various chemokines have been described to promote angiogenesis and metastatic dissemination, which in many cases correlates with bad prognosis. Especially CXCL8 has been reported several times to be a marker of poor outcome in melanoma patients[92,93]. Compared to the often detrimental effects of CXCL8 expression in the tumor cells it was shown for a variety of other chemokines that their high expression improves prognosis due to stronger infiltration by cytotoxic effector cells[92,94]. The outcome of chemokine overexpression is therefore hypothesized to be strongly dependent on the immunogenicity and the entity of the tumor but most importantly on the presence of cognate receptors on the tumor cells.

3.2.4.3 CX3CL1/fractalkine

CX3CL1 is a very distinct chemokine in that it is the only known chemokine that has a three amino acid spacer between its two N-terminal cysteines and in that it can be expressed in a membrane-bound and soluble form, mostly by EC[95]. The actual chemokine domain is located at the N-terminus on top of a mucin-like stalk. Interestingly, both versions exhibit distinct functions. The membrane-bound chemokine mostly functions as an adhesion molecule for CX3CR1-expressing cells such as monocytes, DCs and NK cells whereas the soluble form is a potent chemokine, which induces migration of the formerly mentioned CX3CR1 positive cells[96]. Remarkably, 5 naturally occurring gene polymorphisms exist for CX3CR1, with the V249I and the T280M reportedly exhibiting reduced signaling[97]. Importantly, in a setting of renal transplantation an increased risk of developing cancer was found for homozygous carriers of the V249I and the T280M alleles pointing towards an anti-tumorigenic role of the CX3CL1-CX3CR1 axis. Several proteases have been reported to mediate CX3CL1-shedding, amongst others a Disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), ADAM17 and cathepsin S[98–100]. Notably, CX3CL1 is also heavily expressed in the central nervous system by neurons whereas the receptor is mostly expressed by microglia[101]. Here, neuron derived CX3CL1 confers protection from glutamate excitotoxicity via microglia derived adenosine which ultimately prevents damage[102].

In the periphery, CX3CL1 has been reported to impact the lineage commitment of myeloid precursors to the macrophage/DC lineage and is used to discriminate resident monocytes from inflammatory monocytes blood with the first one being CX3CR1^{high} [103,104]. So it was shown that EC upon inflammation exhibit *de novo* CX3CL1-expression which leads to the extravasation of resident monocytes, that differentiated into macrophages and modulated the immune response in the inflamed tissue[105]. NK cell activation by DCs was also critically impaired, when CX3CL1 expressed by DCs was blocked. NK cells showed extensive KIR phosphorylation and thus Src homology region 2 domain-containing phosphatase (SHP-1) recruitment leading to an abrogation of NK cytotoxicity[106].

Notably, CX3CR1- and CX3CL1-expression have also been reported on various tumor types with contradictory results. So Shulby *et al.* were reporting CX3CR1-positive prostate cancer cells that upon binding of CX3CL1 elicited phosphatidylinositol-3-kinase (PI3K) survival signaling. Also colonization of the bone marrow was found by them to be CX3CR1-mediated[107]. Further, hypoxia was reported to induce CX3CL1-expression on EC which was shown to induce their proliferation and tube formation thus potentially increasing angiogenesis. These data argue for a possible tumor growth promoting role of CX3CL1[108]. For breast cancer two studies were reporting opposite results. Whereas a study performed by Tsang *et al.* reported a worse outcome for CX3CL1^{high} breast cancer bearing patients despite higher counts of tumor-infiltrating lymphocytes (TIL) another study on breast cancer by Park

et al. showed that high levels of CX3CL1 correlate with good prognosis[109,110]. Importantly, it was shown that CX3CL1 is a direct target gene of p53[111]. This suggests that upon DNA damage the up-regulation of CX3CL1 arrests cytotoxic cells at the damaged cells facilitating their elimination by immune cells. Thus, CX3CL1 might be beneficial for clearance of pre-malignant or malignant cells. Indeed, it was reported that high expression of CX3CL1 can lead to better prognosis. Thus, for prostate carcinoma and gastric adenomacarcinoma high levels of CX3CL1 were predictive of prolonged disease-free survival. Of note, in none of the clinical studies CX3CR1-expression by tumor cells was evaluated, possibly explaining the trememdous variance between several studies. Still, several reported data clearly suggest the potential exploitability of the CX3CL1-CX3CR1 axis for cancer immunotherapy[112].

Thus, like many other chemokines, CX3CL1 is a multi-functional chemokine affecting many cell types not only in terms of migration. Therefore, modifying expression levels *in vivo* requires a comprehensive analysis of all processes that potentially are affected to exclude unwanted side effects.

3.3 Natural killer cells

Natural killer cells are important effector cells of the innate immune system that comprise about 5-15% of peripheral blood lymphocytes in humans. As innate cells, they use a broad array of activating and inhibitory germline-encoded receptors for activation and not just one clonally expressed receptor like B or T cells. 1975, in the first back-to-back reports that identified NK cells both groups mentioned the inherent and MHC unrestricted natural cytotoxicity of NK cells against syngeneic and allogeneic tumor cells[113,114]. Besides, they exert various effector functions against stressed, infected or transformed cell, shape adaptive immune responses and contribute to placentation[115,116]. Due to their central role in this thesis the most important aspects of NK cell biology will be discussed in this chapter.

3.3.1 Origin and development of NK cells

Developmentally, there are still some controversies about the origin of NK cells. However, it is widely accepted that NK cells in adults are derived from bone marrow-resident HSCs via a so-called CLP, the common lymphoid progenitor[117]. This lineage$^-$ CD34$^+$ progenitor cell upon signaling via the early acting receptors Fms-like tyrosine kinase 3 (flt3), c-kit, IL-3 or IL-7 *de novo* expresses the IL15 receptor complex consisting of the β- and the common γ-chains CD122 and CD132 respectively, turning the NK cell progenitor into an IL-15R$^+$ NK cell precursor[118–120]. Importantly, IL-15 signaling has been identified to be crucial for NK cell development in various models. Thus, the acquisition and signaling of CD122, the IL-15 receptor β-chain, allows for NK cell differentiation and maturation[120,121]. The source for the IL-15 was determined to be bone marrow stromal cells[120]. Notably, it was shown that IL-15 *in vivo* is presented complexed to CD215, the α-chain of the IL-15 receptor to the dimeric IL-15 receptor (IL-15R β-chain CD122, common γ-chain CD132) on the target cell[122]. The signaling via the IL-15R then induces the final differentiation of mature CD56^{bright} NK cells. However, the exact way of the genesis of CD56^{dim} cells remained elusive for quite some time. Therefore, various models have been suggested:

a) A distinct NK cell precursor that upon cytokine signaling would develop into CD56^{dim} NK cells
b) A possible common progenitor might develop with signals other than IL-15 into KIR$^+$, CD16$^+$ and CD56^{dim} NK cells
c) CD56^{bright} NK cells are direct progenitors of CD56^{dim} NK cells

Interesting observations in this respect were made by Jacobs *et al.* who showed that upon bone marrow transplantation a substantial percentage of all newly developed NK cells displayed a CD56^{bright} CD16$^-$ phenotype (> 50%). CD56^{dim} NK cells were developing later after engraftment of the bone marrow cells[123]. This finding potentially indicated that CD56^{dim} NK cells might develop out of CD56^{bright} NK cells, explaining their slower kinetics of repopulation. Indeed, it could be shown in later studies that CD56^{dim} NK cells most likely develop out of CD56^{bright} cells as already proposed in 1986[124,125].

In general, NK cell development has been extensively worked on. This research lead to the identification of several transcription factors, 2 of which were recently shown to be involved in NK cell differentiation, namely T-bet and eomesodermin (Eomes)[126]. Other transcription factors like Id2,

GATA-3, Ikaros or E4BP4 have also been described, yet their exact interplay, especially spatio-temporarily needs more investigation[127].

Several other regulators of NK cell development have been described, such as Notch signaling and miRNA regulation in humans. The Notch ligands Jagged2, Delta1 and Delta4 enhanced the differentiation of $CD34^+$ hematopoietic progenitor cells into NK cells *in vitro*[128]. Of note, this impact of the Notch pathway is regulated by miRNAs, adding another regulatory element to NK cell differentiation[129]. Also it is noteworthy that even not fully matured NK cells, so-called NK developmental intermediates (NKDIs) can exert potent effector functions[130]. Further, external factors can interfere with proper NK cell development. Importantly, the immunosuppressive factor TGF-β that was mentioned in 3.2.3 has been reported to interfere with NK cell differentiation thus providing a possible link between malignant disease and de-regulated NK cell development[131].

Last, the site of differentiation classically was believed to be the bone marrow although not all developmental stages were detected there. Also, a growing body of evidence is pointing out that stromal cells from spleen, liver or secondary lymphoid tissue can also provide important early differentiation factors such as Flt3 or IL-7. Accordingly, all developmental stages of NK cells have been found in human secondary lymphoid tissue. Thus, it is hypothesized that bone marrow derived progenitors egress the bone marrow and home to various organs such as the uterus, the liver, the spleen or lymph nodes to finish their development[132–134]. Of note, this cascade of events – migration into the primary site of development, maturation, egress – is highly similar compared to the T cell maturation in the thymus. Of note, developing NK cells in the bone marrow in mice have been described to be retained in the bone marrow via the CXCR4-CXCL12 axis. Upon maturation, they egress via the up-regulation of sphingosin-1-phosphate-5 (S1P5) receptor[135]. It is currently unclear if similar mechanisms exist for other organs.

3.3.2 Cytokine-activation of NK cells

As other immune effector cells, NK cells require signaling to acquire their full functional potential and thus differentiate into effector cells. Here, inflammatory cytokines such as IL-2, IL-15, IL-12 or IL-18 but also type I IFNs play pivotal roles. As the production of these stimulatory cytokines is triggered by danger signals, NK cells assure to be only fully activated when for instant pathogens or malignant transformation are detected. This cytokine-mediated activation can be achieved by either DCs, neutrophils or $CD4^+$ T cells[136–138]. Due to the importance of especially IL-12 and IL-18 later in this thesis, in this the most important NK cell-activating cytokines are briefly introduced.

IL-2 and **IL-15** both share very similar receptor complexes and thus signaling pathways[121]. Both receptors share the same β- and γ-chain. Yet, they have distinct α-chains which increase the affinity of the heterodimeric shared receptor for IL-2 or IL-15, respectively. Compared to the α-chain of the IL-2 receptor (CD25) the α-chain of the IL-15 receptor (CD215) does not require the binary complex of the β- and the common γ-chain to exhibit high-affinity binding to its ligand. In the absence of cytokines, janus kinase (Jak)1 associates with the β- and Jak3 with the γ-chain of the receptor. LIgand binding activates the two tyrosine kinases which activate STAT3 and STAT5, respectively. These STATs dimerize and after translocation into the nucleus serve as transcription factors. Also, the β-chain gets phosphorylated which creates src homology 2 (SH2) docking sites triggering Ras/Raf/MAPK pathways ultimately leading to activator protein 1 (AP-1) activation. Further the PI3K-Akt pathway, src kinase

signaling and NF-κB signaling get activated leading to the induction of anti-apoptotic Bcl-2 promoting cell survival. Importantly, IL-15 has been shown to exert activation more potently, when it is presented on trans on the α-chain of the IL-15 receptor by for instance a DC[122]. Of note, cellular sources of IL-2 are activated T cells only whereas IL-15 can be provided mostly by monocytes, DCs and bone marrow stromal fibroblasts. Also, besides NK cell activation IL-15 signaling has been shown to be a critical prerequisite for NK cell development, as IL-15$^{-/-}$ or IL-15R$^{-/-}$ mice lack NK cells under steady state by promoting NK cell development from hematopoietic precursors[120]. Importantly, both cytokines strongly increase cytotoxicity for tumor targets that are resistant against NK cells that haven't been stimulated with cytokines. This is due to a STAT5 mediated up-regulation of perforin but also because of the well-known up-regulation of various activating receptors due to the IL-2- and IL-15-signalling[139].

IL-12 belongs to the family of monokines and thus is produced by by monocytes, macrophages and DCs. Its heterodimeric receptor comprises the β1- and the β2-chain that upon binding to IL-12 gets autophosphorylated at the β2-chain thus recruiting Tyk2 and Jak2 that subsequently activate STAT4 that activates the AP-1 transcription factor. Of note, it was reported that signaling of IL-2 also strengthens the IL-12 signaling cascade in NK cells by up-regulating the IL-12R and STAT4[140]. Due to the potent stimulation mediated by simultaneous signaling by IL-2 and IL-12, joint signaling of the cytokines was found to cause apoptosis of NK cells, possibly indicating a negative regulator of NK immunity. This process was found to be dependent on TNF-α[141]. Notably, IL-12 was shown to be a strong inducer of IFN-γ and increased the cytotoxicity of human and murine NK cells by upregulating perforin levels[142].

IL-18 also belongs to the monokines and thus, like IL-12, is produced by monocytes, macrophages and DCs, but also constitutively by non-immune cells such as keratinocytes or osteoblastic stromal cells. Similar to IL-12, its receptor is a heterodimer that consists of the IL-18Rα- and β-chain but it structure- and functionwise. Upon binding of IL-18 β-chain of the receptor transmits the signal via Myeloid differentiation primary response gene 88 (MyD88) and interleukin-1 receptor-associated kinase (IRAK)/TNF receptor associated factors (TRAF)6 to ultimately activate NF-κB in the respective cell. In addition, STAT3 and MAPK signaling were shown to be involved in the signal transduction. IL-18 signaling alone however has been reported to be an intermediate strong inducer of cytotoxicity and IFN-γ production due to the weak constitutive expression of the receptor[143]. However, expression of the IL-18R is inducible by IL-12 and type I IFNs leading to a synergistic effect between the two cytokines[144]. Accordingly, with IL-12 supplementation *in vitro* or *in vivo* where IL-12 is present IL-18 has been shown to be crucial for NK cell activation[145,146]. Several mechanisms regulating the bioavailability of IL-18 further suggested its importance. So it is synthesized as a pro-protein (proIL-18) that needs either intracellular cleavage by the producing cell via caspase-1 prior to secretion or extracellular cleavage by proteinase-3[147,148]. Proteinase-3 is expressed by activated neutrophils and monocytes which thus assist in NK activation. Also the existence of a soluble binding-protein (IL-18 BP) has been reported that was suggested to regulate the availability of free IL-18 by sequestering excess IL-18[149].

3.3.3 Activating receptors and their ligands

As effector cells of the innate immune system NK cells rely not on a single recombined receptor but on a broad array of germline encoded activating and inhibitory receptors to distinguish "self" from

"non-self" or "altered-self"[116]. A comprehensive overview of various receptors on mouse and human NK cells is displayed in **figure 3.5**. With all other receptors binding to target cell derived ligands, CD16 or constant Fcγ-receptor IIIa (FcγRIIIa) is an exception in a way that it binds to the Fc part of antibodies belonging to the IgG class. It is therefore enabling NK cells to exert ADCC against various antibody coated cellular targets, leading to the exocytosis of perforin/granzyme loaded vesicles.

The other receptors have in common that their ligands, if they have been identified, are stress-induced and thus are expressed or up-regulated upon viral infection, DNA damage or malignant transformation[150].

NKG2D is expressed on almost all NK cells in mice and humans and also on subsets of T cells where it acts as a co-stimulatory molecule. It is a homodimer that signals via DNAX activation protein (DAP)-10 in humans or via DAP-10 or DAP-12 in mice[151]. Notably, in humans the NKG2D signaling leads to cytotoxicity and cytokine secretion[152]. The cellular ligands of NKG2D include MHC class I chain-related gene (MIC)-A, MIC-B and UL16 binding protein (ULBP) 1-6. MIC-A and MIC-B have been described to be up-regulated upon malignant transformation, due to the DNA damage response or upon heat shock response pathway signalling[153]. Sonja Textor from our group could further demonstrate that p53 induction causes an up-regulation of ULBP-1 and -2[154]. Of note, some of the ligands are weakly expressed on non-malignant cells, yet below the threshold for activating NK cells. In mice, the respective NKG2D ligands are the retinoic acid early transcript-1 molecules (Rae-1) α, β, γ, δ, ε, the murine UL16-binding-like transcript-1 (MULT-1) and histocompatibility 60 (H-60). Importantly, the contribution of NKG2D to tumor surveillance has been demonstrated in mouse models of spontaneous prostate adenocarcinoma and B cell lymphoma[155]. Further evidence for the importance of NKG2D in a tumor context comes from the observation that the receptor is targeted in immune-evasion strategies of tumors. Tumor-derived TGF-β has been shown to down-modulate NKG2D levels. Also, it was shown that tumor cells shed MICA thus blocking functional NKG2D receptors with the cleaved soluble ligand and therefore preventing the induction of tumor cell lysis[50].

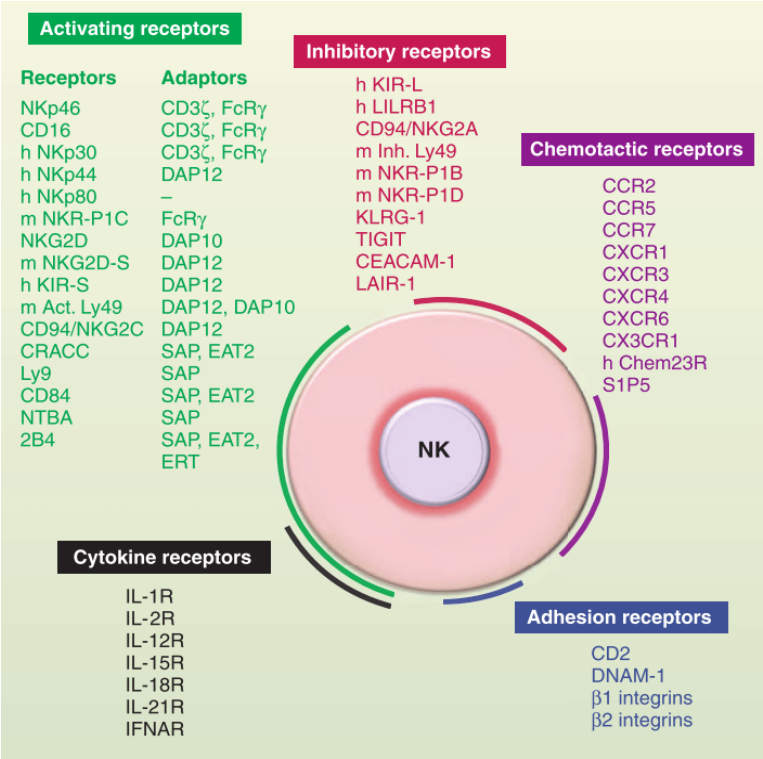


Figure 3.5 – Summary of receptors being expressed by human and murine NK cells. NK cells express various activating and inhibitory receptors tuning their reactivity. In addition they harbor various receptors integrating signals from cytokines or chemotactic proteins or adhesion molecules. Relevant abbreviations are explained in the text, (Vivier et al *Science* 2011; 6013: 44-49)[116].

Another activating, yet co-stimulatory receptor is **DNAM-1**, a protein that is expressed on almost all human and only 25-50% of murine NK cells and in addition on T cell subsets where it was shown to exhibit co-stimulatory functions and on macrophages[156]. The ligands of this receptor are CD112 (Nectin-2) and CD155 (polio virus recptor, PVR) and they were implicated in some anti-tumor reactions in which they mediated tumor regression[157]. Notably, in the same study it was observed that in the absence of DNAM-1, the growing tumor cells were expressing higher levels of DNAM-1 ligands, thus indicating immunoediting mediated by DNAM-1. Further, similarly to NKG2D ligands, DNAM-1 ligands are also up-regulated in the presence of genotoxic stress, for instance inhibitors of replication and can be shed from tumor cells[158].

An entire class of activating NK cell receptors is comprised by the so-called natural cytotoxicity triggering receptors (NCRs). They all belong to the Ig-superfamily. So far, 5 members (NKp30, NKp44, NKp46, NKp65 and NKp80) have been identified. Notably, only NKp46 is expressed on murine NK cells whereas human NK cells express all of the NCRs Of note, only NKp44 requires NK cell stimulation with inflammatory cytokines such as IL-2 or IL-15 to get expressed[159]. The signaling leads for each of them to a cytotoxic response and the secretion of cytokines. Of note, cellular ligands for some of them remain elusive.

NKp30 signals via CD3ζ and the Fc receptor gamma chain (FcRγ) with its ligands being identified as B7-H6 and the HLA-B-Associated Transcript 3 (BAT3/BAG6)[160,161]. BAT3 is an intracellular protein that gets released in response to stress and triggers NKp30 expressed by NK cells outside the target cell. Also B7-H6 has been reported to be shed from the cell surface, again implying an immune-escape mechanism[162]. Further, Nathalie Fiegler in our group found that B7-H6 expression is negatively regulated by histone deacetylase (HDAC) inhibitors[163].

NKp44 associates with DAP-12 and its recently identified cellular ligand is the mixed-lineage leukemia-5 protein (MLL-5)[164]. Notably, an inhibitory cellular ligand, the proliferating cell nuclear antigen (PCNA), has been described that assists tumor cells in evading NKp44 immunity by blocking the receptor[165].

NKp46 equally to NKp30 signals via CD3ζ and FcRγ. Also the binding to heparan sulfate proteoglycans has been reported for both receptors[166]. Besides these interactions no tumor derived ligand has been described so far; yet, hemagglutinin as a viral ligand has been reported to bind to this NCR[167]. NKp46, coded for by the *Ncr1* gene was believed for a long time to be NK cell specific which is why several knock in or knockout mice have been generated using the *Ncr1* promoter. However, later it was found out that it is also expressed on a small population of NK-like T cells and more importantly on lymphoid tissue inducer (LTi) like cells in the gut mucosa[168,169]. Yet, upon *Ncr1* knockout, a worse prognosis was observed in a syngeneic B16 model of melanoma compared to wild-type mice and also in human disease NKp46 signalling was shown to contribute substantially to tumor control[170,171].

NKp65 and **NKp80** exhibit common features such as signal transduction via a hemi-ITAM domain and genetic linkage with the genes coding for their ligands. NKp65 in this respect binds to the keratinocyte-associated C-type lectin (KACL) and NKp80 to the activation-induced C-type lectin (AICL) with the latter being expressed on myeloid cells after TLR stimulation and of note on NK cells after stimulation with IL-12/15/18[172–174].

Notably, as for many tumor entities melanoma metastases have been reported to express abundant NKp44- and NKp46-ligands and these ligands were shown to be important for tumor surveillance by NK cells[171].

3.3.4 Inhibitory and killer Immunoglobulin-Like Receptors (KIRs)

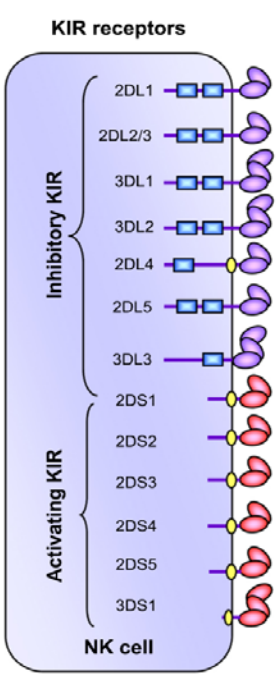
Similar to other cytotoxic cells, the activity of NK cells is tightly regulated by a panel of inhibitory receptors counteracting potential overstimulation and thus preventing immunopathology[175]. The KIR proteins belong to the Ig-superfamily of proteins, harboring either 2 or 3 immunoglobulin (Ig)-like domains and either a short or a long cytoplasmatic tail[176]. They are expressed by NK cells, but also by αβ and γδ T cells. Importantly, only the KIR molecules with a long cytoplasmatic tail signal via ITIMs thus exerting a negative signal, whereas the KIRs with a short cytoplasmic tail signal via ITAM mediated recruitment of DAP12 and thus activate the NK cell (**figure 3.6**)[177]. They comprise the biggest group of inhibitory receptors and detect MHC class I molecules that are expressed on virtually all nucleated cells. Upon perturbation of MHC class I expression like during viral infection or malignant transformation this inhibitory signalling is lost leading to the activation of NK cells in line with the "missing self" theory if activating, i.e. stress-induced, ligands are present[178].

The KIR family in humans, the lectin-like Ly49 molecules in mice and CD94/NKG2A heterodimers in both species are the most relevant and best-studied inhibitory receptors. Of note, the KIR loci in human and the Ly49 loci in mice are highly polymorphic both in terms of gene numbers (14 genes and 2 pseudogenes) and alleles present[179]. Other inhibitory molecules include amongst others for instance Killer cell lectin-like receptor subfamily G member 1 (KLRG1), T cell immunoreceptor with Ig and ITIM domains (TIGIT), NKR-P1A or Leukocyte immunoglobulin-like receptor subfamily B member 2 (LILRB2)[150,180]. These molecules detect cadherins (KLRG1), CD155 and CD112 (TIGIT) or a broad array of HLA-A, -B, -C and -G alleles (LILRB2) and thus complement the capability to distinguish "self" from "altered self" or "missing self". All mentioned receptors signal via ITIMs that subsequently recruit and activate the phosphatases SHP1 and SHP2, thus decreasing the absolute signal strength.

KIR binding takes place at the α1-helix of the HLA protein. Especially the binding of KIR2DL depends on one certain amino acid at position 80 from the C terminus. Whereas KIR2DL1 binds all HLA C alleles that harbor a Lys^{80} residue (C2 epitope) KIR2DL2/L3 detects and binds to all alleles with an Asn^{80} (C1 epitope). The mechanism, determining which NK cell is expressing what KIR molecule is believed to be a random process that is mediated via DNA-methylation of the other KIR loci, thus silencing all other KIR genes but the expressed ones[181].

Importantly, NK cells in one given individual can vary in terms of which KIR alleles are expressed and the level of expression creating different populations of KIR positive NK cells[182]. The fact that KIR alleles are expressed in a largely random manner unlinked from the host's HLA haplotypes is troublesome as this potentially could cause NK cell mediated autoimmunity[179]. Therefore, an education system exists in which developing NK cells are acquiring self tolerance if they fail to bind to self-HLA[183]. This state of hyporesponsiveness not only affects reactivity to self but impacts stimulation with MHC class I negative tumor cells or plate-bound antibodies against various stimulatory receptors. The same phenomena can be observed when mice or human individuals lack HLA class I molecules[184,185]. This HLA-dependent education of NK cells thus has also been termed

licensing and allows the acquisition of the full cytotoxic effector potential only if negative regulatory circuits are properly installed[180].



Number of protein variants	HLA class I ligand
24	HLA-C group-2
11/17	HLA-C group-1, B46, B73, some HLA-C group-2
58	HLA-Bw4
61	HLA-A3, A11 (peptide dependent)
22	HLA-G
17	Not known
55	Not known
7	HLA-C group-2
8	Not known
5	Not known
13	HLA-A11 and subsets of HLA-C
11	Not known
12	Not known

Figure 3.6 – Activating and inhibitory KIR molecules and their respective ligands. HLA-C group 1 refers to HLA-C alleles harboring an Asn[80] whereas HLA-C group 2 refers to HLA-C alleles harboring a Lys[80] (Rajalingam *Korean journal of hematology* 2011; 4:216-28).

Of note, if the acquisition of effector functions upon engagement of a cognate HLA molecule (arming) or the acquisition of hyporesponsiveness if no KIR is engaged (disarming) is the active process is currently under active debate[180]. It was further shown that on one given NK cell the number of KIRs expressed for HLA molecules in one individual correlates with the responsiveness showing that the higher the possible degree of inhibition by self is the more reactivity is granted by the licensing system[186]. Importantly, neither hypo- nor responsiveness are stably imprinted in NK cells. It was shown in a mouse model that 'licensed' NK cells upon transfer into a MHC $I^{-/-}$ host acquire a state of reduced responsiveness[187].

3.3.5 Triggering of NK cell effector function

The integration of the signals of the various introduced activating and inhibitory receptor is decisive for the NK cell to trigger or not to trigger an effector function. This could be either a cytotoxic response or the secretion of cytokines.

Cytotoxic response

Upon recognition, target cells can get killed by one of two means: either via the perforin/granzyme secretion pathway or via membrane-bound death receptors both of which are displayed in **figure 3.7**:

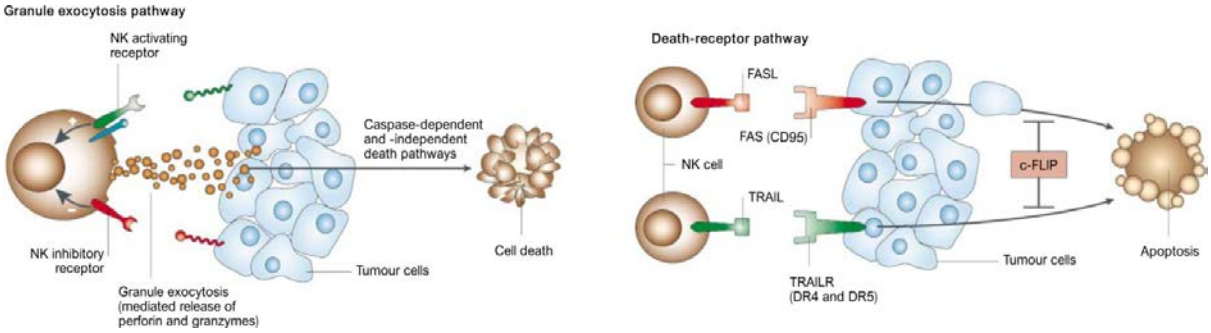


Figure 3.7 – NK cell modes of inducing cytotoxicity. Displayed are schematic representations of how NK cells mediate a cytotoxic response. (Left part) Induction of apoptosis via the release of perforin/granzyme containing lytic granules (right part) death inducing ligands on the surface of NK cells trigger caspase mediated apoptosis in cells expressing the respective receptors; c-FLIP, Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (Smyth *et al. Nature Reviews Cancer* 2002; 11: 850-61).

Like other effector cell such as CTLs NK cells store pre-formed perforin and granzymes in secretory vesicles that upon triggering of the pathway cluster around the microtubule-organizing center (MTOC) which guides the vesicles in a directed way towards the target cell to prevent damaging bystander cells. Both components are cooperating in a way that perforin presumably forms pores in the target cell membrane thereby disrupting the membrane integrity and allowing the entry of the apoptosis inducing granzymes[188]. This family of serin-proteases upon passing the membrane through perforin-induced pores intracellularly activates caspases, which subsequently trigger the apoptotic program in the target cell. Notably, granzymes via the activation of various molecules such as IL-8 or IL-6 also have also been shown to promote inflammation[188,189]. The importance of the lytic perforin/granzyme pathway has been demonstrated in knock-out animals. Here, perforin$^{-/-}$ animals exhibited a less efficient tumor control after transplantation of tumor cells and after MCA treatment[190]. Notably, also ADCC is mediated in a perforin dependent way. Interestingly, cell-surface bound cathepsin B and CD107a protect NK cells and CTLs from taking damage from their own granzyme release[191,192].

The second way of inducing cytotoxicity is via death receptors. Three ligand-receptor pairs, namely FasL-Fas, TNF-TNFR and TRAIL-TRAILR, have been well studied. The ligands induce the recruitment of various adaptors ultimately leading to the formation of the death-inducing signaling complex (DISC) upon binding to their cognate receptors[193]. Subsequently, the caspases 8 and 10 get activated via proteolysis and initiate apoptosis. This pathway similarly to the perforin/granzyme pathway has also been shown to be or high relevance in pre-clinical tumor models[194].

Cytokine secretion

In addition to the cytotoxic response NK cells are important sources of various cytokines such as IFN-γ, TNF-α or IL-10 and of chemokines such as CCL3/4/5 or CXCL8. Of note, some of the chemokines are potent attractors of DCs which is why NK cells and DCs often co-localize in inflamed tissue[195]. IFN-γ due to its pleiotropic functions and its early discovery in NK cells is considered to be the signature cytokine of NK cells which is why they have been assigned to the group I innate lymphoid cells (ILCs) according to a recently proposed classification scheme[196]. Various functions of IFN-γ have been discussed already and will be discussed later. To briefly summarize some of it main functions IFN-γ has been shown to have anti-proliferative effects on tumor cells, exert

anti-angiogenic functions, induces apoptosis sensitivity on tumor cells via upregulation of caspase, FasL and TRAIL and induces MHC class I up-regulation thus promoting CTL immunity just to mention the effects on tumor cells; in addition it activates macrophages and DCs and promotes CTL maturation[197]. Due to its various roles, it is complexely regulated involving induction of expression via IL-12, the effect of which can be further enhanced by IL-1, TNF-α or IL-18. Interestingly, the combination of TNF- α and IFN-γ both of which can be secreted by NK cells have been shown to trigger tumor senescence when they are present simultaneously[198].

Importantly, cytokine secretion is regulated differently as compared to the release of cytotoxic granules. As cytokine secretion is mediated via recycling endosomes it uses a distinct pathway from cytotoxicity which allows for differential regulation[199].

3.3.6 Phenotype and physiological functions of NK cells

NK cells as part of the innate immune system rely on an array of germline-encoded receptors that screen for delicate differences in the levels of activating and inhibitory ligands on their potential target cells. The integrated signals determine ultimately, if the NK cell reacts or not[175]. This general mechanism holds true for balancing the reactivity of both $CD56^{bright}$ and $CD56^{dim}$ NK cells, the two main NK subsets that are discussed in this chapter.

3.3.6.1 Phenotype of NK cell subsets

Around 10% of NK cells in peripheral blood exhibit a $CD56^{bright}$ phenotype. This subset has been described to be less mature and is endowed with less spontaneous killing activity compared to $CD56^{dim}$ cells[125,200]. Yet, it was shown that upon IL-2 or IL-12 activation cytotoxicity can reach similar levels as in $CD56^{dim}$ cells[201,202]. Further, this subset produces significant amounts of inflammatory cytokines and chemokines such as CCL3 and CCL4 shortly after activation[200]. Notably, the combination and concentration of monokines varies for individual effector cytokines such as for the granulocyte macrophage colony-stimulating factor (GM-CSF), IL-10 or IFN-γ, allowing the determination of the predominant effector cytokine according to the monokine milieu[200].

As compared to these cells, the second population with a low expression of CD56 shows higher spontaneous cytotoxicity[201]. In line with this, it was also reported that this subset expresses higher levels of NCRs and importantly significantly higher levels of perforin. Classically, this subset was described to secrete little amounts of inflammatory cytokines, yet a recent study showed that depending on the stimulation this subset can be a very potent producer of IFN-γ and TNF-α as well[203]. However, in several *in vitro* studies the $CD56^{dim}$ cells were less efficient producers of cytokines either after co-culture with LPS-activated macrophages or PMA[200]. Of note, this subset is characterized by the co-expression of CD16, the low-affinity Fcγ receptor IIIa (FcγRIIIa), allowing them to react to target cell bound antibodies, a process termed antibody dependent cytotoxicity (ADCC)[204].

These most important findings and several relevant others have been summarized in **figure 3.8**[205].

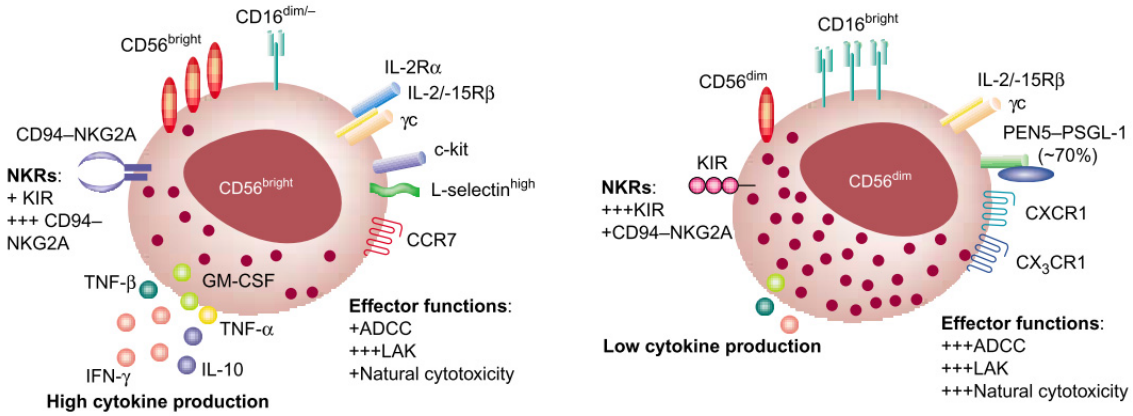


Figure 3.8 – Phenotype and function of the two major human NK cell subsets. NK cells with a bright expression of the CD56 antigen exert classically more regulatory functions due to their high cytokine production capability whereas NK cells with an intermediate expression level of CD56 are more prone to exert cytotoxicity. They differ in terms of the expression of various receptors and molecules as indicated. LAK, lymphokine activated killer, (Cooper *et al. Trends in Immunology* 2001; 11: 633-40).

Importantly, $CD56^{dim}$ NK cells express higher levels of killer cell immunoglobulin-like receptors (KIRs) and the inhibitory LILRB2, whereas they express less CD94-NKG2A compared to $CD56^{bright}$ NK cells. This indicates a different regulatory circuit for the two subsets subsequently allowing differential activation by one given target[205]. Also, only on $CD56^{dim}$ NK cells the expression of CD57 can be seen, a marker for terminally differentiated effector cells further indicating that $CD56^{bright}$ NK cells might be less mature compared to $CD56^{dim}$ cells[206]. In terms of cytokine-receptor expression, it is noteworthy that only $CD56^{bright}$ NK cells seem to express c-kit and the heterotrimeric IL-2 receptor IL-2Rαβγ granting higher responsiveness to IL-2. This was shown to translate into much higher *in vivo* and *in vitro* proliferation after IL-2 stimulation compared to $CD56^{dim}$ cells[202]. Of note, the $CD56^{dim}$ NK cells in general were reported to proliferate poorly, regardless of the stimulation[207]. Importantly, also the IL-18 receptor was found to be expressed at higher levels on $CD56^{bright}$ NK cells[208].

3.3.6.2 Homing of NK cell subsets

Owing to the differential expression of chemokine receptors, the two introduced subsets exhibit significant differences in their homing behavior (**figure 3.9**)[205,209]. Notably, Campbell *et al.* in 2001 based a subtype classification on the expression of chemokine receptors. Thus, the first subset, including the $CD16^{bright}$ cells, expressed CXCR1, CXCR3, CXCR4 and CX3CR1 but not CCR1-7, CCR9 and CXCR5. The second subset includes the $CD16^{dim}$ cells that expressed high levels of CCR7 but low CXCR3 and CXCR4[210]. Inflammatory cytokine signaling has been shown to maintain the expression of constitutively expressed CXCR4 and CCR7 but in addition induces the expression of inflammatory receptors such as CXCR1 or CXCR3[211].

As mentioned, it was shown that L-selectin and CCR7 – typical molecules for homing to secondary lymphatic tissue – are exclusively expressed on $CD56^{bright}$ NK cells. Accordingly, this subset responds to CCL19 and CCL21, chemokines that are abundantly expressed in secondary lymphoid tissue such as lymph nodes and other secondary lymphoid tissue such as the tonsils where $CD56^{bright}$ cells comprise the predominant NK population[210,212]. Possibly, DC derived CXCL10 and NK cell derived CCL3 and CCL4 might cross-recruit NK cells in these tissues into close proximity to the DC allowing the two

populations to activate each other. The amounts of secreted CCL3 exceed after IL-2 stimulation the amount of IFN-γ which is why it was suggested to call CLL3 and not IFN-γ the prototype NK cytokine[213]. Of note, here T cells are waiting to be stimulated, responding to stimulation with IL-2 production that at low levels stimulates the high affine IL-2R of $CD56^{bright}$ cells that in turn respond with high IFN-γ levels skewing the T cell response into a Th1 direction. Thus it is very tempting to assume, that especially the $CD56^{bright}$ cells are involved in shaping adaptive immunity. $CD56^{dim}$ cells on the other hand express high levels of CXCR1 and CX3CR1 thus exhibiting very distinct homing properties from $CD56^{bright}$ cells[210]. The mentioned receptors readily guide them into inflamed tissue whereas under steady-state conditions they make up around 90% of NK cells in peripheral blood or spleen.

For tumor biology, several chemokines have been reported to facilitate NK cell accumulation *in situ*. In a mouse model, Marco Wendel from our group could show that CXCL9, CXCL10 and CXCL11 are crucial in recruiting $CD27^{+}$ murine NK cells, mostly in an IFN-γ-dependent way[94]. Further, CX3CL1 was successfully shown to recruit NK cells in a syngeneic model, reducing tumor load in the respective animals compared to controls[112]. Besides those, amongst others CCL19 and CCL2 have been reported to efficiently recruit NK cells to the tumor thus reducing the tumor burden. In line with the finding that certain chemokines exhibit various functions on NK cells and that extravasating cells need to digest themselves a way through the basement membrane it was reported that CXCL12 induces the expression of marix degrading enzymes on the NK cell surface[214]. Thus, chemokine signaling not only induces migration but also promotes invasion of, amongst others, tumor tissue.

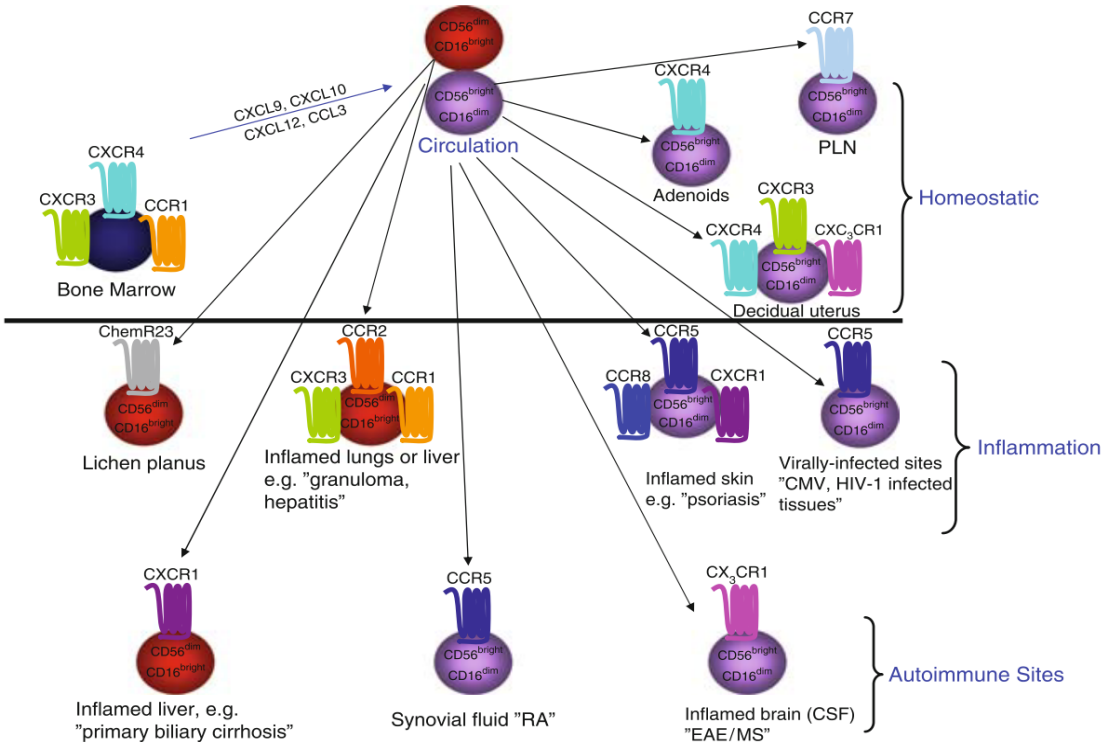


Figure 3.9 – Roles of chemokines and chemokine receptors in health and disease. Schematic representation of some of the most important chemokine-chemokine receptor interactions in NK biology. CMV, cytomegalovirus; EAE, experimental autoimmune encephalomyelitis; HIV-1, human immunodeficiency virus-1; MS, multiple scelrosis; PLN, peripheral lymph node; RA, rheumatoid arthritis (Maghazachi Curr Top Microbiol Immunol 2010; 341:37-58)[209].

Further, during infection, the CCR5-CCL5 axis has been shown to play an important role for the accumulation of NK cells at the respective infected site, for instance during HIV or toxoplasma gondii

infection. Notably, chemokines have been identified that mediate NK cell recruitment in autoimmunity. Thus, it was shown that the CCR5-CCL3/CCL4/CCL5 axis recruits $CD56^{bright}$ NK cells into synovial fluids of patients suffering from rheumathoid arthritis whereas the CX3CR1-CX3CL1 axis as shown to contribute to to NK cell migration into lesions of multiple sclerosis (MS) patients. Accordingly, in $CX3CR1^{-/-}$ mice NK cell recruitment into inflamed brain areas was abrogated leading to more severe symptoms of disease, although other studies show opposite result. As MS is a Th1-driven disease NK cells in those lesions are likely to interfere with Th1 generation. Yet, because of inconsistent data, it is hard to speculate what the mechanism might be.

3.3.6.3 Physiological functions of NK cells

Owing to the two specialized subsets and their presence in various organs NK cells contribute to various physiological processes some of which will be discussed next[115].

NK cells in malignant disease

It has been demonstrated in *in vivo* mouse models via antibody-mediated depletion of NK cells that these cells contribute to immune-surveillance of spontaneously arising, transplanted or carcinogen-induced tumors[178,215]. Although mouse models gave valuable insights into the control of tumor growth by NK cells, the studies most frequently were performed under conditions not clearly dissecting the specific role of NK cells. This is due to the lack of markers that are exclusively expressed on NK cells. In human disease evidence that NK cells in parts can control tumor growth came from epidemiological observations or from the analysis of tumor samples[216]. Several groups analyzed excised tumor samples in terms on NK cell infiltration levels and correlated the NK levels *in situ* with the prognosis of the patients. Importantly, to detect NK cells in all of these studies the CD57 antibody was used; the epitope CD57 only is expressed on about 70% of $CD56^{dim}$ NK cells and besides also on T cell subsets[217–219]. Thus, it will be important to repeat those studies using a marker that specifically and exclusively stains all NK cells. Yet, **figure 3.10** shows that for various cancer entities NK cell infiltration correlated positively with the patient survival.

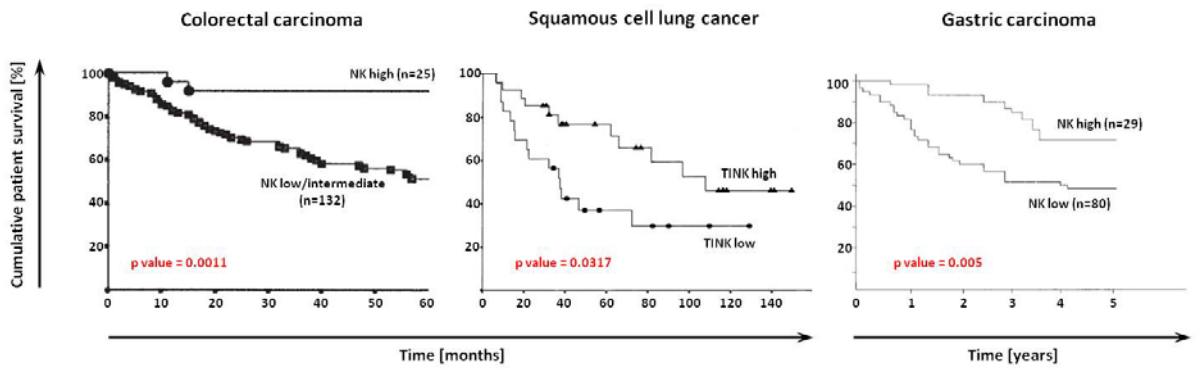


Figure 3.10 – Contribution of NK cells to *in vivo* tumor control. Plotted is the cumulative survival for patients grouped according to the level of NK cell infiltration as determined by CD57 staining. Figures are withdrawn from three publications as cited (**Colorectal carcinoma**: Coca *et al. Cancer* 1997; 12: 2320-8, **Squamous cell lung cancer**: Villegas *et al. Lung Cancer* 2002; 1: 23-8, G**astric carcinoma**: Ishigami *et al. Cancer* 2000; 3: 577-83)[217–219].

Importantly, several studies were pointing out that tumors are enriched for the CD56^{bright} NK cells subset of NK cells[220,221]. Several mechanisms can cause this phenomenon. First, CD56^{bright} NK cells could be preferentially attracted into tumor tissue. Also, infiltrating NK cells could potentially re-differentiate into CD56^{bright} NK cells. Last, the NK cells with the bright expression of CD56 could proliferate more pronouncedly or be more resistant to an apoptosis inducing milieu. In line with the latter it has been shown that CD56^{bright} NK cells are more resistant to apoptosis induction via ROS that are possibly present in the tumor stroma[222]. It furthermore was shown in a mouse model that in general NK infiltration increases with the susceptibility of tumor cells to NK cell recognition[223]. Yet, although many tumor cells express activating ligands for NK cells do not infiltrate properly. Here, it was shown for various cancer entities that NK cells rather do not deeply infiltrate tumor tissue but get stuck in the stroma[224]. Interestingly, published data were showing that upon down-regulation of MHC class I higher infiltration of CD8$^+$ CTLs but reduced numbers of NK cells could be found, implying a selective exclusion of anti-tumor reactive cells. In other studies however, NK mediated control of metastasis could be observed[225].

As discussed, usually only few NK cells properly infiltrate into tumor tissue, not representing the blood resident population distribution. *In situ*, reactivity against tumor targets frequently is lost, representing a major problem for NK cell based immunotherapy as well as for T cell based therapy[226–228]. Notably, in a recent study performed in the Rosenberg group showed that NK cells after re-infusion into melanoma patients persisted but completely lost their anti-tumor reactivity indicating the presence of NK-suppressive mechanisms[229]. They observed a significant down-regulation of the activating NKG2D after *ex vivo* analysis of peripheral blood NK cells. Also the observed shedding of activating ligands off of tumor cells decreased the level of functional receptor molecules as soluble versions block the respective binding sites[50,230]. Further, tumor cells have been shown to releas FasL bearing vesicles that induce apoptosis on Fas expressing immune cells[231]. Also via the expression of B7-H3 and/or nonclassical HLA molecules such as HLA-G or –E tumor cells potentially can avoid NK cell mediated destruction[232,233]. In other studies, lower levels of CD3ζ or Lck have been described in tumor-associated NK cells causing less IFN-γ but higher IL-10 production[234,235]. Impressively, de-regulation of NK cells affects already NK differentiation in the bone marrow, as depicted in a recent study for multiple lineages of tumors, including thymoma, breast cancer, colon cancer, and mela- noma cell lines[236]. As overexpression of IL-15 restored NK cell genesis, it is likely that the disturbed maturation of NK cells (same cytotoxicity, less IFN-γ) was due to interference in the IL-15 signaling pathway in the bone marrow. Also, besides the intracellular signaling machinery, the expression levels of activating receptors are often decreased *in situ*. This can either be due to internalization after constant ligand exposure as it was described for NKG2D and DNAM-1 but also immunosuppressive cytokines like TGF-β that eventually are enriched in the microenvironment are de-regulating the expression of activating receptors on NK cells[237–239]. Besides, the tumor-microenvironment *per se* differs already dramatically from healthy tissue by the means of hypoxia, acidity and glucose levels some of which have been shown to interfere with NK cell activation in general[240].

Also cellular factors interfere with NK reactivity in tumors. T_{reg} and myeloid derived suppressor cells (MDSC) both have been reported to suppress NK cell responses and proliferation in mouse models and in human, in parts via the expression of membrane-bound TGF-β[241,242].

Notably, a sustained supply of NK cell derived IFN-γ could break the tolerance inducing milieu by preventing M2 macrophage differentiation, stimulate DC maturation - thus subsequently prevent T_{reg}

recruitment and TGF-β entrichment - but also could stop neo-vascularization of the developing tumors, rendering NK cells potentially important players in the prevention of tumor outgrowth. Yet, a proper infiltration is required and a sustained activation of these potent effector cells is needed as they succumb quickly in for example T_{reg}- and/or TGF-β-enriched microenvironments. Importantly, cancer stem cells that were often reported to be resistant to classical chemotherapeutical treatments and/or ionizing irradiation have been reported to be sensitive to NK cell mediated lysis rendering NK cells attractive effector cells for immunotherapy[243].

NK cells during infectious disease

As elucidated in mouse models of NK cell depletion or reconstitution, NK cell activity is a critical determinant for the susceptibility to viral infections. Amongst others, herpes simplex virus-1, influenza virus or the murine cytomegalovirus MCMV were shown to be under critical control of NK cell immunity[244]. This was also most impressively demonstrated by a case-study of Biron *et al.* who were reporting the case of a young girl lacking NK cells thus suffering from severe varicella and CMV infections[245]. Of note, in murine CMV infection the respective virus-specific molecule that is detected by the activating Ly49H receptor has been identified as m157[246]. Likewise, $Ly49H^+$ NK cells expand upon virus infection. Importantly, also mice lacking the expression of important effector molecules like the IFN-γ receptor or mutations in humans affecting for instance this receptor dramatically increased the susceptibility towards CMV infection[244,247]. Notably CMV infection also in humans leads to the expansion of a certain NK cells subset that expresses the NKG2C receptor[248].

NK cells as immune-regulatory cells

Due to the secretion of multiple inflammatory cytokines and their ubiquitous presence it is not surprising that NK cells cross-talk with various other effector cells of the innate or adaptive immune system. One of the best-studied interactions is the bidirectional interaction with DCs either in peripheral tissue or secondary lymphoid tissues[249]. Here, NK cells have been shown to promote the maturation of monocyte derived DCs (moDC) via TNF-α, IFN-γ, HMGB1 and also via NKp30 stimulation[250–254]. This maturation led to a substantial increase in the secretion of proinflammatory cytokines of DCs like IL-12 and IL-18 which in turn stimulated $CD4^+$ T cells to develop into IFN-γ producing Th1 cells and augmented IFN-γ production in $CD8^+$ T cells[250,251,253]. Further, activated DCs signaled back to the NK cells. Thus, activated DCs have been described to enhance CD69 expression, proliferation, IFN-γ secretion and cytotoxicity of NK cells via secretion of the monokine IL-12, TNF-α or type I IFNs[249]. For the induction of proliferation, also the contributions of B7 molecules on moDCs and CD28 on NK cells, trans-presented IL-15 as well as secreted IL-12 have been reported in the DC-stimulated NK proliferation[255,256]. In terms of cytokine induction, it has been demonstrated that the CX3CL1-CX3CR1 axis mediates the polarized secretion of IL-12 from DCs towards NK cells that in turn produce IFN-γ[257]. Importantly, DCs also augmented the lytic capability of NK cells which was largely mediated by IL-12 and the CX3CL1-CX3CR1 axis[256,257].

Notably, NK cells also increase the turnover of DC as they exert cytotoxic effects against immature DCs via NKp30 and to a lesser extent via NKp46 and NKG2D[250]. Furthermore, NK cell killing of for example tumor cells *in vivo* releases antigens for DCs cross-presentation that subsequently can

mount efficient CD4 and CD8 T cell responses. In this context it has been shown that apoptotic tumor cells are the best DC-based vaccine to induce antigen-specific T cell responses[258,259].

Besides this cross-talk with DCs, NK cells also directly impact effector cells of the adaptive immune system. Thus it was shown, that NK cell derived IFN-γ directly promotes Th1 differentiation in inflamed conditions[260]. Also, activated $CD4^+$ T cells are direct targets for killing for activated NK cells, especially during blocking CD94-NKG2A what led to the hypothesis that NK cells might prevent autoimmunity by shaping the T cell repertoire[261,262]. Notably, it was shown that administration of daclizumab led to an expansion of $CD56^{bright}$ NK cells which correlated with a decline in circulating $CD4^+$ T cells and CTLs. This NK mediated, cell-to-cell-contact dependent contraction of the T cell compartment led a substantial decrease of lesions in the brain[263].

Similar to immature DCs, activated macrophages can be targets for NK cells. In line with this, NK cells and macrophages co-localize in the red pulp of the spleen and in peripheral tissues. Thus, in one mouse model it was shown that NK mediated elimination of hyperactivated macrophages prevented immunopathology[264].

NK cells during pregnancy

Importantly, a distinct subset of NK cells, the so-called uterine NK cells or uNKs recently has been reported to secrete pro-angiogenic factors such as VEGF or the placental growth factor (PLGF)[265]. The enrichment of this subset is observed in pregnant endometrial tissue where they support the vascularization and thus the nurturing of the developing placenta. Notably, decidual NK cells have also been reported to dampen Th17 responses via IFN-γ secretion thus establishing maternal-fetal tolerance[266].

3.3.7 NK cell "memory"

One major feature that is used to distinguish adaptive from innate immune responses is the efficient formation of an antigen-specific memory-compartment. After their clonal expansion following activation T-cells undergo a contraction phase which eliminates more than 90% of the expanded progeny. Antigen-specific cells that still exist after this contraction phase subsequently form a unique cell subset. They differ from their antigen-un-experienced counterparts in that they re-create themselves, thus are long-lived, and by exhibiting an enhanced response upon re-challenge with their specific antigen. This enhanced secondary response is typically characterized by a fast expansion of the memory cells yielding high numbers of effector cells early after infection and by elevated levels of effector molecules compared to the first immune reaction[267]. This program is imprinted after the original activation. Here, amongst others, epigenetic mechanisms are thought to contribute to the memory-phenotype. As NK cells use germline-encoded receptors they are thought to lack antigen-specificity. As antigen-specificity of effector cells was thought to be crucial for the formation of a memory compartment, NK cells were believed for a long time to be uncapable of mounting memory responses.

However, recently several reports have built a substantial body of evidence demonstrating memory-like features in the NK cell compartment[268–270]. So far, three ways of inducting NK cell memory have been described in mice that are summarized in **figure 3.11**[269].

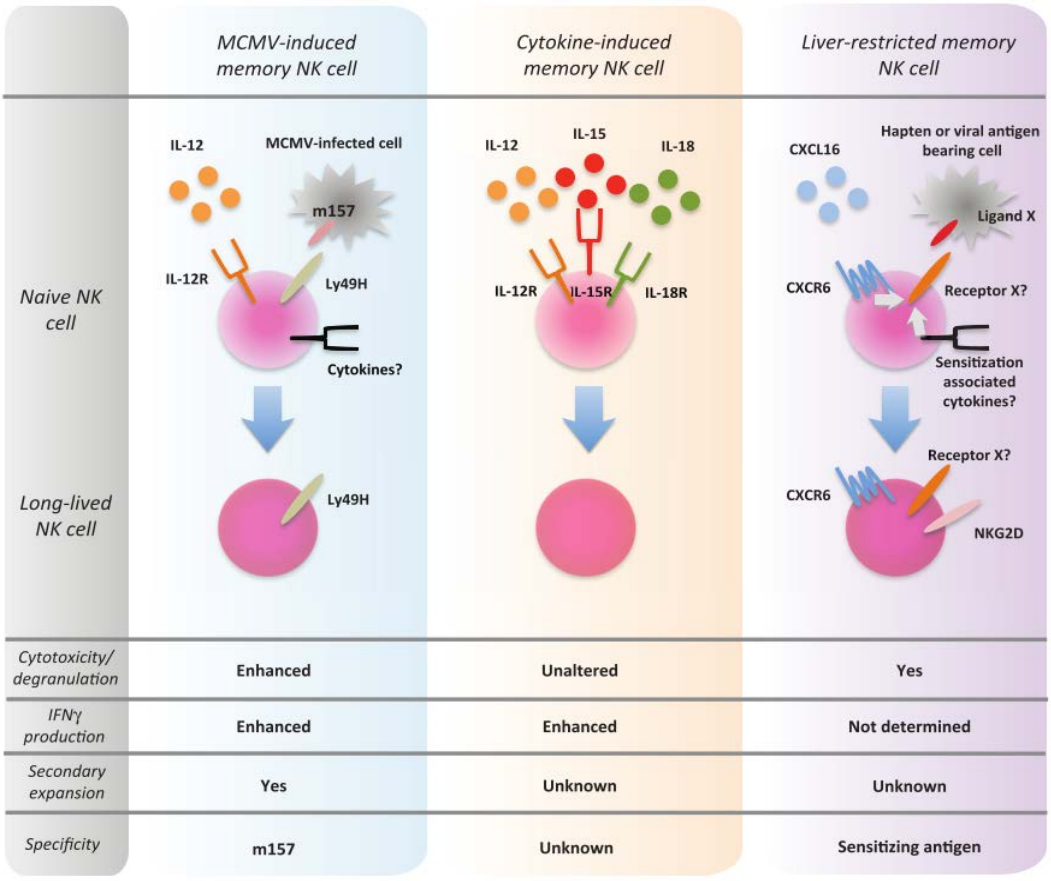


Figure 3.11 – The three reported ways of turning NK cells into memory cells. So far, the recognition of the m157 antigen from MCMV together with IL-12R signaling (left panel), simultaneous IL-12, IL-15 and IL-18 stimulation (middle panel) and the recognition of haptens or certain other specific antigens together with CXCL16 (right panel) triggered memory-induction of NK cells (Min-Oo *et al. Trends in immunology* 2013; 6:251-8).

Of note, the three aforementioned possibilities to induce NK cell memory exhibit differences (see below). Importantly, elevated recall responses of CMV- and hapten-challenged NK cells were shown to be specific for m157-expressing cells or the exact chemical compound used for sensitization which is why the term 'memory' will be used referring to these modes of memory induction. Compared to that, cytokine-activation enhances the reactivity of NK cells towards a broad array of stimuli. Due to the antigen-unspecific nature of this recall-response the term memory-like will be used.

Virus-induced memory

It has been established, that MCMV infection in mice drives the expansion of Ly49H positive NK cells that recognize the viral m157 protein. This phase is followed by a contraction phase generating a pool of survivors that persists for more than 2 months. Notably, the persisting cells are capable of conferring protection against re-challenge by exhibiting robust secondary expansion and enhanced effector functions[271,272]. These processes were shown to be strictly m157 and thus antigen-specific and they critically depended on the presence of the inflammatory cytokine IL-12 and STAT4

signaling[273]. However, the real determinant inducing the infection-mediated memory formation remains elusive. IL-12 might be only important to drive expansion, possibly supported by IL-15. Yet, the importance of antigen-driven expansion still needs to be elucidated. Further factors pre-determining the population after the contraction phase are also yet to be determined. Evidence from T cells with which NK cells share some features are pointing to an involvement of Bcl-2 or Bim[274,275]. Notably, by a comprehensive microarray analysis Ly6C and CD49a were shown to discriminate memory NK and T cells from their naïve or effector counterparts[276]. Similar memory-like responses could be also found in other models of infection[277]. Notably, a model of genital herpes simplex virus-2 (HSV-2) showed elevated antigen-specific recall responses. Yet, the responsible antigen-specific receptor remained elusive leaving the need to clarify what factors in which infection drive NK cell memory formation[278].

Importantly, expansion of NK cells subsets upon infection have also been reported for human patients for instance after human cytomegalovirus (HCMV) infection[279]. The expanded subset expressed high levels of the activating CD94-NKG2C receptor. Notably, a long-lived population of NKG2C positive cells persisted in patients and proliferated rapidly upon reactivation with the progeny exhibiting high effector functions[280]. Interestingly, these features were transferable as after HSCT NKG2C positive NK cells from CMV positive donors expanded in CMV positive recipients whereas NKG2C positive NK cells from CMV negative did not, indicating a recall response against HCMV[281]. Also, upon infection of HCMV positive individuals with hantavirus or chikungunya virus an NKG2C positive subset also expanded and persisted until viral clearance, suggesting a possible role of these cells for the control of various viral infections[282,283].

Memory-like NK cells induced by inflammatory cytokine signaling

The first report on the cytokine-induced generation of a long-lived NK cell population that exerted elevated functionality compared to control cells was published 2009[284]. After an overnight activation of murine splenic NK cells with IL-12, IL-15 and IL-18 these induced memory-like cells exhibited massive *in vivo* proliferation and elevated IFN-γ levels upon *ex vivo* re-stimulation by various means for up to three weeks after transfer. The authors al could demonstrate that the progeny from the stimulated cells also exhibited similar functionality, indicating that the memory-like properties were passed on to the daughter generations. Of note, no markers have been described yet to discriminate cytokine induced memory-like NK cells from naïve or activated NK cells. It is further unclear what the exact mechanism underlying the induction of memory-like functionality is. Clues are withdrawn from T cell immunology. It was shown, that during differentiation and the acquisition of novel functionalities like during Th1/Th2 differentiation epigenetic mechanisms are involved. Thus, it is tempting to speculate that similar epigenetic marks might contribute to the cytokine-imprint in NK cells[285,286].

Importantly, Jing Ni from our group demonstrated a superior anti-tumor effect of cytokine-induced memory-like NK cells in a mouse model of lymphoma[287]. As IL-12/15/18 stimulated cells exhibited a markedly elevated level of CD25 expression, they were sensitive towards IL-2 derived from activated CD4 positive T cells. The cytokine led to a substantial induction of proliferation of IL-12/15/18 pre-activated NK cells. Importantly, relevant data concerning cytokine-induced human memory cells were acquired during this thesis and will thus be introduced later in detail.

Of note, the exact mechanism of the cytokine-mediated imprint to date is unknown. Also, this form of memory-like responses has only been induced and observed *in vitro*. It remains to be clarified if for instance during acute infections high levels of inflammatory cytokines can induce this type of antigen-unspecific memory *in vivo* and if so, what the physiological function of this cell population is.

Liver restricted memory

Pioneering work in the field of hapten-induced memory formation of NK cells was performed by O'Leary *et al.*[288]. They showed that transfer of hepatic but not splenic NK cells from RAG2$^{-/-}$ mice after hapten-sensitization into naïve mice co-transferred contact hypersensitivity. This was found to be specific for the compound used for sensitization of the donor animals. Related compounds used for re-challenge did not induce this type of memory-reaction. Notably, the hapten-specific NK cells were critically dependent upon the chemokine receptor CXCR6 for their function, the ligand of which, CXCL16, is abundantly expressed on liver sinusoidal endothelial cells[289]. Importantly, hapten-stimulation did not lead towards preferential proliferation of the CXCR6 positive subset[288]. This type of reaction was not only inducible with haptens, but also with viral antigens derived from influenza, vesicular stomatitis virus (VSV) or human immunodeficiency virus type-1 (HIV-1)[289]. As introduced, influenza HA binds to NKp46, yet HA-deficient influenza strains still vaccinated animals. Thus, the relevant receptor-ligand pair is completely elusive. Data point towards a role of NKG2D signaling in hapten mediated memory formation yet the mechanism remains to be defined[288]. Of note, no hapten or virus-like-particle (VLP) induced NK cell memory formation to date has been published for human NK cells.

Of note, the three known paths that induce NK cell memory exhibit very unique features like organ distribution of memory cells or effector functions. However, the likewise induced memory or memory-like NK cells share the longevity and the possibility to mount recall responses. Importantly, specific receptors for many memory-inducing agents like haptens are elusive. Furthermore, the molecular mechanism of how NK cells exactly memorize prior stimulations is completely unknown. Importantly, memory or memory-like NK cells exhibit various features that might either be causative for immunopathologies such as inflammatory diseases but on the other hand potentially could be used to treat for example malignant diseases. Here, mouse models are urgently needed to demonstrate the role of memory or memory-like NK cells and to investigate if their elevated functionality can be exploited to treat diseases such as tumors.

3.4 Anti-tumor immunotherapy

In various previous sections the interplay between NK cells or immune cells in general and cancer cells has been introduced (refer to sections 3.1.2, 3.2.3 and 3.3.5). As pointed out, it was recognized by the scientific community that immune cells or immuno-modulatory drugs can help substantially to control tumor growth in cancer patients. Thus several strategies have been investigated to translate basic scientific findings from "bench to bedside" (**figure 3.12**)[290]. This section will introduce different relevant avenues of experimental anti-tumor immunotherapy with a focus on NK cells.

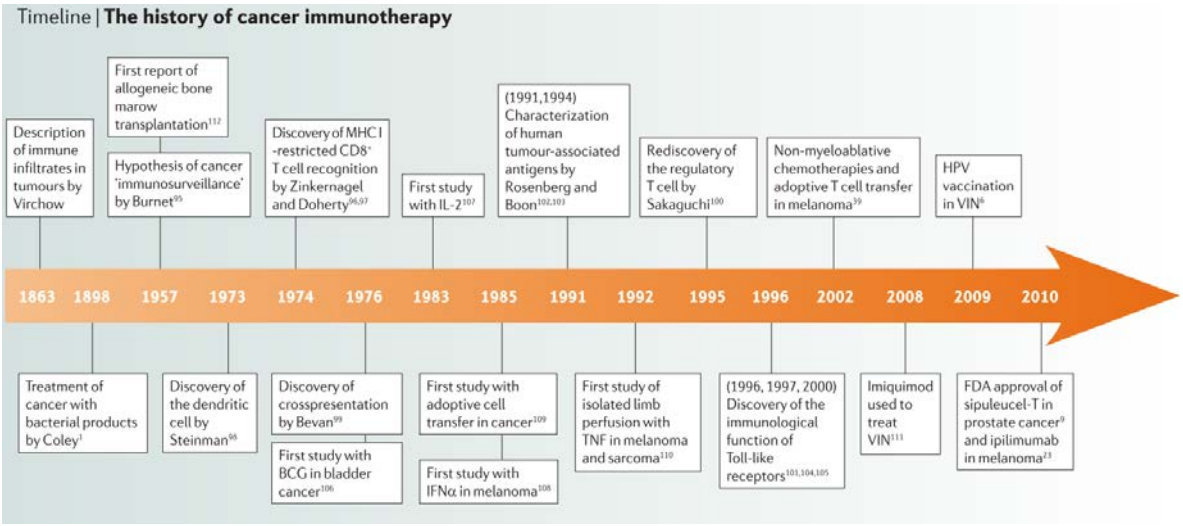


Figure 3.12 – Timeline of the most important breakthroughs in cancer immunotherapy. BCG, bacille Calmette-Guérin; IFNα, interferon-α; IL-2, interleukin-2; MHC, major histocompatibility comples; TNF, tumor necrosis factor; VIN, vulvar intraepithelial neoplasia (Lesterhuis *et al. Nature Reviews Drug Discovery* 2011; 8:591-600).

Vaccine-based strategies

Protective vaccinations so far only could be developed for virus-induced cancers with well-defined viral antigens. One prominent example is the vaccination against the human papillomavirus (HPV), some serotypes of which are known to cause cervical carcinoma[291]. With an estimated 15% of cancers being virally induced, vaccination against immunodominant epitopes of for example hepatitis B virus (HBV) or Epstein-Barr virus (EBV) potentially holds great adjuvant potential. Other tumor-antigens are typically self proteins. They include mutated oncogenes or tumor suppressor genes (specific for tumor cells, for instance mutated p53), overexpressed cellular proteins (for example Her2/neu), tumor antigens produced by oncogenic viruses (for example HPV), oncofetal antigens (for example the carcinoembryonic antigen (CEA)), altered glycolipids or glycoproteins, differentiation antigens (for example tyrosinase or Melan-A) or cancer testis antigens (for example NY-ESO-1). Of note, recently effort was put into categorizing them due to their physiological relevance[292]. Accordingly, in many cases negative selection as part of the TCR quality control can prevent reactive TCRs to pass the quality control in the thymus thus deleting potentially tumor-reactive TCR specificities. Therefore, several approaches such as peptide vaccination, tumor cell vaccines, DNA/RNA vaccines or vaccinations with peptide-loaded DCs failed in inducing substantial clinical responses[293]. Still, the first cell-based vaccine inducing T cell responses against prostate cancer (Sipuleucel-T) was food and drug administration (FDA)-approved few years ago.

Cytokines

Despite low response rates of around 15% and severe side effects both IL-2 and IFN-α have been used for treatment of renal cell carcinoma and advanced melanoma[294,295]. Toxic side effects are tremendous, leading to death in 1.5% of the patients due to capillary leak syndrome and thus organ failure[296]. Yet, in addition to the systemic application of IL-2 and IFN-α local application of IL-2 and TNF-α have been reported to induce beneficial effects for patients with soft-tissue sarcomas or melanoma[297,298]. Of note, IL-2 therapy, regardless if injected locally or systemically, can induce T_{reg}.

Immune-targeted agents

Immune-targeted agents comprise the newest class of therapeutical regimen. One famous example is ipilimumab, a cytotoxic T lymphocyte-associated antigen 4 (CTLA4) blocking antibody. CTLA4 is a potent attenuator of immune responses which is why $CTLA4^{-/-}$ mice die from lymphoproliferative disease shortly after birth. It is mainly expressed on CD4-positive T cells including T_{reg} and CD8-positive CTL[299]. Of note, in a large randomized trial ipilimumab was found to be the first treatment in 30 years to give a survival benefit in metastatic melanoma patients[300]. Thus, ipilimumab recently was FDA approved for the treatment of melanoma. Likewise, several other so-called checkpoint molecules have received attention for blocking mAFbs such as PD1, PDL1 or B7-H3. Notably, blocking an inhibitory axis such as PD1-PDL1 or CTLA-4-B7 molecules can result in autoimmunity[301].

Adoptive T cell transfer

For the infusion of large numbers of T cells several T cell sources such as tumor-infiltrating lymphocytes (TIL) or peripheral blood were tested. Also, geneticaly modified effector cells were evaluated for safety and clinical response. Compared to the previously introduced methods adoptive cell therapy (ACT) is not relying on activating endogenous immune cells but rather supplies exogenously autologous or allogeneic effectors in high numbers. The first study showing in melanoma patients that isolated TIL upon re-infusion into patients exert tumoricidal activity was published in 2002[302]. 50% of the patients who received high-dose infusions of *ex vivo* expanded and activated TIL showed an objective clinical response with 10% obtaining complete response. Based on these results a more targeted approach is appealing. After the characterization of the tumor-specific TCRs with the highest affinity, these could be cloned and used to create TCR-transgenic effector T cells for re-infusion into patients, possibly potentiating the effects that were observed with bulk expanded TIL. First studies using TCR transgenic T cells were performed using T cells recognizing melanoma antigen recognized by T cells 1 (MART1) and/or glycoprotein 100 (gp100) and response rates of 20-30% were obtained[303].

NK cell-based therapy

For therapy, activated autologous or allogeneic NK cells are used. Besides peripheral blood as a source of NK cells, also stem cells or induced pluripotent stem cells (iPSC) have been used for NK cell

generation. Further, NK cell lines were tested some of which were genetically modified to allow them to secrete activating cytokines or to express chimeric antigen receptors[304].

Several models and/or trials that implemented systemic cytokine application to either activate endogenous or transferred NK cells showed that cytokine administration comes with the risk of life-threatening toxic side effects and cytokine-induced NK apoptosis. Still, a safe application of *ex vivo* expanded and activated NK cells showed clinical responses against some entities such as metastatic renal cell cancer (RCC), malignant glioma and breast cancer[305–307]. Yet, Parkhurst *et al.* showed that transferred NK cells persisted but without IL-2 reactivation exerted no cytotoxicity against tumor cell lines *ex vivo*[229]. Similar findings were reported for colorectal carcinoma, non-small cell lung cancer and RCC. In these settings, the presence of matched MHC class I molecules *in situ* was thought to contribute to preventing full functionality of activated, autologous NK cell. The transferred cells during their development were licensed in the patient who receives the cell product and thus express receptors that are inhibiting the NK cell reactivity against the tumor.

Importantly, Ruggeri *et al.* were using KIR-HLA mismatched donors for allogeneic hematopoietic stem cell transplantation (HSCT). As in any given individual a subset of NK cells is present that only expresses one KIR molecule with which it was licensed against an HLA molecule of the donor that is missing in the recipient, an allo-reactive population is transferred. Indeed, in an acute myeloid leukemia (AML) patient group, the KIR-HLA mismatch in graft versus host (GVH) direction in 34 patients showed no rejection, importantly no toxicities and a decreased 5 year relapse rate of 5% as compared to 75% when no mismatch was present[308]. Later on it was shown that KIR2DS1^{+} NK cells from HLA C1 positive donors are the mediators of the anti-AML effect[309]. Yet, controversial data exist emphasizing the need for a comprehensive analysis which donor is the best for which patient; thus Pende *et al.* could show that KIR2DL1 incompatibility is more potent than KIR2DL2/L3 incompatibility with the latter only working if KIR2DS1 is co-expressed[310]. After the success of the aforementioned studies other groups were also trying to exploit the allo-reactivity of KIR ligand mismatched NK donors[229].These trials showed minimal toxicity of allo-reactive NK cells, more pronounced anti-tumor effects against non-small cell lung cancer and persistence of transferred cells in patients with metastatic melanoma, RCC and others[311]. In line with these findings, experiments using anti-KIR monoclonal antibodies (mAbs) that blocked the KIR-HLA interaction were performed. Most importantly, the pan-KIR blocking antibody 1-7F9 increased NK lysis of HLA Cw*03^{+} tumor cells whereas healthy cells were spared, suggesting a therapeutical window for KIR blockade. Also, the survival of animals in a xenograft model of AML was significantly increased by 1-7F9 administration with NK cells compared to NK cells alone, indicating the potency of the blocking[312]. Of note, in another model of EBV-transformed B cells, KIR blocking *per se* did not increase NK cell mediated lysis but improved the effect of ADCC[313]. These results state the superiority of allo-reactive over autologous NK cells. Yet, the exact pre-requisites of an ideal donor and the ideal allo-reactive subset need to be defined more precisely.

The use of NK cell lines as allogeneic NK cell source has several advantages. They can be easily maintained and expanded *in vitro* allowing for the large-scale production of NK cells. Further, they allow for the use of one NK cell product for various application or patients thus eliminating deviations by donor-to-donor variabilities. Of note, the NK-92 line was found to be safe and potentially exhibits beneficial effects in malignant melanoma, RCC and advanced lung cancer patients[314,315]. Thus, so far it is the only cell line that was FDA approved for tumor therapy. Of note, due to their virtual complete lack of KIR molecules NK-92 are potent mediators of allo-reactivity. Due

to the feasibility of NK cell lines, several groups were trying to enhance the endogenous anti-tumor effect of for instance NK-92 cells by genetic means. Cytokine gene transfer of for example IL-2 or IL-15 was shown to increase survival and activation[316]. Also, NK cells were shown to exert increased reactivity against for instance CEA or HER2/neu expressing targets after introduction of a chimeric antigen receptor (CAR) against CEA or HER2/*neu*, respectively[317]. This retargeting could substantially increase their clinical applicability.

Other agents

As against virtually any molecule of choice a specific monoclonal antibody (mAb) can be produced, self-proteins can easily be targeted by the respective mAb. Compared to the restrictions of T cell therapy due to the thymic negative selection of self-reactive TCRs, this confers a major advantage as many tumor-antigens are self antigens. The most widely used antibody is rituximab, a chimeric anti-CD20 antibody used against non-Hodgkin lymphoma (NHL) or chronic lymphocytic leukemia. If applied together with CHOP therapy (cyclophosphamide, doxorubicin, vincristine, and prednisone) it achieves 76% of complete remissions[318]. For the treatment of solid tumors several mAbs have been approved. Cetuximab for instance targets the epidermal growth factor receptor (EGFR) and trastuzumab HER2/*neu*. Notably, the first approved mAb with anticancer-activity that did not target cancer cells directly was Bevacizumab, a mAb directed against VEGF[319]. Besides depleting molecules such as VEGF or blockade of relevant growth receptors mAbs also recruit other effector cells or molecules such as the complement system or Fc receptor expressing effector cells such as neutrophils, monocytes or NK cells that can mediate ADCC. Importantly, whereas natural cytotoxicity was reported several times to be decreased in tumor patients, tumor-infiltrating NK cells were shown to potently elicit ADCC without re-activation[229,320]. This indicates the big potential antibody-therapy could have on NK cell anti-tumor immunity. Further, in pre-clinical models various arming-strategies of antibodies have been tested such as conjugates with radioactive particles or toxins[321]. Also bispecific antibodies were evaluated that for instance bind to CD3 and a tumor-antigen, thus localizing effector cells in very close proximity to their targets[321].

Importantly, also topically applied adjuvants have been tested for tumor-therapy and were approved by the FDA. For instance, imiquimod containing a TLR7 agonist has proven efficacy against pre-malignant lesions, low-grade epithelial tumors and VIN[322].

4 AIM OF THE STUDY

Malignant melanoma currently exhibits a bad prognosis for tumor patients and its incidence is increasing. Few promising treatment strategies exist and despite the identification of many melanoma-associated antigens, T cell-based strategies often are not applicable due to the frequently observed down-regulation of HLA class I molecules. As the selective or complete loss of HLA class I expression renders the tumor cells sensitive towards NK cell mediated lysis, we wanted to investigate innovative approaches to improve existing NK cell-based immunotherapy strategies against malignant melanoma.

So far, little success was achieved in clinical studies using NK cells against non-hematological malignancies leaving an urgent need for improved protocols. Amongst others, there are two explanations why in the performed studies no beneficial effect could be observed:

a) The NK cells did not infiltrate properly into the tumor tissue
b) *In situ* or systemic immunosuppression by tumor-derived factors renders NK cells anergic, preventing an effective anti-tumor response

Thus, to investigate possibilities to overcome these two major restrictions, we focused on the following aims:

a) The identification of chemokines that improve the homing of cytotoxic human NK cells to and their infiltration into tumor tissue.
b) The characterization of a novel activation protocol prior to infusion that allows for more persistent anti-tumor reactivity. Notably, it was reported that murine NK cells exhibited an induced “memory like” phenotype upon short term activation with IL-12, IL-15 and IL-18 mounting elevated responses after re-stimulation[284]. Thus, we wanted to perform a comprehensive phenotypical and functional analysis of IL-12/15/18-stimulated human NK cells focusing on more sustained reactivity and cytotoxicity against tumor cell lines.

Both approaches subsequently should get evaluated in a xenograft mouse model of malignant melanoma that we set up for these studies.

These strategies are instrumental to tackle existing problems with NK cell-based anti-cancer immunotherapy. The findings that will be obtained in this study are potentially of very high clinical relevance as they might help to improve immunotherapy against melanoma and malignant diseases in general.

5 MATERIALS AND METHODS

5.1 Materials

5.1.1 Mouse strains

All mice used were bred in the DKFZ animal facilities. NOD-SCID mice were received from the barrier 2 (DKFZ, Heidelberg) and NSG mice from the barrier 1 (DKFZ, Heidelberg), respectively. Mice were housed under specific pathogen-free conditions and used in experiments typically at 6-12 weeks of age. All experiments were performed according to local animal experimental ethics committee guidelines.

5.1.2 Cell lines

Name	Cell type	Medium
721.221 mutant (parental)	Human EBV transfected B lymphoblastoid cell line	IMDM
721.221 mutant HLA-Cw*03		
721.221 mutant HLA-Cw*04		
721.221 mutant HLA-Cw*07		
721.221 mutant HLA-Cw*15		
A549	Human alveolar adenocarcinoma line	RPMI
HCT116 wtp53	Human colorectal carcinoma line	RPMI
HUVEC	Human umbilical vein endothelial cells	Endothelial Cell Growth Medium
K562	Human myelogenous leukemia line	RPMI
Ma-Mel-86b	Human melanoma line	RPMI
Phoenix-ampho	Human embryonic kidney line	DMEM
SK-Mel-28	Human melanoma line	RPMI
U937	Human histiocytic lymphoma line	RPMI
UKRV-Mel-02	Human melanoma line	RPMI

All cell culture media were supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin and were regularly tested for the presence of mycoplasma using the multiplex contamination assay available at the core facility for genomics at the DKFZ, Heidelberg.

E.coli host strains

Product	Source	Catalogue number
JM109	Promega	P9751
MAX Efficiency® DH5α™ Competent Cells	Invitrogen	18258-012
One Shot® TOP10 Chemically Competent E. coli	Invitrogen	C4040-10

5.1.3 Cell culture products

Product	Source	Catalogue number
15 ml conical tubes	Greiner	188271
24-well flat-bottom with lid – Standard TC	BD	353047
24 well transwell migration plates	Corning	3421
40 µM cell strainer Falcon™	BD	352340
5 ml round-bottom polypropylene test tubes	BD	352008
5 ml round-bottom polystyrene test tubes w/ cell strainer	BD	352235
50 ml conical tubes Falcon™	BD	352070

70 µM cell strainer Falcon™	BD	352350
96-well flat-bottom with lid – Standard TC	BD	353972
96-well U-bottom with lid – Standard TC	BD	353077
Cryovial, 2 ml sterile	Roth	E309.1
Filter tips: 10, 20, 100, 200, 300 and 1000 µl	Biozym	770010,-50,-280,-300,-400
Petri dishes (60 mm)	TPP	93060
Safe lock tubes: 0.5, 1.5 and 2 ml	Eppendorf	13625, 12682, 12776
Serological pipettes: 5, 10, 25 and 50 ml, sterile	Costar	4487-4490
Standard tissue culture flask/filter screw caps – 75 cm^2	TPP	90076
Standard tissue culture flask/filter screw caps – 150 cm^2	TPP	90151
Tissue culture flask/filter screw caps – 182 cm^2	Greiner	660175

5.1.4 Cell culture media and reagents

Product	Source	Catalogue number
Biocoll Separating Solution	Biochrom AG	L6115
Dimethylsulfoxide Hybri Max (DMSO)	Sigma Aldrich	D2650
DMEM (1x) (High Glucose) with L-Glutamine, 4500 mg/L D-Glucose, w/o Sodium Pyruvate	Sigma Aldrich	D6429
D-PBS (1x) w/o Ca, Mg, Sodium Bicarbonate	Sigma Aldrich	D8537
EDTA	BioChrom AG	L2113
Endothelial cell growth medium	PromoCell	C-22010
Fetal Bovine Serum, Origin: EU approved	Gibco	10270
Gelatine	Sigma	G1393
Geneticin (G418)	Gibco	10131-027
Human serum, type AB, converted	PAA	C05-002
Ionomycin	Sigma Aldrich	I3909
LB-medium	Roth	X968.2
L-Glutamine 200 mM (100x), 29.2 mg/ml	Gibco	25030
Penicillin/Streptomycin-Solution: 10,000 U Penicillin, 10,000 µg/ml Straptomycin	Sigma Aldrich	P4333
Phorbol 12-myristate 13-acetate (PMA)	Sigma Aldrich	P1585
Puromycin	Sigma Aldrich	58-58-2
RPMI 1640 (1x) with L-Glutamine	Sigma Aldrich	R8758
Stem cell growth medium (SCGM)	CellGenix	20802-0500
Trypsin-EDTA 0.25%	Gibco	25200
Trypan Blue	Sigma Aldrich	T8154
Trypsin-EDTA (1x) HBSS w/o Ca, Mg with EDTA	Sigma Aldrich	T3924

All sera were heat-inactivated at 56°C for 30 minutes prior to usage in culture media.

For all bacteria cultures 1x LB medium with appropriate antibiotics was used.

5.1.5 Magnetic cell sorting (MACS)

Product	Source	Catalogue number
LS Columns	Miltenyi	130-042-401
Human NK cell isolation kit	Miltenyi	130-092-657

5.1.6 Kits

Product	Source	Catalogue number
Antarctic phosphatase	NEB	M0289S
BCA™ Protein Assay Kit	Pierce	23227
Bioplex cell lysis kit	Biorad	171-304011
HiPure Plasmid Maxi-Prep Kit	Invitrogen	K210007
Human CX3CL1 ELISA	R&D	DY365
Human IFN-gamma ELISA	BioLegend	430101
Luciferase assay system	Promega	E1500
pGEM-T-easy	Promega	A1360
QIAprep spin Mini-Prep Kit	Qiagen	27106
QIAquick GelEx Kit	Qiagen	28706
QIAquick PCR purification kit	Qiagen	28104
T4 DNA ligase	NEB	M0202S

5.1.7 Antibodies

Primary antibodies	Clone	Company	Catalogue number
B7-H6	1.18	G. Moldenhauer, DKFZ	-
CD107a-FITC	H4A3	BioLegend	328606
CD112	L14	-	-
CD155	SKIL4	BioLegend	337610
CD16-APC Cy 7	3G8	BioLegend	302018
CD16-FITC	3G8	BD Pharmingen	555406
CD16-PE	3G8	BioLegend	302008
CD16-PerCP Cy 5.5	3G8	BioLegend	302028
CD25-APC	BC96	BioLegend	302610
CD3-FITC	HIT3a	BioLegend	300306
CD3-PE	HIT3a	BioLegend	300308
CD3-PE-Cy7	SK7	BioLegend	344816
CD45-Pacific Blue	HI30	BioLegend	304029
CD56-APC	HCD56	BioLegend	318310
CD56-PE Cy7	HCD56	BioLegend	318318
CD69-APC	FN50	BioLegend	310910
CX3CL1-PE	51637	R&D	IC365P
CX3CR1-PE	2A9-1	BioLegend	341604
CXCR1-PE	8F1/CXCR1	BioLegend	320608
CXCR2-PE	5E8/CXCR2	BioLegend	320706
CXCR3-AF488	TG1	BioLegend	334907
DNAM-1-PE	TX25	BioLegend	337106
KIR2DL1/S1-FITC	HP-MA4	BioLegend	339505
KIR2DL2/L3-PE	DX27	BioLegend	312605
KIR3DL1-PE	DX9	BioLegend	312707
MICA	AMO1	Bamomab	AMO1-100
MICB	M363	Amgen	-
NKG2D-APC	1D11	BioLegend	320808
NKG2D-Fc	-	R&D	1299-NK-050
NKp30-APC	P30-15	BioLegend	325210
NKp44-APC	P44-8	BioLegend	325109
NKp44-Fc	-	R&D	2249-NK-050
NKp46-AF647	9E2	BioLegend	331910
NKp80-APC	5D12	BioLegend	346707
Pan-MHC class I	W6/32	BioLegend	311412

Isotype antibodies	Clone	Company	Catalogue number
CD99-Fc	-	R&D	3905-CD-050
mIgG1	MOPC-21	BioLegend	400124
mIgG1-AF647	MOPC-21	BioLegend	400130
mIgG1-APC	MOPC-21	BioLegend	400122
mIgG1-FITC	MOPC-21	BioLegend	400108
mIgG1-PE	MOPC-21	BioLegend	400112
mIgG2a	G155-178	BD Pharmingen	554645
mIgG2a-FITC	MOPC-173	BioLegend	400210
mIgG2a-PE	G155178	BD Pharmingen	554648
mIgG2b-FITC	MPC11	BioLegend	400310
mIgG2b-PE	133303	R&D	IC0041P
rIgG2b-PE	eB149/10H5	eBioscience	12-4031-82

Secondary antibodies	Company	Catalogue number
gah-Fc-PE	*Jackson* ImmunoResearch Laboratories	109-116-097
gam-Fc-PE	Dianova	115-116-071

5.1.8 Chemicals and biological reagents

Product	Source	Catalogue number
7-AAD viability staining solution	BioLegend	420403
Agarose	Sigma	A9539
AlamarBlue™ cell viability reagent	Life technologies	DAL1025
Carboxyfluorescein succinimidyl ester (CFSE)	Sigma-Alrich	21888
Chloroquin	Sigma-Aldrich	C6628
^{51}Chromium	Perkin Elmer	NEZ020005MC
Collagenase IV	Cell Systems	LS004188
Colorless RPMI	Sigma-Aldrich	11835-030
CX3CL1	PeproTech	300-31
CXCL10	PeproTech	300-12
CXCL12	PeproTech	300-28A
CXCL8	PeproTech	200-08
DNAse I	Sigma-Aldrich	DN25
dNTPs	Promega	U1511
Ethidiumbromide	AppliChem	A1152.0025
Evan's Blue dye	Sigma-Aldrich	E2129
GoTaq	Promega	M3001
Heparin-sodium, 25.000 units	Braun	1708.00.00
Hyaluronidase type V	Sigma-Aldrich	H6-254
IL-12	Peprotech	200-12
IL-15	R&D	247-IL-005
IL-18	MBL	B003-5
IL-2	Tecin™, teceleukin from Roche, received from NIH	
Isofluran B	Braun	6724123.00.00
Matrigel™	BD	356231
Nuclease-free water	Ambion	AM9937
Polybrene	Sigma-Aldrich	28728-55-4
Stay Bright™ Luciferin	BioVision	7903-1G
TMB liquid substrate system for ELISA	Sigma-Aldrich	T0440
Triton X-100	Sigma-Fluka	T9284
Human IgG	Sigma-Aldrich	I4506

5.1.9 Enzymes and markers

Product	Source	Catalogue number
BamHI-HF	NEB	R3136S
DNA-Ladder	NEB	N3200S
PacI	NEB	R0547S
PvuI	NEB	R0150S
SalI-HF	NEB	R3138S
XhoI	NEB	R0146S

5.1.10 Plasmids

Oligonucleotide	Source
pBabe-puro	Stock of D080, originally from Robert Weinberg
pDONR221-CX3CL1	Gene repository, Genomics core facility, DKFZ, Heidelberg
pGL2A	Günter Küblbeck, DKFZ, Heidelberg
pMX-neo	Stock of D080, originally Cell Biolabs, Inc.
pCL-ampho	Stock of D080

5.1.11 Oligonucleotides

All Oligonucleotides were ordered from MWG, Germany.

Oligonucleotide	Sequence 5' – 3'
CX3CL1 forward	TTTGGGATCCCATGGCTCCGATATCTCTGTC
CX3CL1 reverse	TTTTCTCGAGTCACACGGGCACCAGGACAT
Intra-CX3CL1 sequencing	CTAAGGCTGAGGAACCCATC
Luciferase primer sequencing	GCGTTTTGTTGACTCCATCC
pMX-neo forward sequencing	GGCAGCCTACCAAGAACAAC
pMX-neo revers sequencing	CTGGTTGCTGACTAATTGAGATGC
Psi PlusPack primer	CCGCCTCCTCTTCTTCCATC

5.1.12 Solutions

Solution	Ingredients
10x PBS	1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4 (anhydrous), 20 mM KH_2PO_4
2x HBS	50 mM HEPES with 12 mM dextrose, 10 mM KCl, 280 mM NaCl, 1.5 mM Na_2HPO_4 (pH 7.05)
50x TAE	242 g Tris Base, 57.1 ml 100% acetic acid, 100 ml 0.5 M EDTA (pH 8.0) ad. 1 liter
ACK lysis buffer	0.829 g NH_4Cl, 0.1 g $KHCO_3$, 0.38 mg EDTA ad. 100 ml ddH_2O
$CaCl_2$	2 M $CaCl_2$
DNA loading dye 5x	0.25% (w/v) Orange G, 30% (v/v) glycerol, 10 mM EDTA, 10mM Tris-HCl (pH 7.4)
FACS buffer	1x PBS, 3% FCS, 0.02% (v/v) NaN_3
Freezing medium	1x FCS, 10% DMSO
Homing buffer	RPMI-1640, 0.5% BSA
MACS buffer	1x PBS, 3% FCS, 0.5 mM EDTA
Primary cell culture medium	1x SCGM, 10% human serum, 1% pen/strep

5.1.13 Laboratory equipment, tools and material

Equipment	Name	Company
Analytical scale	Classic Plus PB602-S	Mettler Toledo
	Adventurer OHAUS	
Anesthesia machine	Vapor 19.1	Drägerwerk AG
	XGL-8	Xenogen
Animal irradiator	OB 58/902-1	Buchler
Bacterium incubator	Innova 42330	New Brunswick Scientific
Caliper	Calimax	Wiha
Cell culture incubator	Heraeus BBD6220	Kendro
Centrifuge	Heraeus Multifuge 4KR	Kendro
	Heraeus Multifuge X3R	Thermo Scientific
	Heraeus Multifuge 3SR+	Thermo Scientific
	Heraeus Biofuge pico	M&S Laborgeräte GmbH
	Sorvall Evolution RC	Thermo Scientific
	-	WIFUG
Counting chamber	Neubauer chamber	Blau Brand
ELISA microplate reader	Multiscan Fc	Thermo Fischer
ELISA plate	ELISA plate, Microlon 96 W	Greiner bio one
Flow Cytometer	FACS™ Calibur	BD
	FACS™ Aria I	
	FACS™ Canto II	
Flow hood	Hera Safe	Kendro
	Cell Gard, Lab Gard	Nuaire
Freezer -20°C	Premium, Comfort, Profi line	Liebherr
Freezer -86°C	VIP series	Sanyo
Fridge	Premium, Profi line	Liebherr
	-	VWR, International
Geldocumentation	UV Flächenstrahler	Konrad Benda
Ice machine	Ice maker	Hoshizaki
Imaging machine	IVIS-100	Xenogen
Lumaplate	LumaPlate™-96	Perkin Elmer
Lumaplate reader	TopCount NXT™	Perkin Elmer
Luminescence reader	Orion L microplate Luminometer	Berthold Detection Systems
Magnetic stirrer	MR3001 K	Heidolph
Microscope	Wilovert 30	Hund Wetzlar
Multichannel pipette	Transferpette-12, -8	Gilson
Multistepper	Multipipette plus	Eppendorf
N2 tank	Cryosystem 6000	MVE
Needles	Microlance™ 3 (25 G, 27G and 30G)	BD
One-way scalpel	Feather disposable Scalpel	Feather
PCR machine	PTC-200	MJ Research
pH meter	pH211	Hanna Instruments
Photometer	NanoPhotometer™	Implen
Pipettes	Pipetman (various sizes)	Gilson
Pipetboy	CellMate II	Matrix
Power supply	Power Pack basic	BioRad
Pump	PC2004 Vario	Vacuubrand
	BVC21	Vacuubrand
	Vacusafe	Integra
Red light lamp	Heat Glo 75 W	ExoTerra
Shaver	Aesculap GT415	Braun
Surgery equipment (scissor, forceps…)	-	Dimeda, A. Dumont&Fils
Syringe	Discardit™ II 5 ml	BD
	TBC-Spritze 1 ml	mediware
Thermomixer	Compact, Comfort	Eppendorf
Vortexer	VortexGenie 2	VWR/Scientific Industries
Water bath	Heraeus Julabo TW20	Kendro
White 96 well plate	Costar™ 96-Well white	Corning

5.2 Methods

5.2.1 Cell culture methods

5.2.1.1 Thawing of cells

For thawing of cells, the respective cryovials were withdrawn from the liquid nitrogen tank and thoroughly sprayed with 70% ethanol. Thawing was done by adding pre-warmed medium with a 1 ml pipette and transferring the thawed cell suspension into Falcon tubes with pre-warmed medium. The Falcon tube was filled up with medium and the cells were centrifuged (1200 or 1500 rpm, 5 min, RT). After removal of the supernatant the cells were resuspended in pre-warmed medium and put into appropriate cell culture flasks for culture (37°C, 5% CO_2) for at least 7 days prior to usage.

5.2.1.2 Passaging and antibiotics treatment of cells

The culture medium of adherent cells was decanted or removed with a vacuum pump and trypsin-EDTA or PBS-EDTA was added until the cell layer was properly covered. The flasks were incubated at 37°C until all cells detached upon rocking of the flask. The cells were then transferred into pre-warmed medium in Falcon tubes and were centrifuged (1200 rpm, 5 min, RT). The supernatant was removed from the pellet and the cells got resuspended in fresh medium and either split at appropriate ratios into fresh cell culture flasks for culture (37°C, 5% CO_2) or were used for individual assays.

Suspension cells were resuspended properly by rocking the cell culture flask and an appropriate volume of medium was removed by either decanting or with a vacuum pump. Fresh medium was added so that the cells were diluted in a range from 1:2 up to 1:50.

HUVEC cells have been cultured in flasks that were coated with 1% gelatin for 2 hours. For detachment of HUVEC cells, Trypsin-EDTA at a concentration of 0.25% was used.

To select the three melanoma lines either for pBabe-puro-eGFP-2A-CBGr99 or pMX-neo-CX3CL1 transductants the following titrated concentrations of antibiotics have been used:

	Puromycin	G418 (geneticin)
Ma-Mel-86b	0.75 µg/ml	750 µg/ml
SK-Mel-28	1 µg/ml	750 µg/ml
UKRV-Mel-02	2 µg/ml	750 µg/ml

5.2.1.3 Freezing of cells

Suspensions of the cells were centrifuged (1200 or 1500 rpm, 5 min, RT) and after removal of the supernatant by decanting or with a vacuum pump the cell pellet was resuspended in an appropriate volume of freezing medium and 1 ml aliquots were frozen for 1-3 days at -80°C. Subsequently, the frozen vials were transferred into liquid nitrogen for long-term storage. Typically, a mycoplasma-test according to the guide lines of the core facility for genomics was performed prior to freezing the cells.

5.2.1.4 Counting of cells

If necessary, the respective cell suspension was pre-diluted and an aliquot was further diluted with an appropriate volume of trypan blue solution (0.05 % w/v) mixed 1:1 with PBS for live-dead discrimination. Live cells were counted with a Neubauer counting chamber (0.1 mm depth) and the total cell number was calculated using the following formula:

Total live cell number = Counted cells/counted squares* dilution * 10^4

5.2.2 Molecular biological methods

5.2.2.1 Cloning of the eGFP-2A-CBGr99 insert into pBabe-puro

As shown in **figure 6.1**, the expression cassette for eGFP-2A-CBGr99 luciferase was cut out of the pGL-2A vector kindly provided by Günter Küblbeck (DKFZ, Heidelberg) using BamHI and XhoI to cut the insert and PacI and PvuI to degrade the remaining backbone. The target vector pBabe-puro was digested with BamHI and SalI, creating identical overhangs for XhoI. The digests were performed in a total volume of 20 µl using 5 µg DNA and appropriate buffers according to the manufacturer's instructions. After 4 hours of incubation at 37°C loading dye was added to the restriction products and the mixtures were loaded quantitatively on a 1% agarose gel containing 0.1% ethidiumbromide. The gel was prepared of 1x TAE that was also used as a running buffer (90 volts). An appropriate DNA-ladder mix was added on one lane of the gel to allow for determination of size and quantity of the DNA fragments. Fragments displaying the correct sizes were cut out of the gel using one-way scalpels under UV light (long wavelength, 366 nm) and were subjected to the QIAquick gel extraction kit following the manufacturer's instructions. The concentration of the respective DNA products was determined with a spectrophotometer and used for a ligation reaction with a total volume of 12 µl using 50 ng of vector backbone and a stochiometric vector insert ratio of 1:3. 1 µl of T4 DNA ligase was used per ligation reaction. The Ligation was allowed to proceed o.n. at 16°C. The next day the mixture was heat-inactivated at 65°C for 10 minutes and used for transformation of competent bacteria (5.2.2.3).

5.2.2.2 Cloning of CX3CL1 into pMX-neo

As shown in **figure 6.11**, we first PCR amplified the full length CX3CL1 DNA out of the pDONR221-CX3CL1 with primers introducing a 5'-BamHI and a 3'-XhoI restriction site (see materials section) using the following conditions:

	pDONR221-CX3CL1
1:10 diluted DNA	1 µl
5x Colorless GoTaq buffer	10 µl buffer D
dNTPs	1 µl
For primer	2.5 µl
Rev primer	2.5 µl
GoTaq polymerase	0.5 µl
Nuclease-free water	32.5 µl
25 mM MgCl2	3 µl

	Temp. [°C]	Time [s]
1x	98°C	120
30x	98°C	10
	65°C	30
	72°C	60
1x	72°C	600
1x	4°C	infinite

The samples were supplemented with gel loading dye and separated on a 1% agarose gel containing 0.1% ethidiumbromide and purified out of a gel section as described previously. The purified nucleic acids were then cleaned using the PCR purification kit in order to decrease the concentrations of organic solvents. The respective products were ligated into the pGEM-T vector according to the manufacturer's instructions and the ligation product was used for bacterial transformation (5.2.2.3).

Upon verification of the pGEM-T-CX3CL1 construct via sequencing (5.2.2.4) the plasmid was used to clone the entire ORF into the pMX-neo vector. First, a preparative digestion was performed for 4 hours at 37°C using 5 µg of the respective plasmids and 1.5 µl each of XhoI and BamHI in a 20 µl reaction volume containing 2 µl 10x buffer 4 (NEB). 30 minutes prior to the end of the reaction, 2.5 µl antarctic phosphatase buffer and 2.5 µl antarctic phosphatase (both NEB) were added to the pMX-neo reaction. The respective digests were analyzed via agarose gel-electrophoresis as described and fragments showing the correct sizes were excised out of the gel. The gel extraction and PCR-purification protocol were performed in a row according to the manufacturer's protocol. The cut fragments were used in a ligation reaction (total volume 10µl, 100 ng backbone, backbone-insert ratio 1:3, 1 µl T4 DNA-ligase) over night at 4°C after a 30 minutes pre-incubation at RT. The ligation product was then used for transforming competent cells.

5.2.2.3 Transformation of bacteria and preparation of mini-prep cultures

Chemically competent bacteria were thawed on ice and aliquoted in 50 µl steps per condition. Typically, 7 µl of the ligation reaction or respective controls or 1 µl of plasmid DNA were added to the bacteria and the reaction was incubated for 30 min on ice. The mixture then was put into a pre-warmed waterbath (42°C) for 60 seconds and was cooled on ice again after the heat-shock. 250 µl of SOC medium were added and the cells were incubated for 1 further hour at 37°C prior to plating of the reaction on LB-agar plates containing an appropriate antibiotic. The next day individual colonies were picked with a sterile 200 µl pipette tip and added to 5 ml of LB-medium containing the selection antibiotic for over-night culture. Mini-preps were performed using the Mini-prep kit (Qiagen) according to the manufacturer's instructions and the plasmid DNA was subjected to appropriate analytical control digestions and sequencing as described.

5.2.2.4 Sequencing and maxi-prep

All sequencing reactions have been performed by MWG using appropriate sequencing primers (see materials section) and were evaluated *in silico* with nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Upon confirmation of correct insertion, 2 ml of the respective mini-prep culture were added to 200 ml of LB-medium supplemented with an appropriate antibiotic for selection. After an overnight expansion at 37°C maxi-preps were performed according to the manufacturer's instructions (Invitrogen) and validated via analytical digestions and sequencing.

5.2.2.5 Retroviral transduction of cell lines

$2*10^6$ phoenix-ampho cells were seeded the day prior to transfection in T75 flasks. The next day, the cells received fresh medium containing 25 µM chloroquine and a transfection mix was prepared (9 µl plasmid of interest, 1 µl pCL-ampho, 506 µl ddH_2O, 74 µl $CaCl_2$, 600 µl 2x HBS dropwise while mixing the reaction). The mixture got added dropwise onto the phoenix-ampho cells and after 6 hour incubation time the medium was exchanged. Two days later, virus-containing supernatant was harvested from the phoenix cells, filtered, supplemented with 8 µg/ml polybrene and added to subconfluent target cells of choice. After 1 or 2 days and extensive washing (1200 rpm, 5 min, 25°C), the cells were transferred into the S1 lab and analyzed for transgene expression.

5.2.3 Magnetic-activated cell sorting (MACS)

NK cells were purified out of heparinized fresh peripheral blood or leukocyte concentrates from the blood bank in Mannheim, Germany according to the manufacturer's protocol (Miltenyi). Yet, a 45 minute adhesion step was included prior to magnetic labeling of the cells and typically only 75% of the recommended staining antibodies were used. The purity of the NK cell fraction was determined via CD3 and CD56 staining and usually was > 90%.

5.2.4 Fluorescence-activated cell sorting (FACS)

5.2.4.1 Staining and analyzing of cells

Typically, between $5*10^3$ and $2*10^6$ cells were washed 2x with cold FACS buffer (1200 or 1500 rpm, 5 mins, 4°C) and were resuspended in 50 µl of huIgG or FACS buffer containing appropriate dilutions of primary antibodies or fusion proteins. After 30 minutes of incubation in the dark on ice (60 minutes for fusion proteins), the cells were washed 2x with FACS buffer and if necessary a suitable secondary fluorochrome-conjugated antibody was added for 30 minutes on ice in the dark. The cells were washed twice and transferred in 50 µl of 1:40 diluted 7-AAD into FACS tubes for analysis on a BD FACS Calibur™ or BD FACS Canto II™ after adjustment of the lasers using appropriate single stainings. All displayed results were produced by gating on living cells as determined by 7-AAD staining using the FlowJo 9.3.2 software (Treestar).

5.2.4.2 Sorting of cells

For sorting of pBabe-puro-eGFP-2A-CBGr99 transduced tumor cells, expanded and puromycin-pre-selected tumor cells were harvested using PBS-EDTA, filtered through a 40 µm cell strainer to remove cell clusters and stained with 7-AAD to exclude dead cells. Settings were done with appropriate single stainings and $eGFP^{high}$ tumor cells were sorted through a 100 µm nozzle into 100% FCS for further expansion and long-term culture. The cells were sorted on a BD FACS Aria™ II.

5.2.5 *In vitro assays*

5.2.5.1 *In vitro* quantification of luciferase-activity

$1*10^5$ cells were put into 1.5 ml eppendorf tubes, washed with PBS (2100 rpm, 3 mins) and lysed with 150 µl of lysis buffer (Luciferase Assay System, Promega). After one freeze-thaw cycle (-80°C) the thawed suspension was centrifuged (13.000 rpm, 1 min) and the supernatant was transferred to a clean eppendorf tube and got frozen at -20°C until measurement. For detecting the luciferase intensity, 30 µl of the supernatant were loaded on a white 96-well plate and the signals were read out on a luminometer with the following settings:

Injection volume: 50 µl, delay: 2.05 s, measurement: 10 s

5.2.5.2 CD107a mobilization assay

$1*10^5$ NK cells were co-cultured with tumor cells at an effector:target ratio of 1:2 or 1:1 in RPMI-1640 supplemented with 10% FCS and 1x pen/strep for 30 min in the presence of FITC conjugated anti-CD107a or the respective isotype control. 1 µg GolgiStop (monensin) was added/well to increase accumulation of CD107 protein in the Golgi complex. The assay was continued for 4 further hours prior to staining the cells for flow cytometric analysis. 50 ng/ml PMA and 1 mM ionomycin were used as a positive control.

5.2.5.3 ^{51}Cr release assay

Target cells were harvested with PBS-EDTA or an aliquot was taken from suspension cells and the suspension was centrifuged (1200 or 1500 rpm, 5 min, RT). The pellet was resuspended in 500 µl assay medium (RPMI-1640, 10% FCS, 1x pen/strep) and labeled with 100 µCi ^{51}Cr for 90 minutes. Effector cells were adjusted to $1.5*10^6$ cells/ml for a 50:1 E:T ratio or to less when few cells were available and got titrated in 1:1 dilution steps into the wells of a 96 well plate (round bottom). After excessive washing (1200 rpm, 5 min, RT) the target cells were counted and adjusted to $3*10^4$ cells/ml. 100 µl of labeled target cells were added to the serial dilution of effector cells or to the minimum (assay medium) or maximum (10% triton-x10) lysis wells. The plates were incubated for 4 hours at 37°C and after centrifugation (300 rpm, 3 min, RT), 100 µl of supernatant were transferred onto lumaplates. After drying for 1-3 days, the plates were read out and the specific release was calculated:

Specific release [%] = (mean cpm (sample) – mean cpm (min))/(mean cpm (max) - mean cpm (min))

5.2.5.4 Enzyme-linked immunosorbent assay (ELISA)

IL-15 or IL-12/15/18 stimulated NK cells were used at various timepoints after the pulse in *in vitro* restimulation assays. Co-cultures were set up in a total volume of 200 µl with all stimulation media consisting of SCGM, 10% human serum, 1% pen/strep and 100 IU/ml of IL-2 and either no addition (medium background), 10 ng/ml IL-12 and 50 ng/ml IL-15 or K562 in a E:T ratio of 1:1. After 24 hours, cell-free supernatant was harvested and used for detection of IFN-γ via ELISA (BioLegend).

For the detection of CX3CL1 shedding of the respective transductants, $1*10^6$ cells were seeded in a 6 cm dish in RPMI-1640 with supplemental. After 24 hours, the medium got replaced by migration medium and was conditioned by the tumor cells for further 24 hours. Cell-free supernatant was harvested after centrifugation (1200 rpm, 5 min, RT) and used for the human CX3CL1 ELISA (R&D) or frozen at -20°C.

All samples were used undiluted or were diluted up to 1:2000 with assay reagent. The ELISAs for IFN-γ and CX3CL1 were used according to the manufacturer's instructions. Samples for the CX3CL1 ELISA from tumor biopsies after lysis were subjected to a protein concentration determination assay (BCA-kit, Pierce) and were adjusted to 1 mg/ml prior to usage (refer to section 5.2.5.4).

5.2.5.5 Transwell-migration assay

All chemotaxis assays were performed in 24-well transwell chambers using 5-µm pore polycarbonate filters (Corning). NK cells, typically after 1-3 days in medium containing 200 IU/ml IL-2, were washed with chemotaxis buffer and adjusted to a concentration of $2*10^6$ cells/ml. 600 µl of chemotaxis buffer containing various doses of recombinant chemokines or supernatant from tumor cells cultured for 24 h in chemotaxis buffer (after adjustment to the ideal CX3CL1 concentration) were added to the lower chamber of the transwell. After adding $2*10^5$ NK cells into the upper wells or into input tubes the plates were incubated for 4 h at 37°C to allow for migration. 500 µl of the medium in the lower well containing the migrated NK cells were harvested after thorough flushing of the membrane and the well. The samples were stained together with the input samples for flow cytometric analysis with appropriate antibodies. For internal standardization, equal numbers of beads were added to equal volumes of stained cells. All samples were acquired until a certain number of beads was detected, thus standardizing for the flow rate of the FACS. Plotted is the % specific migration:

Specific migration [%] = (number of cells (sample)-number of cells (medium))/number of cells (input)*100

In some experiments the membranes have been coated with human vascular endothelial cells**.** Therefore, the membranes have been treated with 100 µl 1% gelatin for 2 hours prior to seeding of $1*10^5$ HUVEC cells/membrane. HUVEC medium was added to the upper and lower well until after 4 days the layers were dense as detected by Evans Blue dye test for endothelial cell permeability. Briefly, medium was removed and into the lower well 600 µl colorless RPMI were added, whereas 100 µl of 4% BSA and 0.67 mg/ml of Evans Blue in colorless RPMI were added to the upper well. After 4 hours, the Evans Blue dye concentration in the lower well was detected spectrometricaly at 620 nm and compared to the concentration when no gelatin was present. Permeabilities under 5% were considered to be indicative of a dense HUVEC layer. The specific migration through these coated membranes was calculated as described before.

5.2.5.6 AlamarBlue™ cell viability assay

1.000 cells of various cell lines were added per well of a 96 well flat bottom plate in 200 µl of control medium (RPMI-1640, 10% FCS, 1x pen/strep) or in medium containing various concentrations of chemokines as indicated. After 20 hours, alamarBlue™ dye was added in a 1:10 ratio (20 µl) and the

reaction was incubated for further 4 hours at 37°C. After a total of 24 and 48 hours after seeding the absorbances of the wells were read out at 570 nm.

5.2.5.7 CFSE-labeling of NK cells

NK cells were harvested and adjusted to $1*10^7$ cells/ml in PBS. A solution of 4 µM CFSE in PBS was freshly prepared and mixed in a 1:1 ratio (v/v) with the NK cell dilution. The cells were labeled for 15 minutes in the dark at room temperature while spinning before the reaction was stopped with pure FCS. The cells were washed with PBS (1500 rpm, 5 min, RT) before they were used for injection or for *in vitro* assays.

5.2.6 In vivo assays

5.2.6.1 Intravenous and subcutaneous tumor injections and adoptive NK cell transfer

Tumor cell lines as indicated were harvested in the exponential growth phase with PBS-EDTA. After two washing steps with PBS (1200 rpm, 10 mins, 4°C) the cells were counted twice and adjusted appropriately. For intravenous injections the cells were filtered through a 40 µm cell strainer to get rid of aggregates prior to adjusting the cell concentration. For intravenous (i.v.) injections the animals were pre-warmed with a red light lamp for 5 minutes and later on were fixed in an injection tube. The tail veins were cleaned with spitacid prior to injection of 200 µl of the respective solutions through 30 G needles containing the indicated tumor doses. For subcutaneous injections, the mice were anesthetized with isofluran and the tumor cells were injected into the subcutis of the pre-shaved left flank of the animals. Tumor growth was measured 3 times weekly with a caliper and is displayed as a product of three diameters. In some experiments, matrigel-tumor mixtures were injected. Therefore, matrigel was thawed on ice and tumor cells were mixed 1:1 with ice-cold matrigel prior to injection of the indicated cell numbers in 200-300 µl cell-matrigel solution.

Accordingly, NK cells were washed and adjusted in PBS (1500 rpm, 10 mins, 4°C). I.v. injections were performed as described. For peri-lesional injections, the NK cell solution was injected subcutaneously through a 27 G needle roughly 1 cm away from the tumor plug. In some experiments NK cells were labeled with 2 µM CFSE for 15 minutes in the dark as described prior to i.v. injection into animals.

5.2.6.2 Application of cytokines

When indicated, 20.000 IU of recombinant human IL-2 in 200 µl of PBS or PBS as a control were injected i.p. into NSG mice.

5.2.6.3 Acquiring of bioluminescent signals

Mice were imaged at the indicated time points using the IVIS® imaging system-100 (Xenogen). D-luciferin was dissolved in D-PBS to a final concentration of 30 mg/ml and was injected i.p. at a dose of 150 mg/kg (typically 200 µl) 10 minutes prior to the acquisition of the bioluminescent signal

intensity (BLI). General anesthesia was induced with 5% isoflurane in a chamber and was continued during the acquisition with 2.5% isoflurane via nose cones. After acquiring photographic images of each mouse, luminescent images were acquired with various (60 s - 300 s) exposure times depending on the luminescent intensity of the cell line used. Acquisitions have been repeated until a maximal signal could be detected. Usually, all animals have been injected 2x with 200 µl of luciferin to ensure saturating levels of the compound. The resulting grayscale photographic and pseudo-color luminescent images were automatically superimposed by the IVIS Living Image Software (Xenogen, version 2.50.1). Regions of interest (ROI) were manually drawn around the signal to quantify the emitted photons. Luminescence was integrated over these ROIs and is expressed as relative light units (RLU) [photons/s/cm^2/sr] in all respective experiments.

5.2.6.4 Organ dissection and preparation of single cell suspensions

Blood

The mice were killed by asphyxiation with CO_2 and blood was collected from the retro-orbital vein after removal of the eyeball. Withdrawn blood was supplemented with heparin for flow cytometric analysis. Likewise heparinized blood was added quantitatively into 25 ml 1x ACK for 8 mins before stopping the reaction with excessive amounts of PBS. Unlysed, remaining cells were washed with PBS (1500 rpm, 10 mins, 4°C), counted and used for staining or re-stimulation. For serum preparation, un-heparinized blood was incubated for 2 hours at 37°C, clothed blood was removed from the vessel wall and the samples were centrifuged (3 min, max speed) prior to collection of the serum that was stored at -20°C.

Spleen

Animals were sacrificed by dislocation of the neck. Spleens were removed and stored in ice-cold PBS until further processing. For mechanical disruption, spleens were minced through a 70 µm-pore strainer. The cell suspension got washed (1500 rpm, 10 mins, 4°C), counted and was used directly for staining.

Liver

After sacrifice of the animals as described, the mice were perfused by injecting 5-10 ml of PBS with a 25 G needle into the heart. Livers were removed and cut into small pieces prior to digestion with 0.05% collagenase type IV in PBS (20 mins, 37°C). The digested organs then got minced through a 70 µm-pore strainer and washed with PBS (1600 rpm, 10 mins, 4°C). Afterwards, the cells were minced through a 40 µm-pore strainer and loaded onto 6 ml of lympholyte for density gradient centrifugation (1500 rpm, 26 min, 22°C, acceleration 5, brake 1). The leukocyte ring was harvested together with the serum and washed with PBS (1500 rpm, 10 min, 4°C) prior to counting and staining.

Lung and tumor

Animals were sacrificed and perfused with PBS as described. Lungs and tumors or tumor-matrigel plugs were removed, cut into pieces using forceps and scalpels and were digested with 5 mg/lung of hyaluronidase type V and DNAse I (30 min, 37°C). Digested tissues got minced through a 70 µm-pore strainer and washed with PBS (1500 rpm, 10 min, 4°C). After mincing the suspension through a 40 µm-pore strainer it got loaded onto 6 ml of lympholyte for density gradient centrifugation (1500 rpm, 26 min, 22°C, acceleration 5, brake 1). The leukocyte ring was harvested together with the serum and washed with PBS (1500 rpm, 10 min, 4°C) prior to counting and staining.

In some experiments pieces of tumors have been removed, weighed and used for the preparation of tumor lysates. Therefore, the samples have been treated with the cell lysate kit (Biorad) according to the manufacturer's instructions.

5.2.6.5 *Ex vivo* re-stimulation of blood cells

For some experiments, blood cells have been used for an *ex vivo* re-stimulation assay. After counting, 5.000 bulk blood cells were used per re-stimulation in a total volume of 200 µl (SCGM + 10% human serum + pen/strep + 100 IU/ml IL-2) with either medium, 10 ng/ml IL-12 and 50 ng/ml IL-15 or K562 at a 1:1 E:T ratio. After 19 hours, supernatant was removed for an IFN-γ ELISA. To standardize the IFN-γ levels, the percentage of NK cells as detected by FACS analysis was used to extrapolate all samples to the level of IFN-γ which 50.000 NK cells would have produced.

5.2.7 Statistical analysis

If not stated differently, p-values were calculated using Student's t-test with two-tailed distribution and two-sample equal variance parameters. The Mantel-Cox test was used for comparison of survival curves. P-values for Student's t-test and the Mantel-Cox test were calculated using the GraphPad Prism or Microsoft Excel software. Values of $p < 0.05$ were considered to be statistically significant.

6 RESULTS

6.1 Establishment of xenograft mouse models of melanoma

6.1.1 Generation of pBabe-puro-eGFP-2A-luc

In order to investigate the therapeutic potential of human NK cells against malignant disease *in vivo,* we aimed at setting up a minimally invasive imaging model. As the usage of optical imaging compared to other technologies such as positron emission tomography (PET), magnetic resonance imaging (MRI) or computer tomography (CT) does not require long acquisition times or extensive training, we created a retroviral vector allowing for the overexpression of the green click beetle luciferase[323,324]. In various reports the quantification of luciferase-derived signals enabled researchers to locate and quantify tumor cells or to monitor various cellular processes[325–328]. We decided to use the green click beetle luciferase CBGr99 from the yellow click beetle (*Pyrophorus plagiophthalamus*) as the spectral peak at 543 nm is less absorbed by tissue as compared to the more commonly used firefly luciferase (spectral peak at 560 nm). Therefore, it has been shown by Miloud *et al.* that the CBGr99 exhibits stronger signals *in vivo* compared to other luciferases upon subcutaneous, intraperitoneal or intravenous transplantation of luciferase-positive cells[329]. Finally, the use of the 2A-sequence of the porcine teschovirus-1 (P2A) guarantees for the stochiometric expression of the two genes it links[330,331]. By cloning out the eGFP stop codon and in-frame inserting the 2A-CBgr99 construct a single bicistronic mRNA coding for both proteins is transcribed. The 2A-sequence co-translationaly cleaves itself giving rise to two independent protein products[332]. The efficiency of this process has been reported to be 100%. Of note, the protein being coded for 5' of the 2A sequence carries most of the amino acids coded for by the 2A gene whereas the protein being coded for 3' of the 2A sequence carries a proline at its N-terminus. Thus, using eGFP as a reporter gene, sorting for $eGFP^{high}$ cells also selects for $CGBr99^{high}$ cells.

As shown in **figure 6.1 A**, we used the expression cassette from the pCMV-eGFP-2A-CBGr99 plasmid (i.e. pGL-2A) kindly provided by Günter Küblbeck (DKFZ, Heidelberg). As we wanted to create a retroviral vector with the possibility to select for transductants with antibiotics, we used the commercially available pBabe-puro as a backbone. Using the indicated restriction enzymes (also refer to the respective Materials and Methods section), the expression cassette was inserted into pBabe-puro and the respective pBabe-puro-eGFP-2A-CBGr99 construct was used for transduction of the three melanoma cell lines Ma-Mel-86b, SK-Mel-28 and UKRV-Mel-02. After pre-sort selections with pre-determined doses of puromycin, $eGFP^{high}$ cells were sorted out and we compared the eGFP levels quantitatively to the untransduced parental lines via flow cytometry (**figure 6.1 B**). As shown, high eGFP positivity could be achieved for all three lines.

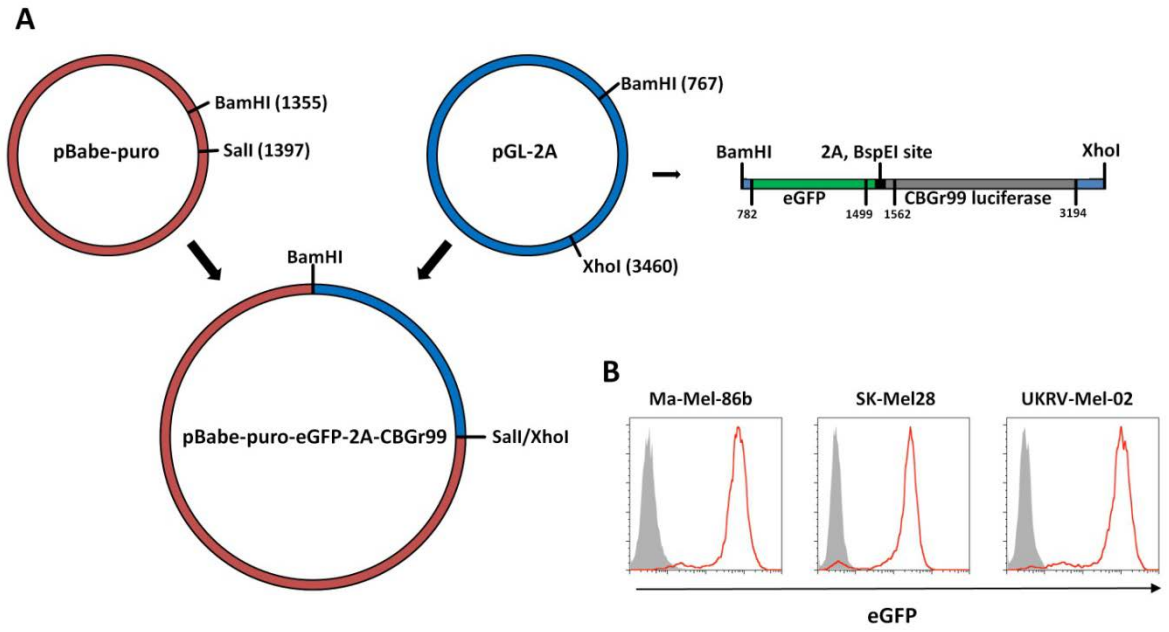


Figure 6.1 – Cloning strategy for pBabe-puro-eGFP-2A-CBGr99. **A)** Restriction digestion of pGL-2A with BamHI and XhoI gives rise to the full length expression cassette eGFP-2A-CBGr99. This insert is ligated into the pBabe-puro backbone after digestion with BamHI and SalI, creating an un-digestable combined SalI/XhoI site. **B)** Exemplary eGFP-expression profiles of three melanoma cell lines after retroviral transduction, puromycin selection and FACS sorting. The red line indicates the signal of the sorted transductants, the grey filled signal the untransduced parental lines. One of two experiments is shown.

6.1.2 Evaluation of two mouse strains for xenotransplantation experiments

To test if the transduced cell lines (refer to 6.1.1) are suitable for *in vivo* bioluminescent intensity (BLI) measurements, we first determined if the transduced CBGr99 gene is functional in the created cell lines. As shown in **figure 6.2 A**, all cell lines exhibited varying but high levels of luciferase activity *in vitro*. Of note, all lines were emitting photons several 1000-folds higher than the background.

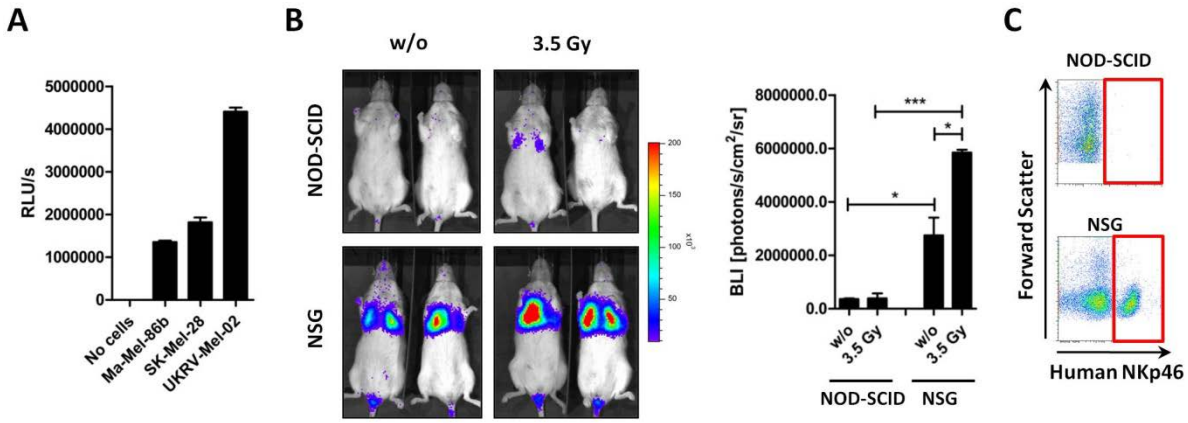


Figure 6.2 – NSG mice show higher engraftment of human tumor lines and NK cells as compared to NOD-SCID mice. A Either lysis buffer or a cell lysate from $1*10^5$ cells transduced with pBabe-puro-eGFP-2A-CBGr99 were subjected to an *in vitro* luciferase quantification assay. Plotted is the mean ± SD (n = 2) of the RLU/s**.** One of two experiments is shown. **B** NOD-SCID or NSG mice have been left untreated or were irradiated 1 day prior to the i.v. injection of $5*10^5$ SK-Mel-28 cells transduced with pBabe-puro-eGFP-2A-CBGr99. Shown are the respective acquired images on day 7 (left panel) and the quantification of the signals (right panel) as mean ± SD (n =2). **C** NOD-SCID or NSG mice have been irradiated 4 hours prior

to the i.v. injection of either $1.75*10^6$ (NOD-SCID) or $0.7*10^6$ NK cells (NSG) that have been activated with 20 ng/ml IL-15 for 16 hours. Until the end of the experiment on day 4 (NOD-SCID) or 6 (NSG), the mice received daily i.p. injections of 20.000 IU IL-2. Shown is the percentage of human $NKp46^+$ cells amongst living cells. w/o without, * $p < 0.05$, *** $p < 0.001$ (student's t-test).

However, the *in vitro* generated data do not clarify if the cells are suitable for *in vivo* applications. We thus next investigated, if the transduced cells generate a detectable signal *in vivo*, what the most permissive mouse strain for human tumor and NK cell engraftment is and if pre-conditioning by whole body irradiation is necessary for successful xenotransplantion. We therefore injected $5*10^5$ SK-Mel-28 transduced with pBabe-puro-eGFP-2A-CBGr99 i.v. into either NOD-SCID or NSG (complete name NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice that either received or not 3.5 Gy whole body irradiation one day prior to tumor injection. NSG mice were derived from NOD-SCID mice by The Jackson Laboratory by breeding in a null-allele for the interleukin-2 receptor gamma chain (IL2Rγ). As both strains lack adaptive immunity and show no complement activity and various defects in innate immunity, for example reduced DC and macrophage function they are both widely used in xenotransplantation experiments[333–335]. Due to the lack of the common γ-chain receptor NSG compared to NOD-SCID mice in addition show various defects in cytokine signaling (affected are IL-2, -4, -7, -9, -15 and -21). Most importantly, they are void of murine NK cells that can potentially reject xenotransplanted cells in NOD-SCID mice. In line with that, it was shown that tumor cells and hematopoietic stem cells engraft more potently in NSG mice[333,336].

As shown in **figure 6.2 B**, NSG mice engrafted tumor cell lines with higher efficiency compared to NOD-SCID mice as detected by luciferase quantification, regardless if the mice have been irradiated or not. Systemic irradiation with 3.5 Gy was enhancing the tumor take to a significant degree in these animals. Of note, we observed the same engraftment promoting effect of γ-irradiation on tumor engraftment in NOD-SCID mice when we increased the tumor dosage (data not shown).

Most importantly, as shown in **figure 6.2 C**, NSG mice were also able to engraft human NK cells more potently as compared to NOD-SCID mice. Of note, the injected cell numbers for IL-15- and IL-12/15/18-stimulated NK cells were not the same, as depicted in the figure legend. However, as fewer injected NK cells persisted better in the blood of NSG compared to NOD-SCID mice after a longer time *in vivo,* we concluded that NSG mice are superior to NOD-SCID mice in terms of engrafting adoptively transferred human NK cells. Since also tumor cells engrafted more potently most of the experiments shown were performed with NSG mice unless stated differently.

To determine if γ-irradiation prior to i.v. tumor injection also impacts the survival of recipient animals, we injected untreated or irradiated NOD-SCID mice i.v. with melanoma cell lines. As shown in **figure 6.3 A**, pre-conditioning with γ-irradiation of the animals decreased the survival time of recipient mice, most likely due to a higher tumor take. Also, only animals that have received irradiation showed macroscopically visible metastases on the lungs (data not shown).

As some of the planned experiments required subcutaneous tumor inoculation, we injected Ma-Mel-86b cells subcutaneously into either γ-irradiated (3.5 Gy) or unirradiated NOD-SCID mice and recorded the tumor growth. As compared to i.v. tumor cell inoculation, irradiation in this model was showing detrimental effects in terms of tumor growth (**figure 6.3 B**). Until three weeks after the injection the tumor was hard to palp in animals that have received 3.5 Gy of total body irradiation. At this time the unirradiated group already showed a tumor size of almost 1 cm^3. These data were

highly reproducible and in any experiment that required a subcutaneous tumor injection the animals thus were not irradiated prior to tumor cell injection.

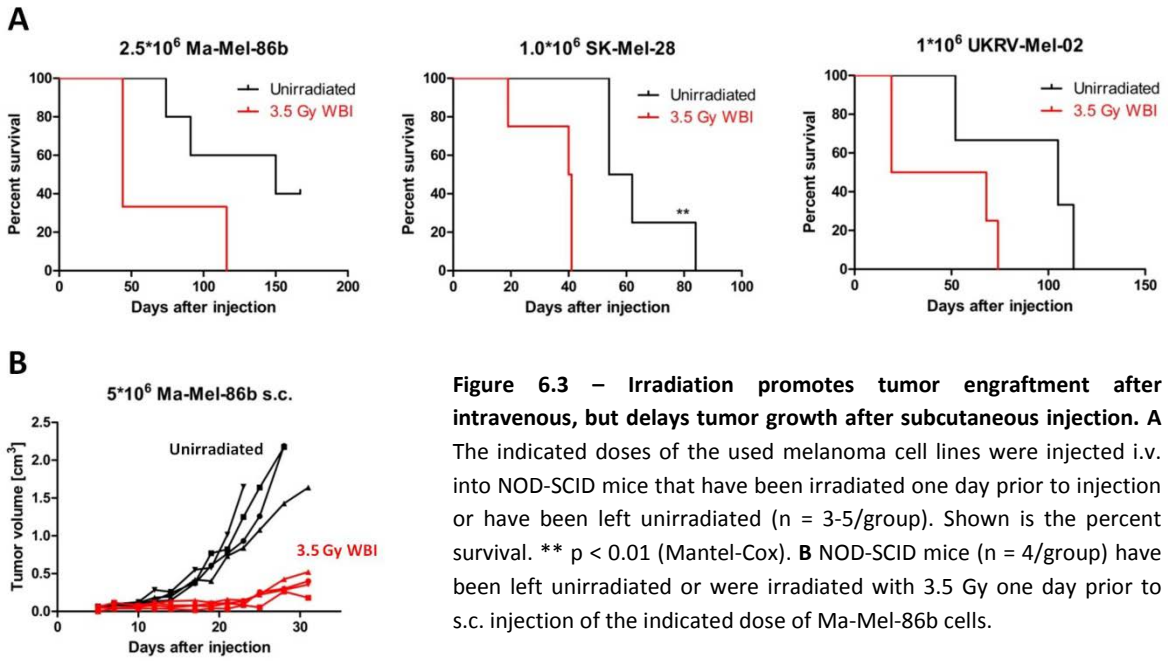


Figure 6.3 – Irradiation promotes tumor engraftment after intravenous, but delays tumor growth after subcutaneous injection. A The indicated doses of the used melanoma cell lines were injected i.v. into NOD-SCID mice that have been irradiated one day prior to injection or have been left unirradiated (n = 3-5/group). Shown is the percent survival. ** $p < 0.01$ (Mantel-Cox). **B** NOD-SCID mice (n = 4/group) have been left unirradiated or were irradiated with 3.5 Gy one day prior to s.c. injection of the indicated dose of Ma-Mel-86b cells.

6.1.3 Characterization of melanoma target cell lines

As discussed previously, melanoma cells often show critical down-regulation of HLA class I expression compared to healthy tissue[337,338]. This leads to diminished T cell recognition but also to less inhibition of tumor infiltrating NK cells rendering them attractive targets for NK cell-based immunotherapy. After we have screened multiple lines, we have decided to use Ma-Mel-86b, SK-Mel-28 and UKRV-Mel-02 based on growth behavior and the expression levels of activating and inhibitory ligands.

The most important activating and inhibitory ligands were analyzed by flow cytometry. The ligand profile of the three lines is shown in **figure 6.4 A**. Of note, all cell lines expressed high levels of DNAM-1-ligands and detectable levels of NKp44-ligands with SK-Mel-28 being the only MHC class I-positive cell line. The expression of DNAM-1- and NKp44-ligands were of particular importance as a previous report showed that the DNAM-1-DNAM-1-ligand and the NCR-NCR-ligand axes substantially contributed to the control of melanoma growth *in vivo*[171].

To investigate if the observed phenotype translates into a solid NK cell-activation upon tumor cell contact, ^{51}Cr release assays were performed that, compared to CD107a mobilization assay, quantitatively detect target cell killing. As shown in **figure 6.4 B** the MHC class I-negative cell lines Ma-Mel-86b and UKRV-Mel-02 exhibited comparably little donor to donor variability whereas the killing of SK-Mel-28 substantially varied between different donors, possibly due to the KIR heterogeneity in between varying donors[339,340].

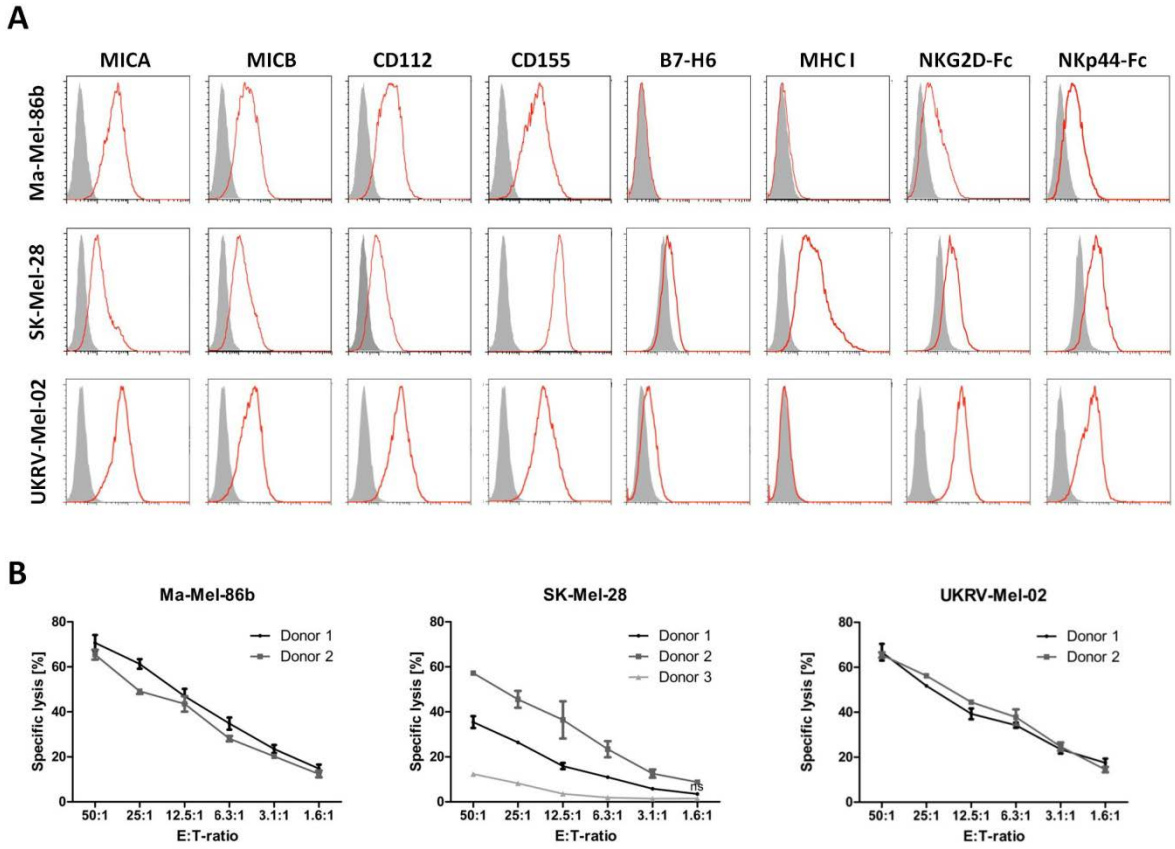


Figure 6.4 – Characterization of activating and inhibitory ligands on three melanoma lines and their susceptibility towards NK cell mediated lysis. A Staining of various activating ligands and MHC I levels on the three melanoma cell lines Ma-Mel-86b, SK-Mel-28 and UKRV-Mel-02. The grey filled signal is derived from the respective isotype staining and the red line represents the specific staining as indicated. All stainings have at least been repeated once. **B** ^{51}Cr release assay with NK cells activated with 200 IU IL-2/ml for 1-3 days. Shown is the mean ± SD (n = 3). In total 5 – 12 donors have been tested.

6.1.4 Titration of CBGr99-transduced tumor lines for intravenous injection

As shown in **figure 6.2 B** the melanoma cells upon transduction with the pBabe-puro-eGFP-2A-CBGr99 plasmid were producing a detectable luminescent signal in the lungs of NSG mice upon i.v. injection. However, as ultimately the model needs to display the anti-tumor effect of adoptively transferred NK cells the correlation of the detected BLI with the amount of tumor cells was determined. Therefore, various doses of the CBGr99-transduced melanoma lines Ma-Mel-86b and SK-Mel-28 were injected i.v. into irradiated NSG mice and the BLI was detected three days after tumor cell inoculation.

As shown in **figure 6.5 A-C** differences in the tumor load could be accurately detected despite the low resolution of the imaging modality. Both lines tested showed a satisfying degree of linearity between the tumor load and the respective signal intensity as depicted by the r^2 value (**figure 6.5 C)**.

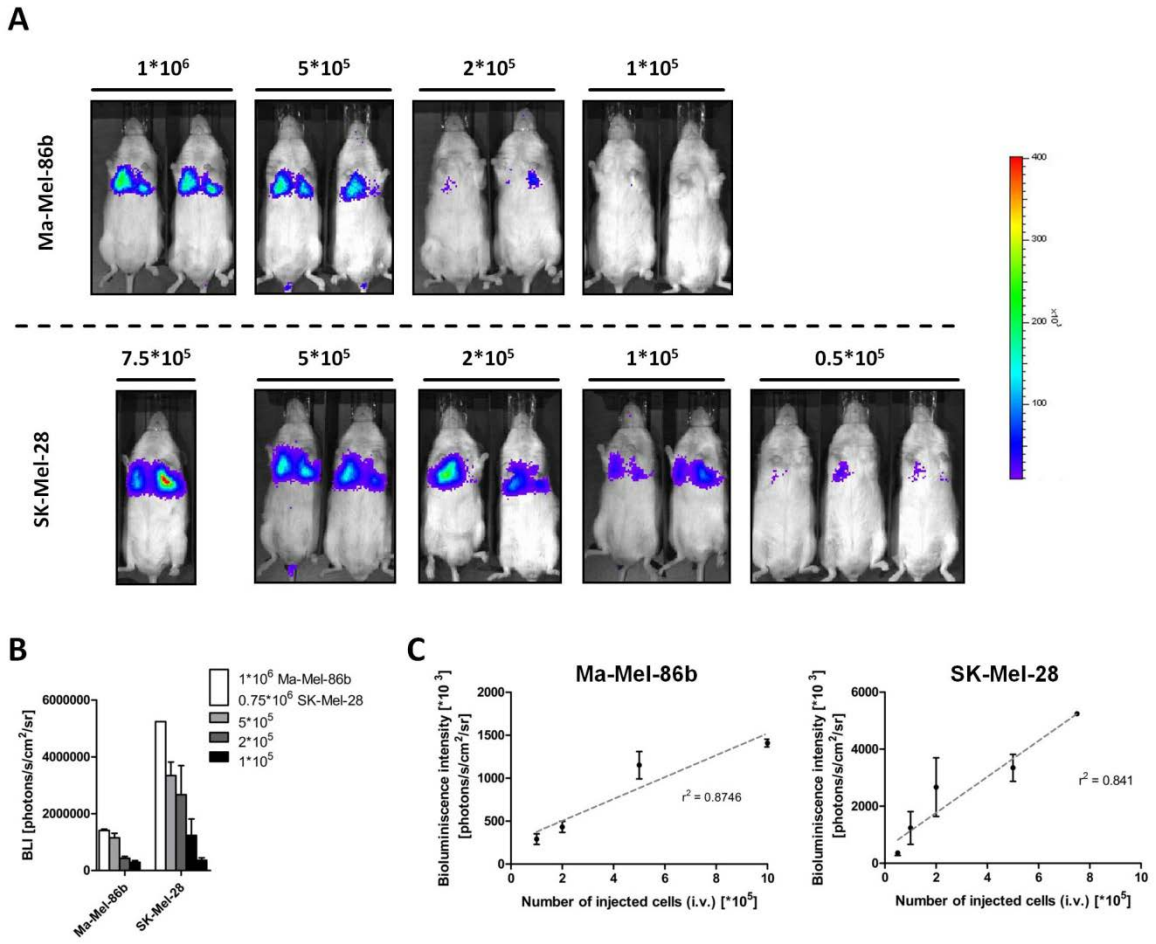


Figure 6.5– The quantification of the BLI correlates with the tumor load *in vivo*. A 1-3 NSG mice per group have been irradiated with 3.5 Gy 4 hours prior to the injection of various doses of CBGr99-transduced Ma-Mel-86b or SK-Mel-28. The images shown were acquired 3 days after the injection. **B** Quantification of A. Shown is the mean ± SD of the BLI (n = 1-3). **C** Linear regression of respective tumor dosage - BLI datasets.

6.2 Improved NK cell surveillance of CX3CL1-positive tumors

6.2.1 Identification of the CX3CL1-CX3CR1 axis as a potential tool for immunotherapy

One of the possibilities to improve existing NK cell-based immunotherapy strategies against malignant disease that needs more investigation is the targeted attraction of cytotoxic effector NK cells into tumor tissue. As described previously, NK cells are not a homogenous cell population but consist of the $CD56^{dim}$ $CD16^{+}$ subset and the $CD56^{bright}$ $CD16^{-}$ subset with the $CD56^{dim}$ subset comprising approximately 90% of the peripheral blood NK cells[186]. As in the context of malignant disease often a) little NK cell infiltration can be observed[341] with b) most of the infiltrating cells belonging to the $CD56^{bright}$ NK cell subset[220,221,342], we wanted to identify a suitable chemokine that is able to potently attract the $CD56^{dim}$ subset in order to restore full NK cell immune-competence *in situ*. Of note, the $CD56^{dim}$ subset has been reported to exert more cytotoxic functions whereas the $CD56^{bright}$ subset classically produces higher levels of various cytokines[205,343].

As several reports were showing that CX3CL1 is one of the chemokines that attracts the $CD56^{dim}$ subset with high specificity, we stained purified human NK cells during culture in IL-2 containing medium at various timepoints for CX3CR1 and analyzed its expression on the two subsets[210].

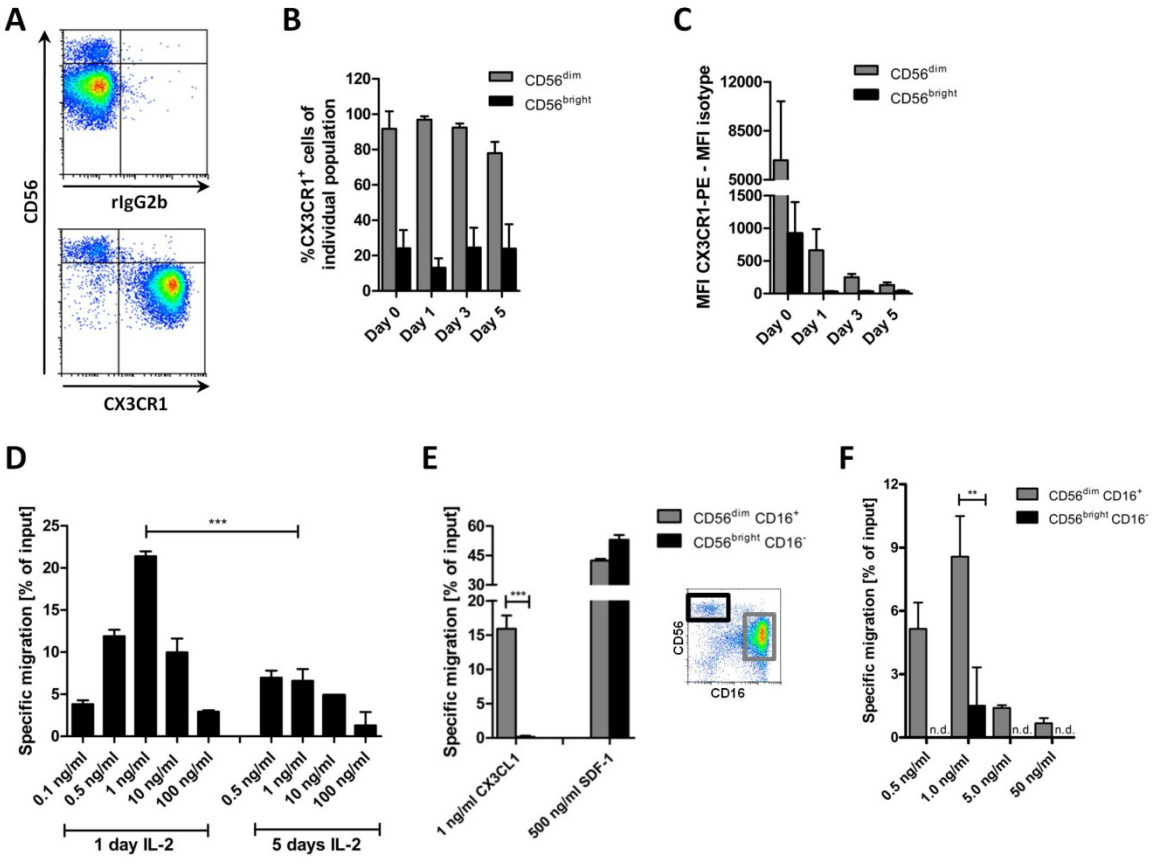


Figure 6.6 – The $CD56^{dim}$ NK cell subset exclusively expresses CX3CR1 and migrates towards recombinant CX3CL1. Do-plots depict the staining of CD56 and CX3CR1 or the respective isotype on NK cells, which have been activated with 200 IU/ml IL-2 for one day. One representative of 5 donors is shown. B Shown is the mean ± SD of the percentage of CX3CR1-positive cells in the indicated subpopulations (n=4-6). **C** Shown is the mean ± SD of the MFI of CX3CR1 for the individual NK cell subpopulations (n=4-6). **D** Transwell migration assay of NK cells activated for 1 or 5 days with IL-2 (200 IU/ml) against recombinant CX3CL1. Shown is the mean ± SD (n = 3) of the specific migration. One representative out of 6

similarily performed experiments is shown. **E** NK cells have been activated for one day with 200 IU/ml IL-2 and were used in a transwell migration assay against 1 ng/ml of CX3CL1. Migrated cells were stained and quantified. Shown is the mean ± SD of the specific migration calculated individually for the two subsets (n = 3). One representative out of 7 donors is shown. **F** Purified NK cells have been used in a trans-endothelium migration assay and the migrated cells were stained and assigned to the two main populations. Shown is the mean ± SD of the specific migration as % of input (n = 3) of one representative out of 3 donors. n.d., not detectable. ** $p < 0.01$, *** $p < 0.001$ (student's t-test).

As shown in **figure 6.6 A-C**, CX3CR1 indeed was expressed exclusively on the $CD56^{dim}$ subset. Of note, the MFI of CX3CR1 dramatically decreased during culture and although the percentage of CX3CR1-positive cells virtually remained stable (**figure 6.6 B**) the MFI, correlating to the CX3CR1-density on the cells, dropped substantially (**figure 6.6 C**). This finding seems to be generally applicable to short term exposure of NK cells to activating cytokines such as IL-2, IL-15, IL-12 or IL-18[344]. Accordingly, we tested if the migration of NK cells is affected after culture periods of 1 or 5 days in IL-2 containing medium. Thus, we performed transwell-migration assays and the specific migration of NK cells towards CX3CL1 was determined. **Figure 6.6 D** shows that short *in vitro* culture periods maintained the specific migration towards CX3CL1 whereas NK cells cultured for 5 days or longer showed a significant decrease in CX3CR1-expression and thus in their migratory response towards CX3CL1. We found that NK cells despite the partial loss of CX3CR1 during the culture migrated equally well towards CX3CL1 as compared to naïve NK cells for up to three days (data not shown). Therefore, we cultured NK cells for a maximum of two days in IL-2 when they were used in *in vitro* or *in vivo* migration assays.

Ultimately, we tested if the NK cells that migrated towards CX3CL1 truly belonged to the $CD56^{dim}$ $CD16^{+}$ subset. Therefore, we performed transwell migration assays and stained the migrated cells for CD56 and CD16. The migrated NK cells were assigned to one of the populations based on their expression levels of the two markers (**figure 6.6 E**) and the specific migration of each subpopulation was calculated (also refer to materials and methods). As shown in **figure 6.6 E**, as compared to stromal cell-derived factor-1α (SDF-1α, systematic name CXCL12), a chemokine known to induce the migration of all NK cells, CX3CL1 specifically attracted the $CD56^{dim}$ subset of NK cells. We next went on to also test if recombinant CX3CL1 is able to recruit NK cells through a dense layer of endothelial cells (human umbilical vein endothelial cells, HUVEC) while maintaining the specificity for the $CD56^{dim}$ subset. As shown in **figure 6.6 F** $CD56^{dim}$ NK cells indeed were attracted towards CX3CL1 through endothelial cells although to a lesser extent as compared to the transwell assay without the HUVEC layer (**figure 6.6 E**).

As we wanted to identify more than one suitable chemokine, we went on and evaluated CXCR1/2 and CXCR3 in terms of subset distribution and their potential to attract the $CD56^{dim}$ $CD16^{+}$ NK cell subset. CXCR1 and CXCR2 were of interest, as a publication by Campbell *et al.* showed that their cognate ligand CXCL8 exclusively attracted the $CD56^{dim}$ subset[210]. Furthermore, Marco Wendel, a former PhD student from our group, identified the CXCR3-CXCL9/10/11-axis as being crucial for NK cell mediated tumor control in a murine tumor model[94]. Thus, we also evaluated the expression of this receptor on human NK cells.

As shown in **figure 6.7 A** and **B**, the receptors for CXCL8 were indeed expressed exclusively on the $CD56^{dim}$ cells, enabling specifically this subset to migrate in a dose dependent manner towards various concentrations of CXCL8.

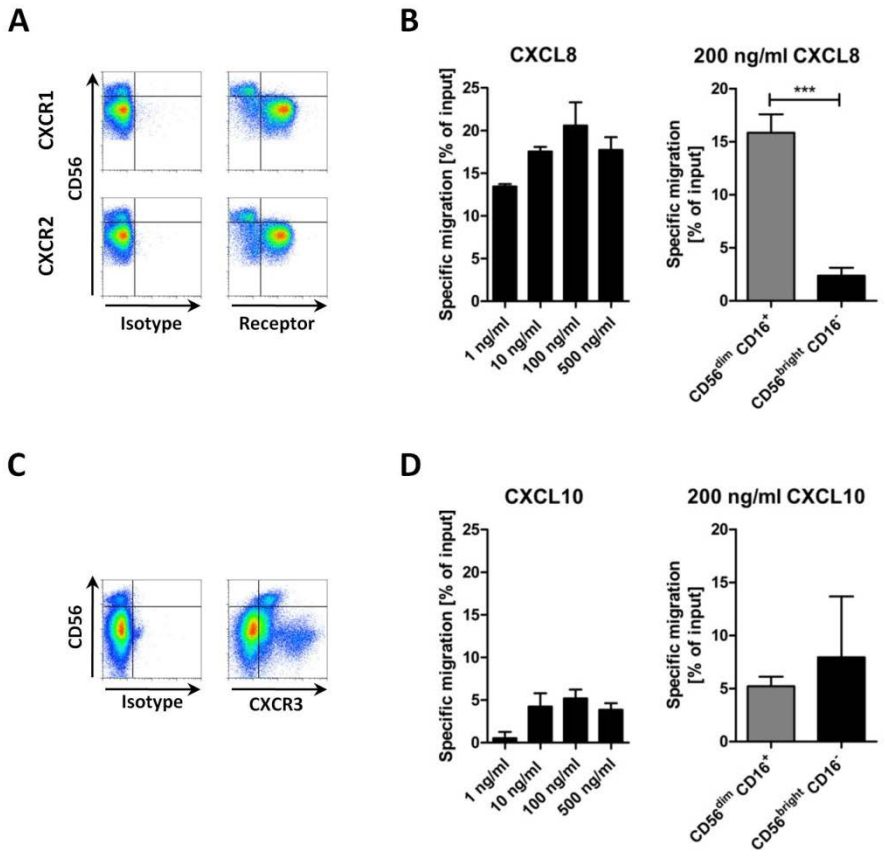


Figure 6.7 – Human CXCL8 but not CXCL10 specifically attracts CD56^{dim} CD16^{+} NK cells. A Purified human NK cells after overnight culture in 200 IU/ml IL-2 were stained for CXCR1 and CXCR2 or with the respective isotype control antibodies. Shown is one representative donor out of eight. **B** Purified human NK cells have been cultured overnight in 200 IU/ml IL-2 and were used in a transwell migration assay against various doses of human CXCL8. Migrated cells were stained and assigned to the CD56^{dim} CD16^{+} or to the CD56^{bright} CD16^{-} subset as described before. Shown is the mean ± SD (n = 2) of the specific migration of all NK cells (left panel) or the specific migration of the individual subsets (right panel). **C** Purified human NK cells after 3 days in culture in 200 IU/ml IL-2 have been stained for CXCR3 or with the respective isotype control antibody. Shown is one representative donor out of eight. **D** The experiment was performed as described under **B**. The NK cells have been cultured for three days with 200 IU/ml of IL-2. Shown is the mean ± SD (n = 2) of the specific migration of one out of two donors. *** $p < 0.001$ (student's t-test).

Importantly, CXCL8, especially under hypoxic conditions, has been reported to mediate pro-tumorigenic functions in terms of tumor cell invasion, migration but also angiogenesis[93]. As therefore, the overexpression of CXCL8 potentially exerts tumor-promoting effects we decided to select CX3CL1 for our experiments.

Compared to CXCR1/2, the expression of CXCR3 was more specific for the CD56^{bright} subsets with only a subpopulation of CD56^{dim} NK cells expressing the receptor (**Figure 6.7 C**). Accordingly, only few CD56^{dim} NK cells got attracted (**figure 6.7 D)**. These data are in line with a report, showing that human CD56^{bright} cells are similar to murine CXCR3^{+} CD27^{bright} cells[345]. In the murine model, the majority of circulating NK cells expresses this receptor which is why it was found to be crucial for mediating NK cell attraction into solid transplanted tumors[94]. Compared to this study, only around 10% of human NK cells in the peripheral blood express this receptor (**figure 6.7 C**). Thus, we excluded CXCR3 in our experiments.

6.2.2 CX3CL1-expression during tumor progression

After the identification of CX3CL1 as a suitable chemokine to attract CD56^{dim} CD16^{+} NK cells, we searched databases and gene arrays for information about how the expression levels of this chemokine either change during tumor progression or if the *in situ* levels differ from neighboring healthy tissue. Interestingly, Hyakudomi *et al.* investigated tissue sections of 158 patients with gastric adenocarcinoma[346]. They found that CX3CL1 levels were showing a significant positive correlation with the size of the CD8^{+} T cell and NK cell infiltrate and further that the CX3CL1^{high} cohort had a significantly better prognosis as compared to the CX3CL1^{low} group in terms of diseasae-free survival (**figure 6.8 A**).

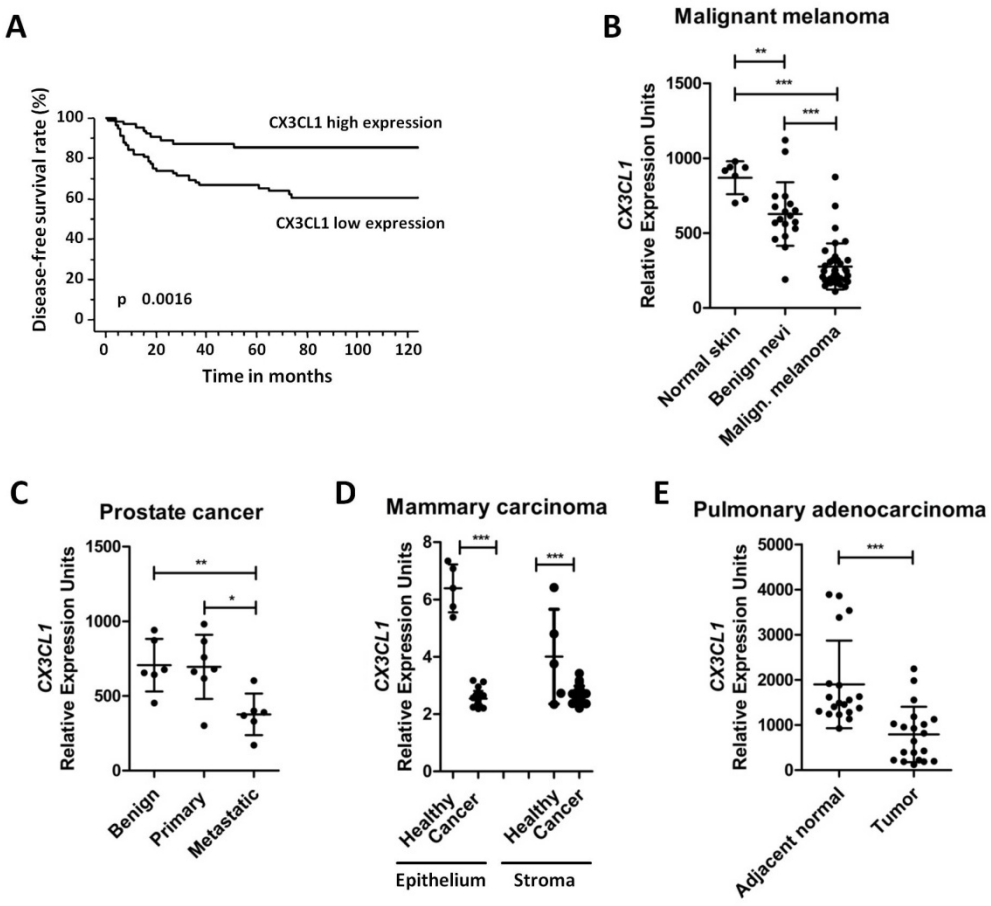


Figure 6.8 – CX3CL1 in various cancer entities frequently gets down-regulated as compared to healthy control tissue. A Modified figure 2 from Hyakudomi *et al.*. Increased expression of CX3CL1 is correlated with a better prognosis and an increased number of both CD8+ T cells and natural killer cells in gastric adenocarcinoma (Hyakkudomi *Annals of surgical oncology* 2008; *15*(6): 1775–82). **B-E** CX3CL1-expression was evaluated in GDS datasets in the GEO database. Individual samples are presented as dots whereas the bars represent the mean ± SD. The data are derived from GDS1375 (melanoma), GDS1439 (prostate cancer), GDS3324 (mammary carcinoma) and GDS1650 (pulmonary adenocarcinoma). Malign., malignant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (student's t-test).

Notably, for several cancer entities the analysis of datasets published in the GEO database revealed that CX3CL1 frequently gets down-regulated during malignant transformation or tumor progression (**figure 6.8 B-E**). This is potentially indicative of a tumor immunoescape mechanism by which tumor cells are trying to avoid the infiltration and subsequent attack by immune effector cells. However, the effects of CX3CL1 might vary from entity to entity or even from patient to patient since, for instance, the analysis of cervical cancer samples did not show any change in CX3CL1 levels compared

to healthy tissue (GDS3233) and colorectal carcinoma (GDS2947) or renal cell carcinoma (GDS2881) even showed elevated levels of CX3CL1 as compared to healthy tissue (data not shown).

6.2.3 Soluble CX3CL1 does not activate NK cells

Various publications show that chemokines, amongst others MIP-1α, MCP-1 and RANTES, can trigger activation and/or proliferation of naïve human NK cells[86,347]. Furthermore, it has been published that some chemokines like CCL19 and CCL21 only exerted a beneficial effect when a primary proliferation stimulus such as IL-2 was present[347]. Therefore, we tested if soluble recombinant CX3CL1 affects NK cell proliferation or if increased levels of the activation marker CD69 could be detected after chemokine addition into the culture medium. As shown in **figure 6.9 A** CX3CL1 was not able to increase the proliferation of purified human NK cells as detected by CFSE dilution, regardless if IL-2 was present or not. This held true for either of the two tested concentrations of recombinant CX3CL1. We furthermore stained NK cells for CD69. However, no significant regulation of the activation marker was detectable (**figure 6.9 B**).

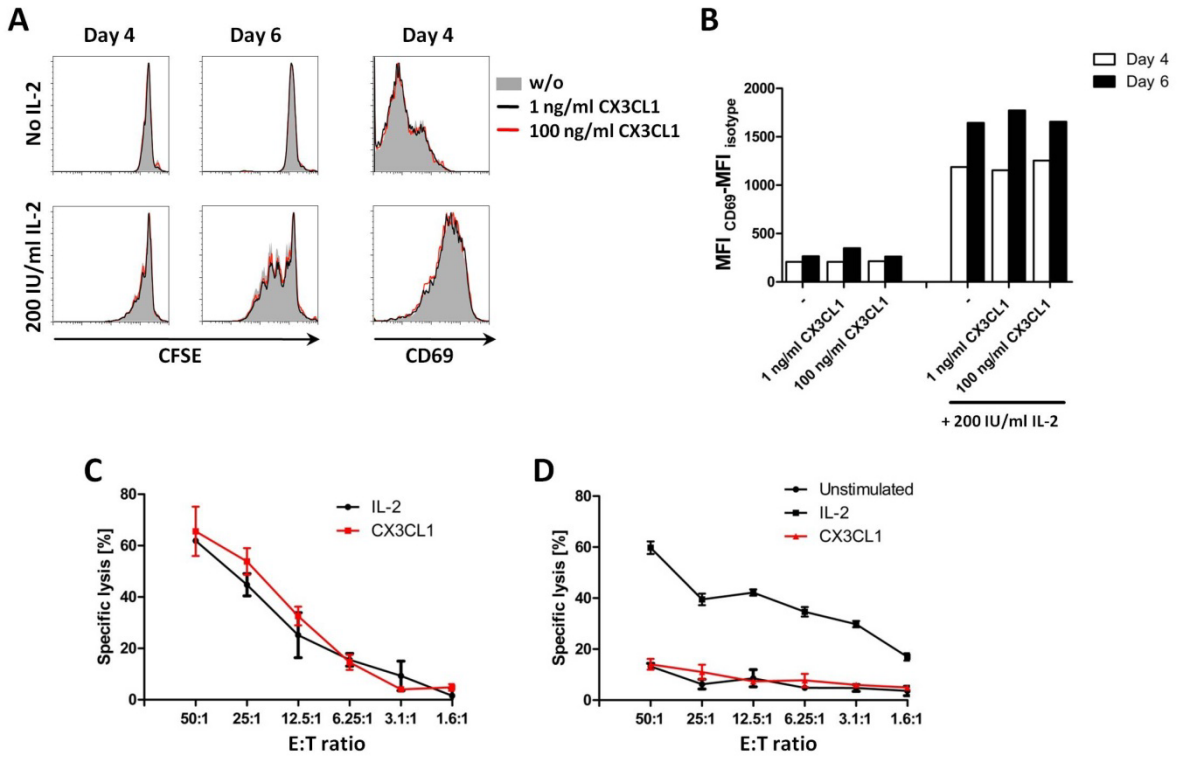


Figure 6.9 – Recombinant soluble CX3CL1 does not activate NK cells. A Purified NK cells have been labeled with 2 µM CFSE and were cultured either in the presence or absence of 200 IU/ml IL-2 for the indicated times with or without either 1 or 100 ng/ml of recombinant CX3CL1. Plotted is the acquired CFSE signal (left panel) or the expression level of CD69 (right panel) for the indicated conditions. Shown is one representative out of two donors. **B** NK cells cultured as described under **A** were withdrawn from the culture and stained either for CD69 or with the respective isotype control antibody. Plotted is the MFI of CD69 corrected for the isotype expression. One of two donors is shown. **C** Purified NK cells have been pre-activated over night with IL-2 or IL-2 plus 100 ng/ml CX3CL1. Ma-Mel-86b melanoma cells were used as targets and plotted is the mean ± SD of the specific lysis. **D** Human NK cells were left untreated over night and were short-term activated on the next day for 2 hours with either 200 IU/ml IL-2 or 10 ng/ml CX3CL1. The CX3CL1 concentration during the kill was adjusted to 10 ng/ml. Shown is one of three target lines.

Since it was also shown that soluble CX3CL1 was able to increase lysis of cellular targets, we performed several ^{51}Cr release assays to see if melanoma cell lines are lysed more efficiently in the presence of CX3CL1[86,87,348]. As shown in figure **6.9 C and D**, soluble CX3CL1 neither increased the killing of NK cells when IL-2 was present as a primary stimulus nor did it activate naïve NK cells compared to the control medium level. The same held true for various cell lines, including amongst others K562 and UKRV-Mel-02 (data not shown).

6.2.4 Generation of CX3CL1-overexpressing cell lines

Since we demonstrated that CX3CL1 exclusively attracted the $CD56^{dim}$ subset and that its expression was reported to a correlate with longer disease-free survival for some cancer entities, we concluded that CX3CL1 is a suitable chemokine for overexpression studies. Thus, next we cloned the full-length CX3CL1 gene into a retroviral vector for overexpression. As depicted in **figure 6.10 A**, the pGEM-T-CX3CL1 vector and the retroviral pMX-neo backbone were digested with BamHI and XhoI and the CX3CL1-ORF was ligated into pMX-neo. The resulting pMX-neo-CX3CL1 or the empty pMX-neo vector were subsequently used for super-infection of the three previously introduced melanoma cell lines (**figure 6.4**) that were already transduced with pBabe-puro-eGFP-2A-CBGr99.

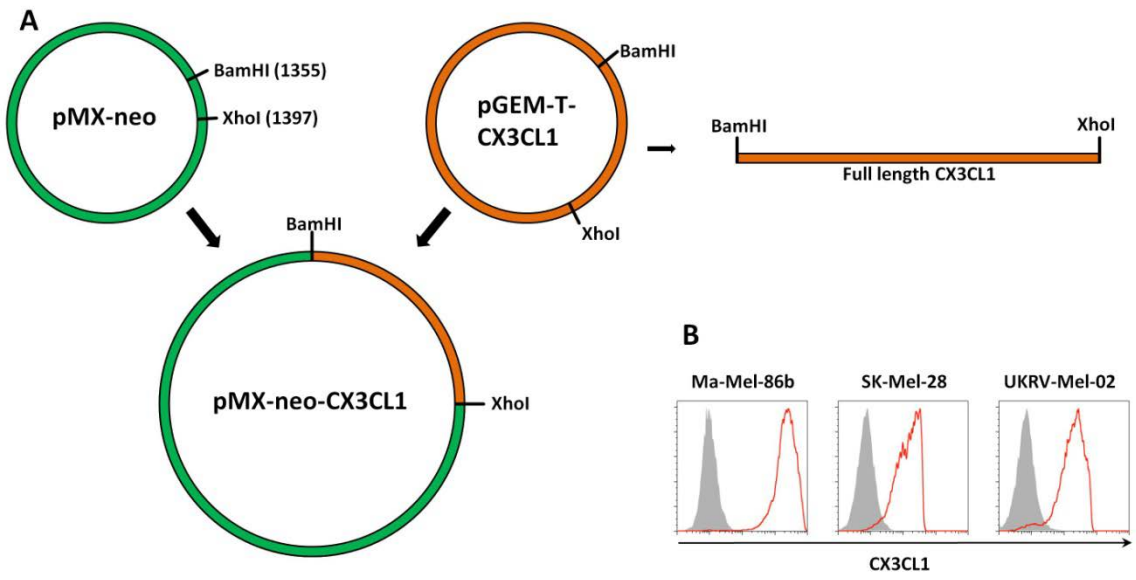


Figure 6.10 - Cloning strategy for pMX-neo-CX3CL1. A The full length ORF for human CX3CL1 was amplified via PCR using the pDONR construct provided by the core facility for genomics at the DKFZ as a template introducing BamHI and XhoI restriction sites 5` and 3' respectively. The PCR product got ligated into the pGEM-T vector according to the manufacturer's protocol. The destination backbone pMX-neo got cut with BamHI and XhoI as was the pGEM-T-CX3CL1 vector and the CX3CL1 gene was ligated into the backbone. **B** Transduced and G418-selected cell lines (all of which were transduced with pBabe-puro-eGFP-2A-CBGr99 previously) were stained for CX3CL1. The grey signal represents the staining of the vector control cells and the red line represents the staining of the pMX-neo-CX3CL1 cells. One representative out of two experiments is shown.

As shown in **figure 6.10 B**, all three tumor cell lines showed a high and uniform expression of CX3CL1 without sorting as determined by FACS staining for the membrane-bound chemokine.

6.2.5 Characterization of CX3CL1-overexpressing cell lines

To determine if the transductants as created under 6.2.4 are also capable of shedding CX3CL1 from the cell surface, thus producing the chemotactic form of the protein, supernatant of vector control cells or the CX3CL1-overexpressing lines was harvested. The CX3CL1-concentrations were measured with an ELISA. As shown in **figure 6.11 A** for two tumor lines, melanoma cells were able to shed CX3CL1. We next adjusted the supernatant to the determined ideal dose of CX3CL1 for NK cell attraction of 1 ng/ml (**figure 6.6 D**) and used the diluted supernatant for a transwell-migration assay to investigate if the expressed chemokine was biologically active. As depicted in **figure 6.11 B**, the adjusted supernatant exerted a potent *in vitro* chemoattraction with high specificity for the $CD56^{dim}$ $CD16^{+}$ NK cell subset.

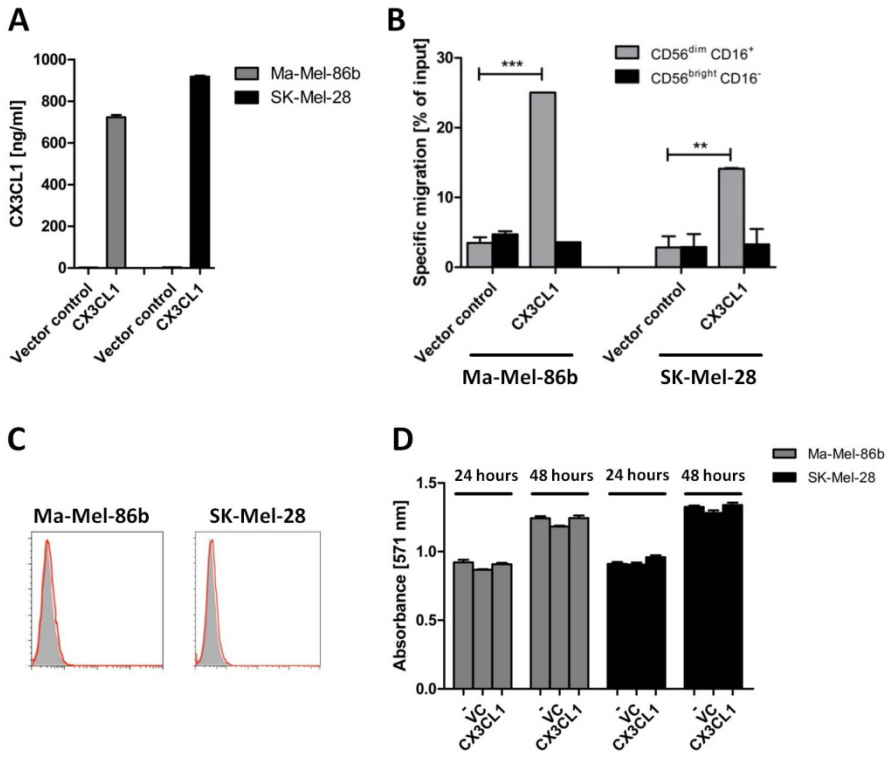


Figure 6.11 – CX3CL1-expressing melanoma cells shed CX3CL1 which potently attracts NK cells while exhibiting similar growth behavior *in vitro* compared to vector control cells. A $1*10^6$ cells of the indicated cell lines were seeded in RPMI cell culture medium. 24 hours later the medium was removed and replaced by migration medium for further 24 hours. A CX3CL1-ELISA was performed according to the manufacturer's instructions using cell-free supernatant. Plotted is the mean ± SD of the concentration of soluble CX3CL1 (n=2). **B** Supernatants created as described were adjusted to the ideal concentration of CX3CL1 of 1 ng/ml with migration medium and used in transwell migration assays. Plotted is the mean ± SD of the specific migration of the indicated NK cell subsets (n=2). **C** Staining of Ma-Mel-86b and SK-Mel-28 for CX3CR1 or with the respective isotype control antibody. One of two experiments is shown. **D** Parental Ma-Mel-86b and SK-Mel-28 or the indicated transductants were seeded at 1.000 cells/well in a 96-well plate. AlamarBlue™ reagent was added and the absorbance at 571 nm was detected after 24 and 48 hours. Shown is the mean ± SD (n=5). ** $p < 0.01$, *** $p < 0.001$ (student's t test).

As discussed previously, various chemokines have been investigated for their capability to induce growth or migration of tumor cells[93,107,349]. In order to exclude similar effects in our system, we stained all three lines for the receptor of CX3CL1, CX3CR1, and did not detect expression (**figure 6.11 C** and data not shown). Accordingly, an effect on tumor growth could be observed with neither Ma-Mel-86b nor SK-Mel-28 (**figure 6.11 D**) nor with UKRV-Mel-02 (data not shown). Of note, upon

intravenous injection of equal numbers of vector control- or CX3CL1-transduced cells the BLI signals from time to time were different for the two transductants (data not shown).

6.2.6 *In vitro killing of CX3CL1-overexpressing cell lines*

As shown in **figure 6.9 C and D**, exogenously added soluble CX3CL1 did not elevate NK mediated killing of tumor targets. However, it also has been reported that cell lines expressing membrane-bound CX3CL1 were killed significantly better as compared to their CX3CL1-negative controls and in addition induced higher levels of IFN-γ in NK cells[350,351]. This was due to an increased adhesion of CX3CR1-positive NK cells to CX3CL1-bearing targets. We thus set up ^{51}Cr release assays to test if our transductants expressing membrane-bound CX3CL1 were inducing higher cytotoxicity by IL-2 activated NK cells. However, of three cell lines we found only Ma-Mel-86b to be stronger killed upon CX3CL1-overexpression (**figure 6.12**). Of note, Ma-Mel-86b cells seemed to exhibit the highest expression of CX3CL1 of the three cell lines (**figure 6.10 B**).

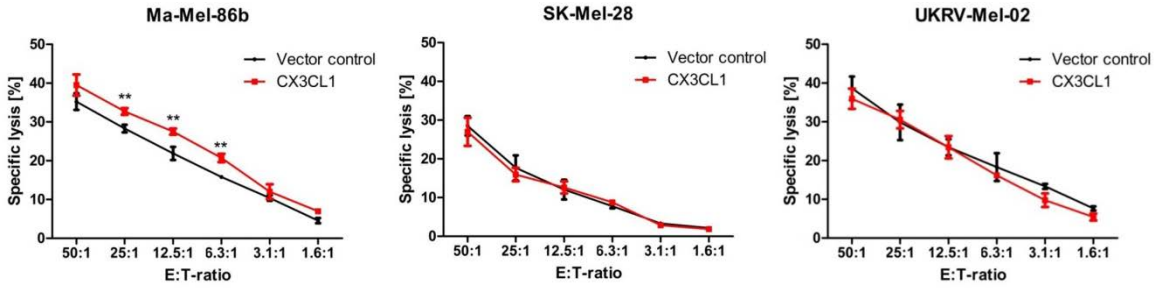


Figure 6.12 – CX3CL1-expression does not increase killing of melanoma cell lines. Human purified NK cells have been activated with IL-2 for 16 hours and were used at the indicated E:T ratios against vector control or CX3CL1-transduced melanoma cells in ^{51}Cr release assays. Plotted is the mean ± SD of the specific lysis (n = 3). One representative of three donors is shown. ** $p < 0.01$.

6.2.7 *In vivo homing of intravenously injected NK cells to subcutaneous tumors*

In a next step, we investigated if the overexpression of CX3CL1 in melanoma cells could mediate the migration of adoptively transferred NK cells *in vivo* into tumor tissue. As previously described for various malignancies, the level of CX3CL1-expression showed a correlation with the degree of NK cell infiltration in the tumors of cancer patients[110,346]. As it was shown in a murine model of lymphoma that the growth of CX3CL1-overexpressing cells was controlled better as compared to control cells, we aimed at showing a) a higher accumulation of adoptively transferred NK cells in CX3CL1-overexpressing tumors and b) a more pronounced control of tumor growth *in vivo* in a xenograft model upon CX3CL1-expression[112]. The results of one of the approaches are depicted in **figure 6.13**. Upon injection of a tumor/matrigel-mixture, the tumor cells were allowed to settle and to engraft for 7 days in order to establish a tumor. After irradiation, NK cells were injected i.v. and supplemented *in vivo* with daily i.p. injections of IL-2 until the end of the experiment. We irradiated the mice as a) we have seen previously that irradiation is an important pre-conditioning regimen that led to substantial colonization of NSG mice with adoptively transferred NK cells (**figure 6.2 C**) and b) several reports indicate that γ-irradiation activates endothelial cells in terms of up-regulated levels of

ICAM-1, VCAM-1 and E-selectin thus supporting the adhesion and transmigration of lymphocytes[352–354].

When we analyzed serum and tumor samples of the respective animals we found that serum samples only displayed slightly elevated levels of CX3CL1 in animals bearing CX3CL1-positive tumors. The tumor biopsies however showed a substantial enrichment of CX3CL1 compared to controls (**figure 6.13 A**). As chemokine receptors are known to be internalized upon engagement of their respective ligands, blood cells were analyzed via flow cytometry for CX3CR1-levels to see if circulating NK cells still expressed this receptor[71]. As depicted in **figure 6.13 B**, NK cells from animals that were transplanted with the CX3CL1-overexpressing tumor cells surprisingly – despite only slightly elevated serum levels of soluble CX3CL1 – almost entirely lost CX3CR1-expression. As NK cells from vector control injected animals still expressed high levels of CX3CR1 (**figure 6.13 B**), high dose IL-2 *in vivo* compared to *in vitro*, if at all, only moderately decreased CX3CR1 levels.

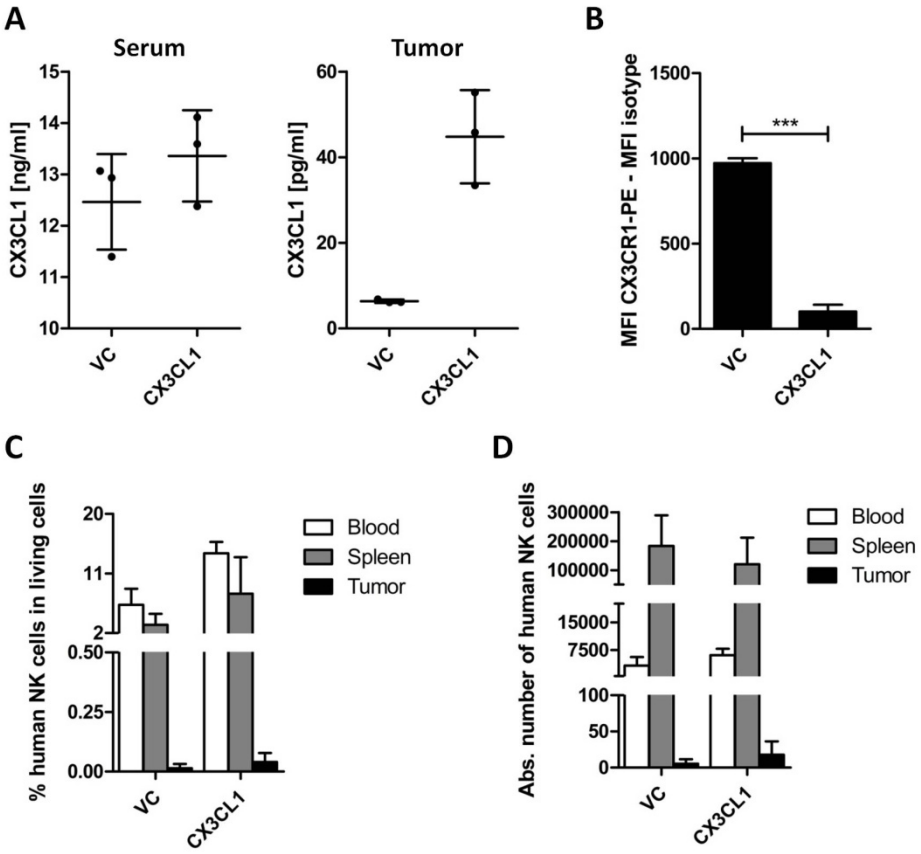


Figure 6.13 – NK cells do not infiltrate into subcutaneous tumors regardless of CX3CL1-expression. 3 mice per group were injected subcutaneously with a 1:1 mixture of $3.3*10^6$ SK-Mel-28 melanoma cells (vector control and CX3CL1) with matrigel. 7 days later all mice received $2*10^6$ NK cells (activated o.n. with 20 IU/ml IL-2) via intravenous injection 4 hours after total body irradiation (3.5 Gy) and 20.000 IU IL-2 i.p. per day until their sacrifice after 2 days. **A** Serum (from 100 µl blood) or tumor lysates (from pieces of 45 mg) were prepared and tested for CX3CL1 levels with a CX3CL1-ELISA according to the manufacturer's instructions. Shown is the mean ± SD of the concentration of CX3CL1 (n=2). **B** Blood cells were either stained for CX3CR1 or with the respective isotype control. Gates were set on human $CD45^+$, $CD3^-$ and $7AAD^-$ cells. Shown is the mean ± SD of the MFI of CX3CR1 corrected for the isotype expression level (n=3). **C and D** Blood (200 µl) was heparinized and spleens and tumors were prepared as a single cell suspension counted and stained for various markers. Shown is the mean ± SD of the percentage of $7AAD^-$ $CD3^-$ $CD45^+$ cells of total living cells (n=3) (**C**) and the absolute cell number of cells with the same marker combination (n=3) (D). *** $p < 0.001$ (student's t-test).

We next stained spleen, tumor and blood cells for human NK cell markers to determine their fraction amongst living cells via flow cytometry. With these percentages, in addition the absolute numbers of NK cells in the investigated organs were calculated. As shown in **figure 6.13 C and D**, neither in terms of percentages nor in terms of numbers a difference could be found in blood, spleen and the tumors. Most importantly, the tumors – vector control (VC)- and CX3CL1-transduced - were displaying a virtual absence of NK cells. We therefore next tested a different route of administering NK cells.

6.2.8 In vivo homing of perilesionally injected NK cells to subcutaneous tumors

As the intravenous injection of NK cells in several experiments did not allow for NK cell infiltration into subcutaneous tumors (**figure 6.13** and data not shown), we performed an experiment in which the NK cells were administered subcutaneously approximately 1 cm away from the tumor injection site **(figure 6.14**). This setup was chosen as incompatibility between murine (endothelial and interstitial cells) and human (NK cells) adhesion molecules might be one explanation for the absence of NK cells in the tumors in the previous experiments. Further, some publications indicate that perilesional effector cell injections in xenograft experiments can lead to tumor control[355,356].

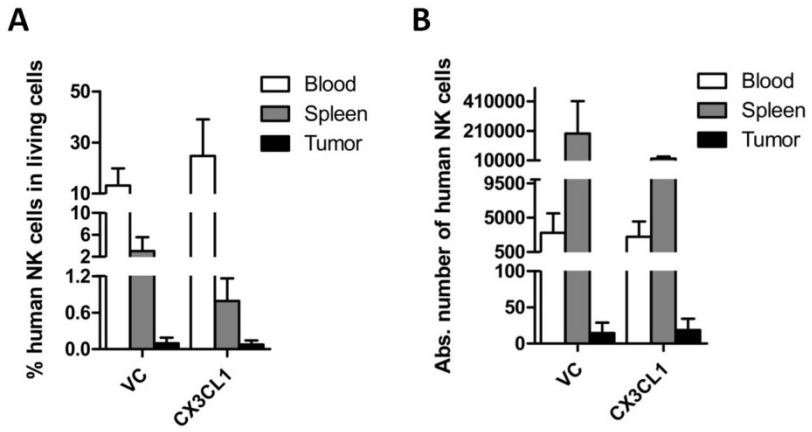


Figure 6.14 – NK cells do not migrate into solid subcutaneous tumors upon perilesional injection. 4 mice per group were subcutaneously injected with a 1:1 mixture of matrigel and $3*10^6$ Ma-Mel-86b melanoma cells (vector control or CX3CL1-overexpressing). 4 days after the injection the mice received a single subcutaneous perilesional injection (ca. 1 cm distance to the tumor) of $1.5*10^6$ naïve NK cells 4 hours after irradiation with 3.5 Gy. The next three days the animals received 20.000 IU IL-2 i.p. and were sacrificed 6 days after the NK cell injection. **A and B** 400 µl of heparinized blood or single cell suspensions of spleens and tumors were counted and stained for various human antigens. Human NK cells were defined as $CD3^-$, $7AAD^-$, $CD45^+$ cells. Shown is the mean ± SD of the percentage of human NK cells in living cells (**A**, n=4) and the absolute cell number of human NK cells in blood, spleens and tumors (**B**, n=4). VC, vector control.

However, as shown in **figure 6.14**, this setup did not allow for the detection of intra-tumoral human NK cells. Like in **figure 6.13**, the adoptively transferred NK cells were detected in blood and spleen. Notably, the tumors were excised carefully and including the close surrounding tissue – the stroma – as it was shown that infiltrating cells, although being attracted to the tumor often reside in proximity to but not in the tumor tissue[357].

6.2.9 Tumor control in vivo mediated by IL-2 activated NK cells

As adoptively transferred human NK cells were not detected in subcutaneously growing tumors, we did not detect an anti-tumor effect of the transferred NK cells in subcutaneously growing tumors *in vivo* (**figures 6.13, 6.14** and data not shown). We thus changed our setup and in a next step and injected the tumor cells intravenously. As compared to the subcutaneous injection of a high number of tumor cells, the intravenous injection of few tumor cells artificially mimics metastatic disease in which single, disseminated tumor cells are transported in the blood or lymph stream to the site of metastasis. Of note, this model is more likely to monitor NK cell mediated effects as NK cells are thought to contribute more to the control of small tumors, hematological malignancies and metastasizing cells as compared to large solid tumors[358,359].

In order to set up the experiment, we used the pBabe-puro-eGFP-2A-CBGr99 transductants that were generated earlier in the project (**figure 6.1, 6.2 and 6.5**) and injected them i.v. into irradiated NSG mice. In a first step, IL-2-activated NK cells and a constant dose of luciferase-positive CX3CL1-negative Ma-Mel-86b were injected at different E:T ratios to find a ratio at which vector control cells are controlled by the co-injected NK cells but still leave a substantial tumor load (**figure 6.15**).

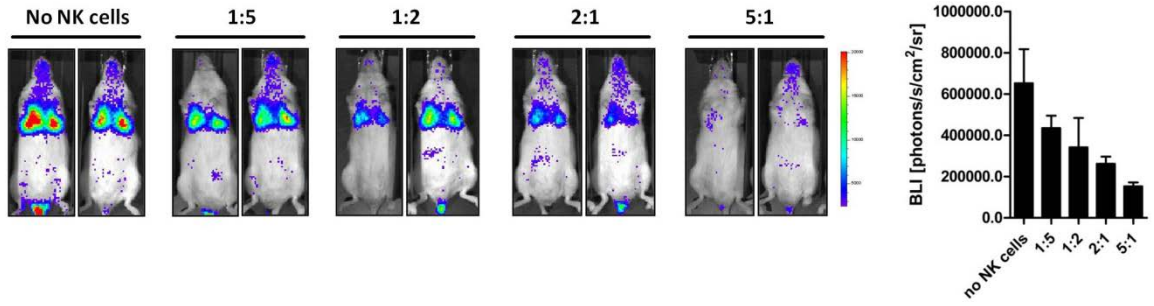


Figure 6.15 – IL-2-activated NK cells upon co-injection with tumor cells decrease the tumor-load in a dose-dependent manner. 4 hours after total body irradiation with 3.5 Gy $7*10^5$ Ma-Mel-86b cells transduced with pBabe-puro-eGFP-2A-CBGr99 and pMX-neo were injected i.v. together with IL-2-activated NK cells (2 days activated with 200 IU/ml IL-2) at the indicated E:T ratios. The signals were quantified and plotted as mean ± SD (n=2).

As depicted, with increasing E:T ratios the tumor engraftment in the lungs as detected by BLI subsequently decreased (**figure 6.15**). Based on this experiment we decided to inject NK cells at a 1:2 E:T ratio in the following experiments.

6.2.10 Impact of CX3CL1-expression on NK cell-mediated tumor control

In order to investigate if CX3CL1-expression in a metastatic disease model can increase the efficacy of adoptively transferred NK cells, identical numbers of either vector control- or CX3CL1-transduced Ma-Mel-86b cells were injected together with IL-2-activated NK cells at an E:T ratio of 1:2 i.v. into irradiated NSG mice.

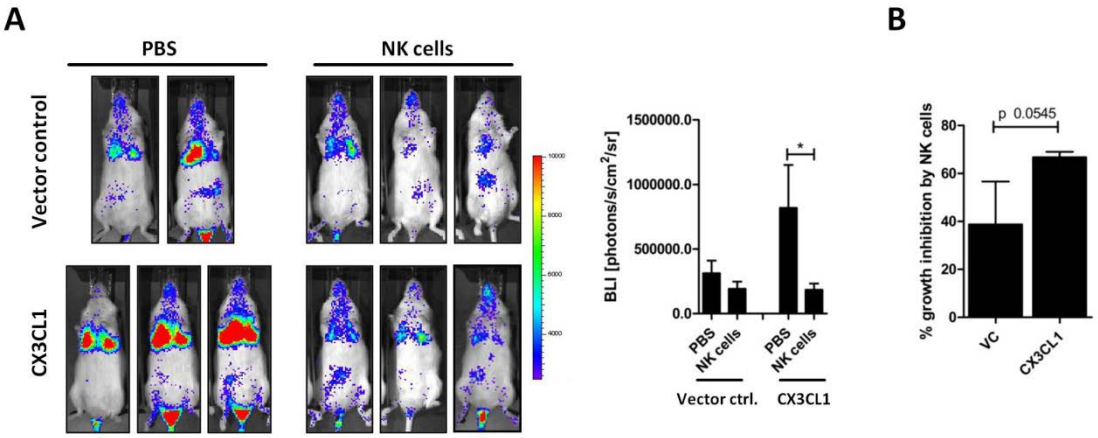


Figure 6.16 – CX3CL1-expression *in vivo* enhances NK-mediated tumor control. 4 hours after 3.5 Gy WBI 7*10^5 Ma-Mel-86b vector control- or CX3CL1-transduced Ma-Mel-86b were injected i.v. into NSG mice. NK cells that were activated with 200 IU/ml IL-2 for two days were co-injected at a 1:2 E:T ratio. **A** Shown is the BLI after 8 days of the animals (left panel) and the mean ± SD of the signal (n=2-3, right panel). **B** Shown is the mean ± SD of the % growth inhibition by NK cells as compared to PBS control animals (n=2-3). ctrl., control; VC, vector control, * $p < 0.05$ (student's t-test).

As depicted in **figure 6.16 A**, both target lines engrafted less efficiently when NK cells were co-injected. As displayed, the Ma-Mel-86b cell line showed varying BLIs for the vector control and the CX3CL1-expressing line, although they were injected at similar numbers. Yet, a quantification of the absolute values of the BLI revealed that only the CX3CL1-expressing cell line was killed to a significant degree by the NK cells (**figure 6.16 A**). Thus, we plotted the % of the BLI decrease under the influence of NK cells relative to the PBS control animals (**figure 6.16 B)**. As shown, although not significant, a strikingly lower tumor burden was observed, when the tumor cells were expressing CX3CL1.

We also performed this experiment with SK-Mel-28 melanoma cells. Of note, this cell line did not exhibit more pronounced killing *in vitro* upon CX3CL1-overexpression (**figure 6.12**). We co-injected the same ratio of IL-2-activated NK cells as previously for the Ma-Mel-86b cells and acquired the bioluminescent signals after 4 days (**figure 6.17 A**). As shown, IL-2-activated NK cells exerted a significant decrease of the tumor load as detected by BLI regardless of CX3CL1-expression. Of note, CX3CL1-expressing tumor cells did not show a more pronounced growth inhibition by NK cells compared to vector control cells when compared to PBS treated animals (**figure 6.17 B**). In order to investigate if CX3CL1 was able to recruit more NK cells into the respective lung tissue, we sacrificed the animals on day 4 after the injection and prepared single cell suspensions out of spleens and lungs which we stained for human NK cells. As shown in **figure 6.17 C,** we observed less NK cells in blood samples and in spleens of animals that were injected with CX3CL1-overexpressing SK-Mel-28 compared to vector control cells. Importantly, in the lungs of three out of 4 animals that have been injected with $CX3CL1^{high}$ SK-Mel-28 we detected almost twice as many NK cells when compared to vector control cells. Exemplary dot plots are shown in **figure 6.17 D**. In addition, we were staining blood NK cells for CX3CR1 to see if circulating cells in this model down-regulated CX3CR1 similarly as observed for the subcutaneous model (**figure 6.13**). Of note, NK cells of all mice still expressed CX3CR1 at high levels, thus enabling them to home towards CX3CL1-expressing cells (**figure 6.17 E**).

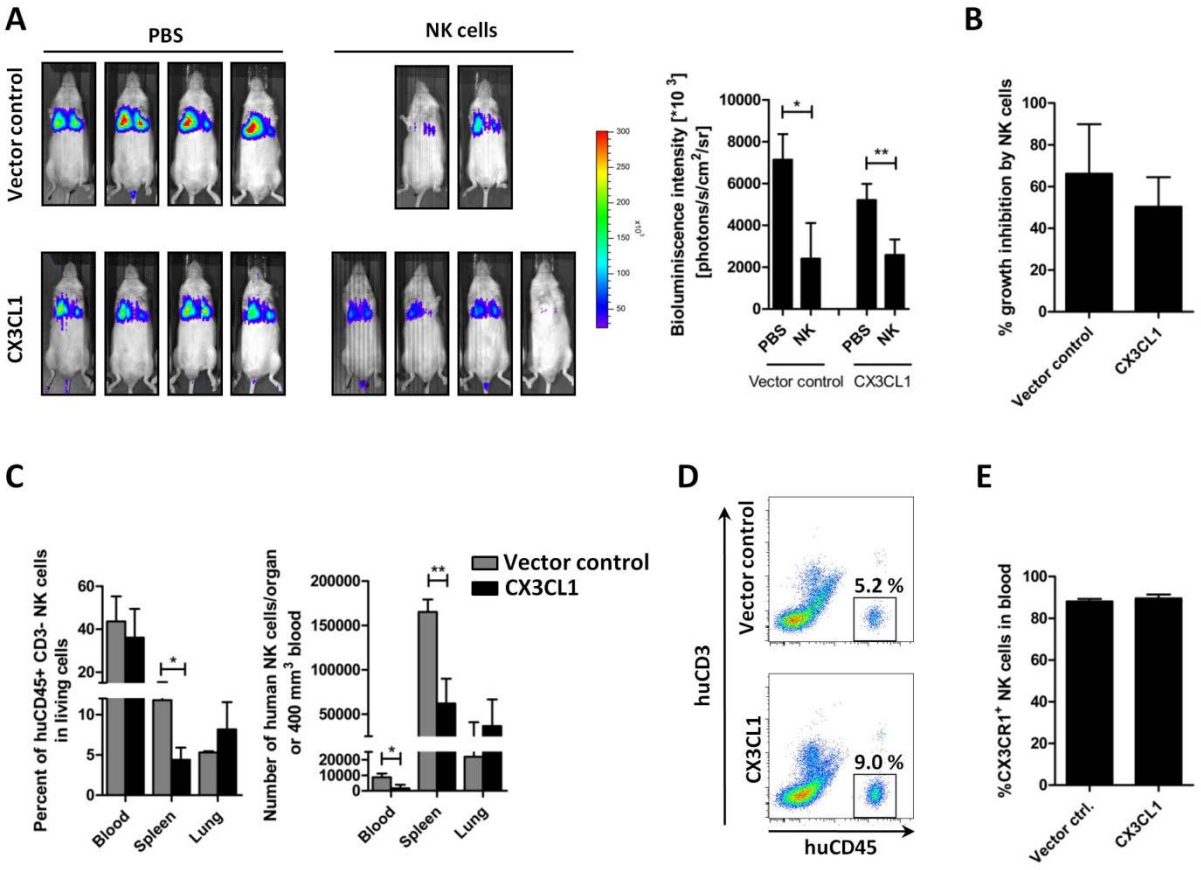


Figure 6.17 – CX3CL1-expression increases NK cell infiltration into tumor-harboring lungs. NSG mice were irradiated with 3.5 Gy 4 hours prior to the i.v. injection of SK-Mel-28 vector control- or CX3CL1-transductants. NK cells that have been activated with 200 IU/ml IL-2 for two days were co-injected at a 1:2 E:T ratio. **A** Shown is the BLI 4 days after injection (left panel) and the mean ± SD of the signal (n=2-4, right panel). **B** Shown is the mean ± SD (n = 2-4) of the percentage of human NK cells amongst living cells and the absolute cell numbers of human NK cells (defined as 7-AAD$^-$, CD45$^+$, CD3$^-$). **C** Representative dot plots showing the elevated levels of NK cells in single cell suspensions obtained from lungs. **D** Percentage of CX3CR1-positive NK cells amongst blood cells. Shown is the mean ± SD (n = 2-4). * $p < 0.05$, ** $p < 0.01$.

6.3 Evaluation of IL-12/15/18-activated NK cells for anti-tumor immunotherapy

6.3.1 In vitro characterization of IL-12/15/18-stimulated NK cells

In 2009, Cooper *et al.* published a report showing that NK cells as part of the innate immune system memorized their history of stimulation, enabling them to react stronger upon secondary challenge[284]. This study was in line with an earlier report from O'Leary *et al.*, who were demonstrating in a model of contact hypersensitivity response that NK cells were able to show elevated secondary or memory-like immune responses against the same antigen that was used to 'immunize' them[288]. However, the mode of activation of the NK cells remained unclear, the memory-like function was only assigned to hepatic NK cells and most importantly it remained elusive if memory formation was NK cell intrinsic. Compared to this study the finding by Cooper and colleagues seemed to be more relevant for tumor therapy as splenic NK cells upon a short pulse with IL-12/15/18 were displaying elevated reactivity not only against the antigen used for initial priming but against various stimuli such as plate-bound α-NK1.1, α-Ly49H or cytokine re-stimulation with IL-12 and IL-15[284].

As to this point no data on the effects of a mixture of IL-12, IL-15 and IL-18 on human NK cells have been reported, we set up experiments like schematically displayed in **figure 6.18 A**.

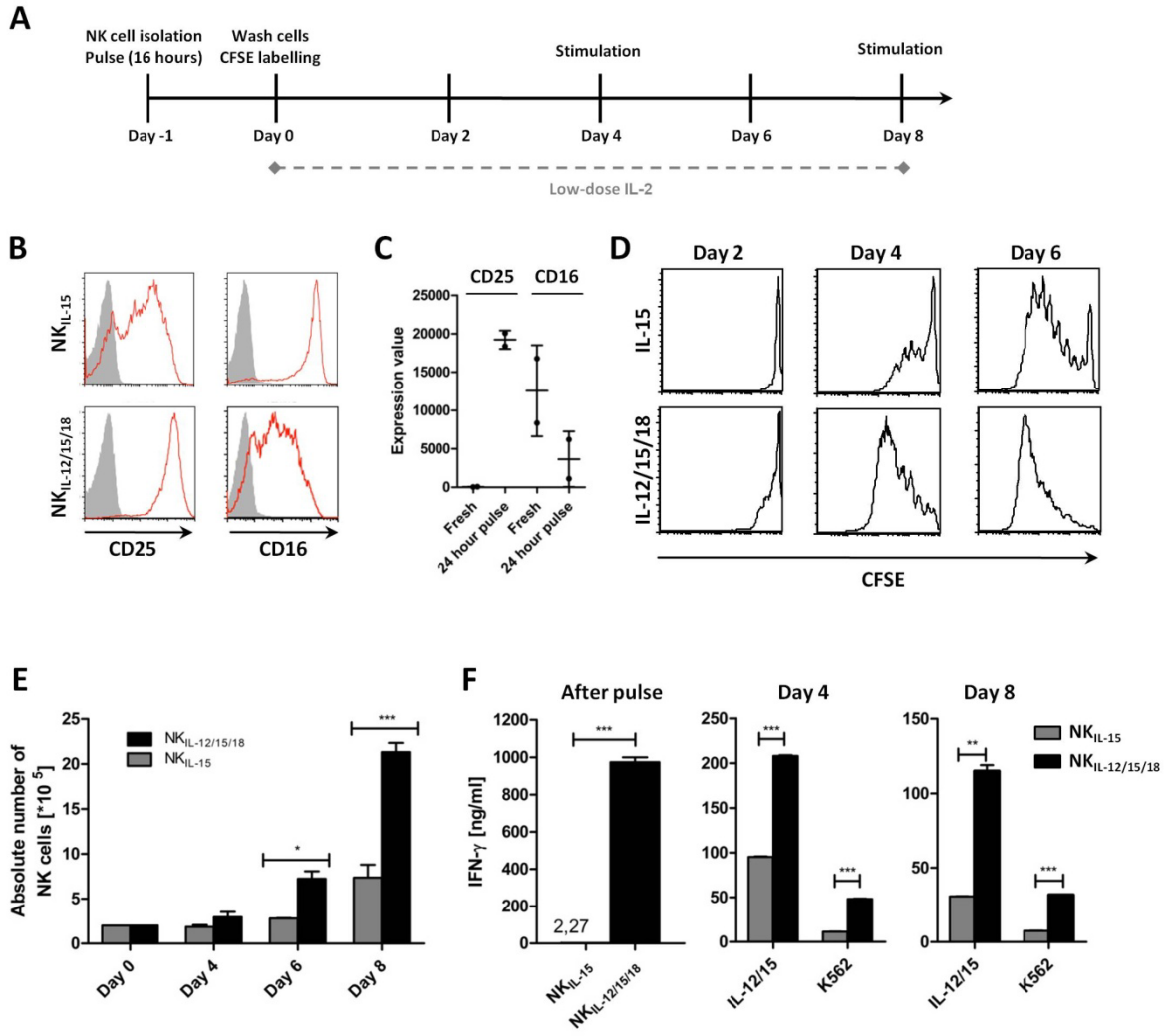


Figure 6.18 – IL-12/15/18-stimulated NK cells show higher IL-2 dependent proliferation *in vitro*, maintaining a higher responsiveness towards various stimuli. A Schematic representation of the experimental setup. **B** Staining of IL-15- and IL-12/15/18-stimulated NK cells for CD25 (left panel) and CD16 (right panel). Grey, filled signals represent the isotype stainings, red lines the respective specific staining. Data are representative of at least 8 donors. **C** mRNA expression levels of CD25 and CD16. Data were extracted from the GEO dataset GSE22919[360]. Plotted is the mean ± SD of relative expression values (n=2). **D** CFSE dilution of IL-15- or IL-12/15/18-stimulated NK cells at various timepoints during culture with 100 IU/ml of IL-2. Data are representative of 4 donors. **E** Plotted is the mean ± SD of the absolute cell number/well at various timepoints during culture. Data are representative of 6 donors. **F** Quantification of IFN-γ either directly after the pulse or after re-stimulation with either 10 ng/ml IL-12 and 50 ng/ml of IL-15 or K562 at a 1:1 E:T ratio after 4 and 8 days in culture. Plotted is the mean ± SD of the IFN-γ concentration as detected by ELISA (n=2). Data are representative of 4 donors. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (student's t-test).

Based on experiments performed by Jing Ni from our group in a C57Bl/6 model we prolonged the pulse from 13-15 hours (Cooper protocol) to up to 16 hours and analyzed the surface expression of various receptors. Of note, the IL-2 receptor α-chain (CD25) got highly up-regulated whereas the FcγRIII CD16 was substantially down-regulated on protein level after 16 hours on IL-12/15/18- compared to IL-15-stimulated NK cells (**figure 6.18 B**). In addition, we analyzed the expression levels in a publically available dataset in which Smith *et al.* performed gene-array analysis to detect differences between naïve NK cells and NK cells after a 24 hour pulse with IL-12/2/18 (GEO data set, accession number GSE22919)[360]. Of note, according to their activation protocol they have used 100 IU/ml IL-2 instead of 20 ng/ml IL-15 and the pulse lasted for 24 instead of 16 hours in our protocols. Still, a strong induction of CD25 and a decrease in CD16 transcript levels could be detected when comparing naïve with cytokine-pulsed NK cells (**figure 6.18 C**), thus confirming our findings.

CD25 represents the α-chain of the IL-2 receptor and upon expression increases the sensitivity of the low affinity IL-2 receptor consisting of CD122 and CD132 to IL-2 by decreasing the K_d of the receptor complex and IL-2[361]. Therefore, we asked if the elevated level of CD25 impacts IL-2 driven NK cell proliferation. In a next step, we thus labeled NK cells after the pulse with CFSE and cultured them in medium containing 100 IU/ml of IL-2. As shown in **figure 6.18 D and E** $CD25^{high}$ IL-12/15/18-stimulated NK cells diluted CFSE more quickly during culture and hence number wise outgrew the IL-15-stimulated control cells. Most importantly, the IL-12/15/18-stimulated NK cells maintained an imprint of the cytokine stimulation and reacted stronger upon stimulation with a combination of IL-12 and IL-15 or K562 cells in terms of IFN-γ production 4 and 8 days after the pulse (**figure 6.18 F**). These data indicate that human IL-12/15/18-stimulated NK cells react with substantial proliferation towards IL-2 while maintaining a more pronounced reactivity in terms of IFN-γ production[287]. Of note, the TNF-α levels were also strongly increased after the pulse (data not shown).

6.3.2 In vivo characterization of IL-12/15/18-stimulated NK cells

In order to test if the effects that we have seen *in vitro* (**figure 6.18**) also relate to an *in vivo* situation, we designed an adoptive transfer experiment which is schematically outlined in **figure 6.19 A**. As depicted, after the stimulation the NK cells were labeled with CFSE prior to injection to later on measure for proliferation in various organs. In order to provide the transplanted NK cells with a survival factor and proliferation stimulus, we injected the mice on a daily basis i.p with recombinant IL-2 or PBS as a control. Importantly, also *in vivo* IL-12/15/18-stimulated NK cells upon IL-2 supplementation proliferated more pronouncedly (**figure 6.19 B**). This could only be seen when IL-2

was injected. In PBS treated animals, no proliferation could be detected after 6 days as detected by CFSE dilution (data not shown).

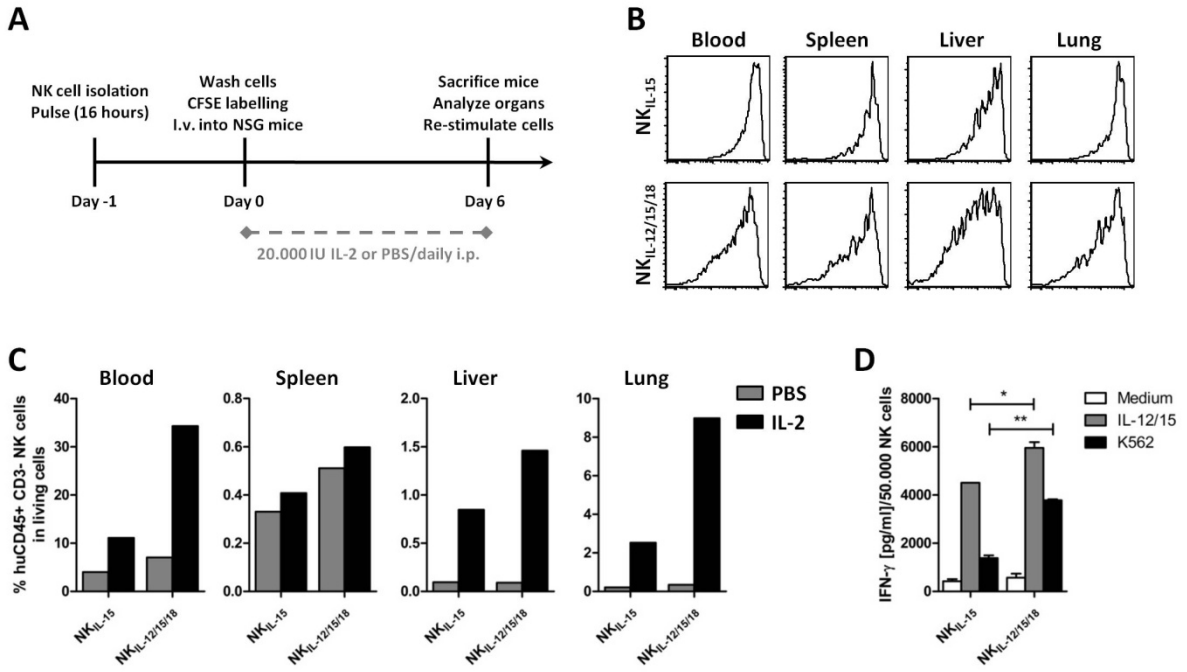


Figure 6.19 – IL-12/15/18-stimulated NK cells proliferate faster *in vivo* showing an elevated IFN-γ response upon *ex vivo* re-stimulation. **A** Schematic representation of the experimental setup. **B** CFSE dilution of IL-15 or IL-12/15/18-stimulated NK cells ($CD45^+$, $CD3^-$, $7AAD^-$) in various organs 6 days after the injection. Data are representative of 3 donors. **C** Detection of living human NK cells ($CD45^+$, $CD3^-$, $7AAD^-$) 6 days after injection in indicated organs. Plotted is one value for PBS and IL-2 injected mice each. Data are representative of 3 donors. **D** *Ex vivo* restimulation of blood cells with either medium, IL-12/15 or K562 cells. Plotted is the mean ± SD of the IFN-γ concentration extra-polated for 50.000 NK cells (n=2). * $p < 0.05$, ** $p < 0.01$ (student's t-test).

As the differences in CFSE dilution not necessarily have to translate into higher cell numbers *in vivo* for instance due to different apoptosis rates, we also determined the percentages of human NK cells in single cell suspensions obtained from the indicated organs (**figure 6.19 C**). Importantly, for all samples analyzed, the percentages of human NK cells were higher in animals that have received IL-12/15/18- compared to IL-15-stimulated NK cells. However, the survival and the proliferation were critically dependent on IL-2. Upon substitution of the IL-2 with PBS only few NK cells were recovered that did not show substantial CFSE dilution (**figure 6.19 C** and data not shown).

One of the striking features of the IL-12/15/18-stimulated NK cells was their elevated response compared to control cells upon re-challenge with either IL-12 and IL-15 or with K562 cells several days after the cytokine pulse (**figure 6.18 F**). To see if also after the transient injection into the bloodstream of NSG mice the cells maintained this elevated responsiveness, we used whole blood cells 6 days after the injection of recipient mice and challenged them with the same stimuli as compared to the *in vitro* data. Strikingly, as shown in **figure 6.19 D,** also after being xenotransplanted into animals and persisting in the circulation for 6 days the elevated IFN-γ response of IL-12/15/18-activated NK cells over IL-15-stimulated NK cells was maintained.

6.3.3 Cytotoxicity of IL-15- and IL-12/15/18-stimulated NK cells

Up to this point, the responses of IL-12/15/18- compared to IL-15-activated NK cells were investigated in the context of cytokine production. In a next step, we performed various ^{51}Cr release assays to determine if the stimulation with Il-12/15/18 also directly impacts cytotoxicity towards a panel of various tumor targets. Of note, in the panel presented in **figure 6.20**, Ma-Mel-86b is the only line lacking MHC class I-expression whereas the other lines express MHC class I at various levels (data not shown).

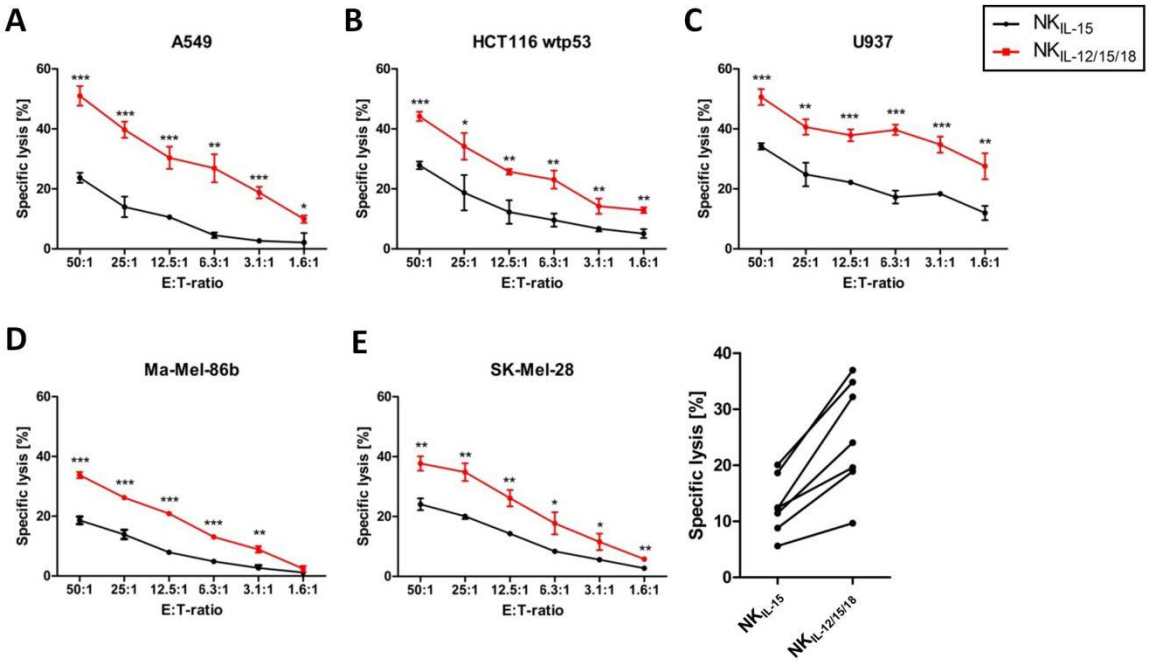


Figure 6.20 – IL-12/15/18-stimulated NK cells show significantly higher killing of various target cell lines *in vitro*. IL-15- and IL-12/15/18-stimulated NK cells were tested in standard 4 hour ^{51}Cr release assays against various MHC I positive and negative cell lines. **A** A549 lung adenocarcinoma **B** HCT116 colon carcinoma **C** U937 histiocytic lymphoma **D** Ma-Mel-86b melanoma **E** SK-Mel-28 melanoma, a representative kill plot is displayed on the left panel and pooled data for 7 donors on the right panel. Plotted is the mean ± SD (n=3) of the specific lysis [%]. Data are representative of at least 2 donors. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

As depicted in **figure 6.20 A-E** for every cell line tested, short term IL-12/15/18-stimulated NK cells at almost every E:T ratio exhibited a significantly stronger lysis as compared to NK cells that have only been activated with IL-15. Some reports pointed out already that IL-18 and IL-12 can elicit more potent cytotoxic responses of either murine or human NK cells or are important to generate fully functional NK cells[142,145,146,362]. However, the used concentrations were different and none of the reports has investigated cytotoxicity of human NK cells in a setting in which the cytokines created a memory-like phenotype.

6.3.4 Impact of IL-12/15/18-stimulation on KIR-expression on NK cells

As shown in **figure 6.20**, we observed increased killing of virtually any tumor target in ^{51}Cr release assays by IL-12/15/18- compared to IL-15-stimulated NK cells. We therefore asked, if this is due to an increased expression of activating or due to a decreased expression of inhibitory receptors or

molecules. Of note, two studies reported that signaling via IL-12 and IL-18 did not impact the expression levels of KIR molecules or slightly increase them[363,364]. However, the Chrul *et al.* data were generated using IL-2 instead of IL-15 and the data from Romee *et al.* using different concentrations of IL-15 and IL-18 as compared to our setup. Also the latter group was using NK cells that have been rested in IL-2 for 7 days. Thus, we analyzed cultures that received the cytokine pulse for either 16 or a prolonged period of 60 hours and stained these cultures together with naïve cells for flow cytometric analysis for KIR2DL1/S1, KIR2DL2/L3 or KIR3DL1 specific antibodies, KIRs that are present on virtually all caucasoid individuals[365]. As shown in **figure 6.21 A and B**, already after 16 hours a small but reproducible down-regulation of KIR2DL2/L3 was detectable that got more pronounced after 60 hours.

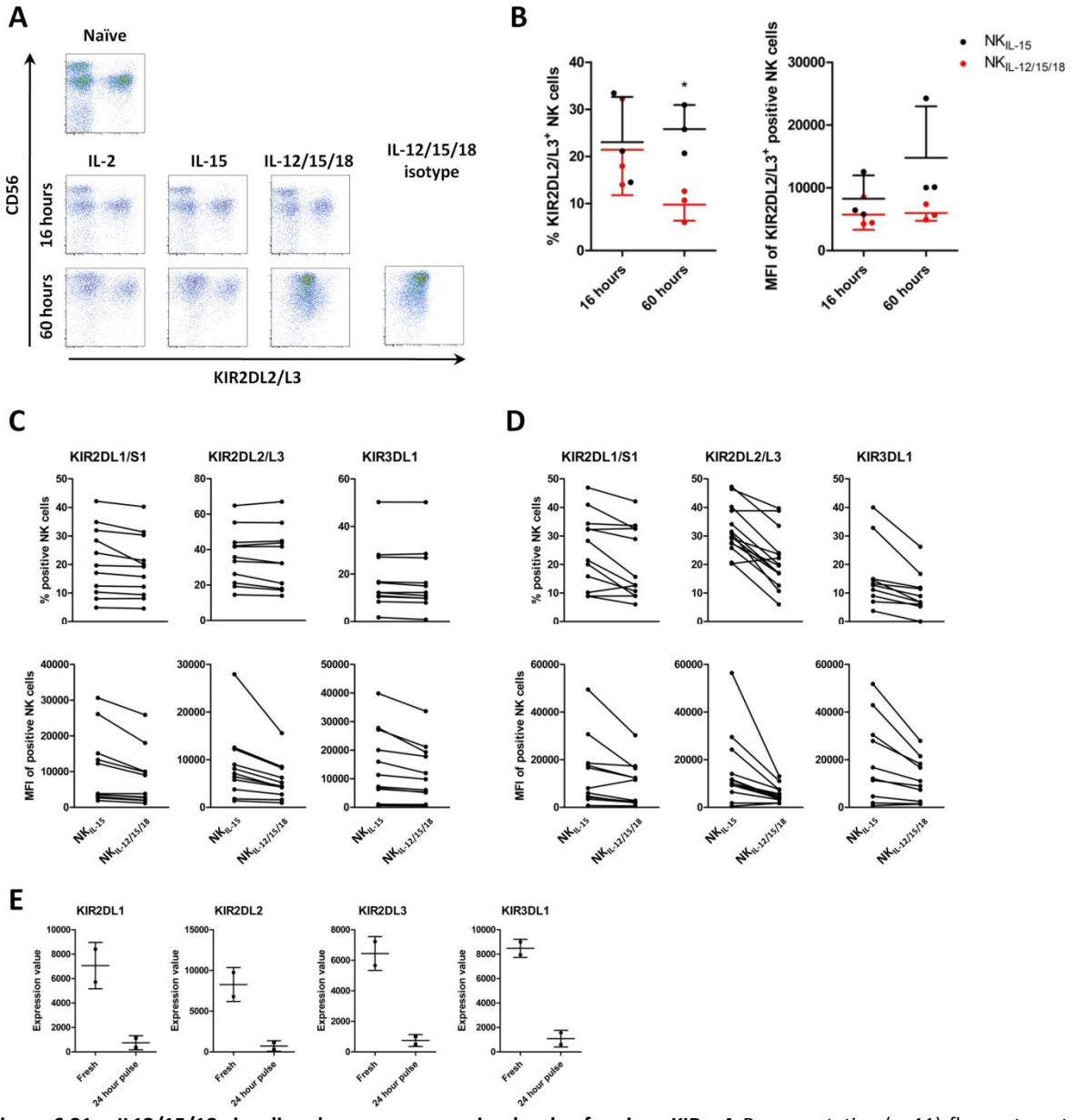


Figure 6.21 – IL12/15/18 signaling decreases expression levels of various KIRs. A Representative (n=11) flow cytometric staining of one donor for CD56 and KIR2DL2/L3 after indicated times of stimulation. **B** Cumulative data for three donors of one experiment. Shown is the mean ± SD of the percentage of KIR2DL2/L3-positive cells (left part) and the MFI of KIR2DL2/L3 of the positive population. One dot represents one donor. **C** Staining of indicated KIRs on purified NK cells after 16 or **D** 60 hours of IL-12/15/18- or IL-15-stimulation (n=10-11). Corresponding cells from one donor are connected with a line. **E** mRNA expression levels of indicated KIRs of either naïve NK cells or cells that were treated for 24 hours with 10 ng/ml of IL12, 100 IU/ml IL-2 and 100 ng/ml of IL-18. Data were extracted from the GEO dataset GSE22919[360]. Plotted is the mean ± SD of relative expression values (n=2).

Of note, the IL-12/15/18-stimulated cells exhibited substantially increased autofluorescence. The isotype control staining depicted in **figure 6.21. A** thus indicates that the IL-12/15/18-stimulated NK cells of this donor indeed virtually lost the expression of KIR2DL2/L3. **Figure 6.21 B** summarizes the findings of three donors of one experiment. Shown are the percentages of the KIR2DL2/L3-positive cells and the MFI of the positive population for the two timepoints analyzed. We concluded that KIR2DL2/L3, especially after prolonged stimulation periods, gets down-regulated by inflammatory cytokine signaling. Notably, after a 16 hour pulse and 6 days in IL-2 containing medium the KIR levels of IL-15- and IL-12/15/18-stimulated NK cells were similar thus arguing for a highly reversible mechanism.

We further analyzed the expression of KIR2DL1/S1 and KIR3DL1. As shown cumulatively for multiple donors in **figure 6.21 C** (stimulation for 16 hours) and **D** (stimulation for 60 hours), after 16 hours usually the MFI for various KIRs already decreased whereas after 60 hours a robust reproducible down-regulation of all three investigated KIRs was detectable on the level of percentage of positive cells as well as on the MFI level.

Furthermore, we looked the respective KIR genes up in the GEO dataset GSE22919 as described[360]. Importantly, after a 24 hour pulse with IL-12, IL-2 and IL-18 the NK cells used in the gene array of Smith *et al.* showed highly decreased mRNA levels for KIR2DL1, KIR2DL2, KIR2DL3 and KIR3DL1, substantiating our findings (**figure 6.21 E**).

Together these data indicate that joint signaling by IL-12, IL-15 and IL-18 could represent a so far un-appreciated mechanism of KIR regulation.

6.3.5 Regulation of activating receptors by IL-12/15/18 signaling

As remarkable changes in the expression profile of three inhibitory KIR molecules were detected (**figure 6.21**) we extended the phenotypical analysis of IL-12/15/18-stimulated NK cells also towards the most important activating receptors. We analyzed cell surface expression levels of activating receptors on purified NK cells via flow cytometry on either naïve cells or after 16 or 60 hours of stimulation with either IL-15 or IL-12/15/18. The impact of inflammatory cytokines on the expression of activating receptors has been elucidated for some receptors and some cytokines. For instance, NKG2D expression has been reported to be up-regulated by IL-2 as are most of the other important NK cell activating receptors[239,366,367]. As an exemplary negative regulator, TGF- β has been shown to down-regulate NKp30, NKp44, NKG2D and DNAM-1[239]. However, only little information is available on the regulation of activating NK receptors in the context of IL-12/15/18-stimulation. One exception is the influence of the three cytokines on NKp80 expression. Klimosch *et al.* recently reported that upon stimulation of freshly isolated NK cells with IL-12/15/18 NKp80 gets significantly down-regulated[174].

As shown in figure **6.22**, the level of almost every receptor analyzed was changed after 16 (**figure 6.22 A**) and 60 hours (**figure 6.22 B**) of stimulation in terms of the percentage of cells staining positive for it and in terms of the MFI of the positive population. In addition, we were looking for the respective transcript levels in the GEO dataset GSE22919 as described[360]. Of note, with the exception of NKp44 and NKp46 the up-regulation of DNAM-1 and the down-regulations of NKG2D, NKp30, NKp80 and CX3CR1 could also be seen on mRNA level (**figure 6.22 C**).

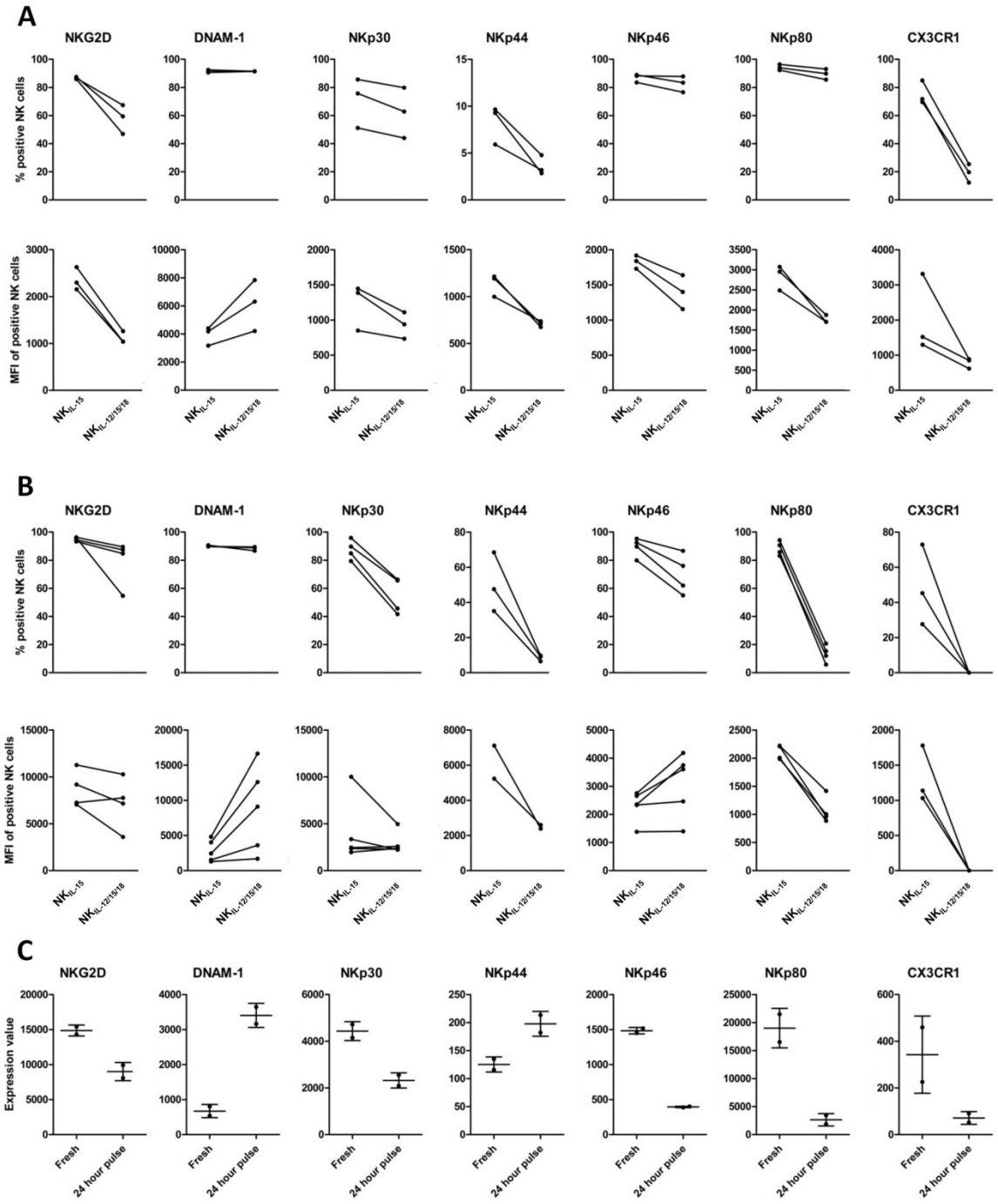


Figure 6.22 – The expression of activating NK receptors is regulated by IL-12/15/18. Purified human NK cells were treated with either IL-15 or IL-12/15/18 and stained for the indicated activating receptors after 16 (**A**) or 60 hours (**B**). Plotted are the percentages of positive cells (upper panel of A and B) and the MFI of the positive population (lower panel A and B). Corresponding cells from one donor are connected with a line (n=2-5). **C** mRNA expression levels of indicated activating receptors. Data were extracted from the GEO dataset GSE22919[360]. Plotted is the mean ± SD of relative expression values (n=2).

As we observed that in general the effect of IL-12/15/18-stimulation on various activating and inhibitory receptors was more pronounced after 60 hours as compared to 16 hours (**figure 6.21** and **figure 6.22**), we were hypothesizing that the killing of target cells would be even more elevated after 60 hours compared to 16 hours of cytokine stimulation (**figure 6.20**). However, IL-12/15/18- compared to IL-15-stimulated NK cells did not exhibit elevated levels of cytotoxicity as observed after 16 hours of stimulation (**figure 6.20** and **figure 6.23**).

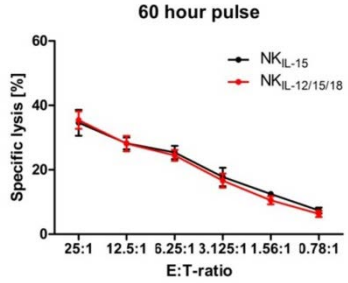


Figure 6.23 – IL-12/15/18-stimulated NK cells do not exhibit more pronounced killing of melanoma cells after 60 hours of stimulation. Purified NK cells were stimulated for 60 hours with IL-15 or IL-12/15/18 and used in ^{51}Cr release assays against SK-Mel-28 targets. Shown is the mean ± SD of the specific lysis (n = 3). One representative of 2 donors is shown.

6.3.6 Impact of decreased levels of KIR2DL2/L3 on NK cell functionality

Since we detected decreased levels of several important inhibitory receptors after the stimulation with IL-12/15/18, we investigated if this phenomenon contributes to an enhanced recognition of MHC class I-positive tumor cell lines *in vitro* (**figure 6.20** and **figure 6.21**). To see, if the reduced levels of KIR2DL2/L3 are causative for an elevated response against targets harboring the cognate ligands (i.e. HLA-C1-positive targets), we performed CD107a mobilization assays against 721.221 cells that have been transduced with various HLA class I molecules that were kindly provided by Prof. Dr. Carsten Watzl (IfADO, Dortmund). The mutant 721.221 (short 221) lymphoblastoid cell line *per se* does not exhibit any HLA class I-expression but is able to express selected HLA molecules upon transfection of the respective heavy chain[368]. Therefore, this model line has been widely used in assays where only distinct, selected HLA alleles were needed to be expressed on a target cell line.

The various 221 cell lines were stained for pan MHC class I-expression and analyzed via flow cytometry (**figure 6.24 A)**. As depicted, compared to the parental mutant 221 cells all cell lines transduced with one HLA class I molecule showed *de novo* surface expression of HLA class I exhibiting different expression levels. These 221 lines were used together with Ma-Mel-86b and SK-Mel-28 cells in a CD107a degranulation assay with NK cells that have been stimulated for 16 hours with either IL-15 or Il-12/15/18. Of note, Ma-Mel-86b and 221 cells did not express HLA class I molecules (**figure 6.4 and 6.24**), whereas SK-Mel-28 expressed HLA-Cw*03 as ligand for KIR2DL2/L3. 221 cells expressing HLA-Cw*03 and -Cw*07 harboring the C1-epitope expressed a cognate ligand for KIR2DL2/L3 whereas HLA-Cw*04 and –Cw*15 expressing 221s bear the C2-epitope and thus are ligands for KIR2DL1.

Importantly, the 16 hour pre-treatment with IL-12/15/18 reduced the surface expression of KIR2DL2/L3 to a significant degree (**figure 6.24 B**). Also, the analysis of the bulk NK cell population showed that NK cells that have been stimulated with IL-12/15/18 compared to NK cells that have only seen IL-15 showed significantly higher CD107a-mobilization regardless of the target cell line in line with cytotoxicity assays performed after 16 hours of stimulation (**figure 6.20** and **figure 6.24 C**). Therefore, we decided to refer to the degranulation of KIR2DL2/L3-negative NK cells as an internal standard to assess the impact of KIR regulation on KIR2DL2/L3-positive cells.

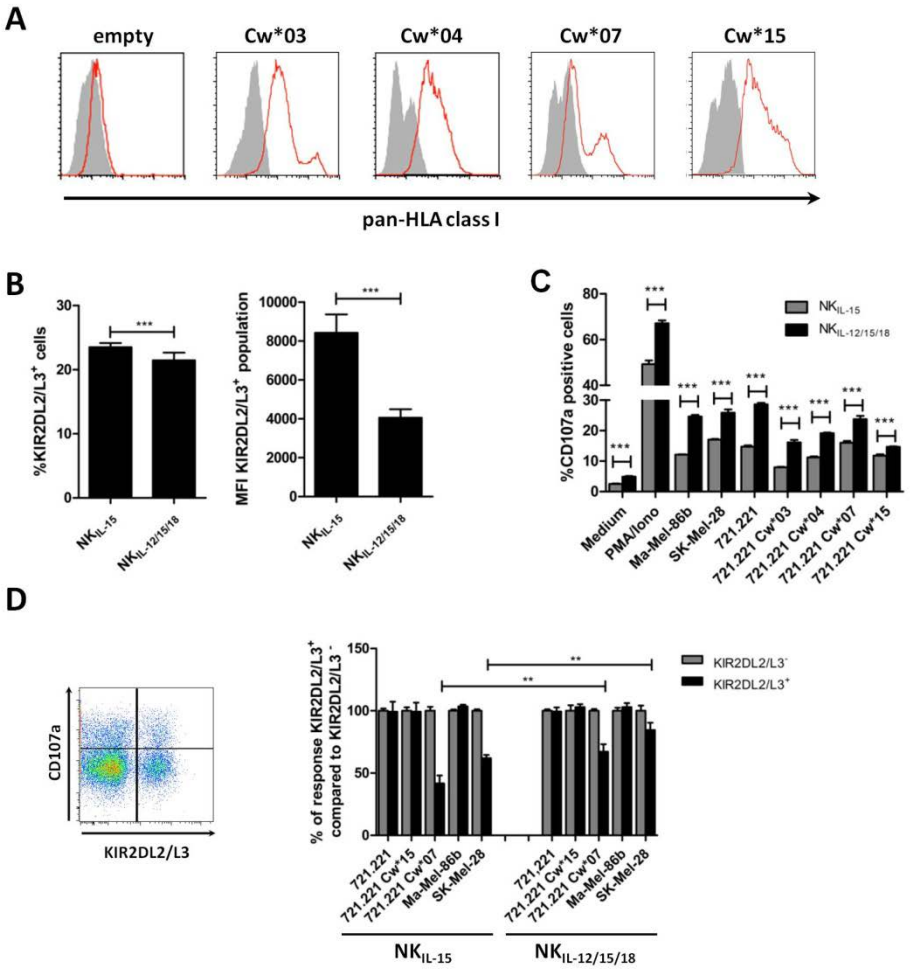


Figure 6.24 – Decreased KIR2DL2/L3-expression correlates to increased reactivity of IL-12/15/18-stimulated NK cells towards HLA C1-expressing target cells. A Detection of total HLA class I on 721.221 cells transduced with indicated HLA C alleles. The grey filled signal represents the isotype staining, the red line the specific HLA staining. **B** Shown is the mean ± SD of the percentage of KIR2DL2/L3-positive cells (left part) and of the MFI of the KIR2DL2/L3-positive population (right part). Values were withdrawn from the stainings after the CD107a mobilization assay. **C** Shown is the mean ± SD of CD107a-positive NK cells ($CD3^-$, $CD45^{bright}$, $CD56^+$) after a 4 hour CD107a mobilization assay (n=3). **D** Representative gating for detecting CD107a-positive KIR2DL2/L3 subsets (left part). Mean ± SD of the % of response of KIR2DL2/L3-positive NK cells compared to KIR2DL2/L3-negative NK cells (n=3). ** $p < 0.01$, *** $p < 0.001$ (student's t-test).

Co-staining of KIR2DL2/L3 and CD107a allowed for the detection of degranulating NK cells in both KIR2DL2/L3-positive and -negative subsets (**figure 6.24 D, left part**). Setting the percentage of CD107a-positive cells from the KIR2DL2/L3-negative subset to 100% created an internal standard against which the KIR2DL2/L3-positive cells could be compared. As shown in **figure 6.24 D** (**right part**), when no MHC class I was expressed by target cells (221 and Ma-Mel-86b) or HLA-alleles that do not bind to KIR2DL2/L3 (221 Cw*15) KIR2DL2/L3-positive cells reacted equally well regardless of the expression levels of the KIR (i.e. independent of the stimulation). However, when target cells expressed relevant ligands for KIR2DL2/L3 (Cw*03 on SK-Mel-28 and Cw*07 on 221 Cw*07), the KIR2DL2/L3-positive NK cells exhibited less CD107a mobilization. Most importantly, IL-12/15/18-activated NK cells showed a significantly smaller drop in degranulation compared to IL-15-activated NK cells, indicating that cytokine-mediated reduction of KIR levels allowed them to decrease inhibition by self thus creating a slightly increased reactivity towards target cells expressing the respective inhibitory ligands.

6.3.7 In vivo killing of IL-12/15/18-stimulated NK cells

Ultimately, we tested if IL-12/15/18-stimulated NK cells exhibit stronger tumor-control in an *in vivo* xenograft model of melanoma. Thus, in a first experiment a constant dose of SK-Mel-28 melanoma cells (transduced as described) was injected i.v. into NSG mice together with varying doses of 16 hour IL-15-stimulated NK cells (**figure 6.25 A**). As shown, the NK cells controlled the tumor load of melanoma cells in the lungs in a dose-dependent way.

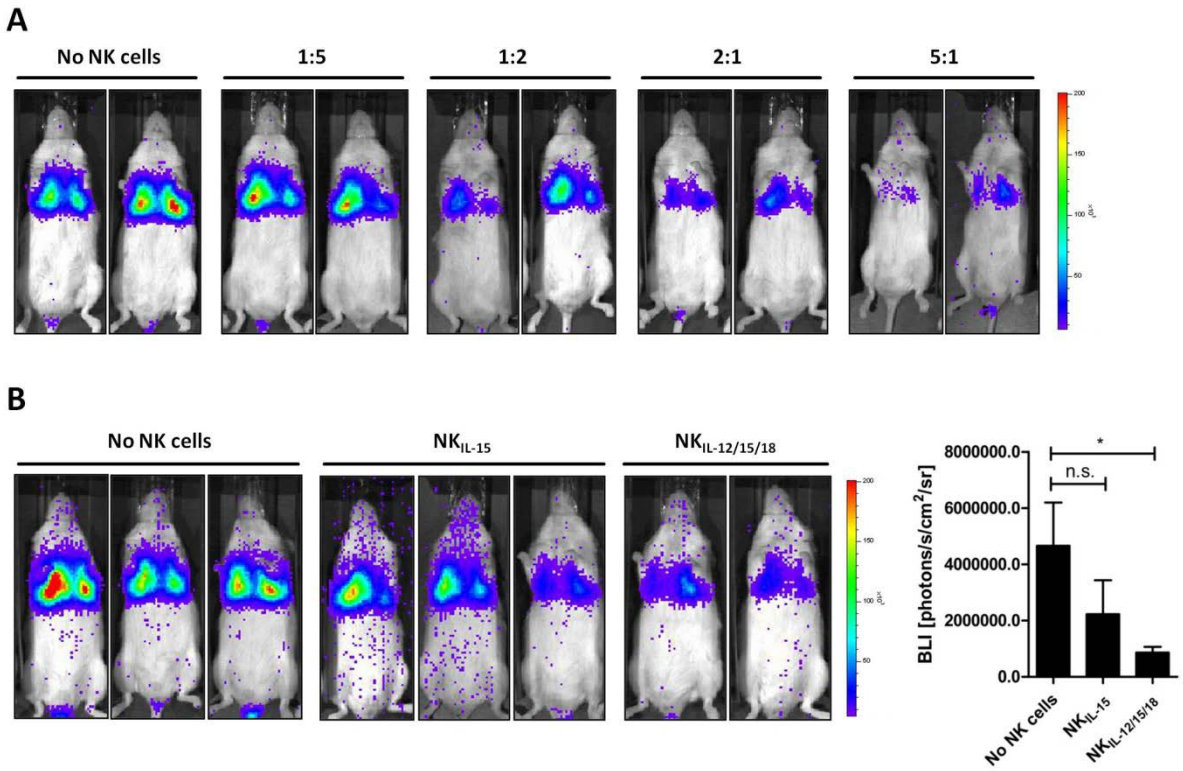


Figure 6.25 – IL-12/15/18- compared to IL-15-stimulated NK cells upon co-injection decrease the tumor load of SK-Mel-28 melanoma cells to a higher degree. A $7*10^5$ SK-Mel-28 melanoma cells transduced with pBabe-puro-eGFP-2A-CBGr99 were injected together with 16 hour IL-15-stimulated NK cells at the indicated E:T ratios i.v. into irradiated NSG mice. The BLI was determined 8 days after the injections. **B** Irradiated NSG mice were injected with $7*10^5$ SK-Mel-28 melanoma cells transduced with pBabe-puro-eGFP-2A-CBGr99 and either IL-15 or IL-12/15/18-stimulated NK cells (16 hour pulse) at a 1:2 E:T ratio. 8 days after the injection the BLI was determined. Shown are the acquired images of the mice (left panel) and the mean ± SD of the BLI (n=2-3). One of two experiments is shown. n.s. not significant, * $p < 0.05$ (student's t-test).

At the E:T ratio of 1:2 IL-15-stimulated NK cells were able to control the tumor engraftment to some extent but still left a significant tumor load. We thus decided to use that E:T ratio in the next experiments. Finally, as shown in **figure 6.25 B**, NK cells that have been stimulated with IL-12/15/18 decreased the tumor load to a significant degree in this experimental setup compared to IL-15-stimulated NK cells.

7 DISCUSSION

Melanoma and other neoplasia are classically treated with chemotherapy, γ-irradiation and, most importantly, surgery. These classical treatment regimens often are not applicable, such as surgery, or can cause severe side-effects in healthy tissue due to a non-specific mode of action. In this context, cancer patients could benefit substantially from immunotherapy as the immune system harbors many potent and, most importantly, highly specific effector cells such as CD8-positive CTLs, NK cells or γδ T cells that are known to lyse tumor cells efficiently *in vitro* and *in vivo*[369–371].

To focus on malignant melanoma, several studies have been performed, in which multiple aspects of immunotherapy have been investigated:

- Antibody therapy – for instance monoclonal antibodies (mAbs) against tumor-specific antigens inducing ADCC or mAbs blocking immunoregulatory molecules, for example CTLA-4 and/or PD-1[372–375];
- Vaccination – tumor antigens are delivered for example as peptides only, intact protein, DNA or mRNA coding for the respective antigens or peptides exogenously loaded on DCs[376,377];
- Cytokine therapy, for instance IL-2, IFN-α or IL-21[378,379];
- Adoptive transfer of effector cells, for instance CD8-positive T or NK cells[229,380].

Most of the studies focused on cells of the adaptive immune system, mostly T cells, leaving several key questions unaddressed that potentially could improve immunotherapeutical strategies harnessing NK cells. However, it is known from epidemiological studies that NK cells contribute to the control of human malignant disease[216]. In line with that, the amount of NK cell infiltration into the tumor tissue has been proposed to be a marker of beneficial prognosis, for instance, in squamous cell lung cancer, gastric adenocarcinoma or colorectal cancer[217–219]. Thus, we wanted to identify factors that potently attract NK cells into tumor tissue to enrich malignant tissue for cytotoxic effector cells. On the other hand, it has been shown that upon adoptive transfer of IL-2-activated NK cells the transferred cells rapidly lose their reactivity *in vivo*[229]. Hence, we aimed at the identification of new activation strategies that allow for a maintained reactivity of the transferred cells.

Both approaches hold great promise for NK cell-based tumor immunotherapy.

7.1 Establishment of xenograft mouse models of melanoma

In order to perform relevant *in vivo* experiments, two models of tumor xenotransplantation were established. To analyze homing of adoptively transferred NK cells, we set up s.c. injections of human melanoma cell lines and evaluated pre-conditioning regimens in two different immunocompromised mouse strains. In addition, we aimed at introducing a model of i.v. tumor injection with the possibility for optical imaging. Since tumor cells upon i.v. injection mimic artificially metastatic disease an experimental tumor model was created that is known to be controlled to some extent by NK cells[358,359].

Establishing the models, we observed a beneficial effect of γ-irradiation on tumor engraftment upon i.v. transfer of tumor cells (**figure 6.2 B** and **6.3 A**). One of several reasons, for instance, could be the clearance of residual murine immune cells by the irradiation. In NSG mice, resident neutrophils, and monocytes but also defective macrophages and dendritic cells, potentially interfered with proper tumor engraftment. In NOD-SCID mice, in addition murine NK cells possibly caused a xeno-rejection of human cells. In line with the presence of additional immune effector cells in NOD-SCID compared to NSG mice, this mouse strain engrafted xenotransplanted cells in general less efficiently compared to NSGs (**figure 6.2 B** and **C**)[333,336]. In addition to the lymphodepleting effect, γ-irradiation has been shown to induce the release of the von Willebrand factor from EC[381]. Due to thrombus formations this could potentially facilitate clogging of the lung capillaries and thus assist tumor cells to get entrapped in the lung microvasculature thus increasing the engraftment[382]. Also, several adhesion molecules like ICAM-1 or VCAM-1 have been shown to be up-regulated on irradiated endothelial cells and might contribute to a higher rate of adhesion and subsequently engraftment of tumor cells in the lung[353]. These effects are not lung specific, but due to the tight vessels, the impact might be most pronounced in this organ. The reason for the observed lower tumor engraftment in irradiated animals after s.c. tumor injection (**figure 6.3 B**) on the other hand could be due to different effects of irradiation on skin- and lung-resident immune cells. Macrophages in the subcutis possibly still were present at the time of injection and eventually exhibited an irradiation-induced activated phenotype, thus clearing tumor cells. In the lungs on the other hand, the alveolar macrophages probably were already depleted at the same time or the effect of the endothelium activation was overcompensating for the macrophage activation.

Also, we observed a better engraftment of SK-Mel-28 cells compared to Ma-Mel-86b cells, as detected by the BLI after injection of identical doses (**figure 6.5**). This effect could be derived from varying expression levels of the used CBGr99 luciferase, as detected *in vitro* (**figure 6.2 A**). Yet, also the bigger cell size of SK-Mel-28 compared to Ma-Mel-86b cells, as observed by microscopy and the forward scatter in flow cytometric analysis, might be a key factor determining the efficiency of engraftment. As the lung capillaries are the first narrow passage that cells have to pass upon i.v. injection, a bigger cell size confers a higher chance of clogging the lung endothelium and subsequently infiltrating into the tissue.

We reported the successful establishment of subcutaneous and intravenous xenotransplantation protocols for human melanoma and NK cells. Importantly, we established a minimally invasive imaging modality, harnessing luciferase-based optical *in vivo* luminescent imaging. As previously reported, NSG mice in our study were a suitable mouse strain for performing these experiments. In addition, we reported that whole body irradiation improved tumor engraftment after i.v. injection of tumor cells but delayed the tumor growth after s.c. injections of melanoma cells.

7.2 CX3CL1 as a tool to attract NK cells into tumors

NK cells are a heterogeous cell population consisting of at least two phenotypically and functionally distinct subsets, the CD56^{dim} CD16^{+} and CD56^{bright} CD16^{-} NK cells[125,205]. Importantly, whereas the CD56^{dim} CD16^{+} subset is considered to be the natural cytotoxic subset, displaying a more mature effector phenotype, it is mostly the CD56^{bright} CD16^{-} subset that can be detected in tumor tissue[205,220,221,342,343]. There are several explanations for the frequently observed, but poorly explored, accumulation of the less cytotoxic CD56^{bright} subset in tumor tissues. Amongst others, a specific attraction of these cells or a better proliferation and/or survival of the CD56^{bright} subset could be possible. Of note, some reports pointed towards a better survival of CD56^{bright} NK cells compared to CD56^{dim} cells in the tumor microenvironment due to a more pronounced resistance towards ROS which are typically enriched in proximity to and also directly in the tumor[222]. Also, in experiments performed with tumor supernatant we detected a preferential accumulation of the CD56^{bright} subset (data not shown). Of note, it was recently shown that also CD56^{dim} cells exerted pronounced cytokine secretion and that a CD56^{bright} CD16^{+} subset was strongly involved in the control of melanoma growth *in vivo*[203,383]. Thus, it is not fully understood which subset might be more relevant for controlling tumor growth *in vivo*. However, in several studies that correlated NK cell infiltration and patient survival, NK cells were identified by the CD57 marker which is expressed on around 70% of all CD56^{dim} NK cells. Still, a correlation could be found between the number of CD57-positive cells, i.e. a subset of CD56^{dim} NK cells, and the survival of the patients[206,217,219]. This, together with the fact, that CD56^{bright} NK cells *in situ* are often already present and CD56^{dim} NK cells outnumber the CD56^{bright} NK cells led us to aiming at recruiting preferentially the CD56^{dim} subset into the tumors to restore full NK cell functionality of both subsets.

7.2.1 The CX3CR1-CX3CL1 axis selectively recruits CD56^{dim} NK cells

As shown in the **figures 6.6** and **6.11**, CX3CL1 was shown to be a chemokine that exclusively attracted the CD56^{dim} CD16^{+} NK cell subset. These findings confirmed published data about the chemoattracting specificities of CX3CL1[210]. Of note, recently a second ligand for CX3CR1 was published, CCL26, that was shown to cooperate with CX3CL1 for CX3CR1 triggering[384,385]. Yet, we were focusing on the CX3L1-CX3CR1 axis. Importantly, it further was reported that CX3CR1-expression on NK cells might be used as a marker to detect mature cytotoxic effector cells as the high expression of the chemokine receptor correlated with the expression of CD57 and low levels of CD62L and CD27[344]. Furthermore CX3CR1-positive NK cells or CD8^{+} T cells were reported to be more cytotoxic as compared to their CX3CR1-negative counterparts[386,387]. In line with these reports, our data indicated that all CD57-positive NK cells express the CX3CR1 receptor with CD57 representing a marker for terminally differentiated effector immune cells (data not shown). Thus, attracting specifically this subset of NK cells, characterized by CX3CR1-expression, could be highly relevant for immunotherapeutic approaches of melanoma and malignant disease in general. Of note, the CX3CL1-CX3CR1 axis also could play an important role for attracting effector cells of the adaptive immune system such as CX3CR1-positive CTLs, thus harboring the potential to recruit cytotoxic cells from both branches of the immune system.

7.2.2 Effects of CX3CL1 on tumor cells and on NK cell reactivity

As already introduced in 6.2.2, it is difficult to exclude side effects of chemokines that might promote tumor growth. Notably, various chemokines have been described to also act on tumor cells and in many cases promoted tumor outgrowth and metastasis[92]. In several studies also CX3CL1 has been shown to exert pro-tumorigenic effects, for instance, in prostate or breast cancer (mouse and human) and pancreatic ductal adenocarcinoma[107,109,388,389]. So Shulby *et al.* showed that CX3CR1 can be expressed by prostate cancer cells, subsequently supporting their invasion into bone marrow. Also, CX3CL1 upon binding to CX3CR1-positive tumor cells elicited PI3K/AKT survival pathway signaling, indicating the potential danger of chemokine-signalling in tumor cells[107]. Similarly, a study from Tsang *et al.* showed that, in a cohort of 753 breast cancer patients, the high expression of CX3CL1 - despite higher TIL levels - correlated with worse overall survival[109]. Conversely, another study on breast cancer - similar to the presented data in **figure 6.8 A** - showed that high levels of CX3CL1 were predictive of a good prognosis[110]. This would be in line with the fact that CX3CL1 was reported to be a direct target of p53, thus suggesting a role in tumor suppression[111]. Importantly, it has been demonstrated that high CX3CL1 levels in the tumor correlate with higher CTL and NK infiltration and thus with better prognosis[110,390]. This is also implied by the presented data in **figure 6.8 B-E** that suggest that tumor cells possibly down-regulate CX3CL1-expression to escape attacks of the immune system. Yet, in a recent study it was shown in a spontaneous mouse model of mammary carcinoma that despite CX3CL1-down-regulation, adenoviral overexpression of CX3CL1 *in situ* enhanced tumor numbers in a dose dependent manner[388]. The example of these studies with controversial results shows the urgency to properly analyze patient material in terms of CX3CR1-expression and subsequently for responsiveness to CX3CL1 *ex vivo* to exclude adverse effects on tumor outgrowth and dissemination. As shown, all the melanoma cell lines we used lacked CX3CR1 and thus did not proliferate in response to CX3CL1 (**figure 6.11 C and D** and data not shown).

Some chemokines have been reported to activate NK cells and/or to drive them into proliferation. In line with this, several studies reported that soluble recombinant[348,350] as well as membrane-bound CX3CL1[350,351] can increase NK cell mediated cytotoxicity to a significant degree. We thus performed experiments in which we investigated the impact of soluble CX3CL1 on the proliferation and cytotoxicity, as well as the influence of membrane-bound CX3CL1 on the cytotoxicity of NK cells (**figures 6.9 and 6.12**). We found that neither form of CX3CL1 activated naïve or IL-2-activated purified NK cells above the medium background. Only for the Ma-Mel-86b cell line a slight increase in killing could be found when membrane-bound CX3CL1 was expressed (**figure 6.12**). We thus performed control-experiments reproducing the published setup and we did not observe increased cytotoxicity. Still, we did not thoroughly exclude the possibility that CX3CR1 might be blocked by serum-components, for instance by serum-starvation of the NK cells. Remarkably, with similar media our migration assays worked thus excluding the presence of compounds abrogating CX3CL1-binding to CX3CR1 or the signaling of the receptor.

7.2.3 In vivo tumor models exploiting CX3CL1-expression

7.2.3.1 Subcutaneous tumor injection

To determine if overexpressed CX3CL1 attracts adoptively transferred human NK cells *in vivo* into tumor tissue, we performed animal experiments in the established mouse model of subcutaneous tumor transplantation (**figures 6.3, 6.13**, **6.14** and data not shown). Importantly, as evaluated by flow cytometry, we did not detect adoptively transferred human NK cells in the tumors. Transferred NK cells were defined as huCD45^{+} and huCD3^{-} and were reliably stained in blood samples and spleens of the respective animals but not in the tumor plugs regardless of CX3CL1-expression. This held true for either intravenous or perilesional injection of NK cells (**figures 6.13** and **6.14**). Controversially, some reports demonstrated a therapeutical effect of adoptively transferred human NK cells against subcutaneously growing tumor cells or detected them in the tumor tissue[391–393]. Although incompatibilities between some human and murine adhesion molecules exist, *in vivo* tumor infiltration seem to be possible[394]. One issue in our experiments could be the injected NK cell numbers, since in some studies compared to our experiments up to 10 times more NK cells were injected. We cannot exclude that a certain NK cell count needs to be exceeded to allow for efficient extravasation of NK cells and their subsequent infiltration into transplanted tumors. However, as we used fresh or short-term cultured NK cells in our experiments we were restricted in this context. As NK-92, a well-characterized NK cell line, did not express CX3CR1 (data not shown) we will screen other lines to switch from polyclonal NK cells to large effector cell numbers using a NK cell line. Another difference compared to many published studies was that we used NSG mice in our experiments whereas most of the other groups used SCID or nude mice. However, since NSG mice are directly derived from NOD-SCID mice that have successfully been used for similar experiments, we have no explanation why we could not detect NK cells in the tumors. Of note, the discrepancy between cell homing and localization in human tissues, as compared to humanized mouse models, is one of the most important issues in pre-clinical mouse models[395].

7.2.3.2 Intravenous tumor injection

As we did not detect an accumulation of adoptively transferred NK cells in subcutaneously transplanted human melanoma cell lines, we decided to switch to a model in which the tumor cells were injected i.v. to mimic metastatic disease (**figure 6.2, 6.5, 6.15, 6.16** and **6.17**). This approach potentially is advantageous since NK cells do not have to migrate through murine tissue, possibly struggling with incompatible adhesion molecules, but make contact with tumor target cells directly in the blood stream of the animals. Likewise, NK cells in a dose-dependent manner decreased the tumor load in the mice, an effect that we observed for Ma-Mel-86b and SK-Mel-28 melanoma cells with IL-2- as well as IL-15-stimulated NK cells (**figure 6.15** and **6.25 A**). Importantly, as shown in **figure 6.16**, we also observed a more pronounced rejection of CX3CL1-positive Ma-Mel-86b tumor cells compared to vector control cells. Controversially, with SK-Mel-28 melanoma cells we could not detect this preferential killing of the CX3CL1-expressing line (**figure 6.17 A**). Due to the sub-optimal condition in the tubes prior to the injection, a killing *in vitro* before the injection could be excluded. In line with this, in previous *in vitro* experiments, we did not observe increased killing of tumor cell lines expressing membrane-bound CX3CL1 (**figure 6.12**). Only Ma-Mel-86b cells out of three tested lines showed a small, yet significant increase of specific lysis in standard ^{51}Cr release assays. Thus, a difference in *in vitro* killing seems to be indicative of a more pronounced NK cell-reactivity towards

CX3CL1-expressing targets *in vivo*. The reasons for this phenomenon remain elusive. Notably, expression levels of CX3CL1 seemed to be higher in Ma-Mel-86b compared to SK-Mel-28 cells (**figure 6.10**). This possibly indicates that a certain threshold of CX3CL1-expression might exist that is decisive if NK cells can control CX3CL1-expressing targets *in vivo* to a higher degree. Other studies reported that membrane-bound CX3CL1 served as a *bona fide* adhesion molecule for CX3CR1-positive effector cells[386]. Thus, depending on the expression level of CX3CL1 this could increase the adhesion of NK cells to target cells under flow which subsequently can lead to a more frequent delivery of lytic hits and thus to a more pronounced control of tumor growth[350]. One further point to explain the discrepancy between Ma-Mel-86b and SK-Mel-28 could be that shedding of CX3CL1 by SK-Mel-28 cells is faster compared to Ma-Mel-86b cells. Importantly, the adhesion of NK cells to CX3CL1-coated membranes was shown to be highly blockable by soluble CX3CL1[350]. Therefore, faster shedding of CX3CL1 from SK-Mel-28 cells could have possibly decreased the adhesion of NK cells and thus annihilated the previously observed beneficial effect. In addition, Zhang *et al.* already proposed that the presence of further co-stimulatory or stimulatory signals on the target cells might be required to translate a $CX3CL1^{bright}$ phenotype into stronger killing by NK cells. Thus, Ma-Mel-86b cells compared to SK-Mel-28 cells might express another ligand that triggers a superior NK cell-response against CX3CL1-positive transductants. Further experiments with an increased panel of CX3CL1-expressing cells will be needed to clarify what the determinants for elevated NK cell-responses against $CX3CL1^{bright}$ targets *in vitro* and *in vivo* are.

Importantly, we were able to demonstrate a higher infiltration of NK cells into the lungs of animals that were injected with $CX3CL1^{high}$ SK-Mel-28 cells compared to animals that were injected with vector control cells (**figure 6.17 C and D**). This experiment demonstrated that the over-expression of CX3CL1 in tumor cells *in vivo* can lead to the accumulation of adoptively transferred NK cells in tumor-harboring organs. Of note, we did not stain single cell suspensions of the lungs for the presence of NK cells when we used Ma-Mel-86b (**figure 6.16)**. It is currently unknown to us why, despite a stronger infiltration of NK cells, CX3CL1-expressing SK-Mel-28 cells were not rejected more potently *in vivo*. Possibly, NK cells that infiltrated into the tumor-harboring lungs rapidly succumbed to immunosuppression by the tumor cells. As SK-Mel-28 cells got killed less efficiently compared to Ma-Mel-86b cells (**figure 6.4**) it is possible that NK cells have longer contact times with SK-Mel-28 cells compared to Ma-Mel-86b cells and are thus more exposed to tumor-derived immunosuppressive factors, such as TGF-β. This could lead to a more pronounced inhibition of NK cells upon co-injection with SK-Mel-28 compared to Ma-Mel-86b cells enabling tumor growth *in vivo* regardless of NK cell infiltration. This hypothesis could be tested by supplementing the animals exogenously with high doses of NK cell-activating cytokines such as IL-2 or IL-15 which subsequently might translate the elevated NK cell numbers into a decreased tumor burden (**figure 6.17 C and D**). Of note, these data obtained from xenotransplantation experiments in general resemble the main observations after transfer of autologous IL-2-activated NK cells into melanoma patients. In both cases, adoptively transferred NK cells persisted but no beneficial effect was observed possibly due to suppression of NK cell responses by tumor-derived factors[229].

Notably, this model of co-injection is not a clinically relevant setting. Therefore, in a next step, we want to set up a therapeutical model in which the tumor cells will be injected prior to the NK cells and hence the NK cells will have to actively migrate to the vector control- or CX3CL1-transduced tumor cells. As pointed out, by supplementation with high dose IL-2 we will be able to more stably maintain the activation of the injected NK cells to get more conclusive results on the combinatorial effects of CX3CL1 gene therapy and the adoptive transfer of NK cells.

7.2.4 Aspects for clinical translation

One possibility to translate these findings into clinics would be the expression of CX3CL1 in the cancer tissue of melanoma-bearing patients using genetically designed adenoviruses as vectors for gene therapy. By molecular biological means the replication of these viral vectors can be restricted to tumor cells[396,397]. This can be achieved by cloning genes essential for the replication of the virus behind a promoter that is, if possible, exclusively used in tumor cells. This technique allowed for the over-expression of certain molecules of interest that can subsequently modulate the tumor microenvironment to improve for instance immune cell infiltration or effector cell activation[398]. For the application in melanoma patients, for instance, a mutated *E1A* gene, which is essential for the replication of the virus, was cloned behind a tyrosinase promoter and was only expressed in cells exhibiting inactive pRb, thus allowing replication, oncolysis and also transgene-expression only in malignant tissue[399]. Thus, a melanoma-specific virus potentially could be created and armed with the full-length CX3CL1 gene to attract $CD56^{dim}$ NK cells into melanoma tissue.

Most importantly, as discussed in 7.1.2, a thorough analysis of patient material *ex vivo* needs to be done in order to exclude growth or metastasis promoting effects of the chemokine. This analysis needs to include staining for CX3CR1 on the tumor cells and, if possible, an *ex vivo* proliferation and migration assay of tumor cells in the presence or absence of recombinant CX3CL1.

Furthermore, CX3CL1 attracted exclusively CD16-positive NK cells. This is of particular interest as in several studies it has been shown that tumor infiltrating NK cells can exhibit decreased expression levels of several activating receptors rendering them inert towards activation by binding their cognate ligands. Despite this profound inhibition it has been shown for various cancer entities that ADCC mediated by the F_c receptor CD16 was not affected and remained functional without IL-2 re-stimulation of tumor infiltrating NK cells. Therefore, the attraction of CD16-positive NK cells should ideally be combined with the application of tumor-antigen specific antibodies to possibly enhance the therapeutic effect of attracted endogenous or adoptively transferred NK cells[229,320].

Of note, besides NK cells also monocytes, Th1 CD4-positive T cells, CD8-positive CTLs and mast cells express CX3CR1, indicating that a broad panel of anti-tumor effector cells can get recruited to $CX3CL1^{bright}$ tumors[400]. Reports, which investigated T cell infiltration into melanoma metastases, suggested a critical role for CCL2-5 and CXCL9 and CXCL10[401]. These chemokines were identified by hierarchical clustering of gene expression of infiltrated and non-infiltrated metastases. Importantly, it is unclear if the expression correlated with a better prognosis. Moreover, as shown for CXCL10 (**figure 6.7**) these chemokines do not always efficiently support the attraction of NK cells or other effector cells. Thus, the usage of CX3CL1 possibly provides the advantage of attracting multiple cytotoxic effector cells of various lineages such as CTLs, γδ T cells and T cells that express high levels of intracellular perforin and granzyme B[386].

We identified CX3CL1 as a promising candidate for immunotherapy attracting a broad array of ant-tumor effector cells. Importantly, the possibility of the attracted NK cells to mediate ADCC should be investigated in terms of combinatorial therapies, using NK cells and antibody therapy.

7.3 Evaluation of IL-12/15/18-stimulated human NK cells for anti-tumor immunotherapy

In *in vitro* assays, pre-clinical *in vivo* models and clinical trials it has been shown that NK cells are capable of controlling tumor growth. Further, several modes of activation have been evaluated in terms of identifying the prime regimen leading to a more pronounced and sustained activation of NK cells[310,340]. Some of the obtained results are summarized in the following table:

Used mode of activation	Important observation	Entity	Reference
IL-2	Other strategies to augment persistence are needed	Recurrent ovarian and breast carcinoma, melanoma	229,402,403
IL-15 (+ hydrocortisone)	IL-2 and IL-15 both better than IL-7	Advanced non-small cell lung cancer	311,403
IL-21 i.v. as bolus	IL-21 is safe and induces higher cytotoxicity *in vivo*	Melanoma and RCC	379,404
HSC	KIR-HLA mismatch in GvH direction extremely beneficial	AML	308
IL-12/15/18	Pronounced proliferation, more sustained reactivity	Lymphoma, leukemia	287,363
IL-12/15/18	Trial, start 09/2013	Relapsed and refractory AML	NCT01898793

Of note, it was recently published for a cohort of melanoma and renal cell carcinoma (RCC) patients that the infusion of pre-activated NK cells – activated *ex vivo* by IL-2 and irradiated autologous feeder cells - led to high numbers of circulating transferred NK cells, yet those cells did not show any clinical effect due to the rapid loss of responsiveness or activation *in vivo*[229]. Parkhurst *et al.* showed in this study that the expression levels of NKG2D on NK cells 1 week after the infusion into melanoma patients were down-regulated, explaining in part the decreased *ex vivo* reactivity against melanoma cell lines, unless the cells were re-activated with IL-2. Thus, regardless of the activation of NK cells prior to infusion, one major obstacle for successful NK cell-based immunotherapy is that the transferred NK cells often do not respond efficiently to tumors *in vivo*. This is partially due to the enrichment of immunosuppressive factors and cells in the tumor microenvironment. For instance, it was shown that T_{reg} inhibit autologous or transferred NK cells at various levels or affect their recruitment to the tumor[94,405,406]. Also, elevated TGF-β or IL-10 serum levels in tumor patients can systemically down-regulate NKG2D and other activating receptors on NK cells leading to decreased responses against tumor cells[239,367].

These data support the need of a more sustained reactivity of the transferred NK cells or a blockade of inhibitory mechanisms to overcome various immunosuppressive factors *in situ* and thus to enhance the therapeutic effect of transferred NK cells. Repeated injections with high dose IL-2 have been shown to be partially efficient, however severe toxic side effects were observed leading to death in 1.5% of the patients due to capillary leak syndrome and thus organ failure[296]. IL-15 compared to IL-2 seems to be less cytotoxic, does not activate T_{reg} and especially in the complexed form with the IL-15Rα is a potent inducer of NK cell activation[407,408]. Therefore, it is currently being tested in clinical trials. Also, efforts have been made to break immunosuppressive microenvironments, for example by antibody-neutralization or blockade of molecules that reportedly contribute to immunosuppression, for instance VEGF or CTLA-4[373,409,410].

7.3.1 Sustained effector function of IL-12/15/18-stimulated NK cells

To overcome immunosuppressive mechanisms exerted on NK cells *in vivo*, we aimed at generating NK cells that maintain their reactivity after the primary activation. Therefore, we used IL-12/15/18 in combination to induce more potent effector cells based on a previous publication from Cooper *et al.*[284]. We showed that 16 hours of stimulation with IL-12/15/18 were sufficient to induce CD25 (IL-2R α)-up-regulation, a process that in murine NK cells was reported to be mainly dependent on IL-12[411]. The elevated expression of the IL-2R α-chain rendered IL-12/15/18-stimulated NK cells highly responsive to IL-2 yielding significantly higher cell recoveries after culture in IL-2 containing medium (**figure 6.18**). Importantly, not only the cell numbers were increased but also the levels of secreted IFN-γ upon restimulation were significantly higher compared to IL-15-stimulated NK cells (**figure 6.18 F**)[287]. These data indicate that for a period of up to 8 days after the cytokine stimulation the cells were still functionally superior. Notably, CD4-positive T cells stimulated with plate-bound α-CD3/CD28 were also able to drive the preferential expansion of IL-12/15/18-stimulated NK cells and to maintain their significantly higher reactivity compared to IL-15-stimulated control cells (data not shown). This was mostly due to the secretion of IL-2 upon stimulation. Remarkably, Romee *et al.* published similar findings in a highly related *in vitro* model of cytokine activation and restimulation[363]. Compared to our setup Romee *et al.* were using IL-15 for the culture of IL-12/15/18-stimulated NK cells. Of note, in earlier experiments we were also using IL-15 instead of IL-2 for the culture of stimulated NK cells and observed similar effects in terms of proliferation and IFN-γ production of NK cells (data not shown). This indicates that usage of the up-regulated IL-2R α-chain was not the exclusive mechanism that drove the proliferation of IL-12/15/18-stimulated NK cells and by which these maintained their higher reactivity. Importantly, no staining of the IL-15R α-chain CD215 was performed to compare the expression on IL-12/15/18- with IL-15-stimulated NK cells. However, since on the mRNA level the expression of the IL-15R α-chain, similarly to the IL-2R α-chain, was highly up-regulated (data not shown) it is likely that the sensitivity of IL-12/15/18-stimulated NK cells is higher towards both IL-2 and IL-15. Due to the similarity of the signaling pathways of IL-2 and IL-15, similar phenotypes were observed after culture of IL-12/15/18-stimulated NK cells in either IL-2 or IL-15. This could potentially be important, since IL-2 *in vivo* is known to exert toxic side effects and to promote T_{reg} expansion[296,412]. Importantly, we observed a more pronounced proliferation of IL-12/15/18-stimulated NK cells together with a stronger induction of IFN-γ upon restimulation also in an *in vivo* mouse model. We injected either IL-15 or Il-12/15/18-stimulated NK cells i.v. into NSG mice (**figure 6.19**) and supplemented the animals repeatedly with IL-2 by i.p. injections. In both the *in vitro* and *in vivo* setup the more pronounced proliferation and the elevated reactivity in terms of IFN-γ production depended on the presence of an activating inflammatory cytokine. We did not test if IL-15 maintains the reactivity of IL-12/15/18-stimulated NK cells *in vivo*. Here, further experiments will be performed to investigate if the use of IL-2 *in vivo* can be circumvented.

Importantly, Romee *et al.* showed in their model, that after 7 days in culture, IFN-γ mRNA levels did not differ in the NK cells regardless of the stimulation (IL-15 and IL-12/15/18) thus making similar observations as Cooper *et al.* with murine cytokine activated NK cells[284,363]. These findings imply that the higher production of IFN-γ might be mainly regulated at a posttranscriptional level. Also, the levels of the IL-12 receptor were similar between IL12/15/18- and IL-15-stimulated NK cells, thus not explaining why upon re-stimulation with IL-12/15 the IL-12/15/18-stimulated NK cells showed a more robust IFN-γ production compared to control cells. Notably, also restimulation with K562 cells induced an increased response. However, the higher levels of IFN-γ production after tumor cell

restimulation could be due to an elevated expression of NKp46 on IL-12/15/18-stimulated NK cells that was described in the same paper[363]. One potential regulatory mechanism for the elevated IFN-γ responses could thus be the repression of translation via miRNAs that were described to regulate IFN-γ protein expression and could be decreased after IL-12/15/18-stimulation[413,414]. A stably decreased expression of such inhibitory miRNAs could for instance result from epigenetic regulation. Furthermore, a more efficient formation of IFN-γ loaded intracellular vesicles could explain the higher secretion of the cytokine despite similar levels of mRNA. However, our preliminary data indicate though that intracellular IFN-γ levels are similar after 8 days in culture (data not shown) thus excluding the possibility of a higher abundance pre-formed IFN-γ-loaded secretory vesicles. Notably, we also detected highly elevated levels of TNF-α in IL-12/15/18-stimulated NK cells (data not shown). This cytokine has been shown to be involved in NK cell apoptosis after cytokine stimulation thus preventing overproduction of IFN-γ[141]. Likewise, TNF-α induction could possibly serve as a cell-intrinsic negative control element to prevent immunopathology. These issues need to be investigated in the future.

As described (section 3.3.7), the term **memory-like** has been introduced for NK cells stimulated with certain cytokines such as a combination of IL-12, -15 and -18 as they exhibit elevated responses against **various stimuli** whereas **virus-induced memory** seems to be **antigen-specific**. Notably, in a murine cytomegalovirus (CMV) infection model it was shown that IL-12 signaling was crucial for the induction of NK cell memory. When IL-12 signaling was abrogated by using IL-12R$^{-/-}$ mice, no expansion of Ly49H^{+} NK cells, which are specific for the viral m157 protein, was detected[415]. Also during human CMV infection or re-activation the expansion of a NKG2C-positive NK cell subset was observed that produced elevated levels of IFN-γ and exhibited features of recall responses thus indicating memory features[416]. To date, the factor driving this expansion is unclear but unpublished data from our group point towards a contribution of IL-12. These data indicate that IL-12 signaling might be one of the key factors for inducing long-lasting NK cell responses. Due to the potential clinical relevance of these findings for NK cell-mediated protection from virus infections but possibly also for the treatment of malignant disease, comprehensive investigations are needed to delineate the exact molecular details granting NK cells this sustained either antigen-specific or -unspecific reactivity.

7.3.2 IL-12/15/18-activated NK cells exhibit increased cytotoxicity and a display a distinct phenotype

To gather comprehensive data on how IL-12/15/18-stimulation potentially impacts NK cell reactivity we did not only monitor cytokine secretion but also performed ^{51}Cr release assays against various tumor targets to measure for cellular cytotoxicity. As depicted in **figure 6.20**, IL-12/15/18-stimulated NK cells exhibited stronger killing against any investigated tumor cell line compared to control cells. Of note, this effect was not dependent on the expression of MHC class I. IL-12, IL-15 and IL-18 have been described individually to act as NK cell activating cytokines yet little or no mechanistic studies have been performed[142,145,146,362,418].

7.3.2.1 KIR phenotype after IL-12/15/18-stimulation

So far, no systematic analysis of the expression levels of various inhibitory and activating NK cell receptors directly after 16 hours of stimulation with IL-12/15/18 has been performed. Importantly, we detected a slight decrease in the expression levels of various KIRs after 16 hours of cytokine stimulation that got more pronounced after 60 hours (**figure 6.21**). As indicated by the analysis of the available gene array data (GEO dataset, GSE22919) this deregulation was also obvious at mRNA level, pointing towards a transcriptional regulation of the three KIR molecules by IL-12/15/18 (**figure 6.21 E**)[360]. Most importantly, to our knowledge, signaling via inflammatory cytokines is a novel regulatory mechanism of KIR expression. In fact, Chrul *et al.* published that the combinatorial treatment of naïve NK cells for 72 hours with IL-12 and IL-18 led to a significant increase of the percentage of KIR2DL1- and KIR2DL2-positive cells compared to naïve NK cells KIRs[364]. Controversially, in our study, NK cells after the stimulation with IL-12/15/18 expressed less KIR than naïve and IL-15-stimulated NK cells (**figure 6.21 A**). The fact that Chrul *et al.* did not observe the substantial down-regulation that we observed might be due to the lack of IL-15 in their setup. Of note, IL-15 has been shown to up-regulate KIR levels, but still its effects in combination with IL-12 and IL-18 as in our setup might have been adverse or simply be dominantly counteracted by the combined signaling of IL-12 and IL-18. Hence, in our setup, IL-12/15/18-stimulation seemed to decrease inhibition by KIR signaling, possibly contributing to the observed significantly higher killing of various MHC class I-positive tumor targets (**figure 6.20**). However, de-sensitization for self HLA and simultaneous up-regulation of perforin and activating NK cell receptors such as NKG2D or the NCRs mediated by IL-15 and IL-18 could potentially have a negative impact in terms of autoimmunity[418]. Notably, our preliminary data suggest that IL-12 or IL-18 alone are sufficient to prevent IL-15-induced up-regulation of KIRs. This is of particular interest, as the two cytokines use very distinct signaling pathways (introduced in section 3.3.2). Thus, for MHC class I-positive targets, IL-12- or IL-18- together with IL-15-stimulation might ideally activate NK cells by inducing relevant cytotoxic molecules such as perforin but simultaneously decreasing the expression of inhibitory receptors. Importantly, further phenotypical and most importantly functional studies are required to determine the cytokine combination and concentration to ideally activate NK cells. Of note, the down-regulation of KIR after stimulation with IL-12/15/18 was reversible. After 6 days in IL-2-containing medium, the levels of the investigated KIRs were detectable at similar levels, indicating a reversible mechanism (data not shown).

There are several mechanisms for the observed KIR regulation in these *in vitro* stimulation experiments. As shown in **figure 6.21 E**, the decreased mRNA levels of the investigated KIR molecules in IL-12/15/18-stimulated compared to naïve NK cells suggest a transcriptional regulatory mechanism. Most importantly, it was recognized shortly after the discovery of KIRs that DNA-methylation as well as histone acetylation and methylation are key mechanisms to regulate the expression levels of various KIR alleles[419,420]. The regulation is recognized to be very complex including promoter elements in intergenic fragments, antisense RNAs and an array of involved transcription factors like c-Myc in multi-promotor-controlled genes[421,422]. Notably, all KIR genes do have a p300/CBP binding site in the distal promoter. This protein upon binding is able to acetylate all 4 nucleosomal core histones and thus could be a powerful inducer of KIR expression[423]. Furthermore, all expressed KIR genes have a potential AML/Runx binding site what led to the hypothesis that AML proteins might also be contributing to regulate KIR expression[424]. Importantly, Runx and p300 are epigenetic regulators and NK cells have been shown to up-regulate previously silenced KIR genes after treatment with the methyltransferase inhibitor 5-Aza-dC[419]. A preliminary search for altered expression levels of DNA methyltransferases or potentially KIR-expression modifying proteins in the

publically available array data (GEO dataset, GSE22919) on IL-12/2/18-stimulated NK cell did not reveal any targets, yet[360]. Notably, not only expression levels of these proteins but also their biological activity that could potentially be regulated by IL-12/15/18 could affect the expression levels of KIR molecules. It will be crucial to stimulate purified NK cells with IL-12/15/18 in the presence of a panel of inhibitors for various DNA or nucleosome modifying proteins, such as DNA methyltransferases or histone methylating, acetylating or phosphorylating proteins. It thus can be analyzed what epigenetic pathways are crucial for decreasing KIR protein levels by interfering with gene transcription. These experiments are ongoing in our laboratory and the data will clarify the contribution of individual epigenetic mechanisms to the observed KIR regulation by IL-12/15/18.

Importantly, the down-regulation of KIR expression levels by inflammatory cytokines - regardless of the mechanism - is of particular interest. Blocking of KIR molecules with mAbs, which decreased inhibition by self HLA, yielded promising results in NK cell-based therapies of leukemia in pre-clinical mouse models[312]. More importantly, absence of KIR signaling introduced by KIR-HLA mismatching in allo-HSCT settings against AML improved the prognosis significantly[308]. If IL-12/15/18-stimulation should represent a possibility to *ex vivo* activate NK cells to transiently exert less inhibition by self HLA, this would be of high translational importance.

7.3.2.2 Activating receptor phenotype after IL-12/15/18 stimulation

To obtain comprehensive data concerning the phenotype of IL-12/15/18-stimulated NK cells, we stained purified NK cells after 16 and 60 hours of cytokine-stimulation for a panel of activating NK receptors. We observed that most of the analyzed receptors (NKG2D, NKp30, NKp44 and NKp80) were expressed less abundantly on IL-12/15/18-stimulated NK cells compared to control cells, with DNAM-1 being the only receptor that showed up-regulation (**figure 6.22**). Several mechanisms can be responsible for the observed regulation of the investigated activating receptors. Yet, the changes we observed on protein level for most receptors were evident as well on the mRNA level of naïve and IL-12/15/18-stimulated NK cells as revealed by analysis of available gene array data (GEO dataset, GSE22919) (**figure 6.22**)[360]. These findings argue for transcriptional regulation mechanisms. Of note, IL-12/15/18-activated NK cells exhibited a decreased expression of NKp44 on protein level compared to IL-15-stimulated NK cells whereas the mRNA expression compared to naïve cells was up-regulated (**figure 6.22 C**). This is in line with a *de novo* induction of NKp44 in IL-12/15/18-treated NK cells (data not shown). Thus, IL-12/15/18-stimulated NK cells up-regulate NKp44 compared to naïve cells albeit to a lower degree compared to IL-15-stimulated cells explaining the seemingly controversial data plotted in **figure 6.22**. In line with the suggested transcriptional regulation of some of the molecules, epigenetic mechanisms have been described to be involved in the regulation of the expression levels of certain receptors in mice or humans[425,426]. NKG2D transcription for instance was described to be negatively regulated by DNA-methylation whereas histone acetylation increased transcription of the gene. Furthermore, in another study, the hypomethylating agent decitabine increased NKp44 and inhibitory KIRs in a dose-dependent manner whereas in the same study decitabine down-regulated NKG2D[427]. Despite the discrepancy with regards to the impact of DNA methylation on NKG2D expression these two studies point out that epigenetic mechanisms might be either causative or contributing to the phenotype that we detected. Further, it has been reported, that recombinant IL-12 or type I IFNs decreased DAP-10 and NKG2D mRNA thus leading to decreased NKG2D levels which is in line with our findings (**figure 6.22**)[428]. Notably, in this study, Muntasell *et al.* did not

observe IL-12-mediated regulation of other activating receptors like NKp30 or NKp46. Still this study further emphasizes the complex regulatory network underlying the expression of activating receptors[429]. It is unclear why DNAM-1 is the only activating receptor being up-regulated by the three cytokines. Again, little information is available on the regulation of expression of this molecule. Deciphering the regulatory mechanisms behind the dynamic expression of activating receptors could greatly contribute to a better understanding of NK cell biology and subsequently to more tailored NK immunotherapies against for example malignant disease.

In addition to the mentioned regulatory elements that act on the suggested transcriptional level, further possibilities exist that might contribute to the regulation of cell surface expression of some of the receptors. For instance, one of the mechanisms that was previously shown to impact the surface expression of CD16 was matrix metalloproteinase 25 (MMP25)-mediated shedding of the receptor from the cell surface[430]. The contribution of similar mechanisms to the down-regulation of other receptors after stimulation with IL-12/15/18 currently cannot be ruled out.

Physiologically, this coordinated down-regulation of activating receptors together with inhibitory receptors on IL-12/15/18-stimulated NK cells potentially prevents immunopathology. Inflammatory cytokines such as DC-derived IL-12 and IL-18 may increase *in vivo* the expression of NKG2D or NCR ligands also on healthy tissue. Moreover, since NK cells after IL-12R and IL-18R signaling down-regulate activating receptors, bystander cells expressing low levels of cytokine-induced activating ligands might be protected from taking collateral damage[431–433]. Furthermore, in line with Klimosch *et al.*, highly cytokine-activated NK cells could down-regulate activating receptors and simultaneously express elevated levels of activating ligands[174]. Killing of highly cytokine-stimulated cells by activated NK cells, a mechanism known as fratricide might further negatively regulate over-stimulated NK cells. Notably, comprehensive stainings of various activating ligands on cytokine-activated NK cells still need to be performed and functional consequences to be elucidated.

7.3.2.3 Functional consequences of IL-12/15/18-stimulation

The phenotypic analysis of several receptors revealed that the different expression levels after 16 hours got even more pronounced after 60 hours of stimulation with IL-12/15/18 or IL-15. Still, already after 16 hours a significant difference in SK-Mel-28 killing has been observed (**figure 6.20**). Thus, we were speculating to see an even stronger killing of SK-Mel-28 cells by IL-12/15/18-stimulated NK cells after 60 hours of cytokine activation. Remarkably, the difference in killing between the IL-15- and IL-12/15/18-activated NK cell populations was virtually gone (**figure 6.23**). Both IL-15- and IL-12/15/18-stimulated NK cells exhibited a specific lysis of around 40% (E:T ratio of 25:1). Seemingly, IL-12/15/18-stimulation did not increase the cytotoxicity towards SK-Mel-28 substantially after more than 16 hours of activation (compare **figure 6.20 E**). However, IL-15-stimulated NK cells were further activated and after 60 hours exhibited similar levels of killing compared to IL-12/15/18-stimulated NK cells. The fact that NK cells after 60 hours of activation with IL-12/15/18 were not exhibiting increased cytotoxicity is intriguing. Possibly, prolonged stimulation further dampened inhibition by cognate HLA molecules via down-regulation of the respective KIRs but also decreased activating signals - via down-regulation of the respective activating receptors – potentially maintaining the ratio of inhibitory and activating signals and thus the lysis efficiency.

As the impact of inflammatory cytokine signaling on the expression of inhibitory KIRs was highly remarkable and so far not described, we wanted to investigate if the decreased KIR levels translate into an increased function of target cells bearing the cognate ligands. Therefore, we made use of 721.221 cells that do not express HLA class I molecules (from now on called 221 cells) or various transductants of 221 cells expressing single HLA alleles. With these cells we performed CD107a mobilization assays and analyzed the results as described previously. Most importantly, the decreased KIR2DL2/L3 levels caused a less pronounced inhibition of KIR2DL2/L3-positive NK cells by C1 ligands (**figure 6.24 D**). As already mentioned, this elevated response against self-HLA expressing cells could have a big impact on NK cell based immunotherapy. For instance, the requirement for suitable donors for allo-HSCT would omit as allo-reactive-like functionality could be introduced in an autologous setting by stimulating NK cells with the three cytokines. Most importantly, one phase I trial featuring IL-12/15/18-stimulated NK cells is already set up under the identifier NCT01898793 at the Washington University School of Medicine in the context of refractory and relapsed AML. It will evaluate the safety of the infusion of IL-12/15/18-activated NK cells as a primary readout and their possible beneficial effect as scondary measures and hence provide the first *in vivo* data generated in human subjects.

7.3.3 IL-12/15/18-stimulated NK cells exhibit stronger anti-tumor effects in vivo

To investigate the impact of the short-term activation of NK cells with IL-12/15/18 in an *in vivo* model, we injected CBGr99-positive melanoma cells together with IL-15- or IL-12/15/18-stimulated NK cells i.v. into NSG mice. Importantly, we found that IL-12/15/18-stimulated cells controlled the engraftment of the melanoma cells in the lungs to a higher degree compared to IL-15-stimulated NK cells (**figure 6.25**). This effect was already observed after 4 days and most likely was due to a) an elevated cytotoxicity of IL-12/15/18-activated NK cells towards SK-Mel-28 cells and b) higher NK cell numbers due to proliferation (not analyzed, compare to **figure 6.19**). It will be interesting to dissect the effects of these two factors.

A combination of the two approaches introduced in this dissertation – CX3CL1-overexpressing tumor cells and IL-12/15/18-stimulated NK cells as effector cells – might be a promising approach. Yet, as CX3CR1 upon IL-12/15/18-stimulation got substantially down-regulated (**figure 6.22**) we did not perform this experiment.

7.3.4 Aspects for clinical translation

Several previous studies showed that the responsiveness of NK cells gradually declined after infusion into tumor patients. In this study, we showed that NK cells activated with IL-12/15/18 exhibited a more sustained reactivity and thus could contribute to more long-lasting NK responses *in vivo*.

Still, several issues need further investigation. First of all, it is to date unclear how the decreased levels of KIR molecules impact the reactivity of stimulated NK cells towards healthy cells. Uninfected and untransformed tissue should theoretically not harbor activating NK cell ligands. However, since inflammatory cytokines might induce low levels of for instance NKG2D ligands in proximity to inflamed tissue, auto-reactivity of KIR^{low} NK cells needs to be excluded prior to clinical application to prevent auto-immunity. Second, we only observed that IFN-γ and TNF-α (not shown) were produced

and secreted at high levels. Yet, in our reductionistic model in which we only transferred NK cells, a cross-talk with other cells is not occuring. Here, it will be important to investigate the impact of elevated levels of IFN-γ on the adaptive immune system. For instance, it potentially potently activates dendritic cells that subsequently drive T cell responses into a Th1 and CTL type of response[434,435]. Importantly, this induction of CTL immunity via DC activation was already reported to be an important feature of IL-18-primed NK cells[436,437]. Indeed, IL-18-stimulation of NK cells after tumor cell contact were shown to attract immature DCs via CCL3 and CCL4 secretion. Subsequently, these helper NK cells induced high expression of CXCR3 ligands in DCs by which CD8-positive T effector cells were recruited and efficiently primed. We further did not perform experiments on the impact of various secreted cytokines on tumor cells. Importantly, it has recently been shown that the presence of the Th1 cytokines IFN-γ and TNF-α can induce senescence in tumor cells[198]. Thus, besides classical cytotoxicity and boosting adaptive responses, the cytokine profile of IL-12/15/18-stimulated NK cells potentially allows them to arrest tumor growth without forming immunological synapses with their targets, rendering them multi-functional in terms of anti-tumor effector mechanisms.

Due to the well-known NK cell-activating properties of IL-12 and IL-18 several studies have been published in which the two cytokines have been combined in *in vivo* models. The injection of therapeutical amounts of the cytokines alone or in combination was reported to cause severe side effects in mouse models and in clinical studies[438–440]. In one setup, 100% of the mice died after 4-8 days of daily injections of IL-12 and IL-18 due to sepsis-like symptoms[439]. Thus, it will be important to either carefully evaluate a proper dosing-schedule or to infuse pre-stimulated, expanded NK cells without the need for exogenous systemic application of IL-12 and IL-18. Importantly, IL-12 and IL-18 also have already been used together in tumor models and proved to be potent inducers of NK cell-depending anti-tumor responses[441]. Of note, in the tumor model mentioned IL-12-expression was restricted to the tumor by genetic means. Therefore these data suggest that the cytokines should not be applied systemically to prevent general inflammation but high levels should be generated exclusively at the tumor site. This could be achieved for instance by using tumor targeted viruses as vectors as introduced in chapter 7.2.4.

In conclusion, stimulation of NK cells with IL-12/15/18 harbors the possibility to generate effector NK cells that respond to low doses of IL-2. Accordingly, these cells rapidly expand when supplied with IL-2 and exhibit sustained reactivity towards various stimuli. Importantly, the unique potential to induce allo-reactive-like behavior in autologous NK cells potentially harbors high relevance for the future design of NK cell-based therapies.

7.4 Therapeutic implications and concluding remarks

With the exception of the treatment of certain hematological malignancies, NK cell-based immunotherapies did only show moderate success[308]. Reasons for the discrepancies between the observed *in vitro* functionality of NK cells against tumor targets and the lack of tumor control *in vivo* could be multifaceted. For instance, autologous NK cells as used in a study performed by Parkhurst *et al.* acquired a self-tolerant and thus quiescent status which was detected by the means of NKG2D downregulation[229]. This could for instance be achieved by a tumor microenvironment enriched for immunoregulatory or -suppressive cells and factors[442–444]. Also, defective homing of cytotoxic effector cells into the tumor could contribute to the low success rate of NK cell-based therapies[224,445].

We wanted to approach these two problems that NK cells in tumor-immunotherapeutical settings are likely to face:

a) Little infiltration into solid tumor tissue. Here, CX3CL1-overexpression in the tumor tissue potentially holds great promise if the patients are selected thoroughly based on tumor-reactivity towards the chemokine;
b) Loss of reactivity due to immunosuppressive milieus *in situ*. We propose IL-12/15/18-stimulation as a superior activation regimen. Not only do NK cells react in a more sustained way towards tumor cells but they also kill tumor targets more efficiently and proliferate robustly upon supplementation with IL-2 or IL-15.

Most importantly, both approaches were tested successfully in pre-clinical *in vivo* mouse models in which for a) upon CX3CL1-expression a better tumor control and higher migration of NK cells into the tumor bearing lungs was detectable and b) IL-12/15/18-stimulated NK cells demonstrated higher efficiency against injected human melanoma cells compared to IL-15-stimulated NK cells. Therefore, we propose that CX3CL1-overexpression *in situ* (for instance via adenoviral tumorspecific vectors) and activation of NK cells with IL-12, IL-15 and IL-18 are promising novel strategies for NK cell based immunotherapy against cancer. These findings should be incorporated into novel strategies harnessing NK cells for immunotherapy of melanoma or malignant disease in general.

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9 ABBREVIATIONS

221	721.221 EBV transformed lymphoblastoid cell line
7-AAD	7-aminoactinomycin D
ACK	Ammonium-Chloride-Potassium
ACT	Adoptive cell therapy
ADAM	A disintegrin and metalloproteinase domain-containing protein
ADCC	Antibody-dependent cytotoxicity
AF647	Alexa Fluor 647
AICL	Activation-induced C-type lectin
AML	Acute myeloid leukemia
AP-1	Activator protein 1
APC	Antigen presenting cells or
ATP	Adenosine triphosphate
BAT-3	HLA-B-Associated Transcript 3
BCA	Bicinchoninic Acid Protein
BCA1	B-cell-attracting chemokine 1
BCG	Bacille Calmette-Guérin
Bcl-2	B-cell leukaemia/lymphoma 2
BCR	B cell receptor
BH3	Bcl-2 homology 3
Blast	Basic local alignment search tool
BLI	Bioluminescent intensity
BP	Binding protein
BSA	Bovine serum albumin
Btk	Bruton's tyrosine kinase
CCRL1	C-C chemokine receptor type 11
CCRL2	C-C chemokine receptor-like 2
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
c-FLIP	Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein
CFSE	Carboxyfluorescein succinimidyl ester
Cm^2	Square centimeter
Cm^3	Cubic centimeter
CMV	Cytomegalovirus
CSF	Cerebrospinal fluid
CT	Computed tomography
CTACK	Cutaneous T-cell-attracting chemokine
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
cpm	Counts per minute
CRP	C-reactive protein
DAG	Diacylglycerol
DAMP	Damage-associated molecular pattern
DAP	DNAX activation protein
DARC	Duffy antigen/receptor for chemokines
DC	Dendritic cell
ddH_2O	Double-distilled H_{2O}
dim	Diminished
DISC	Death-inducing signalling complex
DKFZ	Deutsches Krebsforschungszentrum/German Cancer Research Center
DMEM	Dulbecco/Vogt modified Eagle's minimal essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAM-1	DNAX Accessory Molecule-1

dNTP	Deoxynucleotide
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
ELC	Epstein–Barr-virus-induced gene 1 ligand chemokine
ELISA	Enzyme-linked immunosorbent assay
ENA78	Epithelial-cell-derived neutrophil-activating peptide 78
EOMES	Eomesodermin
ESAM	Endothelial cell specific adhesion molecule
E:T	Effector-to-target
FACS	Fluorescence-activated cell sorting
FcγR	Fragment, crystalizable γ-receptor
FcR	Fragment, crystalizable-receptor
FCS	Fetal calv serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
For	Forward
FSC	Forward scatter
g	Gram(s)
G	Gauge
GAG	Glycosaminoglycans
gah	Goat-anti-human
gam	Goat-anti-mouse
GCP-2	Granulocyte chemotactic protein 2
GDS	GEO data set
GEO	Gene expression omnibus
GLUT-1	Glucose transporter-1
GM-CSF	Granulocyte macrophage colony-stimulating factor
gp100	Glycoprotein 100
GPCR	G protein-coupled receptor
Gro	Growth- regulated oncogene
GvH	Graft versus host
Gy	Gray
h	Hour(s)
HA	hemagglutinin
HBS	HEPES buffered saline
HBV	Hepatitis B virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Her2	Human epidermal growth factor receptor 2
HIF1α	Hypoxia-inducible factor 1 alpha
HIV	Human immunodeficiency virus type-1
HMGB1	High-Mobility-Group-Protein B1
HPV	Human papillomavirus
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
Hsp	Heat shock protein
Hu	Human
huIgG	Human Immunoglobulin
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intracellular adhesion molecule-1
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-γ
IFN-γR	Interferon-γ receptors
Ig	Immunoglobulin
IL	Interleukin

IL-12/15/18	IL-12, IL-15 and IL-18
$InsP_3$	Inositol-1,4,5-trisphosphate
i.p.	Intraperitoneal
IP-10	Interferon- inducible protein 10
iPSC	Induced pluripotent stem cells
IRAK	Interleukin-1 receptor-associated kinase
I-TAC	Interferon-inducible T-cell α chemoattractant
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
IU	International unit(s)
i.v.	Intravenous
IVIS	In vivo imaging system
Jak	Janus kinase
JAM	Junction adhesion molecule
KACL	Keratinocyte-associated C-type lectin
KIR	Killer cell immunoglobulin-like teceptor
KLRG1	Killer cell lectin-like receptor G1
L	Liter(s)
LAK	Lymphokine-activated killer
LB	Lysogeny broth
LFA1	Leukocyte functional antigen 1
LILRB2	Leukocyte immunoglobulin-like receptor B2
LPS	Lipopolysaccharid
mAb	Monoclonal antibody
MACS	Magnetic assisted cell sorting
MAdCAM1	Mucosal vascular addressin cell adhesion molecule 1
Malign.	Malignant
MART1	Melanoma antigen recognized by T cells 1
MBL	Mannose binding lectin
MCA	3-methylcholanthrene
MCP	Monocyte chemoattractant protein
MCMV	Murine cytomegalovirus
MDC	Macrophage-derived chemokine
MDSC	Myeloid-derived suppressor cell(s)
MEC	Mucosae- associated epithelial chemokine
MFI	Mean fluorescence intensity
mg	Milligram(s)
MHC	Major histocompatibility complex
MIC	MHC class I–related chain
µCi	Micro-Sievert
µg	Microgram(s)
MIG	Monokine induced by interferon γ
Min	Minute
MIP	Macrophage inflammatory protein
ml	Milliliter
MLL-5	Mixed lineage leukemia 5
mM	Millimolar
µM	MIcromolar
MMP	Matrix metalloproteinase
moDC	Monocyte-derived dendritic cell
MΦ	Macrophage(s)
MRI	Magnetic resonance imaging
(m)RNA	(messenger) ribonucleic acid
miRNA	Micro RNA
MS	Multiple sclerosis
MTOC	Microtubule-organizing center
MULT-1	Murine UL16-binding protein-like transcript-1
MyD88	Myeloid differentiation primary response gene (88)

NAD^+	Nicotinamide adenine dinucleotide
NAP-2	Neutrophil-activating peptide 2
NCR	Natural cytotoxicity receptors
n.d.	Not detectable
NF-κB	Nuclear factor-κB
ng	Nanogram(s)
NHL	Non-Hodgkin lymphoma
NK cell	Natural killer cell
NKDI	NK cell developmental intermediates
nm	Nanometer
NKR	NK cell receptor
NOD-SCID	Nonobese Diabetic/Severe Combined Immunodeficiency
NSG	NOD-scid gamma
P2A	Porcine teschovirus-1 2A
PAMP	Pathogen associated molecular pattern
PCNA	Proliferating-cell-nuclear-antige
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death ligand 1
PE	Phycoerythrin
PECAM1	Platelet endothelial cell adhesion molecule
PET	Positron emission tomography
PerCP-Cy5.5	Peridinin-chlorophyll-protein-complex-cyanine 5.5
PGE_2	Prostaglandin E_2
PhD	Philosophiae doctor
PI3K	Phosphatidylinositide 3-kinase
PLGF	Placental growth factor
PLN	Peripheral lymph node
PMA	phorbol 12-myristate 13-acetate
pRb	Retinoblastoma protein
proIL-18	IL-18 pro-protein
PRR	Pattern-recognition receptor
PSGL1	P-selectin glycoprotein ligand 1
PTEN	Phosphatase and tensin homolog
Puro	Puromycin
PVR	Poliovirus receptor
RA	Rheumatoid arthritis
Rae-1	retinoic acid early inducible-1
RAG	recombination activating gene
RAGE	Receptor for Advanced Glycation Endproducts
RANTES	Regulated on activation, normal T-cell expressed and secreted
RCC	Renal cell carcinoma
rev	Revers
rIgG	Rat immunoglobulin G
RLU	Relative light unit(s)
ROI	Region of interest
ROS	Reactive oxygen species
rpm	Rotations per minute
RPMI	Roswell park memorial institute medium
RT	Room temperature
s	Second
S1-P5	sphingosine 1-phosphate receptor
s.c.	Subcutaneous
SCGM	Stem cell growth medium
SD	Standard deviation
SDF-1α	Stromal cell-derived factor-1α
SH2	Src Homology 2
SHP	Src homology region 2 domain-containing phosphatase

SLC	Secondary lymphoid-tissue chemokine
SOC	Super Optimal broth with Catabolite repression
SSC	Side scatter
STAT	Signal transducers and activators of transcription
Sr	Steradian
TAE	Tris-acetate-EDTA
TARC	Thymus and activation-regulated chemokine
TCR	T cell receptor
TECK	Thymus-expressed chemokine
Temp	Temperature
TGF-β	Transforming growth factor-β
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIL	Tumor-infiltrating lymphocytes
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TRAF	TNF receptor associated factors
TRAIL	Tumor necrosis factor related apoptosis inducing ligand
T_{reg}	Regulatory T cell
ULBP	UL16-binding protein
UV	Ultraviolet
VCAM1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VIN	Vulvar intraepithelial neoplasia
VLA4	Very late antigen 4
VLP	Virus-like particle
VSV	Vesicular stomatitis virus
VVO	Vesiculo-vacuolar organelles
WBI	Whole body irradiation
w/o	Without
wt	Wild-type

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