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Target Recognition by Natural Killer Cells

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Identification and Characterization of Inhibiting and Activating Factors

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Science may set limits to knowledge, but should not set limits to imagination.

(Bertrand Russell)

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Summary

Natural killer (NK) cells are able to attack and destroy tumorous or virally infected cells without prior sensitization. The processes that regulate their activation and function are still incompletely understood. NK cells do not exress a single clonal receptor like T-cells but many different activating and inhibitory receptors. Most of the inhibitory receptors bind to MHC I or related molecules. Some activating receptors recognize ligands induced upon cell stress or transformation. One group of inhibitory receptors on NK cells is constituted by the NKG2 family. Some of these receptors form antithetic pairs. That means, they recognize the same ligands but signal in a contrary fashion. One of the most important NKG2 receptors is NKG2D. For this receptor an antithetic counterpart has not been described yet.

One part of this thesis included the identification of a possible inhibitory counterpart for NKG2D. Data base searches retrieved the cDNA for CLEC12B. Characterization of the receptor lead to the result that it did not bind the known NKG2D ligands and was not expressed on NK cells. Nevertheless, it is able to confer inhibitory signaling via the recruitment of the tyrosine-phosphatases SHP-1 and SHP-2. Expression of the inhibitory receptor CLEC12B was detected on monocyte-derived macrophages.

Another important group of activating receptors is constituted by the natural cytotoxicity receptors (NCR). They have been implied in antiviral defense and in the recognition of malignant cells. Although the ligand remains unknown it is still possible to detect it by applying functional assays and staining methods using soluble fusion proteins. The role of the NCR ligands has up to now not been examined in human cytomegalovirus (HCMV) infection. Fusion protein staining and functional data presented in this thesis suggest that the NCR ligands are downregulated from the cell surface. This might constitute a new immune evasion mechanism of HCMV. It was discovered that this process is mediated by a viral gene product that is expressed *de novo* upon infection during the immediate early or early phase of infection. The creation of viral deletion mutants helped to exclude non-essential regions of the HCMV genome. Immunofluorescence staining with fusion proteins showed that the ligand is held back in intracellular compartments. For further research the elucidation of the NCR ligands is vitally important. Heparan sulfate structures have been postulated to function as ligands but these results are debated. In this thesis, it was established that heparan sulfate is not the functional ligand for NKp30. The putative ligand was found to involve a proteinacious component that is not GPI-anchored. It was tried to identify the NKp30 ligand with the help of a genomic siRNA library. Cells expressing the NKp30 ligand were transduced with the library and selected for cells with an NKp30 negative phenotype. The siRNAs were rescued from the cell population, amplified and analyzed via an affimetrix genechip. The results show the enrichment of some interesting transmembrane or secreted proteins as possible candidates that might function as ligand for NKp30.

Zusammenfassung

Natürliche Killer (NK) Zellen sind in der Lage transformierte oder mit Viren infizierte Zellen ohne vorherige Sensibilisierung zu erkennen und zu lysieren. Um diese Aufgabe erfüllen zu können, verfügen NK-Zellen über verschiedene Rezeptoren mit aktivierender oder inhibierender Funktion. Die meisten inhibitorischen Rezeptoren binden an MHC I Moleküle. Einige der aktivierenden Rezeptoren erkennen Liganden, die nach Zellstress oder Transformation exprimiert werden. Eine wichtige Gruppe der NK-Zell Rezeptoren ist die NKG2-Familie. Manche dieser Rezeptoren bilden antithetische Paare, die dieselben Liganden erkennen, aber gegensätzliche Signale auslösen. Einer der wichtigsten aktivierenden Rezeptoren dieser Gruppe ist NKG2D, für den bisher noch kein inhibitorischer Gegenpart bekannt ist. Ein Teil dieser Arbeit zielte darauf ab, einen potentiellen inhibitorischen Gegenpart für den NKG2D-Rezeptor zu identifizieren. Eine Datenbanksuche identifizierte die cDNA von CLEC12B als möglichen Kandidaten. Die Charakterisierung dieses Rezeptors zeigte, daß er trotz Homologie zu NKG2D keinerlei Affinität für die bekannten NKG2D Liganden besitzt und nicht in NK-Zellen exprimiert wird. Allerdings ist er in der Lage inhibitorische Signale durch die Rekrutierung der Tyrosin-Phosphatasen SHP-1 und SHP-2 auszulösen. Die Expression von CLEC12B konnte schließlich auf aus Monozyten generierten Makrophagen festgestellt werden.

Eine weitere Gruppe ist die Familie der Natural Cytotoxicity Receptors (NCR) mit den Rezeptoren NKp30, NKp44 und NKp46. Obwohl ihre Liganden bisher noch unbekannt sind, ist es möglich sie mit der Hilfe funktioneller Experimente und löslicher Fusionsproteine zu detektieren. Über die Rolle des NKp30 Liganden während einer Infektion mit humanem Cytomegalievirus (HCMV) ist bisher nichts bekannt. Über Färbungen mit Fusionsproteinen und funktionelle Experimente konnte gezeigt werden, daß im Zuge einer HCMV Infektion die Expression des NKp30 Liganden unterdrückt wird. Dieser Prozess wird durch ein virales Genprodukt ausgelöst, das *de novo* während der frühen Infektionsphase exprimiert wird. Über die Generierung von Deletionsmutanten konnten große Bereiche des HCMV Genoms mit nicht-essentiellen Genen ausgeschlossen werden. Immunfluoreszenz-Färbungen mit Fusionsproteinen zeigten, dass die Liganden von NKp30 in intrazellulären Kompartimenten zurückgehalten werden. Für weitere Forschung ist die Identifikation der NCR Liganden unumgänglich. Es wurde ein siRNA screen etabliert, um den NKp30 Liganden zu identifizieren. Zellen, die den NKp30 Liganden exprimieren, wurden mit einer siRNA Bibliothek transduziert und auf einen NKp30 negativen Phänotyp selektioniert. Die siRNAs wurden aus der entstandenen Zell-Population isoliert, amplifiziert und über einen affimetrix gene chip analysiert. Als Resultat ergaben sich siRNAs, die gegen einige interessante Transmembranproteine gerichtet sind. Diese möglichen Kandidaten stellen die Basis für weitere Untersuchungen dar.

I. Introduction

I.1 Natural killer (NK) cell biology

Our immune system is a sophisticated network of highly specialized cells that combat infections and cancer. Numerous cell types have evolved fulfilling distinct functions in this challenge. NK cells constitute a part of the cells found in the thick of the fray, secreting pro-inflammatory cytokines and destroying infected or transformed cells.

NK cells were first described in 1975 as having the ability to lyse allogeneic tumor cells in mice without prior sensitization (Herberman et al., 1975a; Herberman et al., 1975b; Kiessling et al., 1975a; Kiessling et al., 1975b). Later on it became clear that NK cells were also responsible for the phenomenon of F1 hybrid resistance (Kiessling et al., 1977). Therefore, the term 'natural cytotoxicity' was coined and used to describe this particular cell type that was, unlike the cells of the adaptive immune system, a 'natural killer' (Lanier et al., 1986). Additionally, these cells were discovered to play an important role as a first line of defense against viral infections and other intracellular pathogens. Human NK cells are historically defined as $CD56^+$ and $CD3^-$ (Cooper et al., 2001) but new data suggest that a definition as $NKp46^+$ and $CD3^-$ would be more serviceable over species barriers (Walzer et al., 2007a).

NK cells constitute 5-15% of the human peripheral blood mononuclear cells (PBMC) population and up to 5% of the whole lymphocyte population in lymph nodes. Additionally, NK cells can be found throughout most tissues including liver, spleen and lung. A specialized type of NK cells plays a very important role in the decidua during pregnancy (Tabiasco et al., 2006). Although NK cells show strong similarities with T-cells, they are classified as innate immune cells. In contrast to cells of the adaptive immune system which have to be primed by other immune cells, NK cells can act without prior stimulation. This characteristic makes the innate immune cells the first line of defense against pathogenic intruders until the adaptive immune response is able to step in. This point of view led to the assumption that the innate immune system was evolutionary old-fashioned, just a remainder of early defense mechanisms and a precursor of the adaptive immune system. By now this perception has changed. Mice and humans that lack innate immune cells are not able to control infections long enough to activate the adaptive immune response. They die before these specialized cells are ready to defend them (Mandell et al., 2000). Both parts of the immune system are equally important and work hand in hand to fight infections and cancer.

Human NK cells can be divided into two different subsets according to their expression of the surface markers CD56 and CD16. About 90% of the NK cell population show a $CD56^{dim}$ phenotype while 10% are $CD56^{bright}$. $CD56^{bright}$ cells do not express CD16 and $CD56^{dim}$ cells are high in CD16 expression (Cooper et al., 2001). Both subsets have

been reported to fulfill distinct functions. While the $CD56^{dim}$ subset is responsible for the lysis of tumors and virally infected cells, $CD56^{bright}$ NK cells are producers of important cytokines, including interferon-γ (IFN-γ) and tumor necrosis factor α (TNF-α) (Biron et al., 1999). These cytokines are crucial for the initiation of a protective T-cell response against pathogens and cancer. Furthermore NK cells exist in a closely intertwined relationship with dendritic cells (DCs) including mutual activation based on cytokine production and cell-cell contacts (Degli-Esposti and Smyth, 2005). Thus, based on their lineage relationships, receptor repertoire, and effector functions, NK cells appear to be a transitional cell type bridging the innate and adaptive immune systems. Their importance becomes clear in patients born without NK cells. These patients suffer from recurrent illnesses and have particular problems in controling herpesviral infections despite their functional adaptive immune response (Biron et al., 1989).

NK cells have evolved much later than the first simple phagocytic cells. Although the genes for the lytic enzymes perforin and granzyme and the transcription factors that control their expression can be found in rather simple organisms like fish (Spits et al., 1998) the receptor families or signaling adaptors that characterize NK cells seem to have arisen after the mammalian branching. This finding puts NK cells onto the same evolutionary step as T-cells. In this context it seems that the cytolytic machinery used by NK cells is very old, their recognition mechanisms have only evolved recently (Walzer et al., 2007b).

NK cell development takes place in the bone marrow (Colucci et al., 2003). They arise from $CD34^{+}$ common lymphoid progenitor cells. Although NK cells are part of the innate immune system they do not descend from myeloid progenitor cells which can give rise to dendritic cells or monocytes. Instead they share a common progenitor with T-cells. NK cell development is dependent on the presence of interleukin-15 (IL-15) or IL-2. In contrast to B- and T-cells, NK cells do not undergo somatic recombination. A fact which has been demonstrated by the existence of NK cells in RAG1 and RAG2 knockout mice (Hackett et al., 1986). NK cells do not require a functional thymus for development but an intact bone marrow. Furthermore, recent data suggest that part of the NK cell developmental pathway takes place in the parafollicular T-cell rich regions of secondary lymphoid organs (Freud and Caligiuri, 2006).

NK cells do not only share a common progenitor with T-cells, they also show interesting functional similarities. Various receptors that have been identified on NK cells can also be found on T-cells, although with potentially different functional outcome. For example the family of SLAM-related-receptors (SRR) is found on both NK and T-cells. But while receptors like 2B4 and NTB-A can activate IL-2 stimulated NK cells to lyse target cells, they serve a costimulatory purpose in the T-cell activation process (Bhat et al., 2006). Another similarity is that the mechanism employed by NK cells to lyse target cells resembles that of cytotoxic T lymphocytes. Both cell types have preformed granules in their cytoplasm which

contain toxic enzymes like perforin, granzymes and granulysin. Upon contact with a susceptible cell, the NK cell polarizes its cellular machinery including the granules into the direction of the target cell. Then the granules fuse with the outer membrane of the NK cell and their contents are released into the synaptic cleft between the killer cell and its target. According to the current view, perforin molecules form a pore in the target cell membrane which enables granzymes to diffuse into the cytoplasm of the attacked cell and initiate apoptotic processes. Further mechanisms employed by NK and T-cells include induction of apoptosis via TRAIL or engagement of the Fas receptor on the target cell surface via the Fas ligand (Lieberman, 2003).

NK cell activity has to be inhibited by healthy tissue to avoid damage. This is accomplished by the expression of MHC I molecules. NK cells express inhibiting receptors that recognize MHC I. Here we find yet another similarity but also a striking difference to T-cells. Both cell types have receptors for MHC I molecules but while T-cells monitor which peptide is presented through the MHC I molecule, NK cells just bind MHC I no matter which peptide is presented. If in the course of a viral infection or tumorigenesis MHC I is lost from the cell surface in an attempt to escape T-cell surveillance, the NK cells can be activated to lyse this MHC I low target cell. The same can happen when during a transplantation process the MHC I alleles of the transplant donor do not match the recipient's. NK cells are not inhibited by 'foreign' MHC I and thus able to lyse the respective target cell (Karre et al., 1986). This so called 'missing self hypothesis' as shown in fig. 1 has long been the central dogma of NK cell research. However, NK cells are not overreactive in humans or mice deficient in expression of MHC I or associated proteins (Bix et al., 1991; Furukawa et al., 1999; Hoglund et al., 1991; Vitale et al., 2002) but rather hyporesponsive to MHC I deficient targets. Therefore it seems that the recognition of MHC I by NK cells is not a static system like the conserved pattern recognition receptors of other innate immune cells but more like a flexible machinery acquired during NK cell generation in the host. In the course of their development, NK cells 'learn' which MHC I alleles are expressed as self in their particular surrounding. There are fitting inhibitory receptors on NK cells for the many different possible MHC I alleles. These different receptor alleles seem to be expressed in a stochastic fashion on the developing NK cell. NK cells that happen to lack expression of an inhibitory receptor that fits at least one of the MHC I molecules expressed as self become hyporesponsive. The mechanisms of this process are still debated but summarized under the term 'licensing'. It has been shown that in the blood of healthy donors NK cells can be found that do not express a single one of the known MHC I binding inhibitory NK cell receptors. These NK cells, in contrast to the ones that do express receptors for fitting MHC I molecules, are hyporesponsive as well (Anfossi et al., 2006). Hence, potentially self-reactive NK cells are rendered harmless during their development before they can cause any damage.

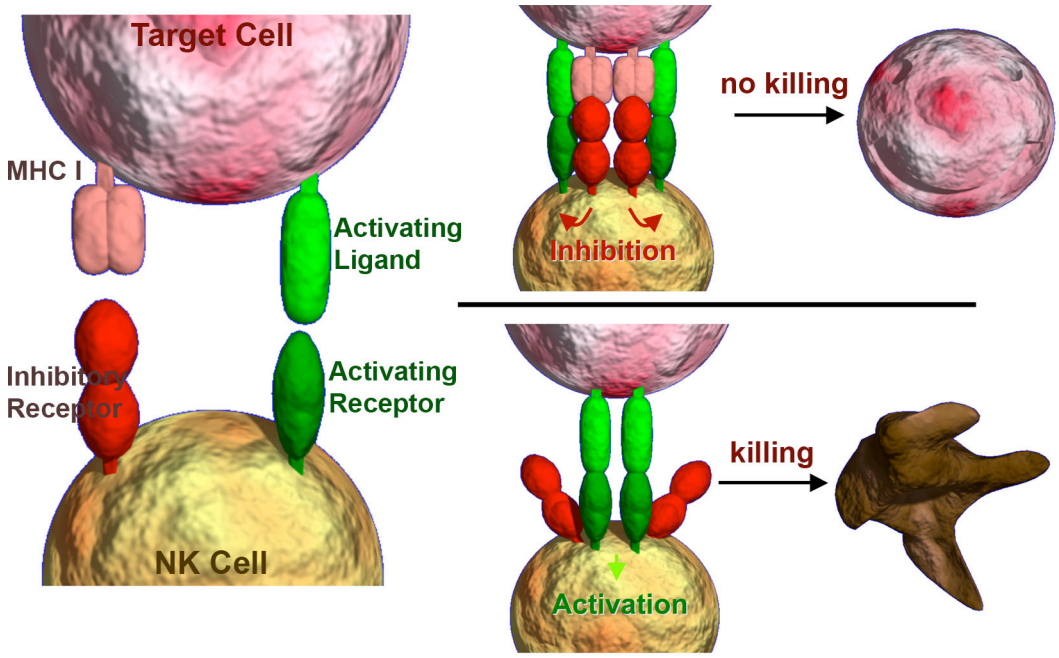

Fig 1: The missing self hypothesis. NK cells express inhibitory receptors for self MHC I molecules. Are these engaged upon contact with a potential target cell, the NK cell is inhibited and the target cell spared fom lysis. If MHC I molecules are downregulated upon infection or tumorigenesis or don't match after a transplantation, the inhibitory receptors are no longer engaged and the NK cell can be activated to lyse the target cell.

The missing self hypothesis and the licensing mechanism are still not the ultima ratio to the regulation of NK cell activity. In recent years it has become quite clear that activating receptors are involved in the regulation of NK cell activity as well. A new term called 'induced self' has been coined to describe this new concept (Watzl, 2003). Target cells that do not express any functional NK cell ligands neither inhibiting nor activating are not attacked at all, for example cells from foreign species like *Drosophila melanogaster*. Stimulating ligands are needed to fully activate an NK cell. Some of these molecules are constitutively expressed on target cells, some only upon infection or cell stress. High expression of these ligands can provide an override mechanism and lead to lysis of the target cell despite self-MHC I presentation (Regunathan et al., 2005).

As displayed in fig. 2, it seems that the gist of the matter of NK cell regulation is a tightly controlled balance of inhibiting and activating stimuli. This balance can be further influenced for example through the presence of proinflammatory cytokines and other soluble agents. Additionally the crosstalk with other immune cells provides an important source for NK cell stimulation. NK cells integrate an abundance of stimulatory and inhibitory signals that lead to the final decision: to kill or not to kill. Elucidation of the underlying mechanisms will greatly advance our knowledge in the fight against cancer and infectious diseases.

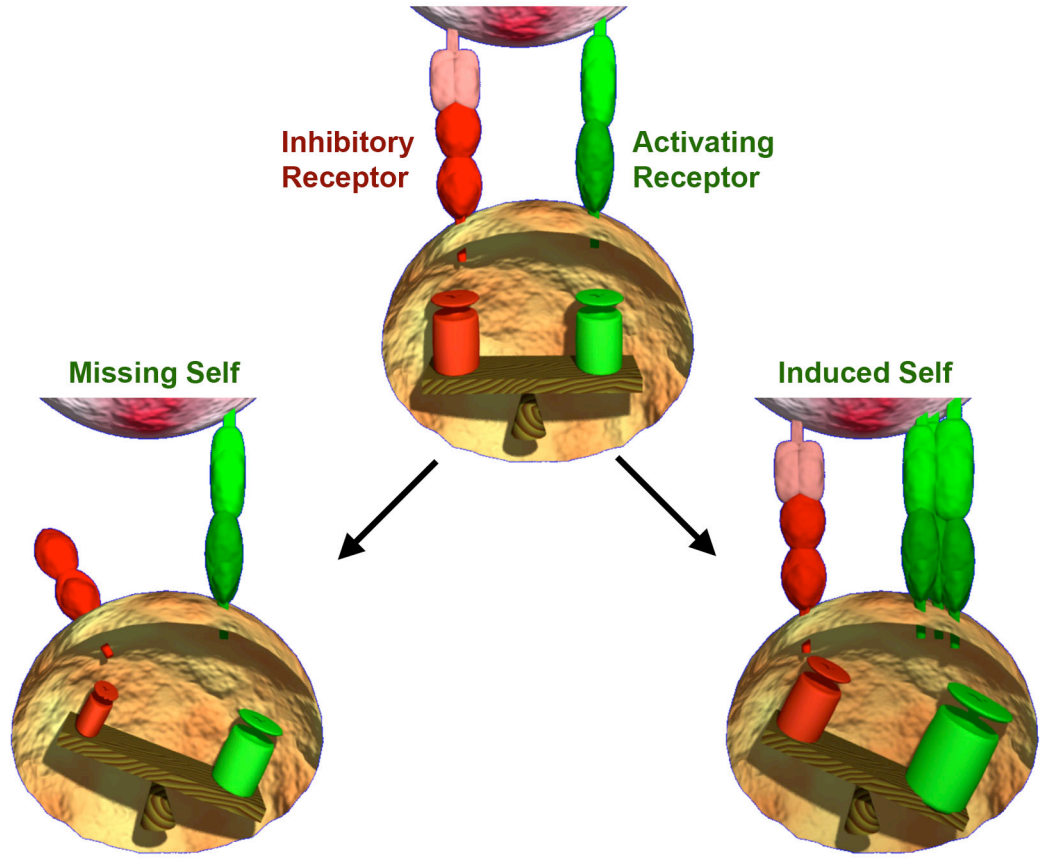

Fig. 2: NK cell regulation requires balance. NK cells engage inhibiting and activating ligands on the target cell surface and are further influenced by their surroundings. If the balance of activation and inhibition is disturbed, the NK cell can be activated to lyse its target ad secrete pro-inflammatory cytokines.

I.2 Inhibitory NK cell receptors

The first inhibitory receptors of NK cells were described in mice (Karlhofer et al., 1992) as C-type lectin like proteins. The Ly49 receptors comprise a small family of type II transmembrane-anchored glycoproteins with considerable allelic polymorphism (Mehta et al., 2001) that recognize MHC I molecules. The inhibitory members of the Ly49 family signal via an immunoreceptor tyrosine-based inhibition motif (ITIM) in their cytoplasmic domains. But apart from inhibitory receptors this family also includes molecules with activating features. These activating receptors lack a cytoplasmic domain and instead pair with the ITAM containing adaptor molecule DAP12 through a charged amino acid in their transmembrane domain and therefore are able to transmit activating signals.

The Ly49 family does not exist in humans. Instead in a remarkable example of convergent evolution human NK cells express inhibitory type I transmembrane receptors of the immunoglobulin (Ig)-superfamily. These so called killer cell Ig-like receptors (KIR) are expressed by NK cells but also by $\gamma\delta TCR^+$ T-cells and memory/effector $\alpha\beta TCR^+$ T-cells (usually $CD8^+$ T-cells and some $CD4^+$ T-cells, but not thymocytes or naive T-cells) (Ferrini et al., 1994; Phillips et al., 1995). KIR receptors may contain two (KIR2D) or three (KIR3D) Ig-

like domains. These receptors, just like receptors of the Ly49 family, can be equipped with different signaling domains: either an inhibitory ITIM in the cytoplasmic tail, or a charged amino acid in the transmembrane region to couple to adaptor molecules with activating potential. Due to these characteristics the receptors are denoted as long (L; KIR2DL or KIR3DL) or short (S; KIR2DS or KIR3DS). The KIR family consists of 15 genes and 2 pseudogenes. Diversity within the KIR family can still be increased by differential splicing and allelic polymorphism (Selvakumar et al., 1997). Different KIRs recognize different human leukocyte antigen (HLA) -A, HLA-B and HLA-C molecules. An overview of the KIR receptors and their corresponding ligands is given in fig. 3. Many HLA-A and HLA-Bw6 allotypes apparently do not have a corresponding KIR, indicating that the KIR repertoire is not all-inclusive for human class I allotypes (Lanier, 1998). Unlike T-cells NK cells bind to HLA molecules on the potential target cell surface regardless of the peptide presented. Although there is evidence that the peptide bound in the MHC peptide groove can affect KIR binding, the functional outcome of these observations is uncertain (Mandelboim et al., 1997).

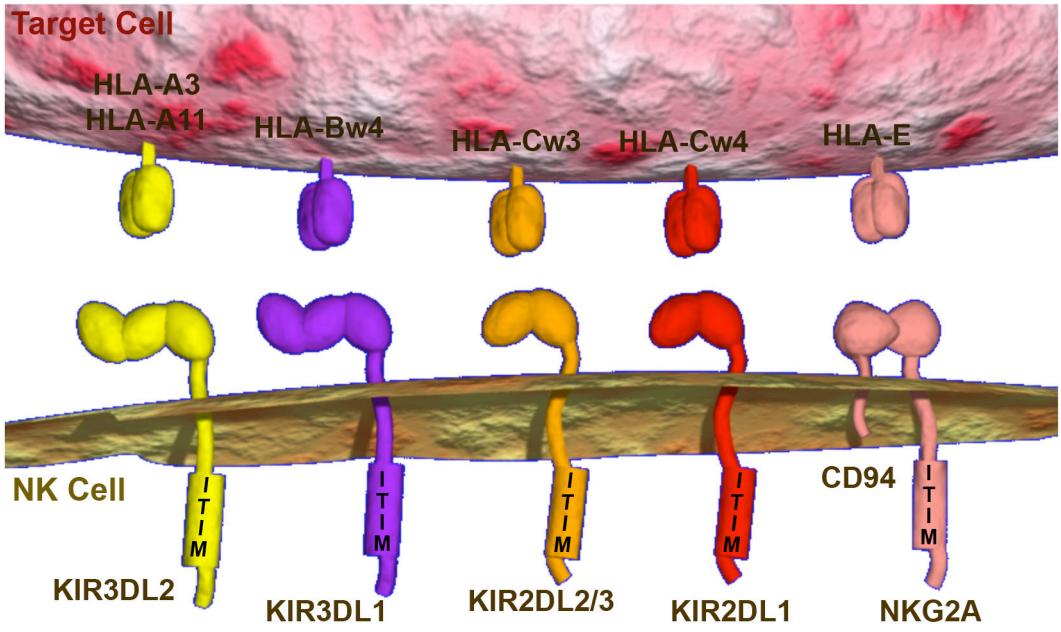

Fig. 3: Inhibitory NK cell receptors and their ligands.

In addition to these Ig-like receptors that recognize conventional MHC I molecules, the CD94/NKG2 family of C-type lectin like receptors is involved in the recognition of the unconventional MHC I molecule HLA-E. HLA-E presents leader-peptides derived from conventional MHC I molecules and thus indicates the overall presence of MHC I. The CD94/NKG2 family has already been identified in primitive organisms like teleostean fish and tunicates (Khalturin et al., 2003; Sato et al., 2003), which hints at an early system of self/nonself discrimination. In humans this family comprises CD94, NKG2A, -C, -E and –F.

Unlike the KIR family these receptors show only limited genetic polymorphism. CD94 is expressed as a disulfide-linked homodimer or as a disulfide-linked heterodimer with NKG2A or NKG2C. Interestingly, only NKG2A leads to inhibitory signals via an ITIM in its cytoplasmic domain. The CD94/NKG2C heterodimer signals in an activating fashion similar to the short KIR receptors. This finding might seem not very sensible for a start. But the inhibitory CD94/NKG2A heterodimer has a higher affinity for HLA-E molecules than the complex involving NKG2C (Vales-Gomez et al., 1999). Furthermore it has been suggested that proteins bound to the HLA-E complex can affect CD94/NKG2A affinity and therefore permit NK cells to be activated by the CD94/NKG2C complex in some cases (Michaelsson et al., 2002). This concept of having several receptors for the same target molecule but with different affinities and signaling properties is again a good example for the tight balance of NK cell regulation.

Both receptor families, KIR and NKG2, can be phosphorylated at the tyrosine residue central to their ITIMs and recruit the SH2 domain containing phosphatases (SHP)-1 and SHP-2 upon engagement (Long et al., 2001). These phosphatases interfere with the recruitment of activating receptors into specialized membrane domains called lipid rafts and therefore abort the initial events of NK cell activation (Watzl et al., 2000).

Apart from the MHC I dependent NK cell inhibitory receptors there are also numerous receptors with inhibitory features that do not bind MHC I molecules. The homophilic receptor carcinoembryogenic antigen cellular adhesion molecule 1 (CEACAM1) has been shown to be upregulated in patients with TAP2 deficiency (Markel et al., 2004). Thus, it seems that expression of the ITIM containing receptor CEACAM1 can prevent autoaggression in the absence of MHC I molecules and therefore functions as an inhibitory NK cell receptor. Although NK cells without adequate MHC I binding receptors are rendered hyporesponsive, expression of CEACAM1 might provide a fail-safe mechanism. Another non-MHC I binding inhibitory receptor belongs to the family of sialic-acid-binding immunoglobulin-like lectins (SIGLECs). SIGLEC7 is the only SIGLEC family member expressed on NK cells. This receptor contains two ITIMs and is known to bind sialic acid residues (Ito et al., 2001). Crosslinking of SIGLEC7 on the NK cell surface leads to inhibition of target cell lysis (Nicoll et al., 2003). Although expression of compatible MHC I molecules still seems to be the main mechanism of NK cell inhibition, these additional inhibitory receptors provide important signals for the NK cell as well.

I.3 Activating NK cell receptors

The group of NK cell activating receptors is much more heterogeneous than the inhibitory fraction. The receptors known to date cover several different families and use

distinct signaling pathways. Fig. 4 illustrates the established NK activating receptors except CD16 as this receptor does not recognize cellular ligands.

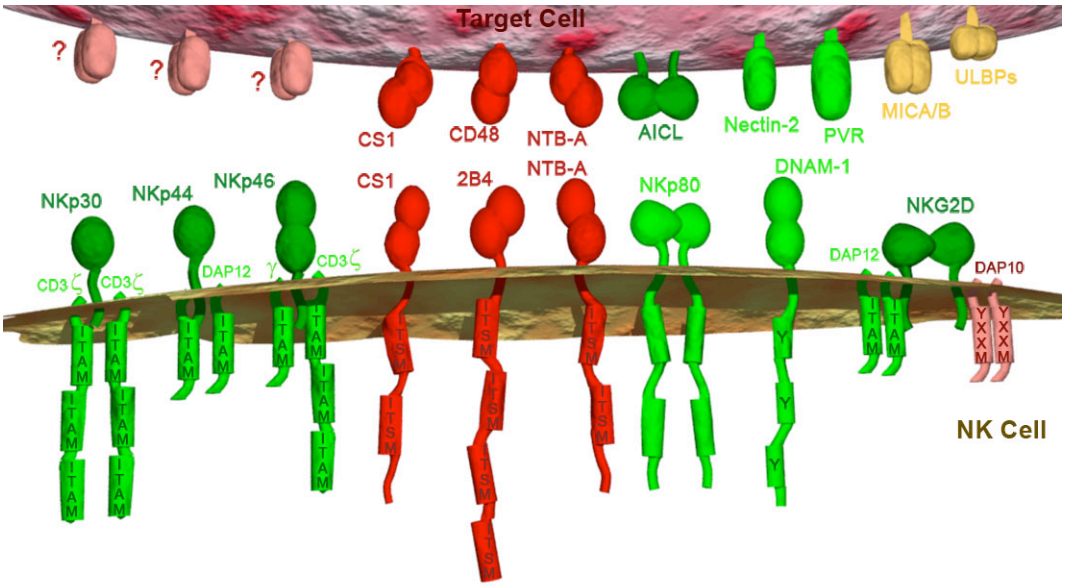

Fig. 4: Overview of the known activating NK cell receptors and their ligands.

Among the activating receptors CD16 takes a special place. This low affinity F_c-receptor is responsible for a phenomenon called 'antibody-dependent cellular cytotoxicity' (ADCC). Upon encounter with an opsonized target CD16 can activate the NK cell to secrete cytokines and lyse the respective target cell. In fresh NK cells CD16 seems to be the only receptor to activate degranulation without co-engagement of several other receptors (Bryceson et al., 2006b). Interestingly it has been described that CD16 induces degranulation without prior polarization of granules. For specific polarization engagement of further activating receptors or adhesion molecules was necessary (Bryceson et al., 2006a). Upon activation of CD16, its adjacent signaling molecules FcεRI-γ or CD3ζ become phosphorylated within their ITAMs and transmit the signal further downstream through various molecules including ZAP70, phospholipase Cγ (PLCγ), phosphatidylinositol-3 kinase (PI3K) and mitogen activated protein (MAP) kinases.

Apart from CD16 and its unique role for NK cell biology, the squad of activating receptors includes NKp30, NKp44, NKp46, 2B4, NTB-A, CS1, NKp80, DNAM-1 and NKG2D as depicted in fig. 4 (Bottino et al., 2005). NKp30, NKp44 and NKp46 belong to the family of natural cytotoxicity receptors (NCR) and will be discussed in detail later on. 2B4, NTB-A and CS1 are members of the SLAM-related receptor (SRR)-family. These receptors are not confined to NK cells but are expressed on different immune cells such as T- and B-lymphocytes, macrophages, monocytes and dendritic cells. SRR consist of two or four Ig-like domains in their extracellular part and are equipped with immunoreceptor tyrosine-based

switch motifs (ITSM). This motif can be used to bind either activatory or inhibitory adaptor proteins and therefore can lead to different signaling outcomes. Except 2B4, the SRR serve as their own ligands because they are homophilic. 2B4 binds to CD48, a glycosylphosphatidyl-inositol (GPI)-anchored member of the CD2 family. On most cell types these receptors seem to serve the purpose of fine-tuning the immune response (Bhat et al., 2006). On NK cells however, these receptors can induce cytotoxicity and cytokine release. Nevertheless, SRR on NK cells can also enhance signals from other activating receptors and therefore fulfill a co-stimulatory function (Bryceson et al., 2006b). NKp80, just like NKG2D is a member of the C-type lectin like family which will be discussed further in chapter I.4. DNAM-1 is a receptor of the Ig-superfamily and recognizes the molecules CD112 (polio virus receptor, PVR) and CD155 (nectin-2). This receptor can activate NK cells but as for the SRR its role seems to be constricted to mainly co-stimulatory functions .

I.4 The lectin like family of receptors

C-type lectin like family members on NK cells are diverse and most of them are not well studied yet. Genes for these receptors are found in the NK Gene complex (NKC) present on human chromosome 12p13. General features of this family include the formation of disulfide-linked homo- or heterodimers and a type II transmembrane orientation (Yokoyama and Plougastel, 2003). The lectin-like receptors encoded in the NKC can have either activating or inhibitory function and play an important role in regulation of NK cell activity. This gene complex also includes the previously mentioned genes for NKp80 and its ligand AICL, as well as the NKG2 family members and CD94. The NKp80 ligand, AICL, is expressed on monocytes, macrophages and granulocytes. Therefore, NKp80 likely is involved in the crosstalk of NK cells with myeloid cells and influences the initiation and maintenance of inflammatory responses (Welte et al., 2006). Other members of the NKC are NKRP1A, LLT1, and CD69. Interestingly in some cases the genes for the receptors and their ligands are found in close proximity as for NKp80 and AICL and for KLRP1A and Clr (Iizuka et al., 2003; Welte et al., 2006). As has already been described the receptor dimers of NKG2A/CD94 and NKG2C/CD94 bind to the same ligands, namely HLA-E molecules, but transmit opposing signals which are important for NK cell regulation.

Another receptor found in the NKC is NKG2D. It shows only minor homology to the remaining NKG2 family members. NKG2D is expressed as a homodimer and not as a heterodimer with CD94. It recognizes several cell surface glycoproteins that are structurally related to MHC I molecules. Among these are MICA, MICB and UL16 binding proteins (ULBP) 1-4. A common feature of these ligands is that they are upregulated on the surface of stressed or virally infected cells. On primary cells the MICA and MICB proteins can be upregulated after initiation of a DNA damage response through irradiation (Gasser et al.,

2005). The MICA and MICB molecules can be found on various tumor cell lines where they are probably induced through heat shock proteins which become expressed upon malignant transformation (Groh et al., 1999). As tumors are flexible systems that adapt quickly to their surroundings they are able to escape this kind of surveillance. For many tumors the secretion of soluble MICA molecules has been reported. This leads to a downregulation of the NKG2D receptor from the NK cell surface and to anergy of the respective NK cell (Salih et al., 2003). Another important stimulus that leads to upregulation of NKG2D ligands on target cells is the infection with human cytomegalovirus (HCMV) (Guma et al., 2006). NKG2D does not have a cytosolic signaling domain but pairs with the adaptor molecule DAP10 through charged amino acids within its transmembrane domain. DAP10 then promotes the activating signal through activation of PI3K and binding of Grb2 and Vav1 (Wu et al., 1999). In mice, NKG2D can also pair with the adaptor protein DAP12, which is not possible in humans (Lanier, 1998). The activation of NKG2D leads to potent cytotoxic response and also to the production of cytokines. Though beneficial in most cases, NKG2D has also been implicated in autoimmune diseases. Patients with rheumatoid arthritis have been found to carry an unusual type of $CD4^+$ T-cells in their inflamed joints that lacks CD28 but expresses NKG2D (Groh et al., 2003). In addition, NKG2D has also been linked to the onset of autoimmune diabetes in NOD mice (Ogasawara et al., 2004). For many members of the KIR and C-type lectin like family twin receptors have been described that bind the same ligand but transmit opposite signals. This has not yet been the case for NKG2D. Despite its importance for NK cell biology this receptor still retains its solitary position with no inhibitory counterpart described yet.

I.5 The family of Natural Cytotoxicity Receptors (NCR)

Apart from NKG2D, the NCR constitute the main triggering receptors on NK cells. The family of NCR comprises three members, NKp30 (NCR3, CD337), NKp44 (NCR2, CD336) and NKp46 (NCR1, CD335) that have been described several years ago (Pende et al., 1999; Pessino et al., 1998; Vitale et al., 1998). All three receptors belong to the Ig-superfamily. Crosslinking of these receptors with antibodies induces a strong cytotoxic response and the secretion of cytokines. In contrast to the other triggering receptors mentioned previously, the expression of the NCR seems to be confined to NK cells. While other more widely expressed receptors like the SRR or DNAM-1 might function as a co-receptor, the NCR are able to provide potent activating stimuli to the NK cell. NKp30 and NKp46 are readily expressed on resting and activated NK cells, while NKp44 is only upregulated after stimulation with IL-2. In mouse only NKp46 has a functional homologue named Ncr1. Both NKp30 and NKp44 do not have homologues in mice. Therefore, *in vivo* research on these receptors is not possible.

The extracellular domain of NKp46 is formed by two C2-type Ig-like domains. The cytoplasmic tail is very short and does not contain any known signaling motif. Instead, NKp46 pairs with the ITAM containing CD3ζ or FcεRIγ adaptor proteins probably through charged amino acids in their transmembrane domain. Ligands for the NCR seem to be widely expressed on many tumor cell lines but also to a lesser extend on primary cells. The identity of the cellular NKp46 ligand on tumor cells was postulated to involve heparan sulfate structures (Bloushtain et al., 2004). Apart from this finding, NKp46 has been described to directly recognize viral gene products on the surface of infected cells. It can be directly triggered to lyse target cells by the presence of viral hemagglutinins, especially that of influenza virus and the sendai virus hemagglutinin-neuraminidase (Mandelboim et al., 2001). Influenza infection in NKp46 knockout mice results in lethal infection but only minor problems in tumor clearance (Gazit et al., 2006). Therefore NKp46 is at least in mice vitally important to combat influenza virus infections. As influenza virus is a very dangerous human pathogen this again emphasizes the role of NK cells as a first line of defense before the onset of the adaptive immune response. The recognition process of hemagglutinins is mediated by sialic acid linked to a sugar residue in the membrane proximal domain of NKp46. (Arnon et al., 2004). Interestingly, the NKp46 mediated interaction is not only important for the clearance of virus infected cells but also for the activation of the adaptive immune response against influenza and other pathogens. Dendritic cells (DCs) infected with influenza virus have been shown to stimulate IFN-γ secretion of NK cells through engagement of NKp46 and NKG2D (Draghi et al., 2007). In addition to the NK-DC crosstalk, NKp46 activation has also been implicated in the lysis of monocytes infected with the intracellular bacterium *Mycobacterium tuberculosis* (Vankayalapati et al., 2002). Here it seems that this effect was mediated through increased expression of the filamentous protein vimentin on the surface of infected mononuclear phagocytes (Garg et al., 2006).

In a fashion similar to NKp46, NKp44 has been shown to be activated by influenza virus hemagglutinins (Arnon et al., 2001). However, its domain structure is quite different. In contrast to NKp46, NKp44 is equipped with a single V-type Ig-like domain in its extracellular sequence. Another difference to NKp46 is that NKp44 is expressed as a disulfide linked homodimer with charged surface grooves in each subunit (Cantoni et al., 2003). Although NKp44 shows an ITIM consensus sequence in its cytoplasmic domain, no signal transduction has been demonstrated to occur through this motif. In fact, this ITIM seems to be non-functional and not able to recruit inhibitory signaling proteins (Campbell et al., 2004). Hence, the main signal transduction process upon NKp44 activation seems to be mediated through its pairing with the small ITAM containing adaptor molecule DAP12. This interaction probably is conferred through a lysine residue within the transmembrane domain of NKp44. Another similarity to the NKp46 receptor is that NKp44 seems to have a preference for binding to

heparan sulfate residues on tumor cells as well (Hershkovitz et al., 2007). Another interesting finding is that the ligand for NKp44 is upregulated on $CD4^+$ T-cells upon infection with human immunodeficiency virus (HIV). This effect is mediated by a peptide found within the envelope protein gp41 of HIV (Vieillard et al., 2005). This leads to lysis of the respective cells by activated NK cells but what might even be more important this fact could also explain the phenomenon of reduced NCR expression and NK cell function in the blood of HIV-patients (De Maria et al., 2003).

The third member of this family is NKp30. This receptor has one V-type Ig-like domain in its extracellular part and pairs with the adaptor molecule CD3ζ through charged amino acids in its transmembrane domain just like NKp46. Crosslinking of NKp30 leads to cytotoxicity and cytokine secretion of NK cells. Again, heparan sulfate residues were postulated as NKp30 ligands on tumor cells but these results are vigorously debated (Bloushtain et al., 2004; Warren et al., 2005). It would seem, as all NCR have been shown to bind heparan sulfate residues that this might function merely as a rather unspecific co-ligand instead of a functional activating molecule. In contrast to NKp44 and NKp46, NKp30 has not been linked to the binding of viral hemagglutinins. However, NKp30 has been implicated in the recognition of *Trypanosoma brucei* infected erythrocytes (Mavoungou et al., 2007). In this case it seems that a protein of *T. brucei* that is inserted into the erythrocyte membrane is able to activate NKp30 specifically. Another pathogen associated activation of NKp30 occurs during the lysis of filovirus infected DCs. This viral family comprises highly pathogenic strains like Ebola and Marburg virus. Early cytokine production is a key determinant for later survival which implicates that activation of the innate immune system is necessary. NKp30 is able to recognize virus like particles of the filovirus type and activate NK cells to produce cytokines (Fuller et al., 2007). Another striking feature of NKp30 is its involvement in the crosstalk between NK cells and DCs. NK cells are able to lyse immature DCs through engagement of NKp30 despite MHC I expression, but mature DCs are protected from lysis (Ferlazzo et al., 2002). Additionally, NK cells that have been stimulated through NKp30 can produce tumor necrosis factor α (TNF-α) which then induces maturation of DCs (Vitale et al., 2005). DCs in return produce cytokines like IL-12 or IL-18 that can stimulate NK cells (Walzer et al., 2005). The mutual control and regulation of DCs and NK cells again is an interesting example how cells of the immune system communicate and work together in numerous ways. Especially NK cells and DCs through their communication among each other are crucial in bridging the innate and the adaptive immune response.

Signal transduction of the NCR shows similarities with signal transduction through the B- or T-cell receptor. All three receptors transmit their signal through ITAMs of the different adaptor molecules they engage. Upon phosphorylation of the ITAMs by the protein tyrosine kinases (PTKs) Lck and Fyn other PTKs like ZAP70 or Syk are recruited to transduce the

signal further downstream. Remarkably, activation of one NCR leads to additional signal transduction through the adaptor molecules associated with the remaining NCR (Augugliaro et al., 2003). That means, there is a highly productive crosstalk that involves the membrane proximal signaling molecules of the NCR but not those of others as CD16 or KIR2DS4. It seems that the NCR, though structurally different, act in concert as a functional coordinated complex on the NK cell surface. This complex might also involve the GPI-anchored protein CD59 that usually functions as a complement inhibitory protein. Though ubiquitously expressed, on NK cells CD59 is physically associated with NKp30 and NKp46 and can function in a co-stimulatory manner (Marcenaro et al., 2003).

Another hint as to the concerted action of the NCR is the concomitant regulation of their ligands through various pathogens. Ligands for all three NCRs are upregulated on cells infected with *Herpes simplex* virus. Here, the viral gene product ICP0 is sufficient to trigger the upregulation of all NCR ligands (Chisholm et al., 2007). The same phenomenon can be observed after infection of fibroblasts with vaccinia virus (Chisholm and Reyburn, 2006). For both observations it is rather clear that not a viral gene product is the respective ligand but that the endogenous cellular ligands are upregulated upon contact of the cellular machinery with the virus. This mechanism seems to constitute an important antiviral defense of the innate immunity. It is therefore crucial to elucidate the nature of the NCR ligands to be able to further explore this.

I.6 A game of hide and seek - NK cells and cytomegalovirus

The β-herpesvirus HCMV has for a long time been a commensal of the human population. About 90% of the population are carriers. Infection usually occurs at young age as a rather symptomless cold-like sickness. Most people are able to control the spread of the virus which then establishes a lifelong latent state without any symptoms. However, HCMV can be reactivated and cause serious illness in immunocompromised patients. This usually happens in elderly people, after organ transplantation or upon HIV induced AIDS. If acquired during pregnancy, HCMV can cause severe congenital disorders such as hearing loss, chorioretinitis and mental retardation (Pass, 2001). Due to a long time of co-evolution HCMV and its human host have achieved a delicate balance allowing the virus to replicate and spread without causing too much harm. The innate immune system and NK cells in particular play an important role in the maintenance of this balance.

HCMV has a large genome of about 230 kbp. A lot of the genes encoded are dedicated to the modification and control of the host cell machinery and immune escape. The complete replication cycle takes place in fibroblasts although monocytic cells are assumed as permanent reservoirs of latent virus. Gene expression during replication follows a tight schedule involving three cascades of immediate early, early and late genes. Much of the

data available today was obtained with the use of laboratory HCMV strains that have undergone genetic changes and deletions through long term *in vitro* culture. This might lead to biased results as in an *in vivo* situation the conditions for successful replication are rather different (Cerboni et al., 2000). However, dissection of the specific pathways involved in immune escape only became possible after the cloning of the genome of the laboratory strain AD169 as a bacterial artificial chromosome or bacmid (BAC) (Adler et al., 2003). This opened up the whole field of reverse genetics for manipulation of the HCMV genome.

HCMV has evolved a whole battery of strategies to evade detection and attack by the host immune system. In turn of course the human immune system has adapted to these immune evasion strategies and is able to control this viral infection. HCMV expresses a remarkable repertoire of regulatory genes that interfere with host signaling pathways. They affect for example apoptosis, cytokine signaling or cell migration. A very important mechanism concerning the T- and NK cell mediated surveillance is the ability of HCMV to downmodulate MHC I and MHC II expression from the surface of the infected cell. This is a very effective way of escaping T-cell control because these cells are not activated through lack of MHC I but through the presentation of foreign peptides on MHC I molecules. The genes implicated in this process so far are *US2, US3, US6* and *US11*. Although other viral gene products can bind MHC molecules as well, these are not necessarily involved in downmodulation. US3 is able to retain MHC I molecules within the endoplasmic reticulum (ER), while US2 and US11 dislocate MHC I from the ER into the cytoplasm where it is degraded. US6 impairs TAP-mediated peptide transport (Furman et al., 2002; Tortorella et al., 2000). Of course, as described in the chapter about inhibitory receptors, according to the 'missing self' hypothesis, the lack of MHC I molecules on the target cell surface would lead to NK cell activation. However, HCMV has also evolved strategies to avoid the activation of NK cells.

It seems that the non-classical MHC molecule HLA-E that is recognized by the inhibitory NK cell receptor CD94/NKG2A is not downmodulated from the surface of infected cells. HLA-E presents leader peptides derived from other processed HLA molecules and therefore is seen as a marker for overall MHC I expression. The HCMV protein UL40 includes a peptide that is loaded onto HLA-E molecules if MHC I leader peptides are in short supply. This loading process occurs in a TAP-independent manner and increases HLA-E expression on the infected cell surface (Llano et al., 2003). Again, this mechanism can be counteracted by the existence of HLA-E specific cytotoxic T-cells. It has been reported that these cells can recognize the UL40 peptide bound to HLA-E and lyse the infected cell (Pietra et al., 2003). Another possibility would be the engagement of the activating receptor pair CD94/NKG2C by the combination of HLA-E/UL40, but this interaction has not been conclusively demonstrated yet.

As just discussed, NK cells do not only receive negative signals from a putative target cell but also activating stimuli. As a logical consequence HCMV might also have evolved means to avoid expression of activating NK cell ligands. It is of note that recently the HCMV gene product UL141 has been demonstrated to interfere with the expression of CD155, the ligand of the activating NK cell receptor DNAM-1. Interestingly, this HCMV protein is not found in the laboratory strains commonly used and has therefore not been implicated in HCMV immune evasion previousy (Tomasec et al., 2005). The interaction of other activating NK cell receptors and their ligands is affected by HCMV infection as well. Upon infection, the stress inducible NKG2D ligands are upregulated on the target cell surface. HCMV counteracts this alarm signal by expression of various proteins. Of the known NKG2D ligands the HCMV gene product UL16 is able to bind to MICB, ULBP1 and ULBP2 but not MICA or ULBP3 and prevent their presentation on the cell surface (Cosman et al., 2001). It has not been shown how this impacts on NK activity in an *in vivo* setting but it seems that this action can inhibit activation of NK cells at least *in vitro* (Dunn et al., 2003).

The interaction of NKG2D and its ligands plays a particular important role in mice. Here, several viral proteins are engaged in the downmodulation of the mouse NKG2D ligands. Mutant viruses that do not express these proteins have severe problems during the replication process because of their increased susceptibility to NK cell recognition (Mocarski, 2004). The viral protein m152 is not only able to inhibit the passage of MHC I molecules to the surface but also the structurally related mouse NKG2D ligand RAE-1. Additionally the m155 gene product is able to suppress presentation of H60. Some mice strains can control HCMV infection better than others. While BALB/c mice are usually highly susceptible to HCMV infection, the mouse strain C57BL/6 can easily clear the virus. This has been ascribed to the presence of the receptor Ly49H. In its presence the NK cell response against MCMV is enhanced enormously. The ligand for this activating mouse NK cell receptor is the viral m157 gene product. This protein shows structural similarities to MHC molecules and engagement by Ly49H leads to strong activation of the NK cell response. The Ly49 family does not exist in humans. Instead NK cells dispose of an array of MHC binding KIR receptors. Viral ligands for these receptors have not been described yet, but they might emerge with further research.

The NCR are very important receptors for NK cell activation. Their yet unknown ligands have been shown to be upregulated during various viral infections like *Herpes simplex* or *vaccinia* virus. HCMV is so far the only virus that has been demonstrated to dispose of a defense mechanism against recognition by NK cells through NKp30. The tegument protein pp65 of HCMV seems to have the ability to bind to NKp30 and dislocate the signaling CD3ζ chain in a way that makes signal transduction impossible and therefore interferes with NK cell activation (Arnon et al., 2005). Although pp65 is not a secreted protein

and high concentrations in the medium are required to mediate this effect it is nevertheless a functional but inhibitory NKp30 ligand. The overall effect of HCMV on the expression of the NCR ligands has not been examined yet. For the residual NCR NKp44 and NKp46 no viral inhibitory proteins are known. It is possible that the increased expression of their ligands might compensate for the inhibition of NKp30.

Aim of the thesis

The general aim of this thesis was to elucidate new molecules and mechanisms involved in the recognition of putative target cells by NK cells.

NK cells express a multitude of receptors with either activating or inhibiting function. Many of these are engaged upon contact with a putative target cell and transmit a precise picture of the target cell status. The final outcome of this signal integration process is the decision whether the NK cell is to be activated to lyse the target cell or not. A fine balance of these signals is required to enable the defense against pathogens and cancer and at the same time avoid autoimmune reactions. Characterization of the molecules that are involved in creating and maintaining this balance will not only advance our knowledge in the field of innate immunity, it might lead to advances in cancer research as well.

The NKG2 family contains inhibiting as well as activating receptors. Interestingly some of these receptors form pairs that have the same ligand but different signaling capacities. NKG2D plays a very important role for NK cell biology as an activating receptor. For NKG2D no inhibitory counterpart has been described to date. One aim of this thesis was to examine whether NKG2D would have an inhibitory counterpart and the characterization of the putative molecule.

The family of the activating natural cytotoxicity receptors including the members NKp30, NKp44 and NKp46 is thought to play an important role in tumor rejection and viral defense. Although the ligands for these molecules are elusive to date they can be detected on the cell surface with functional assays and with the help of soluble fusion proteins. These proteins contain the extracellular domain of the respective receptor and a tag that can be detected with an antibody. As human cytomegalovirus is a highly specialized pathogen that has evolved numerous ways of immune escape, concerning NK cells in particular, it is an interesting questions whether the NCR play a role in the defense against this virus. Therefore the modulation of NCR ligands by cytomegalovirus was to be further analyzed. For further research on the NCR and their impact on NK cell activity it is crucial to elucidate the nature of the unknown NCR ligands. Therefore yet another aim of this thesis is to finally obtain answers to this question of the identity of the NKp30 ligand.

M. Material and Methods

M.1 Material

M.1.1 Antibodies

mouse monoclonal antibodies

name	antigen	source, reference
MOPC21 (IgG1κ)	unknown	Sigma Taufkirchen, Germany
mouse IgG2aκ	unknown	BD Pharmingen, Heidelberg, Germany
anti-ILZ	isoleucin-zipper-motif	(Stark et al., 2005)
anti-CLEC12B (clone 16)	CLEC12B	(Hoffmann et al., 2007a)
anti-his (AD1.1.10)	histidine-tag	Serotec, Oxford, UK
4G10 (biotinylated)	phosphotyrosine	Upstate, Charlottesville, VA, USA
anti-SHP-1	SHP-1 (aa 492-597)	BD transduction labs, Heidelberg, Germany
anti-SHP-2	SHP-2 (aa1-177)	BD transduction labs
anti-NKp30 (p30-15)	NKp30 (CD337) extracellular domain	(Hoffmann et al., 2007b)
10E4 (ascites fluid)	heparan sulfate proteoglycan	United States Biological Swampscott, Massachusetts, USA
anti-NKG2D	NKG2D	R&D systems

rabbit polyclonal antibodies

name	antigen	source, reference
anti-actin	actin C-terminal peptide	Sigma-Aldrich

secondary antibodies

name	antigen	source, reference
goat anti-human IgG (biotinylated)	F_c portion of human IgG	Jackson ImmunoResearch, Laboratories, West Grove, PA, USA
goat anti-mouse IgG , PE	mouse IgG	Jackson ImmunoResearch
goat anti-mouse IgG, HRPO	mouse IgG	Jackson ImmunoResearch
goat anti-mouse IgG, TRITC	mouse IgG	Jackson ImmunoResearch

M.1.2 Bacteria

strain	use	source
E.coli TOP10 one shot	amplification of plasmids	Invitrogen

M.1.3 Buffers, Chemicals and Reagents

M.1.3.1 Buffers

Lysis buffer (eukaryotic cells)	150 mmol/l NaCl 20 mmol/l Tris-HCl, pH = 7.4 10 % (v/v) Glycerol 0.5 % (v/v) Triton X-100 2 mmol/l EDTA 10 mmol/l NaF 1 mmol/l PMSF (for studies on protein phosphorylation) 1 mmol/l Na-orthovanadate
DNA-sample buffer (6 x)	0.25 % (w/v) Bromphenol Blue 0.25 % (w/v) Xylene Cyanol FF 30 % (v/v) Glycerol in H_2O
MOPS buffer (20 x)	Invitrogen, Carlsbad, CA, USA
PBS	137 mmol/l NaCl 8.1 mmol/l Na_2HPO_4 2.7 mmol/l KCl 1.5 mmol/l KH_2PO_4 pH = 7.4
PBST	1 x PBS 0.05 % (v/v) Tween 20
PBST/NaCl	1 x PBS 0.05 % (v/v) Tween 20 0.5 mol/l NaCl
PBS/pervanadate	1 x PBS 10 mmol/l H_2O_2 200 µmol/l Na-orthovanadate
Reducing sample buffer (5 x)	10 % (w/v) SDS 50 % (v/v) Glycerol 25 % (v/v) 2-Mercaptoethanol 0.1 % (w/v) Bromphenol Blue 0.3125 mmol/l TRIS-HCl, pH = 6.8
TAE (10 x)	Invitrogen, Carlsbad, CA, USA

Western blot transfer buffer	24 mmol/l 129 mmol/l 20 % (v/v)	Tris base Glycin MeOH
HBS (2x)	13 g/l (w/v) 16 g/l (w/v) 3 mmo/l pH = 7.0	HEPES NaCl Na_2HPO_4
ILZ wash buffer	50 mM 300 mM 20 mM 0,05% (v/v) pH 8.0	NaH_2PO_4 NaCl Imidazole Tween 20
ILZ elution buffer	50 mM 300 mM 250 mM 0.05% (v/v) pH 8.0	NaH_2PO_4 NaCl Imidazole Tween 20
IgG elution buffer	50 mM pH 3.0	citric acid
antibody wash buffer 1	3 M 50 mM	NaCl Na-borate
antibody wash buffer 2	3 M 10 mM	NaCl Na-borate
antibody elution buffer	100 mM pH 3.0	glycine

M.1.3.2 Chemicals

Most chemicals were purchased from Roth (Karlsruhe, Germany), Sigma-Aldrich (Seelze, Germany), Merck (Darmstadt, Germany).

M.1.3.3 Reagents

Agarose	Invitrogen, Carlsbad, CA, USA
Ampicillin	Roth, Karlsruhe, Germany
BSA	Serva, Heidelberg, Germany
Chondroitin sulfate	Sigma-Aldrich
Chromium-51	Hartmann Analytik, Braunschweig, Germany
DNA ladder (100 bp and 1 kb)	Invitrogen, Carlsbad, CA, USA
Heparan sulfate	Sigma-Aldrich
Heparin	Sigma-Aldrich

Heparinase III	Sigma-Aldrich
Hyaluronidase	Sigma-Aldrich
Interleukin-2 (IL-2)	NIH cytokine repository
LB broth	Invitrogen
Lipofectamin 2000	Invitrogen
Lipopolysaccharide (LPS)	Sigma-Aldrich
Milk Powder	Töpfer, Dietmannsried, Germany
Ni-NTA Agarose	Qiagen, Hilden, Germany
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich
Precision Plus Protein Standard	Biorad, Hercules, CA, USA
Protein A Agarose	Sigma-Aldrich
Protein G Agarose	Invitrogen
Streptavidin HRPO	Jackson ImmunoResearch
Streptavidin PE	Jackson ImmunoResearch
TrypLE Express	Invitrogen
X-ray films	Perbio/Pierce, Rockford, IL,USA

M.1.4 Cells

All media were purchased from Invitrogen; Fetal calf serum (FCS), non-essential amino acids (NEAA) and sodium pyruvate were from Invitrogen; human serum was from PromoCell (Heidelberg, Germany) and horse serum from Biochrom (Berlin, Germany). X-vivo10 medium was purchased from BioWhittaker (Verviers, Belgium).

If not indicated otherwise all cell media were supplemented with 10% (v/v) FCS and 1% Penicillin/Streptomycin (Invitrogen).

cell name	origin	culture medium
primary human NK cell populations, NKpop	isolated from buffy coats	IMDM, 10% human serum, 1% NEAA, 1% sodium pyruvate, 100 U/ml IL-2
primary human macrophages, MΦ	generated from monocytes from buffy coats	X-vivo10, 2% autologous human serum
human foreskin fibroblasts, HFF	kind gift of Hygiene Institute, Heidelberg	DMEM, 10µg/ml ciprofloxacine (Bayer)
NKL	(Robertson et al., 1996)	RPMI, 100 U/ml IL-2
NK92C1	(Gong et al., 1994) (Tam et al., 1999)	Alpha medium, 12,5% FCS, 12,5% horse serum, 50 µM β-mercaptoethanol
Hek-293T	human embryonic kindney cells	DMEM

Phoenix ampho	Hek-293T derived virus packaging cell line	DMEM
721.221	EBV-transformed B-cell line, human	IMDM
CHO	chinese hamster ovary cells	RPMI, 50 µM β-mercaptoethanol
CHO pgsA-745	CHO deficient in protein glycosylation (Esko, 1991)	RPMI, 50 µM β-mercaptoethanol
EM-3	EBV-transformed B-cell line, human	RPMI
Ba/F3	mouse pre-B cell line	RPMI, 50 µM β-mercaptoethanol
Ag8	myeloma cells used for hybridoma generation	RPMI, 50 µM β-mercaptoethanol
P815	F_c-receptor positive mouse mastocytoma line	IMDM
U937	human histiocytic lymphoma cell line (Sundstrom and Nilsson, 1976)	RPMI

M.1.5 Virus strains

Virus strains were a kind gift of Michael Winkler, University Kiel.

name	description
AD169	laboratory hCMV-strain
pHB5	bacmid of AD169, missing US2-US6
WaBAC1	pHB5, missing TRL1-UL20
WaBAC2	pHB5, missing IRL14-US1
WaBAC3	pHB5, missing US7-US25

M.1.6 Constructs

name	comment
pBABE CLEC12B	expression construct for CLEC12B, with his-tag
pRK5 SHP-1	expression construct for SHP-1
pRK5 SHP-2	expression construct for SHP-2
pZipH CS1	CS1-ILZ fusion protein with his-tag
pZipH NKp30	NKp30-ILZ fusion protein with his-tag

pMEG NKp30	NKp30-IgG fusion protein
pMEG CD99	CD99-IgG fusin protein
human 50K lentiviral siRNA library	#SI206A-1 System Biosciences, Mountain View, CA

M.1.7 Enzymes

name	use	source
restriction endonucleases	DNA digest	New England Biolabs, Frankfurt , Germany
T4 DNA ligase	DNA ligation	New England Biolabs
AMV reverse transcriptase	RT-PCR	First strand cDNA synthesis kit, Roche Diagnostics, Indianapolis, IN
Fast Start Taq DNA Polymerase	PCR	New England Biolabs
Titanium Taq DNA Polymerase	PCR	BD clontech, Mountain View, CA
M-MLV reverse transcriptase	RT-PCR	Invitrogen
T4 exonuclease	double strand DNA digest	New England Biolabs

M.1.8 Oligonucleotides

name	sequence (5’ – 3’)
cDNA synthesis	TGCATGTCGCTATGTGTTCTGGGA
siRNA fw	AATGTCTTTGGATTTGGGAATCTTA
siRNA rev	AAAAAGGGTGGACTGGGATGAGTA
siRNA nested fw bio	CTTCCTGTCAGA (5’ biotinylated)
siRNA nested rev	ATAGAAAGAATGCTTATGGACGCTA
CLEC12B fw	CCGCTCGAGCAGATATCTAATGACATTAACTCAG
CLEC12B rev	GCCGCGGCCGCTTAATCCAAATCCTCAGTCTTCAG

M.2 Methods

M.2.1 Molecular Biology

M.2.1.1 Agarose gel electrophoresis

The DNA was mixed with 6x loading buffer and loaded onto the gel. According to DNA fragment size 1 % or 2 % (w/v) agarose gels were cast containing 0.001 ‰ ethidiumbromide. The correct size of the DNA fragment was controlled using a DNA ladder.

M.2.1.2 DNA digest

Between 1 and 2 µg of DNA were incubated with 2 U of the respective restriction endonuclease for at least 1 h at 37°C. Conditions of the reaction were set according to the manufacturer's instructions. The resulting DNA fragments were separated by agarose gel electrophoresis.

M.2.1.3 DNA sequencing

Sequencing reactions were set up in a 20 µl volume with the ABI Big Dye sequencing mix v1.1 according to manufacturer's instructions. After the sequencing reaction the DNA was precipitated from the samples with ethanol. Pellets were solubilised in water and analysed on an ABI Prism 310 Genetic Analyzer.

M.2.1.4 Isolation of DNA fragments from agarose gels

After excision from the gel DNA fragments were extracted using the Qiagen gel extraction kit according to the manufacturer's instructions.

M.2.1.5 Isolation of plasmid DNA

Bacteria were grown in LB medium at 37°C over night with shaking. Cells were pelleted at 6200xg for 10 min. DNA was prepared using the Qiagen Mini, Midi or MaxiPrep DNA isolation kits according to the manufacturer's instructions.

M.2.1.6 Isolation of total RNA from eukaryotic cells

Routinely $1x10^7$ cells were lysed and total RNA was extracted using the Qiagen RNeasy kit according to manufacturer's instructions.

M.2.1.7 Ligation of DNA

The insert and vector DNA were mixed at a molar ratio of approximately 3:1 in 1x ligation buffer in a 10 µl volume. The mixture was incubated with 2 U T4 DNA ligase for 1 h at room temperature and used to transform competent bacteria.

M.2.1.8 Polymerase chain reaction (PCR)

PCR was performed in a 50 µl volume containing 5 µl 10x Thermopol buffer, 0.2 mM of each dNTP, 0.4 µM forward and reverse primer, 5 U Taq-polymerase and up to 5 µl template DNA or cDNA. Cycles were set according to the respective need, including denaturing at 94°C, annealing and elongation steps at 72°C. For amplification of siRNA sequences the suggested protocol of System Biosciences was followed including the use of Titanium Taq polymerase and elongation steps at 68°C.

M.2.1.9 Reverse transcription (RT)

Usually reverse transcription of total RNA was performed using the First Strand cDNA Synthesis kit from Roche. For amplification of siRNA transcripts total RNA was prepared and reverse transcribed according to instructions given in the manual of the siRNA library. Briefly, total RNA was prepared and reverse transcribed using the cDNA synthesis primer included in the kit and M-MLV reverse transcriptase in the presence of DTT.

M.2.1.10 Transformation of bacteria

Top10 bacteria were transformed according to the manufacturer's instructions. Bacteria were grown on LB-agar plates at 37°C for at least 12 h and selected with the appropriate antibiotic.

M.2.2 Cell Biology

M.2.2.1 Cell culture

All cells were grown at 37°C and 5 % CO_2 under sterile conditions. Cells were split on a regular basis every two to three days. Cell culture flasks were exchanged every week. Cells were frozen in FCS containing 10 % DMSO at -75°C and stored in liquid nitrogen. Cell lines were thawed on a regular basis. FCS, horse serum and human serum were heat inactivated by incubation at 56°C for 30 min prior to use.

M.2.2.2 Cell cycle arrest

Cells were incubated over night with 0.1 µM colchicine or 100 ng/ml nocodazole to arrest the mitotic spindle. As a control cells were treated with DMSO or with 10 µM colchicine for 1 h. Cell cycle arrest was confirmed with PI staining of DNA content and flow cytometric analysis.

M.2.2.3 Cell isolation

Natural killer cell isolation

Peripheral blood mononuclear lymphocytes (PBMCs) were isolated from buffy coats via density gradient centrifugation using Ficoll reagent (PAA, Pasching, Germany). NK cells were isolated from the resulting lymphocyte population using the NK cell negative isolation kit from Dynal Biotech (Oslo, Norway). NK cells were only used when more than 90 % $CD3^-$ and $CD56^+$. NK cells were cocultured with irradiated feeder cellsand activated with 2 µg/ml PHA-P and 100 IU/ml IL-2.

Monocyte isolation and macrophage generation

Monocytes were isolated from PBMCs using the monocyte negative isolation kit from Dynal biotech. The resulting populations were at least 90 % $CD14^+$. Monocytes were seeded into 175 cm^2 tissue culture flasks and differentiated into macrophages through plastic adhesion for 10 days.

Depletion of EM-3 cells

EM-3 cells were stained with NKp30-IgG fusion protein and biotinylated goat anti-human secondary antibody. Then NKp30L positive cells were depleted using anti-biotin microbeads (Miltenyi, Bergisch Gladbach, Germany) using the QuadroMACS magnet system. Additionally, cells were sorted on a BD FACSvantage cell sorter.

M.2.2.4 Cell lysis

If necessary, protein phosphorylation was induced using the phosphatase inhibitor pervanadate prior to lysis. For this purpose, $10x10^6$ cells were stimulated with a PBS/pervanadate solution for 10 min at 37°C. After centrifugation, pelleted cells were resuspended in 0.5 % Triton X-100 lysis buffer supplemented with 1 mmol/l PMSF and if necessary 1 mmol/l Na-orthovanadate and incubated on ice for 20 min. Postnuclear supernantant was collected by centrifugation for 10 min at 16000 x g and 4°C.

M.2.2.5 Chromium release assay

$0.5x10^6$ target cells were labeled in 100 µl assay medium (IMDM with 10 % FCS and 1 % penicillin/streptomycin) with 100 µCi (3.7 MBq) of ^{51}Cr for 1 h at 37°C. Cells were washed twice and resuspended at $5x10^4$ cells/ml in assay medium. Effector cells were

resuspended in assay medium and if necessary preincubated with blocking antibodies (10 µg/ml final concentration) for 15 min at 37°C. After preincubation effector cells were mixed at different effector to target (E:T) ratios with 5000 labelled target cells/well in a 96-well V-bottom plate. Maximum release was determined by incubation of target cells in 1 % Triton X-100 solution. For spontaneous release, targets were incubated without effectors in assay medium alone. All samples were done in triplicates. Plates were incubated for 4 h at 37°C. Supernatant was harvested and ^{51}Cr-release was measured in a γ-counter. Percent specific release was calculated as ([experimental release – spontaneous release] / [maximum release – spontaneous release]) x 100. The ratio between maximum and spontaneous release was at least 3 in all experiments. For redirected lysis assay the F_c-receptor positive cell line P815 was used as target. Effector cells were preincubated with antibodies (0.5 µg/ml final concentration) against NK cell receptors.

M.2.2.6 Flow cytometry

Staining with antibodies

Extracellular surface staining was performed in 96-well V-bottom plates. About 1x10^5 cells were resuspended in 50 µl fluorescence-activated cell sorting (FACS)-buffer (PBS / 2% FCS) containing 10 µg/ml of the respective primary antibody. After 20 min incubation on ice cells were washed with 150 µl FACS-buffer, centrifuged and resuspended in 50 µl FACS-buffer with the appropriate secondary antibody (dilution 1:200). After another incubation on ice for 20 min, cells were washed again and resuspended in FACS-buffer containing 2% formaldehyde and analysed using a BD FACScan or a BD FACScalibur. Results were evaluated with the FlowJo sofware available from TreeStar, Ashland, OR.

Propidium iodide (PI) staining

Cells (2x10^5) were washed with stain buffer (PBS / 0.5% FCS) in a FACS-tube. After centrifugation cells were resuspended in 50 µl ice cold stain buffer. Then 150 µl cold ethanol were added slowly while vortexing cells. Afterwards, cells were stored at -20°C for at least 24 h. After another three wash steps, the supernatant was carefully removed and 300 µl of PI staining solution were added (20 µg/ml PI, 100 µg/ml RNase, 0.1% TX-100). After incubation for 30 min at room temperature cells were analysed via flow cytometry.

Fusion protein staining

About 1x10^5 cells were resuspended in FACS-buffer, containing either 1 µg/ml isoleucin-zipper (ILZ)-fusion protein or 10 µg/ml IgG-fusion protein and incubated on ice for 20 min. Then, cells were washed with 150 µl FACS-buffer and resuspended in 50 µl secondary antibody, either 5 µg/ml of mouse anti-ILZ or 10 µg/ml of goat anti-human IgG (biotinylated). After 20 min incubation on ice cells were washed again and resuspended in

fluorophore conjugated third reagent solution, either goat anti-mouse antibody (PE-conjugated) or streptavidin (PE-conjugated). After a last incubation on ice, cells were washed, resuspended in 100 µl of FACS-buffer with 2% formaldehyde and analysed.

M.2.2.7 Immunofluorescence

HFF cells were seeded on 12mm diameter round coverslips in 12-well plates at a density of 70000 cells per well. After CMV infection cells were washed once with PBS / 2mM $MgCl_2$ and fixed for 7 min in 3% formaldehyde, 2% saccharose in PBS at room temperature. Coverslips were washed in PBS three times and permeabilised with PBS, 0.5% TX-100 for 10 min at 4°C. After another three wash steps, cells were incubated with 30 µl per coverslip of 5 µg/ml ILZ-fusion protein in PBS for 30 min on parafilm. As secondary antibody mouse anti-ILZ was used at a concentration of 10 µg/ml and as detection antibody goat anti-mouse (TRITC-conjugated) in a 1:300 dilution. Coverslips were washed, dehydrated for 30 sec in ethanol, dried and mounted onto object slides using fluoromount G (Southern Biotech).

M.2.2.8 Inhibition of CMV replication

Inhibition of immediate early / early phase

HFF cells were seeded into 6-well plates at a density of $2.5x10^5$ cells per well. The next day 3 h prior to and after infection, cycloheximide was added at a concentration of 50 µg/ml. Afterwards, cells were washed and incubated without inhibitor for 3 h. Then actinomycin D was added to a concentration of 20 µg/ml, cells were further cultivated for 14 h and then harvested and analysed.

Inhibition of late phase

For inhibition of the late phase of the viral life cycle, HFFs were seeded, cultivated and infected in the presence of 100 µg/ml phosphonoformate.

UV-inactivation

Cell supernatant containing HCMV particles was plated in 3.5 cm plastic dishes at a layer thickness of 2-3 mm and irradiated for 20 min in a UV Stratalinker 1800 (Stratagene). After irradiation no infectious virus was detectable upon HFF infection.

M.2.2.9 Production of CMV particles

To produce particles of the laboratory strain AD169 and its derivatives, human foreskin fibroblasts (HFFs) were seeded in 175 cm^2 tissue culture flasks and infected with the respective strain. After 1-2 weeks the supernatant was harvested and cleared by centrifugation. Virus titer was determined in an *in vitro* plaque assay detecting production of viral proteins.

Wild type CMV is not found in the supernatant but spreads in a cell associated manner. Therefore, HFFs were seeded into 175 cm^2 tissue culture flasks and already infected HFF were added. The state of infection could be monitored through the appearance of cytopathic effects in the cell layer. When the culture was thouroughly infected, cells were harvested and used in experiments. If not stated otherwise, HFF were infected at a multiplicity of infection of 1 to 1.5.

M.2.2.10 Transfection of eucaryotic cells

Retroviral gene transfer

Retroviral gene transfer was done using the packaging cell line Phoenix ampho. At day one Phoenix cells were plated in a 25 cm^2 tissue culture flask at a density of $2x10^6$ cells in 4 ml medium and grown for 24h. Cells were transfected with the pBABE vector encoding the gene that was to be transduced using Lipofectamin 2000 according to manufacturer's instructions. To increase transduction effiency an expression plasmid for the viral envelope protein VSV-G was added to the transfection mix. Twelve hours post transfection the medium was exchanged with the medium of the cells that were to be transduced and Phoenix cells were grown for an additional 24 h. Supernatant was harvested and cleared by centrifugation. For transduction, $0.5x10^6$ cells were resuspended in the virus containing supernatant, supplemented with 5 µg/ml polybrene and if necessary IL-2 and infected by centrifugation for 1.5 h at 1000xg and 30°C. Spinfection was done in 12-well plates and cells were afterwards grown over night. The medium was exchanged the next day by pelleting cells and resupending them in fresh medium. Cells were plated on a new 12 well plate. After culturing infected cells for one day, puromycin was added at a concentration of 0.5 µg/ml to select for transfected cells. Transfected cells were expanded and if necessary further enriched by FACS.

Calcium phosphate transfection

Hek-293T cells were plated in a 6-well plate at a density of $0.5x10^6$ cells per well in 2 ml medium and grown for 24h. Cells were then transfected by calcium posphate with 3 to 10 µg DNA. For this purpose, 438 µl of sterile water were mixed with 61 µl of a 2 mol/l calcium chloride solution and DNA. 500 µl 2x HBS was added and mixed by bubbling vigorously. The mixture was then applied dropwise to the Hek-293T cells. After 12 h medium was exchanged. After an additional 24h cells were harvested, pelleted by centrifugation and lysed.

M.2.3 Protein-biochemistry

M.2.3.1 Coomassie staining

Gels were washed three times for 5 min in deionized water, incubated for 1h with SimplyBlue Safe stain (Invitrogen, Carlsbad, CA) on a shaking incubator, washed again and dried.

M.2.3.2 Generation of mouse monoclonal antibodies

For the generation of mouse monoclonal antibodies 10 week old female BALBc mice were immunized intraperitoneally with $5x10^6$ Ba/F3 cells transfected with CLEC12B. Injections were repeated after day 21, 35 and 49. On day 63 mice were boosted with 50 µg of purified CLEC12B-IgG fusion protein in PBS and sacrificed three days later. The spleen was removed and spleen cells were isolated, washed three times in PBS and mixed with $4x10^7$ washed Ag8 cells. The cell mixture was pelleted by centrifugation at 1500 rpm for 5 min, the supernatant was aspirated and cells were incubated at 37°C (waterbath, shaking) for 1 min. Afterwards 1 ml pre-warmed PEG 1500 was slowly applied to the cells within 1 min (waterbath, shaking) and the cells were incubated for an additional 1 min on 37°C (waterbath, shaking). Afterwards 10 ml pre-warmed PBS was applied dropwise to the cells within 5 min (waterbath, shaking). 50 ml HAT medium was added and cells were plated on five 96 well flat-bottom plates (100 µl/well). After one week the cells were fed with HAT medium (100 µl/well). After an additional week the supernatant was screened for secreted antibodies by ELISA with CLEC12B-IgG fusion proteins, positive clones were expanded and subcloned until a monoclonal hybridoma could be selected. The monoclonal antibody was purified from the supernant of the hybridoma cells on protein A agarose columns under high salt conditions. The culture supernatant was adjusted to 3.3 mol/l NaCl and 1/10 volume of a 1.0 mol/l sodium borate solution (pH 8.9) was added. The mixture was then passed through a protein A agarose column. The beads were first washed with a buffer containing 3.0 mol/l NaCl, 50 mmol/l Na-borate, pH 8.9, followed by a wash with a buffer containing 3.0 mol/l NaCl, 10 mmol/l Na-borate, pH 8.9. The antibody was eluted using 100 mmol/l Glycine, pH 3.0. The antibody was dialysed against PBS and the concentration was adjusted to 1 mg/ml. Antibodies were frozen at –20°C and working aliquots were stored at –20°C in 50 % glycerol.

M.2.3.3 Immunoprecipitation

Lysate was pre-cleared and incubated for 1 h at 4°C with 2 µg of control IgG1 antibody, followed by 2 µg specific antibody, both coupled to 20 µl of a 50 % slurry of recombinant protein G agarose in PBS. Samples were washed 3 times in ice-cold lysis buffer, beads were dried and stored at –20°C until samples were analyzed by SDS-PAGE/

Western blot.

M.2.3.4 Production of fusion proteins

ILZ fusion proteins were produced as described elsewhere (Watzl, 2006). IgG-fusion proteins were produced and purified as follows: Hek-293T cells were seeded into 5 175 cm^2 tissue culture flasks and transfected with 25 µg per flask of expression plasmid using the calcium phosphate method the next day. After 12-16 h the medium was exchanged and then harvested 2 and 5 days later and cleared by centrifugation. After filtration through a 0.22 µm sterile filter to remove small particles the supernatant was passed over a protein A column. Bound IgG-fusion protein was eluted with 50 mM citric acid pH 3.0. Fractions were neutralized with 1 M Tris pH 8.0. After dialysis against PBS the protein solution was concentrated using Vivaspin 6 columns (Vivascience, Stonehouse, UK) with a molecular weight cutoff of 10000 dalton. Fusion proteins were analysed for purity on coomassie stained SDS-gels.

M.2.3.5 SDS-Polyacrylamid gel-electrophoresis (SDS/PAGE)

After adding 2.5x or 5x reducing sample buffer, samples were boiled for 5 min at 95°C, centrifuged for 1 min and separated on NuPage gels (Invitrogen, Carlsbad, CA) using 1 x MOPS buffer at 150 V for 1h 15 min.

M.2.3.6 Western Blot

After SDS/PAGE proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Corporation, Bredford, MA) for 1.5 h at 200 mA in Western blot transfer-buffer. PVDF membranes were activated with methanol and washed with transfer-buffer prior to use. After Western blotting, membranes were blocked with either 5 % BSA or 5 % skimmed milk in PBST for 1 h at room temperature and incubated with the primary antibody over night at 4°C. The membrane was washed three times with PBST/NaCl and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. Secondary antibodies were diluted 1:20000 in 2.5 % skimmed milk in PBST. After incubation with the secondary antibody, the membrane was washed three times for 10 min with PBST and developed using either Super Signal West Pico or Dura (Pierce, Rockford, IL).

R. Results

R.1 Characterization of CLEC12B

R.1.1 Identification of CLEC12B

For the understanding of NK cell biology it is important to comprehend how the balance between activating and inhibiting signals is accomplished. One possibility is the existence of receptors with the same specificity but opposite signaling capacities. This mechanism has been described for receptors of the C-type lectin like family, which also include NKG2D. In order to identify a possible counter-receptor for NKG2D a database search was conducted for sequences that showed similarity to the NKG2D extracellular domain but contained an inhibitory signaling motif. The search retrieved a mouse cDNA clone (AK016908) coding for an unnamed C-type lectin protein product of 275 amino acids (BAB30491). Using this sequence as bait, a related human sequence designated human macrophage antigen-H (AY358810) was identified. Comparing the sequences, it appeared that human macrophage antigen-H (232 amino acids) was missing exon 6, resulting in a premature stop codon and an incomplete C-type lectin domain. Searching the genomic DNA data base, the missing exon 6, which showed homology to the murine sequence was identified. Using primers spanning exons 1 to 6 we identified the complete cDNA coding for a 276-amino acid type II transmembrane protein (DQ368812) belonging to the C-type lectin family of receptors (Robinson et al., 2006; Sobanov et al., 2001). The extracellular domain of the putative protein showed highest homology to human NKG2D (36% similarity). A cysteine residue in the stalk region might confer dimerization of the molecule as would be typical for C-type lectin like receptors (Robinson et al., 2006). However, unlike NKG2D it did not contain a charged amino acid in its transmembrane region but an ITIM sequence (VTYATL) in the cytoplasmic tail (Fig. 5 A). The genetic locus of this receptor was mapped to human chromosome 12p13.2 and mouse chromosome 6qF3 in the vicinity of other C-type lectin-like receptors (Fig. 5 B). Overall, the receptor showed a high similarity to CLEC12A (Bakker et al., 2004; Han et al., 2004; Marshall et al., 2004) (34% similarity), which was located on the same chromosomal locus. This new receptor was therefore named CLEC12B. Using RT-PCR expression of CLEC12B was found in human cDNA libraries prepared from various tissues except brain (Fig. 5 C). Interestingly, in mammary gland and ovary only a truncated splice variant lacking exon 4 could be detected.

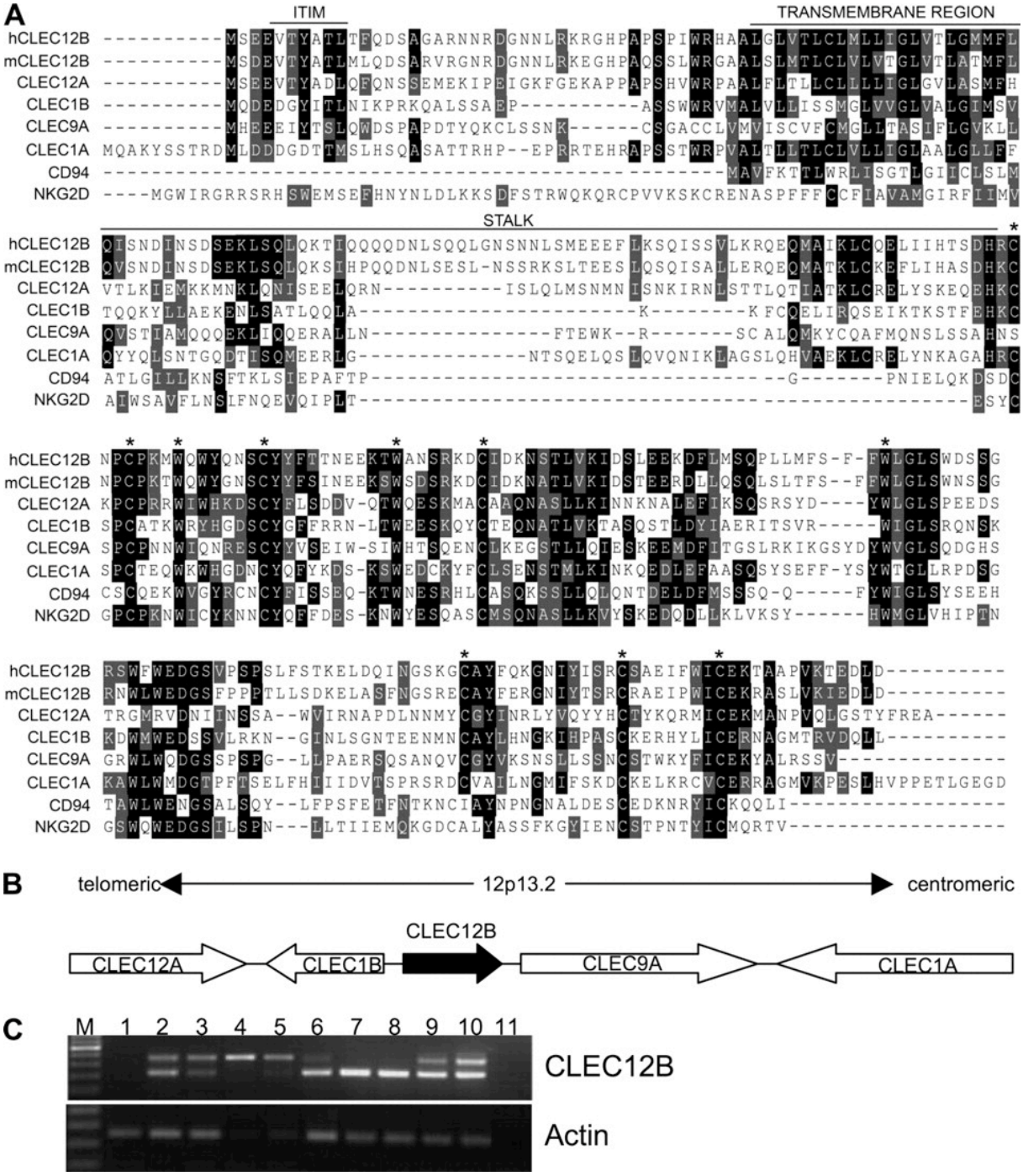

Fig. 5: Characterization of CLEC12B. A, alignment of the predicted amino acid sequence of human and murine CLEC12B with other C-type lectin-like receptors. *Asterisks* indicate the canonic identical amino acids in the C-type lectin-like domain. The intracellular ITIM, transmembrane, and stalk region are indicated. **B**, chromosomal localization of CLEC12B. **C**, PCR analysis of CLEC12B expression (upper panel) using cDNA libraries from human brain (*1*), colon (*2*), heart (*3*), kidney (*4*), liver (*5*), lung (*6*), mammary gland (*7*), ovary (*8*), spleen (*9*), testis (*10*), or water control (*11*). The first round of amplification was performed using primers spanning exons 1 to 6. The resulting product was further amplified using primers spanning exons 3 to 5. The expected amplicon size is 384 bp (*upper band*). The lower band (225 bp) represents a splice variant lacking exon 4. *Lower panel*, actin control.

R.1.2 NKG2D and CLEC12B do not recognise the same ligands

Due to the high similarity between the NKG2D and CLEC12B extracellular domain it might be possible that CLEC12B constitutes an inhibitory counterpart of NKG2D. To test whether it recognizes the same ligands as NKG2D, an IgG-fusion protein of the extracellular domain of human CLEC12B was produced. Ba/F3 cells, transduced to express the human NKG2D ligands MICA, MICB, ULBP1, ULBP2, or ULBP3 were stained at high levels with a NKG2D-IgG fusion protein. However, no binding activity of the IgG-fusion protein containing the extracellular domain of human CLEC12B could be detected (Fig. 6). This demonstrates that there is no apparent overlap in ligand specificity with NKG2D.

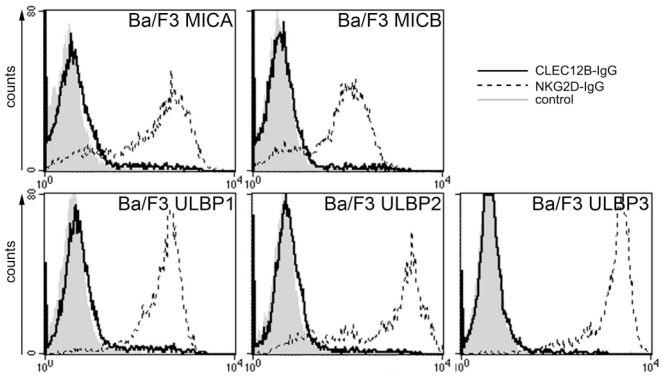

Fig. 6: NKG2D ligands are not CLEC12B ligands. Ba/F3 cells transduced to express the NKG2D ligands displayed in the graphs were stained either with NKG2D-IgG (dashed line) or CLEC12B-IgG (bold line) fusion proteins. Filled gray histograms, control staining using secondary and tertiary reagents only. Results are representative of two experiments.

A common feature of the NKG2 family members NKG2C and NKG2A is, that they can only be expressed at the cell surface in combination with CD94 (Carretero et al., 1997; Han et al., 2004). To test whether this is also true for CLEC12B, the cDNA of CD94 and various other

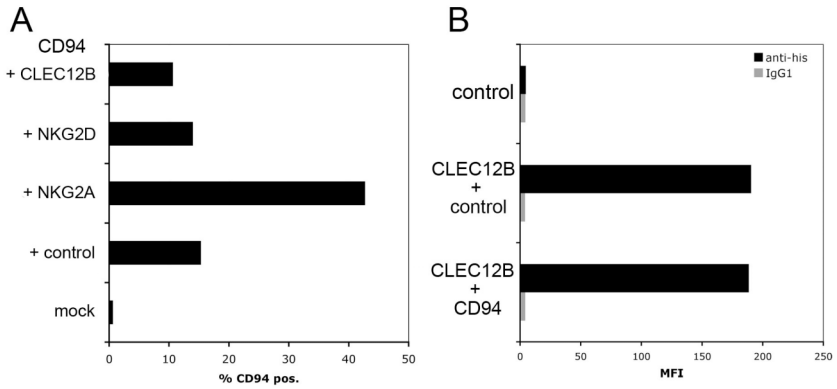

Fig. 7: CLEC12B surface transport is independent of CD94 expression. A, CD94 and NKG2D family members were cotransfected into Hek-293T cells. Surface expression of CD94 was analyzed via flow cytometry. **B**, CLEC12B and CD94 or control DNA were cotransfected into Hek-293T cells and surface expression of CLEC12B was stained with an anti-his antibody and analyzed using flow cytometry.

NKG2 family members were cotransfected into Hek-293T cells. In fig. 7 A CD94 expression at the cell surface has been used as a readout and quantified via flow cytometry. In contrast to NKG2A, CLEC12B is not able to enhance CD94 transport to the cell surface. Vice versa, CLEC12B presentation on the cell surface as measured with an anti-his antibody is independent of CD94 cotransfection (Fig. 7 B).

R.1.3 CLEC12B is expressed on myeloid cells

To examine endogenous CLEC12B expression and biochemical properties, mouse monoclonal antibodies against human CLEC12B had to be generated. Therefore, mice were immunized with Ba/F3 cells transfected with CLEC12B. After a boost with recombinant CLEC12B-IgG fusion protein mice were sacrificed and spleen cells fused to Ag8 hybridoma cells as described in the respective methods section (M.2.3.2). Resulting hybridomas were analyzed for production of a suitable antibody with ELISA, flow cytometry and western blot. Three hybridoma subclones were selected that specifically recognized the receptor in transfected cells either in Western blot (clones 12 and 16, Fig. 8 A and data not shown) or in flow cytometry (clones 30 and 16, Fig. 8 B and data not shown).

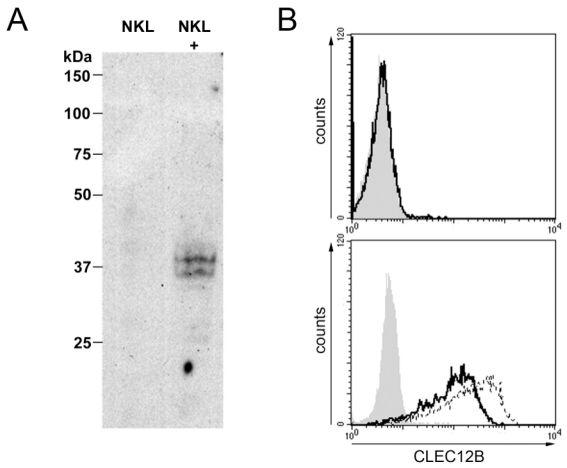

Fig. 8: Characterization of anti-CLEC12B antibodies. A, the anti-CLEC12B mAb clone 12 recognizes CLEC12B by Western blotting. Left lane, untransfected NKL lysate; right lane (+), NKL his-tagged CLEC12B transductant lysate. CLEC12B is detected as a double band. **B**, the anti-CLEC12B mAb clone 30 was tested by flow cytometry on untransfected NKL (upper panel) and NKL transduced with his-tagged CLEC12B (lower panel). Filled gray histogram, isotype control staining; bold line, staining with anti-CLEC12B mAb clone 30; dotted line, staining with anti-His antibody.

CLEC12B was detected as a double band in Western blot analysis of transfected cells, possibly due to differential glycosylation of the receptor.

As several C-type lectin like receptors are expressed on myeloid cells (Robinson et al., 2006) and expression of CLEC12B could not be detected on lymphocytes (data not shown), the human promyelocytic cell line U937 was tested for CLEC12B expression. After PMA stimulation of U937 cells strong CLEC12B expression could be detected on the cell surface via flow cytometry but not on unstimulated cells (Fig. 9 A). The induction of

CLEC12B protein expression was confirmed by Western blot analysis using another anti-CLEC12B-specific mAb (clone 12) (Fig. 9 B). This indicates that CLEC12B can be expressed on monocytic cells after a cell differentiation-inducing stimulus. Interestingly, the endogenous CLEC12B protein was detected as a single band as compared with the double band of the transfected protein. To further characterize CLEC12B expression in primary cells, $CD14^+$ monocytes were purified from human PBMCs. Although no surface expression could be detected on freshly isolated monocytes or any other leukocyte population (data not shown), surface expression of CLEC12B was demonstrated on *in vitro* differentiated human macrophages (Fig. 9 C). This expression was confirmed by Western blot analysis using three independent anti-CLEC12B mAbs (Fig. 9 D and data not shown).

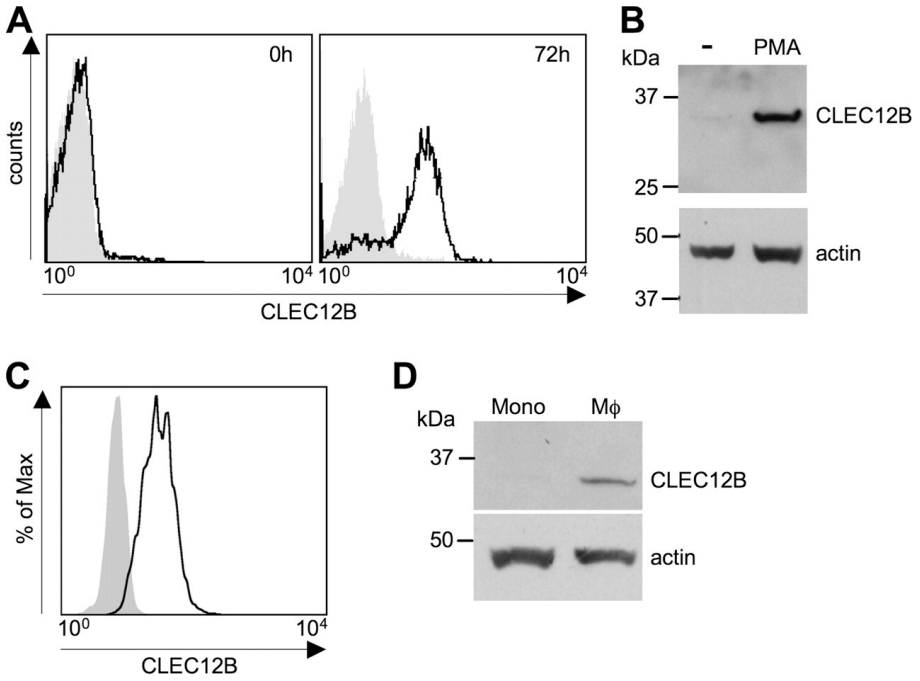

Fig. 9: Expression of CLEC12B. A, CLEC12B expression on U937 cells. U937 cells were stimulated with 10^{-8} M PMA for the indicated times, and cells were stained with an isotype control (filled gray histograms) or anti-CLEC12B mAb clone 30. Results are representative of three experiments. **B**, U937 cells stimulated as described in panel A were lysed and analyzed by Western blotting using the anti-CLEC12B mAb clone 12 (upper panel) followed by anti-actin blotting to control for equal loading (lower panel). **C**, *in vitro* differentiated macrophages were stained with an isotype control antibody (filled gray histogram) or an anti-CLEC12B mAb (clone 30, bold line). **D**, freshly isolated $CD14^+$ monocytes (Mono) or *in vitro* differentiated macrophages (MΦ) were analyzed by Western blotting for the expression of CLEC12B as described in panel B.

R.1.4 CLEC12B can function as an inhibitory receptor

As CLEC12B carries an ITIM sequence in its cytosolic tail an inhibitory function is probable. To test the functionality of the ITIM, the tyrosine residue central to this motif was exchanged with phenylalanine. The his-tagged wild-type (wt) and the mutant receptors were transduced into the NK cell line NKL and surface expression was monitored via flow cytometry (Fig. 10, A and B). In a redirected lysis assay against the F_c-receptor positive cell

line P815 triggering of NKG2D resulted in efficient NK cell cytotoxicity. This activity could be inhibited by co-triggering the CLEC12B wt receptor (Fig. 10 C) but not the mutant receptor (Fig. 10 D). It can therefore be concluded that CLEC12B can function as an inhibitory receptor and that this activity is dependent on the tyrosine residue enclosed in the ITIM sequence.

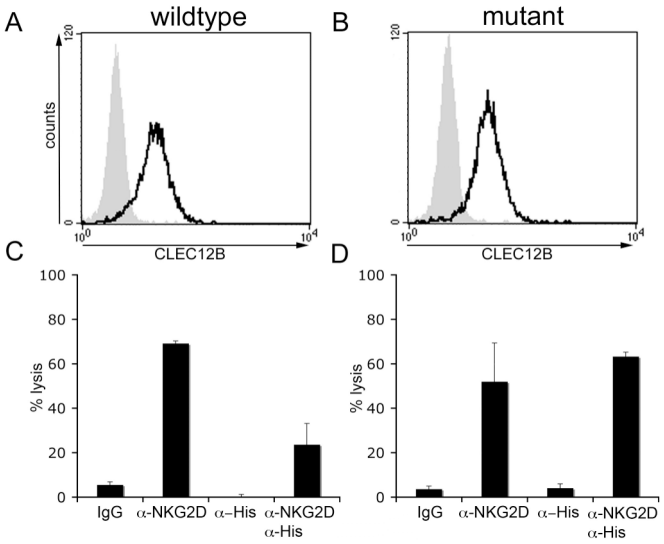

Fig. 10: Functional analysis of CLEC12B. A and **B**, His-tagged CLEC12B wild type (**A**) or ITIM tyrosine-to-phenylalanine mutant (**B**) expression on NKL transductants as determined by anti-his antibody staining for detection of CLEC12B. The use of the anti-CLEC12B mAb clone 30 gave comparable results. **C** and **D**, redirected lysis assay of NKL cells expressing his-tagged CLEC12B wt (**C**) or ITIM mutant CLEC12B (**D**) against P815 cells. Redirected lysis was stimulated by the indicated antibodies (IgG, isotype control antibody). Shown is an effector-to-target ratio of 10:1. Similar results were obtained at an effector-to-target ratio of 5:1 (data not shown). Representative of three independent experiments. Error bars represent SD of triplicates.

R.1.5 CLEC12B signals through recruitment of SHP-1 and SHP-2

ITIMs usually couple to phosphatases to promote inhibitory signaling (Long et al., 2001). To clarify whether this is also true for CLEC12B, Hek-293T cells were transfected with his-tagged wt or mutant human CLEC12B in combination with the phosphatases SHP-1 or SHP-2. Transfected cells were treated with pervanadate to inhibit dephosphorylation and CLEC12B was immunoprecipitated from lysates using an anti-his antibody.

Pervanadate treatment induced detectable tyrosine phosphorylation only in the wt receptor. Interestingly, the lower band of the transfected CLEC12B detected by Western blotting was preferentially phosphorylated (Fig. 11, A and B). Upon phosphorylation the wt, but not the mutant receptor, could recruit SHP-1 and SHP-2 as detected by co-immunoprecipitation (Fig. 11, A and B). This indicates that the inhibitory function of CLEC12B can be mediated by the recruitment of SHP-1 or SHP-2 to its ITIM upon receptor phosphorylation.

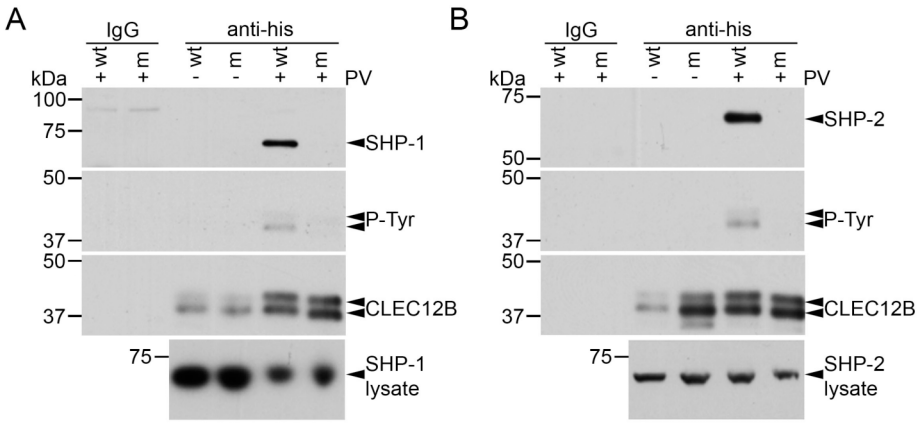

Fig. 11: CLEC12B can recruit SHP-1 and SHP-2. Hek-293T cells were transfected with his-tagged wt or ITIM mutant (m) CLEC12B in combination with SHP-1 (**A**) or SHP-2 (**B**). Cells were treated with (+) or without (-) pervanadate (PV). Lysates were immunoprecipitated using control IgG or anti-his antibodies, and samples were analyzed by Western blotting for co-immunoprecipitated SHP-1 or SHP-2 (top panel), phosphorylated CLEC12B (antiphosphotyrosine (P-Tyr), second panel), and total immunoprecipitated CLEC12B (anti-CLEC12B mAb clone 12, third panel). To demonstrate comparable expression of SHP-1 and SHP-2, lysates were analyzed by Western blotting (bottom panel).

R.2 NKp30 ligands are downregulated upon cytomegalovirus infection

Evolutionary co-development has lead to a sophisticated interplay between the human immune system and HCMV. On the one hand the immune system has developed different means of recognizing and clearing HCMV infected cells on the other hand HCMV has evolved several strategies of immune evasion. NK cells as a first line of defense take a special role in this context (Guma et al., 2006).

R.2.1 Downregulation of NKp30L

To examine if HCMV can influence the expression of the putative cellular ligand for NKp30 (NKp30L), primary HFFs were infected with the laboratory HCMV strain AD169. Three days post infection surface expression of NKp30L was examined by flow cytometry with the help of NKp30-ILZ fusion proteins (Stark et al., 2005). NKp30L expression was readily detectable on the surface of non-infected HFF (Fig. 12 A). HCMV infected HFF showed a clearly reduced surface expression of NKp30L. The same effect was observed when infecting MRC5 lung fibroblasts (data not shown).

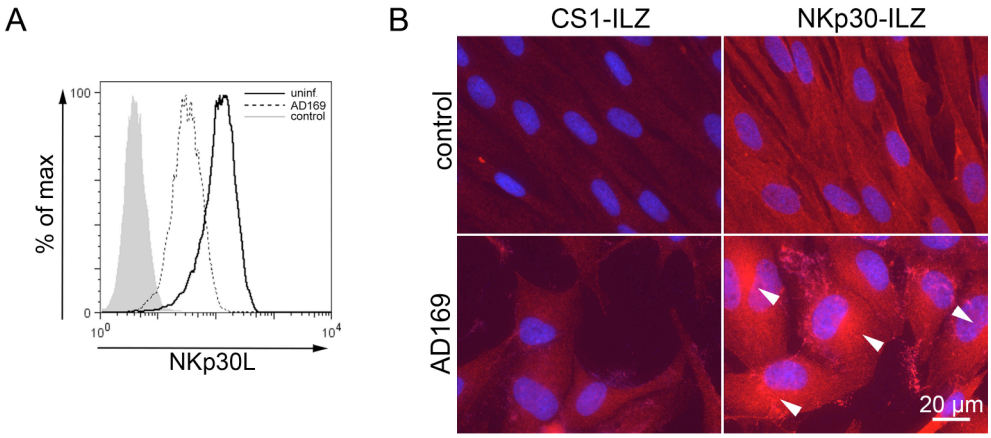

Fig. 12: Modulation of NKp30L in HCMV infection. A, FACS staining of NKp30 ligand (NKp30L) on non-infected and on AD169-infected HFF three days post infection. Grey shadow: control staining with CS1-ILZ; bold line: non-infected HFF; dashed line: AD169-infected HFF. **B**, Immunofluorescence of non-infected and AD169-infected HFF three days post infection. Cells were stained with NKp30-ILZ and with CS1-ILZ as a control. Nuclei were counterstained with DAPI. Arrowheads indicate high density of NKp30L staining. Results are representative of at least three experiments.

To further analyze the reduced surface expression of NKp30L after HCMV infection immunofluorescence staining was performed on non-infected and HCMV-infected HFF. In contrast to a control ILZ-fusion protein, NKp30-ILZ produced a uniform staining in non-infected HFF (Fig. 12 B). In HCMV infected HFF we observed a more clustered staining preferentially in perinuclear regions (Fig. 12 B, arrowheads). This suggests that HCMV

infection results in an intracellular retention of NKp30L, possibly explaining its reduced surface expression.

Laboratory HCMV strains can differ substantially from wild type isolates, a fact which has been demonstrated for the resistance of infected cells to NK cell lysis (Cerboni et al., 2000). To exclude that the observed influence of HCMV on NKp30L is an artifact from the use of the laboratory strain AD169, we performed experiments using two wild type HCMV strains. The clinical HCMV isolates also induced a clear down-regulation of NKp30L on infected HFF (Fig. 13 A and B), demonstrating that this effect on NKp30L surface expression may also be relevant in naturally occurring infections.

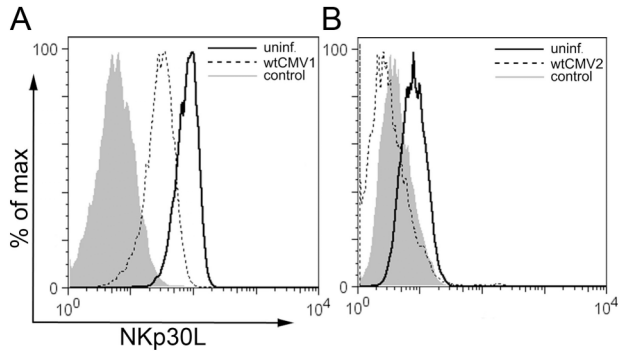

Fig. 13: Wild type HCMV down-modulates NKp30L expression. HFF were non-infected or infected with a HCMV wild type isolate. Three days post infection NKp30L expression was analyzed by FACS staining. Grey shadow: control staining with CS1-ILZ; bold line: non infected HFF; dashed line: wild type-HCMV infected HFF. Results are representative of three (**A**) and two (**B**) independent experiments with two different wild type isolates. Differences in NKp30L staining intensity are due to different HFF preparations.

R.2.2 An immediate early/early HCMV gene product is responsible for NKp30L down-regulation

Down-regulation of NKp30L could either be due to a cellular process initiated upon viral entry like an interferon response (Pichlmair and Reis e Sousa, 2007) or due to a viral gene product that actively interferes with NKp30L presentation. To investigate this, AD169 viral particles were inactivated by UV-irradiation and used to infect HFF. UV-irradiation cross-links the viral DNA, making transcription and replication impossible but leaving the proteinaceous components of the virus intact. This treatment abrogated the effect of HCMV on NKp30L down-modulation (Fig. 14). Hence, some HCMV gene product has to be expressed *de novo* after infection of the cell to cause the observed effect.

In order to identify that putative gene product three HCMV deletion mutants were created, WaBAC1, WaBAC2 and WaBAC3 based on the AD169 bacterial artificial chromosome (BAC) pHB5 (Borst et al., 1999). In this BAC the genes US2 to US6 have been replaced by the BAC-cassette. Fig. 15 A depicts a schematic overview over the HCMV genome and the deleted regions.

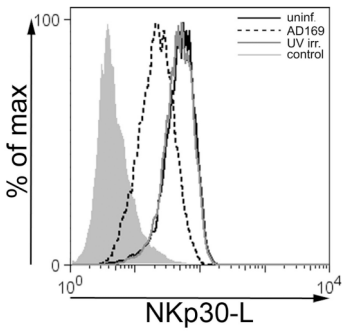

Fig. 14: UV-inactivation of HCMV abrogates NKp30L down-modulation. HFF were infected either with control virus or with UV-irradiated HCMV particles. Staining was done on day three post infection. Grey shadow: control staining with CS1-ILZ; bold black line: non-infected HFF; dashed line: AD169-infected HFF; bold grey line: HFF infected with UV-inactivated AD169 particles.

WaBAC1 lacks the region from *TRS1* to *UL20*. Here, many open reading frames (ORF) can be found whose function has not yet been described. However, this deletion mutant still retained the ability to down-modulate NKp30L surface expression (Fig. 15 B). In WaBAC2 the ORFs comprising *IRL14* to *US6* were deleted. This region encompasses most of the genes implicated in MHC I down-regulation (Mocarski, 2004). As shown in Fig. 15 C, down-modulation of NKp30L was still possible in the absence of these genes. WaBAC3 has a deletion in the region from *US2* to *US25*, which also contains immunomodulatory and many undescribed genes. The resulting virus WaBAC3 showed some growth defects and had to be concentrated to achieve the same MOI as for the other viruses. However, the deletion had no influence on NKp30L down-modulation upon infection (Fig. 15 D). The differences in the extent of the NKp30L down-modulation between the deletion mutants were not significant (data not shown). The creation of further virus deletion mutants was hampered by the fact that essential genes for the replication of HCMV would be affected.

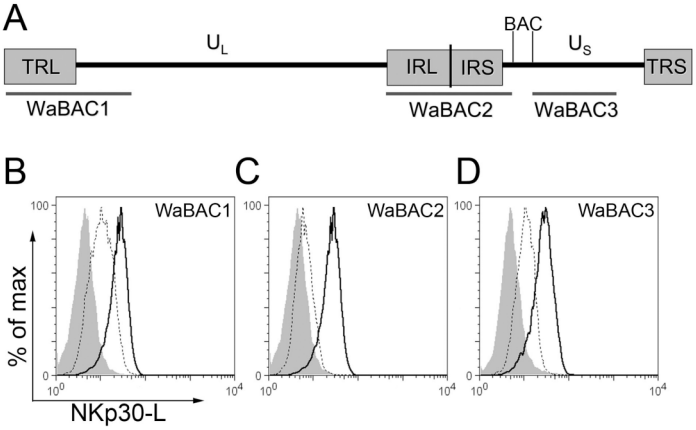

Fig. 15: Analysis of HCMV deletion mutants. A, schematic representation of the HCMV genome and the respective deleted regions. All mutants lack the genes *US2-US6* where the BAC-cassette was inserted (BAC). Black horizontal bars indicate the respective deleted regions. TRL: terminal repeat long; U_L: unique long; IRL: inverted repeat long; IRS: inverted repeat short; U_S: unique short; TRS: terminal repeat short. **B-D**, NKp30L staining after infection with respective virus mutant. Grey shadow: control staining with CS1-ILZ; bold black line: non infected HFF; dashed line: virus infected HFF; **B** WaBAC1: deletion of *TRL1-UL20*. **C** WaBAC2: deletion of *IRL14-US1*. **D**, WaBAC3: deletion of *US7-US25*. Results representative of at least three experiments for each virus mutant.

HCMV genes are expressed in a timely ordered fashion. In order to narrow down further candidates we inhibited different stages of the HCMV replication cycle. First, the translation of immediate early and early genes was inhibited via cycloheximide and actinomycin D treatment. Second, the late replication stage of HCMV was blocked by inhibiting the HCMV polymerase with phosphonoformate. Because of the toxicity of these inhibitors, cells had to be analyzed 20 h post infection to produce reliable results. At this time of infection the effect on the surface expression of NKp30L was detectable (Fig. 16 A), but less pronounced compared to three days post infection (Fig. 12). Blocking late HCMV genes by treatment with phosphonoformate did not affect the down-regulation of NKp30L after HCMV infection (Fig. 16 C). Blockade of the immediate early and early infection phase lead to some reduction of NKp30L surface expression already in non-infected HFF (Fig. 16 B). However, no additional inhibition could be observed after HCMV infection. Fig. 16 D depicts the inhibition of NKp30L expression upon infection calculated from the MFI. Statistical analysis was performed using one-way ANOVA. The inhibition of the IE phase of infection led to a significant reduction of NKp30 downregulation ($P<0.01$). This indicates that an immediate early/early HCMV gene might be responsible for NKp30L down-modulation.

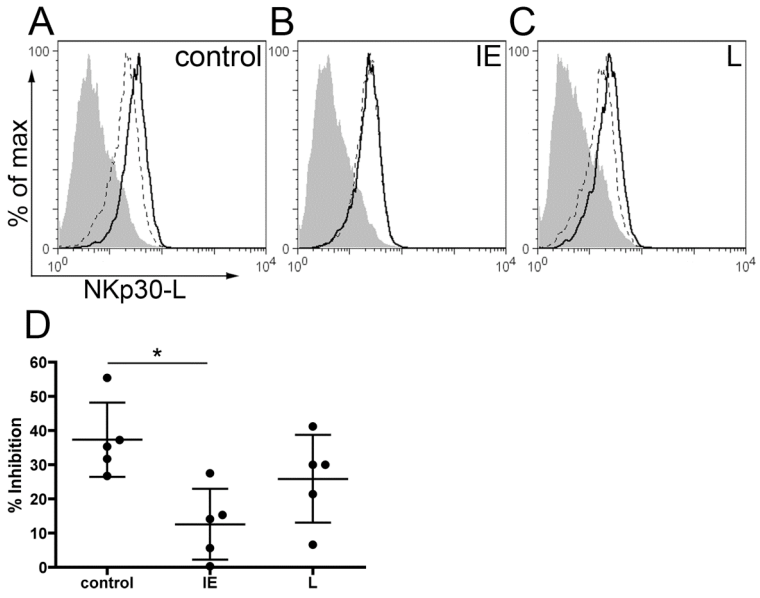

Fig. 16: Inhibition of HCMV infection stages. HFF were treated as described in the materials and methods section to inhibit immediate early and early gene transcription (IE) and late gene expression (L). Cells were harvested 20 h post infection and stained for NKp30L expression. Grey shadow: control staining with CS1-ILZ; bold black line: non infected HFF; dashed line: AD169 infected HFF. Results representative of five independent experiments.

R.2.3 Reduced NKp30-dependent NK cell lysis of HCMV infected fibroblasts

Next, the functional consequences of fibroblast HCMV infection on NK cell killing activity were determined. HCMV infection results in a down-modulation of MHC I surface

expression ((Mocarski, 2004) and data not shown), thereby reducing NK cell inhibition. This might counteract any possible influence of NK cell activation through the modulation of NKp30L expression. In order to reduce the effect of MHC I modulation on NK cell activity we used the NK cell line NK92C1, which lacks the expression of any functional inhibitory KIR or CD94/NKG2A receptors (Komatsu and Kajiwara, 1998). As shown in Fig.17 B, NK92C1 readily lysed non-infected HFF. This lytic activity was partially blocked by the addition of anti-NKp30 antibodies, demonstrating that the lysis of HFF by NK92C1 is dependent on NKp30 triggering. With AD169 infected HFF the lysis by NK92C1 was reduced compared to non-infected cells and anti-NKp30 antibodies no longer blocked lysis (Fig. 17 C). This is consistent with the observed down-regulation of NKp30L in the infected cells (Fig. 17 A) and demonstrates that the reduction in NKp30L as detected by flow cytometry has also functional consequences for NK cell killing.

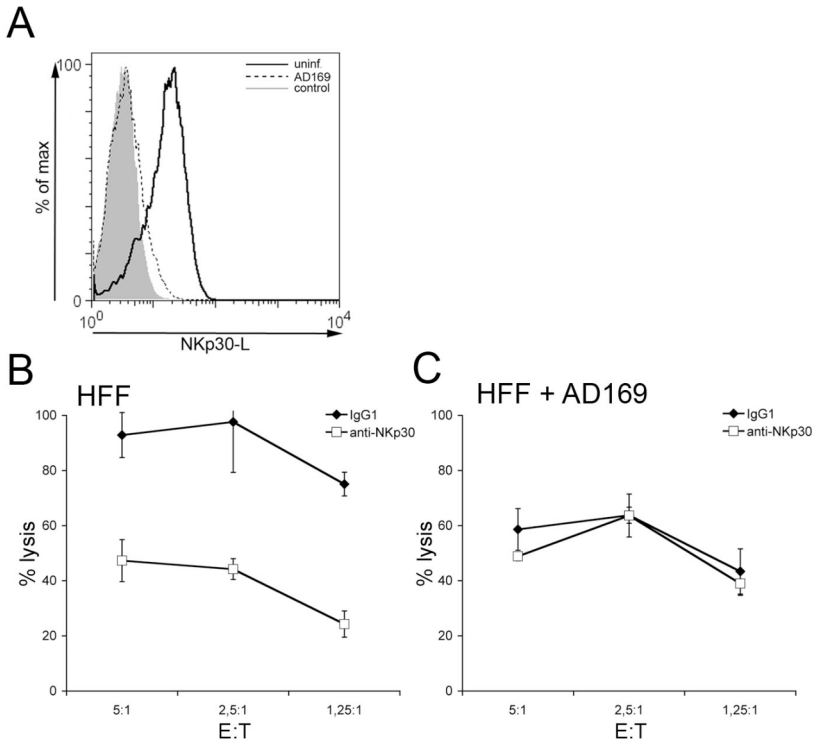

Fig. 17: Down-modulation of NKp30L has functional consequences. A, HFF were infected with AD169 and NKp30L expression was determined three days post infection. Grey shadow: control staining with CS1-ILZ; bold black line: non infected HFF; dashed line: AD169 infected HFF. **B**, **C**, The same cells were analyzed in a chromium release assay using NK92-C1 cells as effectors and non infected (**B**) or AD169 infected (**C**) HFF as targets. IgG control or anti-NKp30 antibody were added to a final concentration of 10 µg/ml. Filled diamonds: IgG-control; open squares: anti-NKp30 antibody. Error bars indicate SD. Representative of four independent experiments.

In another strategy to avoid the effect on MHC I expression by HCMV, the deletion mutant WaBAC1 was used the for infection. In contrast to AD169 this virus was no longer able to down-regulate MHC I molecules from the surface of infected cells. Instead, a slight up-regulation was to be observed, probably due to an interferon-response (data not shown).

This made it possible to use primary IL-2 activated NK cells for the functional assay. As shown in Fig. 18 B lysis of non-infected HFF was less dependent on NKp30 as with the NK92C1 cell line, which is likely due to the presence of the full set of activating receptors on primary NK cells. Nevertheless, lysis was still blocked significantly by the addition of anti-NKp30 antibodies. Similar to the experiment with AD169 and NK92C1 cells, HFF infected with the mutant WaBAC1 HCMV showed reduced lysis by primary IL-2 activated NK cells and this lysis was no longer blocked by the addition of anti-NKp30 antibodies (Fig. 18 C). This change in NK cell reactivity against infected HFF correlated again with the observed down-modulation of NKp30L upon HCMV infection (Fig. 18 A).

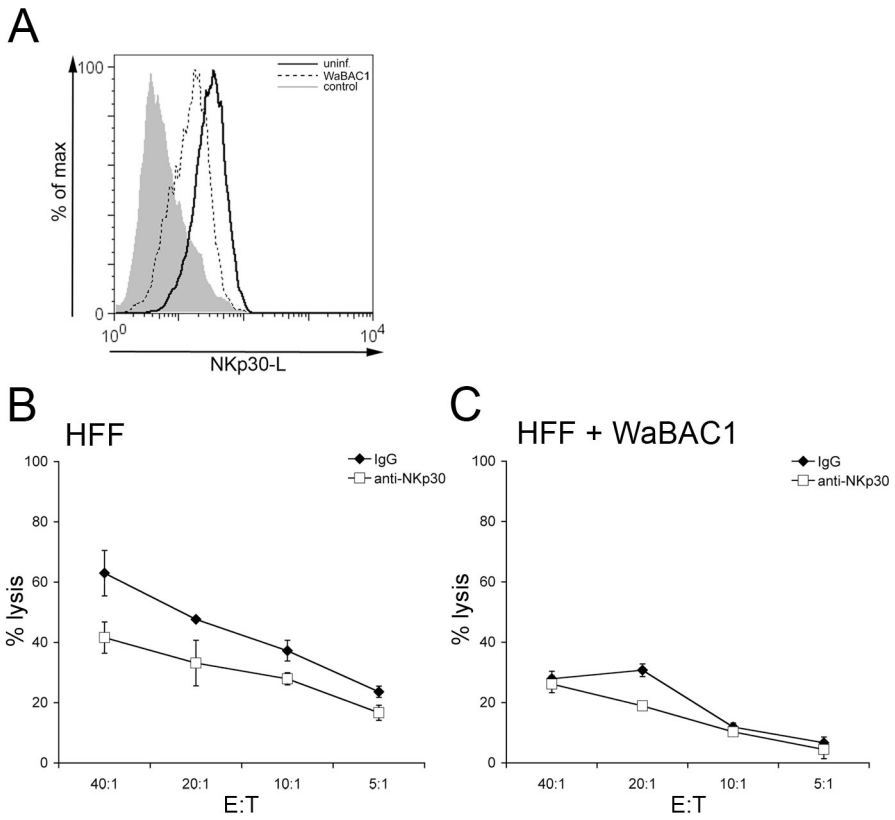

Fig. 18: Reduced NKp30-dependend lysis of HCMV infected HFF by primary NK cells. A, HFF were infected with AD169 and NKp30L expression was determined three days post infection. Grey shadow: control staining with CS1-ILZ; bold black line: non-infected HFF; dashed line: AD169-infected HFF. **B**, **C**, The same cells were analyzed in a chromium release assay using primary IL-2 activated NK cells as effectors and non-infected (**B**) or HCMV-infected (**C**) HFF as targets. IgG control or anti-NKp30 antibody were added to a final concentration of 10 µg/ml. Filled diamonds: IgG-control; open squares: anti-NKp30 antibody. Error bars indicate SD. Representative of three independent experiments

R.3 Identification of NKp30 ligands

R.3.1 Heparan sulfate is not the ligand for NKp30

The involvement of heparan sulfate structures as a possible NKp30 ligand is controversially discussed (Bloushtain et al., 2004; Warren et al., 2005). Both groups used the hamster derived cell line CHO as a model system. Central to this approach are several CHO mutants lacking different proteoglycans (Esko, 1991). To finally shed light on the identity of the cellular NKp30L it was necessary to establish the role of heparan sulfate as NKp30 ligand as part of this thesis.

The first step was to reproduce the results of the previously published papers. As described by Bloushtain et al, staining with NKp30-ILZ fusion proteins was strongly but not completely reduced on the CHO mutant pgsA-745 (Fig. 19 A). This cell line lacks the enzyme xylosyltransferase and is therefore proteoglycan deficient. The same experiment was conducted with CHO cells obtained from Hillary Warren and Angel Porgador with identical results (data not shown). Hence, the differences in the published results are not caused by the use of unequal cell lines. The reduced staining intensity on CHO pgsA-745 might indicate that NKp30 fusion proteins show a high affinity for polysaccharide structures.

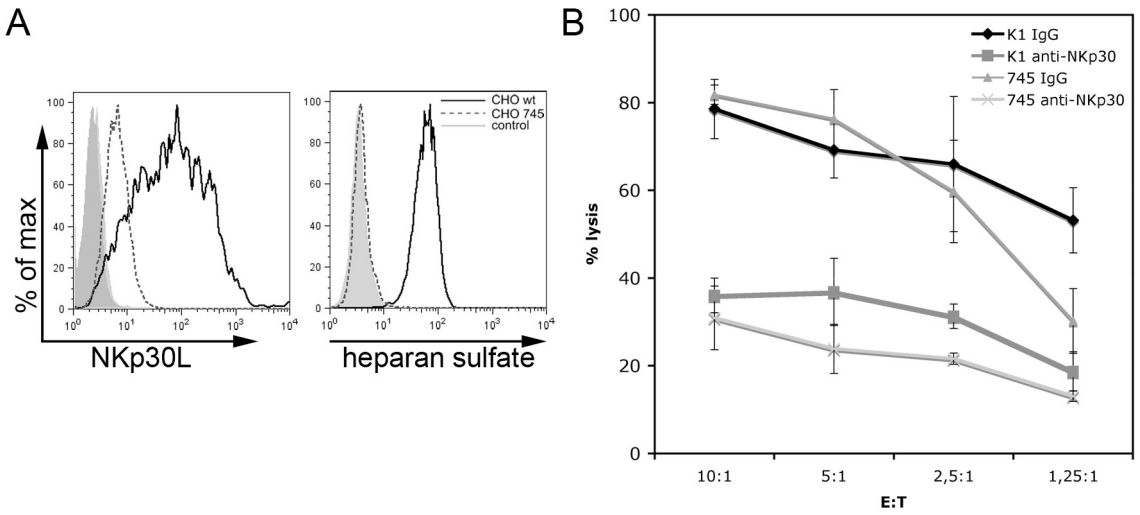

Fig. 19: Experiments in CHO cells are biased. A, wt CHO cells (bold line) and proteoglycan deficient CHO pgsA-745 (dashed line) were stained with NKp30-ILZ fusion proteins (left panel) and with anti-heparan sulfate antibody (right panel). **B**, cytotoxicity assay. Lysis of CHO wt (K1) and CHO pgsA-745 by NK92C1 was blocked by addition of anti-NKp30 or control antibody. Error bars represent SD of triplicates. Shown is one representative experiment of two.

As fusion protein binding does not necessarily indicate the existence of functional NKp30L this was tested via a cytotoxicity assay. Lysis of the wt CHO cells (labeled K1) could be inhibited by addition of anti-NKp30 antibody. Therefore NK cell activation by these cells is dependent on NKp30 engagement. The same result was seen when CHO pgsA-745 were

used as target cells, despite their reduced fusion protein binding capacities (Fig. 19 B). This means that the same amount of functional NKp30L can be found on both cell lines, wt and mutant. In conclusion, it seems that activation of NK cells through NKp30 is not influenced by the lack of proteoglycan structures. The fact that the results from the fusion protein staining did not fit the functional data obtained from the killing assays might be due to unspecific binding of the fusion proteins. This does not seem unexpected as hamster cells are evolutionary far away from human cells and probably display a very different surface structure.

Therefore, the following experiments were carried out on human cell lines. Here, the influence of heparan sulfate residues on fusion protein binding was explored as well. A first attempt to silence xylosytransferase expression via an siRNA approach failed (data not shown). Hence, it was tested whether heparan sulfate would work to block NKp30 activation. NKp30L positive Hek-293T cells were stained with NKp30 ILZ- and IgG-fusion proteins with addition of 10 µg/ml heparan sulfate or chondroitin sulfate as a control. Neither polysaccharide could inhibit the binding of the respective fusion protein (Fig. 20 A) while binding of an anti-heparan sulfate antibody was strongly inhibited at the concentration used (data not shown). In another approach Hek-293T cells were treated with heparinase III that specifically cleaves heparan sulfate residues. Removal of heparan sulfate residues was confirmed by staining with an anti-heparan sulfate antibody (data not shown). As a control, cells were treated with hyaluronidase. No influence on fusion protein binding could be detected (Fig. 20 B).

The data obtained with fusion proteins was further confirmed via functional assays. As shown in fig. 20 C, addition of heparan sulfate to a cytotoxicity assay did not yield a functional effect on the activity of primary NK cells. The same was true for the addition of heparin or chondroitin sulfate. Lysis was also not inhibited when target cells were preincubated with heparinase III or hyaluronidase before addition to the assay (Fig. 20 D). Hence, it is very improbable that heparan sulfate constitutes a functional cellular ligand for NKp30.

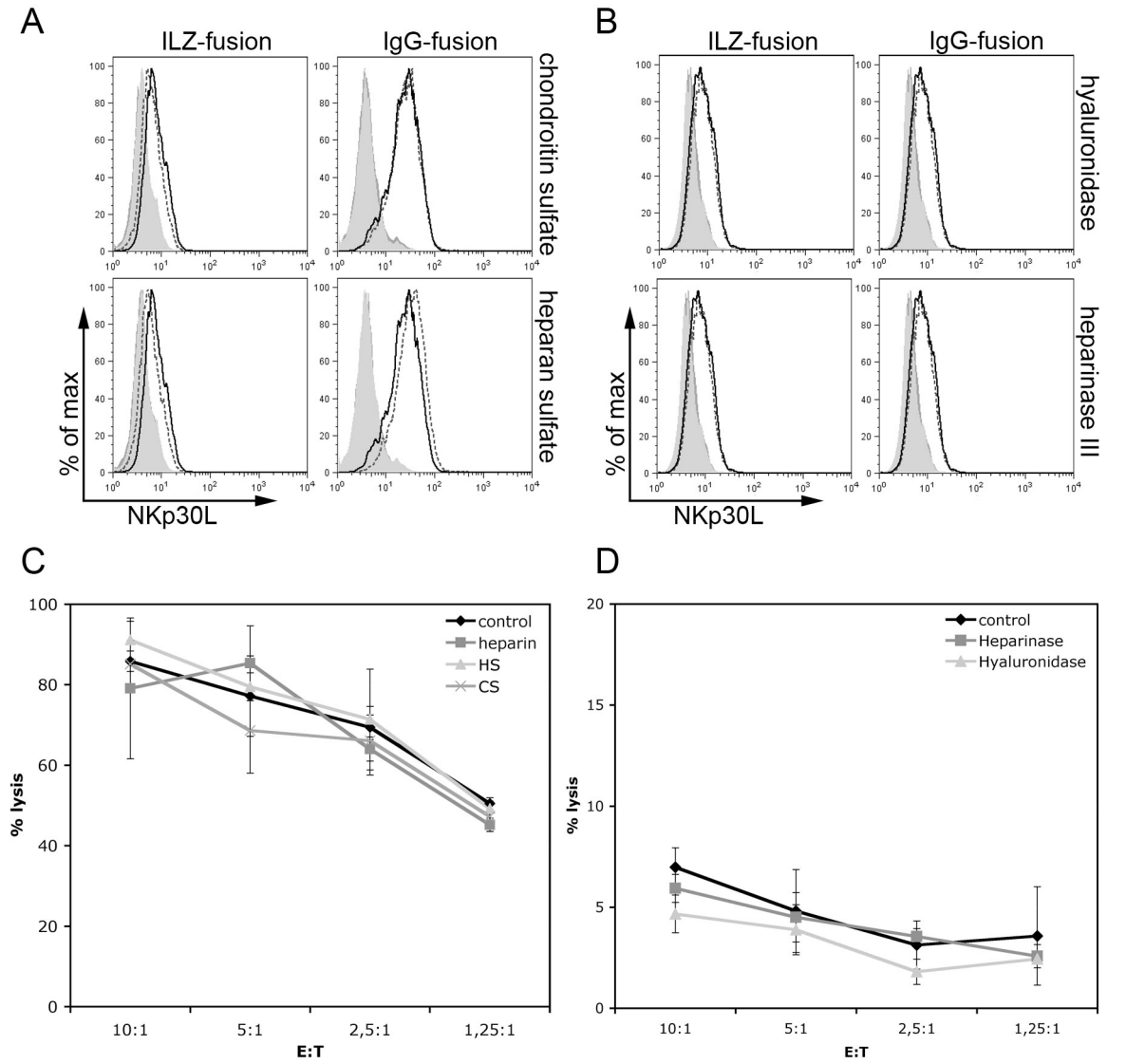

Fig. 20: Heparan sulfate does not mediate functional effects through NKp30. A, Hek-293T cells were stained with NKp30-ILZ or -IgG fusion proteins in the presence (dashed lines) or absence (bold lines) of heparan sulfate (lower panels) or chondroitin sulfate (upper panels) as a control. **B**, Hek-293T cells were treated with hyaluronidase (upper panels) or heparinase III (lower panels) for 2 h at 37°C and then stained with NKp30-ILZ or –IgG fusion proteins. **C**, Heparin, heparan sulfate (HS) or chondroitin sulfate (CS) were added to a cytotoxicity assay of primary NK cells against Hek-293T cells at a concentration of 10 µg/ml. **D**, NK92C1 were used in a cytotoxicity assay against Hek-293T cells that were preincubated with heparinase III or hyaluronidase for 2 h at 37°C. Error bars represent SD of triplicates.

R.3.2 Characterization of NKp30L

If heparan sulfate does not constitute the functional NKp30L, the nature of the NKp30 ligand is still undefined. Further experiments were conducted to find out more about the characteristics of the elusive molecule. On the one hand Hek-293T cells were trypsinized to explore whether NKp30L would also involve a proteinacious component. As shown in Fig. 21A trypsinization reduces binding of NKp30-ILZ fusion proteins to the cell surface considerably. This effect is almost completely reverted when the cells are incubated for 4 h at 37°C before the staining procedure (Fig. 21 B).

These results could also be confirmed with a cytotoxicity assay. As depicted in fig. 21 C, trypsinization of Hek-293T cells led to a significant reduction of NK92C1 activity. Addition of anti-NKp30 antibody reduced the lysis of both targets to almost zero. The remaining lysis of the trypsinized cells most probably is mediated through residual NKp30L that is brought to the surface during the course of the cytotoxicity assay. This might also hint to a rather high turnover rate of the putative ligand.

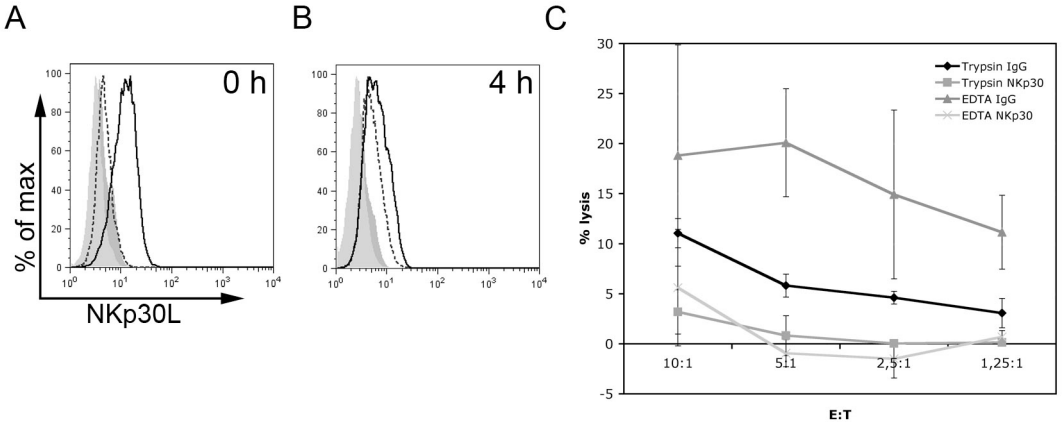

Fig. 21: NKp30L is trypsin sensitive. A, **B**, Hek-293T cells were control treated (bold lines) or incubated with trypsin for 10 min (dashed line) and stained immediately(**A**) or incubated for 4 h at 37°C before the staining procedure (**B**). **C**, Hek-293T cells were control treated (EDTA) or trypsinized and used as targets in a cytotoxicity assay with NK92C1 cells. Lysis was blocked with anti-NKp30 antibody. Error bars represent SD of triplicates.

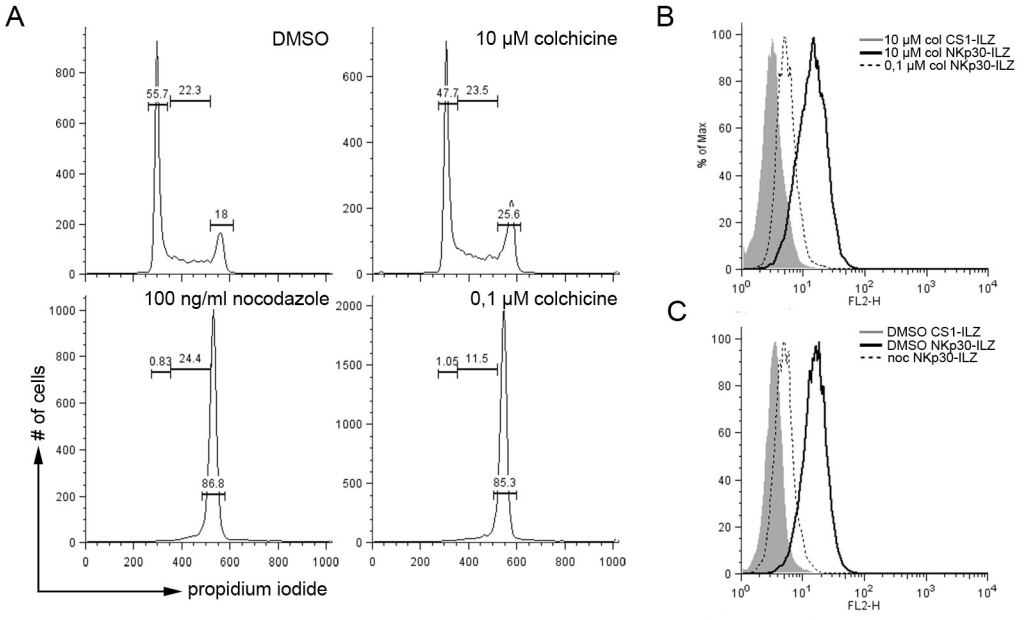

Fig. 22: NKp30L is regulated in a cell cycle dependent manner. Hek-293T cells were arrested in M-phase using 100 ng/ml nocodazole or 0.1 µM colchicine. **A**, PI staining of treated cells to show DNA content. DMSO and 10 µM colchicine for 1 h were used as a control. **B**, NKp30L staining of cells treated with colchicine. **C**, NKp30L staining of cells treated with nocodazole. Representative of at least three independent experiments.

Recently, it has been shown that NK cells preferentially adhere to cells going through mitosis (Nolte-'t Hoen et al., 2007). The NCR NKp46 has been implicated in this process. Hence it

became interesting to query the role of the NCR NKp30 in dividing cells. Hek-293T cells were arrested in M-phase with two different inhibitors, either colchicine or nocodazole. Cell cycle arrest was confirmed through staining with propidium iodide (PI, fig. 22 A). Both colchicine (Fig. 22 B) and nocodazole (Fig. 22 C) treatment led to a reduced surface staining of NKp30L. Thus it seems that contradictory to the NKp46 ligand, NKp30L is downregulated during mitosis.

R.3.3 Identification of NKp30L through an siRNA library

As attempts to identify NKp30L with common methods like expression cloning or biochemical approaches have failed so far, NKp30L might not consist of a single protein component but rather a complex structure or enzymatic modification. Therefore, a siRNA library was chosen as the appropriate method because complex structures can be disrupted upon loss of a single component.

The human Epstein-Barr-virus (EBV)-transduced B-cell line EM-3 was selected for this process as it expresses NKp30L (Byrd et al., 2007) and does not seem to mediate any unspecific effects in fusion protein binding as has been observed for other cell lines. Fig. 23 shows a scheme of the transduction and selection process.

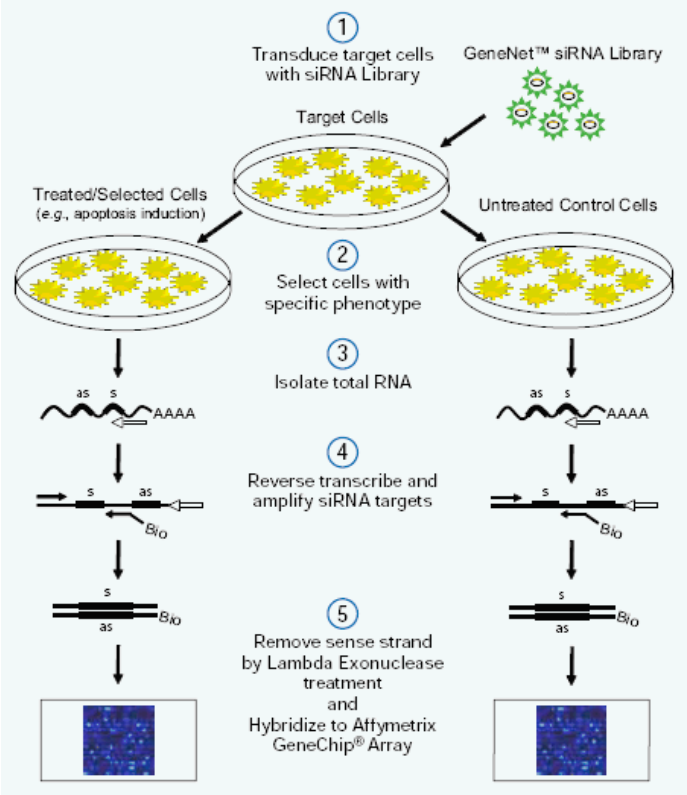

Fig. 23: schematic overview over the transduction and enrichment process of the siRNA library. EM-3 cells were infected with lentiviral particles containing the siRNA libray and a puromycin selction marker. After selection NKp30L negative EM-3 cells were enriched via NKp30-IgG fusion protein staining and subsequent depletion using the Miltenyi MACS technology and FACS-sorting. From the enriched population total RNA was purified and analyzed for enriched siRNAs.

The commercially available siRNA library human 50K was transduced into EM-3 cells with the help of prepackaged lentiviral particles and selected with puromycin (Fig. 23, step 1). The resulting population was enriched for cells that had downregulated NKp30L via NKp30-IgG fusion protein staining and depletion using the MACS system as described in the material and methods section. A second MACS selection step was followed by two further rounds of enrichment by fluorescence activated cell sorting (FACS) (Fig. 23, step 2). Expression of NKp30L in the whole cell population was controlled via flow cytometry as shown in fig. 24 A-C. In addition, subclones were produced through limiting dilution and analyzed for their ligand expression by flow cytometry. To verify flow cytometric data, a cytotoxicity assay was performed. As shown in fig. 24 D, a slight downmodulation of the lytic activity could be observed that also correlated with the respective fusion protein staining.

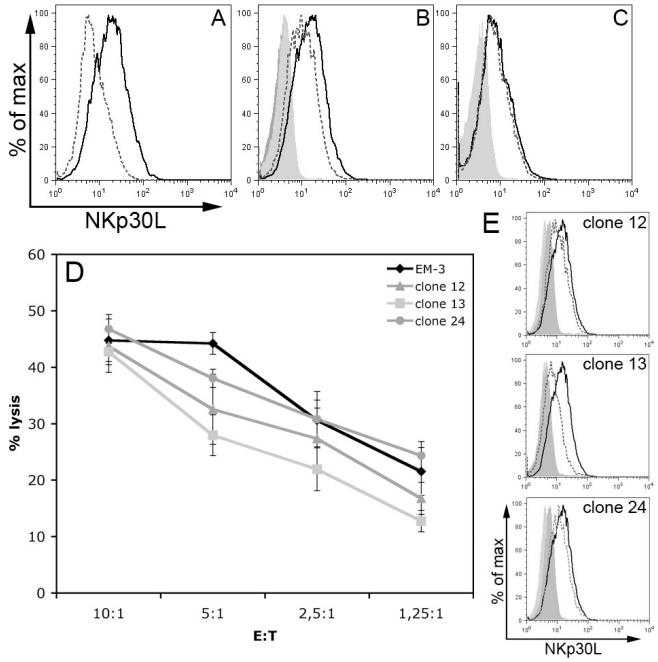

Fig. 24: Depletion of NKp30L positive cells. A-C, NKp30-IgG fusion protein staining at different time points after EM-3 depletion. Bold line, positive fraction; dashed line, negative fraction; grey shadow, control fusion protein staining. **A**, staining directly after the second depletion with MACS beads. **B**, staining 5 days after the second depletion. **C**, 2 weeks after two additional depletions using FACS. **D**, cytotoxicity assay to test for functional consequences of NKp30L downmodulation. Primary NK cells were tested against EM-3 subclones shortly after second depletion. **E** shows the according fusion protein stainings. Error bars represent SD of triplicates.

As can be seen in fig. 24 A-C, the expression level of NKp30L in the depleted cell population returned to that of the control treated population two weeks after the last depletion. The previously generated subclones lost the downmodulation of NKp30L as well (data not shown). The depletion process most probably was successful and siRNAs inhibiting NKp30L expression were enriched. However, after some time the effect of the siRNA might have worn off, probably due to adjustment of the cells to the new conditions.

Following the selection process, total RNA was prepared from the subclones. After reverse transcription into cDNA the siRNA transcripts were amplified via flanking primer pairs and

sequenced. Of 55 clones that grew out, 33 could be sequenced and of these 28 yielded a target sequence. Sequences were aligned to the siRNAs found in the human 50K library and to the human genomic transcript using blast analysis. Some of the target sequences occured several times which indicates that the enrichment process was successful (table 1).

occured	putative target gene
9x	Homo sapiens ADP-ribosylation factor related protein 1 (ARFP1)
5x	Homo sapiens SNW domain containing 1 (SNW1),
3x	Homo sapiens hypothetical protein FLJ21272 (FLJ21272),
3x	Homo sapiens exosome component 2 (EXOSC2)
2x	Homo sapiens apoptotic peptidase activating factor 1
2x	Homo sapiens multiple substrate lipid kinase (MULK),
2x	Homo sapiens lysozyme-like 6 (LYZL6), mRNA
1x	745 275 AA641796 EST
1x	BC027448 Similar to granule cell antiserum positive 8, clone MGC:34919
1x	Homo sapiens chromosome 3 open reading frame 27

Table 1: Summary of the sequences obtained in the siRNA selection procedure. EM-3 cells were lysed and total RNA was prepared. After reverse transcription the siRNA product was amplified by PCR and sequenced. Resulting sequences were aligned using NCBI blast and the data delivered with the library.

The clear advantage of the SBI siRNA library is that the siRNA sequences were designed to fit the affimetrix GeneChip Human Genome U133 Plus 2.0 array. Hence, additionally to sequencing of the subclones, total RNA was isolated from control treated EM3 and depleted EM3 cells. After reverse transcription into cDNA, siRNA was prepared using nested PCR as shown in fig 25.

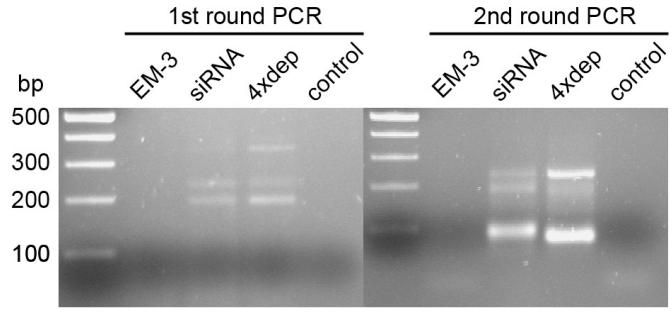

Fig.25: Amplification of siRNA fragments for microarray analysis. PCR-product of the siRNA amplification was loaded onto a 2% agarose gel. After the first round a product of about 200 bp was obtained. The second amplification yielded a fragment of 100 bp that contained only the functional siRNA strand of the hairpin sequence. After exonuclease digestion of the nonbiotinylated strand the single stranded DNA was hybridized to an affimetrix gene chip set. EM3: untransduced EM3 cells; control: no template.

The resulting DNA fragments were subjected to lambda exonuclease digestion to remove the nonbiotinylated antisense strand and the single-stranded and biotinylated product was hybridized to an affimetrix gene chip array. To ensure that results were representative the amplification and hybridization process was repeated and lead to almost identical outcome. The data obtained was analyzed applying the genenet data analysis software provided by SBI. To compare both samples, relative expression of the siRNAs expressed in the control treated cells were compared to the expression in the NKp30L depleted cells as shown in a dot plot in fig 26. In fig. 26 A it is clearly visible that many siRNA species are expressed in the control population but completely absent in the depleted population. These siRNAs were lost during the depletion process and probably play no role for NKp30L expression. Dots found along the red diagonal are expressed equally in both populations. The interesting siRNAs are represented by dots that lie above the red diagonal. These show a higher expression in the NKp30L negative population than in the control. These are the siRNAs that were enriched and are most probably involved in NKp30L expression.

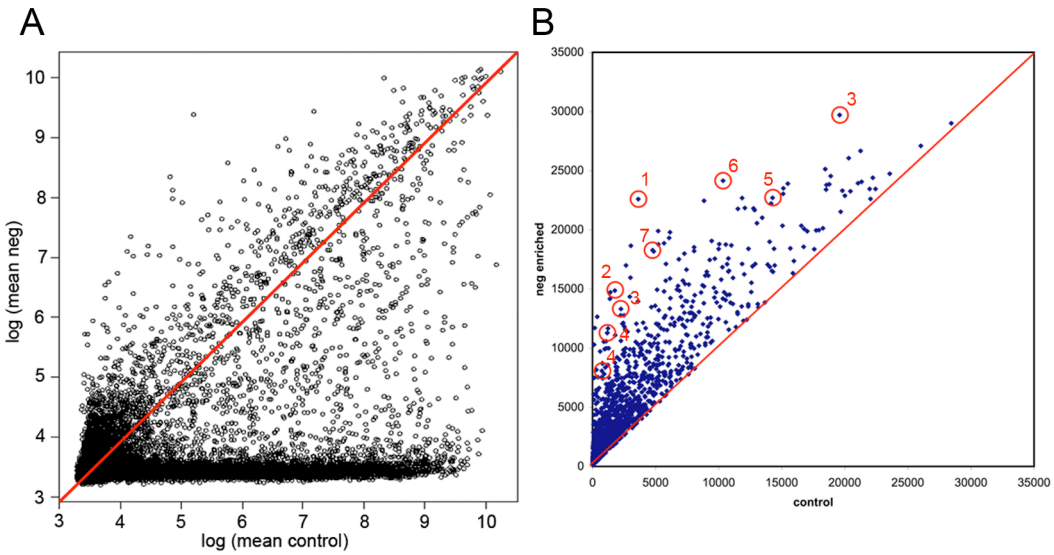

Fig. 26: Dot plot displaying the relative expression levels of detected siRNAs. A, Graph of the relative siRNA expression within the two different populations. Dots found above the red diagonal should be selectively enriched and therefore indicate a possible relation to NKp30L. **B**, siRNA expression is displayed as relative fluorescence value in the control treated cell population (x-axis) versus the selected cell population (y-axis). Due to the size of the data file only positive hits above the red diagonal are shown. Red circles and numbers refer to table 2. Numbers that appear twice denote that two individual siRNAs directed against the same molecule could be detected.

Fig. 26 B shows the distribution of the best hits on a linear scale as derived from another software. Here, only the best 2000 hits are shown, that are found all in the relevant section of the graph.

Depending on where the threshold is set there are 300-500 interesting siRNAs found to be selectively enriched in the NKp30L depleted population. Many of these hits are intracellular proteins that might probably be involved in NKp30L regulation or transport to the

cell surface. Because of the wealth of information obtained by using a microarray based approach it is not possible to list all genes of interest. Table 2 denotes some interesting molecules, either transmembrane, secreted or involved in cancer progression that have yielded a high score in the comparison between the control sample and the depleted sample. Dots represented by these proteins in fig. 26 are marked with red circles.

Two molecules, CD151 and ICAM5 were found to be represented by two different siRNAs that were enriched independently. This increases the probability that these molecules are related to NKp30L. These and the other proteins shown in the table will be subjected to further investigation.

#	Value	GeneBank#	Gene name
1	22604	g4557540	dystonia 1, torsin A
2	14870	g4758811	natural killer cell transcript 4, IL32
3	13313	g12545403	intercellular adhesion molecule 5 (ICAM5)
4	11290	g4757941	CD151 antigen (CD151)
5	22706	g4758109	HLA-B associated transcript-3, BAT3
6	24166	g9966904	Homo sapiens lysozyme homolog (LOC57151), mRNA.
7	18171	g13325360	Homo sapiens, major histocompatibility complex, class I, C

Table 2: Educated guess of interesting molecules retrieved from the siRNA screen after analysis of the microarray data. # refers to numbers given in fig. 26. Value depicts relative expression in the NKp30L depleted cell population.

D. Discussion

D.1 Identification of CLEC12B

Originally identified during a database search, CLEC12B first appeared as a good candidate for an inhibitory counterpart for NKG2D. Due to the high homology in the extracellular domains of the receptors and the converse signaling motifs a functional relationship between these two molecules seemed possible. CLEC12B can be transported to the cell surface in a manner that is independent of CD94 (Fig. 7). Hence, there is no functional similarity between NKG2A or NKG2C and CLEC12B. Using IgG-fusion proteins it became clear that NKG2D ligands are not recognized by the CLEC12B extracellular domain. Of course it cannot be excluded that there are more, yet undescribed NKG2D ligands that might bind to CLEC12B. But, in contrast to NKG2D, expression of the CLEC12B protein could also not be detected on NK , NKT or T cells. Therefore, it seems highly unlikely that CLEC12B would in any way be functionally related to NKG2D.

D.1.1 CLEC12B functions

Examination of the functional properties of CLEC12B lead to the result that this receptor is able to transmit an inhibiting signal via the tyrosine residue embedded in its ITIM. Upon phosphorylation of this motif, the phosphatases SHP-1 and SHP-2 were recruited to the receptor as shown by co-immunoprecipitation. The signal was able to inhibit an activating signal delivered simultaneously through NKG2D. Signals from other activating NK cell receptors such as 2B4 (Bhat et al., 2006) could be influenced by the inhibitory effect of CLEC12B as well (data not shown), demonstrating that CLEC12B can inhibit a broad range of receptor mediated activating signals. In preliminary experiments the function of endogenous CLEC12B was investigated on PMA treated U937 cells. Stimulation of these cells with lipopolysaccharide (LPS) induced the production of tumor necrosis factor α (TNFα). However, engagement of CLEC12B by plate-bound anti-CLEC12B antibody did not significantly inhibit LPS-induced TNFα production (data not shown). The reason for this might be that the plate bound CLEC12B antibody was not able to counteract the signal of the soluble LPS. While plate bound CLEC12B antibody would stimulate the cells only in a specific contact zone the soluble LPS can stimulate the cells all over their surface. It is therefore likely that the local inhibitory effect of CLEC12B engagement was not sufficient to counteract the more global LPS signal. This demonstrates that CLEC12B is only potent in blocking cellular activation when it is co-engaged with an activating receptor in a defined region of the plasma membrane of a cell.

D.1.2 Endogenous CLEC12B expression

By RT-PCR, CLEC12B expression on the mRNA level has been detected in almost every tissue except brain. In contrast to that finding, CLEC12B expression on the cell surface as detected with monoclonal antibodies could only be found on monocyte derived macrophages. This contradiction can be explained with the assumption that macrophages can be found throughout all tissues. Hence, tissue cDNA libraries would also contain macrophage cDNA. This notion would also explain the lack of CLEC12B expression in the brain. In a healthy individual the blood-brain-barrier does not allow immune cells to enter the brain tissue. CLEC12B expression could also not be detected on monocyte derived dendritic cells or blood leukocytes. This leads to the conclusion that CLEC12B is probably exclusively expressed on macrophages that are usually not found in the blood stream.

Another interesting finding was that in mammary gland and ovary only a truncated variant of CLEC12B lacking exon 4 could be detected. As this exon encodes a part of the extracellular domain essential for formation of a functional C-type lectin-like domain, the resulting receptor would probably be nonfunctional. It will be interesting to investigate the regulation of this differential splicing of CLEC12B in different tissues.

CLEC12B may be involved in limiting the activity of monocyte-derived immune cells after cell differentiation and possibly during inflammatory diseases. As there is a CLEC12B homologue in mice it might also be interesting to investigate CLEC12B function in one of the many disease and infection models for this animal. This will probably also give us valuable information about CLEC12B function in human diseases. Another important issue is to identify the still unknown ligand of this receptor. This might give more insight into the biological function of this novel molecule.

D.2 Human Cytomegalovirus downregulates NKp30L

D.2.1 Downmodulation of NKp30L might be a new immune evasion mechanism

The NCR play an important role in the regulation of NK cell activity (Moretta and Moretta, 2004). Their putative cellular ligands can be found on various tumor cells (Arnon et al., 2006) but also on primary untransformed fibroblasts as reported here. Unraveling the identity of these ligands will greatly advance our understanding of NK cell regulation through activating structures on tumors and infected cells. Heparan sulfate structures have been postulated to bind NKp30 (Bloushtain et al., 2004) but other groups could not reproduce these results (Warren et al., 2005). The data presented in this thesis suggests that heparan sulfate is not involved in functional activation of NKp30 during the NK cell-mediated lysis as described in chapter R.3.1.

Fig. 12 shows that NKp30L is downregulated upon HCMV infection. This has not only been demonstrated with the help of fusion proteins but also by functional effects on the cytotoxicity of NK92C1 and primary NK cells. Lysis of HFF infected with HCMV could no longer be inhibited by addition of blocking anti-NKp30 antibody. This means that NK cells were no longer activated through NKp30. Additionally, overall lytic activity was less against infected HFF. Because NKp30 is an important receptor for NK cell activation it is not implausible that a pathogen like HCMV which shares such a long history of co-evolution with humans has evolved an escape mechanism from recognition by this receptor. Clinical HCMV isolates could be shown to downmodulate NKp30L expression as well. Therefore, this phenomenon is not an artifact of the laboratory cultivation of AD169 but seems to play a role *in vivo*.

Modulation of NKp30L by viral infection has been shown for other viruses as well. Infection of HFF with vaccinia virus leads to up-regulation of NCR ligands on the target cell and higher NK cell activity (Chisholm and Reyburn, 2006). Similar findings have been reported for HSV-infected cells (Chisholm et al., 2007). This indicates that there could be an antiviral response in infected cells leading to the up-regulation of NCR ligands. HCMV might have evolved a strategy to evade this response by down-modulation of NKp30L. It has already been described that HCMV expresses an inhibitor of NKp30, pp65/*UL83* (Arnon et al., 2005). This viral tegument protein is reported to be secreted from infected cells and to disrupt NKp30 signaling. However, it is improbable that pp65 is responsible for the effects observed in this study. Cells were washed before staining with NKp30-ILZ. Therefore, secreted proteins would have been removed, making it unlikely that secreted pp65 blocks the binding of NKp30-ILZ. Furthermore, the supernatant of HCMV infected cells did not lead to inhibition of NK cell activity (data not shown). Additionally, pp65 is only strongly expressed in the late infection phase (McLaughlin-Taylor et al., 1994). The experiments in fig. 16 point to an immediate early/early HCMV protein as responsible mediator. As HCMV encodes multiple immunomodulatory proteins it might well be possible that there is more than one mechanism to escape NKp30 activation.

D.2.2 How is NKp30L downmodulated?

Revelation of the HCMV gene product responsible for NKp30L down-regulation might also help in the discovery of the identity of the cellular NKp30L. For heparan sulfate only a minor downregulation could be detected upon HCMV infection. This finding strengthens the theory that heparan sulfate is not involved in NKp30L recognition. Using NKp30-ILZ fusion proteins in immunofluorescence a different staining pattern for NKp30L in non-infected and AD169-infected HFF could be shown (Fig. 12 B). The perinuclear intracellular distribution of NKp30L in infected HFF might be a hint, that the ligand is held back in intracellular

compartments instead of being transported to the cell surface. This strategy is already employed by HCMV, as MHC I molecules are held back and dislocated from the endoplasmic reticulum by gene products of the US region (Mocarski, 2004). In our case, these genes do not seem to be involved in the retention of NKp30L as all three deletion mutants tested did not abrogate NKp30L down-regulation. Therefore we are able to exclude the HCMV ORFs *TRL1* to *UL20* and *IRL14* to *US20*. Furthermore, the additional genes (*UL133* to *UL151*) of HCMV wild-type isolates are excluded, since they are deleted in the laboratory strain AD169 (Cha et al., 1996). Genes lying within the repeat containing regions in WaBAC1 and WaBAC2 cannot completely be excluded as they exist in a second copy within the HCMV genome. Our data show that the putative responsible HCMV gene is a newly produced gene product from the HCMV genome during the immediate early or early infection phase. As many of these genes are essential for HCMV replication, it is not possible to generate simple deletion mutant viruses for these genes. We therefore expressed different HCMV ORFs in the cell lines Hek-293T and HeLa that are also known to carry NKp30L. Among those were the genes encoding the important immediate early proteins IE1 and IE2 (*UL123* and *UL122*). Transfection of these genes had no significant effect on NKp30L expression (data not shown). However, it is also possible that not one single gene mediates the described effect on NKp30L surface expression, but a combination of genes that could compensate for the knockout in a single deletion mutant and which is impossible to recreate by the transfection of individual HCMV genes. Combination of deletion mutants or the expression of several HCMV gene products may be necessary to identify the gene(s) responsible to the down-modulation of NKp30L in future studies. Another possibility would be that the nature of the NKp30L expressed on HFF and tumor cells like Hek-293T and HeLa differ in some way. Then the designated HCMV gene product also might not have an effect on NKp30L expression.

One would predict that murine cytomegalovirus would not need to possess the ability to down-modulate the expression of NKp30L, as the NKp30 and NKp44 receptors do not exist in mice. This is also the reason why it was not possible to recreate our experimental settings in an *in vivo* situation.

Interestingly, we did observe the expression of the putative NKp44 ligand on HFF by using an NKp44-ILZ protein and this expression was also down-regulated upon HCMV infection (data not shown). Possibly, HCMV is also able to downregulate NKp44 employing the same strategy as for NKp30. Due to nonfunctional NKp46 fusion proteins it was not possible to detect any ligand for NKp46 on the surface of HFF, and could therefore not determine if HCMV also has the ability to interfere with NKp46 ligand expression.

HCMV infection leads to the down-regulation of MHCI molecules from the surface of infected cells ((Mocarski, 2004) and data not shown). This would result in a reduced

inhibition of NK cells and enhanced NK cell-mediated lysis of infected cells. To counteract this, HCMV has evolved several strategies. While expressing viral ligands for inhibitory receptors, HCMV gene products also interfere with the expression of ligands for the activating NK cell receptors NKG2D (Dunn et al., 2003; Guma et al., 2006; Welte et al., 2003) and NKp30 as shown here. This would restore the equilibrium of activating and inhibitory signals on the target cell and protect the infected cells from being lysed. The modulation of NKp30L may therefore represent another strategy which contributes to the successful immune evasion of HCMV.

D.3 Characteristics and Identification of NKp30L

D.3.1 Heparan sulfate is not the ligand for NKp30

In a first attempt to establish the role of heparan sulfate as NKp30L, CHO cells were compared to CHO from the laboratories of Hillary Warren and Angel Porgador in respect to NKp30-ILZ fusion protein binding. All three cell lines showed a high affinity for the fusion protein, while the respective xylosyltranferase mutant CHO pgsA745 only bound minor amounts of NKp30-ILZ. In this respect the results of (Warren et al., 2005) could not be reproduced as they could not observe differences in staining pattern for wt and mutant cells. But the results fitted well with what was published by (Bloushtain et al., 2004). To elucidate, whether the staining results for NKp30L would correlate with functional activation of NKp30, cytotoxicity assays were performed. Here, no difference in lytic activity could be seen. Both cell lines were killed in a NKp30 dependent manner as demonstrated by blocking with anti-NKp30 antibodies. This result is contradictory to the NKp30 fusion protein binding assays but has also been observed by (Warren et al., 2005). The contradicting results can probably partly be explained by a evolutionary difference in the cell surface structures between hamsters and humans. A second reason might be that NKp30-ILZ fusion proteins also bind polysaccharide structures and therefore produce high background stainings on CHO cells. Due to these opposing results it was decided to abandon CHO cells as a model system and concentrate on human cells as a more suitable model.

Here, the role of heparan sulfate as NKp30L was established using FACS-based as well as functional assays. Neither addition of heparan sulfate in blocking concentrations nor digestion of heparan sulfate residues from the cell surface could alter fusion protein binding or NKp30 dependent NK cell activity. As the heparan sulfate concentration used was sufficient to block binding of an anti-heparan sulfate antibody to the cell surface it should also have blocked NKp30 binding if it was a specific ligand for this receptor. The same is true for heparinase III digestion of cell surface heparan sulfate residues. Removal of these structures did not lead to a reduced NK cell activity.Therefore it is very unlikely that heparan sulfate is

the functional activating ligand for NKp30. A possible role as a co-ligand cannot be excluded. As the structure of NKp30 has not been solved yet there is no good possibility to predict ligand specificity of NKp30 through computational models.

D.3.2 NKp30L involves a cell cycle regulated protein component

Trypsinization of Hek-293T cells did not only reduce binding of NKp30-ILZ fusion proteins but also activation of NK cells through NKp30. NKp30L expression on the cell surface as determined by fusion protein staining was almost completely restored to normal levels within four hours after trypsin treatment. This effect can be explained by a rather high turnover rate of the putative ligand. Additional data was obtained with experiments conducted with Jurkat cells and GPI-deficient mutants of this cell line. Both the wt and mutant cells showed expression of NKp30L on their surface (data not shown). Therefore it can be concluded that at least one proteinacious component has to be involved in linking NKp30L to the cell surface and that NKp30L does not involve a GPI-anchored protein.

With the help of the inhibitors colchicine and nocodazole Hek-293T cells could effienciently be arrested in M-phase of the cell cyle (Fig. 22 A). NKp30-ILZ fusion protein binding was significantly reduced in mitotic cells as compared to control treated cells. This would mean that NKp30L is downregulated during mitosis. It has been shown that NK cells show increased surveillance of mitotic cells and that this increased affinity depends on engagement of NKp46 (Nolte-'t Hoen et al., 2007). However, it seems probable that different NK cell ligands are cell cycle regulated in different ways such as to keep homeostasis between different activating and inhibiting stimuli. Progression into mitosis leads to major changes within the cell that also include membrane structure and dynamics. Membrane traffic and thus protein turnover is stalled almost completely (Warren, 1993). Possibly this would also affect NKp30L distribution on the cell surface. Another alternative would be that NKp30L regulation is coupled to the cell cycle machinery whose components are turned on and off in cyclic order during cell cycle progression. It will be interesting to further investigate the mechanism of cell cycle regulation of NKp30L and its implications for NK cell activation.

D.3.3 Identification of NKp30L through an siRNA library

Many attempts to identify NKp30L have failed so far. It seems that common methods do not yield valuable results in this respect. In contrast, the rather new method of unbiased screening employing RNAi have lead to the discovery of new signaling pathways and proteins (Hattori et al., 2007). Therefore, an siRNA screen was conducted to identify the putative NKp30L. After transduction of EM3 cells with a library of shRNAs packaged in lentiviral particles, cells with impaired NKp30L expression were enriched. Using reverse transcription and nested PCR the shRNA sequences could be rescued from the transduced

cells.

Analysis of the enriched siRNA sequences was done on two different tracks. On the one hand single cell subclones were generated and the siRNAs expressed in single cells were amplified and sequenced. The second, more global approach was conducted using microarray analysis.

Sequencing of subclones turned out to be rather inefficient as shRNA structures are difficult to sequence and a single mutated or falsely inserted base pair would bias the results obtained with BLAST analysis. Apart from these technical problems, analysis of a small number of subclones cannot be considered representative of a whole cell population. It could merely serve as a control to the microarray experiment. However, some intracellular proteins could be identified that might be involved in the regulation of NKp30L expression.

As the siRNAs were designed to fit the affimetrix genechips it was possible to analyze the overall representation of each siRNA within the whole population. About 300-500 siRNA sequences were found to be significantly enriched within the population with impaired NKp30L expression. Of these, the candidate siRNAs that had the highest expression compared to the control population were determined. Then, several of the most promising targeted proteins that might play a role in NKp30L expression or regulation were selected. When comparing the data obtained from the microarray analysis to the data from the sequencing experiment, it became evident that some siRNAs had been discovered in both approaches. But this was not the case for all sequences. It seems that sequencing of a few subcloned siRNAs leads to a more randomized result and does not yield as evaluable and reproducible datasets as the microarray does. Nevertheless, the recovery of similar targets from both approaches serves as mutual confirmation of the methods used. But because a microarray provides a bigger and more reliable source of information it was decided to concentrate on the evaluation of the data obtained with this method.

Many of the siRNAs found to be enriched were directed against intracellular molecules. This is not surprising as a whole armada of molecules is involved in the expression or modification of a protein. Among the targeted sequences were DNA binding proteins such as transcription factors or DNA methyl transferases. Proteins associated with the cytoskeleton were also identified and interestingly also a valyl-tRNA synthetase. These molecules are all likely to be involved in NKp30L expression and secretion, but as the discovery of a functional NKp30L is most important, membrane proximal and transmembrane proteins were analyzed.

Of all the sequences identified, the siRNA directed against dystonia-1 or torsin-A was most highly enriched in the NKp30L depleted cell population. Although this is not a membrane proximal protein it is nevertheless interesting as this was by far the most enriched siRNA. A mutation of torsin-A is discussed to promote an illness called early onset torsion

dystonia. The mutation causes the protein to aggregate in perinuclear inclusions as opposed to the endoplasmic reticulum localization of the wild-type protein (Ozelius et al., 1997). It seems that functional torsin-A acts as a chaperone in the ER and is involved in protein folding and secretion (Caldwell et al., 2003). Most of the available data concentrates on expression of torsin-A and its mutant isoform in the brain but actually torsin-A is widely expressed throughout the body with a yet undescribed function there. As a member of the family of AAA^+ ATPases its function might be related to the assembly and disassembly of protein complexes (Hanson and Whiteheart, 2005). This putative function might also explain why this protein was found on top of the hit list. It is improbable that torsin-A constitutes NKp30L but it might be very important for its transport to the cell surface or maybe for the assembly of the NKp30L complex.

Interleukin-32 (IL-32) was first described as transcript termed NK4 (Dahl et al., 1992) expressed in NK cells and T cells. However, recent data suggest that IL-32 is also strongly expressed in epithelial cells and EBV-infected B-lymphocytes (Carter et al., 2002; Kim et al., 2005). No definitive function has been assigned to IL-32 until now. Although it can be secreted as a proinflammatory cytokine, it has been described that most of the IL-32 protein is found in the cytoplasm. There, it might be involved in promotion of cell death, as cells transfected to express IL-32 mRNA undergo apoptosis (Goda et al., 2006). A possible mechanism by which IL-32 could function as a proinflammatory cytokine might be release of a membrane bound form of IL-32 by proteinase 3. This enzyme has been shown to bind and cleave IL-32 (Novick et al., 2006). As there is so little information on IL-32 and its function, it might well play a role as NKp30L or enhancing NKp30 signaling when secreted or presented. NK cells are highly important in the control of herpesviral infections. Hence, it would be possible that the secretion of IL-32 of EBV-infected B-lymphocytes provides a danger signal to NK cells and activates them.

Telencephalin or ICAM5 is expressed exclusively in the brain, specifically in the telencephalon. There, it has a crucial function in neuronal development (Furutani et al., 2007). It seems unlikely that a neuronal protein might be involved in NK cell function. In a healthy host NK cells cannot cross the blood-brain-barrier. Furthermore, ICAM5 probably won't be expressed in EM3 cells. The two siRNAs identified in the screen presumably lead to off-target effects. As the family of ICAM proteins shows a high similarity among each other it is possible that the recovered siRNA also had an effect on other ICAM molecules which play an important role for lymphocyte adhesion. It has already been shown that ICAM molecules bind to the receptor LFA-1 (Makgoba et al., 1988). Although this does not exclude the existence of further receptors for ICAM proteins together with the exisiting data it is implausible that NKp30 constitutes a specific receptor for an ICAM molecule.

CD151 belongs to the rather young family of tetraspanins. These very small proteins

are characterized by four transmembrane domains and conserved cysteine residues forming crucial disulfide bonds. Together with integrins, tetraspanins build signaling platforms similar to, but functionally distinct from lipid rafts. As an integrin partner, CD151 modulates integrin-dependent cell morphology, migration, signalling and adhesion strengthening (Hemler, 2005). Mutation of CD151 resulting in a truncated protein leads to hereditary nephritis and a certain kind of skin blistering disease (Karamatic Crew et al., 2004). This finding stresses the importance of tetraspanins in basement membranes. CD151 has also been implicated in cell motility and tumor progression (Liu et al., 2007). Taken together this molecule might be an interesting candidate as ligand for NKp30. As this molecule is linked to tumor invasion of healthy tissues supervision of this marker by NK cells seems plausible.

HLA-B associated transcript-3 (BAT3) or scythe is found among the genes of the MHC cluster, specifically in the MHC III region. It does not show any homology to the common MHC proteins but contains many proline rich sequences. Although BAT3 has been postulated as an NKp30 ligand earlier at the 10th meeting of the society for natural immunology in 2007, no data has been published on that behalf yet. Full length BAT3 seems to be involved in several processes concerning apoptosis. Hsp70 can be inhibited by BAT3 (Thress et al., 2001). BAT3 can also serve as a substrate for caspase 3 (Wu et al., 2004). A BAT3 knockout mouse showed severe developmental defects which can be ascribed to dysregulation of apoptosis and proliferation (Desmots et al., 2005). Another interesting finding is that BAT3 in cancerous tissue is expressed in a splice variant different from the one found in healthy tissue (Roy et al., 2005). This could provide a mechanism through which a normal cell can be distinguished from a healthy cell. Further information on the activity of this protein is scarce and questionable. The position of the BAT3 gene in the MHC locus might point to a function in the immune system that has not yet been elucidated. These facts request that BAT3 should be explored as a possible ligand of NKp30.

The gene for LYZL6 has been discovered in a screen for lysozyme homologues (Zhang et al., 2005). Similar to the original lysozyme, LYZL6 seems to be a secreted protein and has retained its catalytic ability to hydrolyse glycosidic bonds. Its expression is limited to the testis/epididymis tissue, which makes it unsuitable as a possible NKp30L candidate. In this case a cross-reactivity of the respective siRNAs cannot be excluded although it might also be the case that due to the very limited information on this protein a more widespread distribution on stressed cells is possible. Because the gene for LYZL6 is situated within a chemokine cluster (Nomiyama et al., 1999) an immunological function appears feasible but not necessarily that of an NKp30 ligand.

A very interesting result of this screen was that several molecules of the MHC region came up on top of the list. These included not only BAT3, a member of the MHC III family, but also HLA-C, a MHC I molecule. This is an intriguing finding as NK cells are known to bind

MHC molecules with their inhibitory KIR receptors as discussed in the introduction. It would be interesting to speculate that such an important family of activating NK cell receptors as the NCR might bind to MHC related molecules. The interaction of NK cells with MHC molecules seems to be much more complex as previously thought. Several new mechanisms have just been discovered. For example, during the contact of the NK cell with the target cell ligands for various NK cell receptors includig MHC molecules are transferred from the target cell onto the NK cell surface. The detailed mechanism and outcome of this process are still being discussed (Caumartin et al., 2007; Williams et al., 2007). Hence, it might well be possible that NKp30 recognizes certain epitopes presented by MHC molecules *in trans* or *in cis*. It has also been described that MHC II molecules associated with tetraspanin signaling platforms serve a different functional purpose and present distinct peptide epitopes (Kropshofer et al., 2002). Besides, soluble MHC I molecules have been implicated in initiating apoptotic events in NK cells that could be reverted by engagement of KIR receptors (Spaggiari et al., 2002).

Taking into consideration the most interesting hits of the siRNA screen, the ligand for NKp30 presumably is build up within a tetraspanin based signaling platform and could involve molecules originating from genes found within the MHC locus. As NKp30 should be able to detect abnormal or transformed cells it might recognize differentially spliced isoforms of proteins such as BAT3 and others that arise upon progression to cancer.

The results of this screen will be subjected to further investigation. To this end, the function of single siRNAs and their impact on NKp30L expression will be evaluated in EM3 cells. It is also necessary to exclude off-target effectes of the respective siRNAs. If positive results from the screen can be confirmed and siRNAs prove to be specific, the resulting target molecules will be examined for their possible function as NKp30L.

As NKp30 has been discovered several years ago and its ligand has been sought after since then, it is unlikely that the answer will be a simple one. While our knowledge of intercellular interactions becomes more and more complex and involves an ever growing list of molecules it seems that NKp30 offers the opportunity to add to this list a whole new mechanism of interaction. It is still unlikely that a ubiquitously present structure such as heparan sulfate would suffice to unleash the perilous powers of an NK cell. Elucidating the functional cellular ligand for NKp30 will constitute a major advance in the field of innate immunology. This thesis has laid the foundation for the discovery of NKp30L and its mode of function.

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Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
AIDS	aquired immunodeficiency syndrome
BAC	bacterial artificial chromosome
CD	cluster of differentiation
CEACAM1	carcinoembryogenic antigen cellular adhesion molecule 1
DC	dendritic cell
E.coli	*Escherischia coli*
EBV	Epstein-Barr-virus
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
Fig.	figure
GPI	glycosyl-phosphatidylinositol
h	hour
HCMV	human cytomegalovirus
HFF	human foreskin fibroblast
HIV	human immunodefiency virus
HLA	human leukocyte antigen
Ig	immunoglobulin
IL	interleukin
ILZ	isoleucin-zipper
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
ITSM	Immunoreceptor tyrosine-based switch motif
kbp	kilo base pairs
KIR	killer cell Ig-like receptor
MAP	mitogen activated protein
MHC I	major histocompatibility complex class I
NCR	natural cytotoxicity receptor
NK cell	natural killer cell
NKC	NK gene complex
NKp30L	NKp30 ligand
PCR	polymerase chain reaction

PI	propidium iodide
PI3K	phosphatidylinositol-3 kinase
PLCγ	phospholipase C γ
PTK	protein tyrosine kinase
RT	reverse transcription
SHP	SH2 domain containing phosphatase
SIGLEC	sialic-acid-binding immunoglobulin-like lectin
SRR	SLAM-related receptors
TNF-α	tumor necrosis factor α
ULBP	UL16 binding protein

The single amino acid code was used to describe amino acid residues.

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