NK cells in tumor immune evasion: Role of tumor-associated ligands that regulate NK cell function and therapeutical implications

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1 Zusammenfassung

NK Zellen sind Lymphozyten des angeborenen Immunsystems und spielen eine wichtige Rolle bei der körpereigenen Erkennung und Zerstörung von Tumorzellen, bei Anti-Tumor Effekten nach Knochenmarktransplantationen (HSCT) und somit als Effektorzellen bei Immuntherapien. Neben inhibierenden Rezeptoren steuern aktivierende Rezeptoren wie NKp30, NKG2D und CD16 die Aktivität von NK Zellen. Ziel der Arbeit war es zum molekularen Verständnis der unzureichenden Bekämpfung von Tumorzellen durch NK Zellen beizutragen. Dies wurde anhand von Leukämiezelllinien myeloiden und lymphatischen Ursprungs untersucht, da diese von NK Zellen nach Stammzelltransplantationen je nach Ursprung der Leukämie des Empfängers (myeloid, lymphatisch) sehr unterschiedlich bekämpft werden. Im Rahmen dieser Arbeit konnte gezeigt werden, dass der zytotoxische Rezeptor NKp30 maßgeblich dazu beiträgt, dass NK Zellen myeloide leukämische Zelllinien besser lysieren als Zelllinien lymphatischen Ursprungs. So können Exosomen, die Liganden für NKp30 auf der Oberfläche exprimieren, die Resistenz der lymphatischen Leukämiezellen gegen NK-Zellerkennung und Killing überwinden. Zusätzlich verhindert ein verstärktes inhibitorisches *signaling* durch HLA-Moleküle auf lymphatischen Zelllinien eine effektive Lyse.

Darüber hinaus wurde am Beispiel des Hodgkin Lymphoms (HL) gezeigt, dass NK Zellen der Patienten anders als NK Zellen gesunder Spender nicht in der Lage sind Hodgkin Zelllinien zu eliminieren. Dies konnte auf lösliche Faktoren im Serum der Patienten zurückgeführt werden, die auch die Aktivität gesunder NK Zellen beeinträchtigen. Die Ergebnisse der Arbeit zeigen, dass hierbei die löslichen Liganden MICA für den NKG2D Rezeptor und BAT3 für den NKp30 Rezeptor eine wichtige Rolle spielen. Diese Liganden lösen in membrangebundener Form NK Zell Zytotoxizität aus, während sie in löslicher Form eine inhibitorische Wirkung haben und sogar zu einer verringerten Expression von NK-Zellrezeptoren führen. In der Tat zeigen NK Zellen von HL-Patienten eine niedrigere Expression von NKG2D als NK Zellen gesunder Spender. Nach der Therapie des Lymphoms waren die Serumlevel dieser beiden Liganden wieder auf dem Niveau gesunder Probanden.

NK Zellen in Hodgkin Patienten konnten *ex vivo* und *in vivo* erfolgreich mit einem bispezifischen Antikörper-Konstrukt aktiviert werden, das die NK Zellen via CD16

stimuliert und CD30 als Antigen auf Zielzellen bindet. So konnte experimentell eine sehr effiziente Lyse von Hodgkin Zellen erreicht werden.

Zusammenfassend zeigen die Daten, dass die Expression aktivierender Rezeptoren (NKp30 und NKG2D) und der korrespondierenden Liganden Phänotyp und Funktion der NK Zellen beeinflusst und zur Immun-Evasion beiträgt. Die Inhibition der NK Zellen ist jedoch reversibel und kann mit therapeutischen Antikörpern überwunden werden.

NK Zellen stellen somit einen vielversprechenden Ansatz dar, Immuntherapien auf zellulärer Ebene als Kombination oder Alternative zu Standardtherapien zu entwickeln.

2 Abstract

NK cells are lymphocytes of the innate immune system and play a crucial role in tumor immune surveillance, in beneficial graft versus tumor effects upon hematopoietic stem cell transplantations (HSCT) and thereby as effectors in cellular immunotherapies. NK cells are regulated by inhibitory receptors mostly binding to HLA molecules and activating receptors like CD16, NKG2D and NKp30. Aim of this work was to contribute to the molecular understanding of the insufficient control of tumor cells through NK cells. This was examined on the basis of leukemic cell lines with myeloid or lymphatic origin, since these cells, according to their origin, are recognized and killed very differently by donor NK cells upon stem cell transplantation. In this study we could show that the cytotoxic receptor NKp30 contributes decisively to a better lysis of myeloid leukemic cell lines compared to lymphatic leukemic cell lines. Thus, exosomes expressing ligands for NKp30 can help to overcome the resistance of lymphatic leukemia cells against NK cell recognition and killing. Additionally an increased inhibitory signaling via HLA molecules on lymphatic cell lines prevents a more efficient lysis.

Moreover, it was shown in the setting of Hodgkin lymphoma (HL) that patients´ NK cells in contrast to healthy NK cells are not able to lyse Hodgkin cell lines. Soluble factors in the patients´ sera were identified to be responsible for that. Of note, patient sera also suppressed cytotoxic activity of NK cells from healthy donors by downregulation of activating receptors. Results indicate that the soluble ligands MICA for NKG2D and BAT3 for NKp30 play an important role. While these ligands trigger cytotoxicity in their membrane bound form, as soluble factors they have inhibitory impact and can downregulate NK cell receptors. Indeed, NK cell derived from HL patients showed a lower expression level of NKG2D than healthy NK cells. After therapy and clearance of the tumor, serum levels of these soluble ligands were comparable to healthy donors again.

Furthermore, NK cells in HL patients were successfully reactivated *in vivo* and *ex vivo* with a bispecific antibody construct. This construct stimulated NK cells via CD16 and binds CD30 as an antigen on target cells. Thereby, a very efficient lysis of Hodgkin target cells was achieved in experiments.

Taken together, the data indicate that the increased expression of tumor associated ligands for NKG2D and NKp30 in the serum of HL patients influences the phenotype and the function of HL NK cells and hence contributes to a tumor immune evasion. However,

the inhibition of NK cells is reversible and can be overcome with therapeutical bispecific antibodies.
Thus, NK cells seem to be a promising approach for cellular based immunotherapies as an alternative to standard therapies.

3 Introduction

3.1 Natural killer cells

3.1.1 NK cells and their function in immunity and immunotherapies

Natural killer cells (NK cells) belong to the group of lymphocytes (T and B cells) and as such they arise from progenitor cells in the bone marrow. In contrast to T cells and B cells, NK cells do not have several maturation steps in a primary lymphoid tissue. As they also lack antigen specific receptors, they are attributed to the innate immune system (Vivier, Tomasello et al. 2008). NK cells are located largely in the spleen, but also in lymph nodes and in the peripheral blood (Witte, Wordelmann et al. 1990; Fehniger, Cooper et al. 2003). They contribute with approximately 10 % to the peripheral blood mononuclear cells (PBMCs) and were originally defined through their capability of killing virally and tumor transformed cells (Lanier 2005). They play a crucial role in tumor immune surveillance, as they monitor self-cells for any aberrant expression patterns, that indicate stressed or transformed cells.

With T-lymphocytes NK cells share two functions that are crucial for an intact immunity: toxicity against target cells and the secretion of cytokines (Biron, Nguyen et al. 1999). Via their receptors, NK cells can distinguish healthy from non-healthy cells which they can lyse without prior antigen stimulation (Smyth, Cretney et al. 2005). A unique way of lysing transformed cells is the antibody dependent cellular cytotoxicity (ADCC). CD16 (FcγRIII) on NK cells binds the Fc-part of antibodies coating target cells and thereby leads to an activation and lysis (Newman and Riley 2007). For both ways, NK cells use receptors and adhesion structures to build an immunological synapse. This enables the NK cell to exert a directed secretion of cytolytic vesicles containing perforins and granzymes (Orange 2008; Vivier, Tomasello et al. 2008).

Besides that, NK cells can be activated and recruited by antigen presenting cells (APCs) via various chemo- and cytokines (Walzer, Dalod et al. 2005; Lucas, Schachterle et al. 2007). In addition, a crosstalk between NK cells and dendritic cells (DCs) has shown an influence on adaptive immune reactions (Ferlazzo, Tsang et al. 2002; Vitale, Della

Chiesa et al. 2005), as well as the NK cell based secretion of pro inflammatory cytokines like IFN-γ and TNF-α (Cooper, Fehniger et al. 2001).

In some malignancies, NK cells fail to exert their function of surveying stressed and transformed cells. Hodgkin Lymphoma (HL) (see 3.4.2) is a good example for this failure, as NK cells are present in the surrounding tumor tissue, but are obviously not able to control emergence and development of the tumor
Thus, NK cells have become a popular target, when it comes to cellular based immunotherapies. There are several strategies of harnessing and improving NK cell functions in cancer. Some of them aim at trafficking of NK ells, others at susceptibility of target cells towards NK cell mediated apoptosis. But most of those strategies, like in this study, try to modulate the balance between activating and inhibiting receptor signaling of NK cells (see 3.1.2).

3.1.2 Regulation of NK cell activity

With the ability to kill target cells without any antigen specific stimulation or priming, it is important that NK cells can distinguish between healthy and stressed cells. The basic principle is to avoid killing of normal cells, while transformed cells are recognized and eliminated (Lanier 2005). The two regulatory mechanisms comprise “missing-self recognition” and “induced-self recognition” (Fig 3.1). Signal integration of both pathways determines the regulation of the NK cell (Bryceson and Long 2008).

“Missing self” describes the lack of protection of self-cells through a downregulation of HLA class I molecules. Normally, these molecules provide a protective signaling via binding to inhibitory receptors (mainly Killer immunoglobulin like receptors, KIRs) on the surface of the NK cells. However, on transformed cells class I molecules are less expressed on the surface and therefore do not deliver an appearance of a healthy self-cell (Ljunggren and Karre 1990; Bryceson and Long 2008).
“Induced self” in contrast delivers triggering signals via a variety of activating NK receptors, e.g. NKG2D, CD16 and NCRs (NKp30, NKp44 and NKp46, see 3.1.3), that leads subsequently to the elimination of the target cell. On transformed cells, the ligands

for these activating receptors are often upregulated (Groh, Rhinehart et al. 1999; Moretta, Bottino et al. 2001). Since the “missing self” is not necessarily sufficient for lysis of the target cells, the “induced self” signaling finally leads to the activation of the NK cell.

Besides the integration of “missing self” and “induced self”, also synergistic effects among activation receptors seem to be important, the binding of one receptor is not necessarily sufficient. (Bryceson, Ljunggren et al. 2009).

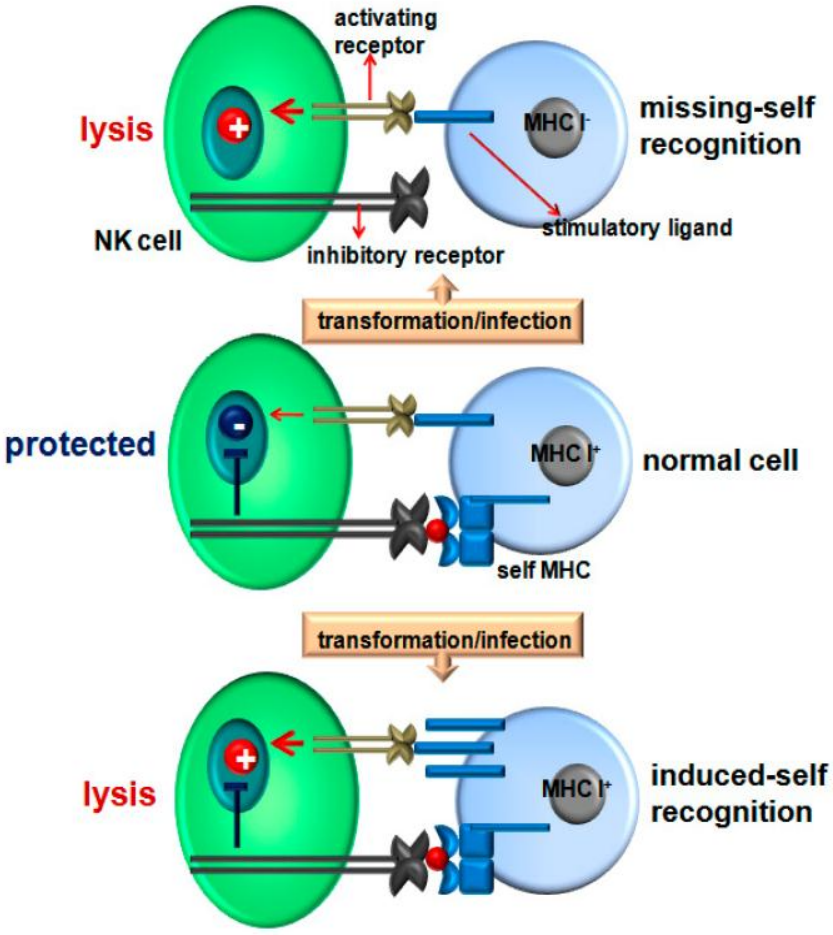


Figure 3.1: Regulation of NK cell activity (Simhadri, 2008)

“Missing-self” and “Induced-self” contribute to the regulation of lysing activity toward a target cell. Inhibitory receptors recognize self MHCs. This negative signaling protects the cell from being killed (middle), unless it is “overruled” by the binding of inducible ligands to activating receptors (bottom). A lack of inhibitory signaling leads to an activation of NK cells (top).

3.1.3 NK cell receptors and their ligands

Decisive for the protection of normal self-cells from NK cell based attacks is the recognition and binding of inhibitory receptors to mainly the polymorphic HLA class I molecules (Ljunggren and Karre 1990) but also other surface molecules like sialic acid or cadherins. Killer cell immunoglobulin-like receptors (KIRs) provide the biggest and obviously most important group of inhibitory receptors that bind to different alleles of HLA class I molecules. (Vivier, Tomasello et al. 2008). They belong to the C-type lectin superfamily and are characterized by immune receptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic moiety. The tyrosine phosphorylation by src kinases subsequent to the ligand interaction leads to the recruitment of SHP-1 and-2 phosphatases. Eventually this results in suppressed cytotoxicity and secretion of cytokines (Lanier 1998).

Mandatory for triggering NK cell effector functions are the activating NK cell receptors. The apparently most important and best examined receptors are the low affinity Fc receptor CD16 or FcγRIII, NKG2D as well as NKp30, NKp44 and NKp46, termed Natural Cytotoxicity Receptors (NCRs).
CD16 binds to the Fc part of IgG antibodies that coat antigens on target cells. Upon ligation, CD16 binds to adapter proteins, whose phosphorylated tyrosines in the immune receptor tyrosine-based activation motif (ITAM) become a target for src family tyrosine kinases (Vivier, Morin et al. 1991). This activates downstream signaling and leads to cytokine secretion and cytotoxicity. Besides that, CD16 is also reported to have broader activating potential in direct NK cell cytotoxicity, independent of antibody binding (Mandelboim, Malik et al. 1999). CD16 also plays an important role in classifying NK cell into subsets: $CD56^{bright}CD16^{dim}$ NK cells show an enhanced cytokine production, while $CD56^{dim}CD16^{bright}$ NK cells produce more cytolytic factors and are describes to be mainly responsible for cytotoxicity (Vivier, Tomasello et al. 2008). Furthermore, CD16 seems to be a receptor that needs little or no support from other activating NK receptors in terms of overcoming the threshold for activation (Bryceson, March et al. 2005; Bryceson and Long 2008; Bryceson, Ljunggren et al. 2009). This makes CD16 a very feasible target for immunotherapeutic approaches and therefore a key receptor in this work.
NKG2D, another well described activating receptor, is expressed on NK cells, but also on CD8+ T cells as a co-receptor (Moretta, Bottino et al. 2001). Its intracellular domain

has no ITAM motif, therefore transmembrane adapter molecules like DAP10 are required. DAP10 is phosphorylated by the phosphatidylinositol-3- kinase (PI-3K) that leads to a cascade ending up with calcium flux and triggered cytotoxicity (Upshaw, Arneson et al. 2006). Known ligands for NKG2D are the MHC class I related molecules MICA and MICB induced upon cellular stress and the UL-16 binding proteins, which are upregulated on some tumor transformed cells (Bauer, Groh et al. 1999; Cosman, Mullberg et al. 2001). By shedding these ligands for NKG2D from the surface, tumor cells exhibit an evasion strategy to escape the immune surveillance of NK cells (Waldhauer and Steinle 2006; Waldhauer, Goehlsdorf et al. 2008; Nuckel, Switala et al. 2010).

More recently, the group of Natural Cytotoxicity Receptors (NCRs) has been defined, comprising NKp46 (NCR1), NKp44 (NCR2) and NKp30 (NCR3). These three receptors are exclusively expressed on NK cells, among which NKp44 only appears on activated NK cells (Sivori, Vitale et al. 1997; Vitale, Bottino et al. 1998; Pende, Parolini et al. 1999). In contrast to NKG2D, but similar to CD16, NCRs associate with ITAM-carrying factors like CD3ζ or FcεRIγ. Via phosphorylation of these ITAMs through Zap70 or Syk, a cascade leads to degranulation of cytokines or cytolytic factors (Moretta, Bottino et al. 2001; Andre, Castriconi et al. 2004). Only few ligands for the NCRs have been identified to date. Viral hemagglutinins bind to NKp46 and NKp44 and the cytomegalovirus tegument protein pp65 binds to NKp30 having an inhibitory effect. (Mandelboim, Lieberman et al. 2001; Arnon, Achdout et al. 2005). Only little information on the cellular ligands for the NCRs has been revealed until now. Both the HLA-B associated transcript 3 (BAT3) and the B7 family member B7-H6 binds to NKp30 and so far unknown ligands for NKp46 are expressed on pancreatic β-cells (Pogge von Strandmann, Simhadri et al. 2007; Brandt, Baratin et al. 2009; Gur, Porgador et al. 2010).

Interestingly, NK cells have a “cross-talk” with Dendritic cell (DCs) via NKp30. They can either stimulate or kill DCs and hence have a direct influence on adaptive immunity (Ferlazzo, Tsang et al. 2002; Vitale, Della Chiesa et al. 2005).

3.1.4 NK cells and their role in hematopoietic stem cell transplantation

In order to replace the hematopoietic system, a leukemic patient often gets a transfer of stem cells following chemotherapy and irradiation. If the donor is for example a sibling or even an unrelated donor, the process is termed allogeneic hematopoietic stem cell transplantation (HSCT). In this setting two reactions can occur: the welcome Graft-versus-leukemia effect (GVL) and the adverse Graft-versus-host disease (GVHD).

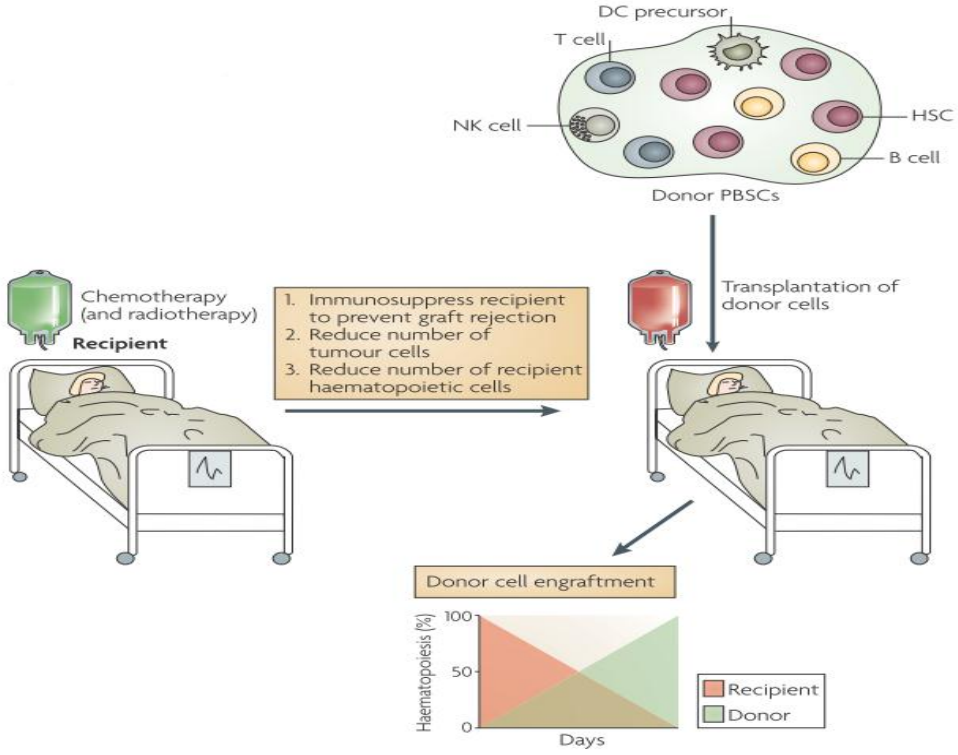


Figure 3.2: Allogeneic stem cell transplantation (Shlomchik 2007), modified

The recipient receives chemo- and radiotherapy in order to kill tumor and to destroy the old "defect" hematopoietic system. This is replaced by the transplant containing hematopoietic stem cells (HSC) and various immune cells including NK cells. After several weeks, hematopoiesis is completely done by cells and tissue of the donor´s transplant.

GVL is mediated by cytotoxic CD8+ T lymphocytes (CTLs) as well as alloreactive NK cells (Ruggeri, Capanni et al. 1999; Pegram, Ritchie et al. 2011). These donor cells recognize tumor cells as foreign in the host and kill them due to their cytotoxic ability.

This can support the treatment thereby making making less severe regimen (irradiation and chemotherapy) possible. More importantly the probability of a relapse can be reduced. (Parham and McQueen 2003).

The main adverse reaction of allogeneic HSCT leads to the GVHD. Donor`s alloreactive CD8+ T cells not only recognize malignant cells in this case, but also attack healthy tissue. In acute GVHD, most affected organs are the liver and skin. With immune suppressive agents or depletion of T cells from the graft, this side effect can be diminished. But T cell mediated GVL is also abrogated (Shlomchik 2007).

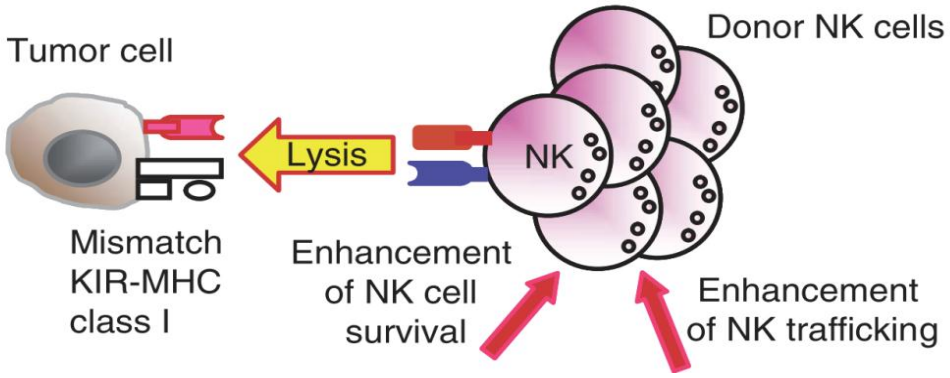


Figure 3.3: Alloreactive NK cells after HSCT (Terme, Ullrich et al. 2008)

Through the mismatch of the KIR on donor´s NK cells and recipient´s MHC on the tumor cells, NK cells become activated. They can kill the tumor cell and proliferate for alloreactive NK cell clones with enhanced trafficking.

The group of L. Ruggeri et al could elucidate the role and the efficacy of alloreactive NK cells in the setting of allogeneic HSCT (Table 3.1). Here, patients with either acute myloid leukemia (AML) or acute lymphoid leukemia (ALL) received transplants mismatched in one HLA haplotype. Due to typing of recipients` HLA-C locus, donor-recipient pairs could be divided into two groups: either with or without KIR-ligand incompatibility. Only in the latter setting, alloreactive NK cell clones occur (Fig. 3.2). Of recipients with AML and a KIR-ligand incompatibility, none experienced a relapse within five years. In contrast, three of four AML patients without that incompatibility and thus not benefiting from NK cell alloreactivity sustained a relapse. This positive effect was

restricted to AML patients. For recipients with ALL, no impact on relapse rates could be observed.

One of the aims of this work is to find putative reasons for these differing cytotoxic abilities of NK cells towards distinct leukemic cells.

Interestingly, NK cell alloreactivity also seems to be responsible for the reduction of GVHD incidence. The reason for this might be the crosstalk and subsequent killing of DCs through NK cells. This might interrupt the cascade of cytokine signaling and interaction between DCs and T cells that normally leads to severe Graft versus host reactions (Ruggeri, Capanni et al. 2002; Parham and McQueen 2003).

Table 3.1: Transplantation outcome in HLA haplotype mismatched transplants with and without KIR ligand incompatibility. (Ruggeri, Capanni et al. 2002), modified

	KIR ligand match	KIR ligand incompatibility
Transplants	58	34
Rejection	15,5 %	0*
GVHD	13,7 %	0*
Relapse ALL	90 %	85 %
Relapse AML	75 %	0**

3.2 HLA-B associated transcript 3 (BAT3)

BAT3 is a gene located in the MHC III cluster on chromosome 6 together with a variety of immunological important factors like the heat shock protein HSP70 or the tumor necrosis factors α and β (TNF α, β). Also the already mentioned NCRs and the NKG2D ligands MICA and MICB are encoded in this cluster. The full length transcript of BAT3 has 3.4 kb and a molecular weight of approximately 130 kDA. Due to its nuclear localization sequence, the protein is predominantly located in the nucleus. Under circumstances and by mechanisms, which are not completely understood yet, it can be released from cells to the extracellular environment. Important for this translocation might be the BAG domain, encoded next to the NLS at the C-terminus. This domain indicates that BAT3 interacts with heat shock proteins of the HSP70 family. (Banerji, Sands et al. 1990; Sasaki, Gan et al. 2007)

For this work, the most important function of BAT3 is the ability to bind and to trigger the activating NK receptor NKp30. For this BAT3 must be released from tumor cells (or also DCs). This happens via the secretion of exosomes (see below), small membrane vesicles that express BAT3 on their surface. Besides this membrane bound form, BAT3 can also appear in a soluble manner, for example in sera of CLL patients. Only the surface-associated protein can activate NK cells, while the unbound BAT3 seems to have a contrary effect and decreases NK cell activity (Pogge von Strandmann, Simhadri et al. 2007; Simhadri, Reiners et al. 2008).

3.3 Exosomes

Exosomes are 50-100 nm small membrane vesicles originating in the endosomal system and secreted by probably all cell types.

Formation of exosomes occurs in so called multi vesicular bodies (MVBs) developing from late endosomes. These MVBs fuse with the plasma membrane to release their exosomes to the extracellular environment.

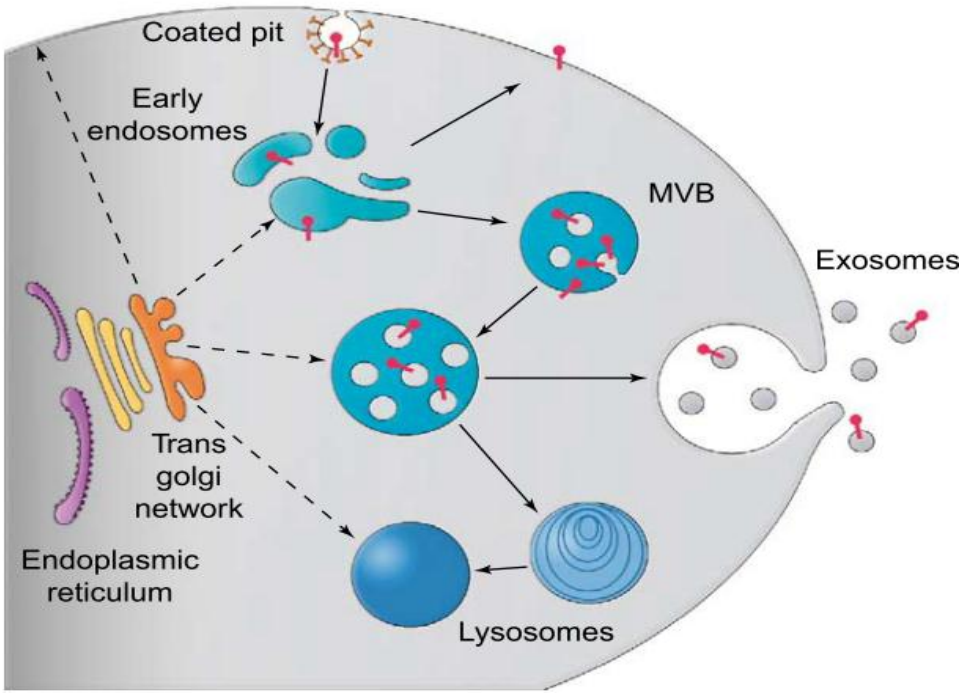


Figure 3.4: Formation of exosomes (Fevrier and Raposo 2004)

Multivesicular bodies (MVBs) are part of the endosomal system. Through budding into the inside, exosomes are generated from the membrane of these MVBs. Later the MVBs merge with the plasma membrane and exosomes are released.

A broad range of molecules is hereby expressed on the surface of the exosomes for example immunological important molecules MHC class I, adhesion molecules like ICAMs and integrins or chaperones like HSP70. Interestingly, after a stress induction like a non-lethal heat shock, release of exosomes can be increased (Simhadri, Reiners et al. 2008) (van Niel, Porto-Carreiro et al. 2006). Depending on the cell type, the expression pattern varies. For instance the tetraspanine CD63 serves as a control marker for the purification of exosomes from the HEK cell line 293T, however it is absent in exosomes of other cell lines.

Functions of exosomes are poorly understood. A main task seems to be the transport of intracellular factors to the extracellular environment. Here, several aims are assumed, ranging from a simple transport and removal of “old” proteins to cell-cell communication. In the case of CD8+ T cells, exosomes can even convey cell death via transporting cytotoxic granules. (Herz, Pardo et al. 2009; Thery, Ostrowski et al. 2009). Exosomes

from tumor cells can carry molecules that account for the recognition of immune cells, for example NK cells. According to their different expression of surface markers, functions can be completely contrary. Exosomes with a high expression of HSP70 can cause enhanced lysis compared to exosomes expressing less HSP70. Other cancer cells like the breast cancer cell line T47d are even capable of diminishing cytolytic activity of NK and T cells via their exosomal release (Clayton and Tabi 2005; Gastpar, Gehrmann et al. 2005).

Ligands for activating NK cells receptors have also been found on exosomes, for instance as the above mentioned BAT3 as a ligand for NKp30 (Simhadri, Reiners et al. 2008). In this thesis, exosomes serve as a tool for carrying membrane associated BAT3. Since for this ligand it is crucial whether it is soluble or not, exosomes are the appropriate vehicle to activate NK cells via NKp30.

3.4 Malignancies suitable for NK cell based immunotherapies

3.4.1 Acute and chronic leukemias

Leukemic diseases are subdivided into a big number of different malignancies. They are often differentiated by their origin and their course. Four of the most common leukemias are the acute myeloid leukemia (AML), the chronic myeloid leukemia (CML), the acute lymphoblastic leukemia (ALL) and the chronic lymphocytic leukemia (CLL). ALL and CLL arise from aberrant cells of the lymphoid side of the hematopoietic system: T cells, B cells and their progenitors. ALL cells are fast proliferating progenitors of B or T cells in the bone marrow. Depending on the stage and characteristics of the malignant cell population, further subdivisions are done. One peak of incidences occurs in childhood at an age of 2-5. AML derives from myeloid blood cells like granulocytes or monocytes. There are several subcategories, basically depending on the cell type the leukemia originated from (Bennett, Catovsky et al. 1976). Its incidence increasess with age and the main symptom is a replacement of healthy bone marrow by the myeloblasts, the malignant cells in AML. Like for ALL patients, an HSCT is a common treatment to replace the defect hematopoietic system.

3.4.2 Hodgkin lymphoma

Hodgkin lymphoma (HL) is a subtype of malignant lymphomas that occurs to a great extent at two peaks: at an age of 15-35 years and over 55 years. Main characteristic is the presence of Reed-Sternberg cells (HRS cells). These large cells contain several nuclei and are usually derived from B lymphocytes. A further hallmark of HL is a large number of immune cells surrounding the HRS cells. All types of lymphocytes plus other cells like macrophages, stromal cells, eosinophils or mast cells contribute to approximately 98-99 % of the tumor (Pileri, Ascani et al. 2002). The main reason for the great number of immune cells seems to be the secretion of a variety of chemokines and cytokines that provide a micromilieu of a maintaining inflammation (Fig 3.4). Despite this great majority of potential tumor threatening cells and a persisting inflammatory milieu, the HRS cells are able to fully evade immune responses.

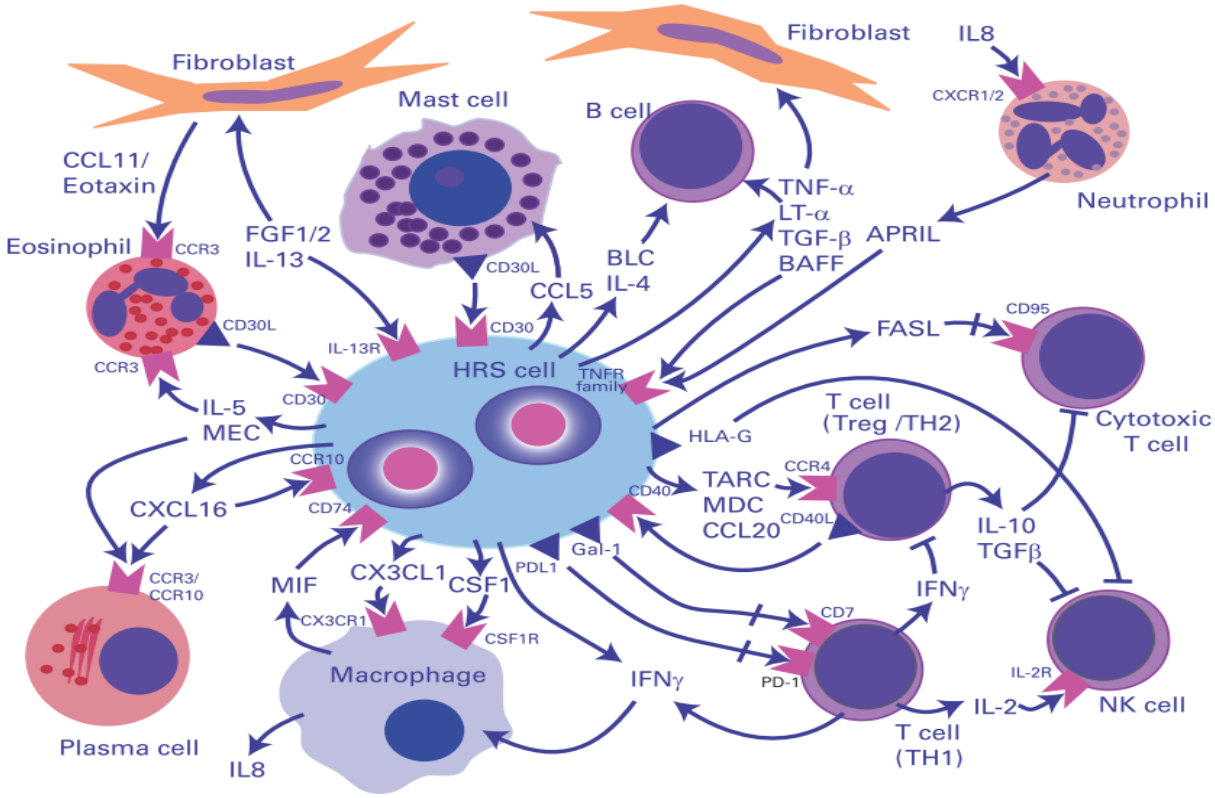


Figure 3.5: Microenvironment and crosstalks in Hodgkin lymphoma (Steidl, Connors et al. 2011)

The binucleated HRS cell is surrounded by bystander cells that are attracted by chemokines. These cells themselves secret further chemokines and cytokines to shape the homeostasis of the infiltrating immune cells.

This attenuation of immunological reactions is not only evoked by the tumor cells, but also by a complex and not fully understood crosstalk among the immune cells themselves (Steidl, Connors et al. 2011). Thus, T helper 2 cells and regulatory T cells account for the most bystander cells in HL, especially in direct proximity to the HRS cells (Ma, Visser et al. 2008). HRS cells also attract macrophages via the secretion of for example colony stimulating factor (CSF1) and other chemokines. Macrophages are believed to be responsible for suppressing antitumor immunity and therefore supporting tumor progression (Qian and Pollard 2010). They also attract further immune cells like neutrophils through IL-8 secretion (Foss, Herbst et al. 1996).

In general, soluble and also shed factors therefore seem to play an important role in the mechanisms of HL tumor formation. One example for this is the macrophage migration inhibitory factor (MIF). MIF is involved in angiogenesis, tumor cell migration and apparently suppresses p53 activity (Hudson, Shoaibi et al. 1999; Fingerle-Rowson, Petrenko et al. 2003). In HL, on the one hand it binds CD74 (the invariant chain of pre MHC class II) on the surface of HRS cells. This protein is implicated in signaling for lymphocyte activation processes (Leng, Metz et al. 2003; Stein, Qu et al. 2004). On the other hand with regard to the role of NK cells in HL, MIF might be involved in tumor escape mechanisms. In ovarian cancer for example, it has been shown, that MIF leads to a downregulation of NKG2D on NK cells and thus might affect anti-tumor activities.

Accordingly, one aspect in this work is the influence of soluble factors or serum of HL patients respectively on NK cell activity and hence a putative reason for impaired anti-tumor efficacy.

With respect to NK cell based immunotherapies, the (re-)activation via activating NK receptors is the second focus of this part.

3.5 Bispecific antibodies and immunoligands

The concept of bispecific antibody and immunoligands therapies is almost as old as the idea and the application of monoclonal antibody therapies. It is based mainly on the principle of retargeting and activation of immune effector cells. For this aim, one moiety of the structure binds to an activating receptor on an effector cell, the other one to an antigen on a target cell. In case of bispecific antibodies, one of the two different antigen determining sites recognizes the target cell, the other one the effector cell. For cytotoxic T lymphocytes, CD3 has become a popular target for bispecific antibodies to be addressed.

Unfortunately, bispecific antibodies go along with a rather low efficacy and some adverse reactions like immunogenicity. (Muller and Kontermann 2010).

As an advanced approach while keeping the principle way of function, bispecific antibody engineering created a broad range of different molecules distinguishable in size and number of binding domains.

So called immunoligands share their basic task with bispecific antibodies, but consist of different structures. Usually the antigen binding moiety is a single chain variable fragment (scFv), consisting of the variable domains of the light and the heavy chain derived from an antibody. The effector cell binding moiety can also be a scFv or a natural ligand to the targeted receptor like in the “ULBP2-BB4” construct. In this bispecific protein, ULBP2 engages NKG2D while the scFv BB4 binds to CD138 on multiple myeloma cells. This led to an activation of NK cells and an effective lysis of CD138 positive multiple myeloma cells (von Strandmann, Hansen et al. 2006).

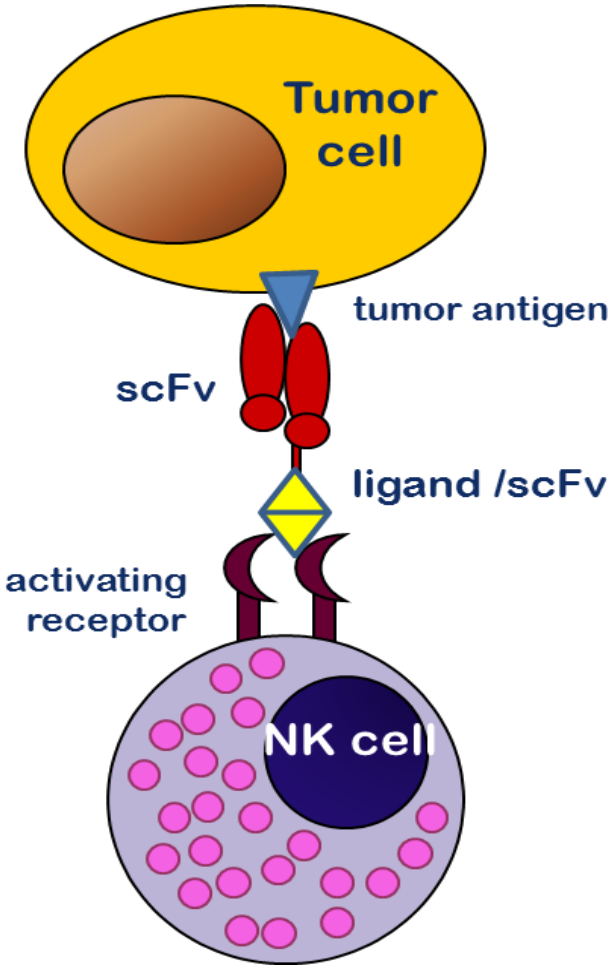


Figure 3.6: Function of immunoligands

Immunoligands bind to target cells and NK cells simultaneously through their two distinct binding moieties. A ligand a single chain variable fragment (scFv), respectively binds an activating receptor on the surface of the effector cell while another scFv binds specifically an antigen on the target cells. Due to that, NK cells are relocated to target cells and trigger cytotoxic activity.

3.6 Aim of the current study

NK cells play an important role in both immune surveillance of tumors and cellular based immunotherapies including the adoptive transfer of allogeneic NK cells in HSCT (Smyth, Hayakawa et al. 2002; Terme, Ullrich et al. 2008; Waldhauer and Steinle 2008). In some settings and under not fully understood preconditions, NK cells fail to execute their function in killing malignant and transformed cells.

For instance, NK cells have the ability to destroy leukemic cells with a lymphatic origin (ALL) upon allogeneic stem cell transplantation. In contrast, myeloid leukemic cells (AML) are resistant to NK cell mediated cytotoxicity.

In the autologous setting, Hodgkin lymphoma is a good example for absent immune surveillance as NK cells are present in the HL microenvironment but fail to control malignant HRS cells.

Aim of this study was (I) to identify molecules involved in the impaired receptor-ligand based target cell recognition for example contributing to different lysis capability against AML and ALL cells in HSCT settings. The main focus laid on differences in target cell phenotypes and their influence on NK cell activity and strategies to overcome tumor cell dependent defects of NK cells.

Furthermore (II) potential reasons for an obvious failure of tumor immune surveillance of NK cells should be illuminated in the setting of HL. Here the state, phenotype and activity of peripheral NK cells was examined and compared to healthy NK cells as well as possible causes for impairments of HL NK cells.

(III) Additionally to the task of finding underlying reasons for deficient NK cell activity, approaches to overcome these handicaps were identified and investigated in order to develop innovative NK cell based immunotherapies.

4 Material and Methods

4.1 Materials

All salts, chemicals and reagents were purchased from Roth (Karlsruhe), Merck (Darmstadt), Sigma-Aldrich (Munich), Invitrogen (Karlsruhe) and BD Bioscience (Heidelberg). Media for cell culture was purchased from GIBCO®-Invitrogen (Karlsruhe). Technical devices are mentioned at dedicated sites.

Table 4.1: Standard buffers

Buffer	Constitution
Blot buffer	3g Tris Base +14,4g Glycin +200ml Methanol +800ml H_2O 250mM Na_2HPO_4
Elisa buffer Coating Blocking Washing	 8,4g $NaHCO_3$ + 3,4g Na_2CO_3 in 1L, pH 9,5 1% BSA in PBS oder 10% FCS in PBS 1 x PBS + 0,05% Tween20
FACS buffer	1 x PBS + 0,2% BSA + 0,2% Sodium azide
LB agar	LB Medium + 1,5% Agar
LB medium	1% Trypton + 1% NaCl + 0,5% Yeast extract
10 x PBS	1,37 M NaCl + 27 mM KCL + 20mM KH_2PO_4 + 100 mM Na_2HPO_4 x 2 H_2O
50 x TAE buffer	2 M Tris acetat +50mM EDTA pH 8.0

4.1.1 Cell Lines

Table 4.2: Cell lines and their physiological origin.

Cell Line	Origin
293T	human fibroblast kidney cell line
697	human B cell precursor leukemia (B-ALL)
HL-60	human acute myeloid leukemia (AML)
K562	human chronic myeloid leukemia (CML)
L428	human hodgkin lymphoma
L540	human hodgkin lymphoma
MOLT-3	human T cell leukemia (T-ALL)
NALM-6	human B cell precursor leukemia (B-ALL)

4.1.2 Vectors

Table 4.3: Vectors for DNA cloning

Vector	Tag	Prokaryotic /eukaryotic selection marker
pcDNA3.1 His	HIS	Ampicillin
pL III	HIS	Ampicillin / Zeozin

4.1.3 Antibodies

Table4.4: Antibodies

Antigen	Fluorchrome	Company	Reactivity	Species
CD3	APC	BD Biosciences	human	mouse
CD3	PE	BD Biosciences	human	mouse
CD9	purified	BD Biosciences	human	mouse
CD16	PE	BD Biosciences	human	mouse
CD16	APC	BD Biosciences	human	mouse
CD45	PacificBlue	Biolegend	human	mouse
CD56	PE	BD Biosciences	human	mouse
CD56	APC	BD Biosciences	human	mouse
CD56	AlexaFl.-647	Biolegend	human	mouse
CD86	purified	Immuno Tools	human	mouse
CD86	FITC	BD Biosciences	human	mouse
CD86	PE	BD Biosciences	human	mouse
CD107a	PE	BD Biosciences	human	mouse
NKp30	PE	Beckman Coulter	human	mouse
NKp44	PE	Beckman Coulter	human	mouse
NKp46	purified	BD Biosciences	human	mouse
NKp46	PE	Beckman Coulter	human	mouse
NKp46	APC	BD Biosciences	human	mouse
NKp46	AlexaFl-.647	Biolegend	human	mouse
hNKG2D	PE	BD Biosciences	human	mouse

NKG2D	Fitc	Abcam	human	mouse
hNKG2A	PE	BD Biosciences	human	mouse
HLA-ABC	purified	BD Biosciences	human	mouse
HLA-DR	purified	BD Biosciences	human	mouse
HLA-ABC	PE	BD Biosciences	human	mouse
HLA-DR	FITC	BD Biosciences	human	mouse
IgG Fc	purified	Dianova	human	goat
IgG	purified	Dianova		goat
IgG H+L	purified	Dianova	mouse	goat
IgG	PE	BD Biosciences	human	mouse
human IgG1	PE	BD Biosciences	mouse	goat
IgG	APC	BD Biosciences	human	mouse
7-AAD	7-AAD	BD Biosciences		
Annexin V	PE	BD Biosciences		
anti-mouse	FITC	BD Biosciences	mouse	goat
anti-mouse	PE	Dako	mouse	goat

4.2 Methods

4.2.1 Standard molecular biological methods

DNA digestion using restriction enzymes

For the cloning of DNA fragments as well as for the testing of plasmids, DNA was digested with appropriate restriction enzymes. For that, few µg were incubated with reaction buffer and 10 units of one or two enzymes and incubated for two hours at a temperature appropriate for the used enzyme. After that, successful cleavage of DNA was tested in an agarose gel.
For cloning scCD16-Immunoligands, scCD16 was designed with 5` NheI restriction site (GCGCTAGC) and a 3` SfiI restriction site (GGCCCAGCCGGCC). The enzymes NheI and SfiI (Fermentas) were used to excise scCD16opt DNA fragment from its origin pBluescript II SK+ vector. Destination vector was “pMS III” already containing the single chains BB4 and RFT5 respectively. This vector was also cut with these two enzymes to open a gap 5` of those single chains for ligating scCD16.

Purification of DNA fragments in agarose gels

Through restriction enzymes digested DNA fragments were separated in a standard 1% agarose gel (TAE buffer, ethidium bromide) with 120 Volts for approximately 30 min. Bands were then excised from the gel with a clean scalpel and eluted with GeneJet™ Gel extraction kit of Fermentas and resolved in water.
For ligations of DNA fragments into vectors, the Rapid DNA ligation Kit (Fermentas) was used according to the manufacturer’s protocol

Bacteria and media

The chemocompetent *E.coli* strain XL10 Gold was used for all cloning strategies. Standard LB media was used with Ampicilin (100 µg/ml) according to the resistance given by the vector. Bacteria were incubated over night at 37°C and 150 rpm.

Transformation of chemocompetent bacteria

To transform chemo competent Bacteria, either 5µl ligation product or 1 µl plasmid were added to 50 µl thawed (on ice) bacteria solution and incubated on ice for 20 minutes. Following a 45 seconds heat-shock at 42°C, bacteria were transferred back on ice and subsequently plated on plates for selection and incubation over night at 37°C.
The next day, clones were picked and used for inoculation of 3ml LB medium for Mini-preparations or 100 ml for Maxi-preparations of plasmid DNA.

Preparation of plasmid DNA

For the preparation of plasmid DNA, either the GeneJET™ Plasmid Miniprep Kit for Minipreps (Fermentas) or the NucleoBond® Xtra Maxi kit for Maxipreps (Macherey-Nagel) were used according to the manufacture`s protocols.
Concentrations of purified plasmids were determined and the DNA was used for transient transfection of 293T cells (human embryonic kidney cell line).

Sequencing of clonings

Samples of DNA were sent to Starseq® in Mainz according to default requirements of the company.

4.2.2 Cell biological, biochemical and immunological methods

Cultivation of mammalian cell lines

All tumor cell lines (table 3.1) used in this study were cultivated in RPMI supplemented with 50 µg/mL penicillin, 50 µg/mL streptomycin, 2mM L-Glutamin and 10% fetal calf serum at 37°C with 5% CO_2. 293T cells were kept in DMEM with the same additions. The viability and the cell count were determined by trypan blue staining.

Cultivation of primary mammalian cells

Primary mammalian cells (NK cells and PBMCs) purified from Buffy Coats or fresh blood samples were cultivated in in IMDM supplemented with 50 µg/mL penicillin, 50 µg/mL streptomycin, 2mM L-Glutamin and 10% fetal calf serum at 37°C with 5% CO_2. When serum-free cultivation was needed, cells were kept in AIM-V lymphocyte medium. For experiments including autologous serum from HL patients, NK cells were kept in IMDM with 10 % autologous serum. Medium for NK cells contained usually 10 U IL-2. For activation experiments, NK cells purified from HL patients` blood were cultivated without IL-2 for one night at most.

Purification of Natural Killer cells via MACS (Magnetic Activated Cell Sorting)

Human NK cells were separated from peripheral blood mononuclear cells (PBMC). PBMCs were purified using Ficoll-Paque density gradient centrifugation with leucosep columns from Greiner (Solingen) or normal falcons from BD (Heidelberg).
For experiments with leukemic cell lines, PBMCs / NK cells were gained from healthy donors` buffy coats. Buffy coats were obtained from the blood bank of the university hospital and were always used the same day.
PBMCs / NK cells from HL patients were purified from EDTA blood. For experiments comparing lysing ability of HL NK cells and healthy NK cells, those healthy cells were also purified from EDTA blood.

Depletion of all non NK cells was performed with the NK cell isolation kit (human) and the autoMACS Pro device (Miltenyi, Bergisch Gladbach). PBMCs were incubated with biotin-labeled antibodies targeting different markers on all peripheral blood cells except NK cells. Subsequently, streptavidin coupled magnetic beads were added to bind the cell surface attached biotinylated antibodies. After washing and centrifugation, NK cells were then separated from labeled non NK cells with the autoMACSpro® (Miltenyi Biotec).
The purity of NK cells should have been at least 90% and this was tested using flow cytometry analysis.

Transfection of 293T cells

For transfection with recombinant BAT3, 1 x 10^6 293T cells were plated in small cell culture flasks (25 $cm^{2)}$ the day before transfection for a final confluence of approximately 70-90 %. For gaining high amounts of the cloned scCD16-Immunoligands from the supernatants of transfected cells, 4x10^6 293T cells were plated in medium cell culture flasks.
For the transfection, the reagents Lipofectamine™ Reagent (Invitrogen) and jetPrime™ (Polyplus transfection) were used and cells were cultivated either in Opti-MEM™ (Invitrogen) for the purification of BAT3 exosomes or DMEM (Gibco) and CD293 (Gibco) for the secretion of scCD16-Immunoligands, respectively .
According to manufacturer's protocol, the dedicated amount of cDNA was incubated with the transfection reagent for 10-20 min and then added to the cells. BAT3 transfected and control cells were cultivated 48h before purification of BAT3 exosomes (see below).
For the expression of scCD16-Immunoligands, 293T were cultivated for two days, then the success of transfection was controlled by expression of GFP in transfected cells.

Purification of BAT3 exosomes

293T cells untransfected and transfected with recombinant were cultivated for 48 h in small cell culture flasks in OptiMEM (Gibco). Then, cells were dissociated with trypsin

and transferred into falcons, in which they were exposed to a nonlethal heat shock at 42°C for 30 minutes followed by a recovery period of 60 minutes at 37°C with 5% CO_2.
Exosomes from the supernatants were then purified by three successive centrifugations: 300 x g (5 min) and 1200 x g (20 min) to eliminate cells and debris, and 100 000 x g for 90 minutes to pellet the exosomes. Before ultracentrifugation, cells were passed through a 0,45 µm filter to deplete larger particles. The exosomal pellet was washed once in approximately 8 ml PBS, centrifuged again at 100 000 x g for 90 minutes and resuspended in 250 µl PBS. Exosomes were stored at -80°C and tested in flow cytometry for BAT3 expression.

Coupling of exosomes and FACS analysis of exosome coated beads

Exosomes were incubated with 20 µl 4.5 micron microsphere polybead carboxylate latex beads ($5x10^7$/ml, Polysciences) for 90 min at 25° C and 700 rpm. The beads were washed once with PBS and then incubated with 1% BSA / PBS for 30 min at 25° C and 700 rpm. After multiple washing steps with PBS and centrifuging 10 sec at 13000 rpm, the beads were resuspended in 100 µl PBS per tube and stained for CD9 (293T cell line) for quantity control and BAT3 to verify the overexpression of BAT3 on the exosomes purified from the BAT3 transfected cells. Beads alone were gated and isotype control antibodies were used as controls for the flow cytometry analysis.

Flow cytometry (FACS analysis)

In principal, all cells were incubated with either unconjugated or with fluorochrome (PE, APC, Cy3, AlexaFluor 647) conjugated primary antibodies (Table 4.4) for 20 min at 4°C and washed three times with FACS buffer (Table 4.1). In case of unconjugated primary antibodies, labeled secondary antibodies against the species of the primary antibody were used, for example goat-anti-mouse-PE.
In case of cells consisting of different types of cells, the population of interest was gated in the SSC (granularity) and FSC (size) dot plot.

Purification of MACS separated NK cells was determined by checking lymphocyte antigens like NKp46 for NK cells, CD3 for T-cells or CD19 for B-cells. Only NK-cells with more than 90 % purity have been used for subsequent experiments.

The expression of ligands for the activating NK cell receptors NKG2D, NKp30 and NKp46 was also checked in the flow cytometer. ALL and AML cell lines and primary cells (blasts from leukemia patients) were incubated with soluble NKG2D, NKp30 and NKp46 receptors that were tagged with an antibody Fc moiety. Those receptors bind ligands (e.g. in case of NKG2D: ULBP1-3, MICA or MICB) on the surface of the cells. As secondary antibody, α-FC-Cy3 was used.
Blasts of fresh patients' blood samples were gated (after lysis of erythrocytes) in a dot plot indicating SSC (granularity) versus CD45. CD45 is a lymphocyte marker that is less expressed on blasts compared to lymphocytes. With these two parameters blasts could be distinguished from lymphocytes and granulocytes and analyzed for expression of ligands for activating NK cell receptors.

Competition binding assay of the αCD16-αCD30 immunoligand on NK and target cells (Flow cytometry)

To validate the binding of αCD16-αCD30 on CD16 and CD30 on target cells, NK cells (CD16+) and CD30+ L428 cells (HL cell line) were incubated with a monoclonal antibody against CD16 and CD30 respectively. Simultaneously, αCD16-αCD30 was added at two different concentrations to compete with the monoclonal antibodies for the binding to the antigens. Goat-α-mouse antibody labeled with PE was used to stain bound monoclonal antibodies and measurement was done with a FACSCalibur (BD).

Detection of immunoligand in Western blot

For verification of expression and secretion of scCD16-Immunoligands, SDS-Page (10 % acrylamide gel) and Western blot was performed with lysates and His-purified supernatants of transfected and non-transfected control cells. Samples were heated at 95°C in Laemmli buffer before loading. Separation was performed at 170 V and with the

"Pageruler Prestaind Protein ladder" (Fermentas) as standard. Proteins and standard were then blotted on nitrocellulose membrane (Protan®, Whatman) at 350 mA for 90 min. For blocking and dilution of antibodies, 1x Roti-block (Roth) was used. For detecting the His tagged protein, the same penta-His antibody (Qiagen) was used as for the flow cytometry binding analysis of the immunoligands. Secondary antibody was always a POD-coupled rabbit polyclonal antibody with mouse reactivity.

Cytotoxicity assay (Europium release assay)

Cytolytic activity of NK cells was assessed in three hour europium release assay in a 96 V-shaped well plate at a total volume of 200 µl Target cells (cell line as indicated) were suspended in labeling solution (Eppendorf hypoosmolar labeling buffer + 10mM DTPA + 10mM EuCL at pH of 7) and shocked via electroporation (Eppendorf Multiporator, Voltage and duration depending on celltype) to label them with europium. After two washing steps with RPMI media and FCS each, cells were adjusted to $5x10^4$/ml.
NK cells were suspended in RPMI with $5x10^5$/ ml for an effector: target ratio of 10:1. Due to the limitation of HL patients` blood sample volume, in those experiments (indicated) NK cells were mostly adjusted to $2{,}5x10^5$/ml for a ratio of 5:1.
Prestimulation of NK cells with BAT3 expressing exosomes were started 48 hours prior to the cytotoxicity assay. Testing of immunoligands was conducted by adding the constructs directly to the NK cells-target cell mixtures 10 µg/ml. In blocking experiments, antibodies against NKp30 and HLA-molecule have been added to the mixtures at 10 µg/ml.
Spontaneous release (SR) and maximum release (MR) were assessed to calculate the relative cytotoxicity.

$$\text{Relative cytotoxicity} = \frac{\text{Experimental value} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} * 100$$

For the MR, cells were incubated in media with 1% Triton-X whereas for SR, target cells were incubated in standard media.
For measurement, cells centrifuged and 20 µl of the supernatant (from each well) were transferred into 200 µl of enhancement solution (Perkin Elmer) and read out via time resolved luminometry with a Wallac Victor^2 (Perkin Elmer).
All values were determined in triplicates.

Mixed lymphocyte reaction (MLR)

NK cells (responder) and PBMCs (stimulator) from different donors were purified as described above. PBMCs were irradiated with 40 Gray (Gy) to abolish metabolism and secretion of cytokines. NK cells from donor A were incubated with irradiated PBMCs from donor B and *vice versa* for 48 h. 1x10^5 NK cells were always used, either incubated with the same number of PBMCs (responder : stimulator ratio 1:1) or with 2x10^5 PBMCs (responder : stimulator ratio 1:2) for an enhanced stimulus to the responder cells. As positive control, cells were treated with 5 µg/ ml concanavalin A (Con A) to control the efficacy of the irradiation. After 48 h incubation, supernatants were collected and used for IFN-γ and TNF-α ELISAs.

Cytokine release assay (ELISA)

For the detection of cytokines secreted by NK cells (IFN-γ and TNF-α) the sandwich ELISA system of R&D was used. A capture antibody was incubated overnight (4°C) with coating buffer in MaxiSorp plates (Nunc). The next day, plates were washed and blocked and then incubated with 100 µl of undiluted sample and standard. After two hours of incubation, four washing steps were conducted before the detection antibody with peroxidase was added. At least seven washing steps were performed before development reaction.
Ultra TMB (Thermo scientific) served as substrate and after change in color 30 % sulfuric acid was added to stop the reaction.
Plates were read out in the uQuant (Biotek) and evaluated with the KC4-software.

Software and evaluations

Evaluation of flow cytometry data was done with “Cyflogic”. For DNA cloning and restriction analysis “Serial cloner 2.1” was used.
Results of NK cell cytotoxicity assays are displayed as means and standard error of the mean (SEM). Significance was calculated with the GraphPad Prism 5 software (Graphpad software) using the Student´s t-test.

5 Results

5.1 NK cell mediated in vitro GVL effects on AML and ALL cells

5.1.1 ALL cell lines are more resistant against NK cell mediated lysis than AML cell line

In stem cell and bone marrow transplantations, respectively, the favorable effect of donor alloreactive NK cells seems to be restricted to AML patients. In this study a significant decrease of relapses was observed. In contrast, ALL suffering recipients could not benefit. As only responsible factor, alloreactivity of transplanted and proliferated donor NK cells was determined. Data of this study suggested, that alloreactive NK cells were responsible for this GVL effect against AML relapse cells but ineffective against ALL occurrence (Ruggeri, Capanni et al. 2002).

To examine these findings in vitro, lysing capability of buffy coat derived NK cells was tested against various cell lines. T B-ALL cell lines (NALM6 and 697, ALL cell lines with B cell origin) and one T-ALL line (MOLT3, ALL cell line with a T cell origin) were available. As control and comparison cell lines the CML line K562 and the AML line HL-60 were used. K562 served as reference cell line and is used widespread as standard target cell line in NK cell based cytotoxicity assays. These cells are lacking of MHC class I molecules. Due to this absence of inhibitory signals, NK cells efficiently kill K562 cells.

Purified NK cells derived from healthy donors were mixed with a steady number of labeled target cells of the leukemic cell line and incubated for three hours (see 4.2.2). These cytotoxic assays were conducted at least three times with NK cells from at least three donors to avoid one time false positive results due to donor specific circumstances. Shown are representative results of one cytototoxicity assay each.

As expected, NK cells efficiently lysed K562 cells with relative cytotoxicity of 88 % at an effector target ratio of five to one. On comparable level, cells of the AML line HL-60 were killed with a percentage of more than 80. T-ALL line MOLT3 was significantly more resistant. Relative killing average was 30 % below the value of K562 and HL-60 cells (Fig. 5.1 A).

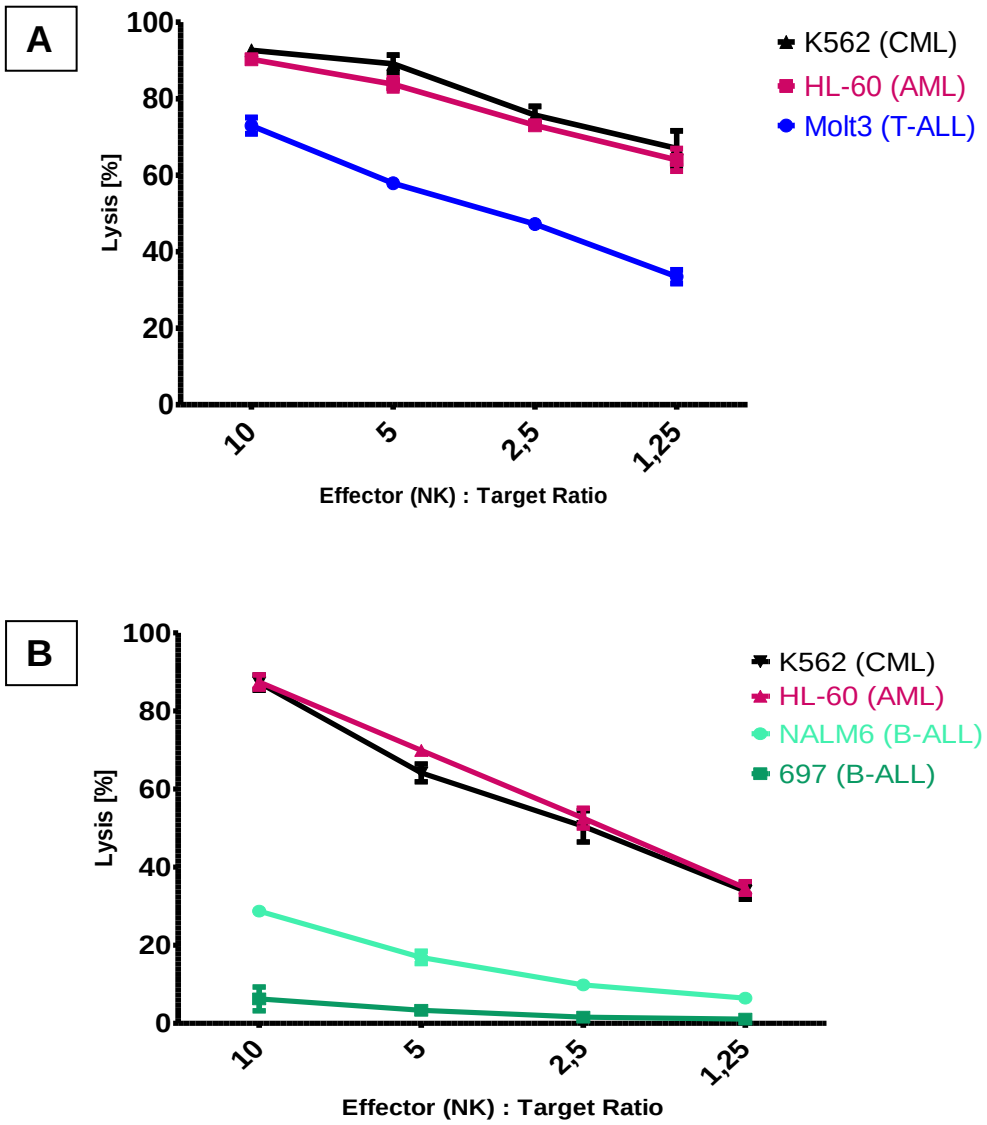


Figure 5.1: NK cell cytotoxicity against AML and ALL cell lines

Two representative cytotoxicity assays with NK cells from healthy donors as effector cells and target cells as indicated. **(A)** HL-60 cell line was compared with the T-ALL line MOLT3, in **(B)** B-ALL line NALM6 and 697 were used. Results are shown as relative lysis and depicted values are means of triplets with standard errors.

Killing of B-ALL cell lines NALM6 and 697 due to NK cells were also determined and compared with the lysis of HL-60 cells. Again, ALL cell lines were significantly less lysed. Relative release of europium from 697 cells was 3 % at an effector target ratio of 5 to 1 whereas 17 % of NALM6 cells were killed.

5.1.2. Expression of ligands for activating NK receptors on leukemic cell lines

To elucidate the reasons for the differing lysing capabilities of NK cells towards the tested
leukemic cell lines, we focused on the surface expression of ligands for activating NK cells receptors. According to the “induced self” principle, ligands for NKG2D, NKp30 and NKp46 should be at least in part responsible for NK cell behavior (Bryceson and Long 2008).
For that purpose, soluble receptors fused to Fc-parts of IgG-antibodies were used. Cell lines were incubated with NKG2D-Fc, NKp30-Fc and NKp46-Fc and subsequently with an α-Fc-Cy3 antibody for analysis in flow cytometry. Thus, the question, whether there are ligands for those receptors and to which extent they are expressed could be addressed.
None of the investigated cell lines showed any binding to an CD16-Fc construct, indicating that no ligands for CD16 are expressed (data not shown).
All tested cells showed expression of ligands for NKG2D. Interestingly, no coherence between sensitivity to NK cell killing and NKG2D ligand expression was visible (Fig. 5.2, green). Strong expressions could be detected on K562, MOLT3 and NALM6 cells. Weaker signals could be observed on HL-60 and 697 cells. In contrast, the expression of NKp30 ligands (Fig. 5.2, blue) matches the results of the cytotoxicity assays. The well lysed K562 and HL-60 cells express ligands for NKp30 on their surface, whereas the ALL cell lines MOLT3, NALM6 and 697 do not. Although not prone for NK cell based killing, only 697 show an expression of NKp46 ligands.

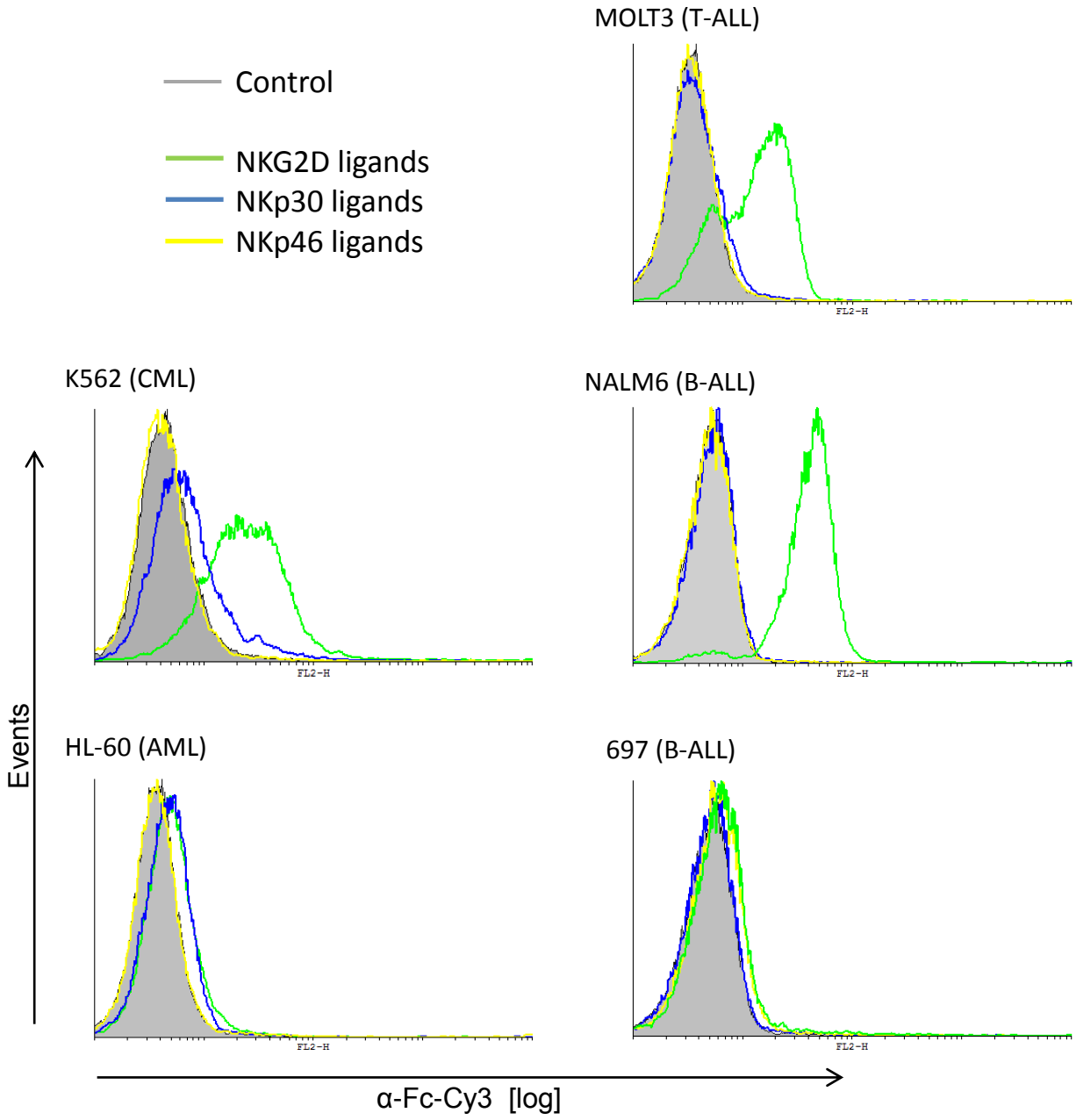


Figure 5.2: Expression of ligands for activating NK receptors on leukemic cell lines

Flow cytometry analysis of two myeloid **(left)** and three lymphatic **(right)** cell lines. Cells were incubated with Fc-tagged receptors (NKG2D-Fc, NKp30-Fc, NKp46-Fc) and labeled subsequently with a Cy3-coupled α-FC antibody. Control measurement (grey) was only incubated with Cy3-coupled α-Fc antibody.

5.1.3 Blocking of NKp30 signaling leads to reduced lysis of AML cells

One obvious difference between the well lysed AML cell line and the three ALL line is the expression of ligands for NKp30. Neither on MOLT3 cells nor NALM6 and 697 any signal could be detected. The next step was to examine, whether the expression of NKp30 ligands on HL-60 contributes to the higher susceptibility to NK cell lysis. An effective strategy to eliminate signaling via a single receptor is the application of a monoclonal antibody. It has been shown that blocking NKp30 can reduce target cell killing to less than 50 % (Pende, Parolini et al. 1999).

For this, purified NK cells were co-cultivated with either MOLT3 cells (ALL) or HL-60 cells (AML) at four different effector : target ratios in a standard europium release assay. At two ratios (5:1 and 1,25: 1), α-NKp30 antibody was added at a concentration of 10 µg/ml to half of the wells with MOLT3 and HL-60 cells. PBS was added to the other wells and served as control. Ratios of 10:1 and 2,5:1 were used as controls and were not supplemented with antibody. This ensures that small differences in lysing rates are not evoked by diluting the ratio.

Adding NKp30-antibody to the NK cells reduced the lysis of NKp30-ligand expressing HL-60 cells the ratio of 5:1 from approximately 80% to 60% and thus being on a par with lysis of MOLT3 cells. At the lower ratio of 1,25:1, the lysis of HL-60 cells decreases from 55% to approximately 30% with the application of the α-NKp30 antibody. In contrast, on both ratios there was no change of cytotoxicity against MOLT3 cell with an application of the α-NKp30 antibody (Fig. 5-3). As well, cytotoxicity against both other NKp30-ligand negative cell lines 697 and NALM6 showed any reduction upon α-NKp30 antibody application (data not shown).

This difference can be explained with the described expression of NKp30 ligands found on HL-60 cells. Thus, blocking of NKp30 mediated signaling would only affect NKp30 ligand expressing cells like HL-60, but not non-expressing cells like MOLT3 cells. The steadiness for the MOLT3 lysis also proves that there is no unspecific reduction of lysis through the α-NKp30 antibody itself.

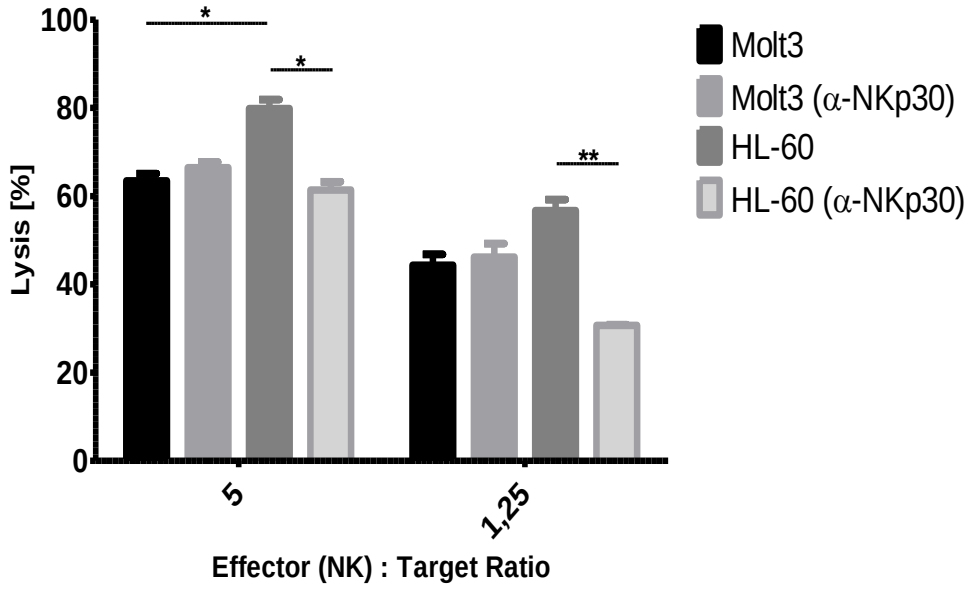


Figure 5.3: Reduction of NK cell cytotoxicity via NKp30 blocking

Cytotoxicity assay of primary NK cells cocultured with europium labeled target cells as described. Before adding target cells, NK cells were either preincubated 15 min with α-NKp30 antibody to block NKp30 mediated signaling (**lighter greys**) or with PBS control (**darker greys**). Shown are the effector to target ratios of 5 and 1,25. Means of triplets are depicted. Significance was determined with the Student`s t test.

5.1.4 HLA expression on ALL target cell lines contributes to their resistance against NK cells

The difference of NK cell mediated lysis of the ALL cell line MOLT3 and the AML cell line HL-60 could be explained with the expression of NKp30 ligands on HL-60 cells.

But it fails to elucidate the resistance of ALL cells lines NALM6 and 697 in cytotoxicity assays (Fig. 5.1, B). Compared with the lysis of MOLT3, 697 and NALM6 showed even less susceptibility to NK cell mediated cell death. This is remarkable, as NALM6 cells showed robust expression of ligands for NKG2D and 697 cells were positive for both NKG2D- and NKp46-ligands.

As NK cells are not only regulated by activating receptors, but also by inhibiting receptors (see 3.1.2) recognizing self MHC I molecules, expression of HLA molecules on the surface of the examined target cell lines were analyzed via flow cytometry.

The expression pattern (Fig. 5.4) reflects the results of the cytotoxicity assays shown in Fig. 5.1. Hardly lysed cell lines NALM6 and 697 showed a higher expression of HLA molecules than the far better lysed HL-60 and MOLT3 cells. Those cells exhibited a comparable low expression of HLA. Accordingly, the best killed cell line K562 is known to lack any HLA expression and therefore very prone to NK cell mediated cell death (Anfossi, Andre et al. 2006).
Also the difference in lysing NALM6 cells and 697 cells correlates with the HLA signal found on those cells. Mean fluorescence intensity values were approximately 50% higher for 697 cells than for NALM6 cells indicating that a higher inhibitory signaling due to HLA expression on 697 prevents them from being killed by NK cells.

To verify, whether inhibitory signaling via HLA molecules are jointly responsible for the resistance of NALM6 and 697 cells against NK cell mediated killing, cytotoxicity assays with blocked HLA signaling were performed. This should provide a reduced inhibitory signaling for NK cells and end up with an increased cytotoxicity. For that purpose, an α-HLA antibody was used as well as a control isotype antibody. To avoid any activation via CD16 (Fc receptor), NK cells were pre-incubated with 30 % human serum to saturate CD16 receptors.
As expected, with the application of an α-HLA antibody, the lysis of NALM6 and 697 target cells increased significantly while the isotype control antibody showed no effect compared to untreated target cells. This indicates that the HLA expression contributes to the low lysis susceptibility towards NK cells cytotoxicity as it can be overcome to some extent.
HLA blocking experiments did not result in a significant higher lysis of the cell lines HL-60 and MOLT3 (data not shown). Either the expression of HLA (Fig. 5-4) is too low to account to any inhibitory signaling or blocking via the α-HLA antibody is just not as effective as necessary to cause any change at this high cytotoxic levels.

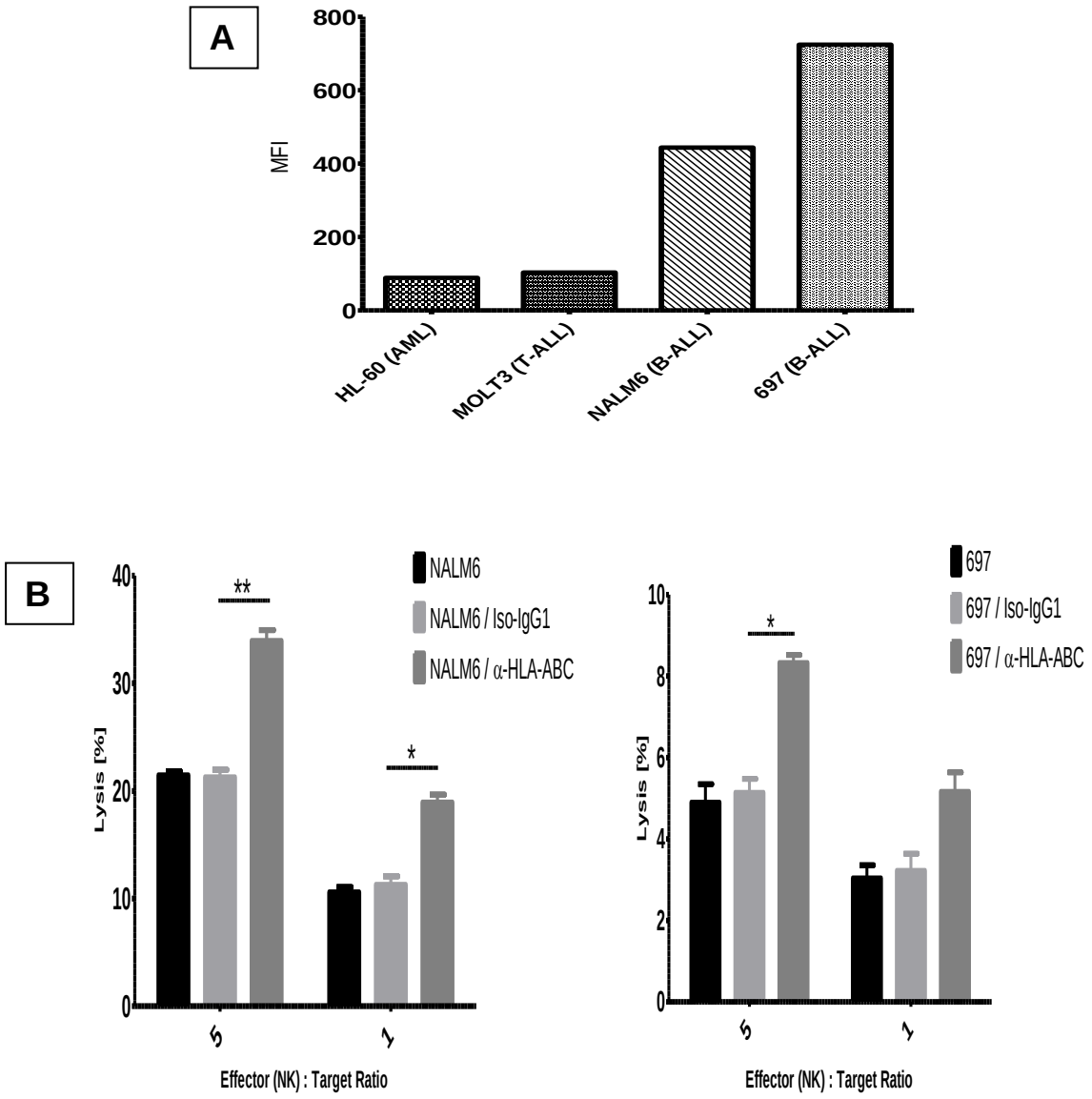


Figure 5.4: HLA expression on the surface of leukemic cells and cytotoxicity upon their blocking

(**A**) One representative flow cytometry detection of HLA molecules on **HL-60, MOLT3, NALM6** and **697** cells. Cells were either incubated with α-HLA mouse antibody or with a PE coupled isotype control. Indicated is the isotype subtracted MFI.
(**B**) Primary NK cells were incubated with europium labeled NALM6 cells (**left**) and 697 cells (**right**) in a standard cytotoxicity assay. First, target cells were pre-incubated with human serum, then with α-HLA antibody (**grey**) or an isotype control (**light grey**) respectively. Shown are the effector target ratios 5 and 1. Depicted are representative assays for both cell lines with means of triplets and Student`s t test evaluated significances,

5.1.5 Increased cytotoxicity towards ALL cells upon external NKp30 stimulation via BAT3 expressing exosomes

Assuming, that the inferior lysing capability of NK cells against ALL cells is due to lacking NKp30-ligand activation and higher inhibitory HLA signaling, additional activation through NKp30-ligands should overcome this “imbalanced” NK cell regulation.

BAT3 (see 3.2) was identified as a cytotoxicity triggering ligand for NKp30 several years ago. In a membrane bound form, it is released by a variety of cells on small vesicles, so called exosomes (Pogge von Strandmann, Simhadri et al. 2007; Simhadri, Reiners et al. 2008). Here BAT3 on exosomes was used as a tool to deliver an external NKp30 stimulus and thus to activate NK cells. Therefore we purified wild type exosomes and exosomes from cells overexpressing BAT3 to ensure that any activating signaling was evoked by the binding of BAT3 to NKp30. The purification happened in a multistep centrifugation protocol including ultracentrifugation with 100.000 x g. Quality of purification was checked by flow cytometry of bead coupled exosomes. To ensure that equal amounts of control and BAT3-exosomes were used, the quantity was determined via staining against the exosomal marker CD9 (Fig. 5.5 A) and exosomes were also stained for BAT3 to verify the overexpression Purified NK cells were incubated with either control or BAT3-overexpressing exosomes for 48 hours prior to the cytotoxicity assay.

NK cell mediated lysis of all ALL cell lines could be increased by preincubation of NK cells with BAT3 overexpressing exosomes (Fig. 5.5 B-D). Killing of MOLT3 cells could be elevated to the level of HL-60 cells (Fig. 5.5 B). The difference in lysing efficacy between the control exosomes and the BAT3-exosomes activated NK cells suggests that the discrepancy is mainly caused by BAT3 and therefore presumably due to NKp30 activation. Control exosomes had also an activating effect on NK cells, which is due to the endogenously expressed BAT3 on exosomes (Simhadri, Reiners et al. 2008).

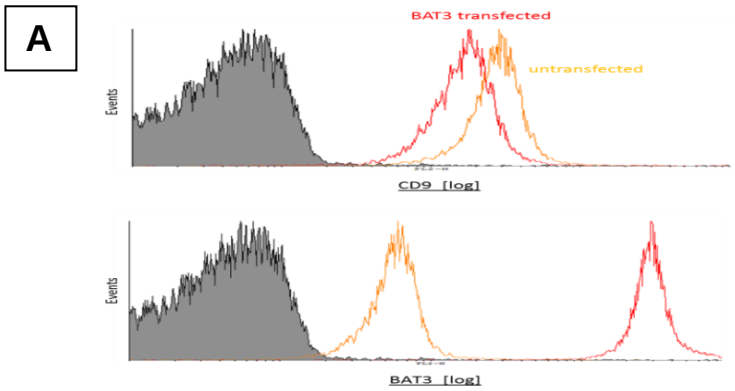
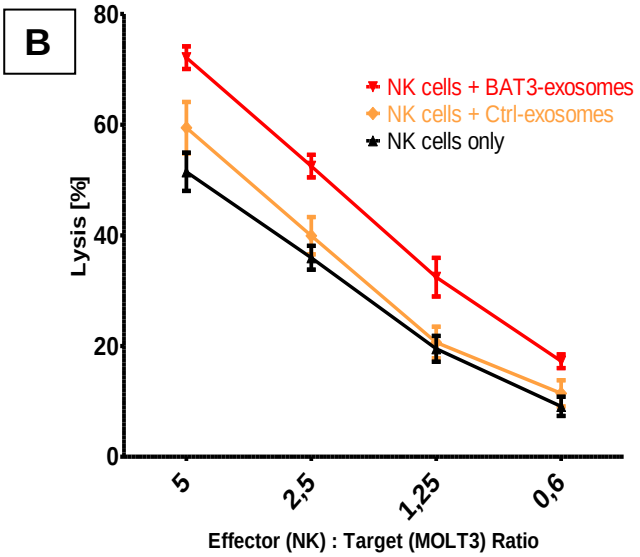
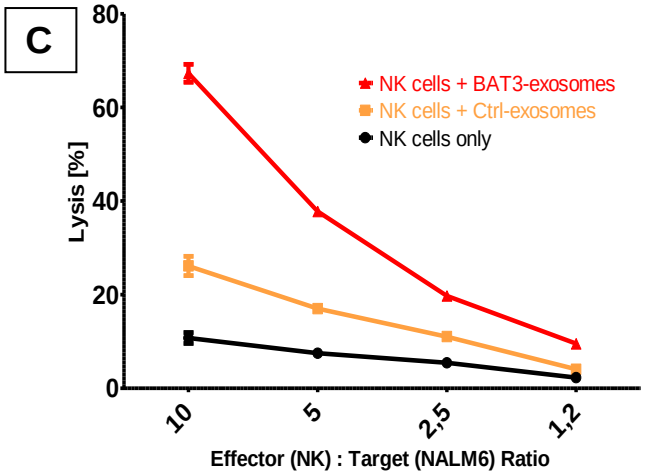


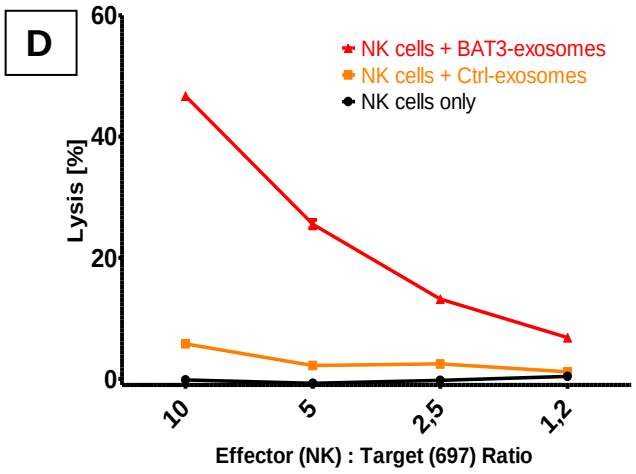
Figure 5.5:
Lysis of leukemic cells through BAT3-stimulated NK cells

(**A**) FACS analysis of CD9 (exosomal marker) stained (**top**) and BAT3 (**bottom**) stained exosomes. Exosomes were purified from supernatants of untransfected (**orange**) or BAT3-transfected cells (**red**), respectively. The grey histogram indicates the isotype control.



(**B-D**) Cytotoxicity assays with indicated target cells at different effector: target ratios. Used NK cells were either left untreated (**black**) or were pre-incubated with control exosomes (**orange**) or BAT3 overexpressing exosomes (**red**) respectively. Shown are representative experiments with means of triplets and standard error.





5.1.6 NK cell mediated cytokine release in a mixed lymphocyte reaction upon NKp30 triggering

NK cell are not only reported to have GVL effects upon allogeneic stem cell transplantation, but also can mediate reduction of GVHD caused by alloreactive T cells (Olson, Leveson-Gower et al. 2010). It is not completely understood, which mechanisms lead to this abrogation of GVH reactions, but donor NK cells are supposed to kill host antigen presenting cells (APCs) and therefore eliminate a cascade leading to GVHD.

One method to simulate an *ex vivo* allogeneic encounter of immune cells is the mixed lymphocyte reaction (MLR). Cells of two different donors are co-cultivated, whereby one population is silenced, usually by irradiation. Then, the activation is measured for example via the secretion of cytokines (Munster, MacDonald et al. 2004; Newland, Russ et al. 2006).

Here we examined the activation of NK cells pre-stimulated with BAT3-exosomes in a MLR. This setting should illuminate the behavior of previously NKp30 stimulated NK cells when encountering allogeneic immune cells like in an HSCT.

In several experiments, PBMCs of one donor have been irradiated and subsequently co-cultivated with purified NK cells derived from another donor in two effector: responder ratios. These ratios serve as verification that irradiated effector cells are responsible for the activation of responder cells. Activation of responder NK cells were determined by measuring IFN-γ and TNF-α secretion into the supernatant.

BAT3-exosome pre-treated NK cells showed a higher release of TNF-α and IFN-γ upon co-culture with allogeneic PBMCs than untreated NK cells. Due to the naturally high release of IFN-γ by NK cells in cell culture, the differences were rather small, but detectable (Fig. 5.6 A). The increase in secretion of TNF-α however was quite obvious. Compared with NK cells either pre-incubated with exosomes or only co-cultivated with PBMCs, the TNF-α release of those NK cells pre-incubated with BAT3 exosomes and mixed with allogeneic PBMCs was approximately three times higher (Fig. 5.6 B), indicating a synergistic effect of the BAT3 pretreatment and the allogeneic stimulus. This shows that NK cells activated via NKp30 prior to the MLR, are more responsive when gathering allogeneic PBMCs.

This might indicate that the GVHD attenuating effects of alloreactive NK cells might also benefit from the additional NKp30 stimulus.

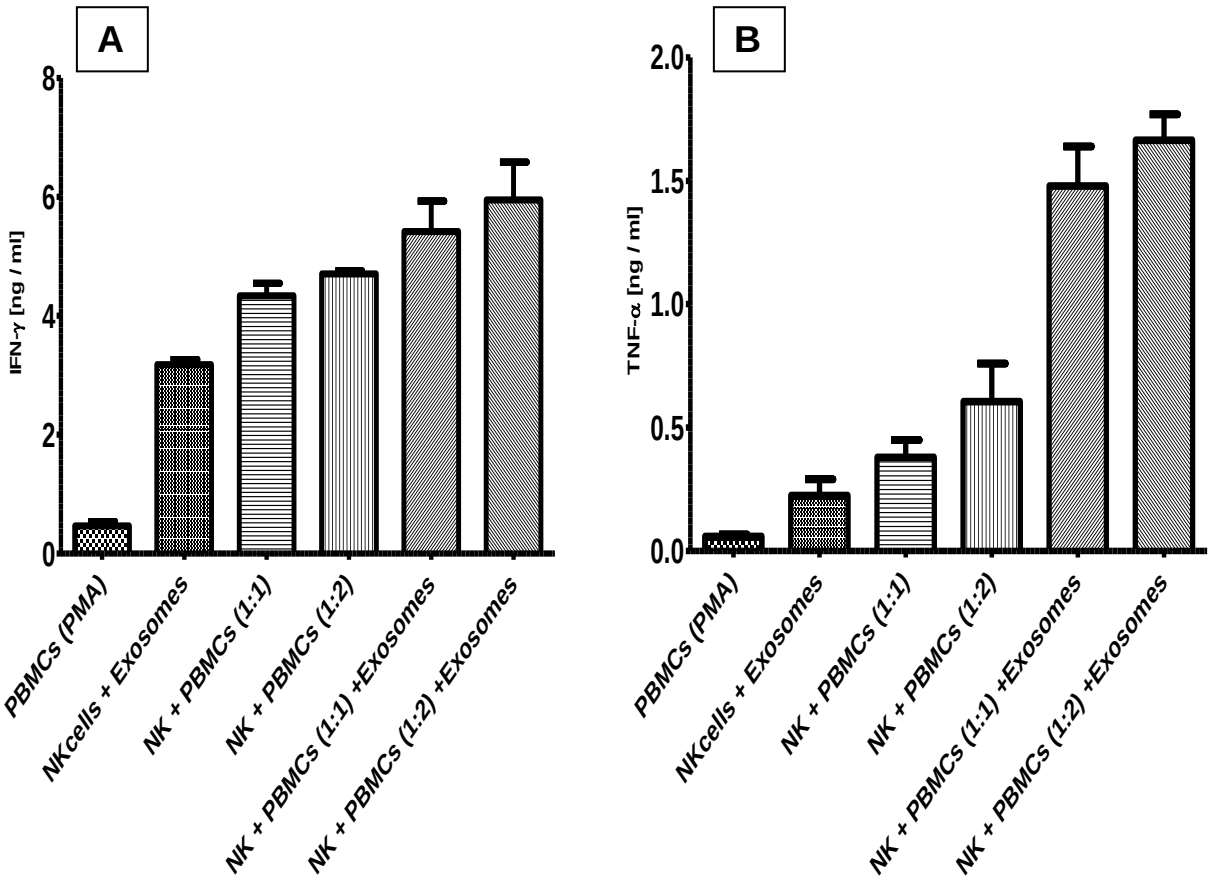


Figure 5.6: Cytokine release assay after mixed lymphocyte reaction

Depicted are the results of ELISAs for IFN-γ (**A**) and TNF-α (**B**) using supernatants of mixed lymphocyte reactions or control settings from PMA treated PBMCs and exosomes-treated NK cells. Shown are means of triplets with standard error.

NK cells do not only play a role in allogeneic stem cell transplantations in leukemic patients but also in the autologous transplantation setting e.g. in Hodgkin Lymphoma patients suffering from a relapse. However, almost nothing is known about the role of NK cells in Hodgkin Lymphoma.

5.2 Impaired functions of peripheral NK cells in Hodgkin Lymphoma patients

The role of NK cells in pathogenesis and failed immune surveillance of Hodgkin Lymphoma (HL) is quite unclear. Therefore, we raised questions concerning any phenotypic changes and a potential impairment of cytotoxicity of HL NK cells compared to healthy NK cells as well as for the feasibility of NK cell based immunotherapies directed against HL cells.

5.2.1 Activating receptor NKG2D is down-regulated on NK cells in Hodgkin Lymphoma patients

We sought to examine, if there are any changes in the phenotype of NK cells in HL patients hinting to any kind of aberration. Hence, we examined NK cell from blood samples received from HL patients and checked them via flow cytometry for a variety of activation markers and cytotoxicity triggering receptors to determine the activity status of those NK cells. For comparison, we did the same with samples from healthy donors.
We found all shown activation marker (e.g. CD69 for early and CD25 for later activation) and most activating receptors (e.g. Fc-receptor CD16 and BAT3 binding receptor NKp30) to be steady when comparing HL with healthy NK cells (Fig. 5.7 A). In contrast, the receptor NKG2D was significantly downregulated on the surface of NK cells in HL patients (Fig. 5.7 B).

A

Marker / receptor	Mean fluorescence intensity
CD25	=
CD69	=
CD71	=
CD16	=
NKp30	=
NKp44	=
NKp46	=
NKG2D	↓

B

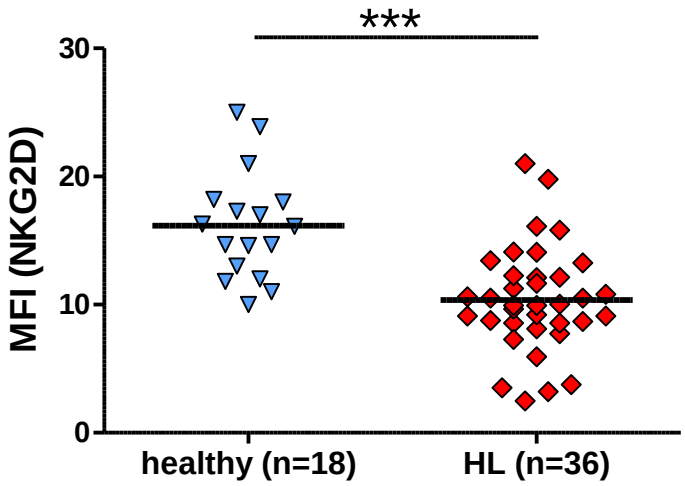


Figure 5.7: Expression of activation marker and cytotoxicity receptors on HL NK cells

(**A**) Table of summarized flow cytometry results of NK cells from 36 HL patients that were screened for marker of different activation states as well as for most important activating receptors and compared to healthy NK cells. Marker and receptors that remained steady are indicated with an equal sign. The changed receptor is indicated with an arrow. (**B**) Depicted are single NKG2D expression levels detected in flow cytometry for 36 HL samples and 18 healthy donors. Significance was calculated with students t test. (in cooperation with Katrin S. Reiners, Internal Medicine I, University hospital, Cologne)

5.2.2 Soluble ligands for NK cell receptors are elevated in HL sera

A variety of tumor cells can shed and release ligands for example for NKG2D from their surface as a strategy to evade tumor immune surveillance. Additionally, soluble ligands for NKG2D are described to have an impairing effect on expression of NKG2D and functionality of T cells (Waldhauer, Goehlsdorf et al. 2008) (Groh, Wu et al. 2002) .

To check if soluble ligands might be responsible for the shown down-regulation of NKG2D on HL NK cells, we analyzed serum levels of known ligands for NKG2D like MICA, MICB and ULPB1-3 via ELISA and compared them with sera from healthy donors. We did the same for the NKp30 ligand BAT3, as this protein in its soluble shape

can also have an inhibiting effect on NK cells function (Pogge von Strandmann, Simhadri et al. 2007).

We found a significant increased level for both the NKG2D ligand MICA and for BAT3 in patients´ sera compared to those of healthy donors (Fig. 5.9). Interestingly, in samples gained from patients after a successful chemotherapy and free of HL, concentrations decreased again to a level comparable to healthy donors (red vs. orange). This indicates that HL cells are responsible for the increased amount of those soluble factors in HL patients´ blood before therapy abolishes tumor burden.

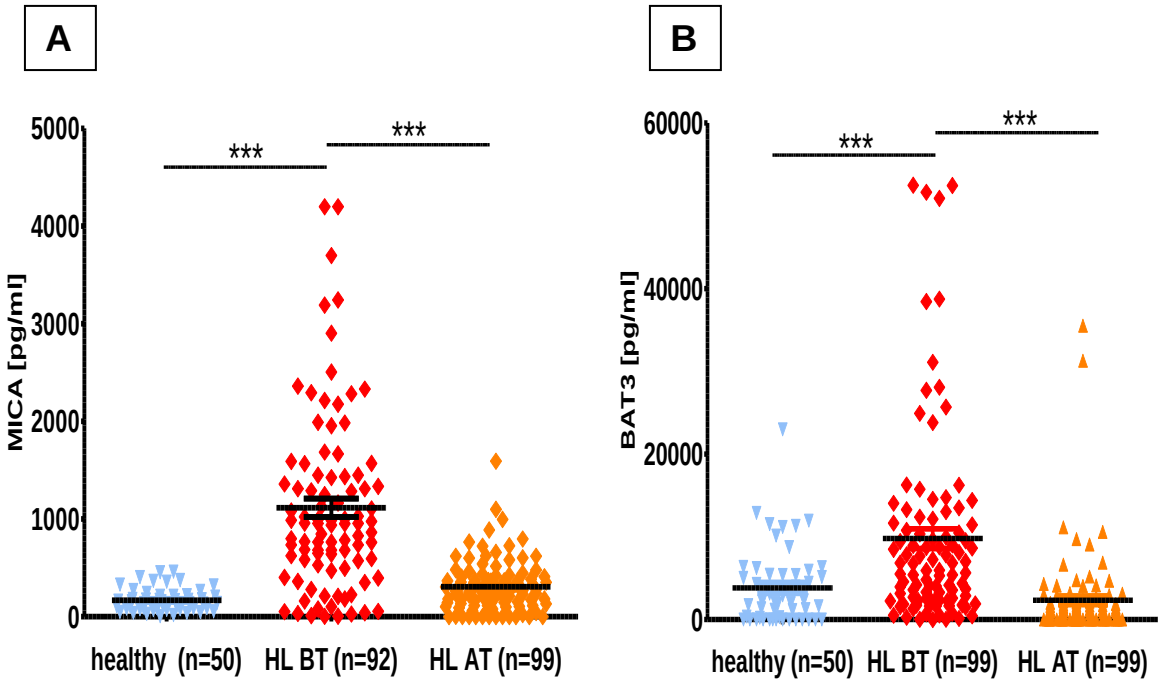


Figure 5.8: Soluble MICA and BAT3 in sera of HL patients

ELISA results of soluble MICA (**A**) and soluble BAT3 (**B**) levels in sera of healthy donors (**blue**), of HL patients before therapy (**red, HL BT**) and after therapy (**orange, HL AT**). Depicted are single values and the mean with standard error. (in cooperation with Maike Sauer, Internal Medicine I, University hospital Cologne)

5.2.3 Hodgkin Lymphoma serum impairs NK cell activity

Data revealed that HL patients´ NK cells express less NKG2D than their healthy counterparts. Matching the theory of down-regulation by soluble ligands, we also found higher levels of MICA and BAT3 in HL sera.
To verify this coherence, the impact of HL serum on healthy NK cells was investigated.
We selected several healthy sera that contain low amounts of soluble MICA and BAT3 and HL sera with high levels of these ligands. Purified healthy NK cells were then incubated over night with medium containing either 20 % healthy serum or HL serum prior to standard cytotoxicity assays and FACS analysis.

Table 5.1: Values of BAT3 and MICA levels in sera used for NK cell incubation

	Healthy serum [pg/ml]	HL serum [pg/ml]
BAT3	23	5015
	0	4113
	5423	7532
	1363	25433
	2929	6736
MICA	70	2331
	75	3191
	68	3699
	108	2506
	168	4198

Upon incubation with several HL sera, NK cells showed a significant reduced expression of activating receptors NKG2D and NKp30 as well as activity marker CD69 and the NK cell marker and adhesion molecule CD56 (Fig. 5.9 A). Measurements for CD16 and NKp46 revealed no significant change. Thus at least some receptors and other surface

molecules seem to be affected by the exposure to HL serum and thus also soluble ligands.

Interestingly, in experiments with HL sera, a down-regulation of NKp30 could be observed, although NK cells derived from HL patients did not show a significant decrease compared to healthy donors.

We also examined whether HL serum might have an effect on functionality of NK cells.

Therefore, we again incubated purified healthy NK cells with normal and HL sera and determined specific lysis in an europium release assay against HL target cells.

Compared with NK cells kept in medium with healthy serum, NK cells exposed to HL serum revealed a reduced cytotoxic potential. This was also observable when NK cells were stimulated with 100 U Il-2 in the overnight incubation (Fig. 5.9 B). Although on a higher lysis level, impairment of the HL serum treated NK cells was evident.

Thus, not only regular NK cell function is impaired by HL serum, but also activation evoked by additional IL2 is attenuated by this serum and presumably its containing soluble ligands.

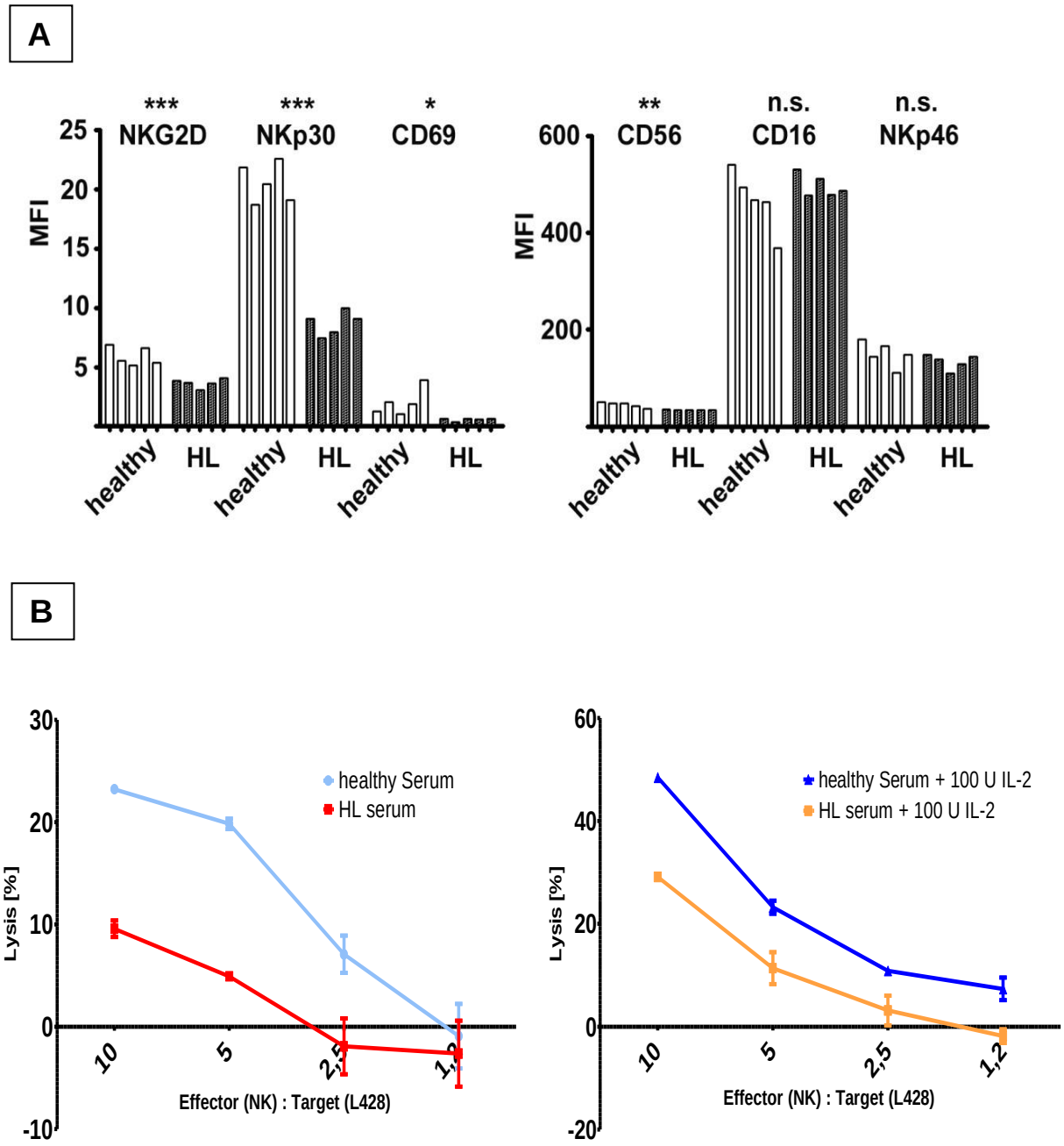


Figure 5.9: HL serum effect on cytotoxic activity of healthy NK cells

(**A**) FACS analysis of NK cell marker and receptors after overnight incubation with either five healthy (**white bars**) or five HL (**black bars**) sera. Shown are MFI values of one representative experiment with NK cells from one donor incubated with five healthy and five HL sera each. (**B**) Cytotoxicity assay of NK cells incubated with either healthy serum (**blue**) or HL serum (**red**) at different effector target ratios with (**right**) or without (l**eft**) additional IL-2. HL cells (L428) were used as target cells.

5.2.4 Cytotoxic functionality of HL patients' NK cells is impaired

Since examined sera of HL patients revealed high levels of putative inhibiting soluble ligands for NK cell receptors and these sera indeed had an impairing effect on healthy NK cells, we checked the cytotoxic constitution of NK cells derived from HL patients.

For comparison, we purified NK cells from EDTA blood samples of HL patients and from volunteer healthy donors in exactly the same way to exclude any influence of the manufacturing process of buffy coats. Then those purified NK cells were used in a standard europium release assay with HL L428 target cells.

As expected, NK cells derived from HL patients showed a significant decreased capability to lyse HL target cells. We tested NK cells gained from eight HL patients and six healthy donors. The latter showed an approx. three-fold higher cytotoxicity towards L428 cells (Fig. 5.10). This matches the results of the experiments, in which healthy NK cells are inhibited after exposure to HL serum with elevated levels of soluble ligands.

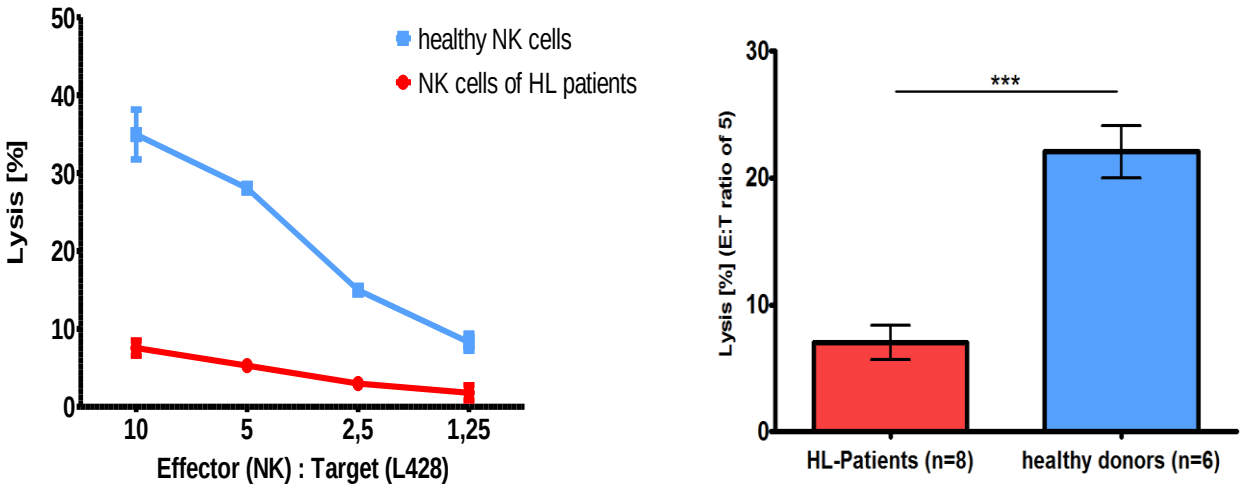


Figure 5.10: Cytotoxicity of NK cells derived from HL patients

Depicted is the relative lysis in one representative experiment (**left**) and the average of six independent experiments (**right**) comparing cytotoxic activity of healthy NK cells (**blue**) and HL NK cells red (**red**). Indicated is the mean with standard error (significance: Student´s t test)

5.2.5 HL NK cell cytotoxicity can be restored

To answer the question whether NK cells might be a feasible target population for immunotherapeutic approaches, the possibility of restoring cytotoxic potential must be illuminated.

A simple but unspecific approach to activate NK cells and thus antagonize the inhibitory effect of the HL serum is the application of Interleukin-2 (IL-2). This T cell secreted factor drives proliferation of NK cells and is crucial for evoking and maintaining NK cell activity (Henney, Kuribayashi et al. 1981; Domzig, Stadler et al. 1983).

Therefore, we treated NK cells gained from HL patients with high doses (50 and 500 U/ml) of IL-2 in overnight culture and used these NK cells for a standard europium release assay.

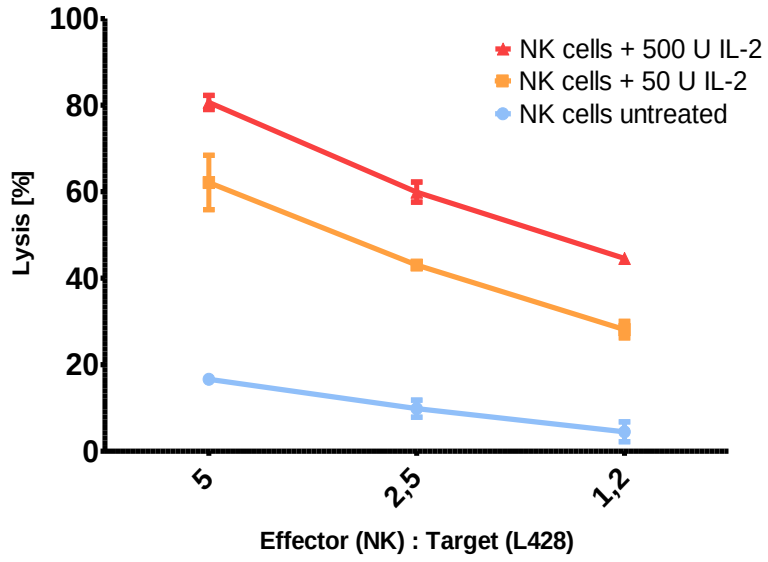


Figure 5.11: Cytotoxicity of HL NK cells upon IL-2 treatment

Cytotoxicity assay of untreated (**blue**) or IL-2 preincubated (**orange, red**) HL NK cells are indicated in a representative experiment. HL L428 cell line was used as target cells. Means of triplets are depicted with standard error.

As expected, IL-2 stimulated NK cells showed a significant higher cytotoxic potential than the untreated ones. 50 Units IL-2 are sufficient to increase the lysis to over 60 %. This proves that the detected inhibition of HL NK cells caused by HL serum is reversible.

As a more NK cell specific and therapy suitable approach, we decided to target the activating receptor CD16 on NK cells to overcome HL specific inhibition.
This receptor might be a promising target, as CD16 seems to be the “most powerful” among the activating NK cell receptors. In contrast to other receptors, the engagement of CD16 is sufficient to trigger cytotoxicity without any costimulation (Bryceson, March et al. 2006).
For that purpose an immunoligand (AFM13, Affimed®) consisting of one scFv against CD16 on NK cells and another scFv against CD30 on HL target cell was used. CD30 is abundantly and selectively expressed on HL cells and therefore a feasible antigen for immunotherapies (Kim, Eow et al. 2003).

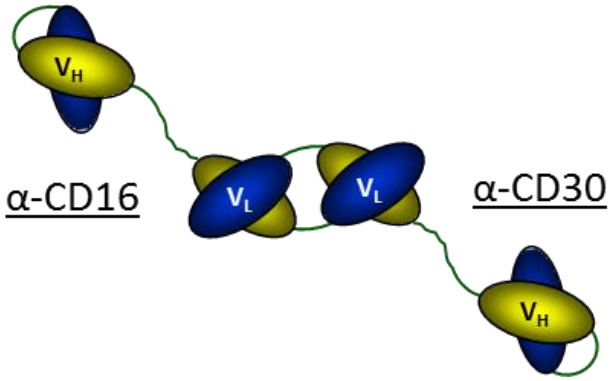


Figure 5.12: αCD16-αCD30 immunoligand (AFM13)

Schematic drawing of the αCD16-αCD30 immunoligand. Both single chains consist of a variable fragment of a heavy chain ($\mathbf{V_H}$) and a light chain ($\mathbf{V_L}$) derived from monoclonal antibodies.

First we characterized this αCD16-αCD30 construct with respect to its binding capacity on NK cells and HL target cell (HL cell line L428). This is a necessary precondition for triggering NK cell cytotoxicity and destruction of the aimed target.

Therefore, a competition experiment was performed. NK cells (CD16 positive) and L428 cells (CD30 positive) were incubated simultaneously with this immunoligand (at 10 and 1 µg/ml) and with monoclonal antibodies against CD16 (for NK cell samples) and CD30 (L428 samples). Then, CD16 or CD30 signals respectively, were determined in FACS analysis after incubation with a PE-labeled secondary antibody.

Figure 5.13 (A) shows that the signal received for CD16 on NK cells is lower when cells are incubated with a αCD16 antibody and the αCD16-αCD30 immunoligand at the same time. Furthermore, a higher concentration of the immunoligand leads to a further reduction of the CD16 signal. This indicates that the αCD16 moiety of the immunoligand and the αCD16 antibody are competing for binding CD16 on the surface of NK cells. Thus, less αCD16 antibody can bind to the surface. As the secondary antibody only recognizes the αCD16 antibody but not the immunoligand, the signal for CD16 went down compared to the PBS control.
The same is observable for CD30 staining on L428 cells (Fig. 5.13 B). When incubated with the immunoligand, a decreased signal for CD30 occurs, as the αCD30 part of construct directly competes with the monoclonal αCD30 antibody.
In conclusion, the αCD16-αCD30 immunoligand seems to bind effectly CD16 on NK cells and CD30 on HL target cells.

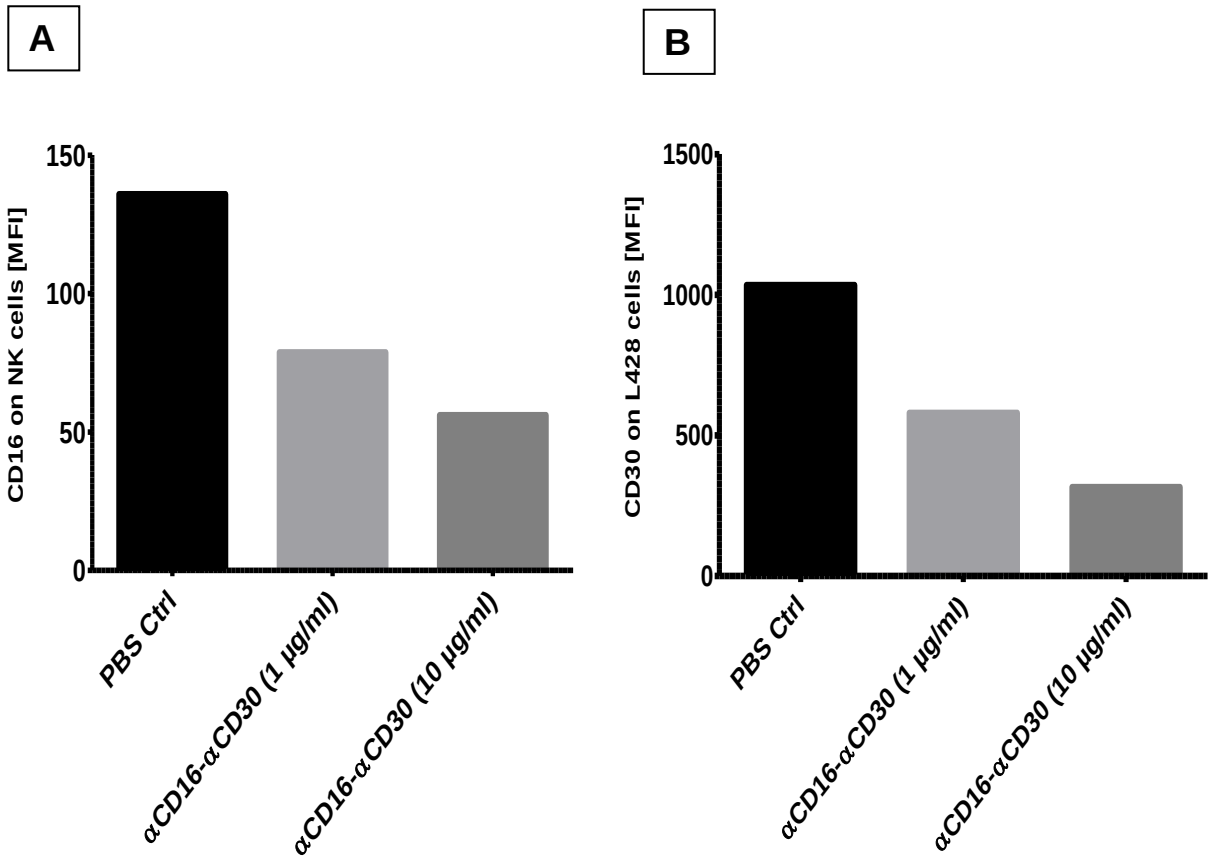


Figure 5.13: Competition assay for binding of αCD16-αCD30 to CD16 and CD30

Mean fluorescence intensity of (**A**) CD16 on NK cells and (**B**) CD30 on L428 cells is depicted. Cells were simultaneously incubated with a primary antibody (αCD16 res. αCD30, 5 µg/ml each) and αCD16-αCD30 construct. This construct was added at two concentrations: 1 µg/ml (**light grey)** and 10 µg/ml (**dark grey**). Staining was performed with a PE labeled goat-anti-mouse antibody. Shown is one representative experiment of three.

The next step was to investigate, whether this bispecific construct would lead to an increased cytotoxicity of NK cells towards HL target cells. Assuming, that it binds simultaneously CD16 on NK cells and CD30 on e.g. L428 cells, a higher cytotoxic impact could be expected.

Thus, we purified NK cells from blood samples, which we received from HL patients before therapy and tested the efficacy of this immunoligand in a standard europium release assay. Unfortunately, the amount of patient blood and hence the number of NK cells was limited.

This restricted the experiments to a maximum of comparison between untreated NK cells and NK cells treated either with the αCD16-αCD30 immunoligand or with a αCD30-αCD30

control construct. Therefore, we preincubated the NK cells with these constructs (at 10 µg/ml) and mixed them with europium labeled L428 target cells at different effector target ratios as seen before.

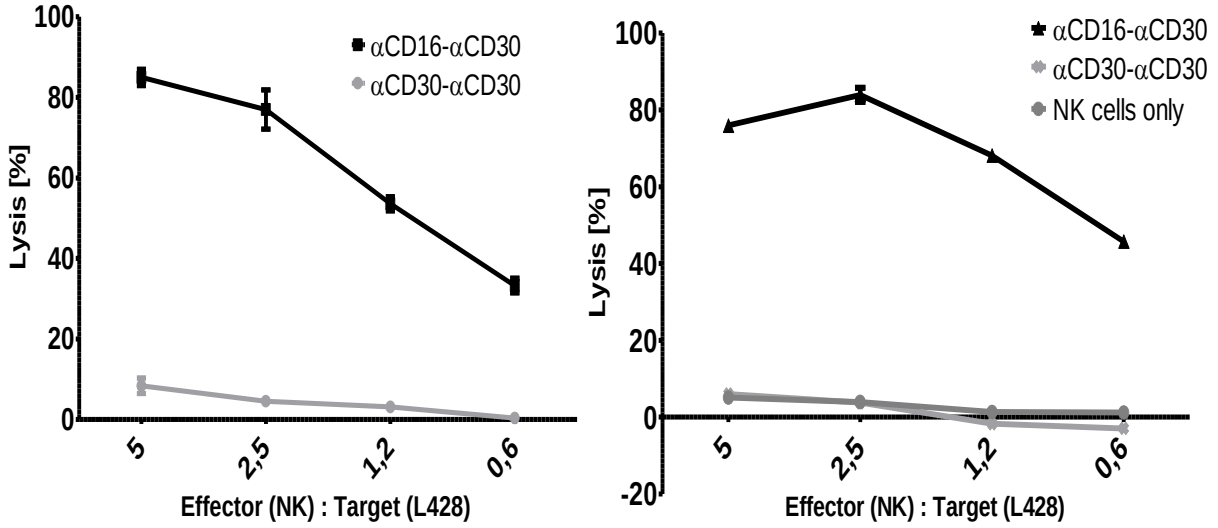


Figure 5.14: Cytotoxicity assay with αCD16-αCD30 treated NK cells

HL NK cells were purified and used for a standard europium release assay. Before co- culturing with L428 target cells, NK cells were briefly pre-incubated with either αCD16-αCD30 (**black**) or αCD30-αCD30 (**light grey**) or were left untreated (**dark grey**, right).
Indicated are means of triplets and shown are two representative experiments out of four.

The αCD30-αCD30 had no triggering effect on the cytotoxicity of HL NK cells (Fig 5.14, right). It was on a par with untreated HL NK cells and comparable to the low cytotoxicity of HL NK cells observed before. In contrast, HL NK cells pre-incubated with αCD16-αCD30 were activated and lysed target cells very efficiently. This strongly suggests that the αCD16-αCD30 construct triggers NK cells via CD16 receptor and presumably delivers a connection to CD30 on target cells.

5.2.6 αCD16-αCD30 immunoligand activates NK cells *in vivo*

After showing activating impact of this αCD16-αCD30 immunoligand in *ex vivo* experiments with HL NK cells, we sought to verify its effect in the *in vivo* environment. Therefore we examined peripheral NK cells gained from HL patients included in the phase I trial of this immunoligand conducted in the German Hodgkin Study Group.

First we controlled the binding efficacy of this construct to the NK cells. We examined NK cells from the blood of HL patients before and 24 h after infusion and checked in flow cytometry, whether these NK cells are “armed” with the construct.

In two patients we found over 50 % more of NK cells positive for “AFM13”, compared to before the start of transfusion (Fig 5.15 A). This proves that the αCD16-αCD30 immunoligand binds to NK cells *in vivo*, which is an essential precondition for subsequent anti-tumor activity. As NK cells were completely CD30 negative, the binding must be occurred via the αCD16 moiety to CD16.

The question that has been addressed next was if there is any impact on NK cell activity induced by the construct. Over a period of 22 days on dose level 4 (0,5mg / kg) several HL patients received four infusions. At all four time points, we checked peripheral NK cells in the blood for their activation status. We determined the level of CD69 (marker for early activation) positive cells before and 24 h and 48 h respectively, after infusing the immunoligand.

At all time points, we saw an increased value of CD69 positive cells 24 h after treatment compared to the results achieved prior to infusion start (Fig 5.15 B). 48 h after infusion, the level of CD69+ NK cells went down again. This is no surprise as CD69 marks only the initial phase of NK cell activation and activation impact of the construct should be terminated 48 h after treatment beginning.

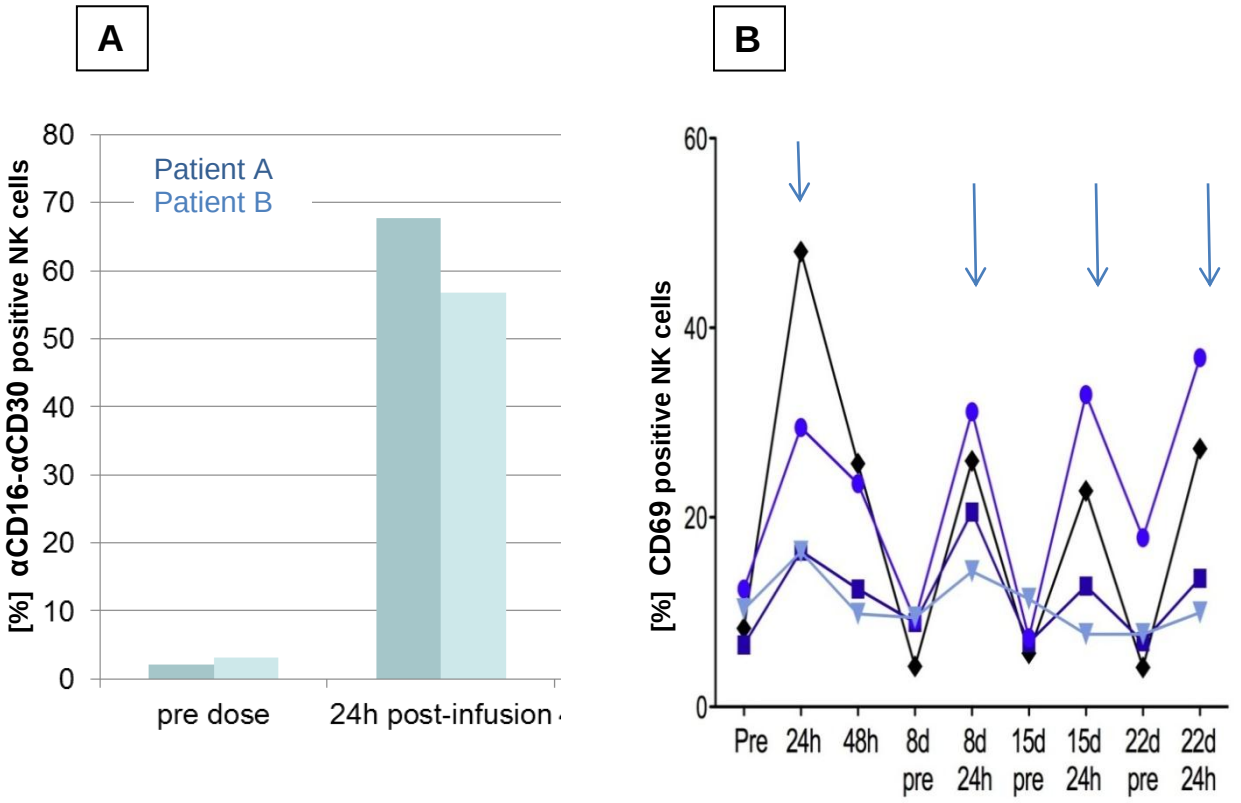


Figure 5.15: *In vivo* binding and activation of the αCD16-αCD30 immunoligand

(**A**) Binding of the αCD16-αCD30 immunoligand (AFM13) on peripheral blood NK cells was examined in flow cytometry for two patients. Depicted are results before start of the treatment and 24 h after end of infusion. (**B)** Indicated are values of CD69+ peripheral NK cells from four patients at several time points of the treatment cycle. Arrows mark measurements 24 h after infusion of the αCD16-αCD30 construct. (in cooperation with Katrin S. Reiners, Internal Medicine I, University hospital, Cologne)

To validate, whether the binding and the obviously increased activation state of NK cells in the HL patients might also lead to an enhanced cytotoxic efficacy towards HL cells, we also purified peripheral blood NK cells just before and 24 h after end of infusion ofthe immunoligand. We then used those NK cells immediately in a standard europium release assay with HL L428 cells as targets.

As seen in previous cytotoxicity assays (5.3.3), the endogenous killing potential of HL patient derived NK cells is extremely low. As expected, NK cells purified 24 h after infusing the immunoligand showed a relative cytotoxicity of 60 % and more (Fig 5.16).

This strongly indicates that the immunoligand armed NK cells on the one hand are activated via CD16 receptor and on the other hand presumably bind to CD30 positive target cells for degranulation and lysing.

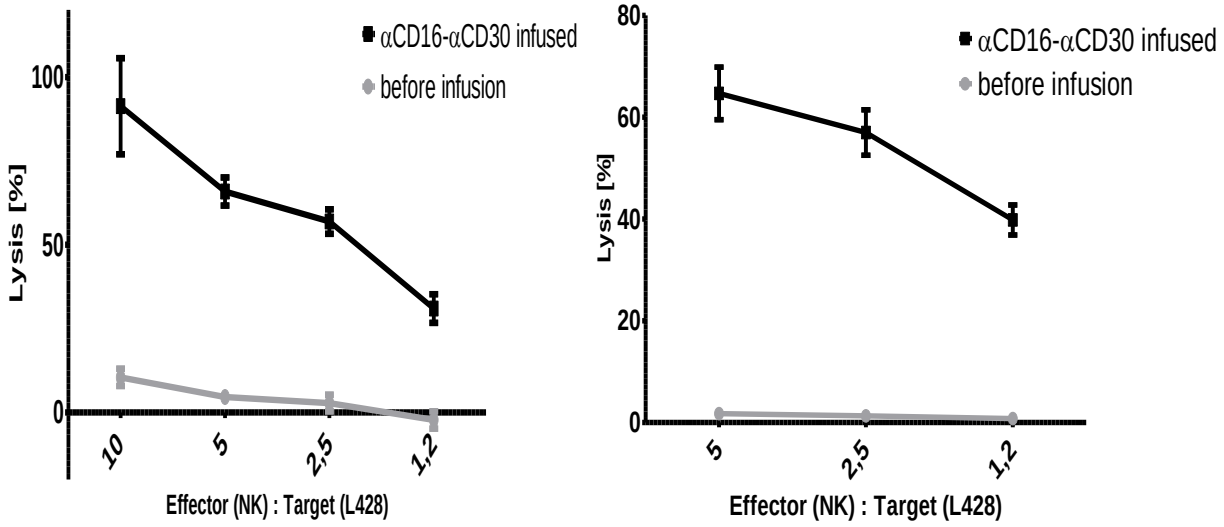


Fig. 5.16: Cytotoxicity of HL NK cells before and after infusing the αCD16-αCD30

Two representative experiments are shown, in which cytotoxicity of HL patient derived NK cells was determined either before starting infusion or 24 h after the infusion of the αCD16-αCD30 immunoligand AFM13. Depicted are means of triplets with standard errors at different effector target ratios. Different starting ratios results from the varying amount of NK cells purified from blood samples.

6 Discussion

6.1 Balance of activating and inhibitory signaling contributes to the distinct lysing ability of NK cells towards leukemic cell lines.

NK cells represent the key component of the innate immune system to recognize and eliminate cancer cells. There is recent clinical and experimental evidence that NK cells are critically involved in the immune surveillance of tumors (Waldhauer and Steinle 2008). Defects in NK cell function including impaired cytotoxicity/cytokine secretion, aberrant receptor expression profile, NK cell number and NK cell anergy are reported in all the haematological diseases malignancies studied so far (Sanchez, Le Treut et al. 2011). Moreover, for diseases such as chronic lymphocytic leukemia a positive correlation of NK cell inhibitory factors to disease stage and bad prognosis is observed (Nuckel, Switala et al. 2010). Moreover, the activity of allogeneic graft-derived NK cells in HLA-mismatched bone marrow or stem cell transplantations settings may critically contribute to the graft-versus-tumor effect (Ruggeri, Capanni et al. 2002; Shlomchik 2007) . These NK cells have the ability to recognize and kill malignant transformed cell. Unfortunately, this ability seems to be restricted. Following clinical investigations, acute lymphatic leukemia cells (ALL) are obviously more resistant against NK cell mediated lysis than acute myeloid leukemia cells (AML) are. Also in preclinical examination, a high killing efficacy against human AML could be demonstrated (Ruggeri, Capanni et al. 1999; Ruggeri, Capanni et al. 2002). Thus, the different activity of NK cells against hematological malignancies seems to be dependent on the tumor type; however the molecular basis or this is not well defined yet.

In this study it was shown for various cell lines that the expression of ligands for the activating NK cell receptor NKp30 contributes decisively to the stronger lysis of myeloid cell lines compared to lymphatic cell lines (Fig. 5.1). Blocking of Nkp30 signaling led to an abrogation of the superior lysing rates of myeloid cells. Ligands for other activating receptors are also expressed, but not in a pattern that did not correlate anyhow with the lysing rates detected in cytotoxicity assays (Fig. 5.2). This suggests a highlighted role for NKp30 signaling in this case. These results were confirmed by the finding that NK cells

treated with NKp30-ligand positive exosomes kill NKp30-ligand-negative cells better (Fig. 5.5).

Regarding the role of NKp30 for NK cell regulation, this receptor has another unique and outstanding function: it is involved in the crosstalk between NK cells and immature dendritic cells (iDCs) (Simhadri, Reiners et al. 2008).

The cross-talk may either results in a reciprocal activation and maturation of both cell types or in NK cell-dependent killing of immature dendritic cells, a mechanisms that limits the supply of antigen-presenting DCs to the immune response (Walzer, Dalod et al. 2005). In this crosstalk, NKp30 on NK cells seems to be the driving and critical receptor for both processes (Ferlazzo, Tsang et al. 2002; Vitale, Della Chiesa et al. 2005; Walzer, Dalod et al. 2005; Simhadri, Reiners et al. 2008).

It is therefore tempting to speculate that the differently expression level of ligands for NKp30 on AML versus ALL is not only responsible for the resistance of ALL cells against NK cell-killing but also one of the molecular reasons for the limited clinical efficacy of allgeneic NK cells for ALL patients. Supporting this suggestion it has been reported that the cross-talk between allogeneic NK cells and (host) dendritic cells (DCs) leads to the elimination of self-antigen-presenting DCs thus counteracting GvHD (Shlomchik 2007).

Vice versa it was recently reported that NK cells from AML patients are characterized by a down regulation of activating NK cell receptors including NKp30 (Sanchez-Correa, Morgado et al. 2011), which might explain why autologous AML-patient NK cells fail to recognize and destroy leukemic cells.

As an additional factor for the differing lysis of AML and ALL cell lines, we identified the expression of HLA molecules on the surface of leukemic cell lines leading to NK cell inhibition (Fig 5.4). The HLA expression pattern correlates with the determined lysis rates of these cell lines mediated by NK cells. While the examined myeloid lines (HL-60) showed only a comparative low expression of HLA molecules on their surface, especially the very weak lysed ALL lines 697 and NALM6 showed a clearly higher presence of these inhibitory molecules (Fig 5.4). Blocking of HLA signaling led to a slightly increased cytotoxicity as expected. As strong activating signals are probably absent due the lack of ligands for NKp30, the impact of abrogating inhibitory signaling is restricted.

Thus, according to the “induced self” and the “missing self” principle, we found two reasons, that could have led to the in some clinical studies observed discrepancy of NK cell mediated Graft versus leukemia (GVL). First, AML cells express ligands for NKp30,

leading to a higher susceptibility towards NK cell based lysis while ALL cells do not express these ligands. And second, ALL cell might express higher levels of HLA molecules, leading to an inhibitory signaling for NK cells and thus to a prevention of lysis.

As several studies in the last couples of years presented controvert data concerning the favorable outcome of NK cell alloreactivity in the recipient, analyses of studies protocols revealed that only minor changes in the study design and execution can led to varying results. Factors responsible for those differences seem to be for instance the stem cell dose, a depletion of T cells to avoid graft versus host directions or the donor source. Also the exact type myeloid malignancy as a target seems to play an important role since AML is divided into several subtypes, which resembles state and origin of the malignant blasts (Bennett, Catovsky et al. 1976; Ruggeri, Mancusi et al. 2007; Gill, Olson et al. 2009). Hence, we tried to confirm our data received from the experiments with leukemic cell lines with primary AML blasts received from blood samples of AML patients. Therefore we analyzed the samples (kindly provided by PD DR. Karl-Anton Kreuzer, Internal Medicine I, Cologne) in FACS analysis (data not shown) and checked again for the expression of ligands for activating NK cell receptors as well as for inhibitory HLA expression. Already our preliminary data with a limited number of AML samples showed a heterogeneity concerning the expression of either ligands for activating NK cells receptors. A potential reason for that could be that the various subtypes of AML have distinct phenotypes regarding their ligand expression.

6.2 NK cell inhibition contributes to the immune escape in Hodgkin lymphoma

A hallmark of Hodgkin Lymphoma (HL) is that the tumor cells, the so called Hodgkin Reed-Sternberg cells (HRS) are surrounded and outnumbered by a variety of immune effector cells. These cells include macrophages, mast cells, eosinophils and lymphocytes including NK cells (Pileri, Ascani et al. 2002). Instead of actively antagonizing the tumor formation, these immune cells contribute to the morphologic constitution and the pathogenesis of HL (Steidl, Connors et al. 2011).

In this work, it could have been demonstrated that peripheral blood NK cells derived from HL patients are clearly less potent in destroying HL target cells (HL cell line L428) than peripheral blood NK cells derived from healthy donors are. It is very likely that if peripheral NK cells in HL patients show an impaired cytotoxic phenotype, NK cells found in HL tissue suffer from the same restrictions.

Since a down regulation of NKp30, NKp44 and NKp46 (NCRs) have been observed in patients with breast cancer and multiple myeloma, we determined the expression pattern of a variety of activating receptors and activation marker. (Carbone, Neri et al. 2005; Mamessier, Sylvain et al. 2011). While the expression of NCRs, CD16 and activation markers like CD69 remained unchanged compared to NK cells from healthy donors, we found a significant lower expression of NKG2D on HL. NKG2D is an important receptor for the role of NK cells in tumor immune surveillance. It is frequently engaged by ligands that are up regulated on the surface of stressed and malignant cells according to the “induced self” principle of the regulation of NK cells (Bottino, Moretta et al. 2006; Waldhauer and Steinle 2008). Unsurprising, NKG2D seems to be a point of attack for evasion strategy of tumors. Some solid tumors and leukemias can secrete big amounts of NKG2D in a soluble form. In these cases, the surface expression is reduced by shedding and therefore tumor cells are less prone to be recognized and lysed by NK cells. Patients with epithelial tumors show decreased expression levels of NKG2D on peripheral blood as well as tumor infiltrated T and NK cells. This down regulation has been accompanied by increased levels of soluble ULBP2 and soluble MICA and MICB, all of them ligands for NKG2D. These usually membrane bound ligands are shed and released by the tumor cells, caused by a higher activity of metalloproteases (Groh, Wu et al. 2002; Salih, Rammensee et al. 2002; Doubrovina, Doubrovin et al. 2003; Salih, Goehlsdorf et al. 2006).

Thus, we checked the serum of about 300 HL patients for elevated levels of soluble ligands for activating NK cell receptors. Indeed we found increased levels of the NKG2D ligand MICA in the serum of HL patients when compared to healthy individuals. Regarding other soluble ligands for NKG2D, we found no significant difference in HL patients. However, we detected higher serum levels for BAT3, a ligand for NKp30. In a membrane bound shape it triggers cytotoxicity, but in its soluble form it suppresses NK cells functions (Pogge von Strandmann, Simhadri et al. 2007).

Moreover we found a “normalized” level of soluble MICA and BAT3 in patients after successful therapy and elimination of the tumor tissue. Interestingly, a small number of patients that showed a sustained, significant elevated level of soluble BAT3 suffered from a later relapse. In this context, we could show in HL tissue stainings that HRS cells express high amounts of BAT3 (data not shown). This could suggest that BAT3 is feasible as a prognostic marker of HL. Soluble ligands for activating NK cell receptor could be a decisive reason for the fact the NK cells in the HL tissue are unable to kill HRS cells. Moreover, the role of soluble factors including chemokines and cytokines is also a crucial one in the pathogenesis of HL, contributing to the permanent inflammation existing in the HL tissue (Steidl, Connors et al. 2011).

Of note there was no significant difference of NKp30 expression level HL NK cells compared to healthy donors (Fig. 5.7). This might be due to the strong dispersion of the values among different donors. In fact when NK cells of one donor, with a given NKp30-expression level are incubated with HL sera characterized by high levels of soluble BAT3 and MICA, a significant downregulation of NKp30 and NKG2D was observed (Fig. 5.9 A).

The latter might add another aspect to the tumor evasion strategy of shed and released surface ligands for activating NK cell markers. According to this down regulation of NKp30 and NKG2D on healthy NK cells, we also detected a negative impact on cytotoxic potential of healthy NK cells when being incubated with HL sera (Fig. 5.9 B). As the inhibitory effect of those soluble ligands are well described (Groh, Wu et al. 2002; Pogge von Strandmann, Simhadri et al. 2007), it is highly likely, that they contribute decisively to the observed inhibition. It also delivers an explanation for the determined impairment of HL patient derived NK cells in comparison to healthy donors.

Thus, soluble ligands shape phenotype and function of peripheral NK cells to contribute to immune evasion and immune tolerance against tumor cells. Similar conclusion was very recently published for breast cancer (Mamessier, Sylvain et al. 2011)

Thus, this is the first study that elucidates the role of NK cells in pathogenesis of HL plus the aberrant expression of soluble ligands for the activating NK cells receptors NKG2D and NKp30 as an evasion strategy in HL.

6.3 A αCD16-αCD30 immunoligand as an effective tool for NK cell based immunotherapy in HL

After we found out that the impairment of NK cells in HL patients is evoked by the serum and high levels of soluble ligands for the activating NK cells receptors NKp30 and NKG2D, we wondered whether this impairment might be reversible. Indeed, incubation with medium doses of IL-2 was sufficient to trigger a robust cytotoxic response against HL target cells, although the effect was attenuated by HL serum. This further tightens the rationale that the impairment of HL NK cells is not due to intrinsic factors but rather caused by their environment.

Moreover, given the fact, that the reduction of NK cells is reversible, this provides the opportunity for NK cell based immunotherapies in HL patients. The activating NK cell receptor CD16 is the most promising choice for stimulation of HL NK cells: one the one hand it is described as the only NK cell receptor whose triggering is sufficient for starting NK cell cytotoxicity without any costimulation of other receptors (Bryceson, March et al. 2006; Bryceson and Long 2008). One the other hand, CD16 is unaffected by the environment in HL patients. Downregulation on HL NK cells was not detected and CD16 expression remained stable on healthy NK cells when being incubated with HL serum. Therefore, we tested the efficacy a αCD16-αCD30 immunoligand (AFM13, Affimed). Beside CD16 on NK cells, it should also bind to CD30 on potential target cells.

CD30 is member of the tumor factor necrosis family and its overexpression on HRS cells is a hallmark HL. It is also known that constitutive CD30 signaling contributes to the proinflammatory tumor microenvironment (Yurchenko and Sidorenko 2010).

Cytotoxicity with HL derived NK cells revealed a high efficacy of this bispecific construct (Fig. 5.14). Killing of target cells was CD16 dependent, since a αCD30-αCD30 construct failed to induce any cytotoxic activity of HL NK cells. It was also dependent on CD30, as

CD30 negative target cells were not lysed by αCD16-αCD30 treated target cells (data not shown).

To confirm these promising *ex vivo* data, we checked the impact on NK cells of HL patients receiving infusions of this bispecific construct. Mandatory for activating and relocating NK cells is an effective binding of the construct. NK cells purified after infusion are armed with the construct. This proves that the binding of the αCD16 scFv works also in the natural environment of peripheral HL NK cells. 20-50 % of these NK cells also showed an expression for CD69, an early activation marker of NK cells. This indicates that these NK cells encountered CD30 positive target cells, since in experiments without CD30 positive target cells, NK cells have not been activated by the construct alone (data not shown).

The relative high percentage of activated peripheral NK cells was quite surprising, because in vitro data suggested that CD16-CD30-dependent activation is only observed in the presence of CD30-positive target cells.

It is thus possible that the construct armed NK cells bound to other CD30 positive immune cells (e.g. activated T or B cells), existing in the peripheral blood. (Alzona, Jack et al. 1994; Locksley, Killeen et al. 2001).Indeed, on the highest dose level (7,0 mg /kg body weight) we found CD30 positive NK cells (data not shown). Thus, there is an opportunity that activated NK cells can kill each other. This would lead to adverse reactions like immune deficiencies and susceptibility to subsequent infections.

Interestingly, studies with another αCD30 therapy approach in HL, revealed severe side effects that might be associated with drug-induced immune suppression via elimination of CD30-positive immune effector cells (FDA 2011, NCT01100562). Brentuximab vendotin is a αCD30 monoclonal antibody linked with an antitubulin agent (MMAE) for enhanced antitumor efficacy. The phase I study with this antibody-drug conjugate achieved complete remission in HL patients (Younes, Bartlett et al. 2010). This underlines the feasibility of developing immunotherapeutical approaches targeted at CD30. However, until now three cases have been reported, in which three patients receiving Brentuximab vendotin suffered from progressive multifocal leukoencephalopathy (PML) (FDA, 2012). This rare disease of the central nervous system is caused by the JC virus. Only individuals with a severe immune deficiency develop PML symptoms. This might be a hint that in these three cases the Brentuximab

vendotin treatment led to an impairment of antiviral immune responses, potentially a consequence of killing CD30 positive immune cells.

These reports and our results illustrate the potency of immunotherapies for the treatment of HL, and particularly for strategies aimed to trigger impaired NK cell function. However, the feasibility of CD30 as a tumor-associated antigen used for re-targeting the immune cells is probably limited, since it is also expressed on non-tumor cells and therefore might be lead to adverse effects. Other promising approaches can be developed on the basis of a better molecular understanding of the interaction of HL tumor cells and NK cells.

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Abbreviations

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APCs	antigen presenting cells
BAT3	HLA-B assoziertes Transkript 3
CD	cluster of differentiation
CD16	activating NK cell receptor
CML	chronic myeloid leukemia
DCs	dendritic cells
Fc	fragment crystallizable
GVHD	graft versus host disease
GVL	graft versus leukemia
HL	Hodgkin Lymphoma
HLA	human leukocyte antigen
HRS	Hodgkin Reed-sternberg
HSCT	hematopietic stem cell transplantation
iDCs	immature dendritic cells
IFN-γ	interferon-gamma
IL-2	interleukin-2
ITAM	immunoreceptor tyrosine based activating motif
ITIM	immunoreceptor tyrosine based inhibitory motif
MHC	major histocompatibility complex
MICA/B	MHC class I chain related A/ B
MIF	macrophage migration inhibitory factor
NK cell	natural killer cell
NKG2D	activating NK cell receptor
NKp30	activating NK cell receptor
NKp46	activating NK cell receptor
TNF-α	tumor necrosis factor-alpha
U	units

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Erklärung

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