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Impact of the NK Cell Receptor LIR-1 (ILT-2/CD85j/LILRB1) on Cytotoxicity against Multiple Myeloma

Dissertation

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2 INTRODUCTION

2.1 Hypothesis

The role of different receptors in natural killer (NK)-cell-mediated cytotoxicity against multiple myeloma (MM) cells is unknown. It was investigated if an enhancement of NK-cell-mediated cytotoxicity against MM could be reached by blocking of the inhibitory leukocyte immunoglobulin like receptor 1 (LIR-1). These investigations revealed high levels of LIR-1 expression not only on the NK cell line NK-92 but also on myeloma cells (MOLP-8, RPMI8226) as well as on a lymphoblastoid cell line (LBCL; IM-9). Subsequent cytotoxicity assays were designed to show the isolated effects of LIR-1 blocking on either the effector or the tumor side to rule out receptr-receptor interactions.

MM is a plasma cell disorder that is characterized by the clonal proliferation of terminally differentiated cells. Due to their high similarity to healthy plasma cells, an effective immune defense seems to be hindered (Cook and Campbell, 1999; Carbone et al., 2005). Despite new therapy approaches, MM is still considered to be incurable. Stem cell transplantation is so far the only option to achieve long time remission, but has the disadvantages of a high therapy-related morbidity and mortality (Palumbo and Anderson, 2011). Following a year or more after reinfusion of cluster of differentiation (CD) 34+ progenitor cells, newly arising B cells and T cells show restricted functions. Only NK cells with their innate capability of defending foreign pathogens return to their post within the first month after stem cell transplantation (SCT), thus being the predominant leukocyte subset within the first 90 days (Ault et al., 1985; Mandelboim et al., 2001; Porrata et al., 2001; Chalifour et al., 2004).

Approaches for an increase of immune reaction against the residing myeloma burden after SCT focus on a disinhibition of NK cells, leading to an increased graft-versus-myeloma (GvM) effect. LIR-1 is one of the major inhibitory NK cell receptors in the early phase after stem cell transplantation (SCT) and might therefore be considered to be an ideal goal for immune modulation (Porrata et al., 2001; Nguyen et al., 2005; Godal et al., 2010).

LIR-1 ligates most classical and non-classical human leukocyte antigens class I (HLA-I), thus being a general sensor for aberrations from the healthy surface pattern (Chapman et al., 1999). As MM retains high levels of HLA-I during growth, the intentional blockade of LIR-1 on NK cells was suspected to increase NK cell cytotoxicity towards a level sufficient to overcome myeloma resistance against lysis.

2.2 Multiple myeloma

2.2.1 Definition, incidence and prevalence

MM is a plasma cell disorder, which is characterized by extended proliferation of terminally differentiated cells that displace healthy bone marrow. They comprise > 10 percent of bone marrow, secrete monoclonal proteins and cause end organ damages.

MM accounts for approximately 13 percent of hematologic cancers and 1-2 percent of all malignancies. Though primarily a disease of the elderly population, still 37 percent of the patients are younger than 65 years, which is the median age at diagnosis. Incidence is stable at 4/100.000 and is twice as often for Afro-Americans compared to Caucasians. The disease is still incurable despite new therapy approaches within the last years (Palumbo and Anderson, 2011).

2.2.2 Diagnostic criteria and pathogenesis

MM is one of the diseases classified as plasma cell disorders that include Waldenström´s macroglobulinemia, primary amyloidosis and the heavy chain diseases (Fauci and Harrison, 2008) .

In 2009, the criteria for diagnosis of MM have been reviewed and were updated in 2011 (Table 1 - Diagnostic criteria for MM) (Kyle and Rajkumar, 2009; Rajkumar, 2011). More than 10% plasma cell infiltrations of the bone marrow, detection of monoclonal proteins in serum and/or urine, as well as end-organ damages define the disease. MM itself can be subdivided by the type of secreted immunoglobulin (Ig) (A, D, E, G, M) and the secreted light chains (λ or κ) or the lack of Ig secretion. Suppression of bone marrow leads to anemia, while high expression of unfunctional immunoglobulins ('paraproteins') causes severe organ dysfunctions such as kidney failure (Rajkumar, 2011). Infections are a severe problem in patients with MM. They harm the patient directly, but also provide a high proliferative stimulus for MM cells via their pathogen associated molecular pattern (PAMP) recognition capability (Jego et al., 2006). Bone lesions are caused by suppression of osteoblasts through changes in the RANK pathway and activation of osteoclasts by macrophage inflammatory protein 1 α (MIP1α) (Roodman, 2010).

Diagnostic criteria for multiple myeloma		
Clonal bone marrow plasma cells		≥ 10%
Presence of serum and/or urinary monoclonal protein (except in patients with true non-secretory multiple myeloma)		
Evidence of end-organ damage that can be attributed to the underlying plasma cell proliferative disorder, specifically		
	hypercalcemia:	
	serum calcium	≥ 11.5 mg/dl
or	renal insufficiency:	
	serum creatinine	> 1.73 mmol/l (>2 mg/dl)
	or estimated creatinine clearance	< 40 mL/min
and	anemia: normochromic, normocytic	
	hemoglobin	> 2 g/dl below the lower limit of normal or < 10 g/dl
and	bone lesions	
	lytic lesions, severe osteopenia or pathologic fractures	

Table 1 - Diagnostic criteria for MM
Diagnostic criteria for MM according to Kyle and Rajkumar (Kyle and Rajkumar, 2009; Rajkumar, 2011).

The most important differential diagnosis to MM is monoclonal gammopathy of undetermined significance (MGUS) with a high prevalence among the elderly that does not require treatment. It is described by <10 percent clonal bone marrow plasma cells and no myeloma related organ or tissue impairment. Serum monoclonal protein must be lower than 3g/dl. The risk for evolving MM is about 1 percent per year, and survival of the MGUS patients is reduced by about 2 years compared to healthy age matched individuals (Fauci and Harrison, 2008; Kyle and Rajkumar, 2009). Smoldering or asymptomatic MM shows serum monoclonal protein ≥3g/dl as well as plasma cell infiltration of ≥10 percent, but lacks end organ damages. Solitary lesions of bone or tissue with clonal plasma cells are defining plasmocytoma, while clonal proliferation as well as the concomitants is missing. Waldenström's macroglobulinemia is an IgM producing gammopathy. The bone marrow infiltrating lymphoplasmacytic cells have a

distinct immunophenotype. As indicated, systemic amyloid light chain (AL) amyloidosis is characterized by amyloid-related systemic tissue damage, while in POEMS syndrome a conglomerate of polyneuropathy, organomegaly, endocrinopathy, MM and skin changes is exhibited (Fauci and Harrison, 2008; Kyle and Rajkumar, 2009).

Staging of MM involves the detection of bone lesions by conventional radiography and optional magnetic resonance imaging (MRI) or fluorodeoxyglucose positron emission tomography/computed tomographic scans (PET-CT) (Rajkumar, 2011).

Today, the International Staging System (SS) and the older Durie-Salmon Staging (DSS) are used together for staging and for prediction of survival (Table 15 - International Staging System for multiple myeloma) (Durie and Salmon, 1975; Greipp et al., 2005).

2.2.3 Etiology

The cause for MM development is unknown. Incidence is elevated in those exposed to high radiation, as well as among farmers, wood workers, leather workers and people exposed to petroleum products. As circulating B cells within patients with MM show to express the same myeloma specific idiotype, the neoplastic event might occur in early B cell differentiation, but has not yet been identified (Fauci and Harrison, 2008).

Development of MM out of a post-germinal-center B cell is commonly assumed to be a multistep process that begins with MGUS and can eventually turn into Plasma-cell leukemia (PCL) (Kuehl and Bergsagel, 2002; Palumbo and Anderson, 2011).

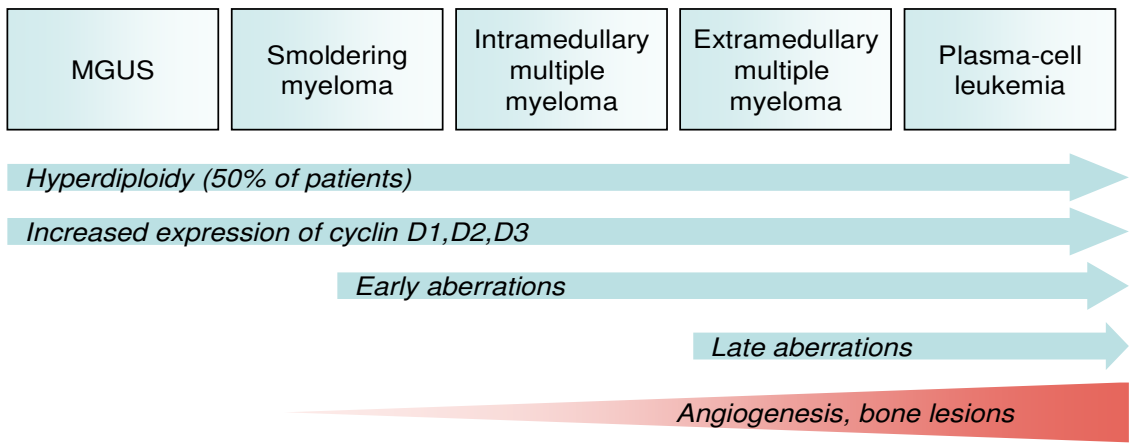


Figure 1 - Stages of development from MGUS to plasma cell leukemia
Commonly assumed stages in development of MM. While hyperdiploidity and increased cyclin D expression can be detected in all stages, other specific mutations or translocations might be acquired later in the course of disease [figure adopted from Kuehl and Bergsagel, 2002; Palumbo and Anderson, 2011].

High- and intermediate-risk aberrations correlate with fast disease progression and reduced therapy outcome. Today, they are defined as deletion (del) of the short arm (p) of chromosome 17 (del17p), translocation (t) between chromosome 4 and chromosome 14 (4;14), t(14;16), t(14;20) and del13p according to the Mayo Stratification for Myeloma and Risk-adapted Therapy (mSMART) (Table 2 - Mayo risk stratification for MM) (Kumar et al., 2009).

The high-risk aberrations all promote tumor growth, either by loss-of-function of tumor suppressor genes or by gain-of-function mutations of so called oncogenes:

Del17p leads to loss of p53, a well described tumor suppressor gene that is involved in DNA excision repair and initiation of cell cycle arrest and apoptosis, which are induced by damaged DNA as well as hypoxia (Levine, 1997; Kumar et al., 2009). P53 deletion can be observed in about 9-55% of patients with advanced stage of MM (stage III, primary PCL, secondary PCL, or relapse) with a wide range between the observers (Drach et al., 1998; Avet-Loiseau et al., 1999). Drach et al. found a reduced median overall survival of patients with p53 deletion, compared to patients without these aberrations, that was 14 months versus 39 months after conventional chemotherapy, respectively.

Del13q14 leads to deletions of the retinoblastoma-1 (rb-1) gene, another major tumor suppressor gene (Zojer et al., 2000).

The three risk translocations in MM involve the immunoglobulin heavy chain region on the long arm of chromosome 14 (14q32):

Translocation (4;14)(p16.3;q32.3) is present in 10-18% of MM. In almost all cases it leads to increased expression of Multiple Myeloma SET domain protein (MMSET) on 4p16.3, while the same translocation activates the tyrosine kinase fibroblast growth factor receptor 3 (FGFR3) on 14q32 in about two thirds of affected myelomas. FGFR3 is so far assumed to act as an oncogene by stimulating cell proliferation (Chesi et al., 1998b; Santra et al., 2003), while MMSET allows adhesion to extracellular matrix and annulling of cell-cycle arrest (Lauring et al., 2008).

t(14;16)(p32;q23) seems to be prevalent only in a small number of MM patients as investigated in a recent retrospective study and was not prognostic for outcome (Avet-Loiseau et al., 2011). On a biological level, it causes expression of the transcription factor and oncogene c-maf on 16q23 (Chesi et al., 1998a).

t(14;20)(q32;q12) is a new high-risk classified translocation resulting in expression of transcription factor V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB). MAFB is usually expressed on hematopoietic cells of the myelo-monocytic lineage and therefore ectopic in MM. The consequence of MAFB expression on plasma cells is not quite clear but expression might hinder normal B-cell

differentiation and proliferation (Hanamura et al., 2001; Boersma-Vreugdenhil et al., 2004).

Mayo Stratification for Myeloma And Risk-adapted Therapy (mSMART)		
High-risk	Intermediate-risk*	Standard-risk*†
FISH del17p t(14;16) t(14;20) GEP: High risk signature	t(4;14) ‡ Cytogenetic deletion 13 Cytogenetic hypodiploidity PCLI ≥ 3%	All others including Hyperdiploidity t(11;14)* * t(6;14)

Table 2 - Mayo risk stratification for MM
Definition of high risk disease, updated June 2010/June2011, version 2.0 (*www.mSMART.org*). High risk disease results in fast progression of MM (Kumar et al., 2009).

* Note that a subset of patients with these factors will be classified as high-risk by GEP
† LDH >ULN and beta-2 M > 5.5 may indicate worse prognosis
‡ Prognosis is worse when associated with high beta-2 M and anemia
**t(11;14) may be associated with plasma cell leukemia
FISH = fluorescence in situ hybridization, PCLI = Plasma cell labeling index, GEP =Gene expression pattern

2.2.4 Myeloma immune evasion

As most of the mechanisms of immortalization described above (2.2.3 Etiology) are quite common among malignancies, they do not explain the outraging success of untroubled expansion (Hanahan and Weinberg, 2000).
MM seems to persist within the so called 'myeloma niche'. Significantly increased cytokine levels characterize the bone marrow of newly diagnosed patients of which interleukin-16 (IL-16) and hepatocyte growth factor (HGF) correlate positively with bone marrow infiltration and might be essential in adhering MM (Cao et al., 2010). By this, MM persistence exhibits clear analogies to viral strategies, that themselves use the induction of cytokine secretion as well as stimulation of bystander cells to create a co-operative environment (Alcami and Koszinowski, 2000).
Furthermore, MM cells interact with the immune cells in their surroundings. They change the CD8/CD4 T-cell ratio towards a decrease of CD4 cells, what is associated

with a lower survival rate and an increased relapse probability rate compared to patients with a normal ratio. In normal T-cell activation, after MHC-II-mediated antigen presentation, an indispensable second co-stimulatory signal needs to be provided by B cells or other antigen-presenting cells via CD80 and its ligand CD28 on the T-cell surface. Interestingly, plasma cells from MM patients, plasmocytomas and human myeloma cell lines (HMCL) do not express co-stimulatory CD80 but do express CD28 levels that are as high as on T cells (Zhang et al., 1998). Additionally MM takes profit of the maintenance of HLA-class-I molecules, by which they retain a surface pattern that does not provoke NK-cell activation (Carbone et al., 2005). Though MM expresses the idiotype (Id), a clone specific antigenic determinant of the produced immunoglobulin, they are poor presenters and remain undetected. Secretion of TGF-β mediates T-cell cycle arrest, and the increased IL-10 serum levels in MM patients efficiently reduce NK-cell responses (Cook and Campbell, 1999; Chalifour et al., 2004).

Both T cells as well as NK cells express FasL after activation and induce apoptosis of their target cell via Fas. Afterwards, the immune cells themselves express Fas and undergo apoptosis to limit the immune reaction (Nagata, 1996). MM express both Fas and FasL on their surface, but have an intrinsic and not yet fully understood resistance towards Fas-mediated apoptosis (Greil et al., 1998; Cook and Campbell, 1999). Therefore, they are able to evade immune cells and what is more, to induce their death. With this mechanism, MM just mimics the behavior of tissues in the so called 'immune-privileged' sites such as eye, brain, placenta, uterus and testis. In those sites, FasL expression on non-lymphoid cells and consecutive killing of invading immune cells prevents destructive immunological responses. The expression of FasL has been shown for various MM cell lines and lymphoblastoid cell lines (LCL), including RPMI-8226 and IM-9, and in vivo assays with T-ALL target cells have proven the ability of MM cells for Fas-mediated target killing (Greil et al., 1998).

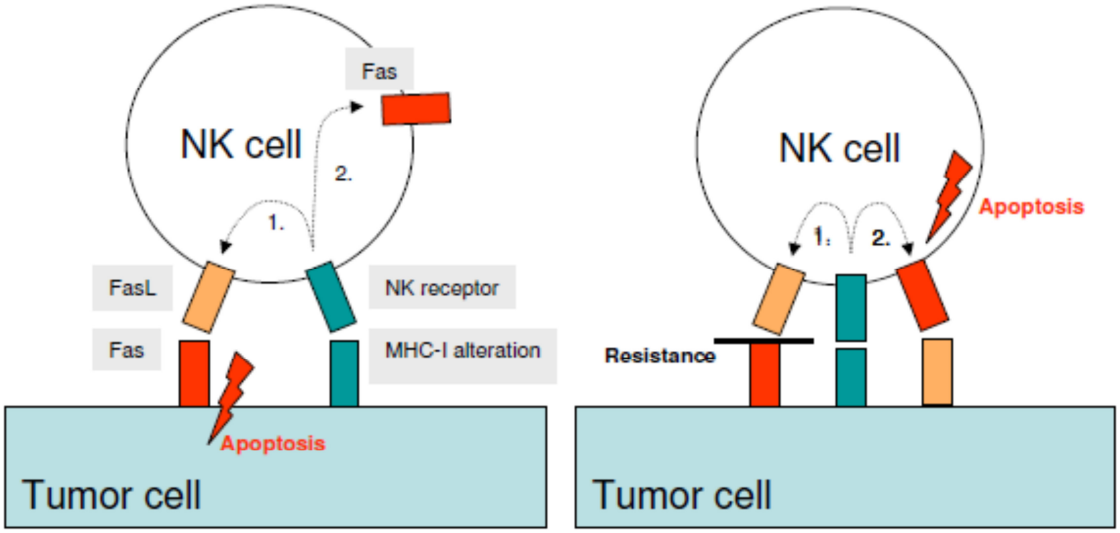


Figure 2 - Mechanism of FasL mediated tumor survival
After MHC or antigen contact, NK cells as well as T cell express apoptosis-inducing FasL. To down regulate immune response, the interaction is followed by Fas induction on the immune cell. MM cells bear Fas and FasL on their surface but are themselves resistant to Fas mediated apoptosis [figure adopted from Nagata, 1996].

Recently detected survival factors for MM cells are constitutively expressed toll-like receptors (TLR), that are functional and stimulate MM growth after PAMP recognition by autocrine IL-6 secretion (shown for primary myeloma cells and HMCL such as RPMI-8226). The pattern of TLR (mainly TLR 1, 7 and 9) is hereby different from that of B cells. Ligands like oligo-DNA with CG palindromes (CpG) protect the MM cells from serum deprivation and dexamethasone-induced apoptosis and stimulate cell expansion, so that infections of MM patients are harmful in two ways: They weaken the patient but furthermore are a stimulus for the growth of plasma cells (Jego et al., 2006).

2.2.5 Therapy

Today, a broad spectrum of therapies is available to prolong the life span of patients. Therapy is generally not started before the disease becomes symptomatic. Besides conventional chemotherapy, new immunomodulatory drugs (ImiDs) like thalidomide, lenalidomide and bortezomib lead to prolonged life spans and therefore limit the indications for stem cell transplantation (SCT), but are still under extensive evaluation (Singhal et al., 1999; Barlogie et al., 2006; Palumbo et al., 2006; Kumar et al., 2009). Patients who undergo SCT receive high-dose chemotherapy to eliminate their own blood formation (myelo-ablative therapy). Reinfusion of previously collected own (autologous) stem cells is well-tolerated, but lacks additional anti-tumor effects. Donor-

derived (allogeneic) stem cells additionally recognize the transplanted patient as 'foreign' and the stem cells offend the residing tumor burden (graft-versus-myeloma effect), but might also damage the healthy tissue (graft-versus-host effect) (Tricot et al., 1996).

Choice of therapy is related to age and risk factors (Table 2 - Mayo risk stratification for MM). While patients younger than 65 years without severe organ dysfunctions might receive induction therapy with thalidomide, lenalidomide or bortezomib, autologous SCT with reduced conditioning regimes is an option for the elderly (Palumbo and Anderson, 2011). It is important to determine early during treatment, if transplantation is eligible, in order to spare stem cell toxic reagents such as melphalan. After conditioning regimes with melphalan with or without total body irradiation (TBI), patients receive single or repeated (tandem) grafts during transplantation. As autologous cells do not exhibit additional anti-tumor effects, high relapse rates (about 80%) within the first five years after transplantation are still a major problem, and optimizing immune response against the residing tumor cells is aim of many investigations (Table 16 - Outcome of MM patients after autologous stem cell transplantation) (Attal et al., 1996; Bourhis et al., 2007).

Allogeneic stem cell transplantation can potentially cure MM, but shows only a median overall survival of 50 months with a median survival of ~60% after 2 years, accompanied with high relapse rates up to 20% within this time span (Gahrton et al., 2001).

2.3 Natural killer cells

2.3.1 Characterization and classification

Natural killer (NK) cells were named for their ability to instantly and efficiently kill tumor cells, bacteria or virus infected cells without the need of prior antigen contact (immunization) (Herberman et al., 1975; Garcia-Peñarrubia et al., 1989; Biron and Brossay, 2001). By this, they were initially considered as a part of the innate immune system, while an advanced view acknowledges their role as an interlink between the innate and the adoptive immune system, due to an immense excretion of cytokines (Lanier et al., 1986; Fehniger et al., 2003; Caligiuri, 2008).

NK cells represent only 10-15 percent of peripheral blood lymphocytes (Lanier et al., 1986). While a unique NK cell marker is still missing, NK cells are merely defined by surface expression of CD56 and lack of CD3 (Robertson and Ritz, 1990). NK-T cells are a CD56+CD3+ subset of T-lymphocytes that is not further discussed within this work (Pittet et al., 2000).

Surface density of CD56 divides two NK cell subsets ($CD56^{bright}$ and $CD56^{dim}$) that have different key aspects in cytokine production and cytotoxicity, reflected by differences in their receptor patterns (Lanier et al., 1986; Cooper et al., 2001). The phenotype of NK-92 effector cells, which were used within the present experiments, resembles mostly the $CD56^{bright}$ subset.

$CD56^{bright}$ cells have no or very low amount of CD16 and add up to 10 percent of NK cells in peripheral blood but 90 percent of the NK cells in the lymph nodes (Campbell et al., 2001; Fehniger et al., 2003). This subsets' preference of the lymph nodes is explained by the particular expression of chemokine receptors and adhesion molecules (Frey et al., 1998; Campbell et al., 2001). Just as known for B and T cells, the high expression of CC chemokine receptor 7 (CCR7) might allow NK cells to migrate into lymph nodes, where the adhesion molecule L-selectin is necessary for homing through high endothelial venules (Warnock et al., 1998; Warnock et al., 2000). L-selectin expression is increased by interleukin (IL)-12, IL-10 and interferon (IFN)-α on both $CD56^{bright}$ and $CD56^{dim}$ cells, while downregulation follows NK cell activation via IL-2, IL-15 and TGF-β, thus increasing the NK cell count in the blood stream during inflammation. Crosslinking of L-selectin leads to activation of lymphocyte function-associated Ag 1 (LFA-1) that is the necessary following step for leukocyte extravasation (Frey et al., 1998).

Especially $CD56^{bright}$ cells are assumed to play a key role in the early immune reaction on pathogens due to expression of high affinity IL-2 receptors (IL-2R$\alpha\beta\gamma$) and growth promoting c-kit tyrosine kinase (CD117) that lack on $CD56^{dim}$ cells (Caligiuri et al., 1990; Matos et al., 1993; Frey et al., 1998; Fehniger et al., 2003).

Interaction between NK cells, dendritic cells (DC) and T cells in the lymph nodes is strong. Upon antigen contact, DC activate NK cells by monokines such as IL-12, IL-15, and IL-18. Vice versa NK cells stimulate DC by subsequent release of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and GM-CSF for proliferation and antigen presentation. IFN-γ primes DC toward an IL-12 producing phenotype that induces strong protective CD8 T cell responses. IL-10 and TGF-β secreted by DC can on the other hand diminish NK cell responses (Fehniger et al., 2003; Mocikat et al., 2003; Caligiuri, 2008).

$CD56^{bright}$ cells also exhibit the same or even higher cytolytic capacity against tumor cells compared to $CD56^{dim}$ cells despite the lack of CD16 (Lanier et al., 1986; Ellis and Fisher, 1989; Caligiuri et al., 1990).

About 90 percent of NK cells in the peripheral blood are characterized by expressing CD56 at a very low surface density ($CD56^{dim}$) while showing high amounts of FcγRIII ($CD16^{bright}$). The later enables them to exhibit strong antibody dependent cytotoxicity

(ADCC) (Campbell et al., 2001; Cooper et al., 2001). Small amounts of L-selectin and a high expression of LFA-1 explain the preference of CD56^{dim} cells to persist in peripheral blood (Frey et al., 1998).

Differences in chemokine receptor patterns lead to the proposal that CD56^{bright} cell interaction might have its focal point in chronic inflammation like monocytes, while the CD56^{dim} cells might be involved predominantly in acute inflammation like neutrophils (Campbell et al., 2001). Another distinction can be seen in the pattern of the subsets` inhibitory NK cell receptors. While CD56^{bright} cells express high levels of the CD94-NKG2A heterodimer but diminishing amounts of KIR and LIR-1, the CD56^{dim} subset exhibits the opposite configuration (Cooper et al., 2001).

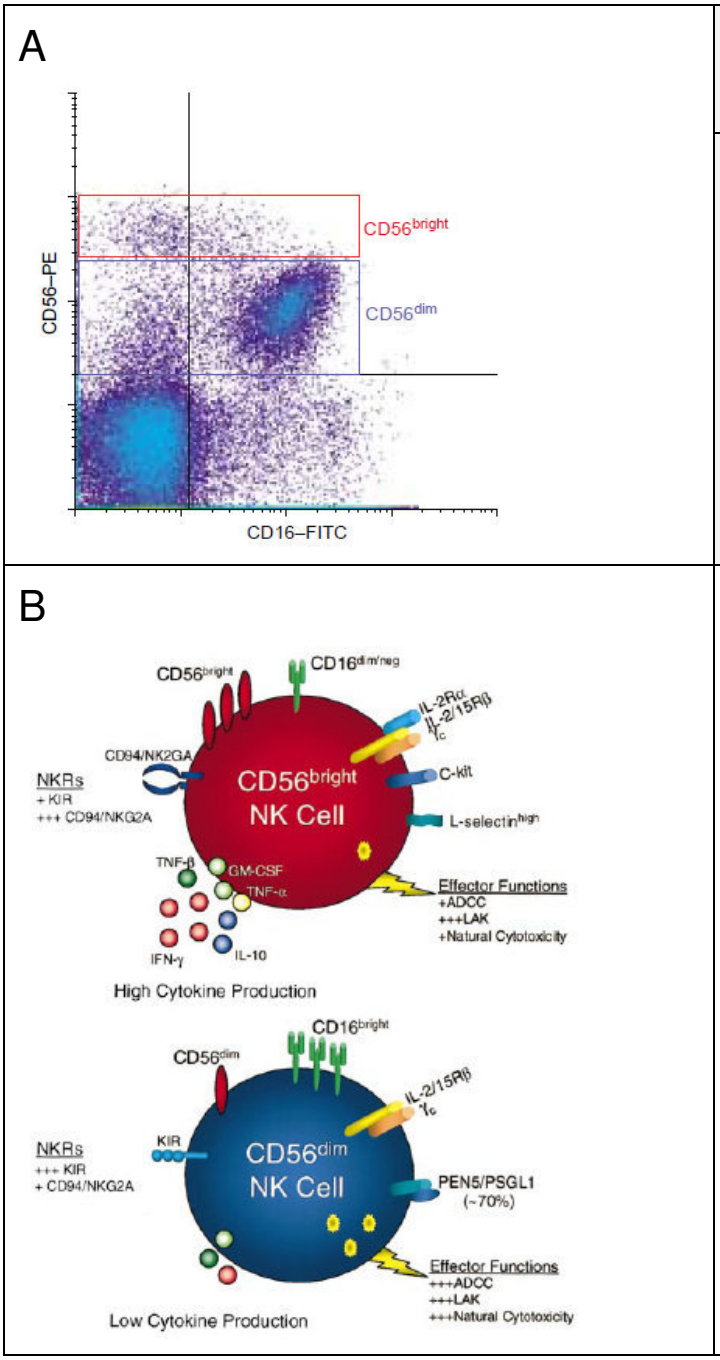


Figure 3 - Human Natural killer cell subsets

(A) Flow cytometric analysis of CD56^{bright} and CD56^{dim} NK cells and (B) depicted surface molecules
[(A)/(B) according to Cooper et al., 2001].

(C) Morphology of bright and dim Giemsa stained CD56+ CD3- lymphocytes. Magnification 6000x [microscopic picture published by Lanier et al., 1986].

2.3.2 Development

Though little is known about the detailed steps of NK cell development, it is assumed today that the initial development occurs in the bone marrow and requires IL-15 (Lanier et al., 1986; Mrózek et al., 1996). For expansion, c-kit ligand (KL) and flt-3 ligand (FL) are necessary (Mrózek et al., 1996; Yu et al., 1998). Most likely, the main process of NK cell development is located in the lymph nodes. Here, almost all CD34+ cells can be defined as $CD34^{dim}CD45RA+\beta_7{}^{high}$. They differentiate to $CD56^{bright}$ NK cells under the influence of IL-2/ IL-15 that is expressed by activated T cells and antigen-presenting cells, but cannot be prompted for progression to $CD56^{dim}$ cells (Freud et al., 2005). The current model for NK cell development assumes a more mature $CD56^{dim}$ subset to evolve from $C56^{bright}$ progenitor cells under the influence of interactions between CD56 and fibroblast growth factor receptor-1 (Chan et al., 2007; Caligiuri, 2008).

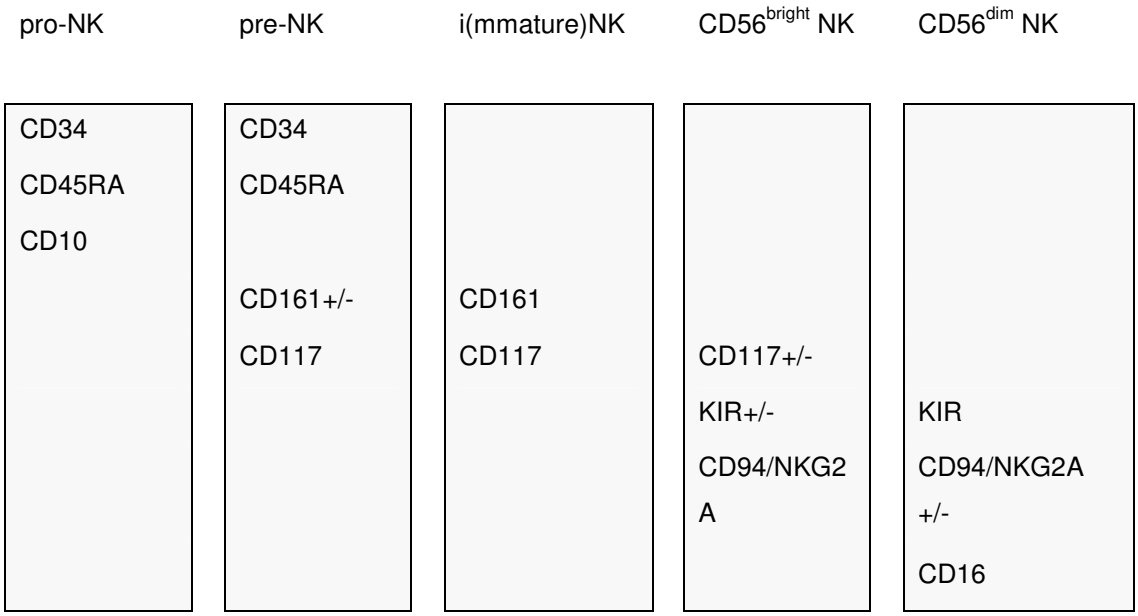


Figure 4 - Development of NK cells
Current model of NK cell development. $CD34^{dim}CD45RA+\beta7^{high}$ NK cell progenitors in the BM might traffic to the lymph nodes and differentiate to $CD56^{bright}$ cells under the influence of IL-2/ IL-15, while $CD56^{dim}$ cells seem to evoke from the bright subset as initially proposed in 1986 (Lanier et al., 1986; Freud et al., 2005; Freud and Caligiuri, 2006; Caligiuri, 2008) [figure adopted from Caligiuri (2008)].

Unlike T cells, NK-cell receptors do not undergo rearrangement, for pathogen recognition of NK cells is not antigen dependent but dictated by the sum of incoming signals via ligands of the target surface (Leiden et al., 1988; Lanier, 2005). During a process called licensing, NK cells with receptors for self MHC-I molecules are positively

selected and stimulated for proliferation. Missing receptors against self MHC-I molecules do not lead to depletion but to a second subset of resting NK cells that might be activated via IL-2 (Kim et al., 2005). Thus, apart from missing-self recognition, activating signals are required to achieve cytolysis or cytotoxicity (Kärre et al.; Ljunggren and Kärre, 1990).

2.3.3 NK cell signaling pathways

Though the signaling pathways of NK cells are not fully understood, there seem to be great similarities to B- and T cells. For the best described groups of activating and inhibitory NK cell receptors, the signaling occurs via cytoplasmic immunoreceptor tyrosine-based inhibitory or activating motifs (ITIMs, ITAMs) (Lanier, 2005).

ITAM-mediated signaling mostly requires the recruitment of adapter molecules and leads to Ca^+ influx, degranulation, and transcription of cytokine and chemokine genes.

The sequence of ITAMs is aspartic acid/ glutamic acid-x-x-tyrosine-x-x-leucine/isoleucine-(x6–8)-tyrosine-xx-(leucine/isoleucine), where x denotes any optional amino acid. The three main ITAM-containing adapter proteins are the gamma chains of high-affinity receptor for the Fc region of immunoglobulin E (FcεRIγ), CD3ζ chain (CD3ζ) and killer cell activating receptor-associated protein /DNAX activating protein of 12kDa (DAP12/KARAP). Tyrosine phosphorylation of the ITAM is followed by recruitment of spleen tyrosine kinase (Syk) and Zeta-chain-associated protein kinase 70kDa (ZAP70) (Lanier, 2005; Vely and Vivier, 2005).

ITIMs consist of an amino acid domain with the conserved sequence (isoleucine/valine)-x-tyrosine-x-x- (leucine/valine) (Muta et al., 1994; Burshtyn et al., 1997).

Crosslinking of inhibitory receptor leads to the inhibition of Ca^+ influx (Muta et al., 1994; Colonna et al., 1997; Lanier, 2005). Src-family kinases recruit SH2-containing protein-tyrosine phosphatase (SHP)-1 and SHP-2 or SH2-containing inositol polyphosphate 5-phosphatase (SHIP) and a direct interaction between inhibiting and activating receptors via SHP has been proposed (Thomas, 1995; Borges and Cosman, 2000). While SHIP exhibits its inhibitory function by preventing Ca^+ mediated signaling, SHP-1 and SHP-2 recruitment leads to a reduction of signal protein phosphorylation (Lanier, 2005; Vely and Vivier, 2005).

LIR-1 signaling is mediated by SHP-1 but not SHP-2 or SHIP (Colonna et al., 1997).

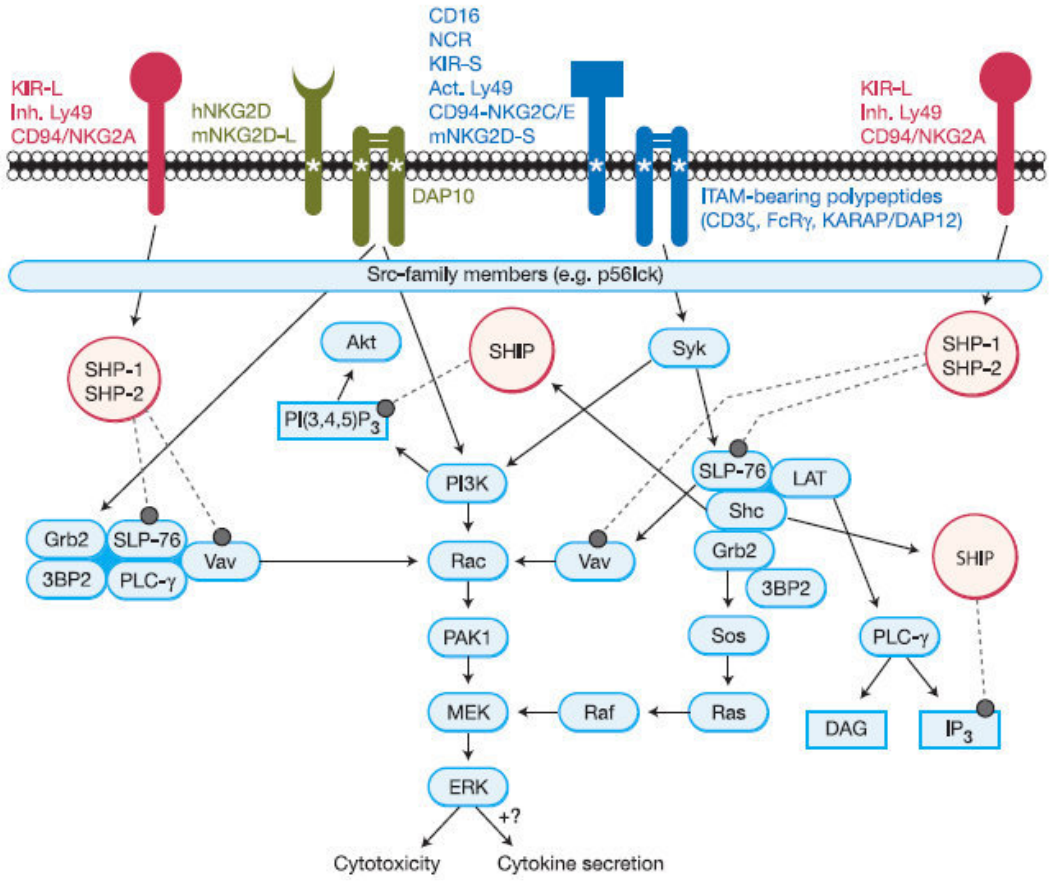


Figure 5 - NK cell effector signaling pathways
Inhibitory MHC-I dependent receptors (red) and activating receptors (blue, green) in human (h) and mice (m). Presence of a transmembrane charged amino acid is marked with asterisks. KIR-S represents KIR with a short intracytoplasmic domain and no intrinsic signaling properties, KIR-L are inhibitory receptors with an intracytoplasmic ITIM. NGK2D splice forms do only exist in mice but not in humans [picture and legend adopted from Vely and Vivier, 2005].

2.3.4 NK cell regulation and defense mechanisms

Natural killer cells originally obtained their name due to the detection of instant lymphocyte-mediated killing of mouse Moloney leukemia cells in vitro that did not involve T cells or B cells (Herberman et al., 1975; Kiessling et al., 1975). Their activation mechanisms allow them to detect and deteriorate foreign or internal pathogens without the necessity of additional co-stimulation or prior immunization. As a part of the innate immune system, they play a key role in the defense against bacteria and viruses. They are the main subset of peripheral blood mononuclear cells (PBMC) that responds to a bacterial stimulus by proliferation and upregulation of activation markers CD25 as well as CD69 (Haller et al., 2000).

Multiple ways of activation are known that lead to cytokine production, distribution of perforin/granzyme containing granulae or induction of apoptosis via TNF death receptor family members like Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) (Figure 6) (Lanier, 2005; Smyth et al., 2005). In many studies the relevance of the presence of accessory cells such as monocytes and DC for successful target lysis has been emphasized, though in certain experimental settings deterioration could be achieved without any bystander cells (Haller et al., 2000; Newman and Riley, 2007; Esin et al., 2008).

The interaction occurs via direct contact with co-stimulatory receptors such as CD28 or the secretion of type I IFNs (IFNα and IFNβ), that are other crucial activation factors for NK cells that lead to the induction of apoptosis-inducing FasL (Haller et al., 2000; Gerosa et al., 2005). While in the early immune response to pathogens, monokines such a IL-12, IL-15 and IL-18 submit dominant stimulatory signals, later on T-cell derived IL-2 becomes a predominant mediator (Morgan et al., 1976; Gillis et al., 1978; Trinchieri et al., 1984; Fehniger et al., 1999). Lately, even in the stimulation via cytokines, the direct contact between NK cells and accessory cells has been proposed to be vital by transferring IL-12 via synapse formation or by re-binding and presenting the soluble IL-15 (Borg et al., 2004; Schluns et al., 2005).

The activation via IL-2 is mediated by different IL-2 receptor complexes. The low affinity IL-2 receptor α chain (IL-2Rα, CD25, binding affinity Kd ~10nM) does itself mediate signal transduction, but requires rather high levels of stimulation. Intermediate affinity to IL-2 is mediated by a complex of IL-2Rβ chain (βc, CD122) and γc (CD132) which together reach a binding affinity of Kd ~ 1nM. The three subunits together mediate high IL-2 affinity (Kd ~ 10pM) (Wang and Smith, 1987). Both $CD56^{bright}$ cells as well as activated T cells express IL-2Rα chain, and are therefore able to take advantage of the high affinity IL-2 receptor (IL-2Rαβγ) that lacks on $CD56^{dim}$ cells (Caligiuri et al., 1990; Nelson and Willerford, 1998).

Activation leads to proliferation and to production of various cytokines and chemokines by the NK cells, such as IL-10, IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein (MIP)-1α, -1β, IFN-γ, thus classifying NK cells as an interlink between the innate and the adoptive immune system (Cuturi et al., 1989; Fehniger et al., 1999; Fehniger et al., 2003). As most of the NK-cell defense mechanisms against tumor cells seem to be designed for bacteria or virus infected cells, they will be described separately within this context (2.3.4.1 Bacterial defense, 2.3.4.2 Viral defense, 2.3.4.3 Detection of infected or altered cells by MHC expression) and be specified in regard to myeloma immune evasion (2.2.4 Myeloma immune evasion).

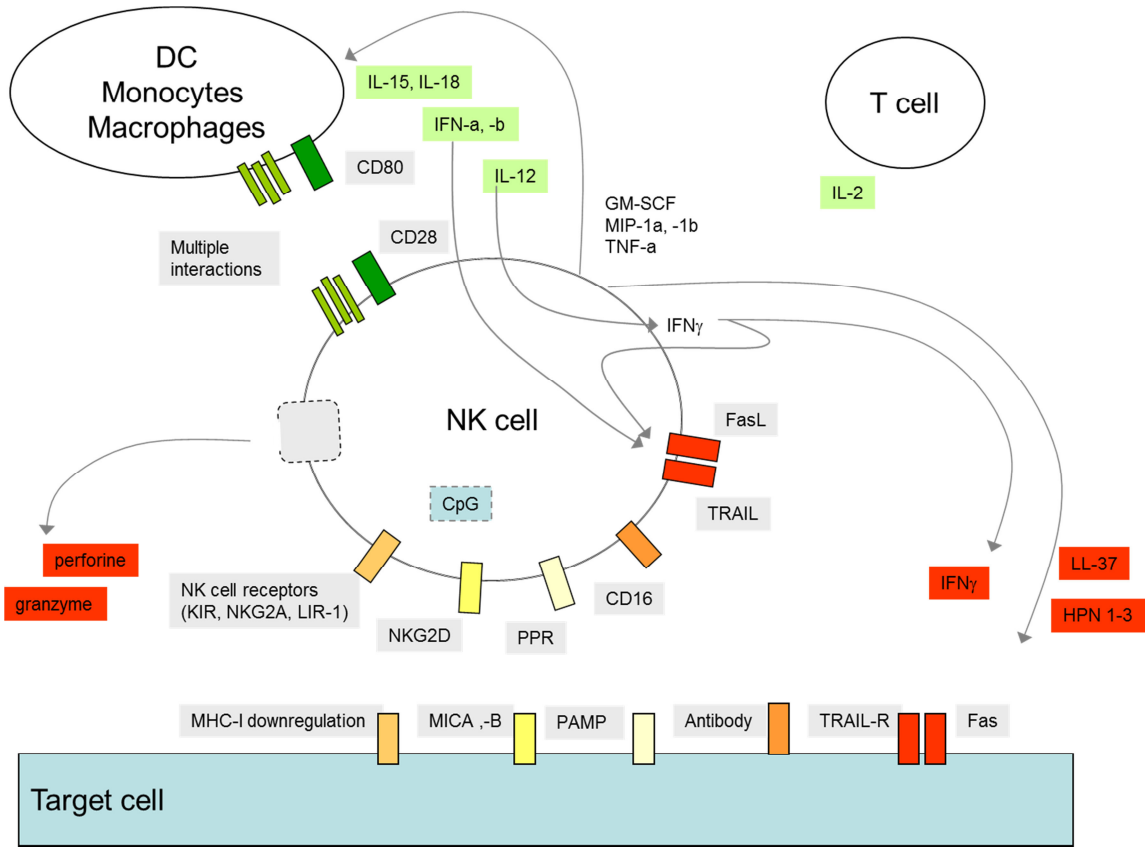


Figure 6 - NK cell activation by accessory cells and target cells

Variant types of NK cell activation mainly lead to a few reactions like FASL or TRAIL induced apoptosis as well as release of toxic substances (like perforine, LL-37 and HPN 1-3) that disrupt the targeted cell membrane (Hristova et al., 1997; Agerberth et al., 2000; Smyth et al., 2005).

Monocytes and DC secrete IL-12,-15 and -18 to stimulate NK cells for production of GM-CSF, MIP1α ,-1β and IFN-γ, of which the latter is mainly induced by DC derived IL-12, while release of T cell growth factor IL-2 also promotes NK cell proliferation (Trinchieri et al., 1984; Fehniger et al., 1999; Haller et al., 2000; Nguyen et al., 2002).

IFN-γ not only induces FasL and TRAIL expression, but exhibits various ways in target cell and virus destruction (Schroder, 2003). On the target side, altered MHC-I expression is a strong but not sufficient stimulus. Stress-induced proteins MIC-A and –B correspond via NK cell receptor NKG2D, while PAMP like CpG and LPS as well as surface bound antigens directly stimulate the NK cell via PPR and FcγIIIR (CD16), respectively (Perussia et al., 1983; Ljunggren and Kärre, 1990; Haller et al., 2000; Chalifour et al., 2004; Sivori et al., 2004; Carbone et al., 2005).

Downregulation of NK cell reaction can be mediated by DC or CD4+CD25^{high} regulatory T cells (Treg) via transforming growth factor (TGF)-β as well as by IL-10, a late

cytokine derived from macrophages as well as T cells and other NK cells. IL-10 inhibits IFN-γ production even in the presence of otherwise stimulating PAMPs. Other inhibitory signals are triggered by human MHC-I molecules as well as viral and bacterial surface molecules in order to evade immune response (Hsu et al., 1992; Chalifour et al., 2004; Ghiringhelli, 2005; Lanier, 2005).

2.3.4.1 Bacterial defense

Extracellular gram-positive as well as gram-negative bacteria are killed by NK cells (Garcia-Peñarrubia et al., 1989). Most invaders are successfully coated by B cell derived antibodies. The free Fc part of the antibody (Ab) corresponds with FcγIII receptors (CD16) to activate NK cells via a mechanism called Ab dependent cellular cytotoxicity (ADCC) (Holm and Hammarström, 1973; Perussia et al., 1983).
NK cells recognize pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PPRs), mostly members of the Toll-like receptor (TLR) family. They express mRNA for TLR 1-8, with highest expression of TLR 2 and 3. Those TLRs are activated by bacterial membrane proteins or bacteria double strand (ds) RNA that can experimentally be replaced by oligo-DNA with CG palindromes (CpG) (Iho et al., 1999; Chalifour et al., 2004; Sivori et al., 2004). Monocytes/macrophages produce NK cell stimulating IFN α/β upon activation by CpG (Yamamoto et al., 1992). Further bacterial stimulation occurs by lipopolysaccharides (LPS) or NK cell contact to bacterially primed monocytes (Haller et al., 2000).
Esin et al. (2008) have demonstrated that NK cells bind directly to mycobacteria and Pseudomonas aeruginosa but not to other bacterial species via NKp44 (but not NKp30 or NKp46). Binding to Mycobacterium bovis bacillus Calmette-Guérin (BCG) leads to upregulation of NKp44, INFγ production, proliferation and cytotoxicity without the need of accessory cells (Esin et al., 2008).
Upon activation, NK cells distribute various other cytokines and release toxic granulae (Fehniger et al., 1999; Haller et al., 2000). They produce IFNγ and α–defensins 1 to 3 (human neutrophil α-defensin, HPN 1 to 3) as well as the antibacterial peptide LL-37 (Agerberth et al., 2000; Chalifour et al., 2004). LL-37 and HPN 1-3 are not only found on NK cells but also on B-cells and γδT cells as well as monocytes and neutrophils, thus providing a general immune response towards invaders (Lehrer and Ganz, 1999; Agerberth et al., 2000).
While cytokine production is predominant in the $CD56^{bright}$ subset, the $CD56^{dim}$ subset preferentially degranulates upon antigen contact and seems to co-stimulate the $CD56^{dim}$ subset (Chalifour et al., 2004; Bottai et al., 2005).

HPN 1-3 are defensins that kill bacteria by disrupting the cell membrane via aggregation to membrane pores (Hristova et al., 1997). Furthermore, α-defensins have a chemotactic effect on monocytes, T-cells, immature dendritic cells and can activate complement factors, thus exhibiting an additional indirect way of immune response (de Yang et al., 2002). LL-37 is an antibacterial protein that shows a broad spectrum of antimicrobial activity against extracellular as well as intracellular bacteria, fungi, and viral pathogens also by disrupting the cell membrane due to its amphipathic properties (Dürr et al., 2006). It is expressed on human lymphocytes and a broad range of tissues such as the epithelial cells of gastrointestinal tract and the respiratory tract. It has chemotactic activity towards CD4+ T cells and can be induced by stimulation via IFNγ or TLR and is therefore a peptide with relevance for the early immune defense (Agerberth et al., 2000; Rivas-Santiago et al., 2008).

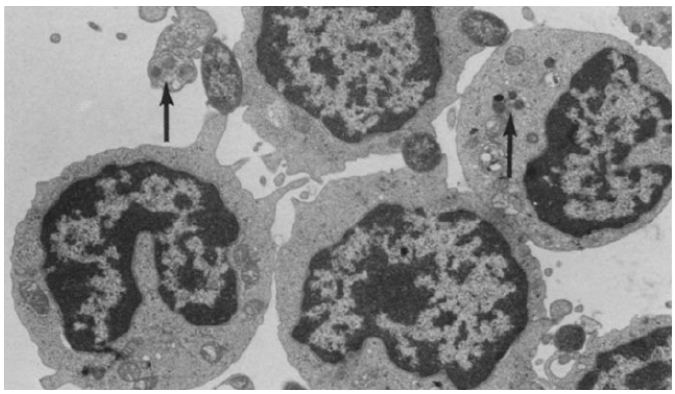


Figure 7 - NK cell degranulation upon bacterial contact
E.coli bacteria associate with the NK cell membrane and induce degranulation. Persistent granules are shown by arrows (x8000) [according to Garcia-Peñarrubia et al., 1989].

2.3.4.2 Viral defense

NK cells are strong combatants against viral offenses: IFN-γ secretion by NK cells is assumed to be a part of the early host defense against infection, while the later provision is due to T lymphocytes in the adaptive immune response, IFN-γ up regulates MHC-I expression of the surrounding cells, marking them as a target for T cells. It exhibits strong antiviral effects and has a pro-apoptotic effect via upregulation of death-associated proteins (DAP) as well as FasL (Schroder, 2003). IFN-γ induces synthesis of anti-viral nitric oxide (NO) and interacts with LRG-47, a 47-kilodalton guanosine triphosphatase (GTPase) family that has been shown to be involved in defense of murine tuberculosis infection (MacMicking et al., 1997; MacMicking et al., 2003). The monokine induced by IFNγ (Mig/CXCL9) is required for viral resistance in the liver and

a potent chemokine for T-cell and monocyte activation (Salazar-Mather et al., 2000; Chalifour et al., 2004; Groom and Luster, 2011).

Just as bacteria, viruses have efficient immune evasion mechanisms that are copied by tumor cells and are extensively studied in human Cytomegalovirus (CMV) infection. CMV down regulates MHC-I expression of the infected cell to evade T cells by retaining the MHC molecules in the endoplasmic reticulum or re-internalizing the heavy chains. It blocks the transporter associated with antigen processing (TAP) and can control the expressed epitopes to remain invisible for the T cells (Ploegh, 1998).
The virus also induces expression of the high-affinity LIR-1 ligand UL18 to inhibit NK cell cytotoxicity and up regulates HLA-E by glycoprotein UL40 for the same reason (Chapman et al., 1999; Tomasec et al., 2000). The CMV encoded protein UL16 can block interaction with the activating NK cell receptor NKG2D, while the main tegument protein (pp65) of HCMV inhibits NK cells by interference with the activating NKp30 (Cosman et al., 2001; Arnon et al., 2005). Other viral defense strategies are the expression of viral hemagglutinins that bind to inhibitory NKp46 and NKp44 (Mandelboim et al., 2001; Arnon et al., 2004).

2.3.4.3 Detection of infected or altered cells by MHC expression

An immense number of inhibitory and activating signals seem to be integrated in the NK cell via a set of receptors that recognize aberrations from the healthy MHC-I pattern of the host. Those alterations are due to intrinsic defects or bacterial and viral infection (2.3.5 NK cell receptors; Table 17 - NK cell receptors). While those reactions were initially assigned to the lack of MHC-I molecules ('missing self' hypothesis), the present theories emphasize the requirement of additional stimulating signals. This is a necessary safety mechanism, for some body tissues like neurons and erythrocytes express low or no MHC-I. A growing number of involved receptors and co-factors are identified for which the respective ligands often remain unknown (Kärre et al.; Ljunggren and Kärre, 1990; Lanier, 2005).

2.3.5 NK cell receptors

NK cell receptors are functionally divided into activating and inhibitory receptors. Their main ligands are MHC-I molecules, while some of the receptors can directly recognize specific antigens on bacteria or damaged cells. Mainly three different subclasses of NK cell receptors (NKR) can be distinguished:

- Type I transmembrane proteins of the immunoglobulin-like receptor superfamily (IgSF), which include killer immunoglobulin like receptors (KIR) and leukocyte

immunoglobulin like receptors (LIR). Both recognize classical MHC-I molecules, while LIR can also ligate non-classical MHC-I.

- Natural cytotoxicity receptors (NCR) that also belong to type I transmembrane proteins but have diverse and poorly defined ligands.
- Type II transmembrane proteins of the C-lectin type superfamily, that are activated by non-classical MHC class I and class-I like molecules (Lanier, 2005).

2.3.5.1 KIR

Killer immunoglobulin like receptors (KIR) belong to the immunoglobulin like superfamily (IgSF). The 15 KIR genes and 2 pseudogenes are located on chromosome 19q13.4. The expression of a distinct gene content leads to at least 37 haplotypes that are further varied by high allelic polymorphisms. Like the other NK cell receptors, no rearrangement but rather a stable expression during cell progeny occurs (Lanier, 2005). On NK cells, the $CD56^{bright}$ subset is the main KIR bearing subset (Cooper et al., 2001).

KIR receptors are classified by the number of extracellular Ig-like domains into KIR2D and KIR3D (Colonna and Samaridis, 1995; Wagtmann et al., 1995). On the cytoplasmic side, receptors with long domains possess one or two ITIMs and are therefore considered inhibitory, naming the receptor KIR2/3DL1 or KIR2/3DL2 (Lanier, 2005). Inhibitory KIR are stimulated by binding to the highly polymorphic regions $\alpha 2$ and $\alpha 3$ of classical MHC-I molecules HLA-A, -B and –C (Borges and Cosman, 2000). Receptors with short cytoplasmic tails associate with the adaptor molecule DAP12 via a lysine residue in their transmembrane region and are assumed to fulfill activating functions. Unlike the inhibitory KIR receptors, their ligands are still unknown and they show only little or no binding to HLA molecules (Lanier, 2005).

The only KIR receptor known today to not fit that pattern is KIR2DL4 (CD158d). Upon stimulation, it is triggering IFNγ production by resting NK cells and additional cytotoxicity in activated NK cells (Rajagopalan et al., 2001; Kikuchi-Maki et al., 2005). Due to its structural characteristics, it shows inhibitory as well as activating functions that seem to work independently in an experimental setting, but physiological stimulation always leads to activation. KIR2DL4 specific is the expression of one cytoplasmic ITIM with a unique C-terminal extension that can bind the phosphatases SHP-1 and SHP-2 as the ITIMs of other KIR plus expression of an ITIM-like motif of unknown function. Furthermore it shows characteristics of activating NK cell receptors but not KIR receptors as there is an arginine rather than lysine transmembrane residue (Faure and Long, 2002). Thereby it associates with FcεRIγ adaptor protein instead of DAP12 (Kikuchi-Maki et al., 2005). Abrogation of the protein adaption still leads to

cytokine production, what indicates a second and direct activation pathway different from the one mediated by FcεRIγ (Miah et al., 2008). The extracellular domains consist of D0 and D2, rather than the expected D1 and D2 domains of other KIR2D receptors (Selvakumar et al., 1996).
The data concerning its ligand are contradictory as some suggest stimulation by soluble HLA-G while others do not (Cantoni et al., 1998; Allan et al., 1999; Ponte et al., 1999; Rajagopalan, 1999; Boyson et al., 2002).

2.3.5.2 LIR-1 / ILT-2 / CD85j

Leukocyte immunoglobulin-like receptor (LIR)-1 is an inhibitory receptor also known as immunoglobulin like transcript-2 (ILT-2) /CD85j or leukocyte immunoglobulin-like receptor, subfamily B member 1 (LILRB1) (Samaridis and Colonna, 1997). It has first been detected in searching for the counterpart of UL18, a cytomegalovirus encoded MHC-I homolog that is expressed on infected cells (Beck and Barrell, 1988; Fahnestock et al., 1995; Cosman et al., 1997).

Classification of LIR molecules

LIR are structurally similar to KIR. Like KIR proteins, LIR belong to the immunoglobulin superfamily (IgSF). Three groups of LIR can be defined according to the presence or lack of ITIMs and the lack of the transmembrane domain, which leads to a single soluble protein. The LIR gene family is located on chromosome 19q13.2-q13.4, close to the genes of human KIR and FcαR (Borges et al., 1997; Cosman et al., 1997; Wagtmann et al., 1997). Their extracellular Ig domains share high sequence identities of up to 84 percent between the family members (Chapman et al., 2000).
LIR are expressed on subsets of NK cells and T cells, as well as on monocytes, B cells, and DC, with the widest distribution for LIR-1. Most LIR are predominantly expressed on monocytes and B-cells (LIR-1,-2,-5,-6) (Borges et al., 1997; Cosman et al., 1997; Samaridis and Colonna, 1997; Godal et al., 2010). NK cells express LIR-1,-2,-4, -5, -7 and -8 with the later only found on NK cells (Borges et al., 1997; Borges and Cosman, 2000).
LIR-1, -2, -3, -5, and -8 show long cytoplasmic domains with two or four immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that deliver inhibitory signals by association with the protein tyrosine phosphatase SHP-1 (Borges et al., 1997; Cosman et al., 1997; Samaridis and Colonna, 1997).
LIR-1 has four Ig domains as well as four cytoplasmic ITIMs of which only one shows the typical sequence (Isoleucin/Valin)-x-Tyrosin-x-x- (Leucin/Valin), that seems to be responsible for SHP-1 binding (Burshtyn et al., 1997; Colonna et al., 1997; Cosman et

al., 1997). There is an LIR-1 variant with a difference of 6 amino acids (ILT-2a) as well as two splice variants (ILT-2b and c) of which one has a cytoplasmic tail without ITIMs. LIR-1 signals via SHP-1 but not via SHP-2 or SHIP (Colonna et al., 1997).

LIR-2 (LILRB2/ILT-4/CD85d) and LIR-3 (LILRB3/ILT-5/CD85a) have four Ig-like domains and three to four ITIMs, respectively (Borges et al., 1997).

LIR-3 cDNA has been shown to exhibit a high diversity. LIR-2 and -3 have at least 6 alternatively spliced forms each, of which some do not have ITIM. LIR-8 (LILRB5/CD85c) has only two ITIMs, whereas LIR-4 (LILRA3, ILT-6, CD85e) is a soluble molecule with no transmembrane domain and with no ITIM (Borges et al., 1997; Colonna et al., 1997).

The only activating members of the LIR family are LIR-6 (LILRA1/CD85i) and LIR-7 (LILRA2/ILT-1/CD85h) with short cytoplasmic domains and a positively charged arginine residue within the transmembrane domain that associate with FcγR that contains a signaling ITAM sequence on the cytoplasmic region. LIR-6a,-6b are splice variants of LIR-6 (LILRA1/CD85i) (Borges et al., 1997; Samaridis and Colonna, 1997). LIR-6 has been shown to bind to HLA-B (Allen et al., 2001), whereas the ligands for other LIR are so far unknown (Samaridis and Colonna, 1997; Borges and Cosman, 2000).

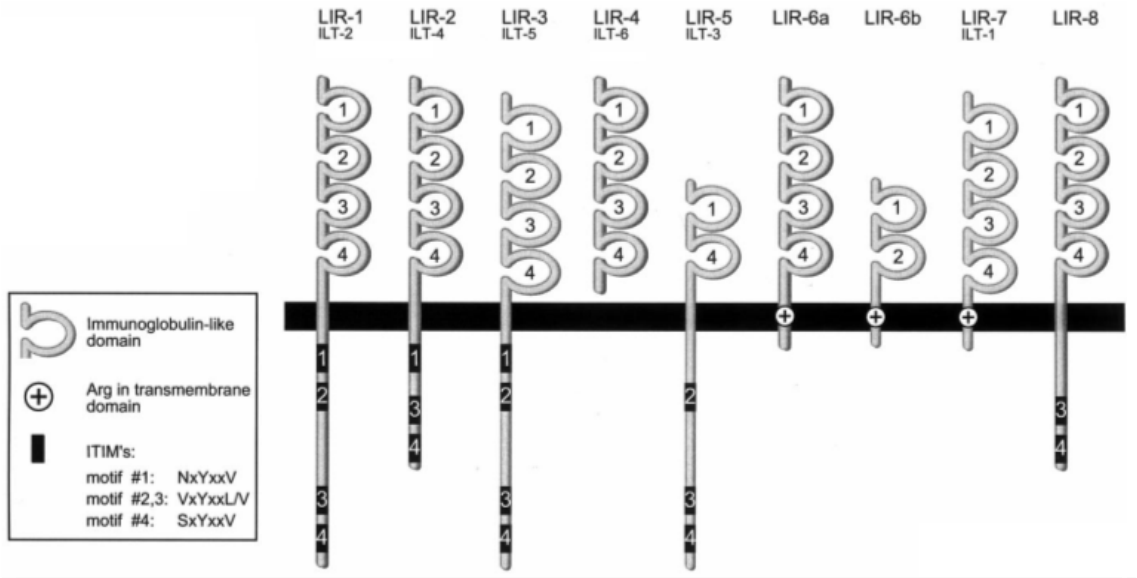


Figure 8 - Leukocyte immunoglobulin like receptors (LIR)
Receptors of the LIR family differ in the number of extracellular and intracellular domains. Out of the eight family members, most exhibit inhibitory intracellular motifs, while LIR-4 is a soluble and LIR-6 and -7 associate with activating FcRγ chain [figure adopted from Borges and Cosman, 2000].

LIR-1

The frequency of LIR-1 expression varies highly among individuals, with a range of 25-75 percent positive staining of NK cells (Davidson et al., 2010). LIR-1 is more likely to be co-expressed on KIR+ than on KIR- NK cells (Godal et al., 2010). It has been proposed, that LIR-1 co-operates with ITIM deficient KIR in a way, that allows stimulated KIR to transduce its signals via a LIR-1 dependent signal way, providing a bypass mechanism in cell that lacks other inhibitory receptors (Kirwan and Burshtyn, 2005). Its expression is known to increase during B-cell maturation, being absent in the pre-B cell stage but ubiquitous on mature B cells (Borges and Cosman, 2000). It is so far the only LIR receptor that has been detected on CD4+ and CD8+ T cells, and Young et al. (2001) proposed a model where LIR-1 expression is followed by KIR expression on cells that survive activation induced cell death by activation through TCR/CD3 complex and then turn into memory T cells. On the other hand, Young et al. found LIR expression on KIR- T cells to render them susceptible to activation-induced cell death (Young et al., 2001).

LIR-1 and LIR-2 have a broad spectrum of ligands, as they bind to HLA-A, -B, -C, -E and G. LIR-1 does not bind to HLA-Cw3 (Colonna et al., 1997; Chapman et al., 1999; Borges and Cosman, 2000). It has a 2-3 fold higher affinity to HLA-G than to classical MHC-I molecules as shown in surface plasmon resonance studies, but lately

HLA-B*2705 rather than HLA-G has been claimed to be the main ligand for LIR-1. As LIR-1 competes successfully with CD8 in MHC-I binding, it might provide an additional inhibitory immune effect beside transmission of NK cell arrest (Shiroishi et al., 2003; Morel and Bellón, 2008).

From the very beginning it has been asked, if LIR have a biological significance, due to the very low affinity to their ligands (Borges and Cosman, 2000; Chapman et al., 2000). HCMV expresses UL18, a MHC class I homolog that has only 25% amino sequence identity with MHC class (Beck and Barrell, 1988). LIR-1, but not the other LIR binds to UL-18 with a 1000-fold higher binding affinity than for class-I MHC proteins which are their main ligands (Borges et al., 1997; Chapman et al., 1999). The high affinity of UL18 to LIR-1 is assumed to be a viral evasion mechanism from immune defense. HCMV induces MHC-I downregulation at the host cell, thus evading T-cells but risking the attention of NK cells that would likely react on this lack of MHC-I, but can be inhibited by UL-18 via LIR-1 (Fahnestock et al., 1995; Wiertz et al., 1997; Chapman et al., 1999).

LIR-1 recognizes both class I molecules and UL18 at their relatively low polymorphic α3 domain with its N-terminal domain D1, what might be the reason for showing binding affinities to a broad range of classical and nonclassical MHC class I molecules. Receptors of the KIR family bind to highly polymorphic regions α1 and α2 in a region located nearby the interdomain hinge of D1 and D2 - therefore being more specific and restricted in their binding capability (Chapman et al., 1999; Boyington et al., 2000; Chapman et al., 2000).

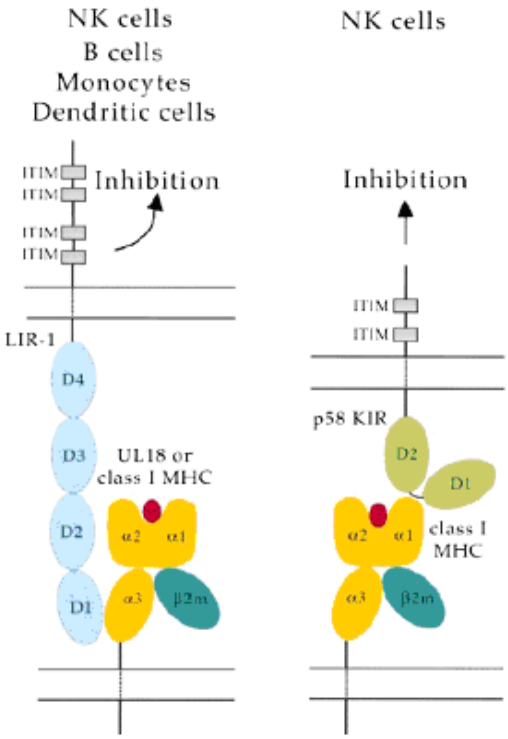


Figure 9 - LIR-1 and p58 KIR binding to MHC-I

LIR as well as KIR molecules bind MHC-I with a low affinity. While LIR recognize the well preserved α3 domain of MHC-I or UL18, KIR cling to the α1-α2 regions of the MHC-I with an interdomain hinge between D1 and D2 domain [figure adopted from Chapman et al., 1999].

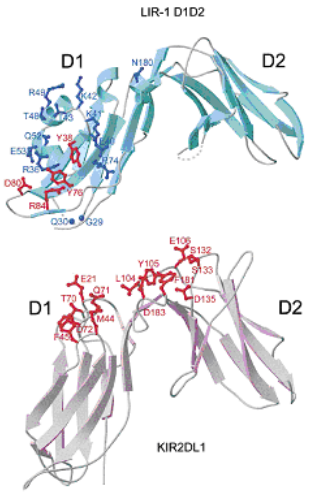


Figure 10 - Ligand binding sites on LIR-1 D1D2 and KIR2DL1

LIR-1 and KIR2DL1 bind to MHC class I molecules by red-marked residues on the left. It is assumed that binding of LIR-1 to MHC-I and UL18 involves the same residues [figure according to Chapman et al., 2000].

Artificial crosslinking of LIR-1 can block B-cell-receptor- and FcR-mediated activation of B cells and monocytes. It can downregulate HLA-DR mediated stimulation of macrophages and DC, whereas MHC-I engagement by LIR-1 has been shown to prevent T-cell and NK-cell signaling (Colonna et al., 1997; Colonna et al., 1998; Fanger et al., 1998; Saverino et al., 2000; Dietrich et al., 2001; Riteau, 2001).

In cytotoxicity assays, an increase of targets cell lysis can be achieved by blocking antibodies against LIR-1 in some but not all cases (Discussion).

There are many indications for other functional capacities of LIR molecules rather than the widely proclaimed direct inhibition of cytotoxicity via HLA. Interestingly, LIR-5 (ILT-3) might be involved in antigen presentation, for after stimulation it is

internalized together with the bound antibody and transported to a compartment, where MHC-II molecules are loaded with peptides (Cella et al., 1997). This function has not yet been described for other LIR molecules (Borges and Cosman, 2000). It has been suggested, that LIR-1 and LIR-2 on DC might prevent them from producing T-cell co-stimulatory factors, thus hindering T cells from autoaggression, which are not fully resistant to self-antigens despite of selection in thymus (Borges and Cosman, 2000).
An increase of LIR-1 on NK cells and T cells has been detected in latent CMV infection and has been proposed to serve as an early marker for CMV infection. Berg at al. (2003) suggested that either CMV induces clonal expansion of LIR-1 positive cells or that LIR-1 could predispose for CMV development (Berg et al., 2003).
LIR-1 as well as its mouse homolog PIR-B are furthermore receptors for bacteria such as S. aureus and E. coli (Nakayama et al., 2007).

2.3.5.3 CD94/NKG2A (CD159a)

Natural killer cell lectin like receptor group 2 (NKG2) receptors belong to the C-lectin like family. The members form heterodimers with CD94 that show inhibitory as well as activating functions.
The genes for CD94/NKG2 are conserved between mice and humans. In humans, one gene for CD94 and five for NKG2 have been detected: NKG2A, B, C, D, E and an F, of which the last one is not found in mice (Vance et al., 1998; Sobanov et al., 1999; Takei et al., 2001). CD94 has a short cytoplasmic domain and is either expressed as a homodimer or as a heterodimer with NKG2 proteins (Pérez-Villar et al., 1996; Vance et al., 1998).
NKG2A and -B are splice variants with the same function. They bind to HLA-E, a non-classical HLA molecule that is loaded with leader peptides of HLA-A, -B, or -C. Therefore, binding of HLA-E serves as a signal for overall HLA expression on the sensed cell. NKG2A has a long cytoplasmic tail containing two ITIMs (Braud et al., 1998; Takei et al., 2001). As a defense strategy in HCMV infection, HLA-E is upregulated by glycoprotein UL40, a homolog for the HLA-E binding peptide (Tomasec et al., 2000).
Inhibitory NKG2A is expressed on about 53% of NK cells and is the dominant inhibitory receptor on KIR- NK cells (Godal et al., 2010). Studies indicate that NKT cells are more likely to express activating CD94/NKG2 heterodimers while NK cells express mostly inhibitory CD94/NKG2A (Takei et al., 2001).
CD94/NKG2A is also expressed on CD8+ T cells and NKT cells (Mingari et al., 1998a; Takei et al., 2001). In trials with murine cytotoxic T cells (CTL), it seems to have an inhibitory function in contact with non-classical MHC-I, but is also shown to take part in

regulation of cytolytic activity and lymphokine production as soon as linked with monoclonal Abs (Le Dréan et al., 1998; Bellón et al., 1999; Speiser et al., 1999; Lohwasser et al., 2001). On CD8+ T cells, expression of NKG2A can be induced by IL-15 and TGF-β (Mingari et al., 1998b; Bertone et al., 1999).

The other transcripts of the NKG2 gene on chromosome 12p12.3-13.1 work as activating receptors as there are NKG2C, -E (splice variant -H) and F. They have a short cytoplasmic tail and adapt to proteins that contain ITAMs (Farag and Caligiuri, 2006). NKG2D is not a member of the NKG2 family. Though the gene is located on the same part of the chromosome as the other NKG2 proteins, it is an activating homodimer with the signaling performed by DAP10 that recruits phosphatidylinositol (PI)-3-kinase which induces cytotoxicity. NKG2D takes an important role in immune defense, for it is known to bind to stress induced MHC class I chain related proteins A (MICA) and MICB, that are highly expressed on certain tumor cells, as well as to the human cytomegalovirus UL16 binding proteins (ULBPs) (Farag and Caligiuri, 2006).

2.3.5.4 NCR

Natural cytotoxicity receptors (NCR) are a group of vigorous activation receptors (NKp46/NCR1, NKp44/NCR2, NKp30/NCR3) that are Ig-like transmembrane glycoproteins but not involved in interaction with HLA. All NCR lack ITAMs and owe their potential to linkage to the adaptor proteins CD3ζ, FcRγ or DAP12. Their surface expression is mostly restricted to NK cells. All NCR have been shown to be strongly involved in tumor cell lysis (Moretta et al., 2001).

The first NCR to be detected in 1997 was NKp46. It was named according to its glycoprotein size of 46kDa and initially believed to be restricted to NK cells, but is also expressed on γδT cells (Sivori et al., 1997; Walzer et al., 2007). Virus detection by NKp46 is based on interaction with viral hemagglutinins as well as signal transduction via FcεRIγ and CD3ζ (Lanier et al., 1989; Mandelboim et al., 2001; Moretta et al., 2001). NKp30 was revealed only shortly thereafter in 1999 and acquires functionality via CD3ζ (Pende et al., 1999). Other than these, NKp44 with its adaptor protein DAP12 is a marker for activated NK cells, being found after IL-2 stimulation but not on resting NK cells (Vitale et al., 1998).

Though similarly named, the recently found activating NKp80 and NKp65 are not classified as NCR, showing a C-lectin-like rather than an immunoglobulin-like extracellular domain (Vitale et al., 2001; Spreu et al., 2010).

A study by Poggi et al. (2005) revealed another important tumor escape mechanism. Though formerly only known as tumor defending structures, NCR interaction seems to

lead to the upregulation of FasL by the tumor cell and the induction of NK-cell apoptosis (2.2.4 Myeloma immune evasion) (Poggi et al., 2005).

2.3.6 Role of NK cells in the treatment of multiple myeloma

The presented experimental results of this work contribute to the efforts that are made to improve the myeloma patients' outcome after SCT. Transplantation is an incisive event, which leaves the recipient in a state of severe immune deregulation for the course of years.

Following a year or more after donation of CD34+ progenitor cells, B cells show high deficiencies in number and production of antibodies. T-cell reconstitution is equally low within the first 3-18 months and shows an inverted CD4/CD8 ratio. The number of circulating CD4+ T cells can be low for years. Only NK cells with their innate functions return to their post within the first month after SCT, thus being the predominant leukocyte subset within the first 90 days (Ault et al., 1985; Porrata et al., 2001). In settings where G-CSF was used to mobilize peripheral blood stem cells (PBSC), all lymphocyte subsets develop faster, but NK cells are still among the first cells to reconstitute (Rondelli et al., 2000). Moreover, immature NK cells in the early phase of development in the bone marrow do not express any inhibitory receptors, while their cytolytic capacity is increased by surface expression of NKp46 and NKp30 (Sivori et al., 2002).

Those NCR as well as the activating receptor NKG2D are considered to be the main receptor entities responsible for the effective killing of the residing tumor burden after SCT, what is called the graft-versus-leukemia (GVL) effect (Gasser et al., 2005; Moretta et al., 2005). On the other hand, NK cells do not seem to be involved in the immune reaction of the transplanted graft against the receiving host. This graft-versus-host disease (GVHD) is an often severe tissue damage that occurs mainly in the gastrointestinal tract, skin and liver. A recent investigation showed that NK cells can even reduce GVHD that is mediated mainly by alloreactive T cells. They induce their apoptosis, while still proceeding against the tumor cells (Olson et al., 2010).

To evaluate the influence of self-MHC-I molecules in resistance of MM against NK-cell mediated lysis, Carbone et al. (2005) investigated the susceptibility of MM cells from bone marrow (BM), pleural effusion (PE) and peripheral blood (PB). They found out that BM and PB cell lines were highly sensitive to killing, representing early stage MM with hardly any expression of MHC class I but high levels of stress induced MICA. The opposite was true for late stage PE cells with a poor vulnerability but high MHC-I and low stress induced proteins. Increase of MHC-I during disease progression could be confirmed with samples from 14 patients and came along with the additional increase

of CD28, a confirmed marker for disease progression. As lysis of PE cells could be restored by either blocking of MHC-I or NCR, NKG2D or MICA, those were claimed responsible for the former NK resistance (Carbone et al., 2005). On the opposite, Frohn et al. have detected strong NK response towards MM cell lines and towards one patient sample despite high HLA-I expression (Frohn et al., 2002).

Taken together, NK cells seem to play an important role in myeloma defense despite the diverse mechanism of MM cells to evade recognition. Keeping or regaining high expression of MHC-I seems to be an effective but not sufficient strategy of MM cells for survival.
To further rule out MHC-I mediated MM escape, interactions with the inhibitory NK cell receptor LIR-1 are of special interest. Due to its broad spectrum of HLA ligands (HLA-A, -B,-C,-E ,-G) the maturation of MM might be associated with an enhanced protection against immune control (Colonna et al., 1997; Braud et al., 1998).

3 MATERIAL AND METHODS

3.1 Material

3.1.1 Cells

Natural killer cell line NK-92 was obtained from American Type Culture Collection (ATCC, Manassas, USA) and cultured in appropriate medium (Table 5 - Culture and freeze media) (Davidson et al., 2010). NK-92 is a cell line that has been derived from peripheral blood of a 50-year-old male with rapidly progressive non-Hodgkin lymphoma. The cell line was established from the patient´s PBMC that contained large granular lymphocytes (LGL) that revealed multiple aneuploidies and structural rearrangements. Those cells were regarded as NK cells due to the expression of CD56 and the lack of CD3. Additional surface markers are CD2 +, CD7 +, CD11a +, CD28 + , CD45 +, CD54+, $CD56^{bright}$, CD1 -, CD3 -, CD4 -, CD5 -, CD8 -, CD10 -, CD14 -, CD16 -, CD19 -, CD20 -, CD23 -, CD34 -, HLA-DR - (Gong et al., 1994). Since NK cells account for only 10-15 percent of total lymphocytes in the peripheral blood, the use of NK cell lines is an established and convenient method for studying NK cell receptors.
NK-92 cells differ from normal NK cells by the lack of CD16, as well as a quite untypical receptor expression: NK-92 are positive for LIR-1 and NKG2A as well as CD94, but express only KIR2DL4 and its homolog NKp49, while other KIR molecules are lacking (KIR3D family, KIR2DL1-3, KIR2DL5, KIR2DS1-5, KIR44, KIR48). Activating Ig-like receptors NKp30 and NKp46 can be detected (Maki et al., 2001; Davidson et al., 2010). The NK-92 phenotype might most likely resemble the $CD56^{bright}$ NK cell subset

with high surface expression of NKG2A and low KIR receptor expression, though this subset is described to show vanishing LIR-1, unlike results in the present study (Colonna et al., 1997; Cooper et al., 2001).
Furthermore, NK-92 cells have been shown to kill the MHC class I negative cell line K562 with a specific lysis of >80 percent at a 1:1 E:T ratio in a 4h chromium release assay and are therefore regarded as a valuable tool for studying the biology of activated NK cells (Gong et al., 1994).

Cell lines RPMI-8226, MOLP-8 (both MM), IM-9 (lymphoblastoid B cell line, BLCL), HL60 (acute myelogenous leukemia, AML) and K562 (chronic myelogenous leukemia, CML) were used as target cell lines in the conducted functional studies. K562 were used as a control cell line, as they are known to be highly sensitive for NK cell mediated killing (Lozzio and Lozzio, 1975; Yan et al., 1998). All tumor cell lines were maintained in RPMI1640 supplemented with antibiotics and fetal calf serum (FCS) (Table 5 - Culture and freeze media).

MOLP-8 is an EBV negative MM cell line established from a 52-year old Japanese patient with Bence–Jones δ/λ type MM. The cell line is positive for cyclic Igλ light chain, δ heavy chain, CD4, CD9, CD10, CD29, CD38, CD39, CD44, CD49b, CD49d, CD49f, CD51, D54, CD56, CD58, CD71, CD126, CD130 and CD138. After co-culture with direct contact to bone marrow stromal feeder cells, MOLP-8 cells turn positive for CD28 like almost all myeloma cells. MOLP-8 chromosomes have structural abnormalities including t(11;14)(q13;q32) that is common in myeloma (Drexler and Matsuo, 2000; Matsuo et al., 2004). MOLP-8 expresses high levels of the short and long splice variants of myeloid cell leukemia-1 protein (Mcl-1S/Mcl-1L) that belongs of the Bcl-2 family (Bae et al., 2000; Legartova et al., 2009). The implications for Mcl-1 on MM are still unclear (Le Gouill et al., 2004). The Mcl-1 gene is located on the 1q21 region, a common site for chromosomal aberrations in MM (Legartova et al., 2009). Hanamura et al. showed that 1q21 amplification increases from the stage of MGUS to MM and to relapse (Hanamura et al., 2006). While the pro-apoptotic short 32kDa variant of the Mcl-1 protein might serve as a prognostic marker for MM, the longer 40kDa variant is also expressed on both MM and lymphoblastoid cells and has anti-apoptotic functions. Mcl-1L can form heterodimers with Mcl-1 and other pro-apoptotic Bcl-2 family members, that antagonize the pro-apoptotic effect (Bae et al., 2000; Legartova et al., 2009).
RPMI8226 is the oldest MM cell line and was established in 1966. It was derived from a 61-year old male and is Epstein-Barr-virus (EBV) negative. Most of the cells resemble

immature plasma cells. The cell line produces up to 20µg λ–type light chains of immunoglobulin per 10^6 cells per day plus another small peptide that could not be characterized at time of exploration but might be an intermediate of light chain synthesis. While IgG heavy chain production of the MM cells was detected within the donor, the cells have lost this productive ability in culture (Matsuoka et al.; Drexler and Matsuo, 2000).

IM-9 is not a MM cell line but an EBV positive lymphoblastoid B cell line (BLCL) derived from a patient with MM (Fahey et al., 1971; Drexler and Matsuo, 2000). In contrast to plasma cells, LCL are capable of producing more than one Ig at the same time, but have little endoplasmic reticulum. Immunoglobulins are only produced during the G1 and S-Phase in contrast to a more constant synthesis within plasma cells (Fahey et al., 1971). They can grow in large dense clumps, while MM cells mostly stay in single cell suspension. Its phenotype resembles B-cells (CD19, CD20) (van Boxel and Buell, 1974; Pellat-Deceunynk et al., 1995; Drexler and Matsuo, 2000).

Cell line	EBV	CD19	CD20	CD28	CD38	Surface Ig
RPMI 8226	-	-	-	+	+	-
MOLP-8	-	-	-	- (inducible)	+	-
IM-9	+	+	+	-	-	A, G, κ

Table 3 - Comparative analysis of myeloma and lymphoblastoid cell lines
Outline of characteristic differences in the expression of surface markers by MM cell lines (RPMI8226, MOLP-8) and lymphoblastoid cell line IM-9 according to precedent publications (van Boxel and Buell, 1974; Pellat-Deceunynk et al., 1995; Drexler and Matsuo, 2000; Matsuo et al., 2004).

HL-60 is a common AML cell line, established in 1976 (Collins et al., 1977). In the present experiments, it was used in order to verify, if certain experimental effects were restricted to MM alone or could be transferred to other tumor entities.

K562 is an erythroleukemic cell line, which has been widely used as a control in NK cell cytotoxicity assays, due to its lack of HLA molecules. It was established in 1975 from pleural effusion cells of a CML patient in terminal blast crisis and is positive for the Philadelphia chromosome. No infection with EBV virus or herpes-like viruses has been detected (Lozzio and Lozzio, 1975; Maki et al., 2008).

K562 cells are commonly described to lack HLA molecules inclusively HLA-E (Drew et al., 1977; Benz et al., 1980). They lack staining with the antibodies W6/32 and B1.23.2 (HLA-A,-B,-C) as well as with 87G (HLA-G1) in flow cytometric analysis as well as with 4H84 (HLA-G α1 domain) in western blot, but Ziegler et al. (1981) showed that K562 express β2m and react with W6/32 in binding assays. Furthermore, SDS-gel electrophoresis with W6/32 showed bands in the height of HLA-A, -B, - and C heavy chains, while others found a band at about 46kDa in W6/32 immunoprecipitation that was claimed to be unspecific (Ziegler et al., 1981; Khalil-Daher et al., 1999). In unstimulated K562 cells, only very low levels of HLA-E mRNA are detectable, but stimulation with IFNγ leads to an immense increase of both HLA-E transcripts of 1.8 and 2.7 kb size. At the time of HLA-E detection, its surface expression could not surely be defined, due to the lack of specific antibodies (Ulbrecht et al., 1992). Palmisano et al. (2005) suggested that the discrepancies in detected surface expression of HLA-E on tumor cells might be associated with culture time, with long-time culture favoring the expression. They could not detect HLA-E on the surface of K562 cells, using the new antibodies MEM-E/06, /07 and /08 that have been confirmed in flow cytometric analysis and at the Third International Conference on HLA-G in 2003. Confirming western blot analysis with MEM-E/02 did not detect HLA-E on K562 cells at a band size of 43kDa despite the detection of HLA-E mRNA transcripts. Generally, mRNA was detected in the whole broad panel of investigated tumor cell lines that did not correlate with western blot or fluorescence-activated cell sorter (FACS) detection (Palmisano et al., 2005).

The present analysis has shown negative surface staining of K562 with antibodies against HLA-I, HLA-E, and –G (Figure 13 - Expression of surface antigens).

JEG-3 is a choriocarcinoma cell line that is commonly used as a control cell line for HLA-G expression and also expresses HLA-C molecules. It does not show any HLA-E on the cell surface, though little mRNA can be detected by PCR (Paul et al., 1998; Apps et al., 2009; Huang et al., 2009).

COS-7 African green monkey kidney cells were used as a positive control for LIR-1 expression after viral transfection with a LIR-1 containing vector (Gluzman, 1981).

PBMC from one donor were obtained after written consent, isolated by standard Ficoll-Hypaque density centrifugation and stored at -80°C after for later use as a positive control for HLA-E expression (Furukawa et al., 1999).

3.1.2 Technical equipment

Cell culture		
Incubator 37°C HERA cell 240	Thermo Fisher Scientific	Waltham, USA
Microscope BIOMED	Esselte Leitz	Stuttgart, Germany
Microscope WILOVERT S	Helmut Hund	Wetzlar, Germany
Ice maker FM-120DE	Hoshizaki	Minamiyakata, Japan
Refrigerator -80°C HERAfreeze HFU 686 Top	Thermo Fisher Scientific	Waltham, USA
Refrigerator -4°C	Bauknecht	Stuttgart, Germany
Centrifuge MULTIFUGE 3 S-R	Heraeus	Hanau, Germany
Bench-top centrifuge BIOFUGE fresco	Heraeus	Hanau, Germany
Water bath 70°C	Köttermann	Uetze/Hänigsen, Germany
Water bath 37°C GFL-1083	Gesellschaft f. Labortechnik	Burgwede, Germany
Vortex device VWR™ LAB DANCER S40	VWR	Batavia, USA
Omnilab	Janke & Kunkel	Staufen, Germany
Shaker IKA-VIBRAX-VXR	Janke & Kunkel	Staufen, Germany
Scale TE412	Sartorius	Göttingen, Germany
PIPETBOY acu	INTEGRA Biosciences	Fernwald, Germany
NALGENE™ Cryo1°C Freezing Container	Thermo Fisher	Waltham, USA
24 Well Cell Culture Plate CELLSTAR®	Greiner Bio-One	Frickenhausen, Germany
96 Well Cell Culture Cluster costar®	Corning Incorporated	Corning, USA
CELLSTAR® Tubes 15mL/50mL	Greiner Bio-One	Frickenhausen, Germany
CELLSTAR® Culture flasks	Greiner Bio-One	Frickenhausen, Germany
CryoTube™ Vials nunc™	Thermo Fisher	Waltham, USA
Eppendorf tubes	Eppendorf	Hamburg, Germany
Serological pipets FALCON®	Becton Dickinson	Franklin Lakes, USA
Biocoll (Ficoll)	Biocompare	Berlin, Germany

Medium preparation		
RPMI 1640	Life Technologies	Carlsbad, USA
DMEM Glutamax	Life Technologies	Carlsbad, USA
HAM´s F12	Life Technologies	Carlsbad, USA
Minimum Essential Medium (MEM) α 22561	Life Technologies	Carlsbad, USA
Equine serum 16050-122 not heat-inactivated	Life Technologies	Carlsbad, USA

Medium preparation		
Fetal bovine serum 10270-106 not heat-activated	Life Technologies	Carlsbad, USA
Fetal calf serum heat inactivated	Life Technologies	Carlsbad, USA
PenStrep 10.000 Units/mL Penicillin 10.000 µg/ml Streptomycin	Life Technologies	Carlsbad, USA
Dulbecco´s Phosphate Buffered Saline 1X (DPBS)	Life Technologies	Carlsbad, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Steinheim, Germany
Myo-Inositol I7508-50G	Sigma-Aldrich	Steinheim, Germany
Folic acid F8758-5G	Sigma-Aldrich	Steinheim, Germany
Interleukin 2, CS-C1061	CellSystems	Troisdorf, Germany
2-Mercaptoethanol	Sigma-Aldrich	Steinheim, Germany

Chromium–release assay		
WALLAC TRILUX 1450 Microbeta Counter	PerkinElmer	Waltham, USA
WALLAC 1450 MicroBeta Windows WS V. 2.70.004	PerkinElmer	Waltham, USA
[Cr51] Sodium chromate (Na_2CrO_4) 74MBq (2mCi)	Hartman analytic	Braunschweig, Germany
Rotiszint eco plus	Carl Roth	Karlsruhe, Germany
Isoplate 96	Perkin Elmer	Waltham, USA
Viewseal 80.0/140 MM	Greiner Bio-One	Frickenhausen, Germany
Eppendorf research plus multi-channel pipette	Eppendorf	Hamburg, Germany
Pipetus®	Hirschmann	Eberstadt, Germany
Eppendorf LoRetention Dualfilter pipet tips	Eppendorf	Hamburg, Germany
Roller, flexible	Hama	Moheim, Germany
Vortex Genie 2 Model No 6560E	Scientific Industries	Bohemia, USA
Contamination monitor CoMo170	Rapp	Gelting, Germany
Incubator 37°C	Heraeus	Hanau, Germany
Centrifuge 5430R	Eppendorf	Hamburg, Germany
Centrifuge 5702R	Eppendorf	Hamburg, Germany
Thermomixer comfort	Eppendorf	Hamburg, Germany
Re-Blot Plus Strong Solution (10x)	Millipore	Temecula, CA, USA

Flow cytometric analysis		
BD FACSCanto II™ flow cytometer	Becton Dickinson	Franklin Lakes, NJ, USA
BD FACSDIVA Software v.6.1.3	Becton Dickinson	Franklin Lakes, NJ, USA
Flow Cytometry Tubes	Sarstedt	Nürnberg, Germany

Western blot		
Amersham Hyperfilm™ MP (18 × 24 cm)	GE Healthcare	Munich, Germany
ECL solution Amersham™	GE Healthcare	Munich, Germany
Rego Intensifying Screen	rego X-Ray	Augsburg, Germany
Hyper processor Amersham Pharmacia	Biotech	Freiburg, Germany
TETENAL SUPERFIX MRP	TETENAL	Norderstedt, Germany
TETENAL ROENTOROLL HC	TETENAL	Norderstedt, Germany
Curix 60 (radiographic development)	AGFA Healthcare	Mortsel, Belgium
Electrophoresis Power Supply Consort EV2	BioPoint	Cambridge, England
XCell SureLock™ Mini-Cell	Life Technologies	Carlsbad, USA
XCell II™ Blot Module	Life Technologies	Carlsbad, USA
NuPAGE® 4-12% Bis-Tris Gel 1.0mm12 well	Life Technologies	Carlsbad, USA
Sponge Pad for XCell II™ Blotting	Life Technologies	Carlsbad, USA
Whatman® Gel Blot Paper GB003	Whatman	Dassel, Germany
Whatman® PROTRAN® Nitrocellulose Transfer Membrane	Whatman	Dassel, Germany
NuPAGE® LDS Sample Buffer(4x)	Life Technologies	Carlsbad, USA
NuPAGE® Sample Reducing Agent (10x)	Life Technologies	Carlsbad, USA
PeqGold Protein Marker V	PEQLAB	Erlangen, Germany
Bio-Rad Protein Assay Dye Reagent Concentrate	Bio-Rad	Hercules CA, USA
GeneQuant Pro photometer	GE Healthcare	Munich, Germany
Albumine bovine serum, Fraction V ≥ 96%	Sigma Aldrich	Steinheim, Germany
Re-Blot Plus strong solution (10x)	Millipore	Temecula, CA, USA

Transformation and transfection		
6 well culture plate	Greiner Bio-One	Frickenhausen, Germany
Opti-MEM	Life Technologies	Carlsbad, USA
One Shot® TOP10/P3 Competent cells	Life Technologies	Carlsbad, USA
SOC medium	Life Technologies	Carlsbad, USA

Transformation and transfection		
FastMedia™ LB Agar Amp IPTG/X-Gal	Fermentas	St. Leon-Rot, Germany
FastMedia™ LB Liquid Amp	Fermentas	St. Leon-Rot, Germany
EndoFree® Plasmid Purification Kit	Qiagen	Hilden, Germany
FuGene® HD Transfection Reagent	Promega	Madison, WI, USA
BioPhotometer 6131	Eppendorf	Hamburg, Germany
Sorvall® 5c Plus	ThermoScientific	Waltham, MA, USA
Certomat IS shaker ((37°C)	B. Braun	Melsungen, Germany
Viability assay		
Trypan Blue Stain 0,4% 15250	Life Technologies	Carlsbad, USA
Neubauer Improved counting chamber	Karl Hecht	Sondheim, Germany

Table 4 - Technical equipment

3.1.3 Medium

Culture medium for MOLP-8	
500 ml	RPMI 1640 1x
20%	Heat inactivated FCS
1%	PenStrep
Culture medium for IM09, RPMI 8226, K562, HL60	
500 ml	RPMI 1640
10%	Heat inactivated FCS
1%	PenStrep
Culture medium for COS-7	
500 ml	DMEM
10%	Heat inactivated FCS
1%	PenStrep
Culture medium for JEG-3	
500 ml	Ham´s F12
10%	FCS
1%	Pen-Strep

Culture medium for NK92	
500 ml	Minimum Essential Medium (MEM) α
12.5%	Equine serum, not heat inactivated
12.5%	Fetal calf serum, not heat inactivated
1%	PenStrep
0.2 mM	Inositol
0.1 mM	2-mercaptoethanol
0.02 mM	Folic acid
200 U/ml	rhIL-2
Freeze medium for tumor cell lines and PBMC	
70%	Culture medium
20%	Heat inactivated FCS
10%	DMSO
Freeze medium for COS-7	
95%	Culture medium
5%	DMSO
Freeze medium for NK92	
40%	Culture medium
50%	FCS
10%	DMSO

Table 5 - Culture and freeze media

3.1.4 Western blot solutions

Lysis buffer		
10 ml	Lysis buffer stock solution	see below
1 tablet	Complete Mini EDTA-free Protease Inhibitor Cocktail Tbl.	Roche Diagnostics, Mannheim, Germany

Lysis buffer stock solution		
10 mM	Tris-HCl (pH 7.9)	
	Trizma®base	Sigma-Aldrich, Steinheim, Germany
	Hydrochloric acid (HCL)	Merck KGaA, Darmstadt, Germany
150 mM	Sodium chloride	Mallinckrodt Baker B.V.,Deventer, Netherlands
1%	NP-40 (Igepal CA-630)	Sigma-Aldrich, Steinheim, Germany
1%	Sodium deoxycholate	Sigma-Aldrich, Steinheim, Germany
0.1%	Sodium dodecyl sulfate (SDS)	BIOMOL, Hamburg, Germany
	Aqua bidest	
Running buffer		
50 ml	NuPAGE® MES SDS Running Buffer (20x)	Invitrogen, Karlsruhe, Germany
950 ml	Aqua bidest	
Transfer buffer		
100 ml	Methanol	Mallinckrodt Baker, Inc, Phillipsburg, USA
50 ml	NuPAGE® Transfer Buffer (20x)	Life Technologies
1 ml	NuPAGE® Antioxidant	Life Technologies
849 ml	Aqua bidest	
TBS-T stock solution (10x) pH7.5		
0.5 M	Tris-HCL	
	Trizma®base (78.8g/L)	Sigma-Aldrich, Steinheim, Germany
	Hydrochloric acid (HCL)	Sigma-Aldrich, Steinheim, Germany
1.5 M	Sodium chloride (87.66g/L)	Mallinckrodt Baker B.V.,Deventer, Netherlands
TBS-T (1x)		
100 ml	TBS 10x	
1 ml	Tween® 20	Sigma-Aldrich, Steinheim, Germany
899 ml	Aqua bidest	
Blocking solution		
3 g	Skim milk powder, spray-dried	Spinnrad, Bad Segeberg, Germany
100 ml	TBS-T (1x)	see above

Table 6 - Western blot solutions

3.1.5 Antibodies and vectors

Transfection			
cDNA	Vector	Transcript	Cat.-No./Distributor
CD85j	pCMV6-AC	Homo sapiens leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 1 (LILRB1), transcript variant 4 (NM_001081639.1)	SC320409 OriGene
CD159a	pCMV6-XL5	Homo sapiens killer cell lectin-like receptor subfamily C, member 1 (KLRC1), transcript variant 3 (NM_213658.1)	SC125810 OriGene

Table 7 - Vector LIR-1 (CD85j) and NKG2A (CD159a – mock transfection)

Western blot			
Target	Size	Clone	Distributor
CD85j	110/90 kDa	VMP55	Santa Cruz
β-Actin	42kDa	C4	Santa Cruz
Goat anti-mouse IgG HRP conjugated		HAF007 (Cat. No)	R&D Systems

Table 8 - Antibodies western blot

Blocking assay				
Target	Isotype	Clone	Solvent	Distributor
CD85j	IgG2b	292319	*1	R&D Systems
CD159a	IgG2b	Z199	*2	Beckman Coulter
HLA-I	IgG1	HP-1F7 anti-human HLA- A,-B,-C,-E,-G	*3	Santa Cruz
IgG Fcγ	$F(ab')_2$	$F(ab')_2$ fragment goat anti-mouse IgG Fcγ frgmnt., polyclonal	*4	Jackson ImmunoResearch
Isotype	IgG2b	20116	*1	R&D Systems
Isotype	IgG1	11711	*1	R&D Systems

Table 9 - Antibodies blocking assays

*1: Lyophilized, diluted with PBS

*2: freeze-dried

*3: PBS, < 0.1% sodium azide, 0.1% gelatin

*4: 0,01M Sodium Phosphate, 0,25 NaCl, pH 7,6

Viability assay				
Target	Isotype	Clone	Solvent	Distributor
HLA-I	IgG1	HP-1F7 anti-human HLA- A,-B,-C,-E,-G	*1	Santa Cruz
Isotype	IgG1	11711	*2	R&D Systems

Table 10 - Antibodies viability assays

*1: PBS, < 0.1% sodium azide, 0.1% gelatin.

*2: Lyophilized, diluted with PBS

Flow cytometry				
Target	Conjugation	Isotype	Clone	Distributor
CD2	PE	IgG1 k	RPA-2.10	BD Pharmingen
CD16	Pacific Blue™	IgG1k	MOPC-21	BD Pharmingen
CD25	FITC	IgG2a	B1.49.9	Beckman Coulter
CD56	APC	IgG1, κ	B159	BD Pharmingen
CD85j	PE	IgG1	HP-F1	Beckman Coulter
CD159a	PE	IgG2b	Z199	Beckman Coulter
HLA-I	unconjugated	IgG1	HP-1F7	Santa Cruz
Anti-IgG1	PE	Goat polyclonal anti-mouse IgG		Santa Cruz
HLA-G	unconjugated	IgG1	MEM-G/09	Abcam
HLA-E	unconjugated	IgG1	MEM-E/08	Abcam
Anti-IgG1	FITC	Goat polyclonal anti-mouse IgG - H&L		Abcam
Isotype	PE	IgG1, κ	MOPC-21	BD Pharmingen
Isotype	PE	IgG2b k	δG9	BD Pharmingen
Isotype	FITC	IgG2a	G155-178	BD Pharmingen
Isotype	APC	IgG1	679.1Mc7	Beckman Coulter
Isotype	Pacific Blue™	IgG1, κ	MOPC-21	BioLegend
Isotype	unconjugated	IgG1	11711 (MAB002) (HLA I expression)	R&D Systems
Isotype	unconjugated	IgG1	X40 (titration HLA I)	BD Pharmingen
7AAD	PerCp Cy5	7-Amino-Actinomycin D		BD Pharmingen
Anti A,B reagent		IgM	polyclonal	Ortho-Clinical Diagnostics

Table 11 - Antibodies flow cytometry

3.2 Methods

3.2.1 Cell culture and isolation of PBMC

As described above (3.1.1 Cells), all cell lines were maintained in appropriate media (Table 5 - Culture and freeze media). Cells were kept in culture flasks in a humidified incubator at 37°C with 5% CO_2 and splitted every second to third day. NK-92 received supplementary 200U IL-2 /ml with each fresh medium provision. COS-7 cells are adherent cells that required trypsinizing for subculturing. PBMC from one donor were isolated by Ficoll-Hypaque density centrifugation and stored at -80°C before use as control cells for HLA-E expression in flow cytometric immunophenotyping.

3.2.2 Flow-cytometric analysis

Flow-cytometric analysis of cell surface antigens was done initially before the start of cytotoxicity assays and after a period of 4 weeks without changing of settings. Staining antibodies and isotype controls are listed above (Table 11 - Antibodies flow cytometry). Clone MEM-G/09 was used for HLA-G detection. The clone reacts with the native form of HLA-G1 and soluble HLA-G5, but not with HLA-G2, -G3, and -G4 (Menier et al., 2003; Palmisano et al., 2005; Maki et al., 2008).

MEM-E08 was chosen as the anti-HLA-E antibody (Ab) and is assumed to exhibit a remarkably small cross-reactivity, as confined at the Third International Conference on HLA-G (Paris, July 2003) and in different studies (Palmisano et al., 2005; Coupel et al., 2007; Lo Monaco et al., 2008).

20µl Anti A,B reagent was used in each sample to block unspecific bindings. Dead cells were stained with 7-Amino-Actinomycin D (7AAD). Cells were incubated with the Ab or isotype control for 30 minutes at 4°C and washed with PBS. Unconjugated anti-HLA Abs were marked with fluorescent secondary Abs that were recommended by the manufacturer and were washed after an incubation of additional 15 minutes at room temperature. All samples were stained with 7AAD and fluorescence was measured on BD FACSCanto II™ flow cytometer. Analyzing software was BD FACSDIVA Software v.6.1.3. To exclude harmful effects in blocking assays, analysis of anti-HLA-I toxicity was performed with three different Ab concentrations. Secondary Ab was always used in an amount of 8 µg/sample; 7AAD for dead cell staining was used in an amount of 5 µl in every sample.

group	a (25:1)		b (1:1)	c (manufacturer)	
subgroup	*1*	2	1=2	2	*1*
µl	*300*	1000	1000	1000	*300*
number of cells	*2 x 10^5*	6.66 x 10^5	7.5 x 10^4	6.66 x 10^5	*2 x 10^5*
µg anti-HLA-I	*10*	33.3	1	0.66	*0.2*
factor	*50*	**50**	**13**	**1**	*1*

Table 12 - HLA-I titration for flow cytometric analysis

groups a = free chosen setting*
b = relation cells/Ab according to 1:1 E:T ratio similar to present CRA
c = conditions recommended by manufacturer

subgroups: 1 = experimental setting in CRA,
2= amounts calculated for 1000µl cell suspension and used in FACS analysis

factor: 1 = recommended relation of Ab for number of cells (1µg/10^6 cells)
others = x-times higher amount of Ab

* breaking down this concentration results in a 25:1 (exactly 26.6:1) E:T ratio as commonly used in CRA with an amount of 5000 target cells and 125.000 effector cells per 200 µl well. The used amount of 33.3 µg Ab/ml exceeds the common concentrations of 10-20 µg/ml.

Only the centrally marked steps of titration were performed. Columns at left and right show comparatively calculated titration steps.

3.2.3 Viability assay

For further examination of HLA-I toxicity, direct influence of the Abs to the cells was determined within a titration assay. Toxicity was tested exemplarily with IM-9, MOLP-8 and K562. Cells were adjusted to required concentration 3.75 x 10^5 cells/ well with 500µl assay medium consisting of RPMI160 with 10% FCS and 1% PenStrep. Cells were incubated with different amounts of HLA-I antibody for one hour. Afterwards, the content was transferred to FACS tubes and was washed with PBS for 5 minutes at 1400rpm. Supernatant was discarded. Pellets were resolved within the residual 200µl of fluid 200µl. 20µl of this cell suspension were investigated for viable cells after staining with 10µl trypan blue.

	group (E:T)	5:1	10:1	1:1	M. (18:1)	*CRA (0:1)*
Model	*assumed target cell no.*	*3.75x10^4*	*3.75x10^4*	*3.75x10^4/ml*	*3.75x10^4*	*5x10^4/ml*
	assumed total cell conc.	*0.45x10^6/ml*	*0.83x10^6/ml*	*7.5x10^4/ml*	*6.66x10^5/ml*	*5x10^4/ml*
	assumed total Ab conc.	*20 µg/ml*	*20 µg/m*	*1 µg/ml*	*0.66 µg/ml*	*1 µg/1000µl*
Viability assay	cell no.	3.75x10^4	3.75x10^4	3.75x10^4	3.75x10^4	*3.75x10^4*
	µl	500	500	500	500	*500*
	total µg anti-HLA-I *	1.7	0.9	0.5	0.0375	*0.75*
	Factor	**45**	**24**	**13**	**1**	***20***

Table 13 - HLA-I titration for viability assay

group: Sample group by standard E:T ratios in cytotoxicity assays as well as recommended relation of cell number and antibody as stated by manufacturer (M). Relations of present CRA are not equally adopted but can be classified on the basis of "factor" between group 1:1 and 10:1

assumed amounts: Cell numbers and Ab concentrations deduced from standard experimental settings and manufacturers recommendations as well as adapted from own CRA setup

assay cell no.: Number of cells/well in this assay

*: Amount of Ab used after adjustment to assay cell number but without adjustment to amount of medium.
[(assay cell no.)/(assumed total cell con.) x (assumed total Ab concentration)]

factor: factor by which used cell/Ab relation exceeds manufacturers recommendation:
[assay total µg anti-HLA-I) / (0.0375)]

3.2.4 Transfection of COS-7 with LIR-1

As a positive control for western blot experiments, COS-7 cells were transfected with pCMV6-AC vector encoding CD85j (LIR-1) (Colonna et al., 1997). All steps were performed according to the manufacturer's instructions.

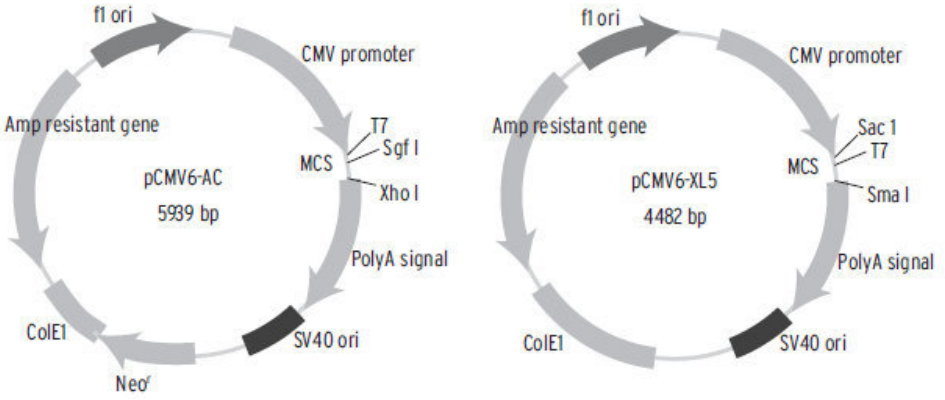


Figure 11 - Physical map of pCMV6-AC (LIR-1) and pCMV6-XL5 (NKG2A/mock transfection) Map of pCMV6-AC as provided by the manufacturer (OriGene). The CMV promoter is used to express the cloned cDNA and is followed by multiple cloning sites (MCS). Poly A is a human growth hormone located downstream of the insert. SV40 ori, ColE1 and f1 ori are mammalian, bacterial and filamentous phage origins of replication, respectively. While the ampicillin resistant gene allows selection of the plasmid in E.coli during transformation, the neomycin resistant gene allowed the creation of a stable cell line (COS-7) expressing LIR-1. pCMV6-XL5 was obtained from the same manufacturer and used for comparative mock transfection of COS-7. The plasmid encodes for Homo sapiens killer cell lectin-like receptor subfamily C, member 1 (KLRC1), transcript variant 3 (NKG2A).

To obtain large amounts of plasmids, transformation of competent E.coli cells (One Shot® TOP10/P3 Competent cells) was performed. One vial of cells was thawed on ice and plasmids were dissolved in 100 µl aqua bidest 1 µl plasmid dilution was transferred to one vial of E.coli, mixed and incubated on ice for 30 minutes. Additional incubation was done for 30 seconds at 42 °C water bath and cells were cooled down on ice. 250 µl of SOC medium was added and cells were incubated additional 60 minutes at 37 °C in a shaking incubator. Subsequently, cells were plated on previously prepared LB agar plates. Incubation was done overnight in a 37°C humidified incubator.

Purification of the expanded plasmids was done with Qiagen EndoFree Plasmid Maxi Kit. Briefly, two colonies of the transformed E.coli cells were picked with a pipette tip and transferred to a 10 ml Falcon containing 3 ml LB Liquid Amp. After 4 hours of incubation in a 37°C shaking incubator, the liquid was completely converted to 200 ml of the same prewarmed medium in an Erlenmeyer flask. Following incubation was

done overnight under the same conditions. The whole medium was centrifuged afterwards in appropriate flasks for 15 minutes at 5000xg at 4 °C. Supernatant was dismissed and the pellet was resuspended in 10 ml Buffer P1. 10 ml Buffer P2 was added, and incubated at room temperature for 5 minutes. 10 ml chilled Buffer P3 was added. The lysate was carefully mixed by shaking the flask and transferred to a QIAfilterCartridge. After additional 10 minutes, the cell lysate was filtered to obtain DNA. 2.5 ml Buffer ER was added to the filtered lysate, gently mixed and incubated on ice for 30 minutes Meanwhile, the Qiagen-tip 500 was equilibrated with 10 ml Buffer QBT and the tip was allowed to drain completely. Subsequently, the filtered DNA-containing lysate was applied to the Qiagen-tip and collected for further use.

The DNA was precipitated by adding 10.5 ml isopropanol and by subsequently centrifugation of the tube at 4000xg for 60 minutes. Supernatant was discarded and the pellet was washed with 5ml 70% ethanol. After an additional centrifugation at 4000xg for 10 minutes, the pellet was air-dried, resolved in Buffer TE and stored at 4 °C overnight. After measuring DNA content, the DNA was frozen at -80°C in 1.5 ml Eppendorf tubes for further use.

Transfection of COS-7 cells was performed as described before (Carretero et al., 1997; Colonna et al., 1997). Cells were plated out in a 6-well plate the day before transfection at a density of $3x10^5$ / 2000 µl cell culture medium (Table 5 - Culture and freeze media) per well, to obtain 50-80% confluency on the following day. 2 µg purified DNA and 3 µl FuGene HD Transfection Reagent were added to 100 µl OptiMEM and incubated for 15 minutes at room temperature (RT). This solution was sufficient for transfection of one well and was added drop-wise without prior change of COS-7 culture medium. Cells were harvested at the desired density the next day. Since no functional assays with the need of a permanent LIR-1 expression were required, additional selection with neomycin was spared. Lysates were prepared immediately as described (3.2.5 Western blot) and used in western blots.

3.2.5 Western blot

Western blot was used as a common method to analyze expressed surface molecules of effector and target cells (Renart et al., 1979; Towbin et al., 1979; Burnette, 1981b).

Cell lysates were prepared by disrupting about $1\text{-}5x10^6$ cells in 80-100 µl buffer within 30 minutes of soft shaking at 4 °C. Protein-containing eluent was obtained by subsequent centrifugation at $1.3x10^4$ rpm at 4 °C and dismissing the precipitate. Lysate was stored in Eppendorf tubes at -80 °C for further use.

Protein content of the lysates was measured by standard Bradford assay, in which protein content of the samples was compared to a standard curve, established prior the

assay by measurement of several concentrations of bovine serum albumin (BSA) binding to Coomassie Brilliant Blue with a resulting shift in absorption between 465 and 595 nm (Bradford, 1976). For measurement of samples 30 µl of 0.1 M HCL and 240 µl aqua bidest were added to 30 µl lysate. 25 µl of this specimen were further diluted with 875 µl Coomassie Brilliant Blue. After 5 minutes of incubation in a dark place, samples were photometrically measured and protein content was determined by the standard curve.

Western blot is a method that allows the detection of distinct proteins by their individual size (Burnette, 1981a). During electrophoresis on sodium dodecyl sulfate-polyacrylamide gels, the proteins are separated by their size. Submission to a nitrocellulose membrane is followed by visualization on film material.

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 5-15 µl lysate were mixed with 2 µl NuPAGE® Sample Reducing Agent and 5 µl NuPAGE® LDS Sample Buffer within Eppendorf tubes. After short centrifugation at $1.3x10^3$ rpm, samples were incubated for 10 minutes at 70°C and transferred to the gel. The gel-chamber was filled with running buffer containing 500 µl antioxidant. PeqGold Protein Marker V was used as reference for protein size. Electrophoresis was done at 195 V constant current for 60 minutes.

After protein separation, gels were transferred to a blotting chamber, enabling the transfer of the proteins to a nitrocellulose membrane. Layers of paper and sponges soaked with transfer buffer provided adequate supporting material for plane surface of gel and membrane, respectively, and facilitated the transfer by sufficient pressure under 25 V constant current.

Membranes were coated with 3% blocking solution for 1 h and subsequently incubated with the appropriate antibody at a concentration of 1:500 – 1:2500 overnight at 4 °C under gentle agitation.

In the next morning, four washing steps were accomplished with TBS-T solution at 15 minutes, respectively. As a secondary antibody, 2.5 µl-3.5 µl anti-mouse IgG were diluted in 10 ml 3% blocking solution. Incubation was done for 1 hour under agitation and followed by four washing steps with 25 ml TBS-T each. ECL solution was freshly prepared according to the manufacturer's instructions within a 15 ml Falcon and directly given onto the drained membrane. After one minute of incubation, the membrane was taken out and dried on absorbent paper for transfer to a film cassette. The film was exposed in the darkroom with reasonable exposure times. After developing the membranes of interest with anti-LIR-1 Ab, Reblot stripping solution was used according to the manufacturer´s instructions to prepare the membranes for re-incubation with housekeeping β-Actin (ACTB) antibody. Reblot solution (10x) was

diluted in aqua bidest. Membranes were washed in TBS-T and aqua bidest for 5 minutes, respectively. Diluted stripping solution was added for a period of 20 minutes. Afterwards, two more washing steps with aqua bidest and TBS-T followed before another hour of blocking and subsequent ACTB incubation and film development.

3.2.6 Chromium-release assay

Introduction

Radioactive chromium as sodium-51-chromate (51Cr(VI), half-life time of 26.5 days) has already been used since 1950 as a cell label (Gibson and Weiss, 1946; Sterling and Gray, 1950). The use of radioactive release of 51Cr from lysed cells as a marker for cytotoxicity was introduced in 1961 (Goodman, 1961). It is commonly assumed, that water soluble 51Cr(VI) enters the cell unidirectional through the same non-specific anion channel as sulfate and phosphate ions (SO42-, HPO42-), due to their structural similarity (Connett and Wetterhahn; Levina A). Its influx follows Michaelis-Menten-kinetics and is increased at higher temperatures. Chromium VI is reduced to chromium III in the cytoplasm and cannot pass the cell membrane. After membrane damage, 51Cr(III) can be detected by gamma- or beta counters (Connett and Wetterhahn; Levina A; Lilien et al., 1970).

Physiologically, Cr(III) is supposed to be an important factor for glucose metabolism as it improves glucose tolerance and increases circulating insulin and glucagon *in vivo* (Anderson et al., 1991; Mertz, 1993).

Standard chromium-release assays

Cytolysis was determined in a 4h chromium-release assay (CRA) according to standard protocols (Goodman, 1961; Newman, 1982; Le Gal et al., 1999).

Target cells and NK-92 were splitted the day before the functional assays to standardize the chromium uptake and cell viability. NK-92 cells were freshly supplied with medium enriched with 200U rIL-2 ml^{-1} at a density of approximately $5x10^4$ ml^{-1} for optimal growth conditions.

Directly before the assay, $1x10^6$ targets cells were pelleted within assay medium (RPMI1640, 10% FCS, 1% PenStrep), supernatant was dismissed and cells were resuspended within the residual liquid. Target cells were labeled with 100 µCi 51Cr for an incubation period of 1.5 h at 37 °C in a humidified incubator with 5% CO_2.

After washing the tumor cells for 5 minutes at 300xg with PBS and assay medium, respectively, cells were resuspended to a dilution of $5x10^3$ cells/100 µl. NK-92 and targets cells were coincubated at effector:target (E:T) ratios of 10:1, 5:1, 2.5:1, 1.2:1 and 0.6:1 in U-bottomed microtiter plates at a total of 200 µl assay medium.

Maximum lysis and spontaneous release of 51Cr were induced by adding 100 µl dilution of 5% Triton-X / PBS or assay medium to 100 µl target cells, respectively. Background radiation was measured from pure assay medium. All samples were plated out in triplicates. Prior to the 4 h incubation period, plates were centrifuged 1 minute at 300xg to facilitate effector-target contact. Additional centrifugation for 5 minutes at the same speed was done after the 4 h incubation period and 25 µl supernatant was transferred with a multi-channel pipette to a 96-well plate. To each well, 150 µl scintillation liquid was added. Plates were closed with Viewseal foils. The suspension was mixed thoroughly for 15 minutes at 19 °C on an Eppendorf thermomixer and then measured on WALLAC TRILUX 1450 Microbeta Counter with 2 minutes counting time and a measurement delay of 10 minutes to avoid chemiluminiscence effects.
Percent specific lysis was calculated as follows:

$$\frac{\text{(c.p.m. experimental release – c.p.m. spontaneous release)}}{\text{(c.p.m. maximum release – c.p.m. spontaneous release)}} \text{ x100}$$

Results are shown as the means of triplicates of at least three independent experiments. In all experiments, spontaneous release (SR) was <20% (Whiteside et al., 1990).

3.2.7 Blocking experiments

NKG2A and LIR-1 interaction with their respective ligands was done according to standard protocols with appropriate isotype controls. HLA-A, -B, -C, -E, and –G engagement was blocked by pan-HLA Ab clone HP-1F7 (Riteau, 2001; Bryceson et al., 2009; Godal et al., 2010).
To prevent ADCC, $F(ab')_2$ fragments against the Fc region of the blocking Abs were used in experiments with blockade of target cell receptors as well as in blocking NK-92 surface molecules, due to little but detectable expression of CD16 and in order to provide comparability between all experimental settings (Le Gal et al., 1999; Riteau, 2001). Controls without $F(ab')_2$ are explicitly named. Figures D, E and F depict a model of the intended interaction (Figure 12 - Experimental setup of blocking assays).

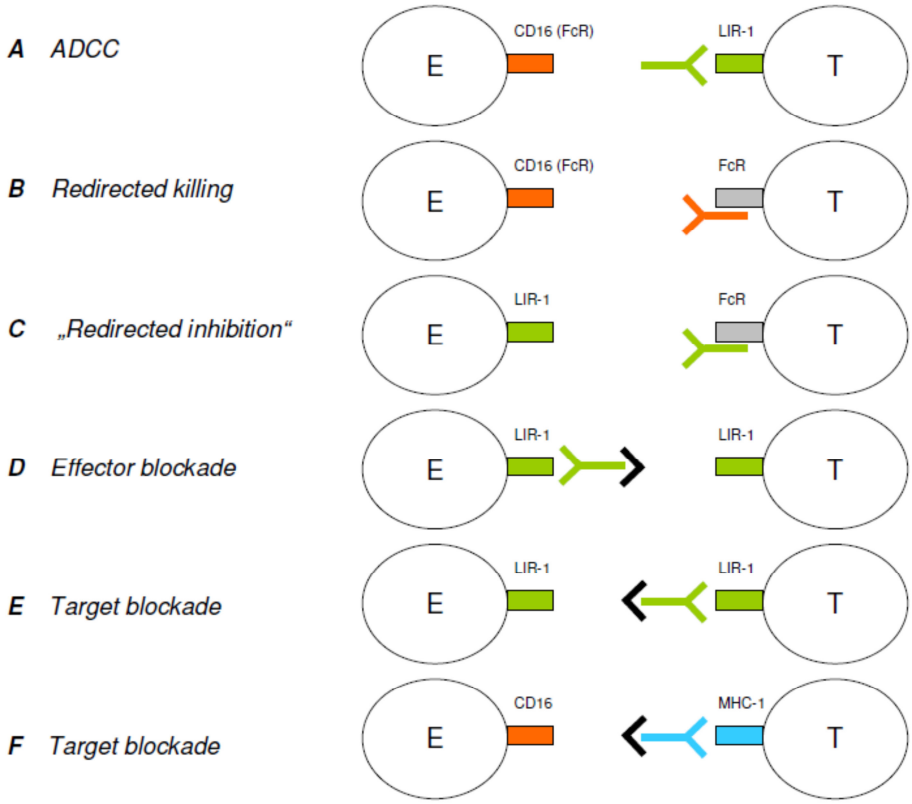


Figure 12 - Experimental setup of blocking assays

In all experiments, prevention for ADCC (A) was taken into consideration. As NK-92 do not bear CD16 on their surface, activation via redirected killing (B) could be excluded.

To crosslink Ab against LIR-1, pre-incubation of target cells with LIR-1 was considered (C) but excluded, for some of the targets were bearing LIR-1, also. Therefore, anti-LIR-1 was preincubated with the effector or target cell and subsequently coated with $F(ab')_2$ against the Fc region of IgG (D, E). The same procedure was chosen for MHC-I blockade, despite the lack of CD16 on NK-92 (F).

Abbreviations: E= Effector, T=Target, FcR= Fc Receptor

Symbols:


= Receptor


= Antibody


= $F(ab')_2$

Blocking of effector cells

CRA were performed as described above. Heat inactivated human serum was prepared from donated blood, which was obtained after written formal consent. Serum tubes were centrifuged for 10 minutes at 1000xg. The serum was transferred to Falcon tubes and heated in a 56 °C prewarmed water bath for 30 minutes and mixed gently

every 5 minutes to ensure uniform heating. Serum was cooled down and aliquots were stored at -20 °C until further use.

NK-92 cells were preincubated with RPMI1640 supplemented with 1% PenStrep and 10% heat inactivated human serum for 30 minutes and kept within the same medium during an additional incubation period with Ab concentrations of 0.1 µg ml^{-1}, 1 µg ml^{-1} and 10 µg ml^{-1}, respectively. Target cells were marked with 100 µCi 51Cr as done before. NK-92 cells were washed twice to avoid interactions of the Ab with the later on coincubated target cell line. Cells were adjusted for a fix E:T ratio of 1.2:1, using $5x10^3$ target cells / 100 µl as before. In half of the experiments, this step was followed by additional preincubation with 11 µg / ml $F(ab')_2$ 15 minutes prior to coincubation of NK and tumor cells. In samples classified as 'untreated', no $F(ab')_2$ was used (Rouas-Freiss et al., 1997; Riteau, 2001).

Spontaneous release (SR) was always <20% for all cell lines except from MOLP-8, which showed a constantly high SR up to 36% (Whiteside et al., 1990).

Toxicity of antibodies

To evaluate the toxicity of the Abs, in four experiments NK-92 were radioactively labeled (51CrNK) and incubated with antibodies under the same conditions as unlabeled cells, thus using different ab concentrations as well as treatment with or without $F(ab')_2$. As the conditions for ^{51}CrNK cells were set up at the same day and performed in parallel to the experimental series of the effector blockings with $F(ab')_2$, systemic errors such as cell growth performance or cell viability could be eliminated.

51CrNK showed SR < 6% for all conditions with a standard deviation (SD) < 3%. No differences related to parameters $F(ab')_2$, antibody or concentration could be detected (Riteau, 2001).

Blocking of tumor cells

Target cells were incubated with 1 µg ml^{-1} of the respective antibody at a cell density of $5x10^4$ cells ml^{-1}. For pan HLA-I staining, this concentration was still 20-times higher than the recommended concentration for staining with the same antibody in flow cytometry assays according to the manufacturer. Procedure and incubation times were the same as described for NK cells. Radioactively labeled target cells were preincubated with medium, supplemented with 10% human serum before blocking. $F(ab')_2$ was used at a concentration of 1.7 µg / ml for all samples, inclusively the 'untreated' control.

Tumor cell incubation with Abs led to SR of <25% for MOLP-8 and RPMI8226, and up to 11% for all other cells.

3.2.8 Statistics

To control for indirect effects, statistical interpretation was done by multivariate Analysis of Variance (ANOVA). In all calculations, specific lysis was defined as the dependent variable. Antibodies, concentrations of the Abs, of the targets and the use or no use of $F(ab')_2$ were defined as independent variables. Wherever appropriate, interdependencies between the variables were taken into account.

All calculations were done by SPSS (IBM® SPSS® Statistics Version 19, Release 19.0.0).

Due to the complexity of the setting, assistance and counseling was provided by the Department of Medical Biometry and Epidemiology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany.

4 RESULTS

4.1 Preliminary studies

4.1.1 Expression of LIR-1 and NKG2A on NK-92 and tumor cells

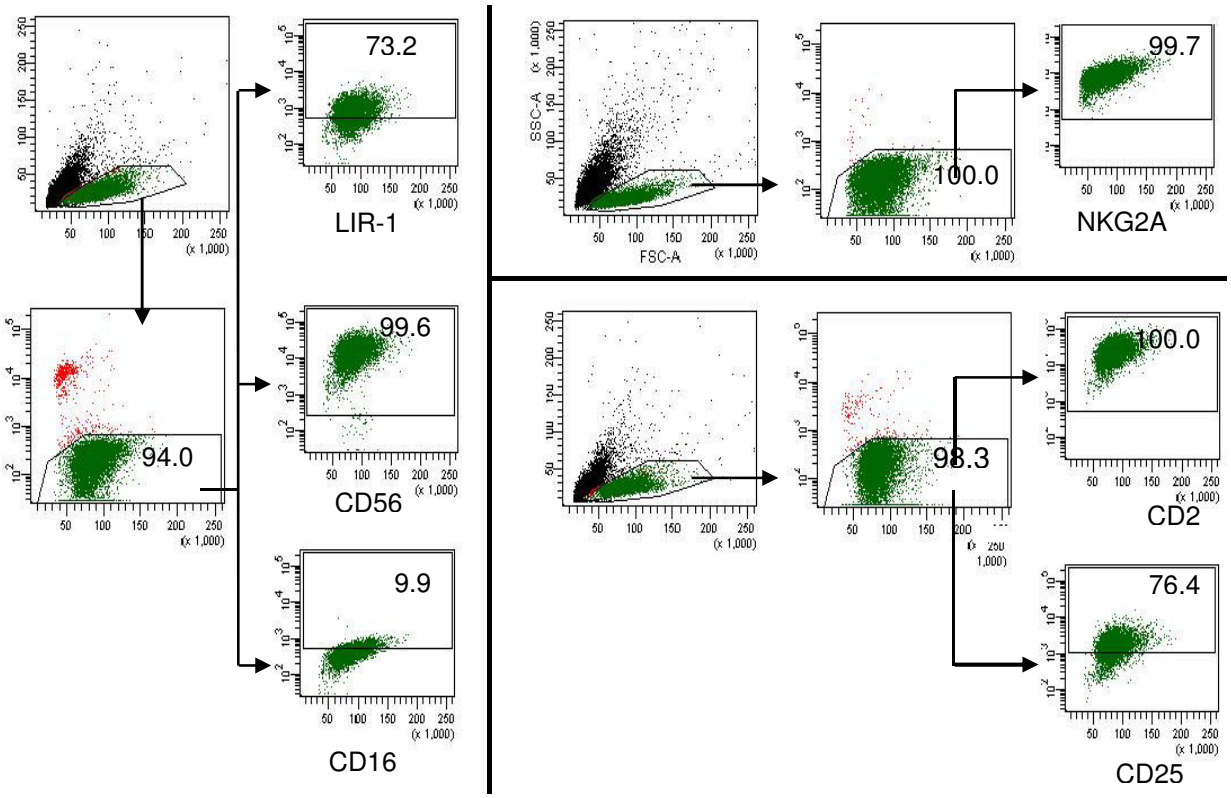
In order to evaluate the expected binding properties of NK-92 and tumor cells, flow cytometric analysis of surface antigens was performed. Analysis of all receptors was repeated after a course of four weeks to exclude changes caused by cell culture. As shown in Figure 13 - Expression of surface antigens, receptor expression on NK-92 and tumor cells varies strongly between cell lines but only little during time.

As expected, NK-92 seem to resemble the $CD56^{bright}$ subset of NK cells with a high expression of activation marker CD25, diminishing amount of FcγRIII (CD16) and bright staining for NKG2A. Unlike $CD56^{bright}$ cells and in contrast to results by other authors, this subset was found to express high levels of LIR-1 that is usually associated with high KIR levels on $CD56^{dim}$ cells (Colonna et al., 1997; Cooper et al., 2001; Godal et al., 2010). NK-92 has been described before to lack most KIR molecules except from KIR2DL4 and its homolog NKp49 (Maki et al., 2001; Davidson et al., 2010).

Noteworthy, no target cell line expressed NKG2A, whereas LIR-1 was present on myeloma cells as well as on IM-9 but not on K562 or HL60. CD56 was present on all lines except from IM-9 and K562 as assumed (van Camp et al., 1990).

To validate these findings, LIR-1 expression was additionally investigated by western blot analysis. As a control cell line for further analysis, COS-7 were transfected with vectors containing mock DNA and DNA for LIR-1. Untransfected COS-7 cells served as auxiliary negative controls. As expected, western blot analysis confirmed the previous findings, and the strength of band representation reflected flow cytometric staining intensity (Figure 14 - LIR-1 expression on NK-92 and target cells).

A


73.2
LIR-1
94.0
99.6
CD56
9.9
CD16
SSC-A
FSC-A
100.0
99.7
NKG2A
98.3
100.0
CD2
76.4
CD25

B

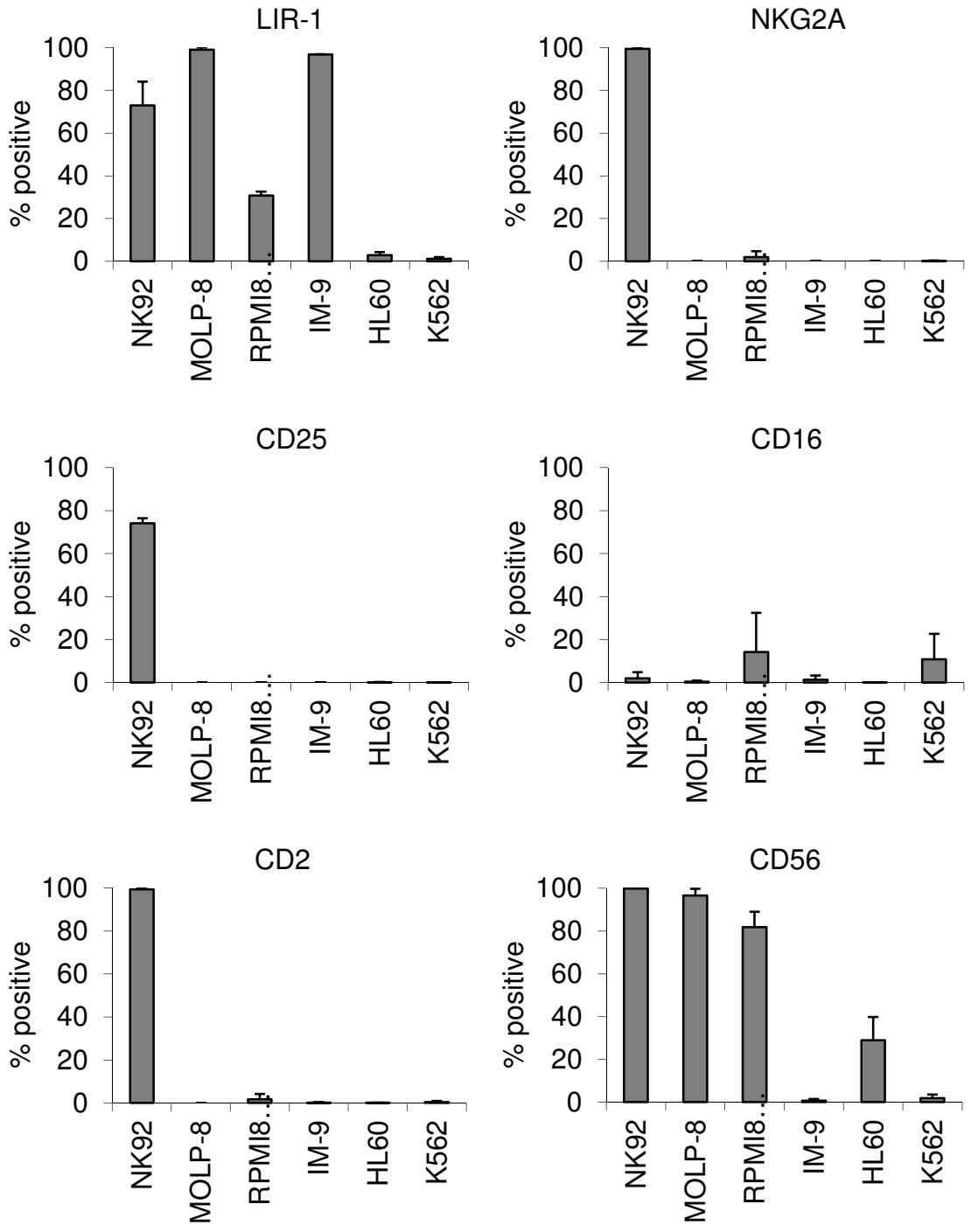


Figure 13 - Expression of surface antigens

A. Flow cytometric phenotyping of all cell lines was done with mABs against LIR-1, CD56, CD16, CNKG2A, CD2 and CD25 as shown for NK-92.

B. Surface antigen expression on NK-92 and tumor cell lines. Values given as % positive staining after subtraction of isotype control.

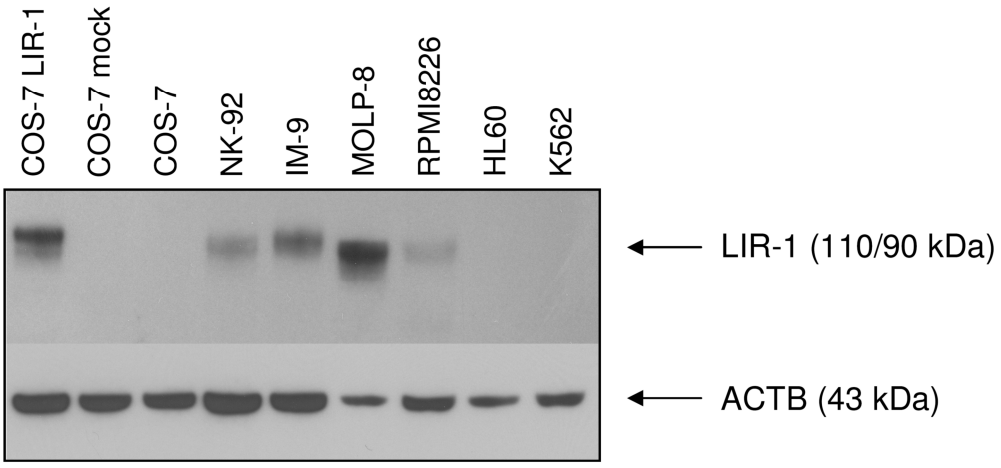


Figure 14 - LIR-1 expression on NK-92 and target cells

Western blot analysis of LIR-1 expression on NK-92 cells and tumor cell lines. Untreated as well as LIR-1 or mock transfected COS-7 cells negative and positive controls, respectively. After film development, the membrane was stripped and re-incubated with ACTB antibodies as a loading control. 4 µg anti-LIR-1 and 10 µg anti-ACTB (1:1000) were used for antigen detection. Lanes contended 35 µg of total protein. As also shown by flow cytometric analysis, NK-92 as well as IM-9 and MOLP-8 express high levels of LIR-1, whereas no detection of LIR-1 was possible on HL60 and K562 cells.

4.1.2 Titration of HLA class I antibodies

Total HLA-I expression was evaluated via FACS based analysis with K562 as negative controls, whereas PBMC and JEG-3 cells were used as positive controls for HLA-E and HLA-G expression, respectively. While K562 were HLA class I negative as expected, all other cell lines were HLA-I positive. This correlated with the common investigations made with MM cell lines and patient derived MM cells from different stages of disease (Carbone et al., 2005; Palmisano et al., 2005).

In Figure 15 - Titration of HLA-I surface antigens, exemplary results from HLA-I staining in FACS analysis are shown. For all cell lines, HLA-I Ab was used in three different concentrations to evaluate toxicity. In all cases, the highest concentration led to a considerable proportion of dead cells, even leading to almost total annihilation of IM-9 cells. The lowest concentration resulted in sparing cell viability, while efficiently staining the desired surface antigens. Interestingly, the highest concentration (a) resembles a commonly used amount (20 µg / µl) in CRA with a 50-times higher than recommended Ab usage, while (b) still exceeded the manufacturers proposal by factor 13 and reflects the setup of later on conducted experiments (Table 12 - HLA-I titration for flow cytometric analysis). Further examination of dead cell staining

additionally underlined the correlation of cell death with Ab concentration (Figure 16 - Dead cell staining in HLA-I titration for flow cytometric antigen detection).

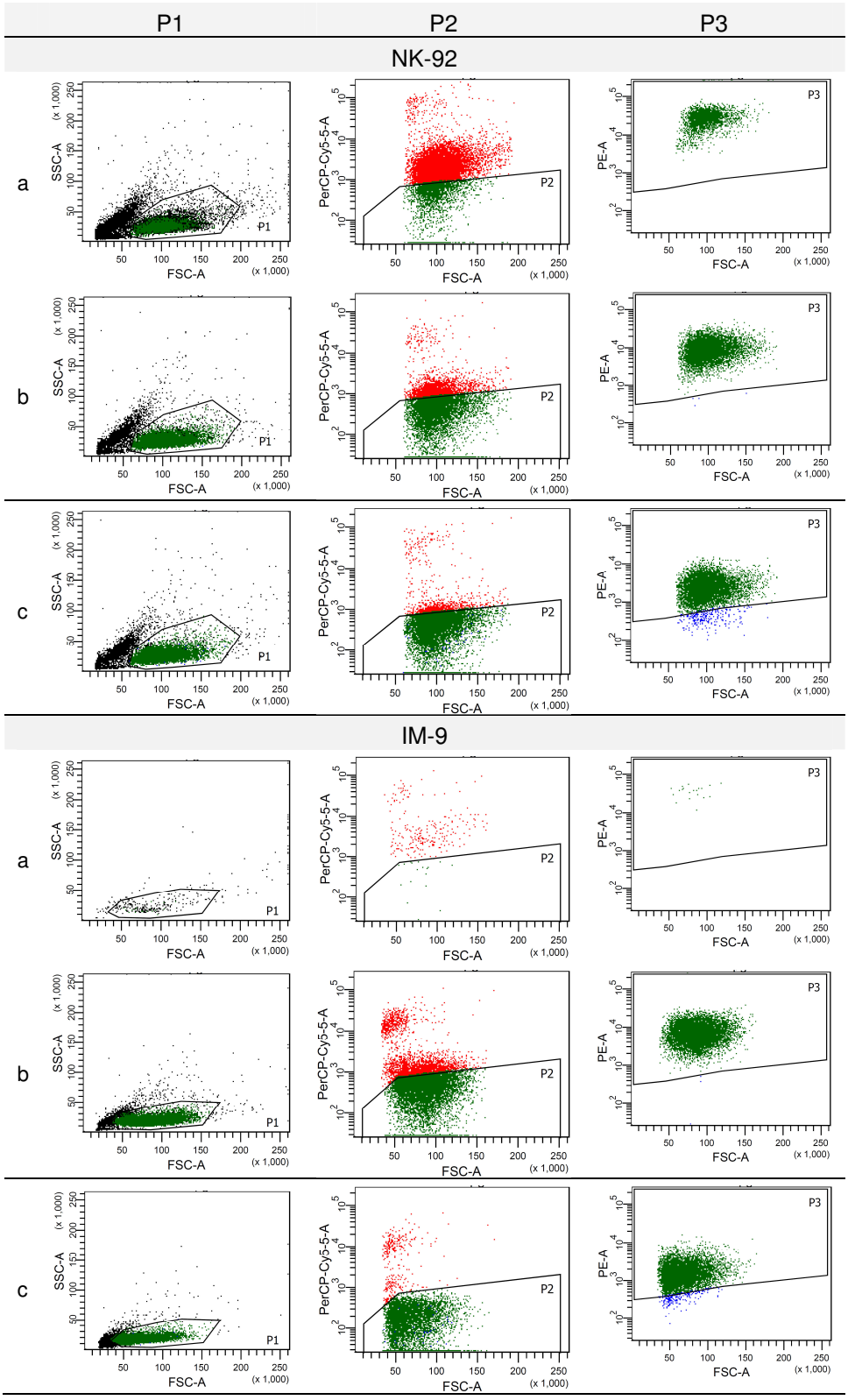


Figure 15 - Titration of HLA-I surface antigens (continued on the following page)

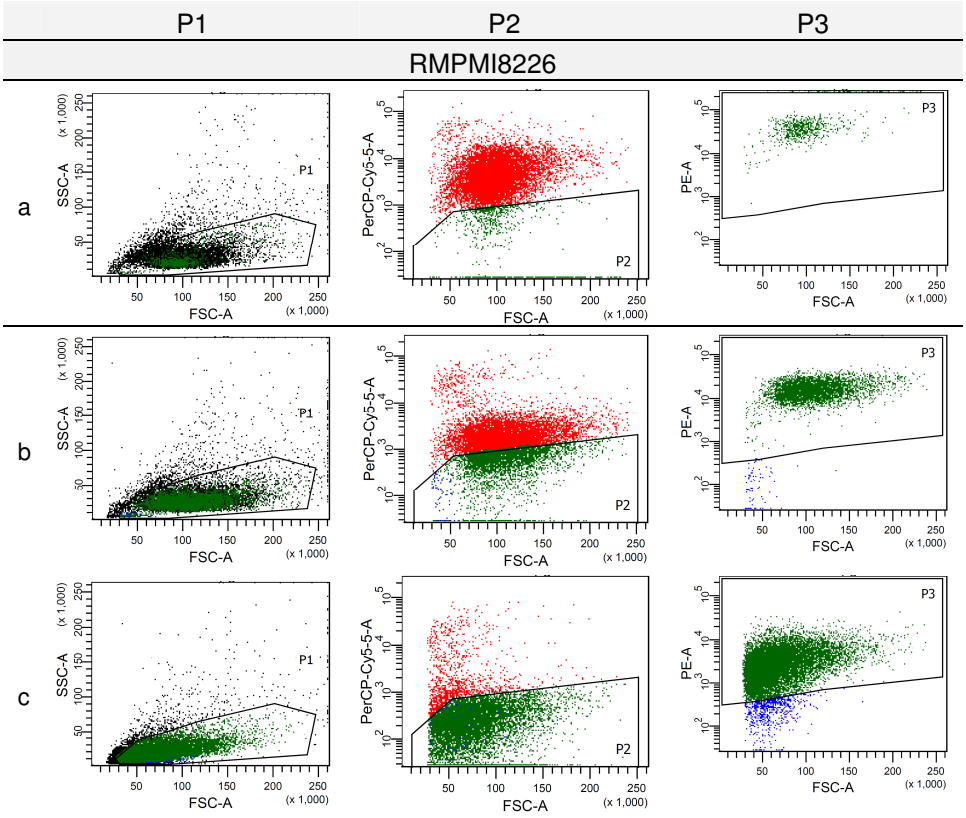


Figure 15 - Titration of HLA-I surface antigens

Evaluation of HLA-I expression of NK-92 and all other experimentally used cell lines. Here, a representative selection of results is shown. All cells were treated with three different concentrations of HLA-I antibody, respectively, while amount of 7AAD for dead cell staining (5 µl / sample) and secondary fluorescent antibody (8 µg / sample) remained stable.

concentration a = 27:1 (E:T) with 33.3 µg / ml Ab (conditions similar to standard CRA)

concentration b = 1:1 (E:T) with 1µg / ml Ab (conditions similar to actual CRA)

concentration c = 27:1 (E:T) with 0.66 µg / ml Ab (manufacturer´s proposed cell/Ab ratio)

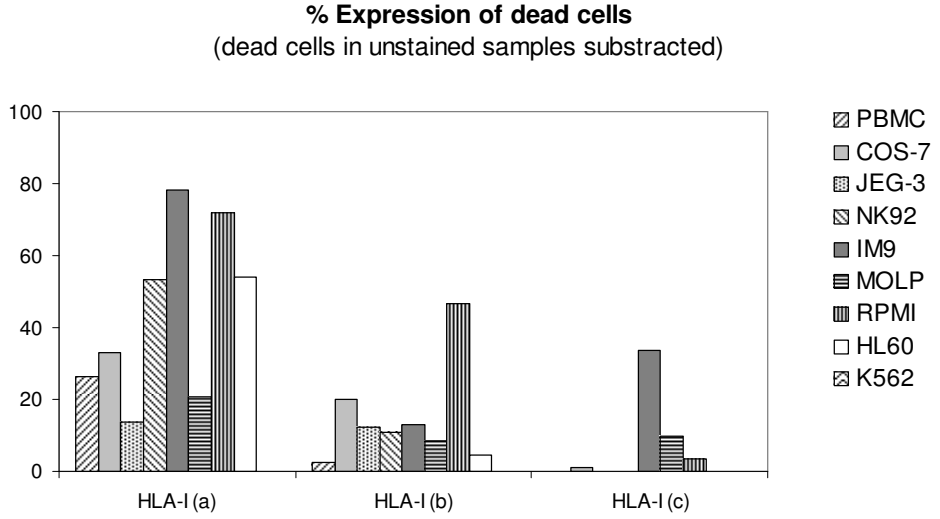


Figure 16 - Dead cell staining in HLA-I titration for flow cytometric antigen detection
Concentrations as described in Figure 15 - Titration of HLA-I surface antigens. Dead cells were stained with 7AAD. After gating out viable (P1) and non-7AAD labeled cells (P2), all other cells were regarded as dead (gating strategy also shown in Figure 15). To evaluate the effect of the used antibodies, the amount of dead cells in unstained samples was subtracted from the results. While IM-9 cells seemed to be highly responsive towards the used HLA-I antibody, a clear decline of toxicity with decrease of antibody amount could be detected in all other samples.

To clarify, whether the toxicity resulted from the amount of antibody or other side effects, a simple viability assay was performed, in which again the antibody concentration was adjusted to the cell number. The same HLA-I antibodies were used throughout FACS analysis, viability assays and later on conducted blocking assays. In the present viability assay, no toxicity could be detected, and cells proliferated extensively within the short incubation time (Figure 17 - Viability assay). The easiest explanation would be that the cross-linking by secondary fluorescent antibodies was the factor of influence that resulted in apoptosis. Whether or not a similar effect by $F(ab')_2$ fragments in subsequent CRA played a role could not be ruled out.

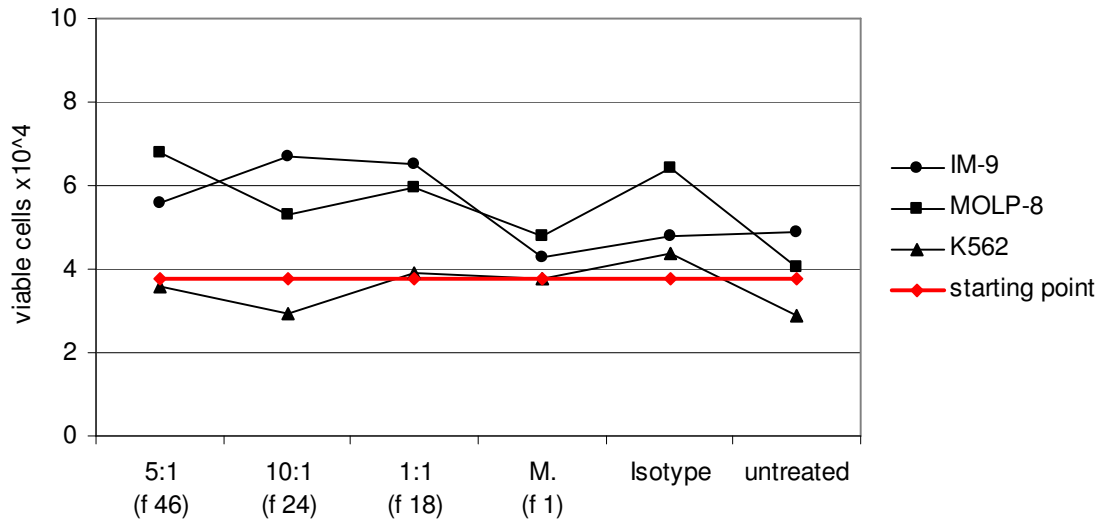


Figure 17 - Viability assay

Cell viability measured by trypan blue staining after 1 hour of incubation with anti-HLA-I Ab. Titration according to Table 13 - HLA-I titration for viability assay. The same number of target cells was incubated with a decreasing amount of Abs, reflecting the cell:mAb [(E:T) : mAb] ratio within the later on conducted blocking assays . All samples were incubated in 500 µl assay medium. Isotype control consisted of 1.5 µg IgG1 / 500 µl.

(f x) = factor, by which the recommended amount of Ab was increased in relation to the cell number

M. = manufacturer´s recommended cell/Ab ratio of 1 µg / 1x10^6 cells

'Starting point' denotes the number of cells present at the beginning of the assay. Obviously, exposure to the Abs did not hinder cell growth within the short time until counting cells.

Relations of the presently conducted CRA are not equally adopted, but can be graded between groups 1:1 and 10:1 (equal to f 20).

4.1.3 Expression of HLA class I molecules on NK-92 and tumor cells

LIR-1 has a broad spectrum of ligands, due to its binding to highly preserved parts of MHC-I molecules (Chapman et al., 1999). As these binding properties are weak and HLA-G is one of the strongest binding partners, expression was also evaluated (Chapman et al., 1999; Borges and Cosman, 2000; Chapman et al., 2000; Shiroishi et al., 2003). Only JEG-3 were positive for HLA-G. Thus, interaction of LIR-1 in later on conducted CRA was restricted to other binding partners. HLA-E as the ligand for NKG2A lacked on all cell lines except of a minor staining on IM-9. As HLA-E is so far the only known ligand for NKG2A, no increase of cytotoxicity could be expected in subsequent blocking assays (Braud et al., 1998). While PBMC are known to express HLA-E, their staining remained undetectable (Furukawa et al., 1999; Palmisano et al., 2005).

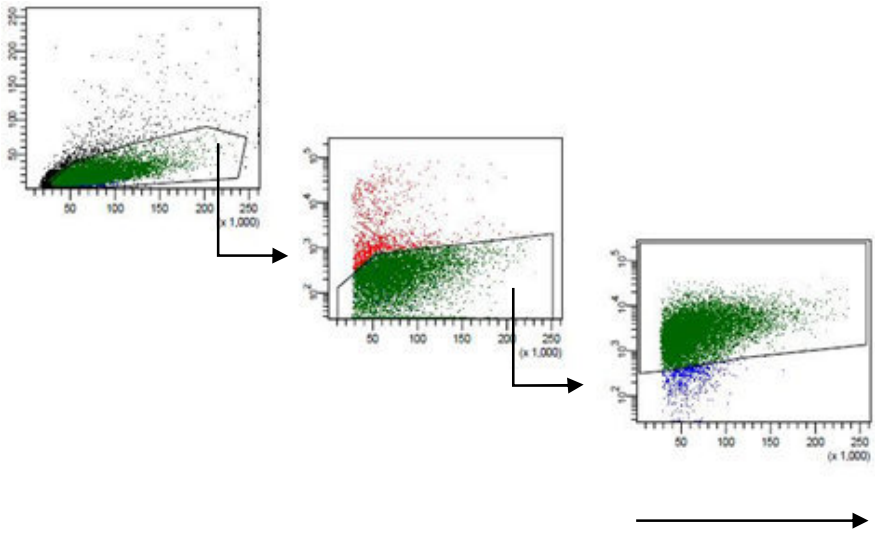


Figure 18 – Gating strategy for HLA surface antigen expression
A. Representative flow cytometric gating strategy for analysis of HLA expression on all used cell lines (shown for HLA class I expression on RPMI8226). All cells were stained with anti-HLA-I (5 µg), anti-HLA-E (1.3 µg) or anti-HLA-G IgG1 (1µg), followed by secondary goat anti-mouse IgG1 FITC (8µg).

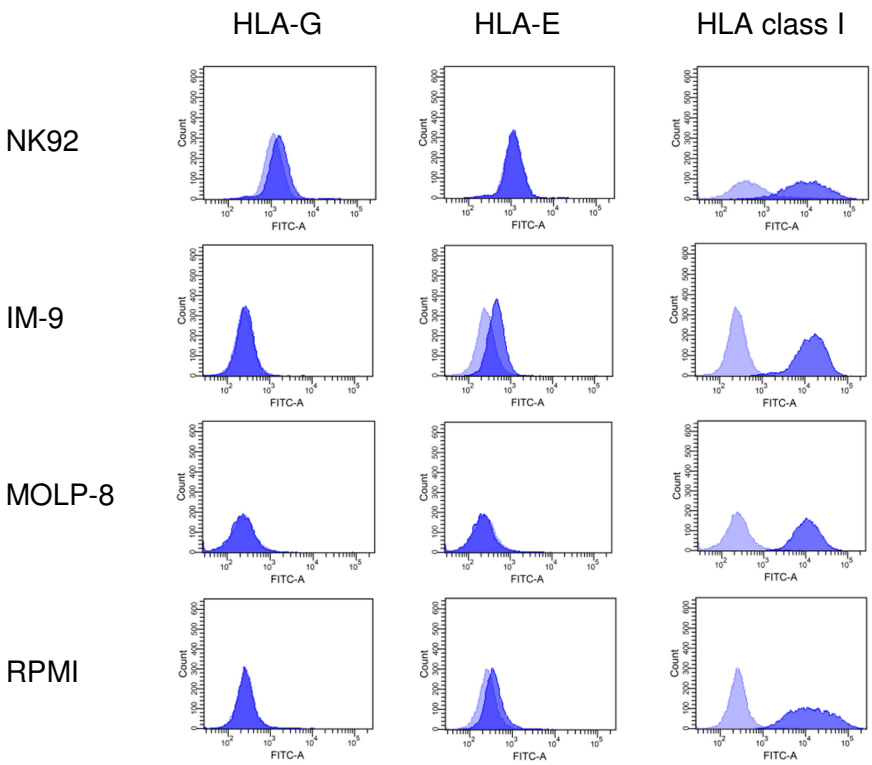


Figure 19 - Cumulative view of HLA surface antigen expression
(continued on the following page)

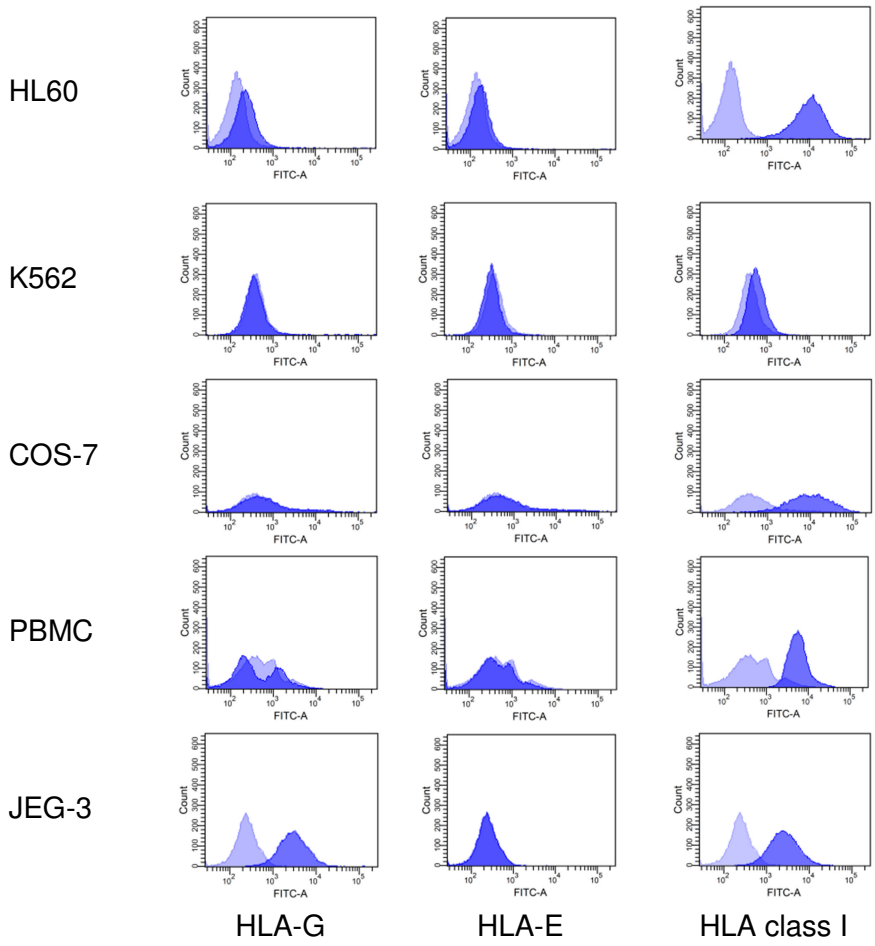


Figure 19 - Cumulative view of HLA surface antigen expression
HLA class I molecules were detectable on all cell lines except of negative control K562.
JEG-3 cells were strongly positive for HLA-G, while detection on NK92 and HL60 was marginal. HLA-E as the ligand for NKG2A lacked on all cell lines except of a minor staining on IM-9.

4.1.4 Myeloma cells are highly susceptible to NK-92 mediated killing

Cytotoxicity of NK-92 against a panel of tumor cell lines was investigated in CRA at 5 different E:T ratios as described above (3.2.6 Chromium-release assay) (Whiteside et al., 1990). MM cell lines and IM-9 were efficiently lysed by NK-92, with highest results for IM-9 cells (E:T 10:1; specific lysis 69.9 ± 6.3%) followed by MOLP-8, K652 and RPMI8226 at 21-30%. HL60 could be regarded as resistant to lysis even at the highest E:T ratio of 10:1 (specific lysis 4.5% ± 2.5%) (Figure 20 - NK-92 mediated killing of target cell lines is dependent on applied E:T ratio). NK-92 culture conditions and conductance of the assays were considered applicable for subsequent blocking assays. Interestingly, sensitivity to lysis could not be equated with spontaneous release

(SR) of 51Cr, what could have been expected at least for MOLP-8 cells, which were used despite of high SR (17.3 ± 1.6%). In fact, SR did not correlate with cell viability or sensitivity to NK cell mediated lysis, what was true for all performed experiments (data not shown).

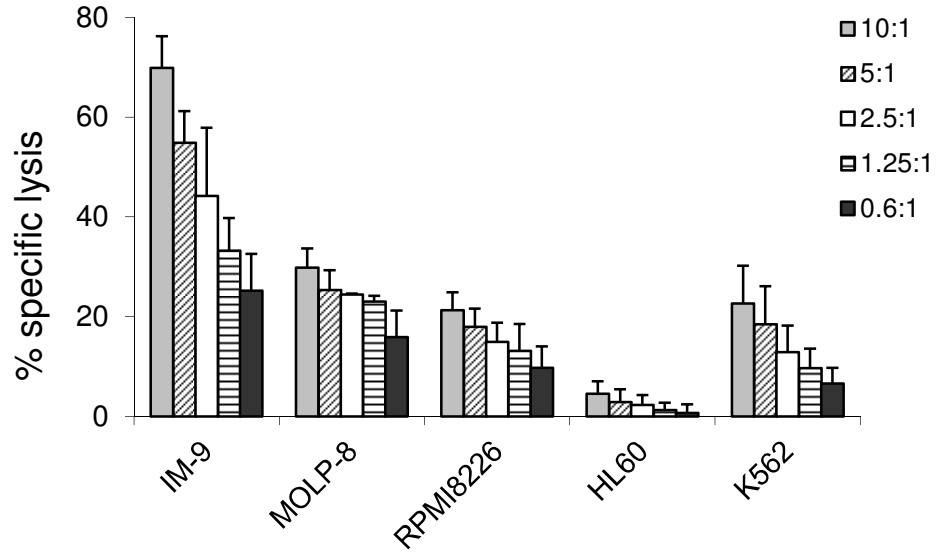


Figure 20 - NK-92 mediated killing of target cell lines is dependent on applied E:T ratio
CRA revealed a clearly E:T dependent level of NK-92 mediated cytotoxicity. Results of CRA are represented as means of at least three independent experiments ± SD. Values in the table below are given as % specific lysis. AML cell line HL60 was almost resistant to NK-92 mediated killing. NK-92 cells were stimulated with 200 U/ml IL-2 the day before the assay.

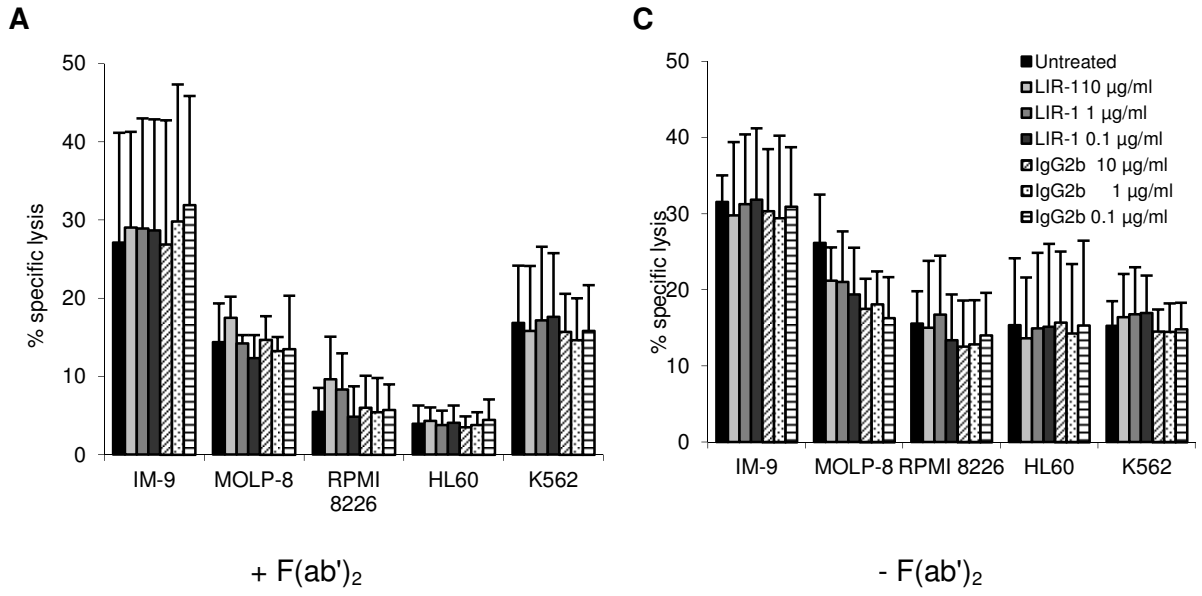
4.2 Blocking assays

4.2.1 Blocking of neither LIR-1 nor NKG2A on NK-92 cells increases target cell lysis

To evaluate the influence of LIR-1 in myeloma cell lysis, mAbs were used to blockade receptor-ligand interactions. As target cells lacked the expression of the HLA-E, blocking of NKG2A was used as a negative control. Taking into account, that LIR-1 was present not only on NK cells, but also on most targets, experimental settings were designed to restrict the influence of the blocking Abs mostly to NK-92. Therefore, mAbs were incubated with effector cells alone, followed by extensive washing. All experiments were performed at least three times, each in a series with or without use of $F(ab')_2$, respectively, in order to prevent ADCC. Although other authors restrict the use of $F(ab')_2$ to target blocking, this decision was made to exclude any systematic errors when comparing the outcome with that of later target blocking. NK-92 is commonly described as lacking the FcγRIII, but little staining of CD16 was done by

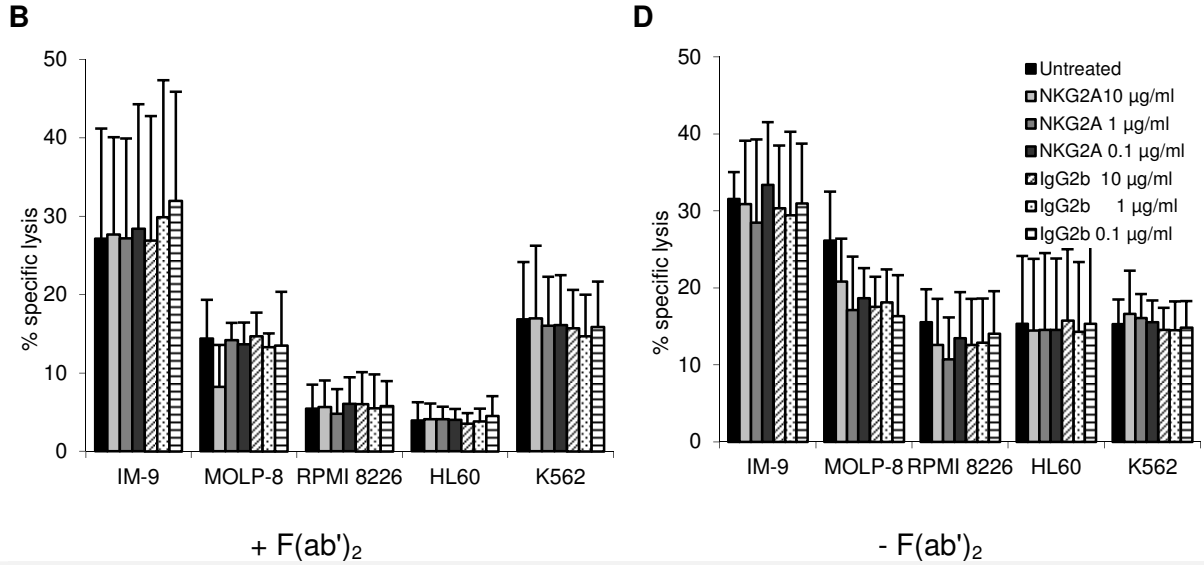
FACS, and influence of other FcR like CD32 and CD64, as well as further unknown interactions with blocking Abs could be excluded by this procedure (Gong et al., 1994). Toxicity of any of the used reagents was excluded (Figure 24 - Neither antibodies nor $F(ab')_2$ show toxic effects on NK-92 cells).

LIR-1



+ $F(ab')_2$ - $F(ab')_2$

NKG2A



+ $F(ab')_2$ - $F(ab')_2$

Figure 21 - Blocking of CD85j and CD159a at NK-92 effector cells does not increase specific lysis

Blocking assays were performed as 4-hour CRA. NK-92 were preincubated with human serum and treated with the respective mAb (0.1, 1 and 10 µg/ml). E:T ratio of 1.25:1 was used throughout all experiments with a total of $5x10^3$ target cells/200 µl. Results are shown as means of triplicates of at least three independent experiments.

(A/B): $F(ab')_2$ was added after the last washing step prior to co-incubation with target cells and maintained within the medium throughout the whole experimental period.

(C/D): Samples without $F(ab')_2$

In samples classified as 'untreated', neither $F(ab')_2$ nor Abs were used. SR was always <20% for all cell lines except from MOLP-8, which showed a constantly high SR up to 36%.

Statistical analysis was performed by ANOVA and did not show any significant effect of Ab use.

No significant increase of tumor cell lysis could be achieved by any of the blocking Abs despite high concentrations (Figure 21 - Blocking of CD85j and CD159a at NK-92 effector cells does not increase specific lysis). Results are shown as means of at least three independent experiments. E:T ratio was 1.2:1 in all experiments. SR was always <20% for all cell lines except from MOLP-8, which showed a constantly high SR up to 36%. This finding implicated a high sensitivity towards any further treatment, what was contrary to the obtained effect.

What first might appear as a protective effect of the applied $F(ab´)_2$ towards a reduced lysis of target cells, must be considered to be a side effect caused by cell culture period, for it included the results for the untreated sample, that did not obtain treatment with either $F(ab´)_2$ or Abs.

This culture effect is highlighted in Figure 22. As specific lysis of target cells by NK-92 was found to be independent from Ab concentration and type, results are presented as means of the used concentrations (0.1 / 1 / 10 µg ml^{-1}) or in a separate bar as means of concentration and Abs (CD85j, CD159a, IgG2b). For MOLP-8, RPMI8226 and HL60, a significant influence of $F(ab´)_2$ towards a decreased lysis seemed relevant, but as said before, could not be taken into account. Only experiments with K562 were performed on the same day with and without additional $F(ab´)_2$ treatment. Their equal outcome confirms the thesis of culture side effects being responsible for significant changes in experimental results. All other tests were done with a delay of one to two weeks. As described above (Figure 13 - Expression of surface antigens), FACS analysis before and at the end of the assays did not reveal changes in surface molecules that were detected with a four week delay and included activation marker CD25.

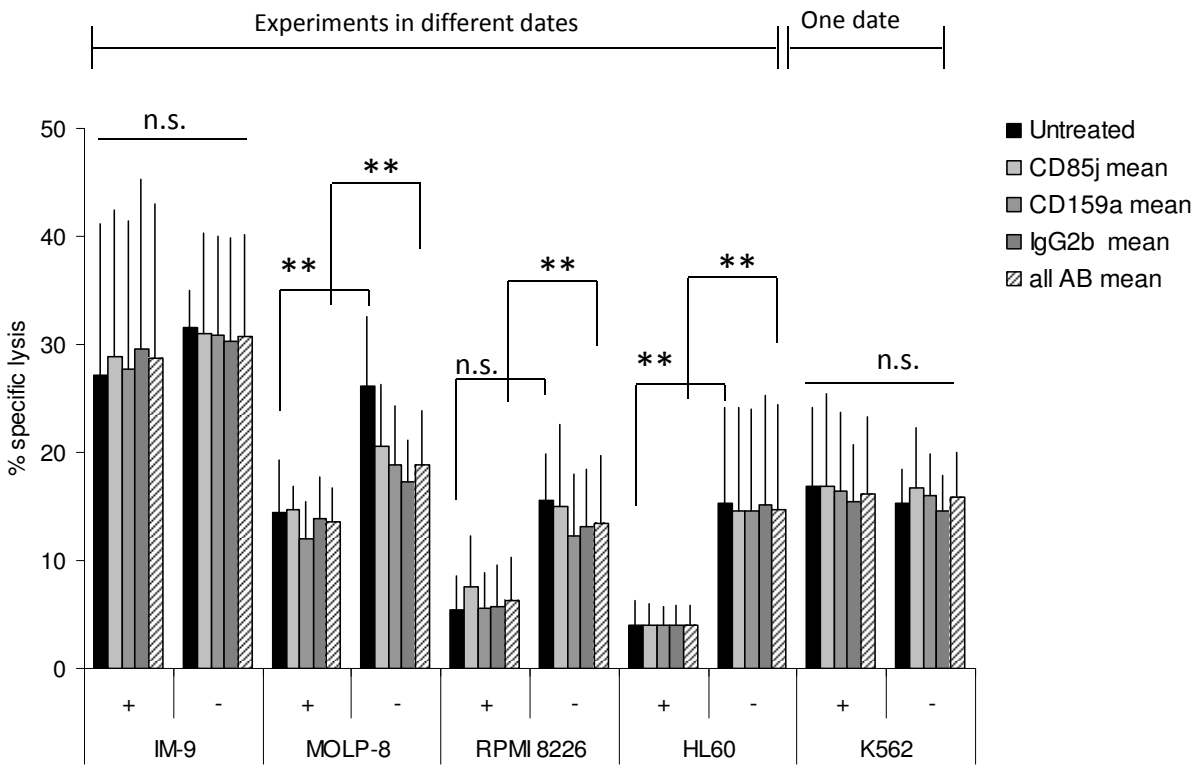


Figure 22 - False appearance of $F(ab')_2$ influence in blocking of NK-92 cells

Statistical analysis (ANOVA) reveals a seemingly protective effect by $F(ab')_2$ fragments against NK-92 mediated lysis. As influence of the type of antibody and the used concentration were excluded, experimental data were merged.

Results were highly significant (untreated ± $F(ab')_2$ / Ab use ± $F(ab')_2$):

IM-9 p=0.317 / p=0.211; MOLP-8 p=0.017 / p=0.002; RPMI8226 p=0.052 / p<0.001; HL60 p=0.009 / p<0.001; K562 p=0.692 / p=0.771.

The same settings were used throughout all experiments. In the experimental row, K652 tests were performed at the same day and within the same panel. For all other target cells, tests including $F(ab')_2$ were performed first and were followed by those without additional treatment 1 week later. Though IM-9 renders more susceptible to lysis to a non-significant degree, time is the factor most likely to be taken into account, for targets incubated with 'untreated' NK cells showed the same changes in sensitivity towards lysis.

4.2.2 Blocking of neither LIR-1 nor HLA-I on target cells increases target cell lysis

According to the experiments done before, antibodies were used to block LIR-1 receptors in the tumor cells. Unlike experiments, where anti-HLA-I Abs are commonly used as a positive control to reach a maximum value for NK cell mediated lysis, in this setting no alteration of lysis was expected, since blocking of LIR-1 as the only relevant inhibitory NK cell receptor on NK-92 had already not shown any influence in tumor cell lysis. Neither blocking of LIR-1 nor HLA-A,-B,-C,-E and -G showed any influence on tumor cell lysis (Figure 23).

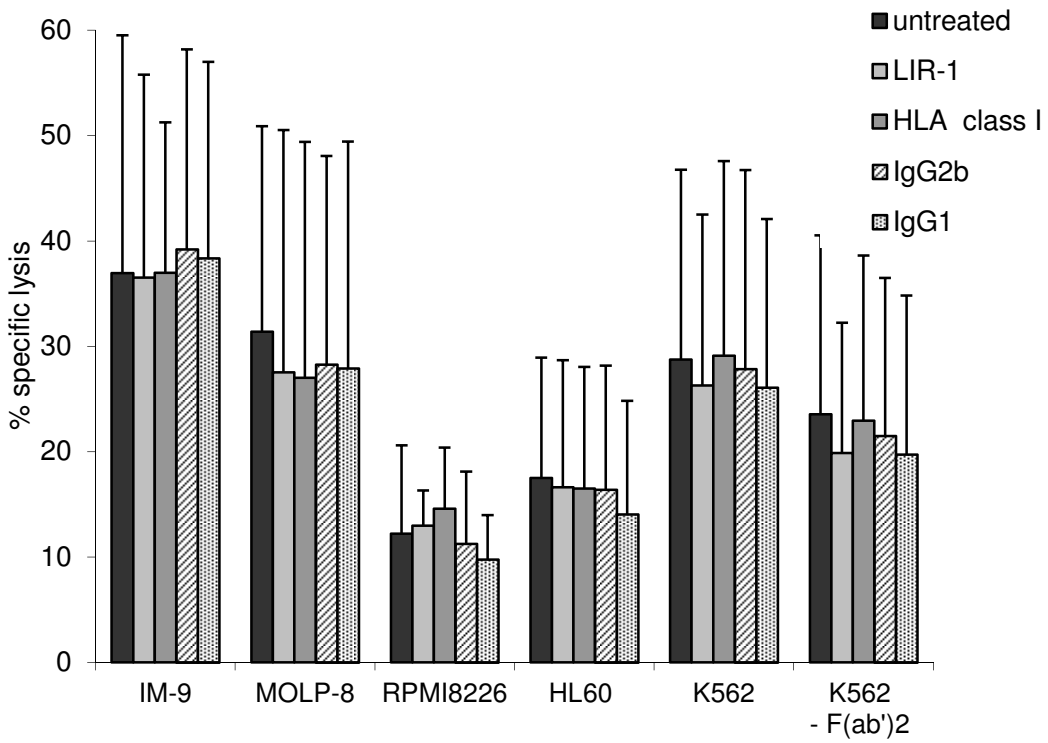


Figure 23 - Blocking of LIR-1 and HLA on tumor cells does not lead to increased lysis by NK-92 cells.

LIR-1 (CD85j) and HLA class I molecules of the tumor cell lines were blocked as before on the NK-92 cells. After incubation and extended washing, standard CRA were performed.

Neither LIR-1 nor HLA blocking showed no significant increase of tumor cell lysis. All experiments were performed with the use of $F(ab')_2$ to prevent ADCC. Statistical analysis performed by ANOVA excluded any significant effect of either isotype or Ab on target cell lysis, compared to untreated samples. Discrete investigation of K562 treatment, performed as a comparison of cumulated values for all Ab-incubated samples with or without $F(ab')_2$, excluded any influence of additional $F(ab')_2$ on the results ($p= 0.218$).

4.3 Evaluation of reagent-based side effects

4.3.1 Ab or F(ab')$_2$ have no toxic effects on NK-92 under experimental conditions

To rule out toxic effects of blocking Abs to NK-92 under experimental conditions, NK-92 were labelled with 51Cr and incubated with the respective Ab in parallel to the conducted experiments. In four independent experiments, each assessed as a means of triplicates, no harmful effect of anti-LIR-1, anti–NKG2A or F(ab')$_2$ could be observed (Figure 24 - Neither antibodies nor F(ab')$_2$ show toxic effects on NK-92 cells).

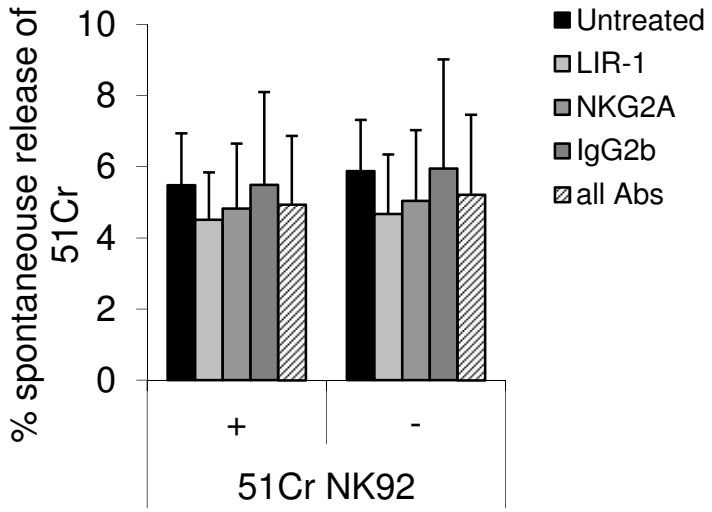


Figure 24 - Neither antibodies nor F(ab')$_2$ show toxic effects on NK-92 cells
NK-92 were labeled with 51Cr and incubated with Abs against LIR-1, NKG2A or IgG2b. Isotype control with (+) or without (-) F(ab')$_2$ under the same conditions as the unlabeled effector cells in the blocking experiments. SR is given as mean of four independent experiments for untreated NK cells and as mean of an experimental series with concentration of 10, 1 and 0.1 µg/ml. 'All Ab mean' integrates those results. F(ab')$_2$ concentration was 11 µ/ml. Spontaneous release was <6%. As this control panel was performed in parallel to regular NK-92 blocking experiments, interferences by reagents, temperature, incubation period, cell viability or technical equipment can be excluded.

4.3.2 F(ab`)$_2$ might balance tumor lysis pattern

Nevertheless, there is an influence of the F(ab')$_2$ fragments, for they seem to stabilize the results. The findings did not reach the level of significance but should be regarded relevant. Usually no prevention from ADCC is included in assays that only block NK-cell receptors. Figure 25 (B) represents the results of such a standard assay compared to (A) in which this prevention is included. Apart from outliers, use of F(ab`)$_2$ seems to even out Ab effects in the blocking experiments, leading to values that oscillate close to the origin in both directions (A). Sparing those fragments decreases relative lysis, thus exhibiting a protective effect to the targets that is predominant in MOLP-8 and RPMI8226 cells. Only K562 cells render more susceptible to NK mediated lysis by usage of F(ab`)$_2$ though not at a significant level (B).

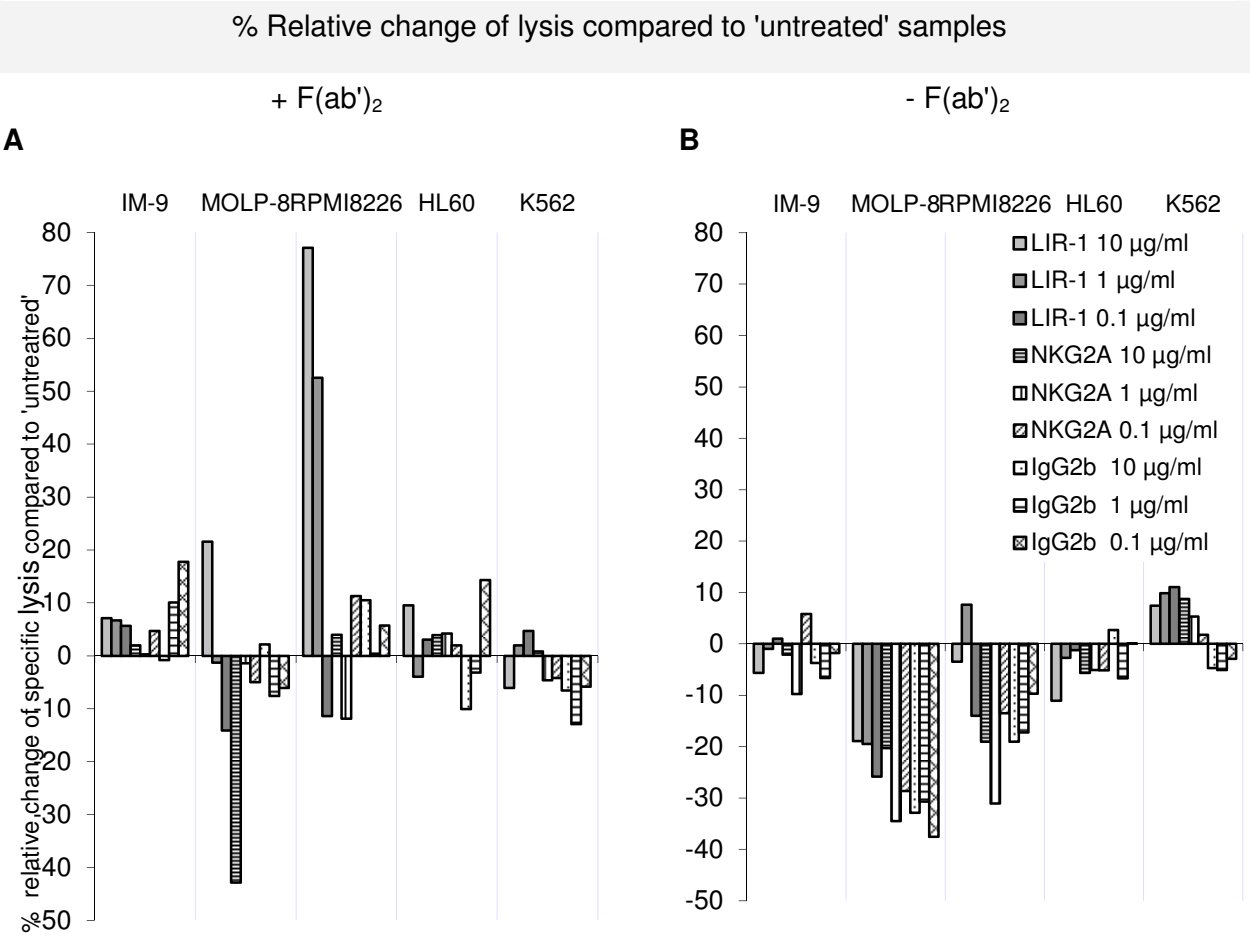


Figure 25 - Use of F(ab`)$_2$ after blocking NK-92 receptors seems to balance lysis pattern

Values show the % relative increase or decrease of target cells lysis in a standard 4-hour CRA, calculated as follows:

% relative change of specific lysis = [(%specific lysis 'antibody`) - (%specific lysis 'untreated`)] / [%specific lysis 'untreated'].

Though no concentration of the used mAbs showed a significant change in tumor lysis, interesting differences in the resulting lysis-patterns could be detected.

(A) Apart from outliers, use of F(ab`)$_2$ evens out mAb effects in the blocking experiments, leading to values that oscillate close to the origin in both directions.

(B) Sparing F(ab`)$_2$ decreases specific lysis, predominantly for MOLP-8 and RPMI8226 cells.

5 DISCUSSION

5.1 Evaluation of the present experiments

5.1.1 Characteristics of the experimental setup

The aim of the present investigations was to evaluate, if LIR-1 works as an inhibitory receptor concerning NK-92 mediated cytotoxicity against different tumor cell lines, especially MM. Secondly, presence of LIR-1 on the tumor cells was to be evaluated regarding its influence on immune escape. For this reason, standard 4h CRA were performed under various conditions, modulating the receptor interactions with their respective ligands. Hereby, influence of NKG2A as the second predominant inhibitory NK cell receptor on NK-92 was ruled out by a parallel series of CRA.
To my knowledge, these experiments provide the first data concerning the influence of isolated LIR-1 inhibition on NK cells towards an alteration of MM lysis. Additionally, the experiments revealed a considerably high expression of the receptor on MM and IM-9 cell lines (Figure 14 - LIR-1 expression on NK-92 and target cells). For the first time, LIR-1 was not only modulated on the effector cells, but was rather treated with blocking agents to specifically aim the tumor surface.

NK-92 cells are commonly considered as appropriate NK cell representatives (Gong et al., 1994). They are positive for LIR-1 and NKG2A, but express no KIR molecules except from KIR2DL4 (Maki et al., 2001; Davidson et al., 2010). Inhibitory influence of KIR2DL4 on the results was taken as negligible. It has structural characteristics and functions of inhibitory as well as activating NK cell receptors, but physiological stimulation always leads to activation, that might be triggered by soluble HLA-G, which also lacked on the target cells (Cantoni et al., 1998; Ponte et al., 1999; Rajagopalan, 1999; Faure and Long, 2002).
Thus, inhibition of NK-92 mediated lysis could be expected mainly from the other two dominant NK cell receptors NKG2A and its splice variant –B, as well as the LIR family, of which LIR-1 is the receptor with the brightest distribution among human tissues (Borges et al., 1997; Cosman et al., 1997; Samaridis and Colonna, 1997; Godal et al., 2010). Taking this into account, MM cells and additional control cell lines were chosen, that did or did not express LIR-1 on their surface.
Target cell lines were mainly negative for HLA-E, the so far only known ligand for NKG2A, so that NK-92 could virtually be considered NKG2A negative despite their surface expression of this receptor (Braud et al., 1998; Lee et al., 1998). FACS analysis showed vanishingly low HLA-E staining IM-9. Therefore and as a negative

control, blocking experiments also included the inhibition of NKG2A interactions. As expected, no influence of NKG2A in the present setting could be detected.

5.1.2 LIR-1 does not influence cytotoxicity

LIR-1 interaction was investigated by selectively blocking either the receptor on the effector or on the target cell side. No enhancement of tumor cell lysis was detected in either of both cases. This finding was confirmed in at least three experiments for each side of interaction that was treated with blocking antibodies. Experiments were repeated with or without the application of reagents that were supposed to prevent any possible ADCC, even though no expression of FcγR is described for NK-92 (Maki et al., 2001). Those reagents did not exhibit any effect on lysis, but seemed to even out variations in lysis pattern, possibly due to diminishing impacts on the antibodies` binding capacities. Blockade of all HLA-I molecules on the target cell side did also not enhance lysis. If LIR-1 is the only relevant inhibitory receptor in this experimental setting as stated above, and the blocking of LIR-1 did not alter cytotoxicity, no other result would have been expected for HLA-I blockade.

5.1.3 Lack of LIR-1 mediated inhibitory influence is in consistency with available data

By now, involvement of LIR-1 in protecting the fetus from abortion has become common immunological knowledge, and so is the adoption of this mechanism by tumor cells by expressing the LIR-1 ligand HLA-G (Paul et al., 1998; Ponte et al., 1999; Sheu and Shih, 2010). Furthermore, viruses express highly affinitive LIR-1 ligands for protection against immune defense, but LIR-1 serves as an effective receptor for bacterial detection (Cosman et al., 1997; Nakayama et al., 2007).

Data about the specific role of this receptor in other contexts are rare and more conflicting. While LIR-1 inhibition of T-cell and monocyte activation has been shown before and will not further be discussed, only a few studies focus on NK-cell modulation, and even less refer to experimental settings in which HLA-G was not present on the target cell (Fanger et al., 1998; Saverino et al., 2000; Dietrich et al., 2001).

While the available publications emphasize data that confirm an important inhibitory effect of LIR-1, a more questioning view reveals quite a few dissonances in the underlying information. Those issues refer to the number of experiments mentioned that did not deliver the pronounced outcome, to the comparability of NK cell lines to donor derived NK cells (dNK) and to the status of outcomes with transfected cell lines:

Only one recent study used tumor cells as targets in cytotoxicity assays with NK cells in the investigated context, while all others derived their data out of experiments with HLA-transfected 721.221 (EBV transformed B cell line), murine cells (P815) or immature dendritic cells (iDC) (Godal et al., 2010).
Godal et al. (2010) made an attempt in revealing the influence of LIR-1 on dNK. Flow cytometry based blocking and degranulation assays with freshly isolated and HLA-G negative AML and ALL blasts revealed only slight effects of LIR-1 blockade towards an increase of lysis or degranulation of NK cells. Sole LIR-1 influence reached a significant increase of lysis only in the case of one AML sample before dividing those donor NK cells in KIR+ and KIR- subsets, but no increase afterwards. The other AML sample as well as the ALL samples did not render susceptible to lysis due to LIR-1 blockade. Similar but slightly better results were obtained for induced degranulation. In an investigation with NK cells, isolated 100 days after allogeneic SCT, in which NKG2A and LIR-1 were the predominant inhibitory NK cell receptors, degranulation after exposure to the targets could be increased by NKG2A blockade and to a much smaller extend by LIR-1 blockade. These findings would suggest that LIR-1 does only serve as a weak inhibitory NK cell receptor in the absence of HLA-G on tumor cells, but might be relevant in situations with low KIR expression as seen within the first months after SCT.

Other available publications used merely HLA-transfected 721.221 cells as targets to examine cytotoxicity, but often had the benefit of comparing results from NK cell lines to the performance of NK cells derived from healthy donors.
In 2008, Yawata et al. already conducted a series of degranulation assays of dNK against HLA-I deficient 721.221 in order to investigate the involvement of distinct receptors in 'missing-self' recognition. Also not being able to identify any involvement of LIR-1, their explanation for the findings based on the hypothesis, by which NK cells need to be licensed by their respective MHC-I ligands in order to later on recognize this missing-self. Another factor of influence was found in the weak binding of LIR-1 to the donors` own HLA-I pattern, as HLA-G with its stronger affinity to LIR-1 is not constitutionally expressed on tissues (Yawata et al., 2008). This hypothesis has lately been doubted, for NK cell licensing has been shown to not necessarily require exposure to MHC class I ligands during NK development in the bone marrow (Elliott et al., 2010). Furthermore, others have convincingly shown influence of HLA-G in tumor resistance, which could not occur if licensing would require presence of HLA-G or if high binding affinities (Riteau, 2001; Maki et al., 2008; Favier et al., 2010).

More successful LIR-1 mediated inhibition was achieved by Morel et al. (2008) who tested the cytotoxicity of dNK and NK-92 against 721.221-G and –B in chromium based assays with subsequent use of Abs. It is essential to mention, that their pre-studies found only 15% of LIR-1+ dNK to be stimulated by target contact and these responsive dNK were chosen for the subsequent studies. Also, comparative analysis of NK-92 and dNK in different types of assays were conflicting, for activated LIR-1 could only induce inhibition for either one of the two effector types in all scenarios tested (Morel and Bellón, 2008).

No such conflict was observed by Favier et al. (2009), who incubated dNK and NKL with 721.221-G1 and used blocking Abs against LIR-1 and HLA-G (Favier et al., 2010). The same was true for Vitale et a. (1999): They incubated dNK with different HLA transfected cell lines in standard CRA and increased lysis by LIR-1 blockade, but unfortunately, no explicit prevention of ADCC was mentioned, leaving the results vague to a certain extend (Vitale, 1999). At last, Colonna et al. (1997) showed the expected LIR-1 influence in different kinds of experimental setups with NKL, serotonin releasing RBL cells, as well as dNK and donor derived LIR+ T cells (dTK).

Interestingly, in all assays with either dTK or dNK against 721.221-B*2705, α-LIR-1 could only partially revert transfection induced inhibition, indicating either incomplete binding of α-LIR-1 to the receptor or the presence of other receptors apart from LIR-1 that link to the ligand. Moreover, in rADCC assays with dNK against P815, only few clones were inhibited by LIR-1 out of no known reason.

Here again, NK cell clones have shown to exhibit less predictable outcomes than cell lines. Colonna et al. assumed different LIR isoforms to be responsible for the outcome, of which not all are detected by the Ab, or that LIR-1 expression might be modulated during culture period, what has been ruled out for the present experiments (4.1.1 Expression of LIR-1 and NKG2A on NK-92 and tumor cells).

Summarizing the available data, NK cell lines seem to be more reliable than dNK in producing the expected results, when it comes to the evaluation of LIR-1 mediated downregulation of cytotoxicity or degranulation. The results for polyclonal dNK show high variances between the different clones that are mostly not characterized in detail. Successful LIR-1 mediated inhibition by HLA-transfected 721.221 has abundantly been shown, but HLA-B*2705 seems to be of higher influence than HLA–G1. Involvement of additional undetected receptors could not always be excluded, and different types of assays performed with the same effector and target cells could lead to highly diverging results. Investigations of cytotoxicity of dNK and donor derived tumor cells are rare and no sufficient information is so far available about the role of LIR-1 in the absence of

HLA-G on donor derived tumor cells or tumor cell lines. Current opinion about the inhibitory influence of LIR-1 is predominantly based on investigations at the feto-maternal interface or has been tried to be adopted to artificial situations in which only a single HLA molecule was present on the target cells- mostly HLA-G or –B on 721.221. The performed experimental settings do not sufficiently cover the extensive binding capacities of LIR-1 to HLA class I.

To my knowledge, so far no functional investigations about the role of LIR-1 on tumor cells have been performed apart from the present studies. As LIR-1 is a very common receptor throughout most human tissues, advanced knowledge about its functional impact needs to be acquired.

5.1.4 Pre-existing maximum NK cell mediated cytotoxicity is likely to conceal discrete inhibitory effects

An alternative explanation for the present results would state that LIR-1 influence could not be measured, for a maximum level of activation had already been achieved before blocking the inhibitory receptor. Former results of Navarro et al. (1999) point to that assumption (Navarro et al., 1999). While anti (α)-LIR and α-HLA-E totally restored cytotoxicity of the NK cell line NKL against 721.221 transfected with HLA-B*2705 (721.221-B*2705), the single effects of α-LIR-1 / α-NKG2A / α-HLA-E or α-HLA-G in trials against 721.221-G1 were hardly above the control level (721.221-G1 co-express NKG2A ligand HLA-E). Only additive use of the mentioned antibodies completely restored lysis. As the lysis pattern of dNK against 721.221-G1 could also not be explained by any influence of LIR-1, Navarro et al. proposed inhibitory KIR2DL4 to be the dominant influence in this scenario (Navarro et al., 1999).

Strong activation masking the inhibitory influence of LIR-1 has also been claimed responsible for a failure in visible effects of LIR-1 activation in rADCC assays by Morel et al. (2008), who investigated discrepancies in the NKp30 driven cytotoxicity of NK-92 and dNK (Morel and Bellón, 2008). This hypothesis is supported by the distinct phenotype of NK-92. Its expression profile is unusual, compared to NK cells, as it has only few inhibitory receptors (LIR-1, NKG2A and KIR2DL4), of which only LIR-1 exhibits its function within the present context. Moreover, the detected number of activating receptors on NK-92 is large (NKp30, NKp46, 2B4, NKG2D, -E, CD28) (Maki et al., 2001). NK-92 was maintained in culture medium with fresh supplementation of IL-2 the day before the assays, leading to a pre-activated condition. It has been shown before, that the high potential activating receptor NKp44 is induced by IL-2 (Vitale et al., 1998).

5.1.5 MM evasion mechanism might outweigh approaches of NK cell modulation

It is possible that successful resistance mechanisms of the tumor cell lines that could not be overcome by the conducted experimental modulations. Though NK cells have been described as being capable of inducing lysis or apoptosis in MM as well as lymphoblastoid cell line IM-9, this lysis never exceeds a certain level despite the application of sometimes very high E:T ratios that are required to overcome MM resistance (Davies, 2001; Zheng et al., 2002; Maki et al., 2008).

MM cells evade detection not only by maintenance of HLA class I expression, but furthermore by acquisition of diverse strategies of immune evasion: By adopting CD28, they promote their own growth and reinterpret infections of the host as further growth stimuli (Zhang et al., 1998; Jego et al., 2006). Far from contenting with a safe niche within the marrow, they adopt FasL in order to actively offend their assaulters (Nagata, 1996; Cao et al., 2010).

5.2 Functional aspects of LIR-1 according to previous publications

5.2.1 Modulation of immune functions

Though LIR-1 surface expression did not change during a four week culture period, it does increase during B cell maturation (4.1.1 Expression of LIR-1 and NKG2A on NK-92 and tumor cells) (Borges and Cosman, 2000). On CD4+ and CD8+ T cells, LIR-1 renders the cells susceptible to activation induced cell death, but after surviving, LIR-1 expansion has been proposed to proceed KIR expression before turning into memory T cells (Young et al., 2001; Vivier and Anfossi, 2004). Lately, NK cells have been found to acquire memory functions and it appears rewarding to investigate, if memory function in NK cells correlates with LIR-1 acquisition as found in B- and T cells (Sun et al., 2009).

DC have also been shown to upregulate LIR-1 during maturation. Furthermore, artificial LIR-1 ligation leads to a modulated DC phenotype and a lack of activity that could be reverted by blockade of CD80 or depletion of regulatory T cells. It has been proposed, that LIR-1 has a high impact on regulating the balance between activation and inhibition in immune responses (Young et al., 2008).

Significant increase of LIR-1 on PBMC has been observed within the context of HCMV infection after lung transplantation, a situation that would likely be suitable for the generation of immunological memory (Berg et al., 2003).

While HCMV infection induced LIR-1 expression on PBMC, downregulation was observed on NK cells by HIV infected monocyte-derived DC (Berg et al., 2003; Scott-Algara et al., 2008). Different than expected, this poor LIR- surface expression was

correlated with poor HIV control, whereas a high LIR-1 level led to effective lysis of the infected cells. Astonishingly, the interaction of LIR-1 with the target cells involved direct contact, but was not mediated via MHC-I molecules but via a so far unknown ligand (Scott-Algara et al., 2008).

These data suggest a more refined function of LIR-1 than initially suspected. Upregulation does not necessarily favor suppression of immune function but might correlate with acquisition of memory and even starting a coordinated defense, in which DC might play a key role. Unknown LIR-1 ligands as well as later discussed complex formations should be considered vital in understanding these new arising aspects.

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5.2.2 Extension of KIR binding range

LIR-1 is most likely co-expressed with KIR rather than with NKG2A on NK cells and even though there are increasing numbers of anti-KIR Abs available today, co-expression with KIR cannot always be totally exluded (Navarro et al., 1999; Vitale, 1999; Kirwan and Burshtyn, 2005; Godal et al., 2010). Kirwan et al. (2005) have therefore conducted experiments with KIR-transfected NK-92 to reveal further information about possible co-operation between KIR and LIR receptors. By transfecting truncated KIR2DL1 without ITIMs, they proofed the extracellular regions to be sufficient for inhibition of lysis. This inhibition was mediated via SHP-1, thus using the same pathway as other inhibitory NK cell receptors. LIR-1 interaction was found to be very likely to explain the results, as the W6/32 Ab (that selectively blocks LIR-1-HLA interactions) was found to restore lysis of previously truncated-KIR transfected NK cells. Co-operative signaling only occurred for truncated KIR2DL1, while the wildtype receptor did not recruit LIR-1. The conclusion driven by Kirwan et al. offers a sustainable thesis for at least parts of the inconsistency in the discussed findings. The authors proposed, that LIR-1 might co-operate with KIR to extend the functional range of its binding. Furthermore, LIR-1 expression might be kept at a threshold, that allows broad rather than specific receptor interaction, while co-expression with KIR might increase binding of LIR-1 to specific HLA molecules due to clustering of MHC-I by KIR (Kirwan and Burshtyn, 2005).

5.2.3 Co-ligation or complex formation might be required for efficient LIR-1 stimulation

The earlier mentioned recruiting of LIR-1 by truncated KIR2DL1 might hint at a general capability of LIR-1 to increase its potential via mechanisms of accumulation (Kirwan and Burshtyn, 2005). The best described LIR-1 ligand HLA-G has been shown to generate covalent dimers and trimers at the cell surface, a unique characteristic among

MHC-I molecules. It has been proposed, that these complexes increase the avidity towards LIR-1, explaining the discrepancy between the relatively low affinity of LIR-1 to its ligand and the high relevance within the context of fetal tolerance (Gonen-Gross et al., 2003).

Vice versa, the extremely high avidity of the HCMV expressed MHC-I homolog UL18 to LIR-1 might lead to a recruitment of additional LIR-1 molecules in a specific area of the cell surface, resulting in a complicated pattern of interaction rather than a distinct one-to-one relation (Chapman et al., 2000). In experimental settings, cross-linking of LIR-1 is also required for activating the receptor (Saverino et al., 2000; Morel and Bellón, 2008).Conclusively, homo- or heterotypic complex forming of the LIR-1 receptor rather than just an increase of avidity by ligand-complexes might be the key factor that regulates the degree of NK cell inhibition. By this, LIR-1 might work as a rheostat of NK cell activation. This hypothesis would perfectly fit the unique capability of LIR-1 to sense the overall HLA-I expression on human tissues by its broad spectrum of ligands. An increase during aging as well as memory acquisition would elevate the necessary threshold for activation during a parallel process of increase of activating NK cell receptors caused by repeated pathogen contact.

Thus, an effective strategy of NK cell evasion by tumor cells would lead to the expression of LIR-1 recruiting complexes. It would be ineligible for a healthy organism to form those complexes, since the balanced NK cell receptor pattern detects minimal deviations from normality. While the fetus is highly dependent on immune evasion, a balanced sorting of aged or transformed cells is vital for maintaining homeostasis.

In the present experiments, LIR-1 has been the only functional inhibitory NK cell receptor, and no HLA-G expression was found on the targets. Conceivably, these circumstances prevented initial activation of the receptor, leading to the observed unresponsiveness to blockade.

5.3 Methodical aspects

5.3.1 System complexity

NK cells do abundantly interact with especially T cells and DC (chapter 2.3.4). Their regulation is far from being understood, but involves innate as well as adoptive features. In vitro assays can therefore only provide a first clue that needs to be verified in clinical studies. So far, NK cell assays usually do not integrate DC within cytotoxicity assays against tumor cells due to matters of complexity. Due to unsuccessful experiments in trying to provoke NK cell degranulation, Bryceson et al. (2009) already discussed whether attempts to overcome complexity were the right way to gain new insights (Bryceson et al., 2009).

NK-92 is a widely used reference cell line for NK cell assays, and is considered to reliably reflect their functions. They were derived from a patient with rapidly progressive NK cell lymphoma whose PBMC contained LGL with multiple aneuploidies and structural rearrangements (Gong et al., 1994; Maki et al., 2001). The cells are known to express a rather untypical receptor pattern as they are positive for LIR-1 and NKG2A, but are negative for KIR and CD16 (Morel and Bellón, 2008). By this, they provide a system, in which the two main inhibitory receptors stand alone without any interaction with the broad pattern of KIR molecules. The common acceptance of NK-92 cells as NK cell representatives must therefore be taken with caution.

In 2008, Maki et al. were the first to test the influence of HLA-G in tumor resistance. As the major LIR-1 ligand, it was investigated in a setting with donor-derived NK cell as well as NK-92 cells. They targeted MM and CLL cells that were also either donor derived or in case of MM also represented by cell lines. About 70% of MM and CLL donor samples were primarily resistant to killing by donor NK cell in CRA. While MM lacked HLA-G surface expression, the prevalent HLA-G on CLL cells allowed increased lysis after HLA-G blockade. Though no direct conclusions about the influence of LIR-1 should be drawn, the study revealed interesting insights in the comparability between cell lines and donor cells. While MM was relatively resistant to NK-92 mediated lysis (maximum of 15% specific lysis), results with donor NK cells were even inferior. On the other hand, all MM cell lines as well as IM-9 were highly susceptible to NK-92 mediated lysis (Maki et al., 2008). These findings strongly suggest that for the field of NK cell studies, findings that are based on cell line studies might not be applicable to *in vivo* surroundings.

Another bias to donor derived cells became obvious after immunophenotyping the used MM cell lines. While HLA-G has been shown to be increased in patients with MM compared to healthy controls, in a recent study all investigated MM cell lines were HLA-G negative (Leleu et al., 2005). For future analysis, the present experiments should be repeated with a comparative setting of cell lines as well as donor derived cells as proposed before (Robertson and Ritz, 1990).

Although being aware of problems concerning the comparability of NK cell lines to donor derived NK cells (dNK), I have chosen an experimental setting that allows studying isolated influence of LIR-1 on cytotoxicity. The major goal was to provide a model system to overcome the common practice of using transfected target cell lines in cytotoxicity assays. Knowing the necessity of future efforts to confirm the present findings in a brighter panel of cells and cell lines, these results might provide a first step towards a new understanding of LIR-1.

5.3.2 Maintenance of antibodies within the culture medium

Due to the investigated expression of LIR-1 on effectors as well as target cells, careful elimination of a dual blockade had to be undertaken, in order to differentiate potential effects of increased lysis.

While it is common to use blocking Abs for modulating NK cell functions, they are usually maintained within the culture medium throughout the whole assay period (Saverino et al., 2000; Riteau, 2001). Here, maintenance of the Ab within the culture dish was not considered necessary to obtain reliable results. Within flowcytometric immunophenotyping of LIR-1, the same preincubation time with subsequent washing delivered bright and unequivocal staining, proving an appropriate binding capacity of the Ab.

5.4 Conclusions

Within the present study, no alteration of NK mediated cytotoxicity against MM was observed after blockade of LIR-1 on either the effector or the target cell side. Being the only functional inhibitory receptor within this setting, major known side effects by e.g. KIR have been ruled out. Considering the grave divergence between outcomes of investigated cell lines and donor derived cells, the actual findings are not assignable to in vivo situations. Nevertheless, this unexpected outcome opens the door to fruitful discussions about the complexity of LIR-1 interactions and its potential role within tumor defense. Together with NKG2A, it has been shown to be the major inhibitory influence in the early phase after SCT that hinders the emerging NK cell subset in fully expressing its graft-versus-leukemia effect (Nguyen et al., 2005; Godal et al., 2010).

5.5 Perspectives

Next step in revealing the influence of LIR-1 should be the comparative analysis of cell lines and donor derived NK and tumor cells regarding their performance in cytotoxicity assays. Besides CRA, rADCC with P815 as well as flow cytometry based assays should be included within the experimental panel to even out the known variations within the previous analyses.

According to the previous remarks about complex forming of LIR-1 or it´s ligands, artificial co-ligation with KIR by GAM antiserum or bimodal Abs might be a way to overcome resistance of donor derived MM cells towards NK mediated lysis. In another step, MM specific antigens would be an interesting target in developing new kinds of bimodal antibodies that induce complex-forming of LIR-1 on the NK cells (Atanackovic et al., 2010; Atanackovic et al., 2011). The present experiments did not reveal

information about the function of LIR-1 on the tumor cells that was detected for MM and IM-9 but not for HL60 or K562. It is likely, that in this case, LIR-1 is a residue of the healthy cell´s receptor pattern. Calcium mobilization assays could prove the functionality on the receptor on tumor cells. LIR-1 would then be likely to hinder their growth and therefore be a target for an activating Ab in palliative treatment or even a new parameter for evaluating chemosensitivity of tumor cells.
It is very likely, that the present presumptions about functions of LIR-1 within the immune regulations are far behind the real impact.

6 SUMMARY

Within the present work, all efforts made led to the rejection of the hypothesis of LIR-1 being a relevant inhibitory NK cell receptor in the context of MM immune evasion.
Experiments with well documented monoclonal antibodies were performed to neutralize specific receptor-ligand interactions of interest. Silencing LIR-1 influence on neither the effector nor the target side led to any significant increase in tumor cell lysis. As anticipated, blocking of NKG2A on NK-92 and HLA-I on target cells did not alter the results either and was therefore regarded as a valid experimental control among others that were carefully implemented to confirm the findings. To my knowledge, this has been the first approach to conduct a functional investigation of LIR-1 on NK cells with the objective to promote myeloma cell lysis.
Furthermore, the present work takes account of the broad distribution of LIR-1 among human tissues in a unique way. For this purpose, LIR-1 expression on target cells was investigated and revealed broad staining on MM as well as on lymphoblastoid cells, but could not be detected on AML and erythroleukemic cells. For the first time, LIR-1 was then selectively blocked on the side of the tumor cells to directly compare the receptors role on NK cells and tumor cells.
As discussed extensively, the rare and conflicting data about LIR-1 on NK cells suggest it to exhibit other important functions rather than directly influencing cytotoxicity. While some authors more or less confirm common presumptions about LIR-1 (Vitale, 1999; Favier et al., 2010; Godal et al., 2010), others put far more emphasize on the incoherent outcomes in different kinds of cytotoxicity assays and mention unsatisfactory results (Colonna et al., 1997; Morel and Bellón, 2008; Yawata et al., 2008).
Only in the exceptional involvement of NK cells at the maternal-fetal interface as well as in contact with HLA-G bearing tumor cells, the role of LIR-1 seems to be sufficiently documented and predictable to some extent. Most studies concerning LIR-1 on NK cells used transfected 721.221 cells as target cells to ‘rule out complexity’, and little

effort has so far been undertaken to adopt the results to a more sophisticated context. Most of all, investigations of cytotoxicity do mostly not involve donor derived cells, leading to an immense bias between the experimental settings and the natural activity of the aims of interest.(Robertson and Ritz, 1990; Maki et al., 2008). Although the present study cannot totally overcome this situation, it provides a first step towards this direction by a close evaluation of both sides of the cell-cell interaction.

Conclusively, homo- or heterotypic complex forming of the LIR-1 receptor rather than just an increase of avidity by ligand-complexes might be the key factor to regulate NK cell inhibition (Gonen-Gross et al., 2003; Kirwan and Burshtyn, 2005). By this, LIR-1 might work as a rheostat of NK cell activation as it has previously been suggested for dendritic cells (Young et al., 2008). This hypothesis would perfectly fit the unique capability of LIR-1 to sense the overall HLA-I expression on human tissues by its broad spectrum of ligands. An increase during aging as well as memory acquisition (Sun et al., 2009) would thereby increase the threshold for activation that might be a necessary process during a parallel increase of activating NK cell receptors that unfold their capacity in repeated pathogen contact. Still unknown ligands apart from MHC-I might play an important role within these interactions (Scott-Algara et al., 2008).

As a consequence, an effective strategy of NK cell evasion by tumor cells would lead to the expression of LIR-1 recruiting complexes. It would be ineligible for a healthy organism to form those complexes, since the balanced NK cell receptor pattern detects minimal deviations from normality. While the fetus is highly dependent on immune evasion, a balanced sorting of aged or transformed cells is vital for maintaining homeostasis.

It is very likely, that functions of LIR-1 are highly underestimated and unfold their meaning in rather unexpected ways.

7 ATTACHMENTS

7.1 Myeloma staging systems

Durie-Salmon Staging System for multiple myeloma

Stage	MM cell mass (cells x $10^{12}/m^2$ body surface	Criteria	Median survival (months) according to % tumor regression after initial response to treatment	
I*	<0.6	All of the following 4 criteria	≥75%	300
	(low)	1. Hemoglobin value > 10 g/ dl	<75%	120
		2. Serum calcium value normal (< 12mg/dl)	≥50%	191
		3. On roentgenogram, normal bone structure or solitary bone plasmocytoma only		
		4. Low M-component production rates		
		a. IgG value < 5 g/100 ml		
		b. IgA value < 3 g/100 ml		
		c. Urine light chain M-component on electrophoresis < 4 g/24 hours		
II	0.6-1.2	Not stage I or III	≥75%	79
	(intermediate)		<75%	63
			≥50%	67
			<50%	37
III	>1.2	One or more of the following	≥75%	120
	(high)	1. Hemoglobin value <8.5 g/100 ml	<75%	15
		2. Serum calcium value > 12 mg/dl	≥50%	66
		3. Advanced lytic bone lesions	<50%	10
		4. High M-component production rates		
		a. IgG value >7 g/dl		
		b. IgA value > 5 g/dl		
		c. Urine light chain M-component on electrophoresis >12g/24 hours		
Subclassification		A = Relatively normal renal function (serum creatinine value <2.0mg/dl)		
		B = Abnormal renal function (serum creatinine value ≥2.0mg/dl)		

Outcome based on serum creatinine levels

Stage	Median Survival (months)
I A	61
II A, B	55
III A	30
III B	15

Table 14 - Durie-Salomon Staging for multiple myeloma

Data inclusively the median survival were taken from the original publication of Durie and Salmon (1975) and completed with outcome according to serum creatinine level (Durie and Salmon, 1975; Fauci and Harrison, 2008). New approaches in therapy were not taken into account.

*In low cell mass category, ≥ 75% tumor regression includes all cases with disappearance of M-component from serum and/or urine. There were no patients with < 50% tumor regression.

International Staging System for multiple myeloma

Stage	Criteria	Median Survival (months)
I	Serum $ß_2$-microglobulin < 3.5 mg/L Serum albumin ≥ 3.5 g/dL	62
II	Not stage I or III: serum ß2-microglobulin < 3.5 mg/L serum albumin < 3.5 g/dL; or serum ß2-microglobulin 3.5 to < 5.5 mg/L irrespective of the serum albumin level.	44
III	Serum $ß_2$-microglobulin ≥ 5.5 mg/L	29

Table 15 - International Staging System for multiple myeloma
ISS staging system as published in 2005 (Greipp et al., 2005).

7.2 Outcome after autologous stem cell transplantation

Outcome of MM patients after autologous stem cell transplantation

Outcome	DSS % (95% CI)	ISS % (95% CI)	P-value
Non-relapse mortality at 1 year			
Stage I	2 (0–8)	4 (2–6)	0.49
Stage II	3 (1–6)	6 (4–10)	0.07
Stage III	6 (4–8)	3 (1–6)	0.11
Progression-free survival			
Stage I			
3 years	60 (46–74)	46 (40–53)	0.09
5 years	47 (32–62)	31 (24–37)	0.05
Stage II			
3 years	45 (39–52)	42 (36–47)	0.42
5 years	29 (22–36)	24 (18–30)	0.33
Stage III			
3 years	37 (33–42)	33 (26–41)	0.39
5 years	20 (15–24)	18 (11–25)	0.62
Overall survival			
Stage I			
3 years	89 (79–96)	73 (67–78)	0.003
5 years	81 (68–92)	55 (48–62)	<0.001
Stage II			
3 years	72 (66–78)	70 (64–75)	0.55
5 years	59 (51–66)	56 (49–62)	0.55
Stage III			
3 years	64 (59–68)	59 (51–66)	0.25
5 years	44 (39–50)	39 (31–48)	0.33

Table 16 - Outcome of MM patients after autologous stem cell transplantation
Comparison of outcome according to DSS and ISS classification of 729 patients with MM after ASCT according to Hari et al. (2009). Patients received ASCT within 12 months after diagnosis and were not pre-treated with lenalidomide nor bortezomib. Conditioning regimes were melphalan or busulfan and cyclophosphamide-based. After statistical analysis, concordance between DSS and ISS was low (p=0,085; 95% CI, 0.043-0.126). Only 36% of the patients were classified within the same stages of both systems. The difference in overall survival of the patients in ISS stage I to stage II is not significant. While ISS delivers easily reproducible and achievable data, the DSS allows distinction between MGUS, smoldering myeloma and MM and low DSS stage better discriminated the cohort with better prognosis. Higher stages in both systems predicted treatment failure (Hari et al., 2009; Kyle and Rajkumar, 2009).

7.3 NK-cell receptors

Activating Receptor	Ligand	Inhibitory Receptor	Ligand
Immunoglobulin like superfamily			
KIR2DL4 (CD158d)	HLA-G (soluble)	KIR2DL1 (CD158a)	Group 2 HLA-C Asn77Lys80
KIR2DS1 (CD158h)	Group 2 HLA-CAsn77 Lys80	KIR2DL2 (CD158b1)	HLA-C Ser77Asn80
KIR2DS2 (CD158j)	Group 1 HLA-CSer 77 Asn80	KIR2DL3 (CD158b2, CD158c)	Group 1 HLA-C Ser77Asn80
KIR2DS3	unknown	KIR2DL5	HLA-C
KIR2DS4 (KIR2DS4del/ins) (CD185i)	unknown	KIR2DL5A	unknown
KIR2DS5 (CD158g)	unknown	KIR2DL5B	unknown
KIR2DS6 (KIR3DP1, KIR3DS2P, CD158c)	unknown	KIR2DL6 (KIR2DP1)	unknown
KIR3DS1 (CD158e2)	unknown	KIR3DL1 (CD158e1)	HLA-Bw4
		KIR3DL2 (CD158k)	HLA-A3, -A11
		KIR3DL3 (CD158z)	unknown
		KIR3DL7	unknown
LIR-6 (LILRA1, CD85i)	HLA-B	LIR-1 (LILRB1, ILT-2, CD85j)	HLA-A,-B,-C,-E,-F,-G, UL18
CD85f (ILT11)	unknown	*LIR-2 (LILRB2, ILT-4, CD85d)*	HLA-A,-B,-C,-E,-F,-G
CD85g (LILRA4, ILT7)	unknown	LIR-3 (LILRB3, ILT-5, CD85a)	unknown
LIR-7 (LILRA2, ILT-1, CD85h)	unknown	LIR-4 (LILRA3, ILT-6, CD85e)	unknown
CD85b (ILT8)	unknown	LIR-5 (LILRB4, ILT-3, CD85k)	unknown
CD85L (ILT-9)	unknown	LIR-8 (LILRB5, ------, CD85c)	unknown
CD85M (ILT-10)	unknown		
C-lectin superfamily			
CD94/NKG2C (CD94/CD159c)	HLA-E	CD94/NKG2A,B (CD94/CD159a)	HLA-E
NKG2D (CD314)	MIC-A, MIC-B, ULBP1-6		
NKG2E/H	unknown		
NCR			
NKp30 (CD337)	BAT3, B7-H6		
NKp46 (CD335), NKp44 (CD336)	viral hemagglutinins		
NKp65	KACL		
Others / Cofactors			
Cytokine / monokine / chemokine receptors			
Toll-like receptors			

Table 17 - NK cell receptors

Classification of major activating and inhibitory receptors on NK cells. Letters printed in italics show LIR that belong to the class of receptors but are not expressed on NK cells (Colonna et al., 1997; Samaridis and Colonna, 1997; Fehniger et al., 1999; Vivier and Anfossi, 2004; Lanier, 2005; Farag and Caligiuri, 2006; Fuchs and Colonna, 2006; Guo and Qian, 2010; Orr and Lanier, 2010).

8 ABBREVIATIONS

721.221	EBV transformed B cell line
51Cr	sodium chromate (IV)
α	Anti
Ab	Antibody
Abs	Antibodies
ACTB	β-Actin
ADCC	Antibody-dependent cytotoxicity
AL	Amyloid light chain
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ANOVA	Analysis of variants
ATCC	American Type Culture Collection
BCG	Mycobacterium bovis bacillus Calmette-Guérin
BLCL	Lymphoblastoid B cell line
BM	Bone marrow
CCR7	CC chemokine receptor 7
CD	Cluster of differentiation
CD3ζ	CD3 ζ-chain
CMV	Cytomegalovirus
CpG	CpG oligodesoxynucleotides (oligo-DNA with CG palindroms)
CRA	Chromium-release-assay
DAP12	DNAX activating protein of 12 kDa
dNK	Donor-derived NK cells
DSS	Durie-Salmon Staging
dTK	Donor-derived LIR-1 positive T cells
EBV	Epstein-Barr-virus
E:T	Effector : target
FACS	Fluorescence-activated cell sorter
FasL	Fas ligand
FcεRIγ	Gamma chains of high-affinity receptor for the Fc region of immunoglobulin E
FcR	Fc receptor
FCS	Fetal calf serum
FGFR3	Fibroblast growth factor receptor 3
GM-CSF	Granulocyte macrophage colony-stimulating factor
GVM	Graft-versus-myeloma
GVL	Graft-versus-leukemia
GVHD	Graft-versus-host disease
HLA	Human leukocyte antigen
HMCL	human myeloma cell lines

iDC	Immature dendritic cells
IFN	Interferon
Ig	Immunoglobulin
IgSF	Immunoglobulin-like superfamily
IL	Interleukin
ILT2	Immunoglobulin like transcript 2
ImiDs	Immunomodulatory drugs
ISS	International Staging System
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KARAP	Killer cell activating receptor-associated protein
KIR	Killer immunoglobulin-like receptor
LCL	Lymphoblastoid cell line
LFA-1	Lymphocyte function-associated Ag 1
LILRB1	Leukocyte immunoglobulin-like receptor, subfamily B member 1
LIR	Leukocyte immunoglobulin-like receptor
LPS	Lipopolysaccharides
MAFB	Maf musculoaponeurotic fibrosarcoma oncogene homolog B
Mcl-1	myeloid cell leukemia-1 protein
MGUS	Monoclonal gammopathy of undetermined significance
MHC-I	Major histocompatibility class I
MICA / B	MHC class I chain related protein A / B
MIP1α	Macrophage inflammatory protein 1 α
MM	Multiple myeloma
MMSET	Multiple myeloma SET domain protein
mSMART	Mayo Stratification for Myeloma and Risk-adapted Therapy
NCR	Natural cytotoxicity receptors
NK	Natural killer
NKG2A	Natural killer cell lectin like receptor group 2A
NKL	NK cell line
P815	FcR+ murine cells
PAMP	Pathogen associated molecular pattern
PE	Pleural effusion
PPRs	Pattern-recognition receptors
PBMC	Peripheral blood mononuclear cells
PBSC	Peripheral blood stem cells
SCT	Stem cell transplantation
SHP	SH2-containing protein-tyrosine phosphatase
SHIP	SH2-containing inositol polyphosphate 5-phosphatase
SR	Spontaneous release
Syk	spleen tyrosine kinase

TAP	Transporter associated with antigen processing
TGF	Transforming growth factor
TLR	Toll like receptors
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cells
ZAP70	Zeta-chain-associated protein kinase 70kDa

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Main parts of this work have been published:

Heidenreich S, zu Eulenburg C, Hildebrandt Y, et al. Impact of the NK Cell Receptor LIR-1 (ILT-2/CD85j/LILRB1) on Cytotoxicity against Multiple Myeloma. Clinical and Developmental Immunology 2012;2012:13.

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Research Article

Impact of the NK Cell Receptor LIR-1 (ILT-2/CD85j/LILRB1) on Cytotoxicity against Multiple Myeloma

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The role of different receptors in natural-killer- (NK-) cell-mediated cytotoxicity against multiple myeloma (MM) cells is unknown. We investigated if an enhancement of NK-cell-mediated cytotoxicity against MM could be reached by blocking of the inhibitory leukocyte immunoglobulin-like receptor 1 (LIR-1). Our investigations revealed high levels of LIR-1 expression not only on the NK cell line NK-92, but also on myeloma cells (MOLP-8, RPMI8226) as well as on a lymphoblastoid cell line (LBCL; IM-9). Subsequent cytotoxicity assays were designed to show the isolated effects of LIR-1 blocking on either the effector or the tumor side to rule out receptor-receptor interactions. Although NK-92 was shown to be capable of myeloma cell lysis, inhibition of LIR-1 on NK-92 did not enhance cytotoxicity. Targeting the receptor on MM and LBCL did not also alter NK-92- mediated lysis. We come to the conclusion that LIR-1 alone does not directly influence NK-cell-mediated cytotoxicity against myeloma. To our knowledge, this work provides the first investigation of the inhibitory capability of LIR-1 in NK-92-mediated cytotoxicity against MM and the first functional evaluation of LIR-1 on MM and LBCL.

1. Introduction

Understanding of NK cell function has undergone a long process since their identification in 1975 [1]. NK cells have initially been regarded as part of the innate immune system, not allowing any modulation of action with respect to their changing microenvironment. Their pattern of inhibitory and activating receptors was considered to be sufficient to adequately detect tumor cells by the lack of human leukocyte antigen (HLA) class I molecules. Those tumor cells were killed instantly and without any obvious need of coactivation by other cells of the immune system [2]. This unique feature among lymphocytes has now been understood to be only the basic function of response, which is completed by diverse interactions with especially dendritic cells (DC) and T cells [3]. NK cells do extensively communicate with their surroundings, and their still-not-fully-deciphered set of receptors detects changes in the normal surface pattern on all types of tissues.

NK cell receptors are functionally divided into activating and inhibitory receptors. Their main ligands are major histocompatibility complex I (MHC-I) molecules, while some of the receptors can directly recognize specific antigens on bacteria or damaged cells. Mainly three different subclasses of NK-cell receptors (NKRs) can be distinguished.

LIR and killer immunoglobulin-like receptors (KIRs) are type I transmembrane proteins of the immunoglobulin-like receptor superfamily (IgSF). Both recognize classical HLA class I molecules, while LIR can also interact with nonclassical HLA class I and bacteria with low binding affinities [2, 4–6]. The second group of natural cytotoxicity receptors (NCRs) also belongs to type I transmembrane proteins but has poorly defined ligands. Type II transmembrane proteins of the C-lectin type superfamily include natural killer cell

lectin-like receptor group 2 (NKG2) receptors that form heterodimers with CD94 [2].

LIRs are expressed on subsets of NK cells and T cells, as well as on monocytes, B cells, and DC, with the widest distribution for LIR-1 [7–10].

LIR-1 is an inhibitory receptor also known as immunoglobulin-like transcript 2 (ILT-2)/CD85j or leukocyte immunoglobulin-like receptor, subfamily B member 1 (LILRB1) [7]. It has first been detected in searching for the counterpart of UL18, a cytomegalovirus encoded HLA class I homolog that is expressed on infected cells [8, 11, 12].

MM is an incurable disease that is characterized by the clonal proliferation of terminally differentiated plasma cells [13, 14]. Stem cell transplantation (SCT) is so far the only option to achieve long time remission of the disease [15]. To improve the outcome of MM patients, approaches like immunomodulation and cellular therapy are under investigation. NK cells are an attractive candidate for immune therapy. They kill tumor cells without antigen-specific priming [2] and are the the predominant lymphocyte subset within the first 90 days after transplantation [16–19]. LIR-1 is one of the main inhibitory NK cell receptors in this early phase after SCT [10, 16, 20].

We therefore investigated the influence of LIR-1 on myeloma defeat. Hereby, we studied the effects of LIR-1 blocking of NK-92 as well as on a panel of tumor cell lines including MM. To our knowledge, these experiments provide the first data concerning the influence of isolated LIR-1 inhibition on NK cells with respect to myeloma cell lysis. Moreover, they provide the first functional study of LIR-1 on MM and on other tumor entities, taking into account its broad distribution among tissues.

2. Material and Methods

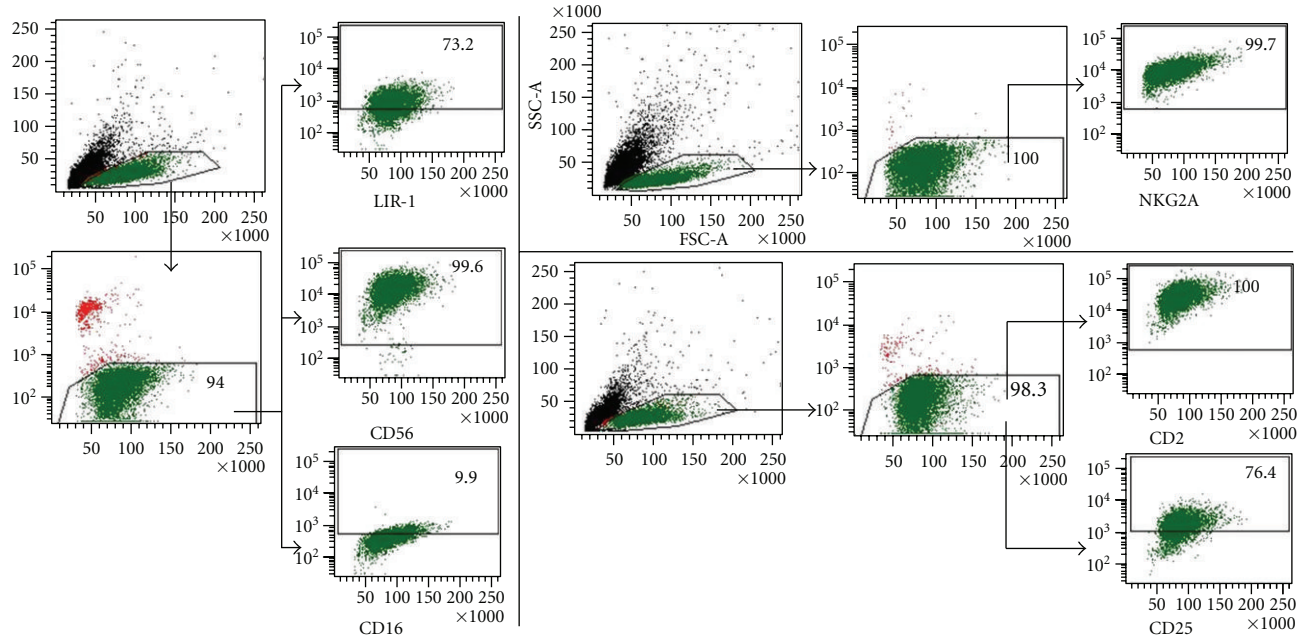
2.1. Cells. Unless otherwise stated, all media and supplements were obtained from Life Technologies. Natural killer cell line NK-92 was cultured in alpha-MEM supplemented with Earl's Salts and L-Glutamine, 12.5% equine serum, 12.5% fetal calf serum, 0.2 mM inositol (Sigma-Aldrich), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), 0.02 mM folic acid (Sigma-Aldrich), and 1% PenStrep. Cells were splitted every third day and received 200 U/mL rhIL-2 (CellSystems) with the fresh medium. Myeloma cell line MOLP-8 was cultured in RPMI1640 with 20% FCS and 1% PenStrep while IM-9, RPMI 8226, HL60, and K562 received the same medium and antibiotics but only 10% FCS. COS-7 cells were cultured in DMEM with 10% FCS and 1% PenStrep. JEG-3 was grown in Ham's F12 with 10% FCS and 1% PenStrep.

2.2. Flow Cytometry. Monoclonal antibodies (mAb) were phycoerythrin- (PE-) conjugated CD2 (RPA-2.10, BDP harmingen), CD159a (Z199, Beckman Coulter), CD85j (HP-F1, Beckman Coulter); Pacific Blue-conjugated CD16 (MOPC-21, BD Pharmingen); fluorescein isothiocyanate- (FITC-) conjugated CD25 (B1.49.9, Beckman Coulter) and anti-IgG (goat polyclonal anti-mouse IgG, Abcam); allophycocyanin (APC)-stained CD56 (B159, BD Pharmingen) as well as appropriate isotype controls. Unconjugated anti-HLA-I (HP-1F7) was obtained from Santa Cruz, anti-HLA-G (MEM-G/09) and -E (MEM-E/08) were obtained from Abcam. 7-Amino-Actinomycin D (7AAD, BD Pharmingen) was used to analyze dead cells. 20 μL Anti-A, B reagent was used in each sample to block unspecific bindings (Ortho-Clinical Diagnostics). Cells were incubated with the antibody or isotype control for 30 minutes at 4°C, washed with PBS and, if appropriate, stained with a secondary antibody followed by an additional washing step. All samples were additionally stained with 7AAD. Fluorescence was measured on a BD FACSCanto II flow cytometer and BD FACSDIVA Software v.6.1.3 (Becton Dickinson) was used for data analysis.

2.3. Transfection of COS-7 Cells with LIR-1. As a positive control for western blot experiments, COS-7 cells were transfected with pCMV6-AC vector encoding for LIR-1 (OriGene) or pCMV6-XL5 as a mock control (OriGene), using FuGene HD Transfection Reagent (Promega) [21, 22]. All steps were performed according to the manufacturer's instructions. The vector was multiplied by transformation of E.coli cells (One Shot TOP10/P3 competent cells) with subsequent purification of the plasmids (Qiagen EndoFree Plasmid Maxi Kit).

2.4. Western Blot Analysis. Western blot was used to analyze expressed surface molecules of effector and target cells [23, 24]. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Gels (Life Technologies) were blotted onto nitrocellulose membranes (Whatman). Membranes were blocked for one hour with TBS-T buffer (0.05 M Tris-HCL, 0.15 M sodium chloride, 0.1% Tween 20) containing 3% nonfat dry milk. Incubation with the anti-LIR-1 (VMP55, Santa Cruz) was done overnight at 4°C under gentle agitation. After extensive washing with TBS-T, secondary one-hour incubation with horseradish-peroxidase-conjugated goat anti-mouse IgG (R&D Systems) was completed with additional washing. Membranes were stained with enhanced chemiluminescence agent (GE Healthcare) and exposed to X-ray film (GE Healthcare). To confirm equal loading of all gel chambers, membranes were stripped from the specific antibody using Re-Blot solution (Millipore), followed by additional staining of β-Actin (ACTB, C4, Santa Cruz).

2.5. Cytotoxicity Assays. Cytolysis was determined in 4-hour chromium-release assays (CRAs) according to standard protocols [25–27]. Briefly, NK-92 and target cells were seeded out in fresh medium one day before functional assays. The next day target cells were labeled with 100 μCi sodium-51-chromate (51Cr) for 1.5 hours at 37°C in a humidified incubator with 5% CO_2. Two washing steps were performed with PBS (Life Technologies) and assay medium (RPMI1640, 10% FCS, 1% PenStrep; Life Technologies), respectively. Cells were resuspended to a dilution of 5×10^3 cells/100 μL. NK-92 and tumor cells were coincubated at various effector : target (E : T) ratios in U-bottom microtiter



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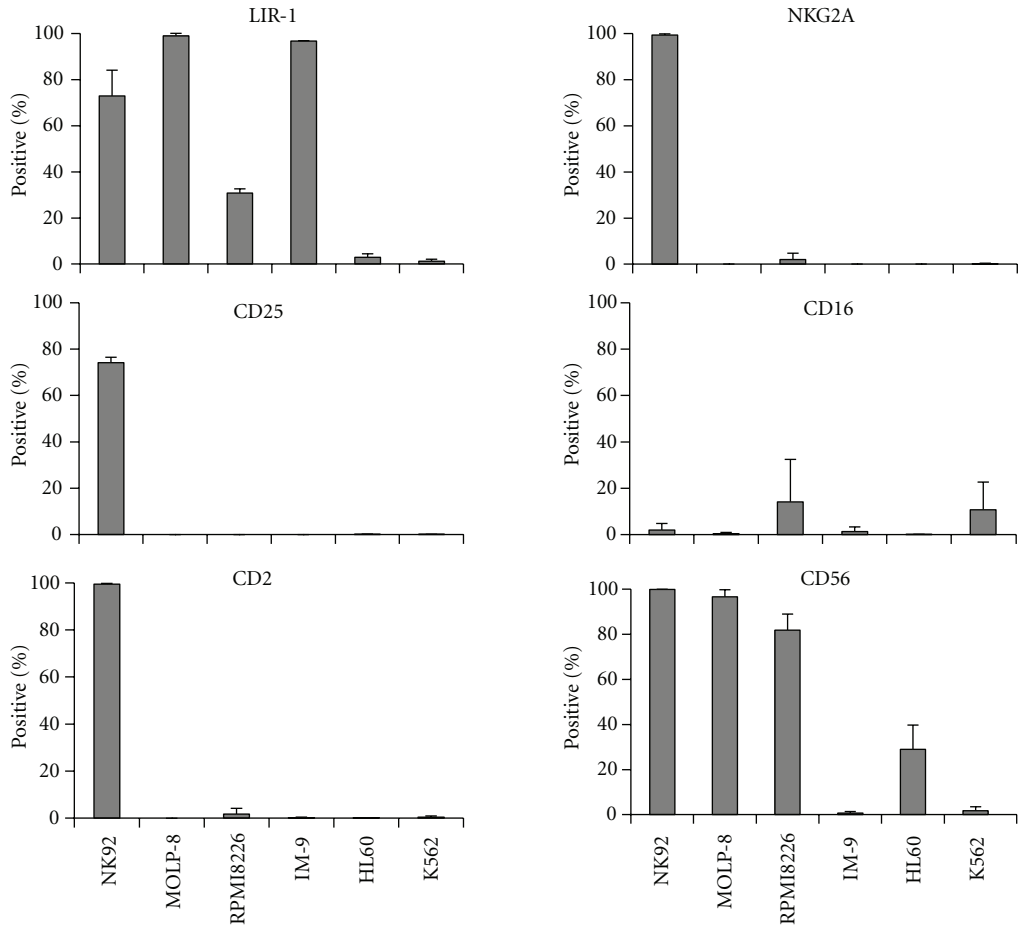


(b)

Figure 1: Expression of surface antigens. (a). Flow cytometric phenotyping of all cell lines was done with mABs against LIR-1, CD56, CD16, CNKG2A, CD2, and CD25 as shown for NK-92. (b). Surface antigen expression on NK-92 and tumor cell lines. Values given as % positive staining after subtraction of isotype control.

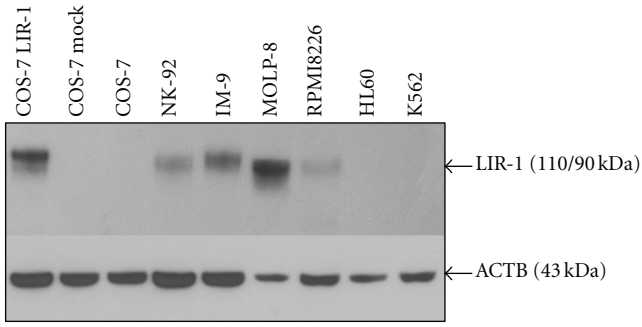


Figure 2: LIR-1 expression on NK-92 and target cells. Western blot analysis of LIR-1 expression on NK-92 cells and tumor cell lines. Untreated as well as LIR-1 or mock transfected COS-7 cells negative and positive controls, respectively. After film development, the membrane was stripped and reincubated with ACTB antibodies as a loading control. 4 μg anti-LIR-1 and 10 μg anti-ACTB (1 : 1000) were used for antigen detection. Lanes contended 35 μg of total protein. As also shown by flow cytometric analysis, NK-92 as well as IM-9 and MOLP-8 expresses high levels of LIR-1, whereas no detection of LIR-1 was possible on HL60 and K562 cells.

plates at a total of 200 μL assay medium. Maximum lysis or spontaneous release (SR) of 51Cr were induced by adding 5% Triton-X or assay medium to 100 μL target cells, respectively. All samples were plated out in triplicates. Prior to the 4-hour incubation period, plates were carefully centrifuged to facilitate E : T contact. After incubation and additional centrifugation, 25 μL supernatant were transferred to a 96-well plate (Isoplate 96, PerkinElmer). To each well, 150 μL scintillation liquid were added (Rotiszint eco plus, Carl Roth). Plates were closed with Viewseal foils (Greiner Bio-One). Suspension was mixed thoroughly for 15 minutes at 19°C on an Eppendorf thermomixer and then measured at Wallac Trilux 1450 Microbeta Counter, Windows WS V. 2.70.004, PerkinElmer). The percentage of specific lysis was calculated as follows:

$$\left[\frac{\text{c.p.m. experimental release} - \text{c.p.m. SR}}{\text{c.p.m. maximum release} - \text{c.p.m. SR}}\right] \times 100. \quad (1)$$

Results are shown as the mean of at least three independent experiments. In all experiments, SR was <20% [28].

2.6. Blocking Experiments. Blocking antibodies were anti-NKG2A (CD159a, IgG2b, Z199 BeckmanCoulter) anti-LIR-1 (CD85j, 292319, IgG2b, R&D Systems) and anti-HLA-I (HP-1-F7, IgG1, Santa Cruz), which blocks HLA-A, -B, -C, -E, and -G engagement [10, 29, 30]. IgG1 (11711) and IgG2b (20116, both from R&D Systems) were used as isotype controls. $F(ab')_2$ fragments (Jackson ImmunoResearch) were used to prevent ADCC [27, 30]. Controls without $F(ab')_2$ are explicitly named. Toxicity of any of the used reagents was carefully ruled out (Figure 8).

2.7. Blocking of Effector Cells. CRA were performed as described above. Heat inactivated human serum (HS) was obtained after informed consent from healthy volunteers. NK-92 cells were preincubated in RPMI1640 with 1% PenStrep and 10% HS for 30 minutes and kept within the same medium during additional 30 minutes of incubation with mAb concentrations of 0.1, 1 and 10 μg/mL, respectively. Target cells were prepared as described above. NK-92 cells were washed twice to avoid interactions of the mAb with the later on coincubated target cell line. Cells were adjusted for a fix E : T ratio of 1.25 : 1, using 5×10^3 target cells/100 μL as before. In some of the experiments, this step was followed by additional preincubation with 11 μg/mL $F(ab')_2$ 15 minutes prior to coincubation of NK and tumor cells. In samples classified as "untreated," no $F(ab')_2$ was used [30, 31]. SR was always <20% for all cell lines except from MOLP-8, which showed a constantly high SR up to 36% [28]. To rule out toxicity of the mAb, in four experiments NK-92 were radioactively labeled (51CrNK) and treated as the unlabeled cells in a parallel series to the blocking assays. 51CrNK showed SR < 6% for all conditions with a standard deviation (SD) < 3%. No differences related to parameters $F(ab')_2$, antibody or concentration could be detected within statistical analysis [30].

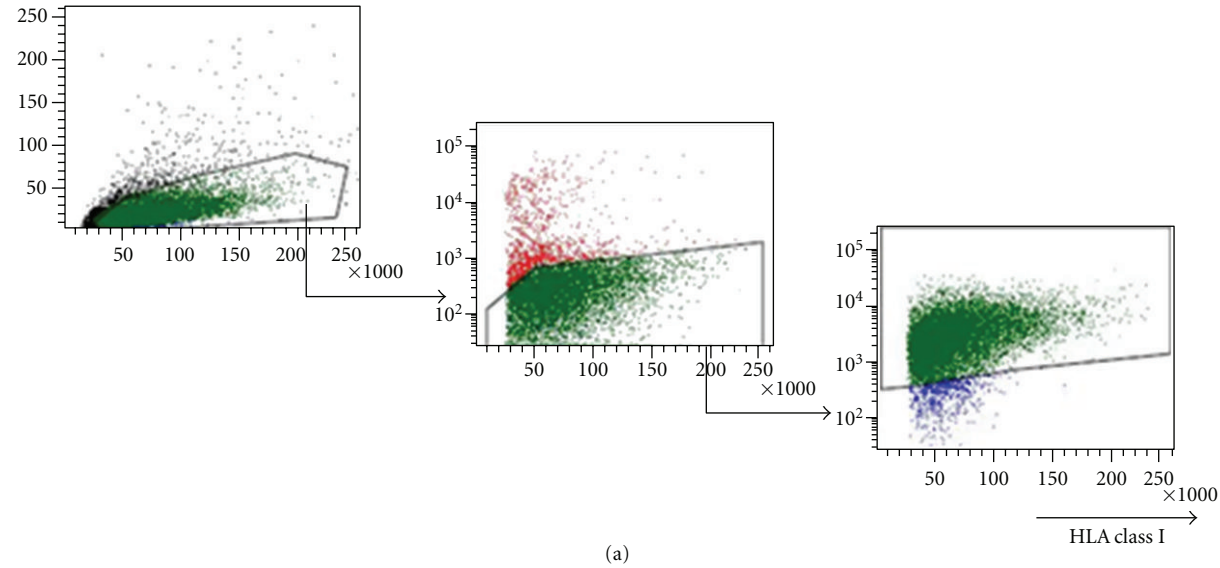
2.8. Blocking of Tumor Cells. Target cells were incubated with 1 μg/mL of the respective mAb at a cell density of 5×10^4 cells/mL. Procedure and incubation times were the same as described for NK cells. $F(ab')_2$ was used at a concentration of 1.7 μg/mL for all samples, inclusively the "untreated" control. Tumor cell incubation with mAb led to SR of <25% for MOLP-8 and RPMI8226 and up to 11% for all other cells.

2.9. Statistics. To control for indirect effects, statistical interpretation was done by multivariate Analysis of Variance (ANOVA). In all calculations, specific lysis was defined as the dependent variable. Antibodies, concentrations of the mAb, the targets and the use or no use of $F(ab')_2$ were defined as independent variables. Wherever appropriate, interdependencies between the variables were taken into account. All calculations were done by SPSS (IBM SPSS Statistics Version 19, Release 19.0.0).

3. Results

3.1. Characterization of NK-92 and Tumor Cell Lines. NK-92 was found to express high levels of LIR-1 based on flow cytometric analysis (Figure 1). We confirmed a high expression of NKG2A and CD25, as well as small amounts of FcγRIII (CD16) [10]. Hereby, but not regarding LIR-1 expression, NK-92 cells share important similarities with the $CD56^{bright}$ subset of NK cells [32].

LIR-1 was present on myeloma cells as well as on IM-9, but no target cell line expressed NKG2A. Western blot analysis confirmed the pattern of LIR-1 expression, and the strength of band representation reflected the staining intensity detected by flow cytometry (Figure 2). LIR-1 or mock-transfected as well as naïve COS-7 served as positive and negative controls, respectively. For evaluation of HLA class I expression, K562 served as negative controls. All cell lines except K562 were HLA class I positive which correlated with earlier investigations for MM (Figure 3) [13, 33]. LIR-1



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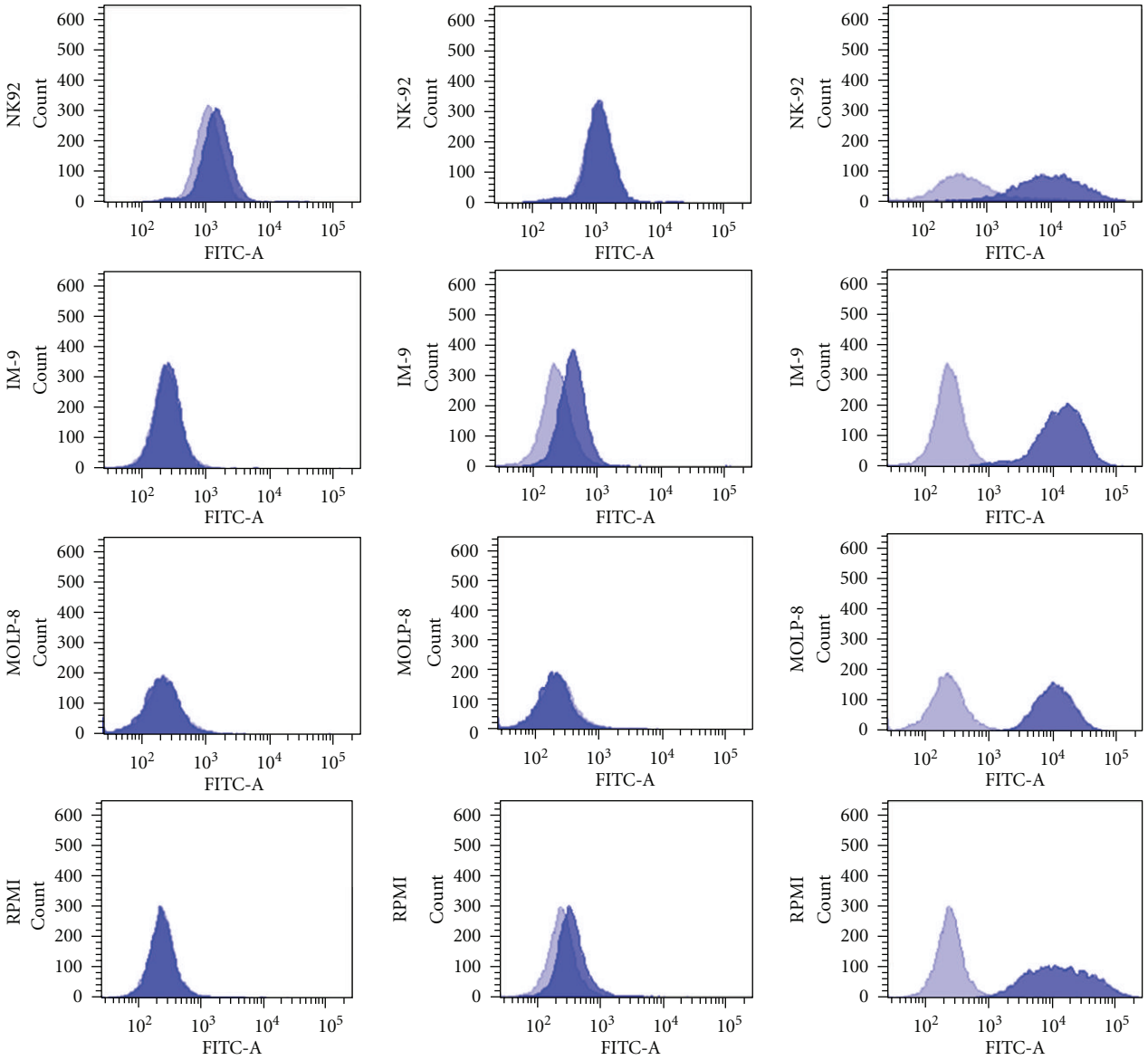
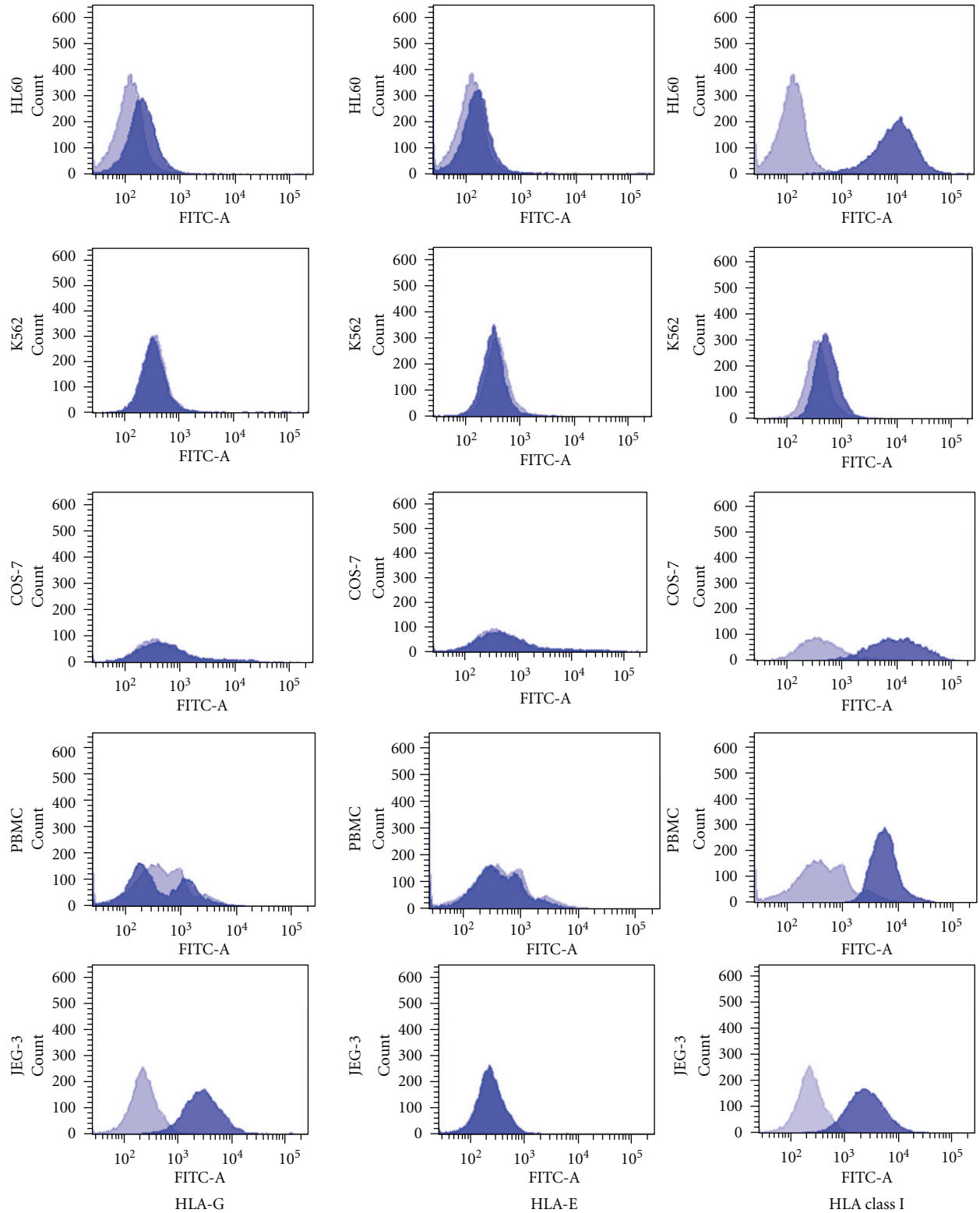


Figure 3: Continued.



(b)

Figure 3: HLA expression. (a). Representative flow cytometric gating strategy for analysis of HLA expression on all used cell lines (shown for RPMI8226). All cells were stained with anti-HLA-I/-E/-G IgG1, followed by secondary goat anti-mouse IgG1 FITC. (b). HLA class I molecules were detectable on all cell lines except for negative control K562. JEG-3 were strongly positive for HLA-6, while detection on NK92 and HL60 was marginal. HLA-E as the ligand for NK62A lacked on all cell linesexcept on minor staining on IM-9.

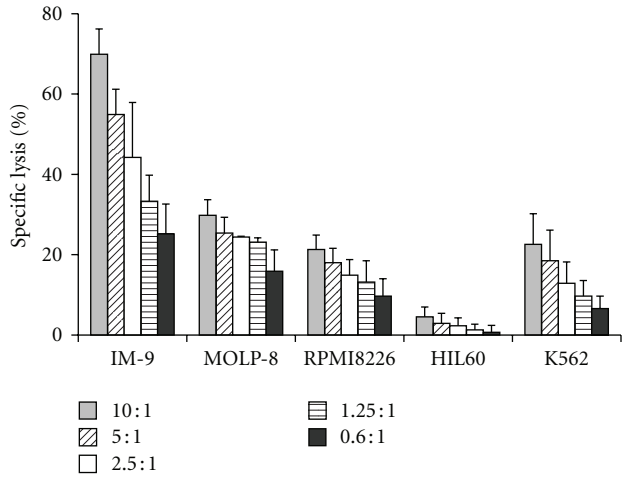


Figure 4: NK-92 mediated killing of target cell lines is dependent on applied E : T ratio. CRA revealed a clearly E : T-dependent level of NK-92 mediated cytotoxicity. Results of CRA are represented as means of at least three independent experiments ± SD. Values in the table below are given as % specific lysis. AML cell line HL60 was almost resistant to NK-92 mediated killing. NK-92 cells were stimulated with 200 U/ml IL-2 the day before the assay.

has a broad spectrum of ligands, but its binding properties are weak. As HLA-G is the strongest binding partner, we evaluated HLA-G expression on all cell lines [4, 6, 34, 35]. Only JEG-3 were positive for HLA-G. Thus, interaction of LIR-1 in later-on conducted CRA was restricted to other binding partners.

The choice of NK-92 and those distinct tumor cell lines instead of primary cells allowed an isolated view on the inhibitory capacities of LIR-1. No increase of cytotoxicity due to blockade of NKG2A could be expected in subsequent blocking assays, for no target cell line expressed the only known NKG2A ligand HLA-E [36]. Furthermore, NK-92 has been described before to lack inhibitory KIR molecules [37, 38].

Other inhibitory LIRs that can be found on NK cells are only LIR-3 (ILT5) and LIR-8 as well as soluble LIR-4 for which the ligands are not yet detected [6].

LIR-1 could therefore be considered to be the only known major inhibitory receptor in this context and was expressed at high levels (Figures 1 and 2). Influence of so far unknown inhibitory receptors was ruled out by selective blockade of LIR-1.

Due to these findings, we considered the use of NK-92 and the chosen tumor cell lines as an ideal system to study the discrete influence of LIR-1 on modulation of NK-cell cytotoxicity.

3.2. Myeloma Cells Are Highly Susceptible to NK-92 Mediated Killing. Cytotoxicity of NK-92 against a panel of tumor cell lines was investigated in CRA at different E : T ratios (Figure 4) [28]. MM cell lines and IM-9 were efficiently lysed by NK-92, with highest results for IM-9 (E : T 10 : 1; specific lysis 69.9 ± 6.3%) followed by MOLP-8 (29.8 ± 3.9%), K652 (22.6 ± 7.6%), and RPMI8226 (21.3 ± 3.6%). HL60 was almost resistant to lysis (4.5 ± 2.5%).

3.3. Blocking of LIR-1 on NK-92 Does Not Increase Target Cell Lysis. To evaluate the influence of LIR-1 in myeloma cell lysis, mAbs were used to block LIR-1 receptor-ligand interactions. As target cells lacked the expression of the HLA-E molecule, blocking of NKG2A was not expected to alter the results but was conducted as a negative control. No significant increase of tumor cell lysis could be achieved by any of the mAbs despite high concentrations (Figure 5).

As specific lysis of target cells by NK-92 was found to be independent from mAb concentration and type, results are presented as means of the used concentrations (0.1/1/10 μg/mL) or in a separate bar as means of concentration and mAbs (CD85j, CD159a, and IgG2b) (Figure 6). For MOLP-8, RPMI8226 and HL60, a significant influence of $F(ab')_2$ towards a decreased lysis seemed to be relevant, but it could not be taken into account.

What first might appear as a protective effect of the applied $F(ab')_2$ towards a reduced lysis of target cells could also be observed in the untreated sample and must therefore be considered to be a side effect caused by cell culture procedure. Only experiments with K562 were performed at the same day with and without $F(ab')_2$ and the observed specific lysis of K562 was the same for both experimental rows. This confirms the thesis of culture side effect to be responsible for significant changes in experimental results.

It is possible that LIR-1 influence could not be measured, if a maximum level of NK-activation had already been achieved before blocking of the inhibitory receptor [38, 39]. Interleukin (IL)-2 requirement during cell culture, induction of the high potential activating receptor NKp44 by IL-2 [40], and origin of NK-92 from rapidly progressive NK cell lymphoma [38, 41] favor a preactivated condition.

3.4. Blocking of Neither LIR-1 Nor HLA Class I on Target Cells Increases Target Cell Lysis. As expected, HLA-A, -B, -C, -E, and -G blockade on tumor cells did not show any influence on lysis (Figure 7) since blocking of LIR-1 as the only relevant inhibitory NK cell receptor on NK-92 had already not modulated cytotoxicity. We also decided to selectively block LIR-1 receptors on the tumor cells before coincubation with NK-92. Though not being likely to directly change NK-cell properties, LIR-1 expression might contribute to MM resistance in so far unknown ways as its role in immune regulations is still fairly unknown (discussed below). As LIR-1 surface expression increases during B-cell and DC maturation [42], it might be directly involved in cell-cell interactions that promote survival and growth. In our experiments, LIR-1 expression on target cells seemed not responsible for a resistance to lysis (Figure 7).

3.5. mAb or $F(ab')_2$ Have No Toxic Effects on NK-92 under Experimental Conditions. To rule out toxic effects of mAb, NK-92 were labeled with 51Cr and incubated with the respective mAb in parallel to the conducted experiments.

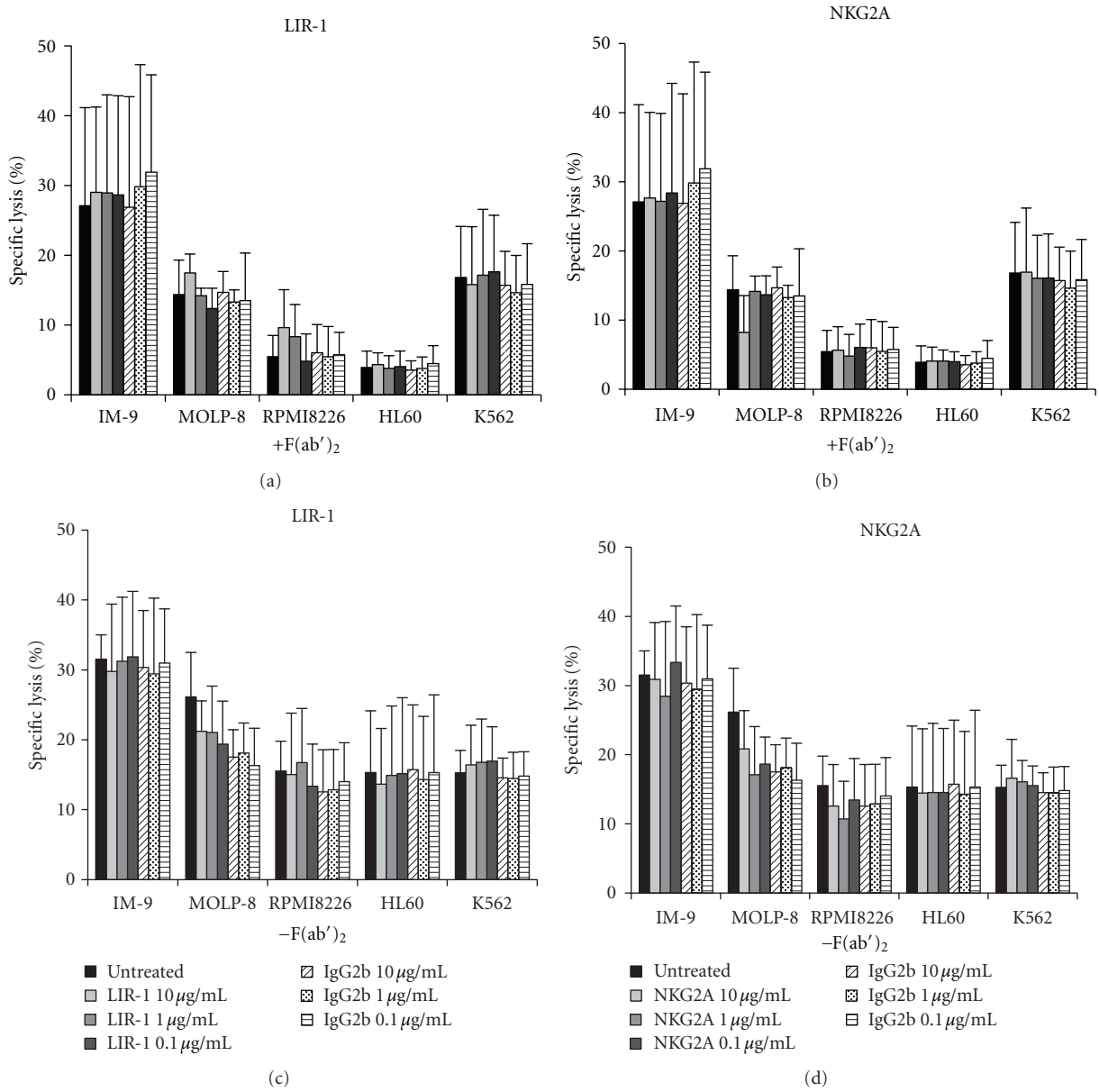


Figure 5: Blocking of CD85j and CD159a on NK-92 does not increase specific lysis. Blocking assays were performed as 4-hour CRA. NK-92 were preincubated with human serum and treated with the respective mAb (0.1, 1 and 10 μg/mL). E : T ratio of 1.25 : 1 was used throughout all experiments with a total of 5×10^3 target cells/200 μl. Results are shown as means of triplicates of at least three independent experiments. (a/b): F(ab′)$_2$ was added after the last washing step prior to coincubation with target cells and maintained within the medium throughout the whole experimental period. (C/D): Samples without F(ab′)$_2$. In samples classified as "untreated", neither F(ab′)$_2$ nor Abs were used. SR was always <20% for all cell lines except from MOLP-8, which showed a constantly high SR up to 36%. Statistical analysis was performed by ANOVA and did not show any significant effect of Ab use.

In four independent experiments, no harmful effect of anti-LIR-1, anti-NKG2A or F(ab′)$_2$ could be observed (Figure 8).

3.6. F(ab′)$_2$ Stabilize Pattern of Tumor Lysis. Although not significantly affecting tumor lysis, there seemed to be an important influence of the F(ab′)$_2$ fragments (Figure 9). They stabilized the results even though prior evaluation of surface molecules did only show very low amounts of CD16 (see above). Apart from outliers, use of F(ab′)$_2$ seemed to even out mAb effects in the blocking experiments, leading to values that oscillate close to the origin in both directions (a). Sparing those fragments decreased relative lysis, predominantly relevant for MOLP-8 and RPMI8226 cells. Only K562 cells rendered more susceptible to NK mediated lysis with a lack of F(ab′)$_2$ though not at a significant level (b).

4. Discussion

The aim of the present investigations was to evaluate if LIR-1 on NK cells inhibits NK-92 mediated cytotoxicity

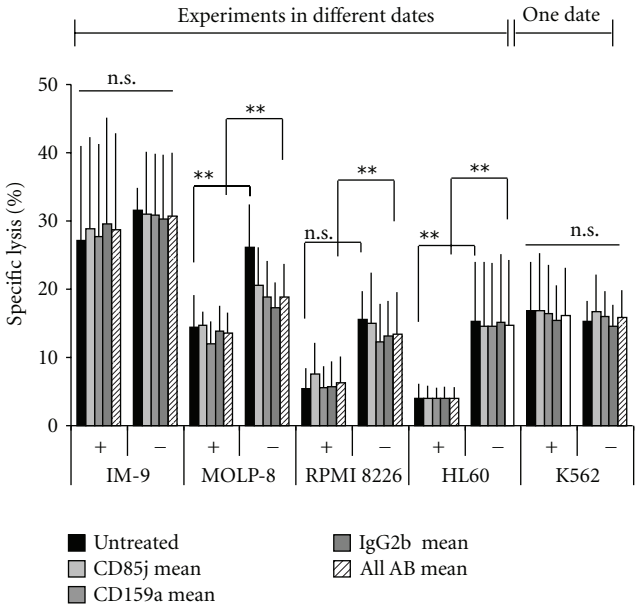


Figure 6: Use of $F(ab')_2$ does not significantly influence cytotoxicity of NK-92 cells. Statistical analysis (ANOVA) reveals a seemingly protective effect by $F(ab')_2$ fragments against NK-92 mediated lysis. As influence of the type of antibody and the used concentration were excluded, experimental data were merged. Results were highly significant (untreated ± $F(ab')_2$/Ab use ± $F(ab')_2$): IM-9 $P = 0.317/P = 0.211$; MOLP-8 $P = 0.017/P = 0.002$; RPMI8226 $P = 0.052/P < 0.001$; HL60 $P = 0.009/P < 0.001$; K562 $P = 0.692/P = 0.771$). The same settings were used throughout all experiments. In the experimental row, K652 tests were performed at the same day and within the same panel. For all other target cells, tests including $F(ab')_2$ were performed first and were followed by those without additional treatment 1 week later. Though IM-9 renders more susceptible to lysis to a nonsignificant degree, time is the factor most likely to be taken into account, for targets incubated with "untreated" NK cells showed the same changes in sensitivity towards lysis.

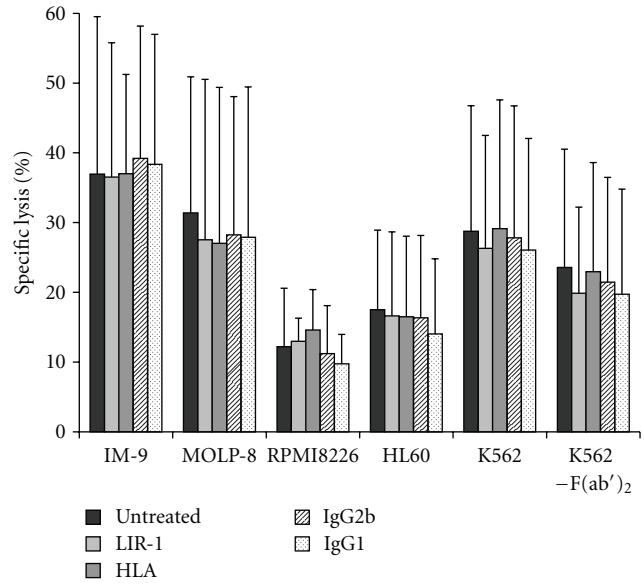


Figure 7: Blocking of LIR-1 and HLA on tumor cells does not lead to increased lysis by NK-92 cells. LIR-1 (CD85j) and HLA class I molecules of the tumor cell lines were blocked as before on the NK-92 cells. After incubation and extended washing, standard CRA were performed. Neither LIR-1 nor HLA blocking showed significant increase of tumor cell lysis. All experiments were performed with the use of $F(ab')_2$ to prevent ADCC. Statistical analysis performed by ANOVA excluded any significant effect of either isotype or Ab on target cell lysis, compared to untreated samples. Discrete investigation of K562 treatment, performed as a comparison of cumulated values for all Ab-incubated samples with or without $F(ab')_2$, excluded any influence of additional $F(ab')_2$ on the results ($P = 0.218$).

against different tumor cell lines. Secondly, presence of LIR-1 on the target cells was validated concerning its influence NK cell mediated lysis. LIR-1 was assumed to be the only relevant inhibitory receptor on NK-92 as stated above (characterization of NK-92 and tumor cell lines) [38]. Surprisingly, no inhibitory influence of LIR-1 on NK-92 within cytotoxicity assays against different tumor cell lines could be detected after treatment with specific antibodies against LIR-1 (Figures 5–7).

By now, involvement of LIR-1 in protecting the fetus from abortion has become common immunological knowledge, and so has the adoption of this mechanism by tumor cells by expressing the LIR-1 ligand HLA-G [43–45]. Furthermore, viruses express highly affinitive LIR-1 ligands for protection against immune defense and LIR-1 serves as a receptor for bacterial detection [5, 8]. Data about the specific role of this receptor in "normal" action of lymphocytes, especially NK cells, are more conflicting and rare. While LIR-1 inhibition of T cell and monocyte activation has less refer to experimental settings in which HLA-G was not present on the target cell [46–48]. Available publications emphasize data that confirm an important inhibitory effect of LIR-1, but a more questioning view might refer to the number of experiments mentioned that did not deliver the pronounced outcome. We will give a short overview of available functional studies of LIR-1 on donor-derived NK cells and NK cell lines in order to demonstrate the conflicting knowledge that is available.

Godal et al. made an attempt in revealing the influence of LIR-1 on dNK in cytotoxicity against HLA-G negative AML and ALL blasts. Their results indicate that LIR-1 does only serve as a weak inhibitory NK cell receptor in the absence of HLA-G on tumor cells, but might be relevant in situations with low KIR expression as seen within the first months after stem cell transplantation (SCT) [10].

Other authors used merely HLA-transfected 721.221, murine cells (P815) or immature dendritic cells (iDC) as target cells, but often had the benefit of comparing results from NK cell lines to the performance of NK cells derived from healthy donors. In 2008, Yawata et al. conducted a series of degranulation assays of dNK against HLA class I deficient 721.221 in order to investigate the involvement of distinct receptors in "missing-self" recognition and were not able to identify any involvement of LIR-1 [49]. More successful LIR-1 mediated inhibition was achieved by Morel and Bellon who

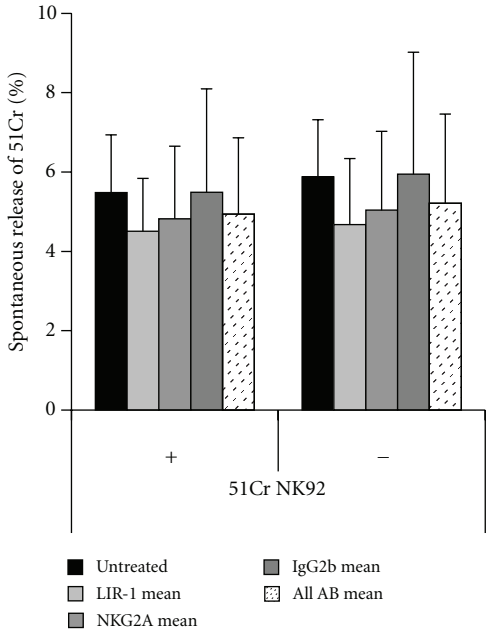


Figure 8: Neither antibodies nor $F(ab')_2$ show toxic effects on NK-92 cells. NK-92 were labeled with 51Cr and incubated with Abs against LIR-1, NKG2A, or IgG2b. Isotype control with (+) or without (−) $F(ab')_2$ is under the same conditions as the unlabeled effector cells in the blocking experiments. SR is given as mean of four independent experiments for untreated NK cells and as mean of an experimental series with concentration of 10, 1 and 0.1 μg/mL. "All Ab mean" integrates those results. $F(ab')_2$ concentration was 11 μ/mL. Spontaneous release was <6%. As this control panel was performed in parallel to regular NK-92 blocking experiments, interferences by reagents, temperature, incubation period, cell viability, or technical equipment can be excluded.

tested the cytotoxicity of dNK and NK-92 against HLA-G and -B transfected 721.221 in CRA with subsequent use of blocking antibodies [39]. Their prestudies found only 15% of LIR-1 positive dNK to be stimulated by target contact and these responsive dNK were chosen for the subsequent studies. Comparative analysis of NK-92 and dNK in different types of assays were conflicting.

No such conflict was observed by Favier et al., who incubated dNK and the NK cell line NKL with 721.221-G1 and used blocking Abs against LIR-1 and HLA-G [50]. The same was true for Vitale et al. They incubated dNK with different HLA transfected cell lines in standard CRA and increased lysis by LIR-1 blockade, but unfortunately, no explicit prevention of antibody-dependent cellular cytotoxicity (ADCC) was mentioned [51]. At last, Colonna et al. showed the expected LIR-1 influence in different experimental setups with NKL, serotonin releasing RBL cells, as well as dNK and donor derived LIR+ T cells (dTK) [21]. Interestingly, in all assays with either dTK or dNK against 721.221-B*2705, anti-LIR-1 could only partially revert transfection-induced inhibition, indicating either incomplete binding of anti-LIR-1 to the receptor or the presence of other receptors apart from LIR-1 that bind to the ligand.

Moreover, in reverse ADCC (rADCC) assays with dNK against P815, only few clones were inhibited by LIR-1. Here again, NK cell clones have shown to exhibit less predictable outcomes than cell lines [21].

Summarizing the available data, NK cell lines seem to be more reliable than dNK concerning LIR-1 mediated downregulation of cytotoxicity. The results for polyclonal dNK show high variances between the different clones that are mostly not characterized in detail. Successful LIR-1 mediated inhibition by HLA-transfected 721.221 has abundantly been shown. Involvement of additional undetected receptors could not always be excluded, and different types of assays performed with the same effector and target cells could lead to highly diverging results. Investigations of cytotoxicity of dNK and donor-derived tumor cells are rare and no sufficient information is available about the role of LIR-1 in the absence of HLA-G. Current opinion about the inhibitory influence of LIR-1 is predominantly based on investigations at the feto-maternal interface or has been gained from settings in which only a single HLA molecule was present on the target cells—mostly HLA-G or -B on 721.221. The performed experiments do not sufficiently cover the extensive binding capacities of LIR-1 to HLA class I.

Although we are aware of problems concerning the comparability of NK cell lines to donor-derived NK cells (dNK), we have chosen an experimental setting that allows studying isolated influence of LIR-1 on cytotoxicity. Our major goal was to provide a model system to overcome the common practice of using transfected target cell lines in cytotoxicity assays. Being aware of the necessity of future efforts to confirm the present findings in a brighter panel of cells and cell lines, these results might provide a first step towards a new understanding of LIR-1.

Available data do not sufficiently support the direct implication of LIR-1 in NK cell inhibition. Upregulation of the receptor does not necessarily favor immunosuppression but might correlate with the acquisition of memory. LIR-1 surface expression increases during B cell and DC maturation [42] as well as during cytomegalovirus infection [52] and acquisition of T-cell memory [53, 54]. We suppose that an increasing LIR-1 expression also correlates with the acquisition of NK cell memory [55], supported by the surveillance that a high LIR-1 level on NK cells leads to an effective lysis of HIV-infected DC [56].

LIR-1 seems to have a high impact on regulating the balance between activation and inhibition during immune responses [42]. It might do so by cooperation with other receptors like KIRs, which favor a clustering of MHC-I [57]. As HLA-G is the only LIR-1 ligand that generates covalent dimers and trimers at the cell surface, aggregation might be a precondition to receptor's activation. It has been proposed that these complexes increase the avidity towards LIR-1, explaining the discrepancy between the relatively low affinity of LIR-1 to its ligand and the high relevance within the context of fetal tolerance [58]. Homo- or heterotypic complex formation of the LIR-1 receptor and its ligands might be a key factor that regulates the degree of NK cell inhibition. By this, LIR-1 might work as a rheostat of NK cell activation. This hypothesis would fit the capability of LIR-1

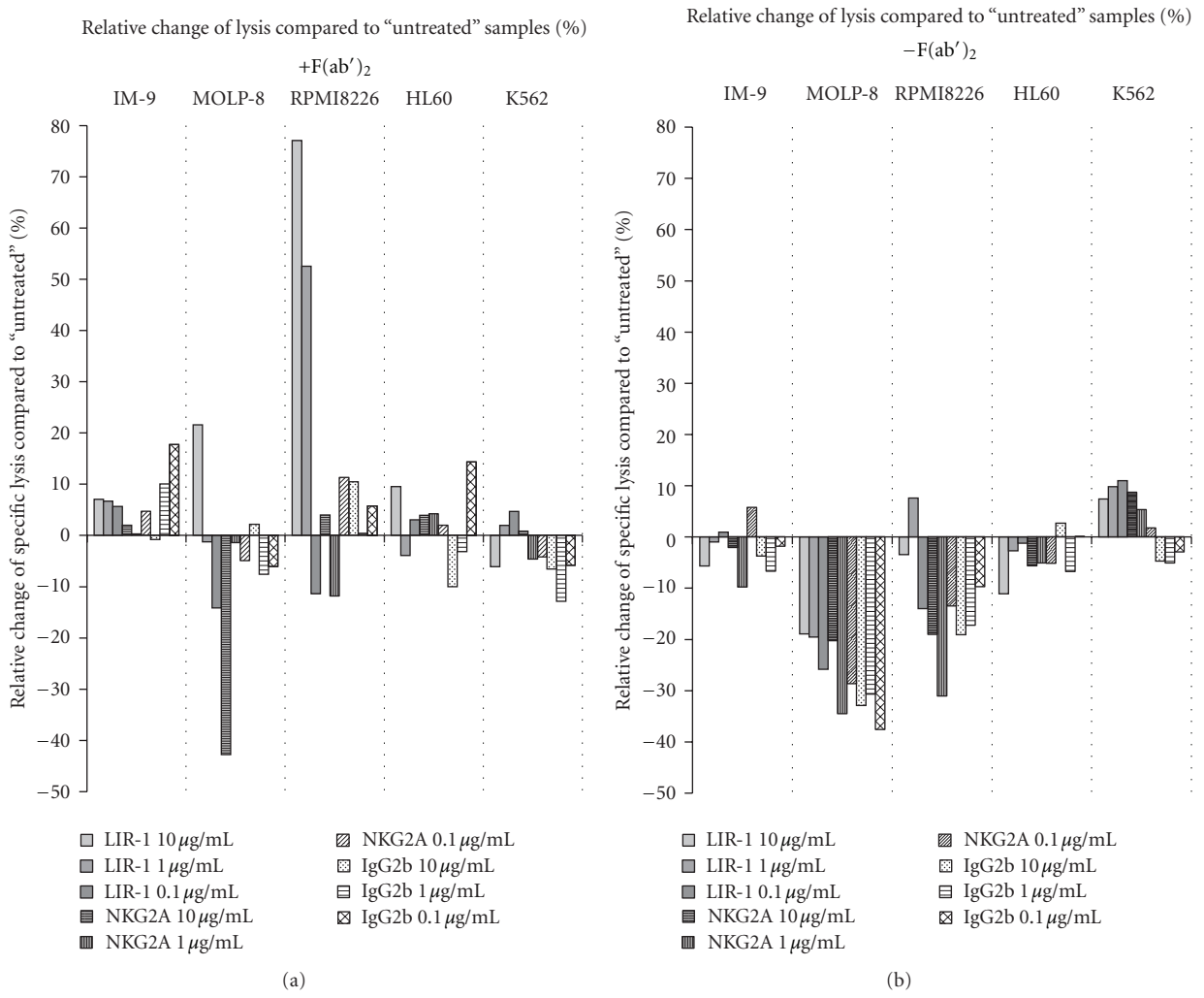


Figure 9: Use of $F(ab')_2$ after blocking NK-92 receptors seems to balance lysis pattern. Values show the % relative increase or decrease of target cells lysis in a standard 4-hour CRA, calculated as follows: % relative change of specific lysis = [(% specific lysis "antibody") − (% specific lysis "untreated")]/[% specific lysis "untreated"]. Though no concentration of the used mAbs showed a significant change in tumor lysis, interesting differences in the resulting lysis-patterns could be detected. (a) Apart from outliers, use of $F(ab')_2$ evens out mAb effects in the blocking experiments, leading to values that oscillate close to the origin in both directions. (b) Sparing $F(ab')_2$ decreases specific lysis, predominantly for MOLP-8 and RPMI8226 cells.

to sense the overall HLA class I expression on human tissues. An increase during aging as well as memory acquisition could elevate the necessary threshold for activation during a parallel process of increase of activating NK cell receptors caused by repeated pathogen contacts.

5. Conclusions

Within the present study, no alteration of NK mediated cytotoxicity against MM was observed after blockade of LIR-1. Being the only functional inhibitory receptor within this setting, major known side effects by, for example, KIR have been ruled out. This unexpected outcome opens the door to fruitful discussions about the complexity of LIR-1 interactions and its potential role within tumor defense. It is very likely that the present presumptions about functions of LIR-1 within immune regulation are far behind the real impact. We hypothesize that LIR-1 has a key role as a rheostat of NK cell modulation and is strongly involved in the acquisition of NK cell memory.

Conflict of Interests

The authors have no financial conflict of interests.

Acknowledgments

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